Universidad Autónoma de Madrid

Programa de doctorado en Biología

Biological and pathological role of A-type lamins in T-cell mediated immune response

Doctoral Thesis

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Madrid, 2018

Departamento de Biología

Facultad de Ciencias

Universidad Autónoma de Madrid

Programa de Doctorado en Biología

Biological and pathological role of A-type lamins in T-cell mediated immune response

Memoria presentada por la Licenciada en Biología:

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para optar al título de Doctor por la Universidad Autónoma de Madrid

Doctorado en Biología con Mención Internacional

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Doctor en Fisiología por la Universidad Autónoma de Madrid

Este trabajo se realizó en el Centro Nacional de Investigaciones Cardiovasculares (CNIC)

Madrid, 2018

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Fdo.: Dr. José María González Granado

A Salva,

a mis padres, Pilar y Jose,

y a mi hermano, Pablo.

“La vida no es fácil para ninguno

de nosotros. Pero… ¡qué importa!

Hay que perseverar y, sobre todo,

tener confianza en uno mismo”

Marie Curie

AGRADECIMIENTOS

En primer lugar, quiero darle las gracias a José María por darme la oportunidad de hacer la tesis doctoral bajo su dirección. Gracias a él he podido cumplir mi sueño de trabajar en el CNIC, el centro de investigación que vi construir a través de mi ventana, mientras estudiaba esos apuntes infinitos de la que sigo considerando la carrera más bonita que se puede estudiar. Nunca olvidaré su llamada telefónica a la que contesté entre sonrisa y sonrisa, ¿en serio? Gracias por sus consejos, su apoyo, sus críticas, y sus grandes, aunque a veces abrumadoras, ideas experimentales. Espero que se me haya pegado algo de su incansable curiosidad científica. Gracias también por interesarse tanto por mi formación y animarme a que me fuera de estancia; por desgracia no todos los jefes ponen interés en esta parte de su trabajo, y por todo ello, siempre le estaré enormemente agradecida.

Gracias a Virginia por todo el apoyo experimental, y también personal. Esta tesis es en parte suya, ya que sin ella muchos de los experimentos no podría haberlos llevado a cabo. De ella espero que se me haya pegado algo de sus ganas de trabajar y sus escasos no por respuesta. Ha sido un placer trabajar contigo.

Gracias al grupo de Vicente Andrés, por acogerme, apoyarme y enseñarme tanto. He tenido la suerte de compartir estos años de trabajo con excelentes personas. Gracias a María Jesús por sus genotipados que hacen posible el trabajo del resto del grupo. Gracias a Pilar por sus sabios consejos y charlas desde la poyata. Gracias a Magda por contestar todas mis dudas y por ofrecerme siempre sus buenísimos chocolates polacos. Gracias también a Bea por sus consejos y ayuda. Gracias a Álvaro por su apoyo, su alegría, sus chistes y sus cánticos; irradias felicidad y eso genera un gran ambiente de trabajo, gracias. Gracias también a mis compis de tesis: a Alberto, por ayudarme día a día con cada duda del labo, y por sus malas apuestas que tres veces nos llevaron a La Chalana; a Amanda, por su amistad aunque casi algo más jaja, y por sus clases de patinaje; a Víctor por sus interminables charlas científicas casi filosóficas, y su especial sentido del humor; y a mi cordobesa favorita, Rosa. Contigo he compartido menos tiempo en el labo, pero has sido la mejor compañera de ordenador (porque siempre estabas jaja). Ahora en serio, gracias por escucharme, por aconsejarme, por tus dudas que también me hicieron aprender, por tu constante sonrisa, y por tu palabrerío andaluz que me tanto me hizo reír. Gracias también a Lara, Cris Rius, Elba, Cris González, Ana, Lara, Yazan, y Pedro. Gracias a Javi por todos los momentos de cafés, comidas o desayunos; sabes hacer desaparecer las preocupaciones del trabajo, con tus conversaciones que aunque algo escatológicas, resultan divertidas. También quiero agradecer a “las compis de enfrente”, Rocío y Ana Marcos, por todas las conversaciones y momentos compartidos. Y a mí querida Irenita claro. Gracias por tu dulzura, tu apoyo y comprensión, tu compañía de cada día, tus detalles de cumple, por nuestros desayunos de pan con chocolate que tan buenos recuerdos me traían y traerán, y en definitiva, por cada momento. De ti me llevo una gran amistad.

Gracias también a Francisco Sánchez Madrid, por darme la oportunidad de formar parte de sus labmeetings y acogerme en su laboratorio del CNIC. Ha sido una gran experiencia profesional vivir ese ambiente de excelencia científica. Sois científicos brillantes y muy trabajadores, y ha sido un placer trabajar a vuestro lado. Gracias a Laura especialmente, por toda su ayuda y consejos, y por nuestras conversaciones para curar la IBD y la ansiedad al mismo tiempo.

Gracias a Jesús Vázquez y a Juan Antonio López de la unidad de Proteómica del CNIC, por llevar a cabo el ensayo experimental y resolver todas mis dudas durante nuestra colaboración científica. Gracias también a todo el personal de las Unidades de Celómica, Microscopía, Animalario, Cultivos Celulares, Vectores Virales, y Genómica, pues me ayudaron en múltiples ocasiones.

También tengo que agradecer muchísimo a Philippe Collas, por abrirme las puertas de su laboratorio en Oslo, y hacerme pasar una de las mejores experiencias profesionales y personales de mi vida. Gracias por hacerme sentir como en casa, por su tiempo invertido en mi proyecto, y por valorar tanto mí trabajo. Nunca antes me habían hecho sentir tan valorada profesionalmente, y gracias a ello, aprendí a creer más en mí y en casa cosa que hago. Gracias también a Kristin y a Anita, por enseñarme tanto y ser tan amables conmigo desde el día que llegué. También gracias al resto de compañeros que me ayudaron en cada duda y en cada momento que lo necesité (Tarvesh, Nolwenn, Cohen, Erwan, Inwasti, Frida, Torunn, Sumithra, Annael, Marie-Odile, and Jonas). Y muchas gracias a Anna, por ser mi amiga y compañera desde mi primer día en Oslo. Lo expresaré también en inglés para que ellos puedan entenderme mejor:

I also want to thank Philippe Collas for opening me the doors of his laboratory in Oslo, and give me one of the best professional and personal experiences of my life. Thank you for making me feel at home, for your time invested in my project, and for valuing my work so much. Before of my stay there, I had never felt myself so professionally valued, and thanks to that, now I believe more in myself and my work. Thanks also to Kristin and Anita for teaching me so much, and being so kind with me from the day I arrived. Also thanks to the rest of my colleagues who helped me in every doubt and in every moment I needed it (Tarvesh, Nolwenn, Cohen, Erwan, Inwasti, Frida, Torunn, Sumithra, Annael, Marie-Odile, and Jonas). And thanks off course to Anna, for being my friend and partner from the moment I arrived in Oslo.

Además me gustaría hacer una mención especial a mis tíos, Marisa y José Ramón. Gracias Marisa por introducirme en el mundo científico, no sé si ha sido tu figura profesional o la genética lo que me ha impulsado a trabajar en esto, pero sin duda sé que has sido uno de los factores responsables de que haya llegado hasta aquí. Gracias a los dos por guiarme, escucharme, y ayudarme siempre que lo he necesitado. Es un privilegio contar con vuestro apoyo, y me siento muy afortunada de teneros como referente científico. Suponéis para mí un gran modelo profesional a seguir.

Por otro lado, quiero nombrar a las personas que han compartido cada momento de mi vida profesional y personal, y que han hecho posible que yo llegara hasta aquí. Gracias a mis padres, por apoyarme en cada uno de los pasos que he dado, por vuestra paciencia, y por saberme llevar, que, a veces, no es nada fácil. Gracias a mi padre por insistir tanto en que estudiara y me esforzara, gracias a ello aprendí que el conocimiento es una de las llaves que llevan a alcanzar el sentido de la vida y la felicidad. Gracias a mi madre por creer siempre en mí, por cada palabra de ánimo, y por hacer desaparecer muchas veces mis nervios, miedos e inseguridades. Has sido y eres, mi amiga incondicional, mi apoyo, y la mejor madre que podría desear. Gracias igualmente a mi hermano, por todo su cariño y admiración que me animan a creer más en mí. Gracias por tu sentido del humor, así como por tu positivismo y filosofía de vida, que tanto me han ayudado y me ayudan. Ojalá acabe algún día siendo un poquito como tú, aunque me conformo con seguir de cerca esa pasión que pones en cada cosa que haces, ya que me suscita afán de superación. Gracias a mi abuela "Cachi", por enseñarme que el conocimiento es la llave de la independencia y la libertad, y que en la vida debemos perseguir nuestros sueños. Gracias también al resto de mi familia y amigos/as, que de una manera u otra me han regalado miles de palabras de apoyo y momentos de felicidad durante estos años.

Por último, pero para nada menos importante, gracias a Salva. Gracias por completar mi vida. Me aportas tanto cada día que me resulta muy complicado expresar en unas pocas líneas, todo lo que supones para mi vida personal y profesional. Desde que te conozco te admiro y eres mi modelo a seguir en muchas facetas de la vida. Gracias a ello y a tu apoyo, sé que sin ti, no podría haber llevado igual este duro, aunque apasionante trabajo. Me impulsas a trabajar con tesón, a ser constante y no tan nerviosa, a poner lo mejor de mí en cada cosa, a ser menos impaciente y más segura de mí misma, a controlar mis impulsos, y en definitiva, a disfrutar más de cada experiencia de la vida. Por eso, jamás podré llegar a agradecerte todo tu apoyo. Gracias por ser mi compañero de aventuras y enseñarme que, creer en uno mismo y ser feliz, son la clave del éxito.

Gracias a todos por estos casi 4 años, pues han sido, sin duda, inolvidables.

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Abbreviations

1. ABBREVIATIONS

APC: antigen-presenting cell

a.u.: arbitrary units

Blimp-1: B-Lymphocyte-Induced Maturation Protein 1

BM: bone marrow

BMDCs: bone marrow derived dendritic cells

BrdU: 5-bromo-2-deoxyuridine

CD4-*Lmna*$^{-/-}$: CD4-CRE$^{tg/wt}$- *Lmna*$^{flox/flox}$ mice

ChIP: Chromatin immunoprecipitation

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

DAPI: 4',6-Diamidine-2'-phenylindole dihydrochloride

DCs: dendritic cells

DMSO: dimethyl sulfoxide

EDTA: ethylenediaminetetra-acetic acid

Eomes: Eomesodermin

ERK: extracellular signal-regulated kinase

FACS: fluorescence-activated cell sorting

F-actin: actin filaments

FBS: fetal bovine serum

Foxp3: Forkhead box P3

GITR: glucocorticoid-induced TNFR family related gene

Gzm: Granzyme (A and B)

HGPS: Hutchinson-Gilford Progeria Syndrome

IBD: inflammatory bowel disease

ICOS: Inducible T-Cell Costimulator

i.d.: intradermal/ intradermally

IFNγ: interferon gamma

Ig: immunoglobulin

IL: interleukin

IL-2R: interleukin 2 receptor

INM: inner nuclear membrane

i.p.: intraperitoneal/intraperitoneally

IS: immune synapse

LADs: lamin-associated domains

. **iTreg:** induced regulatory T-cell

LADs: lamin-associated-domains

***Lmna*$^{-/-}$:** lamin A/C complete knockout mice

MFI: mean fluorescence intensity

MHC: Major Histocompatibility Complex

MIIA: myosin IIA

MLN: mesenteric lymph nodes

MTOC: Microtubule-Organizing Center

mTOR: mammalian target of rapamycin

NKs: natural killer cells

NL: nuclear lamina

nTreg: natural regulatory T-cells

ONM: outer nuclear membrane

PBS: phosphate buffered saline solution

PcG: Polycomb group

PeLN: peripheral lymph nodes

p.f.u.: plaque-forming units

PI3K: phosphatidylinositol 3-kinase

PLN: popliteal lymph nodes

PP: Peyer's Patches

Prf: Perforin (-1)

qPCR: quantitative polymerase chain reaction

RA: retinoic acid

RAR: retinoic acid receptor

RARE: retinoic acid response element

RORγT: RAR-related orphan receptor γ

RT: room temperature

RT- qPCR: reverse transcription-qPCR

SMAC: supramolecular activation cluster

T-bet: T-box transcription factor

TCR: T-cell receptor

Teff: effector T-cells

TF: transcription factor

TGFβ: Transforming Growth Factor β

Th: T-helper

Treg: T-regulatory

Tr1: regulatory T-cells 1

TSS: transcription starting site

VACV: Vaccinia virus

WT: wild type

Summary/ Resumen

2. SUMMARY

A-type lamins (lamin A/C) are intermediate filament proteins that conform with B-type lamins the nuclear lamina (NL). The NL is localized just below the inner part of the nuclear envelope. Thus, the NL interacts with chromatin and transcription factors, modulating epigenetics and gene expression, among other essential functions as cell migration, proliferation, differentiation, and cell cycle progression. However, the most well-known function of lamin A/C is the maintenance of nuclear structure. Little information is known about the expression and function of A-type lamins in immune cells, and specifically in $CD4^+$ T lymphocytes. $CD4^+$ T-lymphocytes are one of the main components of the adaptive immunity, a very complex and a highly specialized immune response that defends the organism against infections. These lymphocytes need to interact through their T-cell receptor (TCR) with an antigen-presenting cell to become active, forming what is called immune synapse (IS). Once the TCR recognizes an antigen, lamin A/C has been shown to be expressed in the $CD4^+$ T-cell, enhancing a proper IS formation and thus $CD4^+$ T-cell activation. Due to its important role in T-cell activation, we hypothesized that lamin A/C might have also a role in proliferation, differentiation and effector function of $CD4^+$ T lymphocytes. We have corroborated that lamin A/C significantly enhances T-cell activation *in vivo*, but it does not regulate T-cell proliferation. Interestingly, our results indicate that lamin A/C significantly determines the T-helper (Th) phenotype commitment. Hence, we have observed *in vitro* and *in vivo* that lamin A/C enhances Th1 differentiation, without affecting Th2 and Th17 phenotypes. Moreover, lamin A/C improves Th1 cells effector function against vaccinia virus (VACV) and *Leishmania major* infections in mice by enhancing $CD4^+$ T cell cytotoxic capacity and Th1 effector response. Furthermore, *Lmna*$^{-/-}$ $CD4^+$ T-cells protect from inflammatory bowel disease (IBD) development in mice enhancing regulatory T-cells (Treg) differentiation, and improving their suppressive function. The molecular mechanism by which lamin A/C determines Th fate is the upregulation of the Th1 master regulator (*T-bet*), and the downregulation of Treg master regulator (*Foxp3*). In more detail, lamin A/C epigenetically modifies the *T-bet* promoter enhancing its gene transcription. However, lamin A/C does not induce epigenetic changes in *Foxp3* promoter. Besides, it is known that retinoic acid (RA) can regulate lamin A/C expression in leukocytes. Additionally, it has been described that $CD103^+$ dendritic cells (DCs), mainly located in the mesenteric lymph nodes, release RA. We have demonstrated in mesenteric lymph nodes that the RA released by $CD103^+$ DCs downregulates lamin A/C in $CD4^+$ T-cells upon antigen recognition, enhancing Treg differentiation. In contrast, in spleen and peripheral lymph nodes, $CD103^-$ DCs are predominant and do not produce RA, facilitating lamin A/C expression in $CD4^+$ T-cells upon antigen recognition, and thus, Th1 differentiation. By this physiological mechanism, lamin A/C levels can be modulated in different anatomical sites, in accordance with immunological requirements to control naïve T cell differentiation. Altogether, our findings set A-type lamins as key regulators of Th differentiation, and thus potential therapeutic targets for IBD and infectious diseases.

RESUMEN

Las laminas de tipo A (lamina A/C) son filamentos intermedios que forman, junto a las laminas de tipo B, la lamina nuclear (LN). La LN se localiza en la cara interna de la envoltura nuclear, interaccionando con la cromatina y factores de transcripción, y modulando la epigenética y expresión génica entre otras funciones esenciales como migración, proliferación, diferenciación, y progresión del ciclo celular. Sin embargo, la función más conocida de la lamina A/C, es mantener la estructura nuclear. La expresión y función de las laminas de tipo A en células inmunes, y más específicamente en los linfocitos T CD4^{+}, es bastante desconocida. Los linfocitos T CD4^{+}, son componentes principales de la inmunidad adaptativa, respuesta inmune compleja y muy especializada que defiende a nuestro organismo frente a infecciones, e interviene en procesos inflamatorios y enfermedades autoinmunes. Estos linfocitos, necesitan interaccionar mediante el receptor de células T (TCR) con la célula presentadora de antígeno formando la sinapsis inmune (IS), para pasar así al estado activo. Una vez que se produce el reconocimiento antigénico, se expresa la lamina A/C en el linfocito CD4^{+}, facilitando la formación de la IS y su activación. Debido a su importante papel en la activación de células T, hipotetizamos que la lamina A/C debía tener un papel en la proliferación, diferenciación, y función efectora de los linfocitos T. Hemos corroborado que la lamina A/C facilita la activación de la célula T en modelos *in vivo*, pero no regula su proliferación. Nuestros resultados indican que la lamina A/C modifica significativamente el compromiso fenotípico Th. Así, hemos observado *in vitro* e *in vivo* que la lamina A/C facilita la diferenciación a Th1, sin afectar a la diferenciación hacia los fenotipos Th2 y Th17. Igualmente, la lamina A/C mejora la función Th1 frente a infecciones por el virus vacuna y *Leishmania major* en ratón. A su vez, hemos observado que las células T CD4^{+} *Lmna*$^{-/-}$ protegen de la enfermedad inflamatoria intestinal (IBD) en ratón, facilitando la diferenciación de células Treg, y mejorando su función supresora. El mecanismo molecular por el que la lamina A/C determina el destino de las células T, es aumentando la expresión del factor de transcripción regulador de los linfocitos Th1 (*T-bet*) mediante modificaciones epigenéticas, y disminuyendo la del factor de transcripción regulador de las Treg (*Foxp3*). Por otro lado, es sabido que el ácido retinoico (RA) puede regular la expresión de la lamina A/C. Asimismo, se conoce que las células dendríticas (DCs) CD103^{+}, mayoritariamente localizadas en el intestino, son capaces de producir RA. Nosotros hemos demostrado que el RA liberado por las DCs CD103^{+} del intestino en el momento del reconocimiento antigénico, disminuye la expresión de lamina A/C en los linfocitos CD4^{+}, facilitando así, la diferenciación hacia células Treg. Sin embargo, en el bazo y ganglios periféricos predominan las DCs CD103^{-} que no producen RA, lo que permite mayor expresión de lamina A/C en los linfocitos CD4^{+}, y promueve la diferenciación de las células T CD4^{+} naïve hacia Th1. Por tanto, este parece ser un mecanismo fisiológico por el cual se regulan los niveles de lamina A/C en el organismo para regular la diferenciación del linfocito T. En definitiva, nuestros estudios sitúan a las laminas de tipo A como reguladores clave de la diferenciación Th, siendo dianas terapéuticas en potencia para el tratamiento de la IBD y enfermedades infecciosas.

Introduction

3. INTRODUCTION

3.1. A-type lamins.

The DNA is compartmentalized in the nucleus of the mammalian cell and separated from the cytoplasm by the nuclear envelope. The nuclear envelope is a complex structure composed by two membranes, the outer- (ONM) and the inner-nuclear membrane (INM). Just at the internal layer of the INM, is where is located the nuclear lamina (NL) (**Figure 1**) (Hetzer M.W., 2010).

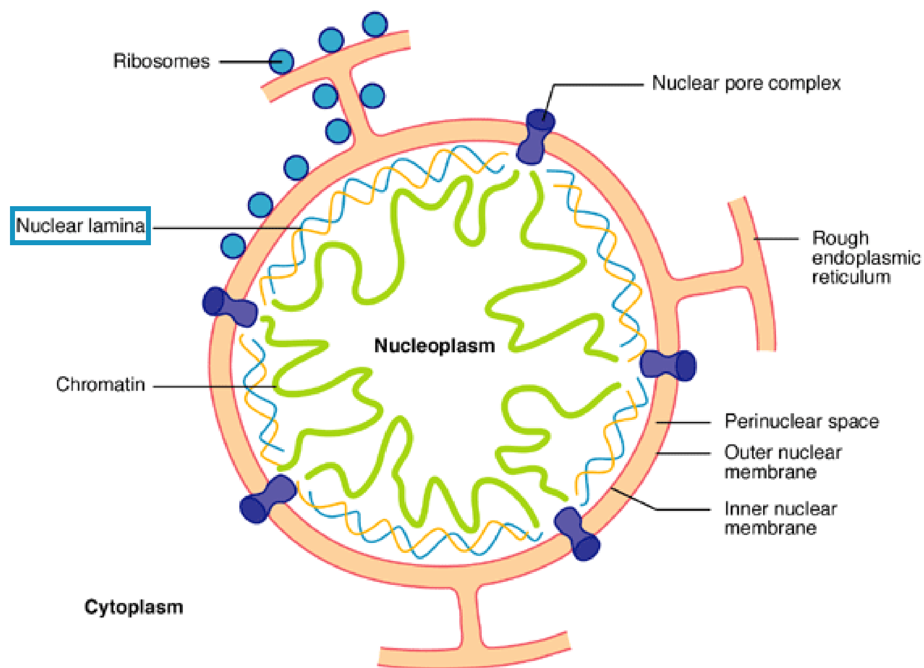

Figure 1. NL localization in the mammalian nucleus. The nuclear envelope defines the nucleus in the mammalian cell, separating the DNA from the cytoplasm components. It consists in two membranes, the ONM and the INM, and the space in between called the perinuclear space. The ONM is contiguous with the rough endoplasmic reticulum and is covered by ribosomes. In the internal part of the INM is where is located the NL, interacting with the chromatin and TFs, as well as with the nuclear pore complexes (modified from Maidment S.L. & Ellis J.A., 2002).

The main components of the NL are the lamins, intermediate-filament-proteins (IFPs) classified as type V. These type V-IFPs are characterized by a nuclear localization signal (NLS), which labels these proteins to be imported to the nucleus (Dechat T. *et al*, 2010). The lamins are classified in two groups: A-type and B-type lamins. A-type lamins are expressed in differentiated cells, and are codified by an unique gene (*LMNA*) that suffers an alternative splicing generating two transcripts: lamin A and lamin C (**Figure 2**). Therefore, it is very common to call them jointly as lamin A/C (from now on). On the other hand, B-type lamins are three ubiquitous IFPs that arise from two different genes: *LMNB1*, coding for lamin B1, and *LMNB2*, coding for lamin B2 and B3 (Moir R.D. *et al*, 2000). These lamins are part of the nucleoskeleton, but also interact with the cytoskeleton through nesprins and SUN domain proteins

forming all together the LINC (linker of nucleoskeleton and cytoskeleton) complex. So that, the most well-known function of lamins is the maintenance of nuclear architecture, regulating also cell processes as proliferation, migration, and differentiation (Schirmer E.C. & Foisner R., 2007). In addition, lamins also interact with the nuclear pores complexes modulating molecular trafficking between cytoplasm and nucleus. Moreover, lamins also interact with chromatin and transcription factors (TFs), acting as a scaffold, modulating chromatin organization and epigenetic changes, and therefore, gene transcription (**Figure 1**). These last features determines the great importance of the NL in cell biology, although it has other essential functions as DNA synthesis and repair, signal transduction and cell cycle progression (Andrés V. & González J.M., 2009).

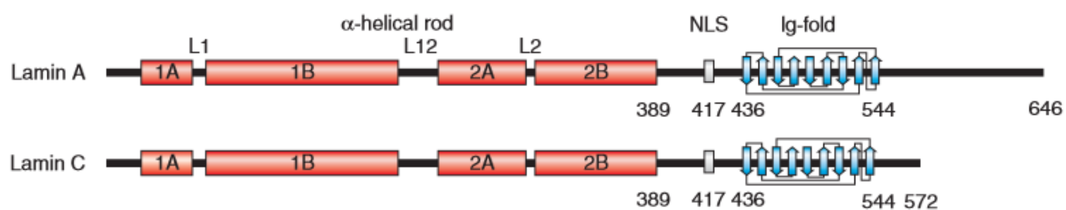

Figure 2. Schematic illustration of lamin A and lamin C polypeptide chains. The lamin protein structure is composed by a short amino terminal head domain, a central helical rod domain, and the carboxy-terminal domain containing the NLS and the Ig-fold. (Dechat T. *et al*, 2010).

There is little information about the regulation of *LMNA* expression. It has been described that retinoic acid (RA), the acid form of vitamin A, can control lamin A/C expression. Lebel S. *et al* reported in 1987, that RA induced lamin A/C expression in mouse embryonic carcinoma cells. However, Olins A.L. *et al* published in 2001, that RA downregulates lamin A/C in the leukemic cell line HL-60. RA is a potent regulator of cell proliferation and differentiation, being essential in embryonic development. It acts through nuclear retinoic acid receptors known as RARα, RARβ, and RARγ. These RAR are bound to DNA and retinoic X receptors (RXRα, RXRβ, and RXRγ) forming heterodimers that work as transcriptional regulators (Germain P. *et al*, 2006). This regulatory regions in the DNA are called RA response elements (RAREs). Interestingly, it has been described a RARE in the *LMNA* promoter (Okumura K. *et al*, 2000). Therefore, RA seems to regulate *LMNA* transcription. Even though it has been reported that A-type lamins also modulate the RA pathway, regulating RARγ nuclear translocation. Moreover, lamin A/C transcription is mechano-regulated depending on the tissue stress and matrix stiffness (Philip J.T. & Dahl K.N., 2008; Swift J. *et al*, 2013). Thus, *LMNA* expression is a complex process that depends on the tissue environment, and in which RA seems to have an important role (Swift J. *et al*, 2013). Hence, more research is needed to further decipher lamin A/C and RA feedback in different cell-types and tissue environments.

Additionally, the research in A-type lamins has been quite relevant due to the discovery of several spontaneous mutations in *LMNA* gen that cause mono-genetic rare diseases, some of them with similar

pathologic features: lipodystrophy, skeletal and muscular dystrophy, and dilated cardiomyopathy. For this reason, almost all the scientific research related with lamin A/C is focused in the study of A-type lamins in adipocytes (Lund E. & Collas P, 2013), stem cells (Espada J. *et al*, 2008; Dechat T. *et al*, 2010), cardiomyocytes (Afilalo J. *et al*, 2007), osteoblasts and muscle cells (Duque G. & Rivas D., 2006; Akter R. *et al*, 2009; Rauner M. *et al*, 2009). Many of these disorders are commonly known as laminopathies (Worman H.J., 2012). One of the most studied, is the Hutchinson-Gilford Progeria Syndrome (HGPS), a premature aging disease that cause the death in children of only 14 years of age (Merideth M.A. *et al*, 2008). Only one laminopathy has been reported with an immunologic defective phenotype. This laminopathy is known as Pelger-Hüet anomaly and is characterized by a defect in neutrophil differentiation because of mutations in lamin B receptor gene (*LBR*) (Worman H.J. & Bonnè G., 2007). In addition, it has been described the repression of lamin A/C gene in B-cell lymphomas, acute lymphoblastic leukemia, and non-Hodgkin's lymphoma (Agrelo R. *et al*, 2005; Stadelmann B. *et al*, 1990). Apart from these, there are few publications relating lamin A/C and immune cells. Hence, the role and the molecular mechanism that regulate the expression of lamin A/C in the immune system are poorly understood.

3.2. The adaptive immune response.

The immune system comprises many different cell types and biological processes to protect the organism against infections and diseases. These cell types and processes are classified in two types of immune response: the innate and the adaptive immunity. The innate immunity is a generic response that provides a rapid defense against pathogens or harmful circumstances for the body. On the other hand, the adaptive immunity is a highly specific immune response, composed by complex mechanisms that try to maintain the health state in the organism. The lymphocytes are main components of the adaptive immune response. B-lymphocytes carry out the humoral response producing antibodies against pathogens, and T-lymphocytes play the cell-mediated response (Delves P.J & Roitt I.M., 2000) (**Figure 3**).

To become active, naïve T-lymphocytes need to recognize through the TCR a peptide-charged MHC of an antigen-presenting cell (APC), forming what is called the immune synapse (IS). The IS is a transient cell-to-cell communication structure formed by a central supramolecular activation cluster (cSMAC) that contains the TCR and associated molecules. This cSMAC is surrounded by the peripheral SMAC (pSMAC) composed by adhesion molecules as integrins. Furthermore, there is a distal SMAC (dSMAC) surrounding the latter, which contains phosphatases that can act as inhibitors of the IS formation (Davis S.J. & van der Merwe P.A., 2006). The IS takes place in secondary lymphoid organs, where activated APCs migrate to interact with the lymphocytes. Once this interaction has been performed, T-cells will proliferate and differentiate towards different T-helper (Th) phenotypes in the case of $CD4^+$ T-lymphocytes, or towards a cytotoxic phenotype in the case of $CD8^+$ T-lymphocytes (Delves P.J. & Roitt I.M., 2000) (**Figure 3**). Th cells are called helpers because they promote B-cell

antibody production and they are also required for the generation of cytotoxic response of CD8⁺ T-cells. Moreover, CD4⁺ T-cells control the expansion and recruitment of other components of the innate immune response. CD4⁺ T-cells can be considered the main guardians of the correct behavior of immune system (Zhu J. *et al*, 2010).

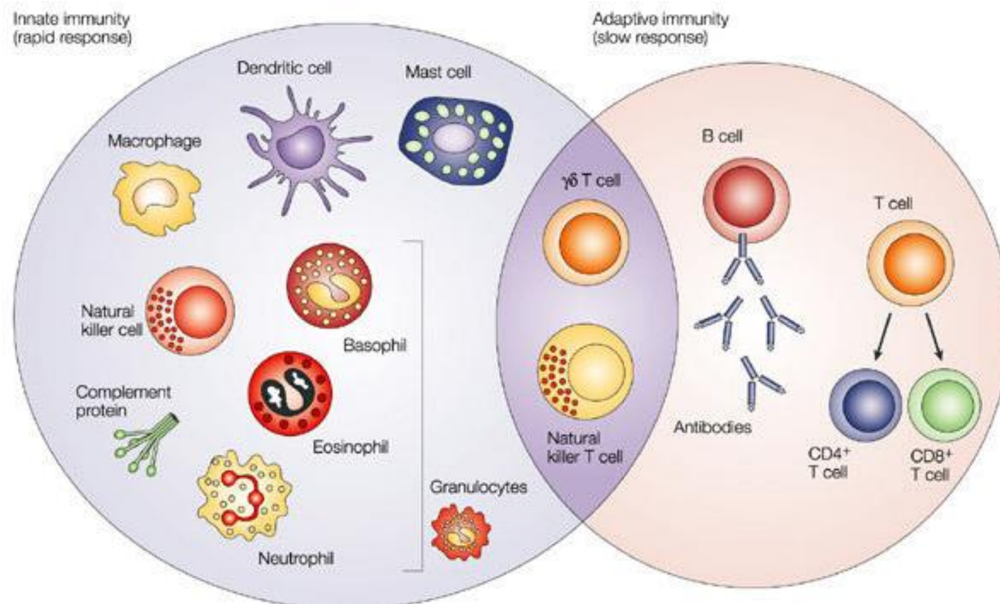

Figure 3. Components of the immune system. The innate immunity is the first line of defense against pathogens and is composed by several types of cells: granulocytes (basophils, eosinophils, and neutrophils), DCs, macrophages, natural killer cells (NKs), mast cells and soluble factors as complement proteins. The adaptive immunity acts later but in a more specific manner. It consists in CD4⁺ T-cells and CD8⁺ T-cells, which compose the cellular response, and B-cells that are in charge of antibody production. NKs and γδ T-cells are cytotoxic cells in the middle of innate and adaptive immunity (Dranoff G. *et al*, 2004).

3.3. A-type lamins and the adaptive immune response.

The role of A-type lamins in the immune system has been poorly studied. The few studies published about this topic before 2014, mainly report the lack of lamin A/C or a low expression in hematopoietic cells and CD4⁺ T-cells (Guilly M.N. *et al*, 1987; Paulin-Levasseur M. *et al*, 1988; Guilly M.N. *et al*, 1990; Rober R.A. *et al*, 1990). However, since 2014, and thanks to J.M. González-Granado and collaborators work, it is known that lamin A/C is transiently expressed in mouse and human CD4⁺ T-cells upon TCR-dependent antigen recognition. Therefore, the expression of lamin A/C is promoted by TCR activation, and it lasts for 3 days since antigen-recognition in CD4⁺ T-cells. This study describes also the importance of lamin A/C in CD4⁺ T-cell activation by the upregulation of CD69 and CD25 (IL-2R). Moreover, the lamin A/C modulates the IS dynamics, controlling the timing and the proper conformation of the interaction with the APC. CD4⁺ T-cells lacking lamin A/C barely interact with APCs, having smaller cSMAC diameters and reduced numbers of CD3-containing microclusters at the

membrane contact area, compared with lamin A/C expressing T-cells. Furthermore, these lamin A/C-dependent differences in T-cell activation are related also with changes in actin filaments (F-actin) polymerization, and microtubule-organizing center (MTOC) translocation towards the IS. All these processes are accompanied by changes in TCR signaling with increased amounts of phosphorylated Vav1, myosin IIA (MIIA), and extracellular signal-regulated kinase 1 (ERK1) and ERK2 (ERK1/2) in lamin A/C expressing T-cells (González-Granado J.M. *et al*, 2014). Therefore, the lamin A/C transient expression in $CD4^+$ T-cells upon antigen recognition is not a random event. On the contrary, it is an essential step that rigorously coordinate the complex process of T-cell activation (**Figure 4**).

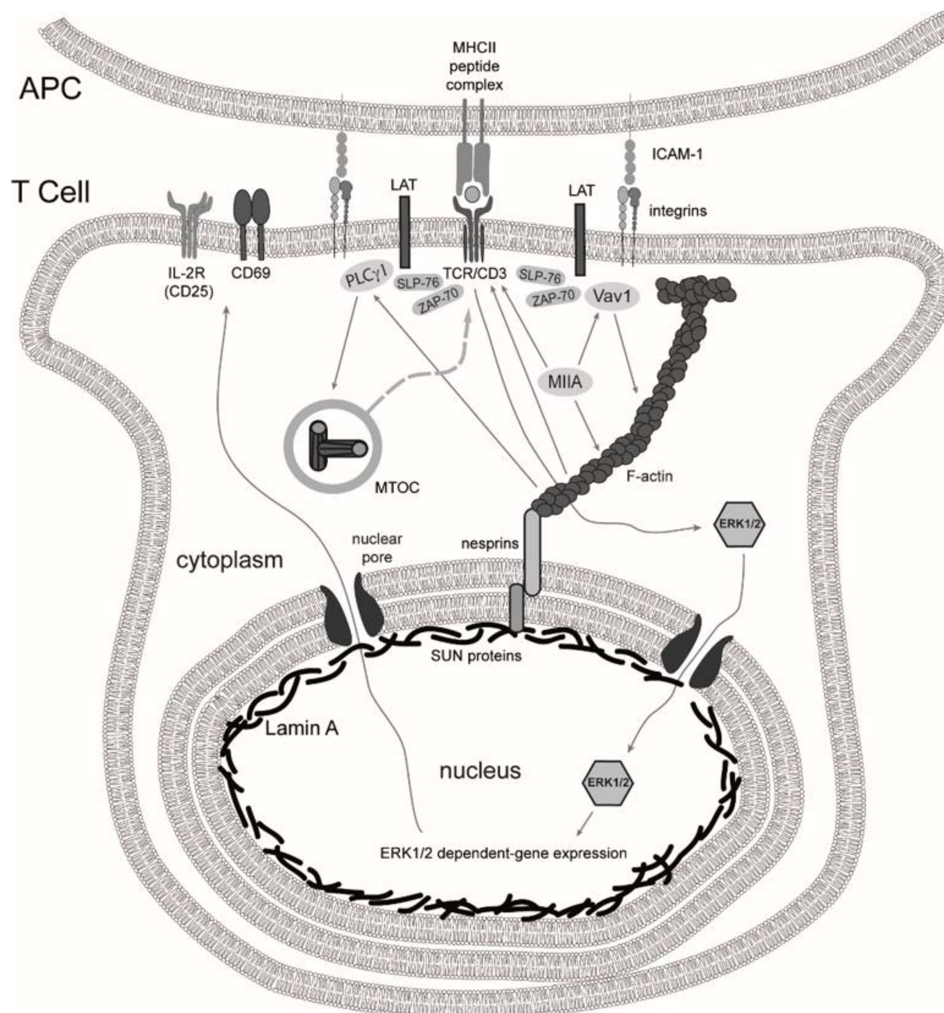

Figure 4. Lamin A/C modulates key points of TCR-dependent T-cell activation. Lamin A/C promotes an increase in TCR/CD3 clusters at the cSMAC of the IS, promoting the phosphorylation of downstream molecular targets as: ZAP-70 (ζ-Chain-associated protein of 70 kD), LAT (linker of activated T cells), SLP-76 (SH2 domain-containing leukocyte protein of 76 kD), MIIA, and PLCγ1 (Phospholipase C gamma 1) which enhances MTOC polarization towards the IS. A-type lamins also induce VAV-1 phosphorilation, favoring the F-actin polymerization, a fundamental step in IS. Besides, lamin A/C enhances the membrane expression of T-cell activation markers as CD69 and CD25 (IL-2R), depending on ERK1/2 phosphorylation (Rocha-Perugini V. & González-Granado J.M., 2014).

3.4. The Th differentiation: Th1 and Treg lymphocytes.

The Th differentiation depends on a strict program of signals that naïve CD4+ T-lymphocytes receive from the microenvironment, among other factors. These signals are cytokines, growth factors, and molecules released by DCs in the moment of the IS formation, which initiate downstream signaling cascades responsible of the Th fate. The most studied effector CD4+ T-lymphocytes, have been Th1, Th2, and Th17 phenotypes, controlling specific responses against different types of infections and autoimmune diseases (Nakayama T. & Yamashita M., 2010). The interleukin (IL)-12 is the main cytokine that promote Th1 differentiation inducing the expression of T-box transcription factor (T-bet). T-bet is the master regulator of Th differentiation, enhancing interferon gamma (IFNγ) production, and suppressing the development of Th2 and Th17 cells by the inhibition of GATA3 and RORγt respectively (**Figure 5**). Other important TFs in the regulation of Th1 development are STAT1, STAT4, Eomes, Runx3, and Hlx. (Luckheeram R.V. *et al*, 2012). Once T-cells are differentiated into Th1, they will carry out their effector function. Th1 lymphocytes are responsible of the defense against intracellular pathogens infections and of some autoimmune diseases. The majority of CD4+ T-cells that are generated in response to viral infections are Th1-type and produce large amounts of IFNγ and express T-bet, as vaccinia virus (VACV) infection (Matsui M. *et al*, 2005). In addition, Th1 cells are implicated in the regulation of bacterial infections and other intracellular pathogens as the protozoon *Leishmania major*, which strongly promotes a Th1 specific response (Biedermann T. *et al*, 2001). Furthermore, Th1 lymphocytes are also involved in some intestine inflammatory diseases as Crohn's Disease (Neurath M.F. *et al*, 2002).

Another type of CD4+ T-cells are the regulatory T-cells (Tregs), capable of regulating the proliferation or differentiation of other immune populations as DCs, CD8+ and CD4+ T-cells, in order to maintain the immune system homeostasis especially in inflammation contexts (Shevach E.M., 2011; Bin L. & Song Guo Z., 2015). Regarding CD4+ T-cells, Treg lymphocytes are specialized in the inhibition of exacerbated proliferation of Th1 and Th17 populations (**Figure 5**). Thus, Tregs are critical for autoimmune pathologies, allergies, chronic inflammation contexts, and allograft rejection (Joller, N. *et al*, 2014). The main specific marker of Tregs is the forkhead box P3 (Foxp3) TF, but also CD25 (IL-2R) surface receptor is commonly used to determine Treg phenotype (Walker L.S., 2004). Tregs can be categorized in two groups: natural Tregs (nTregs) and induced Tregs (iTregs). nTregs are physiologically produced in the thymus and require cell-to-cell interaction to suppress the proliferation and cytokine production of cell targets. However, iTregs are those generated in the periphery or in culture, which only need to release soluble factors to apply their immunosuppressive function. The best studied Treg subsets are: Treg 1 (Tr1), Th3, and CD4+ CD25+. Tr1 are CD4+ T-cells characterized by high IL-10 production, and precisely, they are generated by chronic activation in the presence of IL-10. Nonetheless, the Th3 Tregs are CD4+ and produce TGFβ, IL-4, and IL-10. They can be induced *in vitro* or *in vivo* after the exposure of naïve T-cells to TGFβ. CD4+ CD25+ are the most widely known Tregs

because of their role in autoimmunity protection (La Cava A., 2009; Joller N. *et al*, 2014). It has been demonstrated that the upregulation of this Treg population regarding percentage or cell functionality, is beneficial for the treatment of autoimmune diseases (Mottet C. *et al*, 2003). Nonetheless, the inhibition of their function or proliferation results also helpful for immunity against tumors and pathogenic microorganisms (Ward-Hartstonge K.A. & Kemp R.A., 2017). Thereby, a healthy organism is characterized by an immune homeostasis state, based on a correct balance between effector T-cells (Teff) and Tregs, being this balance the key of a proper immune response (Kondělková K. *et al*, 2010).

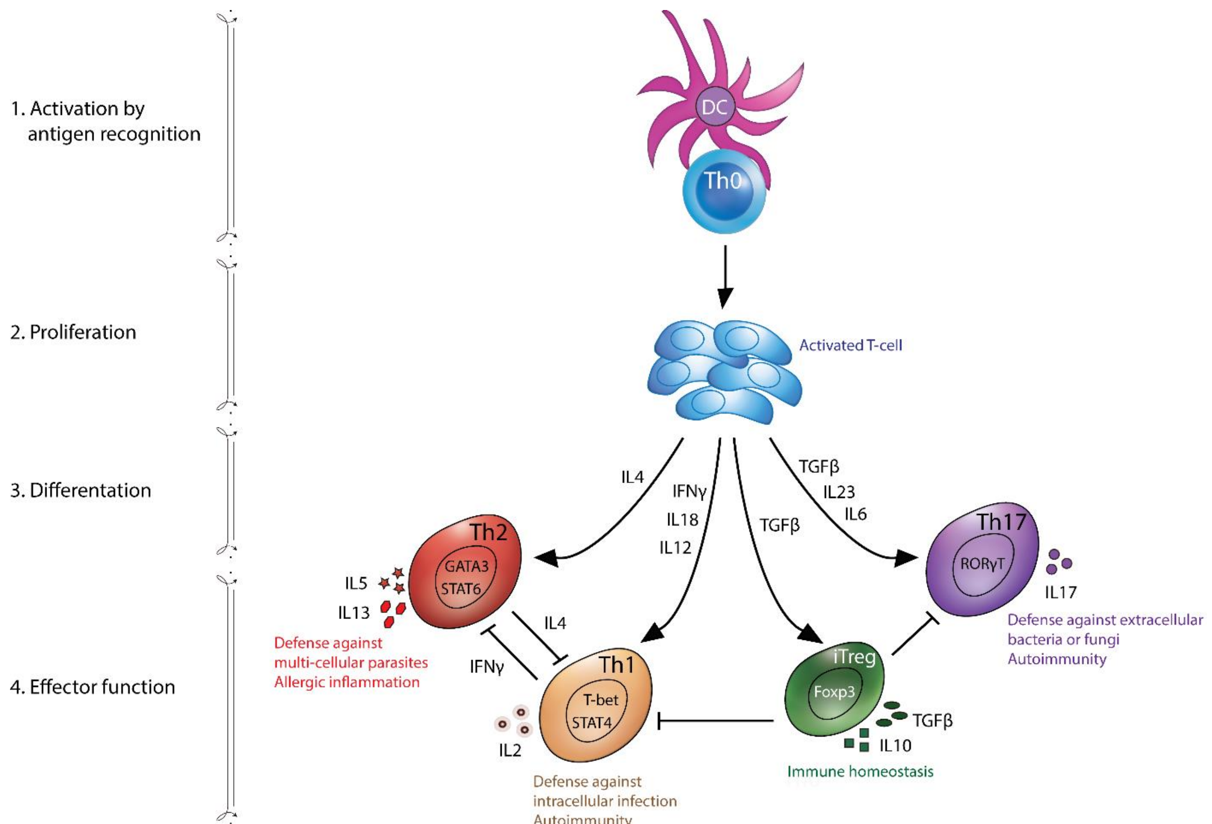

Figure 5. Phases of effector CD4⁺ T-cell response. To carry out their effector function, the CD4⁺ T-cells need to pass through a serial steps: 1) activation through antigen recognition, interacting with the APC; 2) clonal expansion or proliferation; 3) differentiation towards different Th phenotypes (Th1, Th2, Th17, iTreg) depending on the microenvironment signals that they receive; and finally 4) development of their specific function when it is needed in the organism. Th1, Th2, Th17 and iTregs are the main Th subsets. IFNγ, IL12, and IL18 induce T-bet and STAT4 expression leading to Th1 differentiation. Th1 cells produce IL2 and IFNγ to deal with intracellular pathogen infections. IL4 promotes GATA3 and STAT6 expression, which determines Th2 fate. Th2 cells secret IL5 and IL13 and protect from multi-cellular parasite infections and allergic inflammations. Th1 and Th2 inhibit each other releasing IFNγ and IL4 respectively. TGFβ, IL23, and IL6 induce Th17 differentiation and the master regulator gene of this process is RORγT. The function of Th17 cells is to defend against extracellular bacteria or fungi infections. They are also implicated in the response against autoimmune diseases as well as Th1. The master regulator of iTregs is Foxp3. TGFβ promotes Foxp3 expression and it is released by iTregs in addition to IL10. iTregs are responsible of immune homeostasis, being able to impede Th1 and Th17 proliferation.

3.5. Vaccinia virus and *Leishmania major* infections.

Two intracellular pathogens that specifically induce Th1 response are VACV and *Leishmania major*. VACV is a poxvirus that has been widely used in humans as a protective vaccine against smallpox. Thanks to it, nowadays the smallpox is an eradicated disease in humans, and this virus is only still used for research purposes (Ahmed C.M. *et al*, 2007). Tail scarification infection in mice has provided a useful model to study complications derived from VACV immunization, since it is used as human smallpox vaccination. VACV infection by tail scarification in C57BL/6 WT mice is localized and controlled by the host immune system, since Mota B.E.F and collaborators (2011) described no signs of weight loss and no viral load in internal organs. Nevertheless, the same model of infection in a T- and B-cell immunodeficient mice (*Rag1*$^{-/-}$) leads to a systemic disease, with a high dissemination of VACV that finishes with the mortality of 100% of the mice 12 days after the infection. CD4^{+} T-cell adoptive transfer extends lifespan by accelerating viral clearance at least in part through a mechanism mediated by Th1 cells (Mota B.E.F. *et al*, 2011). Therefore, the adaptive immunity in poxvirus infection in mice is a determining factor. Upon intraperitoneal (i.p.) infection a strong humoral response is needed to solve the infection, and CD8^{+} T-cell cytotoxic response is only necessary when the antibody response is abrogated (Bray M. & Wright M.E., 2003). It exists still controversy about which cell type is the main responsible of VACV viral clearance. Some authors attribute the key role to T-cells (Freyschmidt E.J. *et al*, 2007), and others to B-cells and antibodies (Worthington M., 2003; Fang M. & Sijal L.J, 2005). Other studies using localized poxvirus inoculation, corroborates that Th1 response is critical for the control of this infection (Xu R. *et al*, 2004). Tian T. and colleagues reported in 2009, that the *in vivo* overexpression of the Th1 cytokine IL-1α, leads to a higher control of VACV viral replication in comparison to WT mice.

Besides, *Leishmania major* is an obligated intracellular protozoon that also provokes a Th1 specific response (Biedermann T. *et al*, 2001). This parasite is transmitted by the bite of an infected female sand fly, causing a severe disease called leishmaniasis (Mougneau E. *et al*, 2011). The annual global incidence is still of 0.2–0.4 million cases for visceral leishmaniasis, and 0.7–1.2 million cases for cutaneous leishmaniasis (Okwor I. & Uzonna J., 2016). Most of the studies with *Leishmania* have been carried out in mice. C57BL/6 WT mice develop self-healing skin lesions at the place of parasite inoculation, while BALB/c mice cannot control parasite proliferation, and they finally die (Sacks D. & Noben-Trauth N., 2002). The experimental model that mimic the natural transmission is based in the inoculation of only 10–1000 parasites; however, supra-physiologic high doses have been widely used (2×10^{5} – 2×10^{7}). Independently of the inoculation dose, healing in cutaneous leishmaniasis is critically Th1 immune response dependent (von Stebut E. & Udey M.C., 2004). If Th1 immunity fails to control *Leishmania* infection, the disease progress until the death of the individual (Belkaid Y. *et al*, 2002). The main responsible cell-types against this serious infection are CD4^{+} Th1 cells, but also CD8^{+} Tc1 cells (Xu R. *et al*, 2004). Both T-cell populations produce high levels of IFNγ, which activates macrophages

and induces them to produce nitric oxide. In the end, nitric oxide facilitates the elimination of the intracellular amastigotes (Bogdan C. *et al*, 2000). Thus, it has been reported that BALB/c mice manifest a more pro-Th2 immune response, which could suppress Th1 function, and because of that they cannot resolve the infection (Sacks D. & Noben-Trauth N., 2002). Likewise, polarization of Th responses in human cutaneous leishmaniasis has been reported to be towards Th1 or Th2 depending on the patient (von Stebut E. & Udey M.C., 2004).

There is still no specific treatment for leishmaniasis, and the therapeutic drugs as rifampicin, dapsone, intraconazole, and amphotericin B, among others, produce significant toxicity and they are not completely effective (Faghihi G. & Tavakoli-kia R., 2003). In addition, leishmaniasis is spreading to several non-endemic areas of the world due to co-infections with HIV. It has been characterized over 20 species of this protozoon. For this reason, it has been developed several vaccines against this pathogen, but they do not protect completely against the disease (Okwor I. & Uzonna J., 2016). Therefore, more research is needed to find a cure of this major public health problem worldwide.

Conversely, as it has been mentioned before, an exacerbated Th1 differentiation can cause autoimmune diseases as multiple sclerosis, type I diabetes, and IBD, instead of being beneficial against infections. These excess of Th1 cells induce chronic inflammation and tissue damaging, common features of these autoimmune diseases (Dardalhon V. *et al*, 2008).

3.6. The inflammatory bowel disease.

The inflammatory bowel disease (IBD) is an autoimmune disease characterized by a chronic inflammation of the intestine. The inflammatory process is caused by an autoreactive immune system that attacks tissues of the digestive tract, causing lesions of different severity (Podolsky D.K., 1991). Within this group are included the ulcerative colitis and Crohn’s disease. Both of them comprises common features, but they have also some different symptomatic pathological patterns which suggests distinct underlying pathogenic mechanisms (Podolsky D.K., 1991). There have been described several risk factors that contribute to intestine inflammation. Environmental factors as pollution, changes in diet, exposure to chemicals, smoking and lifestyle, have been related with IBD development. As well, dietary habits and microbiota have been closely linked to this disease. Besides, family studies have demonstrated that genetic factors can predispose to IBD, changing for example, the intestine barrier permeability (Hanauer S.B., 2006). Furthermore, as other autoimmune diseases, the adaptive immune response has a main role in the pathogenesis of this disease. As previously mentioned, an exacerbated T-cell differentiation towards Th1, Th2 and Th17, promotes IBD development, while Treg lymphocytes regulate the inflammatory process inhibiting Th functions (Neurath M.F. *et al*, 2002; Izcue A. *et al*, 2006) (**Figure 6**). Th1 population is highly increased in Crohn’s Disease, while Th2 population is more abundant in ulcerative colitis. Th1 frequency can increase from a normal ~20% level to a highly increased level (~70% of intestinal $CD4^+$ T cells) in a severe Crohn’s Disease (Niessner M. & Volk B.A., 1995; Kosiewicz M.M. *et al*, 2001; Kang S.G. *et al*, 2007).

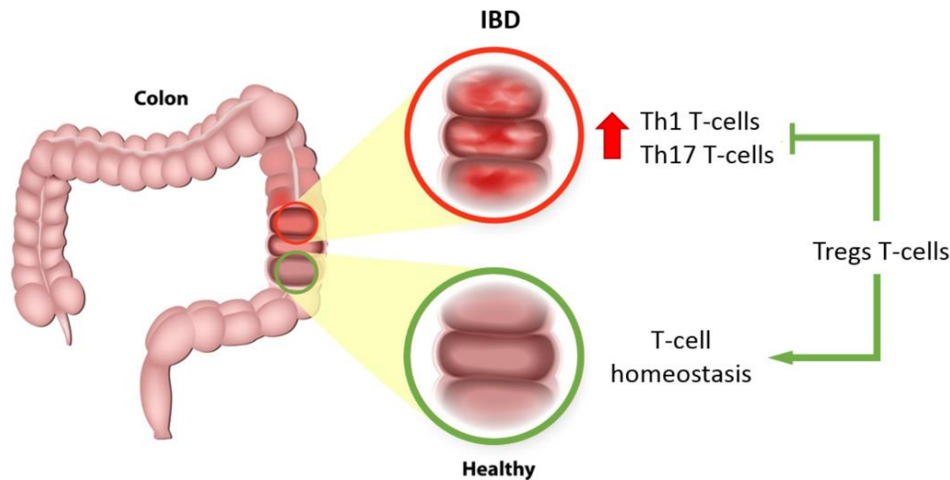

Figure 6. Adaptive immune response in Crohn's disease. The adaptive immune response has a main role in the development of IBD. Treg lymphocytes differentiation and functionality is extremely important to maintain the T-cell homeostasis in the gut. This is because Treg T-cells inhibits the exacerbated proliferation and differentiation of Th1 and Th17 in Crohn's Disease, thus protecting from inflammation (modified from www.hycultbiotech.com).

A well-stablished IBD mouse model that simulates human Crohn's disease consists in the adoptive transfer of CD45RB^{hi} CD4^{+} CD25^{-} T-cells into a T- and B-cell immunodeficient *Rag1*$^{-/-}$ (Morrissey P.J. *et al*, 1993; Powrie F. *et al*, 1993). In this model, because of the absence of Treg population inhibitory function in the colon, immune homeostasis breaks down and IFNγ- and TNFα-inflammatory responses, involving Th1 and Th17 cells among others, are favored mainly in the gut because of the high bacterial load. The intestinal microbiota induces a constant antigen-presentation process, which leads to a chronic intestine inflammation in the absence of Treg cells (Kiesler P. *et al*, 2015). Other organs as spleen or liver, characterized also by a high immune content and a constant antigen-presentation process, can develop, as well, a chronic inflammation (Izcue A. *et al*, 2006). Therefore, this model represents a systemic disease in which Treg, Th1 and Th17 are the main characters.

IBD pathologies have been related also with environmental factors, genetic susceptibility, and changes in the microbiota populations. Moreover, IBD has a high prevalence and incidence in young adults (20-39 years). It affects to 2.2 million people in Europe, while in Spain there are 181.4 cases per 100.000 habitants (Vanhove W. *et al*, 2016; Young C.E., 2016). Therefore, it is a global disease with an increasing number of diagnosed patients every year. Nevertheless, the current treatments are far from being completely effective. For this reason, this disease causes so much money expenditure in surgeries, hospitalizations and ineffective treatments (Ernst & Young, 2016). In addition, almost all of these treatments have serious side effects (De Souza H.S. *et al*, 2016; Kim D.H. *et al*, 2017); RA for example, has been reported to downregulate inflammatory responses shifting the Treg/Th17 profile in human and mice ulcerative colitis, however it produces several side effects (Bai A. *et al*, 2009; David M. *et al*,

1988) so it is necessary to discover new therapeutic targets that improve the quality of life and reduce the mortality of these patients.

3.7. Retinoic acid and immune homeostasis.

As it was mentioned before, RA (last metabolite of vitamin A pathway) controls lamin A/C transcription (Okumura K. *et al*, 2000), and in addition, it is known to be a key regulator of Th proliferation and differentiation (Olson I.L. *et al*, 1982; Ross A.C. *et al*, 2012; Reis B.S. *et al*, 2013). Moreover RA synthesis is dynamically controlled at inflammatory scenarios, where RA signaling on T-cells has been reported (Aoyama K. *et al*, 2013). The role of RA in immune homeostasis has been widely study in several immune populations (monocytes, macrophages, DCs, B-cells and T-cells), since vitamin A-deficient individuals show difficulties to control infectious diseases (Stephensen C.B., 2001). Investigators have described three major roles of RA in cell-mediated immunity: impairment in Th1 polarization by blocking IFNγ and IL-12 secretion, reduced APC function, and enhanced Th2 differentiation. These results are consistent with lower expression levels of T-bet and IL-12R, and higher expression levels of GATA-3 and IL-4R derived from RA effect. Additionally, RA also inhibits Th17 differentiation, producing a reduction in RORγT expression (Pino-Lagos K. *et al*, 2008). Furthermore, RA is not only a transcriptional regulator, recent studies have shown that RA-RAR drives epigenetic changes and chromatin remodeling in embryonic stem cells (Kashyap V. *et al*, 2013; Urvalek A.M. & Gudas L.J., 2014). Indeed, Brown C.C. *et al* published in 2015, that RA signaling through RARα activates Th1 enhancers and represses Th17-cell genes.

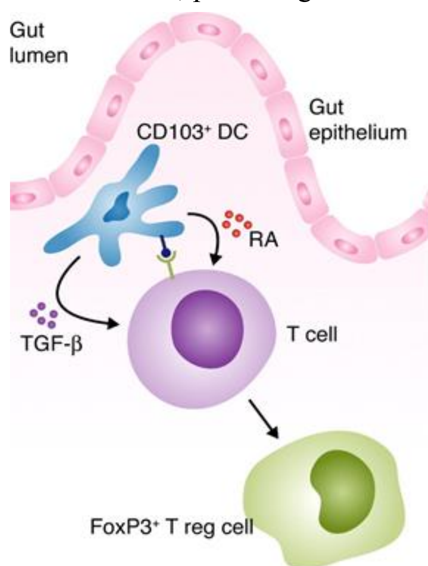

Figure 7. RA promotes Foxp3$^+$ Treg polarization in the gut. The specialized CD103$^+$ DC population produce RA and TGFβ in the gut. Because of that, the CD4$^+$ T-cells that recognize an antigen presented by this specific DC population are more prone to be differentiated into Foxp3$^+$ Treg (modified of von Boehmer H., 2007).

Additionally, several studies have demonstrated that RA remarkably increases Treg differentiation and improves their suppressor activity (Benson M.J. *et al*, 2007; Mucida D. *et al*, 2007; Schambach F. *et al*, 2007; Nolting J. *et al*, 2009). Thereby, RA greatly enhances Foxp3 expression in CD4$^+$ T-cells (Elias K.M. *et al*, 2008; Xiao S. *et al*, 2008) and IL-10 secretion (Kang B.Y. *et al*, 2000). Many of the studies about the role of RA on Tregs development are focused on the gut. This is because RA also enhances gut-homing markers expression as α4β7 and CCR9 on T-cells. Hence, RA-treated CD4$^+$ T-cells migrate preferentially to the gut in comparison with untreated CD4$^+$ T-cells (Iwata M. *et al*, 2004). In line with this, it has been previously reported that DCs from the gut, mesenteric lymph nodes (MLN) and Peyer's patches (PP), induce the same gut-homing markers on T-cells, while DCs from peripheral lymph nodes (PeLN) do not (Mora J.R. *et al*,

2003). Taking together all these results and the discovery that RA is released by DCs from the gut, MLN and PP, and not by DCs from PeLN or spleen (Iwata M. *et al*, 2004), it seems clear that RA has a main role in the Th phenotype and homing in the gut. Furthermore, the $CD103^{+}$ DC population is specifically responsible of this RA production in the gut, MLN and PP (**Figure 7**), whereas $CD103^{-}$ DCs do not produce RA. Thus, $CD103^{+}$ DCs are preferentially located in these intestinal regions, while $CD103^{-}$ DCs are more abundant in PeLN and spleen (Coombes J.L *et al*, 2007; Esterházy D. *et al*, 2016).

The fact that RA promotes not only Treg differentiation, but also Treg immunosuppressive function, determines that RA has been considered as a possible treatment for autoimmune diseases, and particularly, for gut autoimmune diseases as Crohn's disease (Pino-Lagos K. *et al*, 2008). RA treatment has been tested in mice colitis models, showing a great amelioration of the symptoms (Klopcic B. *et al*, 2008; Hong K. *et al*, 2014). In addition, retinoids have been widely used in the last decade to treat especially skin diseases. However, systemic administration of retinoids has been associated with mucocutaneous side effects, liver toxicity and irregularities of serum lipid profiles, which might be related to greater risk of suffering coronary heart disease (David M. *et al*, 1988). Therefore, it is needed to further investigate the RA signaling in order to develop new therapeutic drugs for Crohn's disease and other autoimmune diseases.

3.8. Epigenetics, T-cell differentiation, and A-type lamins.

As it was mentioned before, the polarization towards the different subtypes of T-cells depends on a strict program of gene expression regulated by various events, such as the microenvironment of cytokines at the time of antigen recognition (Nakayama T. & Yamashita M., 2010). Another significant element in T-cell differentiation commitment is the epigenetic regulation (Morinobu A. *et al*, 2004) which causes modifications in chromatin involving the inheritance of phenotypes without alterations in the DNA sequence (Hirahara K. *et al*, 2011). Several studies have demonstrated the relevance of these modifications in differentiation towards Th lineages (Koyanagi M. *et al*, 2005; Mukasa R. *et al*, 2010) (**Figure 8**).

The main structural unit of the chromatin is the nucleosome. So that, chromatin is a chain of nucleosomes connected by a linker DNA. Every nucleosome comprises two replicas of histones H2A, H2B, H3 and H4. N-terminal tails of these histone proteins can be covalently modified through acetylation, methylation and phosphorylation, which determines the status of the chromatin. Thus, histone modifications determine the condensed or relaxed chromatin status, which correlates with the repressed or active transcription, respectively. The most well-known histone modifications are: H3K4me3, H3K27me3, H3K27ac, H3K4me1, and H3K9me3. H3K4me3 is associated with active transcription regions in promoters, while H3K27me3 is related with repressed gene expression H3K27ac is a mark of active enhancers, as well as H3K4me1. H3K9me3 is always located in heterochromatin

permanent repressed regions. DNA methylation is another modification that limits the accessibility of transcription machinery, thereby affecting also the gene expression (Hirahara K. *et al*, 2011). These epigenetic processes decisively control Th cell identity. Thus, during Th differentiation one set of genes is going to be epigenetically activated while the others are going to be silenced (Ansel K.M. *et al*, 2003). Accordingly, it has been shown the existence of H3K4me3 permissive marks in proximal promoters of cytokine genes in their respective Th lineage (e.g. *Ifng* in Th1 cells), and H3K27me3 repressive marks in other subsets (e.g. *Ifng* in Th2 cells) (Wei G. *et* al, 2009). Therefore, chromatin remodeling by histone modifications is essential to promote Th phenotype stability (**Figure 8**).

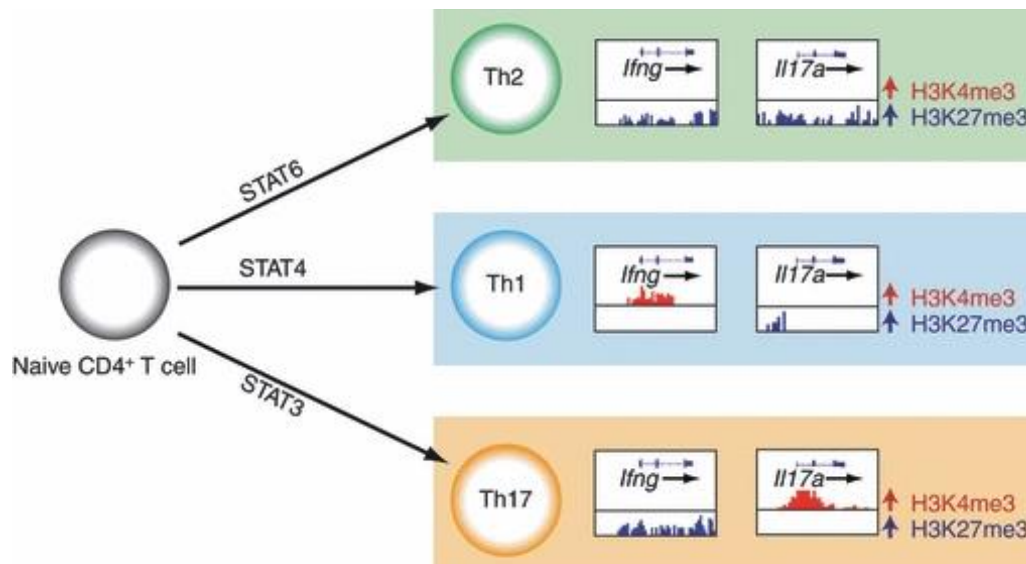

Figure 8. Epigenetic modifications in cytokine genes drive Th phenotype stability. TFs as STAT6, STAT4 and STAT3 are essential to promote Th2, Th1 and Th17 differentiation respectively, but also epigenetic modifications determine the Th fate. H3K4me3 active transcription mark is denoted in red, and H3K27me3 repressive modification is denoted in blue. H3K4me3 is highly present in the *Ifng* promoter in Th1 cells, while in Th2 and Th17 cells, the *Ifng* promoter only contains H3K27me3 modification. The same pattern is observed in the *Il-17a* locus; it contains H3K4me3 in Th17 cells, and H3K27me3 mark in the other Th subsets (Hirahara K. *et al*, 2011).

A-type lamins have a major role in chromatin organization. Lamin A/C is not only present in the nuclear lamina, it also localizes at the nucleoplasm forming oligomers that can cross-link chromatin directly binding the DNA or interacting through H2A/H2B histone proteins (Bronshtein I. *et al*, 2015). The chromatin interactions with A-type and B-type lamins have been called lamina-associated domains (LADs). LADs cover more than one –third of mouse and human genome in individual cell types, making LADs one of the most prominent features of the epigenome. In mammalian cells, there are cell-type invariant LADs (constitutive LADs), and others that are present only in certain cell types (facultative LADs) (van Steensel B. & Belmont A.S., 2017). LADs are generally gene poor and heterochromatic regions; however, LADs may contain both inactive and active genic regions (Oldenburg A.R. & Collas P., 2016). Thus, it has been described that lamin A/C also anchors euchromatin through lamina-

associated polypeptide 2 alpha (LAP2α) (Gesson K. *et al*, 2016). Therefore, lamin A/C is vital in chromatin structure maintenance, but it can also affect gene expression modulating epigenetics and chromosome positioning. For example, in cells derived from patients suffering diseases caused by *LMNA* mutations (e.g. fibroblast form HGPS patients), it has been observed a general loss of heterochromatin accompanied by epigenetic changes as an overall decrease in H3K27me3 (Dechat T. *et al*, 2009).

Moreover, lamin A/C interacts with Polycomb group (PcG) proteins, which are epigenetic repressors that control a wide number of genes during differentiation processes. Thereby, lamin A/C regulates PcG dynamics in the nucleus changing muscle cell differentiation pattern (Cesarini E. *et al*, 2015). Summarizing lamin A/C is a key regulator of epigenetic changes and cell differentiation (Oldenburg A., 2017), and epigenetic modifications are crucial in Th differentiation process (Morinobu A. *et al*, 2004). Nevertheless, it remains unknown if lamin A/C has a role in epigenetic changes related with Th fate commitment.

Objectives

4. OBJECTIVES

In vitro results from our laboratory have shown that lamin A/C is involved in naïve $CD4^+$ T-cell activation. We have thus postulated that lamin A/C might have a role in proliferation and polarization of naïve $CD4^+$ T-cells, and in the development of immune responses against infection and inflammation. To address our hypothesis, we have proposed the following objectives:

1) To assess the role of lamin A/C in $CD4^+$ T-cell development and generation.

2) To analyze the function of lamin A/C in naïve $CD4^+$ T-cell activation, proliferation, and differentiation towards Th effector lineages.

3) To study the relevance of lamin A/C function in the regulation of immune responses against infection and in inflammatory diseases.

4) To decipher the physiological mechanism that regulates lamin A/C expression in $CD4^+$ T-cells upon antigen-recognition.

5) To determine if lamin A/C produces epigenetic changes that compromise the Th phenotype fate.

Material & methods

5. MATERIAL AND METHODS

5.1. Mice.

Lmna$^{-/-}$ mice have been described previously (Sullivan T. *et al*, 1999). C57BL/6-Tg (TcraTcrb) 425Cbn/J mice (OTII mice) which express a TCR specific for the OVA peptide (amino acid residues 323 to 339) in the context of I-Ab, and CD4-CRE mice were obtained from the Jackson Laboratory (stock number 004194 and 017336, respectively). C57BL/6J 129S7-Rag1tm1Mom/J (*Rag1*$^{-/-}$) mice which lack B and T lymphocytes, were purchased as well from Jackson Laboratory. Female mice of 8 to 12 weeks of age were used. C57BL/6-CD45.2^{+} *Lmna*$^{-/-}$ OTII mice were generated by crossing C57BL/6-CD45.2^{+} OTII mice with C57BL/6-CD45.2^{+} *Lmna*$^{-/-}$ mice. C57BL/6-CD45.1^{+} CD45.2^{+} WT mice were generated by crossing C57BL/6-CD45.2^{+} mice with C57BL/6-CD45.1^{+} mice. C57BL/6-CD45.1^{+} and C57BL/6-CD45.1^{+} CD45.2^{+} WT mice were used as recipients for adoptive transfer. C57BL/6-CD45.1^{+} CD45.2^{+}/OTII WT mice were generated by crossing C57BL/6-CD45.2^{+} /OTII mice with C57BL/6-CD45.1^{+} /OTII mice. *Lmna*$^{flox/flox}$ mice were kindly provided by Y. Zheng (Kim Y. *et al*, 2013). C57BL/6 CD4-CRE- *Lmna*$^{flox/flox}$ mice were generated by crossing C57BL/6-CD4-CRE with C57BL/6-*Lmna*$^{flox/flox}$ mice. Foxp3-IRES-mRFP (FIR) mice were obtained from Jackson Laboratory (stock number 008374) and were crossed with the C57BL/6 CD4-CRE- *Lmna*$^{flox/flox}$ mice. C57BL/6 transgenic β-actin DsRed mice (ACTB-DsRed*MST) 1Nagy/J; Jackson Laboratory) was also used. All mice were bred in specific pathogen-free conditions at Centro Nacional de Investigaciones Cardiovasculares (CNIC).

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.

5.2. Antibodies.

Anti-lamin-A/C (n-18), anti-p-Erk1/2, anti-PCNA, and PE-conjugated anti-lamin A/C were obtained from Santa Cruz Biotechnology. Alexa Fluor 488-conjugated anti-lamin-A/C and Alexa Fluor 488-conjugated mouse immunoglobulin (Ig) G1 isotype control were obtained from Cell Signaling. Anti-CD3, anti-CD28, FITC-conjugated-CD45.1, PerCPCy5.5- conjugated anti-CD45.1, APC-conjugated anti-CD45.1, v450-conjugated anti-CD45.2, FITC-conjugated anti-CD45.2, PE-conjugated anti-IFNγ, APC-conjugated anti-IFNγ, V450-conjugated anti-CD4, APC-conjugated anti-CD25, V450-conjugated anti-CD45.1, and PerCPCy5.5-conjugated anti-CD45.2 were from Tonbo Bioscience. PercpCy5.5-conjugated anti-CD64, PE-conjugated anti-CD103, and FITC-conjugated anti-CD11c were from Biolegend. PECy5-conjugated anti-CD45.1, PECy7-conjugated anti-CD45.1, PE-conjugated anti-Tbet, FITC-conjugate anti-CD8, PE-conjugated anti-CD4, FITC-conjugated anti-Foxp3, FITC-conjugated anti-CD69, PE-conjugated anti-T-bet, FITC-conjugated anti-Ki67, PE-

conjugated anti-IL-4, and biotinylated antibodies against B220, CD19, MHCII, CD11c, CD11b, CD44, CD49b, IgM CD25, and CD8α, Alexa Fluor 488-conjugated anti-rabbit IgG were from BD Biosciences. H3K4me3, H3K4me1, H3K27me3, H3K27ac were from Diagenode.

5.3. T-cell isolation, activation and polarization.

$CD4^+$ $CD25^-$ T-cells from spleens were purified by negative selection on separation columns (Miltenyi Biotec) after labeling the cells with a cocktail of biotinylated antibodies against B220, CD19, MHCII, CD11c, CD11b, CD44, CD25, CD49b, IgM, and CD8α and a solution containing streptavidin-bound magnetic microbeads (Miltenyi Biotec). For polarizing experiments, $CD4^+$ $CD25^-$ T-cells from WT/OTII and *Lmna*$^{-/-}$/OTII mice were stimulated with irradiated autologous WT APCs previously incubated with OVA peptide (10 µg/mL) for 30 min at 37ºC. $CD4^+$ $CD25^-$ T-cells from WT and Lmna-/- mice were stimulated with plate-bound anti-CD3 (10 µg/mL) and soluble anti-CD28 antibodies (2 µg/mL).

Polarizing conditions were as follows: IL-12 (10 ng/mL), anti-IL-4 (4 µg/mL), and IL-2 (10 ng/mL) for Th1 polarization; IL-4 (10 ng/mL), anti-IFNγ (4 µg/mL), and IL-2 (10 ng/mL) for Th2 polarization; TGFβ (20 ng/mL) and IL-2 (10 ng/mL) for Treg polarization; anti-IFNγ (4 µg/mL), anti-IL-4 (4 µg/mL), TGFβ (20 ng/mL), IL-23 (10 ng/mL), IL-6 (20 ng/mL) for Th17 polarization. Antibodies and cytokines were from BD bioscience and Tonbo Bioscience, respectively. Cells were cultured in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), 2 mM ethylenediaminetetra-acetic acid (EDTA), 100 mg/mL penicillin, 100 mg/mL streptomycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 55 µM β-2-mercaptoethanol, 1 mM sodium pyruvate, and 2 mM L-glutamine for the indicated times.

5.4. Adoptive transfer.

Splenic $CD4^+$ $CD25^-$ T-cells from $CD45.2^+$ *Lmna*$^{-/-}$, $CD45.1^+$ $CD45.2^+$ WT, or $CD45.2^+$ WT mice were isolated by negative selection on MACS separation columns (Miltenyi Biotec) after labeling the cells with a cocktail of biotinylated antibodies against B220, CD19, MHCII, CD11c, CD11b, CD44, CD25, CD49b, IgM, and CD8α, as well as with a solution containing streptavidin-bound magnetic microbeads (Miltenyi Biotec). Adoptive transfer experiments were performed by inoculating $CD45.1^+$ or $CD45.1^+$ $CD45.2^+$ WT recipient mice with $1{\times}10^6$ isolated $CD4^+$ T-cells through the retro-orbital sinus. In some experiments, $CD45.1^+$ $CD45.2^+$ WT recipient mice were inoculated with a 1:1 mix of $2{\times}10^6$ $CD25^-CD4^+$ T-cells obtained from the spleens of $CD45.2^+$ *Lmna*$^{-/-}$ and $CD45.1^+$ WT mice. In VACV experiments, adoptive cell transfer was performed 24 hours before inoculation with the VACV (Iborra S. *et al*, 2016). Similar adoptive transfer was performed with $CD4^+$ T-cells isolated from $CD45.1^+$ $CD45.2^+$ WT/OTII, $CD45.1^+$ WT/OTII, or $CD45.2^+$ *Lmna*$^{-/-}$ mice.

5.5. Bone marrow-derived DCs generation.

Bone marrow-derived DCs (BMDCs) were generated as described in (Rocha-Perugini V. *et al*, 2017). BMDCs were obtained from bone marrow cell suspensions after culture on non-treated cell culture dishes in complete RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/mL penicillin, 100 mg/mL streptomycin, 55 μM β-2-mercaptoethanol, and 20 ng/mL GM-CSF (granulocyte macrophage colony-stimulating factor) (PeproTech, London, UK). Cells were collected at day 9 and BMDCs were isolated as $CD11c^{+}$ $MHC\text{-}II^{+}$ $Ly6G^{-}$ cells. Maturation was induced after overnight incubation with LPS from *Escherichia coli* O111:B4 (1 μg/mL; Sigma-Aldrich).

5.6. Bone marrow transplantation.

WT recipient mice received 13 Gy of total body irradiation administered in two treatments from a ^{137}Cs source. Bone marrow (BM) cells from $CD45.2^{+}$ WT or $CD45.2^{+}$ *Lmna*$^{-/-}$ mice were transplanted into $CD45.1^{+}$ WT recipients by injection into the retro-orbital sinus immediately after irradiation. A mix 1:1 of bone marrow cells from $CD45.1^{+}$ WT and $CD45.2^{+}$ *Lmna*$^{-/-}$ mice were transplanted into $CD45.1^{+}$ $CD45.2^{+}$ WT recipients by injection into the retro-orbital sinus immediately after irradiation. Approximately eight weeks after transplantation, the chimeric condition of the mice was assessed by flow cytometric analysis of blood cells stained with a combination of fluorescently labeled anti-CD45.1 and anti-CD45.2 antibodies to detect T-cells from donor and from recipient mice, which confirmed that more than 90% of the cells analyzed were derived from the transplanted BM cells.

5.7. Proliferation assays.

24 hours before the sacrifice of the mice, a single dose of 1 mg BrdU (5-bromo-2-deoxyuridine, BD Pharmingen) was injected i.p.. To assess BrdU incorporation, MLN, peritoneal exudate and spleen were stained for CD4, fixed and permeabilized using the BD BrdU Flow Kit (BD Pharmigen) according to the manufacturer's instructions. Cells were incubated at 37º C for 60 min in 30 µg of DNase, followed by staining with anti-BrdU-FITC for 40 min, washed and analyzed by flow cytometry. Peritoneal exudate and MLN cells were fixed and permeabilized using Foxp3 fixation/permeabilization buffer (BD Pharmingen) and stained for PCNA-FITC and Ki67-FITC.

5.8. Vaccinia virus *in vivo* infection and viral titration.

The VACV strain Western Reserve (WR, ATCC number VR-1354) was a gift from Jonathan W. Yewdell and Jack R. Bennink (NIH, Bethesda, Maryland, USA). VACV was propagated in BSC-40 cells and purified by sucrose gradient ultracentrifugation. WT or OVA-expressing VACV (1×10^{6} plaque-forming units) were administered by i.p. injection or intradermal (i.d.) injection in the footpad (Iborra S., *et al* 2016).

Animals were anesthetized with an i.p. injection of Ketamine (100 mg/kg body weight) and Xylazine (10 mg/kg) diluted in sterile phosphate buffered saline (PBS). Thirty 1 cm scarifications were made with a 26 G syringe along the base of the tail, avoiding bleeding (Melamed S. *et al*, 2007). Then 10 ml of

PBS containing 10^7 VACV plaque forming units (p.f.u.) were added to the area and allowed to air dry. For mock infection only PBS was added.

Animals were euthanized and the tail aseptically removed, weighed, frozen at -80ºC, and stored at -80ºC until use. The samples were homogenized with a Tissue Ruptor (QIAGEN, USA) in 0.5 ml DMEM containing 100 U/ml penicillin and 100 µg/ml streptomycin. The homogenates were sonicated for 3 min at 40% amplitude, freeze-thawed twice (-80ºC/37ºC), sonicated again under the same conditions, and then serially diluted in DMEM without FBS. To quantify p.f.u., the dilutions were added to monolayers of CV-1 cells seeded on 24-well plates. The cells were preincubated for 1 day at 37ºC and 5% CO_2, the dilutions (0.2 ml) were added to each well, and the cells were further incubated under the same conditions for 1 h. After this, 0.5 ml DMEM containing 0.5% FBS were added to each well. After 24 h, the cells were stained for 5 min with crystal violet solution (0.5% crystal violet, 10% ethanol, and 1% paraformaldehyde) and washed again. Viral plaques were counted and plaque number was multiplied by the reciprocal of sample dilution and converted to p.f.u/g (Mota B.E. *et al*, 2011).

5.9. *Leishmania major in vivo* infection.

In vivo experiments were carried out using *L. major* Friedlin strain FV1 (MHOM/IL/80/Friedlin), generously provided by Dr. D. Sacks (NIH). For *Leishmania* challenge, parasites were kept in a virulent state by passage in mice. Parasites were cultured and differentiated as described Iborra S. and collaborators (2005). Mice were infected by i.d. injection of 10^3 or 5×10^4 metacyclic *L. major* promastigotes into the dermis of both ears. Lesion size in the ear was determined with digital callipers (Duratool) (Iborra S. *et al*, 2011). The limiting dilution assay was used to determine parasite number (Iborra S. *et al*, 2005). Parasite load was expressed as the number of parasites in the whole organ.

5.10. Inflammatory bowel disease mouse model.

Intestine inflammation was induced by the inoculation of, at least, 5×10^5 of $CD4^+CD25^-$ T-cells from CD4-CRE$^{+tg/wt}$- *Lmna*$^{flox/flox}$ and CD4-CRE$^{wt/wt}$- *Lmna*$^{flox/flox}$ mice into a T- and B-cell immunodeficient *Rag1*$^{-/-}$ mice through the retro-orbital sinus (Powrie F. *et al*, 1993; Morrissey P.J. *et al*, 1993). Mice were monitored during 8 weeks to assess colitis development. Body weight and symptomatology was checked twice a week. Disease severity was considered according to a clinical score defined as follows:

- Weightloss: 0 (no loss), 1 (1–5%), 2 (5–10%), 3 (10–20%), and 4 (>20%).
- Stool consistency: 0 (normal), 2 (loose stool), and 4 (diarrhea).
- Rectal prolapse: 0 (absent), 2 (low), and 4 (pronounced).
- Rectal bleeding: 0 (absent), and 4 (present).
- Spine curvature: 0 (absent), 2 (low), and 4 (pronounced).

5.11. Histopathological analysis.

After 8 weeks of IBD development, mouse colons were collected and fixed in 4 % paraformaldehyde for 48 h, and transferred to 70% ethanol. Colons were cut into six portions of 5 mm approximately. Three of these portions, a proximal one, a distal one, and one from the medial part, were dehydrated to xylene, and embedded in paraffin. The leftover portions were used for mRNA expression analysis. Transverse colon sections (5 µm) were cut and stained with hematoxylin-eosin to evaluate inflammation severity. Images were obtained with an Olympus BX41 microscope. Two images of serial sections cut with a 100-µm separation, were evaluated for each part of the colon (six images per mouse). Colitis severity was assessed taking into account the following features: leukocyte infiltration, goblet cell depletion, epithelial hyperplasia, crypt damage, and submucosal inflammation, with a score from 0 to 4. Ulceration was also considered with a score defined as: 0 (absent), 2 (present), and 4 (prominent). Scores were analyzed by two blinded observers, and they were summed to give a final histological score for each parameter.

5.12. *In vivo* cytotoxicity assays.

Splenic CD45.2$^+$ WT or CD45.2$^+$ *Lmna*$^{-/-}$ CD4+/OTII T-cells were isolated as described in the adoptive transfer section. Recipient *Rag1*$^{-/-}$ mice were inoculated intravenously with 2×10^6 of either naïve CD45.2/WT or CD45.2/*Lmna*$^{-/-}$ CD4+/OTII T-cells and infected with VACV-OVA i.p. After 5 days, splenocytes from CD45.2/WT/dsRED and CD45.1/WT mice were isolated and loaded or not with OVA-OTII peptide, respectively. An approximately 1:1 mix of 5×10^6 CD45.2/WT/dsRED and CD45.1/WT splenocytes was inoculated intravenously in the recipient *Rag1*$^{-/-}$ mice, which were previously inoculated and vaccinia infected. After 16 h, animals were analyzed to determine the killing capacity of CD4/OTII cells in the spleen by flow cytometry. Killing capacity was determined as the ratio between CD45.2/WT/dsRED/+OVA and CD45.1/WT/-OVA cells and the percentage of CD4/OTII T-cells. Adoptively transferred mice with only CD4/OTII cells, and transferred mice with splenocytes in the absence of CD4/OTII cells were used as controls.

5.13. *In vitro* Treg suppression assays.

CD4$^+$ CD25$^-$ T-cells were isolated from CD4-CRE$^{+tg/wt}$-*Lmna*$^{flox/flox}$ and CD4-CRE$^{wt/wt}$-*Lmna*$^{flox/flox}$ mice spleens and polarized towards Treg phenotype as it is indicated in the 5.3 section. After 6 days of *in vitro* differentiation, CD4$^+$ CD25$^-$ T-cells were isolated from CD45.1/WT mice spleens and Treg polarization was checked by flow cytometry. *In vitro* generated Tregs were stained with FITC-conjugated anti-CD4, APC-conjugated anti-CD25, and 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (ThermoFisher) and selected by fluorescence-activated cell sorting (FACS). CD45.1/WT CD4$^+$ CD25$^-$ T-cells were stained with CellTrace Violet (ThermoFisher) following manufacturer's instructions. Then, the Treg:Tnaïve co-culture was performed in serial ratios (1:2, 1:4, 1:8, 1:16, 1:32, 0:1) in the presence of soluble anti-CD3 (10 µg/mL) and soluble anti-CD28 antibodies (2 µg/mL).

5.14. Retinoic acid treatment *in vitro* and *in vivo*.

RA powder (Sigma-Aldrich) was diluted in Dimethyl Sulfoxide (DMSO) and added to *in vitro* cultures at day 0 in a final concentration of 10 nM. After 48 h of incubation, RA was washed. In parallel, non-treated cells was cultured under the same conditions adding instead DMSO.

Mice IBD induced were i.p. injected with 30 µg/mL dose of RA diluted in Corn Oil (Sigma-Aldrich) every day during the first two weeks of the disease mouse model.

5.15. Colon lymphocytes isolation.

Colons were extracted from IBD induced mice, and washed with HBSS buffer (ThermoFisher) supplemented with 100 mg/mL penicillin and 100 mg/mL streptomycin. Then, colons were longitudinally cut and included in HBSS with 100 mg/mL penicillin and 100 mg/mL streptomycin, and 5mM 1,4-Dithiothreitol (DTT) (Sigma-Aldrich), and incubated during 20 min at room temperature (RT) to destroy the intestine mucus. After, colons were washed and incubated again with HBSS and 2mM EDTA during 20 min at 37ºC to detach the intestinal epithelium. Then, colons were washed again, cut into small pieces, and included in HBSS supplemented with 100 mg/mL penicillin, 100 mg/mL streptomycin, 1% IV Collagenase (Roche), and 10 mM DNase I during 30 min at 37ºC (Sigma-Aldrich). Colons were filtered through a 70-µm cell strainer, and the recovery was centrifuged at 1500 rpm for 5 min at 4 °C to obtain a cell pellet.

5.16. DCs and $CD4^+$ T-cells *in vitro* co-cultures.

MLN and PeLN were isolated from CD45.2/WT mice. Then MLN and PeLN were cut into small pieces and incubated during 10 min at 37ºC in HBSS with 1% Liberase TL (Roche) and1000 U/Ml of DNase I. After the incubation, the cell suspension was filtered through a 70-µm cell strainer, and centrifuged at 1800 rpm during 5 min at 4ºC. The cell pellet was stained with the following antibodies and reagents: FITC conjugated anti-CD11c, PercpCy5.5 conjugated anti-CD64, PE conjugated anti-CD103 and DAPI. Two DCs populations were selected by FACS: CD11c $CD64^-$ $CD103^+$ and CD11c $CD64^-$ $CD103^-$. Naïve $CD4^+$ T-cells were isolated as is indicated in 5.3 section from CD45.1/OTII/WT mice spleens. At that point, co-cultures of DCs:T naïve were carried out with a 1:5 ratio respectively.

5.17. Chromatin immunoprecipitation- quantitative PCR.

$CD4^+$ $CD25^-$ T-cells were isolated and incubated *in vitro* as is indicated in the 5.3 section. After 48 hours of *in vitro* activation, alive $CD4^+$ T-cells were selected by FACS. Then, cell pellets were crosslinked following the protocol previously described by Oldenburg A.R. and Collas P. (2016), and snap frozen and preserved at -80ºC. Non-crosslinked cell pellets of each sample were also collected to develop reverse transcription (RT) - quantitative polymerase chain reactions (qPCR) as a control. When all the needed samples were collected, chromatin immunoprecipitation (ChIP)- qPCRs were carried out with crosslinked pellets following the next steps of the protocol (Oldenburg A.R. & Collas, 2016).

The conditions for amplification were: denaturation at 95°C for 3 min [95°C for 10 s, annealing at 60°C for 15 s, extension at 72°C for 20 s] (40 cycles), 95°C for 1 min. The primers were designed with the NCBI Primer-designing tool, and gene sequences were obtained from NCBI database. Promoter regions of the studied genes were selected from the transcription starting point (TSS) backwards until the 5' end of the gene sequence (**Figure 9**). The sequences were obtained from UCSC Genome Browser database. RT-qPCR was developed as is described in the next 5.18 section, with non-crosslinked pellets. Primers used for ChIP-qPCR are listed below in the Table 1. The expression of each studied gene was analyzed by the comparative Ct method with Biogazelle qBasePLUS software using the housekeeping

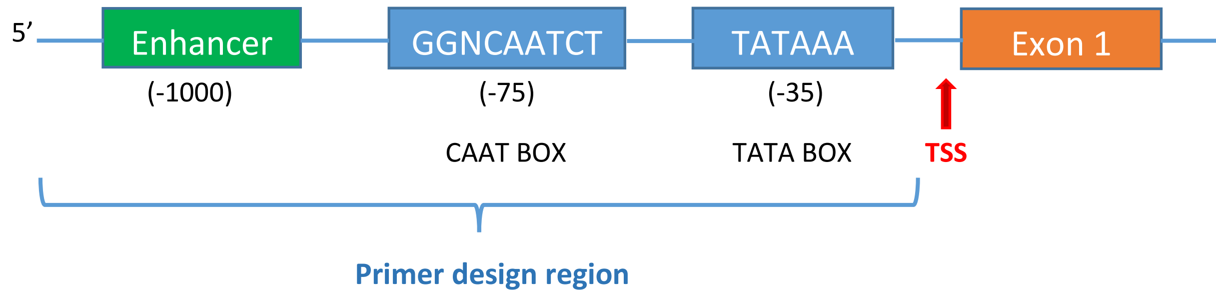

Figure 9. Schematic representation of the promoter region of a eukaryotic gene. The TSS (indicated with an arrow) is the point in which the transcription of a gene starts. Upstream of the TSS, and closed to it, is where is located the TATA box, in the -35 pb position (approximately). The TATA box is the TATAAA sequence, where TATA-binding proteins bind the DNA helping the subsequent binding of the RNA polymerase. The promoter region of eukaryotic genes are very complex and have other components, as the CAAT box located in the -75 pb position (approximately), and regulatory elements as enhancers that can be around the -1000 pb position. The promoter region used for the designing of primers for ChIP-qPCR is indicated.

Gene	5'-3' Forward sequence	5'-3' Reverse sequence
***Cd69* promoter**	TCAGACAGCAGGGAAAACCC	AATTTATGCTCCAGCGGCCT
***Cd25* promoter**	CTGCCAAGAAGTGCTTGCTC	TGGGCAGTGTTTGGTGAGAA
***Il-2* promoter**	ATGCTTTCTGCCACACAGGT	CTCTTCAGCATGGGAGGCAA
***T-bet* promoter**	CCGTAGTTATTGGAATAGAACAGC	ATACCAACCGGTGTCTGTGT
***Foxp3* promoter**	CCCAGGAGGCCATTAACAGG	TTTGGCCCCATGCTATGGTT
***Gata3* promoter**	AGAAATCTCAAGCGCTGCCT	CCGGCTAACAAGAAGCTGGA
***Rorγt* promoter**	AGCTGCTTGGCTCAGCATAA	TTGCCTGCGTCATTCTGACT
***Ube2b* promoter**	TCACGTGACCTGCTCTGATG	GCATCCGGAGCCTGAGATTT
***Gapdh* promoter**	GTTGCACTGGCCTAGCAAAG	GACCGGGATTCTTCACTCCG

Table 1. List of the primers used for ChIP-qPCR technique.

genes *Ube2b* (Ubiquitin Conjugating Enzyme E2 B) and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) as internal controls. Results are represented as fold change relative to control conditions.

5.18. Reverse transcription-quantitative PCR.

Total RNA was isolated with Qiazol Lysis Reagent (Qiagen) and isopropanol precipitation or with the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA concentration and purity were assessed from the ratio of absorbance at 260 and 280 nm. Complementary DNA (cDNA) was synthesized from total RNA (0.1 to 1 µg per reaction) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers and RNase Inhibitor, according to the manufacturer's protocol. Quantitative PCR was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) usign the PCR Power SYBR Green PCR Master Mix (Applied Biosystems), with technical triplicates. The conditions for amplification were as follows: denaturation at 95°C for 10 min [95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 40 s] (40 cycles), and 95°C for 1 min, followed by dissociation curve analysis. Primer sequences are listed in Table 2. The results were analyzed as is indicated in the previous section 5.16 using as internal control the housekeeping genes *Hprt1* (hypoxanthine phosphoribosyl transferase 1) and *Gapdh*. Results are represented as fold change relative to control conditions.

Gene	5'-3' Forward sequence	5'-3' Reverse sequence
Cd69	ATCTCTCCGTGGACCACTTG	CACAGCCCAAGGGATAGAAA
Cd25	AACAACTGCAATGACGGAGACAT	CAGCTGGCCACTGCTACCTT
Il2	TTCAATTGGAAGATGCTGAGA	ATCATCGAATTGGCACTCAA
Tbet	GAAAGGCAGAAGGCAGCAT	GAGCTTTAGCTTCCCAAATGAA
Foxp3	CTCGTCTGAAGGCAGAGTCA	TGGCAGAGAGGTATTGAGGG
Eomes	GCCTACCAAAACACGGATA	TCTGTTGGGGTGAGAGGAG
Ifnγ	TGGCTCTGCAGGATTTTCATG	TCAAGTGGCATAGATGTGGAAGAA
Il12	CTGCTTGACTCTGACATCT	CCACTGCTGACTAGAACTC
Tgfβ	ACCATGCCAACTTCTGTCTG	CGGGTTGTGTTGGTTGTAGA
Il35	CTTACAGGCTCGGTGTGGC	GTGACATTTAGCATGTAGGGCA
Il10	CCCTTTGCTATGGTGTCCTT	TGGTTTCTCTTCCCAAGACC
Il17a	AGAATTCATGTGGTGGTCCAG	ACTACCTCAACCGTTCCACG

Rorγt	GGTGACCAGCTACCAGAGGA	AGCTCCATGAAGCCTGAAAG
Il23	CAACTTCACACCTCCCTAC	CCACTGCTGACTAGAACT
Il6	GACAAAGCCAGAGTC CTTCAGAGA	CTAGGTTTGCCGAGTAGATCTC
Il22	CCGAGGAGTCAGTGCTAAGG	CATGTAGGGCTGGAACCTGT
Lmna	TGAGTACAACCTGCGCTCAC	TGACTAGGTTGTCCCCGAAG
Lmnb1	CAACTGACCTCATCTGGAAGAAC	TAAGACTGTGCTTCTCTGAGC
Hprt1	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Gapdh	CTACACTGAGGACCAGGTTGTC	GGTCTGGGATGGAAATTGTG
Gzma	GACTGCTGCCCACTGTAACG	TCAATATCTGTTGTTCTGGCTCCTTA
Gzmb	TGTCTCTGGCCTCCAGGACAA	CTCAGGCTGCTGATCCTTGATCGA
Prf1	GCGTCTCCAGTGAATACAAAG	TACTTCGACGTGACGCT
Blimp1	ACACACAGGAGAGAAGCCACATGA	TCGAAGGTGGGTCTTGAGATTGCT
Icos	GCTCGGCCGATCATAGGATGT	CCTCCACTAAGGTTCCTTTCTTG
Gitr	CAAGGTTCAGAACGGAAGT	GAAGATGACAGTCAAATG
Ctla4	GCTTCCTAGATTACCCCTTCTGC	AGGTGCCCGTGCAGATGGAA
Pd1	CGTCCCTCAGTCAAGAGGAG	GTCCCTAGAAGTGCCCAACA
Cd49b	CCGGGTGCTACAAAAGTCAT	GTCGGCCACATTGAAAAAGT
Lag3	TCACTGTTCTGGGTCTGGAG	GGTAAAGTCGCCATTGTCTC
Rarα	GGCGAACTCCACAGTCTTAATG	GCTGGGCAAGTACACTACGAAC
Rarβ	GGGGTATACCTGGTACAAATTCTGA	CAGCTGGGTAAATACACCACGAA

Table 2. List of the primers used for RT-qPCR technique.

5.19. Flow cytometry.

$CD4^+$ T-cells were stimulated with PMA (20 ng/ml) plus ionomycin (1µg/ml) for 6 hours. Brefeldin A (Sigma-Aldrich) was added for the last two hours to allow intracellular cytokine accumulation. Surface antigens were stained with antibodies, then fixed and made permeable with either a Cytofix/

Cytoperm kit (BD Biosciences), and then intracellular cytokines and transcription factors in cells were stained. For Foxp3 staining Mouse Foxp3 Buffer set (BD Biosciences) was used. For lamin A/C intranuclear staining 0.5% Triton X-100 permeabilization was used during 5 min. The antibodies were used following concentrations suggestions of manufacturers. Data were acquired on FACSCantoII or LSRFortessa flow cytometers (BD Biosciences), and analyzed with BD FACSDIVA (BD Biosciences) or FlowJo (Treestar Inc.) software.

5.20. Immunofluorescence and microscopy.

$CD4^+$ T-cells were plated onto Poly-L-Lys-coated slides (50 µg/ml; Sigma-Aldrich) during 1 h at 37ºC. Next, samples were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100/PBS solution during 5 min at RT. Alexa488 conjugated anti-mouse lamin A/C (1:100, Cell Signaling), and DAPI (1:500, ThermoFisher) were incubated during 1 hour at RT.

Samples were mounted in Fluoromount-G imaging medium, and immunofluorescence images were acquired with a Zeiss LSM700 confocal microscope (Plan-Apochromat 63×1.4, 40×1.3, 25×1.2 oil objective; Carl Zeiss, Oberkochen,Germany). Images were analyzed using ImageJ Fiji software and Imaris software (Bitplane) by an observer blinded to genotype.

5.21. Retroviral production and transfection.

Plasmids pBABE-puro-GFP-wt-lamin A and pBABE-GFP were obtained from Addgene, and Phoenix-Eco retrovirus producer cell line were kindly provided by Dr. Daniel Martín Pérez (Viral Vector Unit of CNIC). The day before plasmid transfection, Phoenix-Eco cells were grown in DMEM medium (GIBCO) supplemented with 10% FBS, 100 mg/mL penicillin, and 100 mg/mL streptomycin (complete DMEM). The next morning, Phoenix-Eco cells were passed 1:5 adding 20 mL of complete DMEM to each p150 plate. After 5-6 h of incubation at 37ºC and 5% CO_2, 36.25 µg of each plasmid was added separately to 75 µl of $ClCa_2$ 2.5 M and 1250 µl of HBS (Hepes-Buffered Saline) buffer 1X (pH 7.1). The mix was vortexed 5 sec at 1600-1800 rpm, and incubated 20 min at RT. Next, each mix was added slowly to the cells. After 16-18 h of incubation at 37ºC and 5% CO_2, the cell medium was changed to 12.5 mL of fresh complete DMEM and cells were incubated again at 37ºC and 5% CO_2 for 48 h. Then, supernatants containing the retroviruses of interest were used to infect $CD4^+$ T-cells, previously activated with anti-CD3/anti-CD28 for 48 h. Activated T-lymphocytes were resuspended in each supernatant in a concentration of 1×10^6 of T-cells/mL, adding 6 µg/ml of Polybrene transfection reagent (Merck), and centrifuging 90 min at 2200 rpm and RT. Then, T-lymphocytes were washed and resuspended in fresh RPMI medium.

5.22. Quantitative proteomic analysis.

$CD4^+$ T-cells were extracted and *in vitro* incubated during 48 hours as is described on 5.3 section. The same FACS selection described before in 5.17 section was developed. For proteomics analysis,

samples were treated with 50 mM Tris-HCl – 2% SDS – 10 mM TCEP (Tris-2-carboxyethyl) phosphine hydrochloride, Pierce) for protein extraction. Protein concentration was determined by using a Direct Detect IR spectrometer (Millipore). For the quantitative differential analysis by LC-MS/MS by using isobaric tags (TMT 10-plex), about 100 µg of total proteins were digested. Samples were denatured by adding 20% SDS to 0.2% SDS and 500 mM iodoacetamide (IAA) to 50 mM IAA and boiling 5 min. After incubation at the dark for 30 min at RT, samples were diluted in 7 M urea in 0.1 M Tris-HCl (pH8.5) (UA), and loaded onto 10 kDa centrifugal filter devices (NanoSep 10k Omega, Pall Life Sciences). The buffer was replaced by washing filters with UA, and proteins were then reduced with 10 mM TCEP for 30 min, washed with Hepes buffer 50mM and alkylated using 50 mM MMTS (methyl methanethiosulfonate, Pierce) in UA for 20 min in the dark. The excess of alkylating reagent was eliminated by washing three times with UA and three additional times with 50 mM ammonium bicarbonate. Proteins were digested overnight at 37ºC with modified trypsin (Promega) in 50 mM ammonium bicarbonate at 30:1 protein:trypsin (w/w) ratio. The resulting peptides were eluted by centrifugation with 50 mM ammonium bicarbonate (twice) and 0.5 M sodium chloride. Trifluoroacetic acid (TFA) was added to a final concentration of 1% and the peptides were desalted onto C18 Oasis-HLB cartridges and dried-down for further analysis. For stable isobaric labelling, the resulting tryptic peptides were dissolved in 100 mM Triethylammonium bicarbonate (TEAB) buffer, and the peptide concentration was determined by measuring amide bonds with the Direct Detect system (Millipore). Equal amounts of each peptide sample were labelled using the 10-plex TMT Reagents (Thermo Fisher) according to manufacturer's protocol. Peptides were labelled with TMT reagents previously reconstituted with 70 µl of acetonitrile, and after incubation at RT for 2h, reaction was stopped with 0.5% TFA, incubated for 30 min, and peptides were combined. Samples were concentrated in a Speed Vac, desalted onto C18 Oasis-HLB cartridges and dried-down for further analysis. For increasing proteome coverage, TMT-labeled samples were fractionated by high-pH reverse phase chromatography (High pH Reversed-Phase Peptide Fractionation Kit, Pierce) and concentrated as before.

Labelled peptides were analyzed by LC-MS/MS using a C-18 reversed phase nano-column (75 µm I.D. x 50 cm, 2 µm particle size, Acclaim PepMap RSLC, 100 C18; Thermo Fisher Scientific) in a continuous acetonitrile gradient consisting of 0-30% B in 360 min, 50-90% B in 3 min (A= 0.1% formic acid; B=90% acetonitrile, 0.1% formic acid). A flow rate of 200 nL/min was used to elute peptides from the nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on an Orbitrap Fusion mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution=70,000) followed by the MS/MS spectra from the Nth most intense parent ions were analyzed along the chromatographic run. Dynamic exclusion was set at 40s. For peptide identification, all spectra were analyzed with Proteome Discoverer (version 2.1.0.81, Thermo Fisher Scientific) using SEQUEST-HT (Thermo Fisher Scientific). For database searching at the Uniprot database containing all sequences from human and contaminants (may 14, 2016; 70611 entries), the parameters were selected

as follows: trypsin digestion with 2 maximum missed cleavage sites, precursor and fragment mass tolerances of 2 Da and 0.02 Da, respectively, TMT modifications at N-terminal and Lys residues as fixed modifications, and methionine oxidation, carbamidomethyl cysteine and MMTS modified-cysteine as dynamic modification. Peptide identification was performed using the probability ratio method and false discovery rate (FDR) was calculated using inverted databases, and the refined method with an additional filtering for precursor mass tolerance of 15 ppm. Identified peptides had a FDR equal or lower than 1% FDR. Only those peptides were used to quantify the relative abundance of each protein from reporter ion intensities, and statistical analysis of quantitative data were performed using the WSPP statistical model previously described. In this model proteilog2-ratios are expressed as standardized variables, in units of standard deviation according to their estimated variances (Zq values).

Functional protein analysis of the whole set of quantified proteins was performed using a novel algorithm, system biology triangle (SBT) developed specifically for the analysis of coordinated protein responses in high-throughput quantitative proteomics experiments. This algorithm correlates the performance of a group of proteins inside of a category (biological process) in terms of their quantitative behavior (relative abundance); thus, changes can be detected in functional biological processes far beyond individual protein responses. As a result of this coordinated behavior, a Z value is assigned to each category. To identify the significant biological process altered in disease progressions, groups were compared to the control group applying p< 0.01.Variations in the abundance of annotated functional categories were visualized by comparing the cumulative frequency (sigmoid) plots of the standardized variable with that of the normal distribution, as in previous works. Individual protein changes were also considered for further analysis.

5.23. Statistical analysis.

Statistical analyses were performed with Prism GraphPad or Microsoft Office Excel. Unless otherwise stated, statistical significance was calculated by two-tailed Student's t-test. When specified, one-way ANOVA or two-way ANOVA with Bonferroni's post-hoc multiple comparison test was used. Significance of differences was calculated as follows: *P<0.05, **P<0.01, ***P<0.001, and **** P<0.0001.

Results

6. RESULTS

6.1. Analysis of lamin A/C deficiency effect in CD4$^+$ T-cell development.

CD4-CRE$^{tg/wt}$- *Lmna*$^{flox/flox}$ (CD4-*Lmna*$^{-/-}$ from now on) was generated in order to knockdown lamin A/C specifically in CD4$^+$ T-cells. Lamin A/C expression levels were analyzed by immunofluorescence at 48 hours (h) in *in vitro* activated CD4$^+$ T-cells with anti-CD3/CD28 antibodies (**Figure 10A**). Results showed a significant decrease of lamin A/C expression in CD4$^+$ T-cells from CD4-*Lmna*$^{-/-}$ mice in comparison to CD4$^+$ T-cells from CD4-CRE$^{wt/wt}$- *Lmna*$^{flox/flox}$ (WT from now on) mice. Mean fluorescence intensity (MFI) was also analyzed by flow cytometry (**Figure 10B**) showing as well a significant decrease of lamin A/C expression per cell in *Lmna*$^{-/-}$ mice. However, lamin A/C knockdown in CD4$^+$ T-cells was not complete, and we observed some lamin A/C$^+$ cells coming from *Lmna*$^{-/-}$ mice, particularly in flow cytometry analysis (**Figure 10B**).

CD4-*Lmna*$^{-/-}$ mice showed the same physical appearance and body weight as WT mice (**Figure 11A**). Additionally, organs of the immune system as spleen and thymus present the same size, weight and morphology in CD4-*Lmna*$^{-/-}$compared with WT mice (**Figure 11B**). Moreover, basal T-cell populations were analyzed in thymus, spleen and lymph nodes (LN) in CD4-*Lmna*$^{-/-}$ and WT mice in order to corroborate that lamin A/C downregulation does not affect T-cell development and selection in thymus. No differences were observed in percentages of CD4$^+$ CD8$^-$, CD4$^-$ CD8$^+$, and CD4$^+$CD8$^+$ populations in any of the organs that were analyzed (**Figure 12**).

Not all the experiments were performed with CD4-*Lmna*$^{-/-}$ mice model, BM adoptive transfer technique from full *Lmna*$^{-/-}$ knockout (*Lmna*$^{-/-}$ from now on) was also used to carry out several experiments, in order to study the behavior of lamin A/C deficient CD4$^+$ T-cells. To can guarantee that this model is valid to perform *in vitro*, and particularly, *in vivo* experiments, we carried out immune chimeras with CD45.2-*Lmna*$^{-/-}$ and CD45.1-WT BM in a 1:1 ratio, into a CD45.1/CD45.2 WT recipient mice. One month after the adoptive transfer, the percentage of CD4$^+$ and CD8$^+$ T-cells from the two genotypes was analyzed. Our results showed that BM reconstitution worked nicely, and that T-cells population percentages were similar in *Lmna*$^{-/-}$ and WT adoptively transferred mice (**Figure 13**). This experiment demonstrates that lamin A/C does not affect T-cell development.

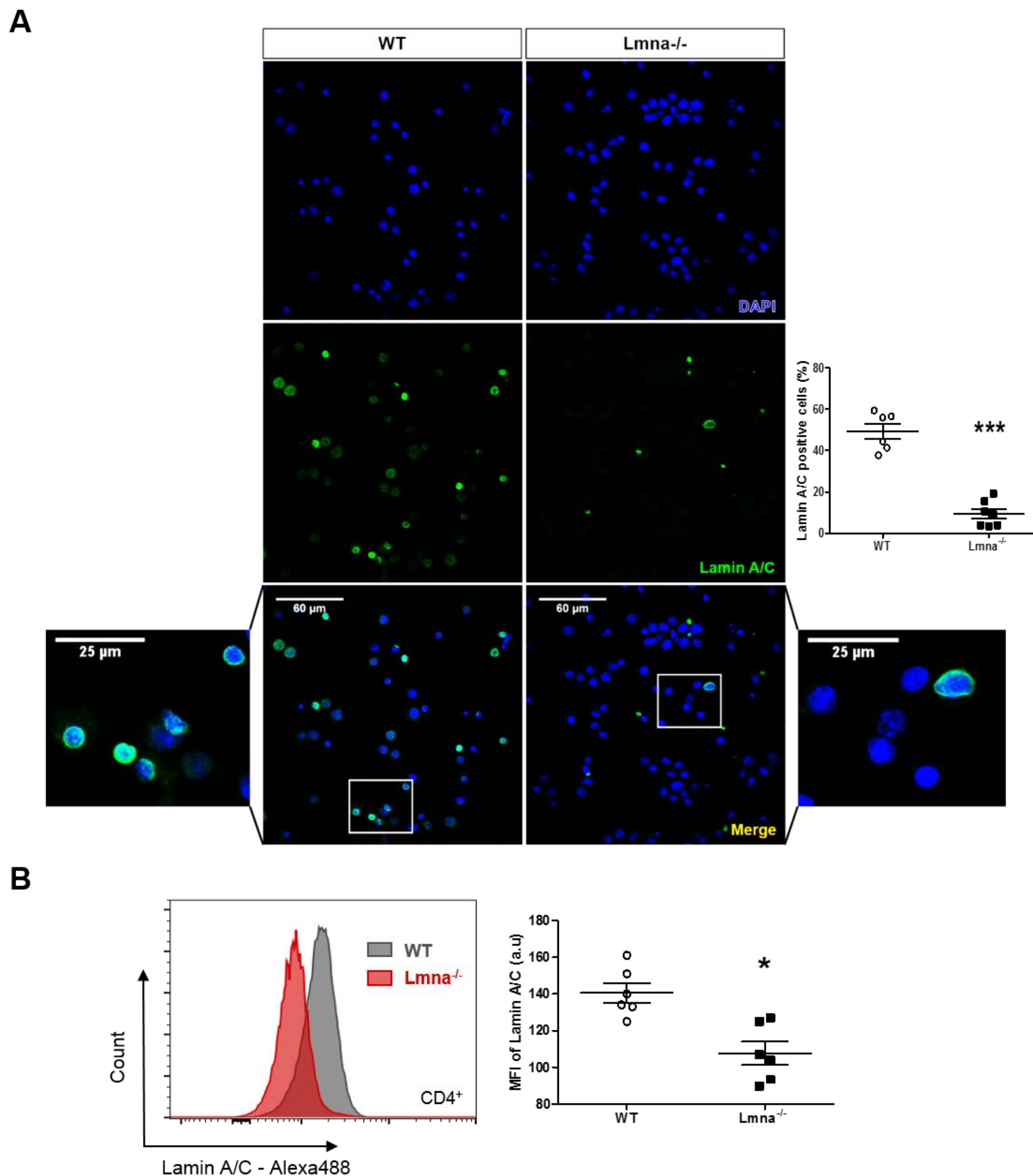

Figure 10. Lamin A/C downregulation in CD4$^+$ T-cells of CD4-CRE$^{tg/wt}$- *Lmna$^{flox/flox}$* (*Lmna$^{-/-}$*) mice. Naïve CD4 $^+$ T-cells were isolated from WT and *Lmna$^{-/-}$* mice spleens, and stimulated with anti CD3/CD28 antibodies for 2 days. **(A)** Immunofluorescent labelling of the nuclear envelope protein lamin A/C (green) in 48 h-activated CD4$^+$ T-cells counterstained with DAPI (blue). Merge of both colors is also shown. Scale bars of 60 µm and 25 µm. Graph shows the percentage of lamin A/C expressing cells quantification. **(B)** Representative histogram and graph of lamin A/C mean fluorescence intensity (MFI) analysis by flow cytometry. (n=3 pool of 2 mice of each genotype). Data are means ± SEM of 6 samples per genotype analyzed by unpaired Student's t-test. *P<0.05; ***P<0.001.

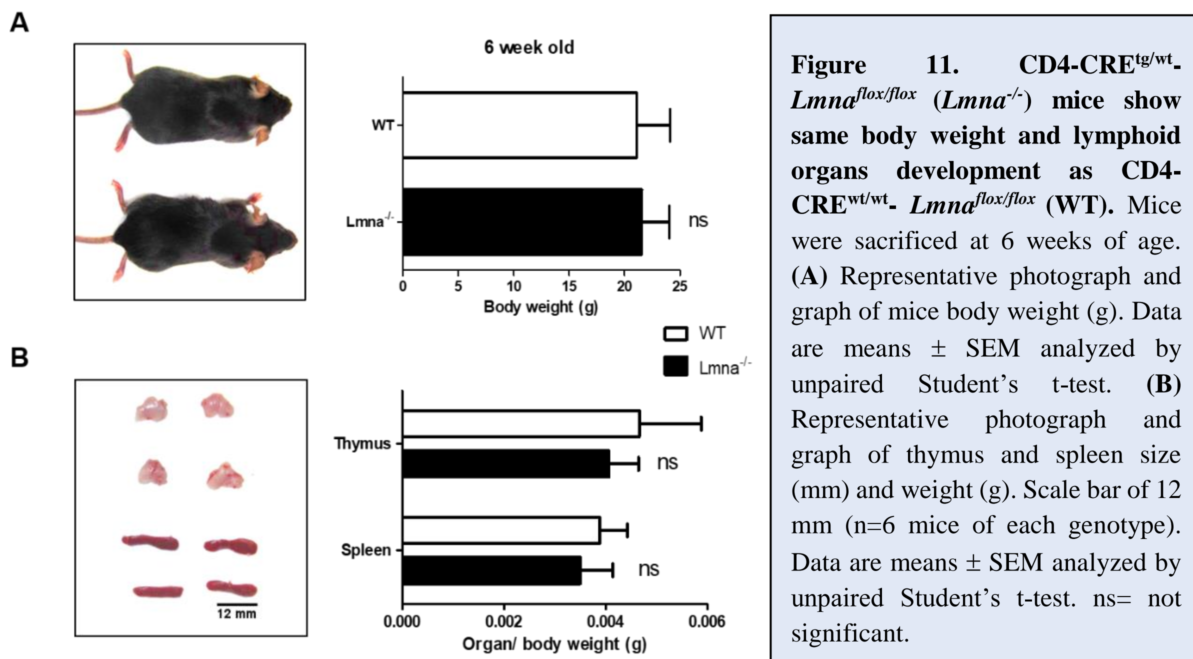

Figure 11. CD4-CRE$^{tg/wt}$- *Lmna$^{flox/flox}$* (*Lmna$^{-/-}$*) mice show same body weight and lymphoid organs development as CD4-CRE$^{wt/wt}$- *Lmna$^{flox/flox}$* (WT). Mice were sacrificed at 6 weeks of age. **(A)** Representative photograph and graph of mice body weight (g). Data are means ± SEM analyzed by unpaired Student's t-test. **(B)** Representative photograph and graph of thymus and spleen size (mm) and weight (g). Scale bar of 12 mm (n=6 mice of each genotype). Data are means ± SEM analyzed by unpaired Student's t-test. ns= not significant.

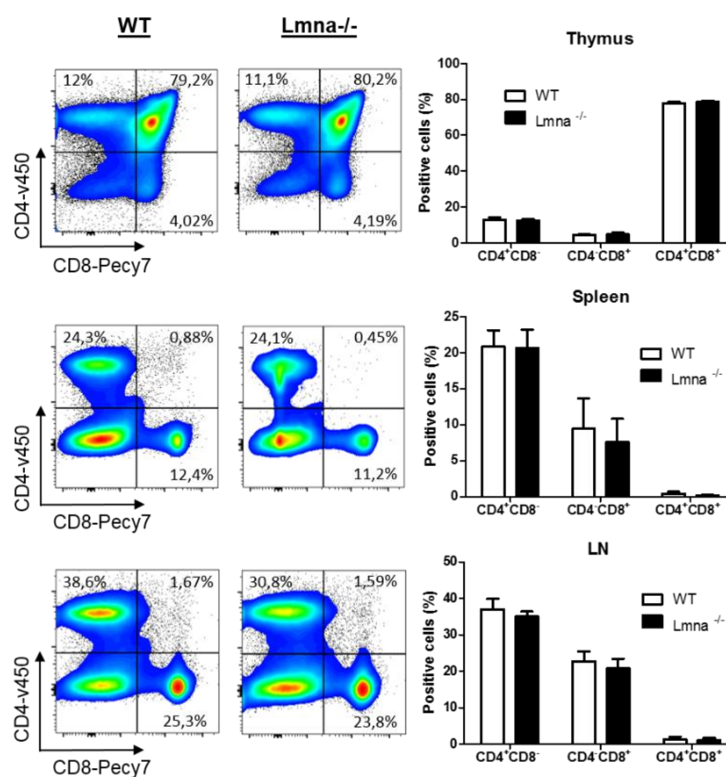

Figure 12. CD4-CRE$^{tg/wt}$- *Lmna$^{flox/flox}$* (*Lmna$^{-/-}$*) mice show same basal T-cell populations as CD4-CRE$^{wt/wt}$- *Lmna$^{flox/flox}$* (WT). Mice were sacrified at 6 weeks of age, and T-cells from thymus, spleen and lymph nodes (LN) were analyzed by flow cytometry. Representative plots and graphs of CD4^{+}CD8^{-} CD4^{-}CD8^{+} CD4^{+}CD8^{+} T-cell populations of thymus, spleen and LN (n=6 mice of each genotype) are shown. Data are means ± SEM analyzed by one-way ANOVA with Bonferroni's multiple comparison test.

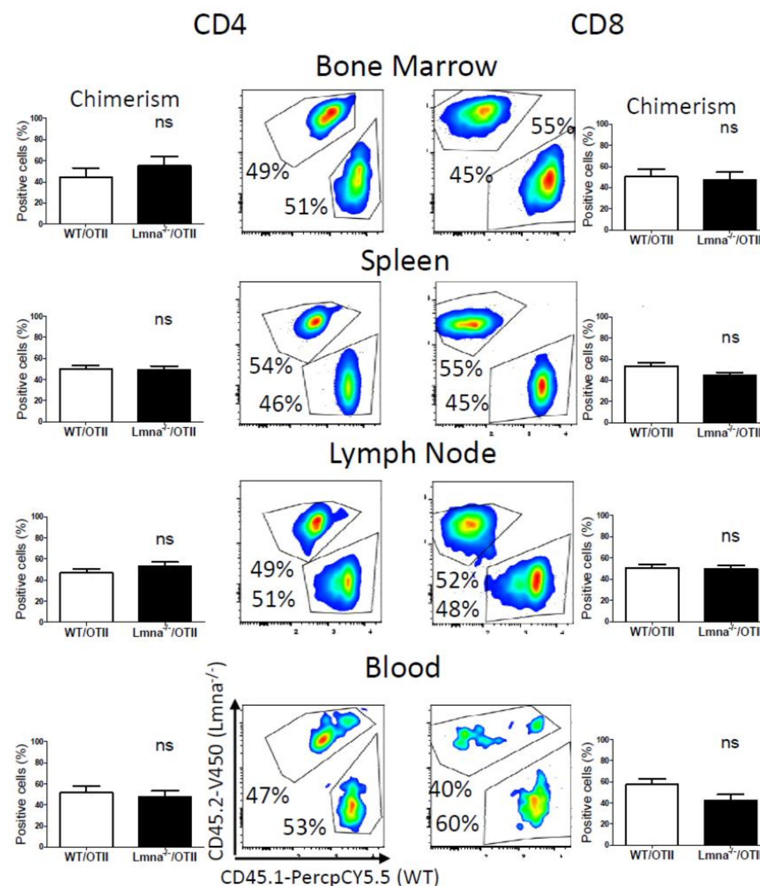

Figure 13. Bone marrow chimeras show that lamin A/C deficiency in immune cells does not affect to CD4^{+} T-cell development. Irradiated WT CD45.1^{+}/CD45.2^{+} recipient mice were adoptively transferred with a 1:1 mix of bone marrow from CD45.1^{+}/OTII WT and CD45.2^{+}/OTII *Lmna*$^{-/-}$(full knockout) mice. One month after transplantation, chimerism in CD4^{+}CD3^{+} and CD8^{+}CD3^{+} T-cells was analyzed by flow cytometry using anti CD45.1, CD45.2, CD4, CD8 and CD3 antibodies. Cells were isolated from bone marrow, spleen, lymph nodes, and blood. Data are means ± SEM from two independent experiments analyzed by Student's *t*-test (n=7 mice of each genotype). ns= not significant.

6.2. Characterization of lamin A/C role in T-cell activation and proliferation.

6.2.1. Lamin A/C enhances CD4^{+} T-cell activation *in vitro* and *in vivo*.

Lamin A/C is an important regulator of CD4^{+} T-cell activation, facilitating IS formation and upregulating CD69 and CD25 membrane receptors expression (González-Granado J.M., *et al* 2015). Now, we have corroborated lamin A/C expression pattern in CD4^{+} T-cells isolated from mice spleen and lymph nodes. As it was previously described (González-Granado JM, *et al* 2015), lamin A/C is expressed upon TCR activation raising its maximum peak of expression after 48 h, and afterwards, lamin A/C is downregulated again (**Figure 14A**). Moreover, we have corroborated *in vitro* and *in vivo* that lamin A/C upregulates CD69 and CD25, surface markers of a proper T-cell activation. *Cd69* and *Cd25* expression were measured at mRNA level by RT-qPCR in CD4^{+} T-cells *in vitro* activated with anti-CD3/CD28 antibodies during 48 h (**Figure 14B**). But also, its expression was analyzed at protein level

by flow cytometry in a VACV-OVA infectious mouse model. For these experiments, *Lmna*$^{-/-}$ and WT mice were back-crossed with OTII mice, which express a TCR specific for OVA peptide. A 1:1 mix of naïve CD4^{+} T-cells from *Lmna*$^{-/-}$/OTII and WT/OTII mice was adoptively transferred into WT recipient mice before intradermal infection with VACV-OVA. Surface expression of the T-cell activation markers CD69 and CD25 was measured 48 h later (**Figure 14C and D**). Accordingly, compared with their WT counterparts, *Lmna*$^{-/-}$ CD4^{+} T-cells in draining lymph nodes expressed lower levels of CD69 and CD25.

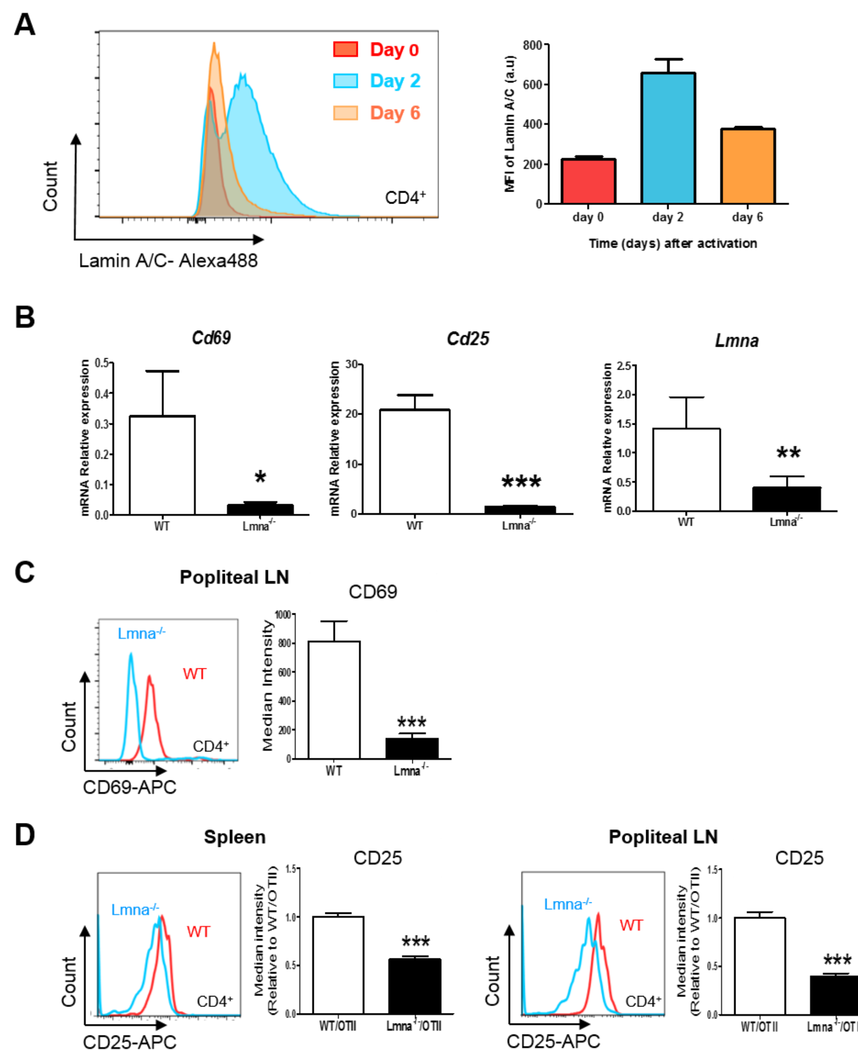

Figure 14. *Lmna*$^{-/-}$ CD4^{+} T-cells show impaired *in vitro* and *in vivo* activation in response to VACV infection. (**A**) Lamin A/C expression in WT and *Lmna*$^{-/-}$ CD4^{+} T-cells 48 h after stimulation with anti-CD3/CD28 antibodies (n=4 mice of each genotype). **(B)** RT-qPCR analysis of the indicated genes in WT and *Lmna*$^{-/-}$ CD4^{+} T-cells stimulated *in vitro* with anti-CD3/CD28 antibodies (n=2 pools of 4 mice of each genotype). **(C-D)** WT CD45.1^{+}/CD45.2^{+} mice were adoptively transferred with a mix of CD4^{+} T-cells from CD45.1^{+}/OTII WT and CD45.2^{+}/OTII *Lmna*$^{-/-}$ mice, and then intradermally infected in the footpad with VACV-OVA for 2 days. Spleen and popliteal lymph nodes were analyzed, and the expression of **(C)** CD69 and **(D)** CD25 was quantified (n=6 mice of each genotype). Data are means ± SEM analyzed by unpaired Student's *t*-test. *P<0.05; **P<0.01; ***P<0.001.

6.2.2. Lamin A/C deficient naïve CD4+ T-cells display normal early TCR-dependent signaling in contrast to lamin A/C-expressing activated CD4+ T-cells.

We have previously shown that A-type lamins are expressed in T-cells upon antigen recognition from 6h to 48 h in a transient manner (González-Granado J.M. *et al*, 2015). Although we did not observe any effect of lamin A/C for naïve T-cell development, we also performed new experiments to assess if the absence of lamin A/C could regulate TCR-dependent signaling in naïve CD4+ T-cells, before its transient expression (**Figure 15**).

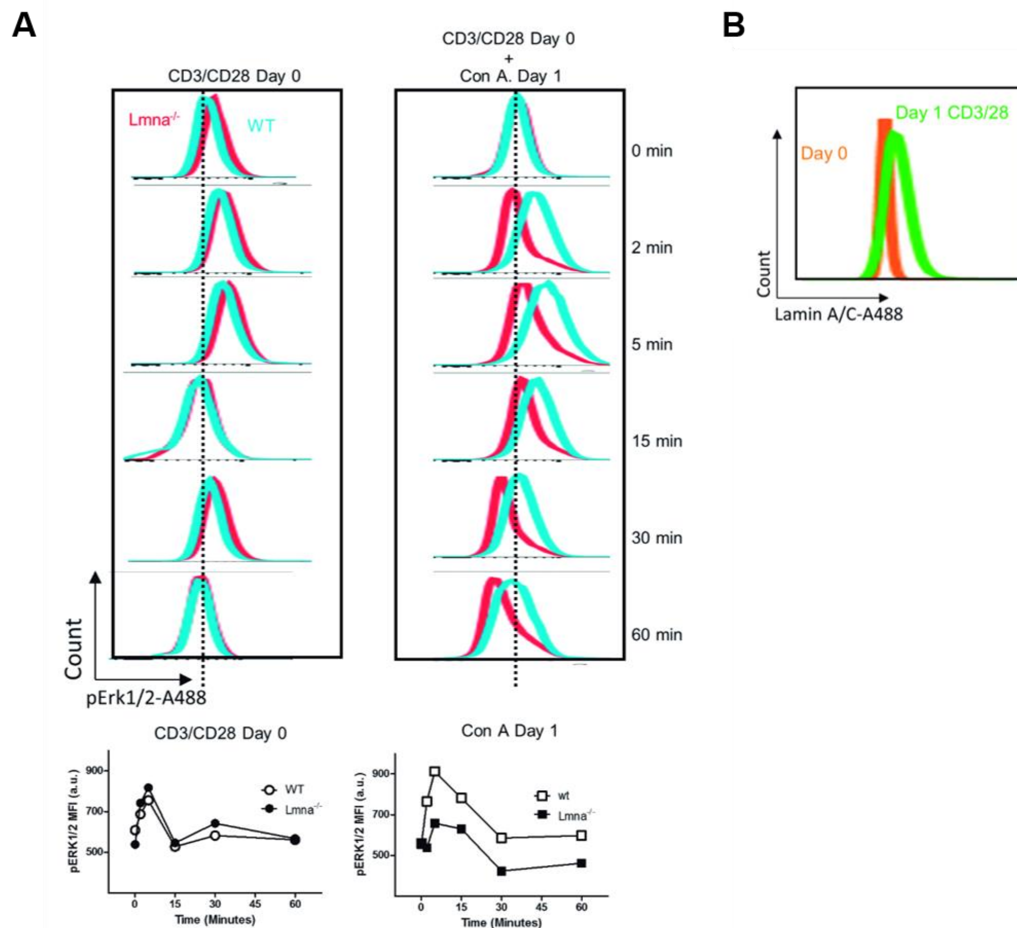

Figure 15. Naïve CD4+ T-cells from *Lmna-/-* mice display normal early TCR-dependent signaling. (A) Intracellular levels of phosphorylated ERK1/2 was measured at short times after stimulation with anti-CD3/CD28 (left panels), and at short times after a second activation challenge with concanavalin A performed 1 day later (right panels). Data show a representative experiment (n =3 mice of each genotype). **(B)** Lamin A/C expression in unstimulated CD4+ T-cells (day 0) or in CD4+ T-cells that were stimulated with antibodies against CD3 and CD28 (1-day CD3/CD28) (n=5 mice of each genotype). Data are means ± SEM analyzed by two-way ANOVA with Bonferroni's multiple comparison test.

After TCR stimulation, WT and *Lmna-/-* naïve CD4+ T-cells showed similar levels of phosphorylated ERK1/2 (**Figure 15A, day 0**), indicating that lamin A/C is not involved in this early activation process.

A second TCR stimulation after 48 h was also induced, moment in which lamin A/C is already expressed in WT CD4+ T-cells (**Figure 15B**). At this moment, levels of phosphorylated ERK1/2 were increased in WT CD4+ T-cells. Phosphorylated ERK1/2 was induced also in *Lmna*-/- cells, but at lower level compared with WT. So that, lamin A/C deficiency in activated CD4+ T-cells diminishes early TCR-dependent signaling (**Figure 15A, day 1**).

6.2.3. Lamin A/C deficient CD4+ T-cells exhibit standard T-cell proliferation.

Next, we investigated if lamin A/C could regulate T-cell proliferation which occurs after antigen recognition and activation. However, proliferation after *in vitro* activation with anti-CD3 and anti-CD28 antibodies for 48 h, was similar in *Lmna*-/- and WT CD4+ T-cells (**Figure 16A**). Moreover, *Il2* expression analyzed by RT-qPCR was alike between activated *Lmna*-/- and WT CD4+ T-cells (**Figure 16B**).

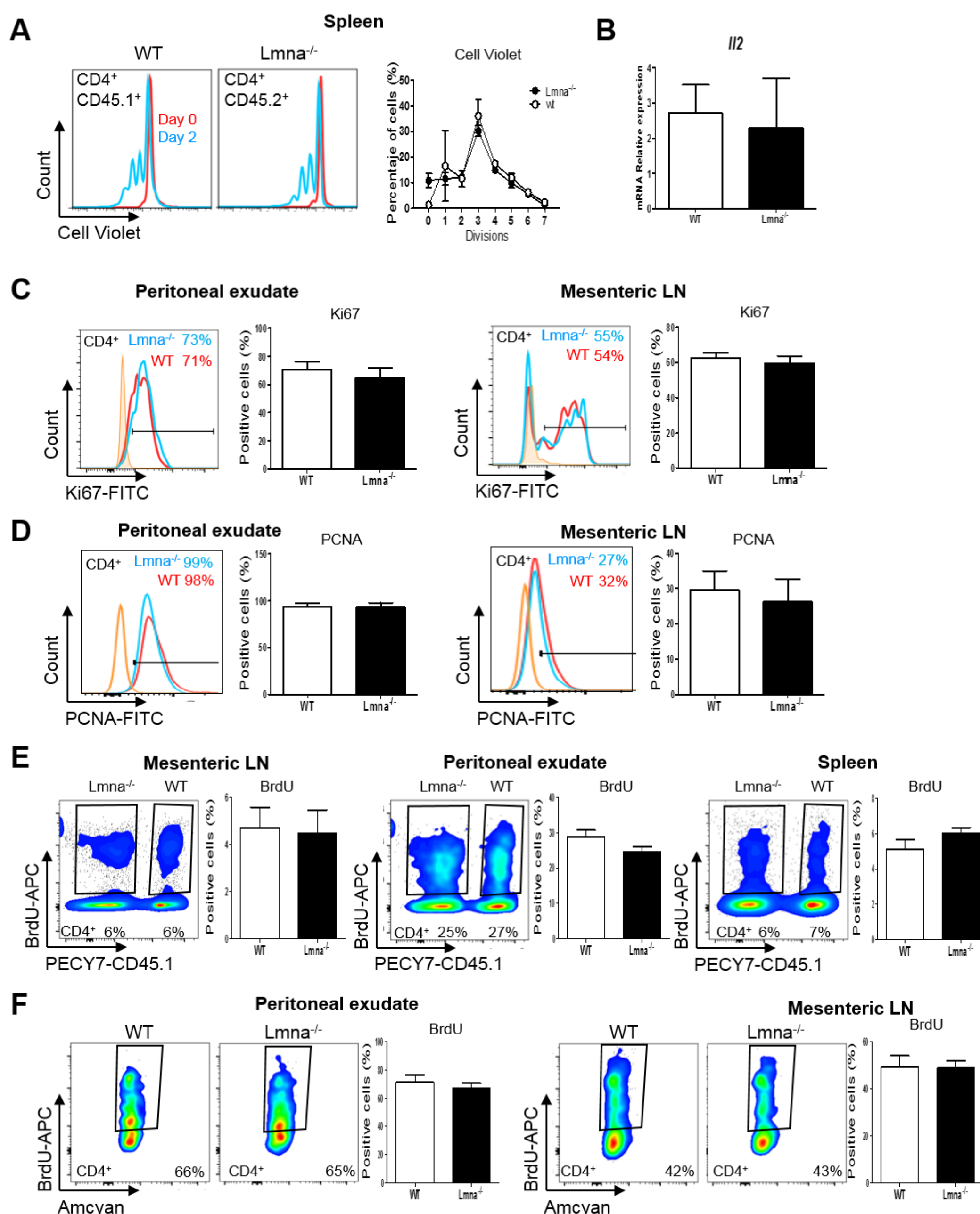

Figure 16. *Lmna*$^{-/-}$ CD4$^+$ T-cells show similar *in vitro* and *in vivo* proliferation rate. **(A)** Splenocytes from CD45.1$^+$ WT/OTII and CD45.2$^+$ *Lmna*$^{-/-}$/OTII mice were mixed, stained with cell violet, and cultured in the presence of OVA for 2 days. CD4$^+$ T-cell proliferation was quantified and the number of cell divisions is shown in the graph. Data are means ± SEM of six independent experiments analyzed by two-way ANOVA with Bonferroni's multiple comparison test. **(B)** RT-qPCR analysis of *Il2* gene in WT and *Lmna*$^{-/-}$ CD4$^+$ T-cells stimulated *in vitro* with anti-CD3/CD28 antibodies (n=2 pools of 4 mice of each genotype). **(C-D)** CD4$^+$ T-cells from CD45.1$^+$ WT and CD45.2$^+$ *Lmna*$^{-/-}$ mice were mixed 1:1 and adoptively transferred to CD45.1/CD45.2 WT mice. Recipient mice were then intraperitoneally infected with VACV for 7 days. The peritoneal exudates and mesenteric lymph nodes (LN) were analyzed, and the percentage of **(C)** Ki67$^+$ or **(D)** PCNA$^+$ CD4$^+$ T-cells was quantified (n=5 mice of each genotype from two independent experiments). Data are means ± SEM analyzed by unpaired Student's *t*-test. **(E)** CD45.1$^+$ WT recipient mice were transplanted with a 1:1 mix CD45.1$^+$ WT and CD45.2$^+$ *Lmna*$^{-/-}$ bone marrow and infected intraperitoneally with VACV. Mice were injected with BrdU, and 24 h later the percentage of BrdU$^+$ CD4$^+$ T-cells was quantified in mesenteric LN, peritoneal exudate, and spleen (n=3 mice of each genotype from one representative experiment). Data are means ± SEM analyzed by paired Student's *t*-test. **(F)** CD4$^+$ T-cells from CD45.2$^+$ WT or CD45.2$^+$ *Lmna*$^{-/-}$ mice were mixed 1:1 and adoptively transferred to CD45.1$^+$/CD45.2$^+$ WT mice. Recipient mice were infected intraperitoneally with VACV for 6 days and injected with BrdU. After 24 h, the percentage of BrdU$^+$ CD4$^+$ T-cells was quantified in the peritoneal exudate and mesenteric LN (n=5 mice of each genotype). Data are means ± SEM analyzed by paired Student's *t*-test.

Similarly, after VACV infection, WT and *Lmna*$^{-/-}$ mice had similar levels of proliferating CD4$^+$ T-cells, as shown by Ki67 expression (**Figure 16C**), PCNA expression (**Figure 16D**), and BrDu incorporation (**Figure 16E-F**). These experiments demonstrate that A-type lamins regulate T-cell activation without affecting CD4$^+$ T-cell proliferation.

6.3. Analysis of lamin A/C role in Th1 differentiation an effector function.

6.3.1. Lamin A/C regulates Th1 differentiation *in vitro*.

To analyze the role of A-type lamins in T-cell differentiation, naïve CD4$^+$ T-cells were isolated from *Lmna*$^{-/-}$/OTII or WT/OTII mice and co-cultured with OVA-loaded WT APCs in the absence of polarizing cytokines. Compared with WT CD4$^+$ T-cells, fewer *Lmna*$^{-/-}$ CD4$^+$ T-cells were IFNγ$^+$, indicating the importance of lamin A/C for Th1 differentiation (**Figure 17A**). This difference was not abolished by addition of IL-2 (**Figure 17B**). We next directed Th1 or Th2 differentiation *in vitro* by incubating WT and *Lmna*$^{-/-}$ CD4$^+$ T-cells with anti-CD3 and anti-CD28 antibodies, polarizing cytokines, and Th1 or Th2 blocking antibodies. Interestingly, *Lmna*$^{-/-}$ CD4$^+$ T-cells produced fewer Th1-cells than WT cells but similar numbers of Th2 cells (**Figure 17C**). Th1 differentiation triggered by co-culture with OVA-loaded WT APCs in the presence of Th1 polarizing cytokines was also lower in CD4$^+$ T-cells from *Lmna*$^{-/-}$/OTII mice than in WT/OTII T-cells (**Figure 17D**). These experiments suggest that lamin A/C is an important intrinsic regulator of T-cell differentiation following TCR stimulation.

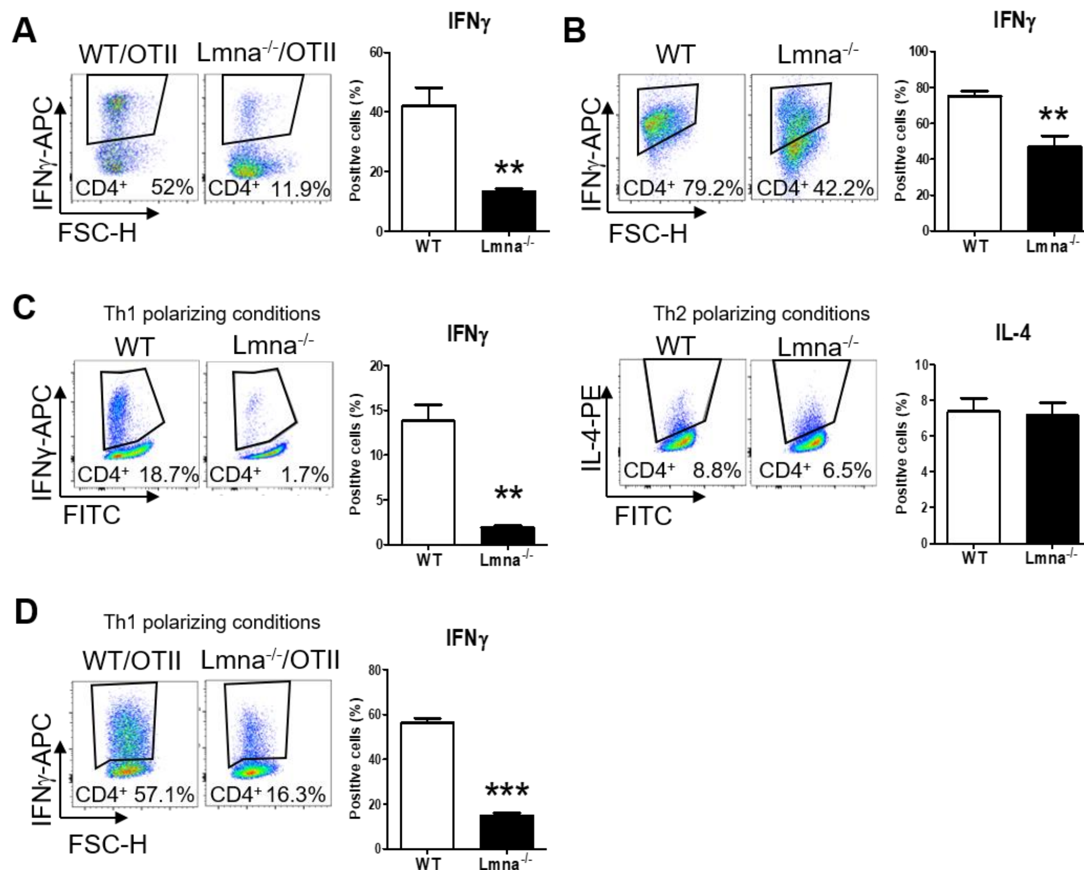

Figure 17. Impaired *in vitro* Th1 differentiation of CD4$^+$ T-cells from *Lmna$^{-/-}$* mice. Percentage of IFNγ$^+$ and IL4$^+$ CD4$^+$ T-cells isolated from WT or *Lmna$^{-/-}$* mice. **(A)** CD4$^+$ T-cells from WT/OTII or *Lmna$^{-/-}$*/OTII mice were cocultured with OVA-loaded WT APCs for 4 days. Data are means ± SEM of at least three independent experiments analyzed by unpaired Student's *t*-test. **(B)** CD4$^+$ T-cells were stimulated with anti-CD3/CD28 antibodies for 4 days in the presence of IL-2. Data are means ± SEM of at least five independent experiments analyzed by unpaired Student's *t*-test. **(C)** CD4$^+$ T-cells were stimulated with anti-CD3/CD28 antibodies for 4 days in the presence of cytokines and antibodies to trigger Th1 or Th2 differentiation *in vitro*. Data are means ± SEM of at least four independent experiments analyzed by unpaired Student's *t*-test. **(D)** CD4$^+$ T-cells from WT/OTII or *Lmna$^{-/-}$*/OTII mice were cocultured with OVA-loaded WT APCs for 4 days in the presence of cytokines and antibodies to trigger Th1 differentiation. Data are means ± SEM of three independent experiments analyzed by unpaired Student's *t*-test. **P<0.01; ***P<0.001.

6.3.2. Lamin A/C enhances Th1 responses *in vivo*.

To investigate the role of lamin A/C in Th1 differentiation *in vivo*, mice were infected with VACV, which provokes a robust Th1 immune response in mice (Swain S.L. *et al*, 2012; Munier C.M. *et al*, 2016). At 3 days after i.p. VACV infection, the frequency of IFNγ$^+$ CD4$^+$ T-cells in MLN and peritoneal exudate was lower in *Lmna$^{-/-}$* mice than in WT mice (**Figure 18A-B**). To study the role of lamin A/C specifically in the immune system, we reconstituted lethally irradiated WT CD45.1 mice with CD45.2 BM from WT or *Lmna$^{-/-}$* mice for 2 months. Confirmation of bone marrow reconstitution with anti CD45.1 and CD45.2 antibodies revealed no significant differences between genotypes. After i.p.

infection with VACV, we analyzed the frequency of IFNγ^{+} and IL-4^{+} in CD4^{+} T-cells. *Lmna*$^{-/-}$-reconstituted mice had proportionately fewer IFNγ^{+} T-cells, and similar numbers of IL4^{+} T-cells with respect to WT-reconstituted mice, in both spleen and peritoneal exudate (**Figure 18C**). To trigger antigen-specific Th1 responses *in vivo*, we reconstituted WT mice with either WT/OTII or *Lmna*$^{-/-}$/OTII bone marrow, and then, infected the mice i.p. with VACV-OVA. As before, the *Lmna*$^{-/-}$/OTII-reconstituted CD4^{+} population included a lower proportion of IFNγ^{+} cells than WT/OTII-derived CD4^{+} cells (**Figure 18D**).

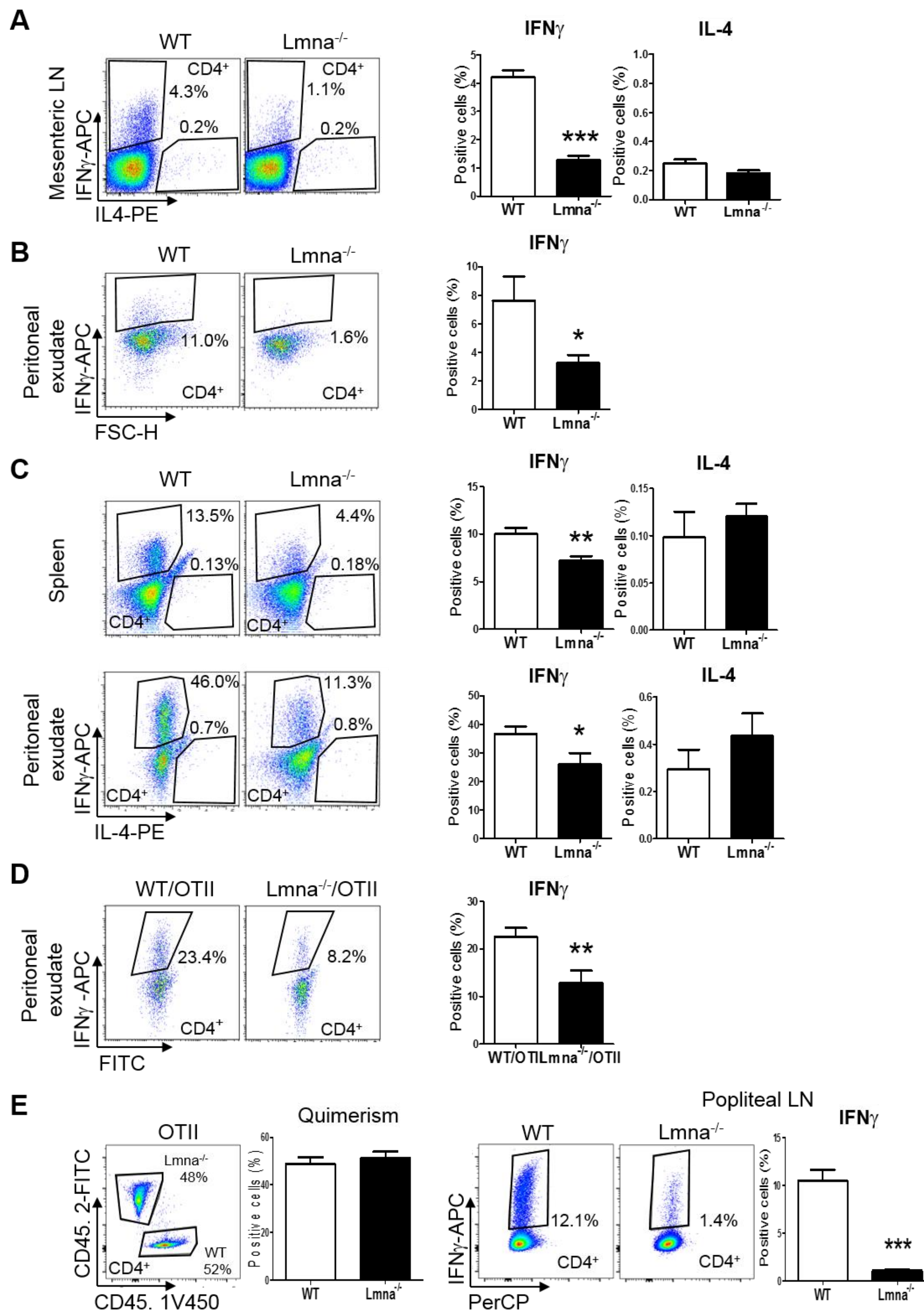

Figure 18. Impaired *in vivo* Th1 differentiation of naïve CD4$^+$ T-cells from *Lmna*$^{-/-}$ mice. Percentage of IFNγ$^+$ or IL4$^+$ CD4$^+$ T-cells upon VACV infection. **(A-B)** WT and *Lmna*$^{-/-}$ mice were intraperitoneally infected with VACV, and after 3 days mesenteric LN **(A)** and peritoneal exudate **(B)** were analyzed, (n=11 WT and 7 *Lmna*$^{-/-}$ mice). **(C)** Irradiated CD45.1$^+$ WT mice were reconstituted with CD45.2$^+$ WT or CD45.2$^+$ *Lmna*$^{-/-}$ bone marrow, and intraperitoneally infected with VACV. After 5 days, spleens and the peritoneal exudate were analyzed by flow cytometry (n=8-11 mice from two independent experiments). **(D)** Irradiated CD45.1$^+$ WT mice were reconstituted with CD45.2$^+$ WT/OTII or CD45.2$^+$ *Lmna*$^{-/-}$/OTII bone marrow and infected intraperitoneally with VACV-OVA. After 5 days, peritoneal exudate was analyzed (n=9 mice from two independent experiments). **(E)** Irradiated CD45.1$^+$/CD45.2$^+$ WT mice were reconstituted with a mix of CD45.1$^+$ WT/OTII with CD45.2$^+$ *Lmna*$^{-/-}$/OTII BM. Five days after VACV-OVA i.d infection in the footpad, popliteal lymph nodes were analyzed (n=9 mice from two independent experiments). Data are means ± SEM analyzed by paired Student's *t*-test. *P<0.05; **P<0.01; ***P<0.001.

Moreover, reconstitution with a mix of bone marrow from WT/OTII and *Lmna*$^{-/-}$/OTII mice produced a lower percentage of IFNγ$^+$ CD4$^+$ T-cells in *Lmna*$^{-/-}$/OTII than in WT/OTII cells (**Figure 18E**). Our results thus indicate that lamin A/C depletion in the hematopoietic compartment impairs Th1 differentiation *in vitro* and *in vivo*.

The observed phenotype could in principal be related to the absence of lamin A/C in any radiosensitive immune cell. Therefore, to directly assess the importance for Th1 differentiation of lamin A/C in CD4$^+$ T-cells, we adoptively transferred WT mice with CD4$^+$ T-cells from WT/OTII or *Lmna*$^{-/-}$/OTII mice. Th1 differentiation from *Lmna*$^{-/-}$ CD4$^+$ T-cells was weaker than from their WT counterparts, producing a lower percentage of IFNγ$^+$ CD4$^+$ T-cells (**Figure 19A**). Similar results were obtained when WT recipient mice were transferred with a mix of CD4$^+$ T-cells from WT/OTII and *Lmna*$^{-/-}$/OTII mice followed by VACV-OVA inoculation, either i.p. (**Figure 19B**) or subcutaneously in the footpad (**Figure 19C**). These results indicate that lamin A/C expression specifically regulates CD4$^+$ T-cell differentiation toward Th1 cells *in vivo*.

We next investigated whether the action of lamin A/C in CD4$^+$ T-cell polarization was related to antigen presentation by DCs. WT mice first received subcutaneous injections of LPS-matured OVA-loaded BMDCs, which trigger Th1 differentiation (Sen D. *et al*, 2010), and 18 h later received intravenous injections of WT/OTII or *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells. After 7 days, Th1 differentiation of adoptively transferred T-cells was analyzed in draining lymph nodes. *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells produced fewer IFNγ$^+$ CD4$^+$ T-cells in the presence of LPS-treated BMDCs than their WT/OTII counterparts (**Figure 19D**). In other experiments, WT mice received subcutaneous injections of splenic CD11c$^+$ DCs incubated in medium containing LPS or papain for 4 h to potentiate antigen-dependent T-cell differentiation to Th1 or Th2, respectively (Sen D. *et al*, 2010; Sokol C.L. *et al*, 2008). As before, 18h later, the mice received intravenous injections of WT/OTII or *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells, and Th1 and Th2 differentiation was analyzed in draining lymph nodes after 7 days. Again, *Lmna*$^{-/-}$ CD4$^+$ T-cells produced fewer IFNγ$^+$ CD4$^+$ T-cells in the presence of LPS-treated BMDCs than their WT counterparts;

in contrast, in the presence of papain-treated BMDCs, the proportion of IL4^{+} cells were similar in WT and *Lmna*$^{-/-}$ CD4^{+} T-cells (**Figure 19E**).

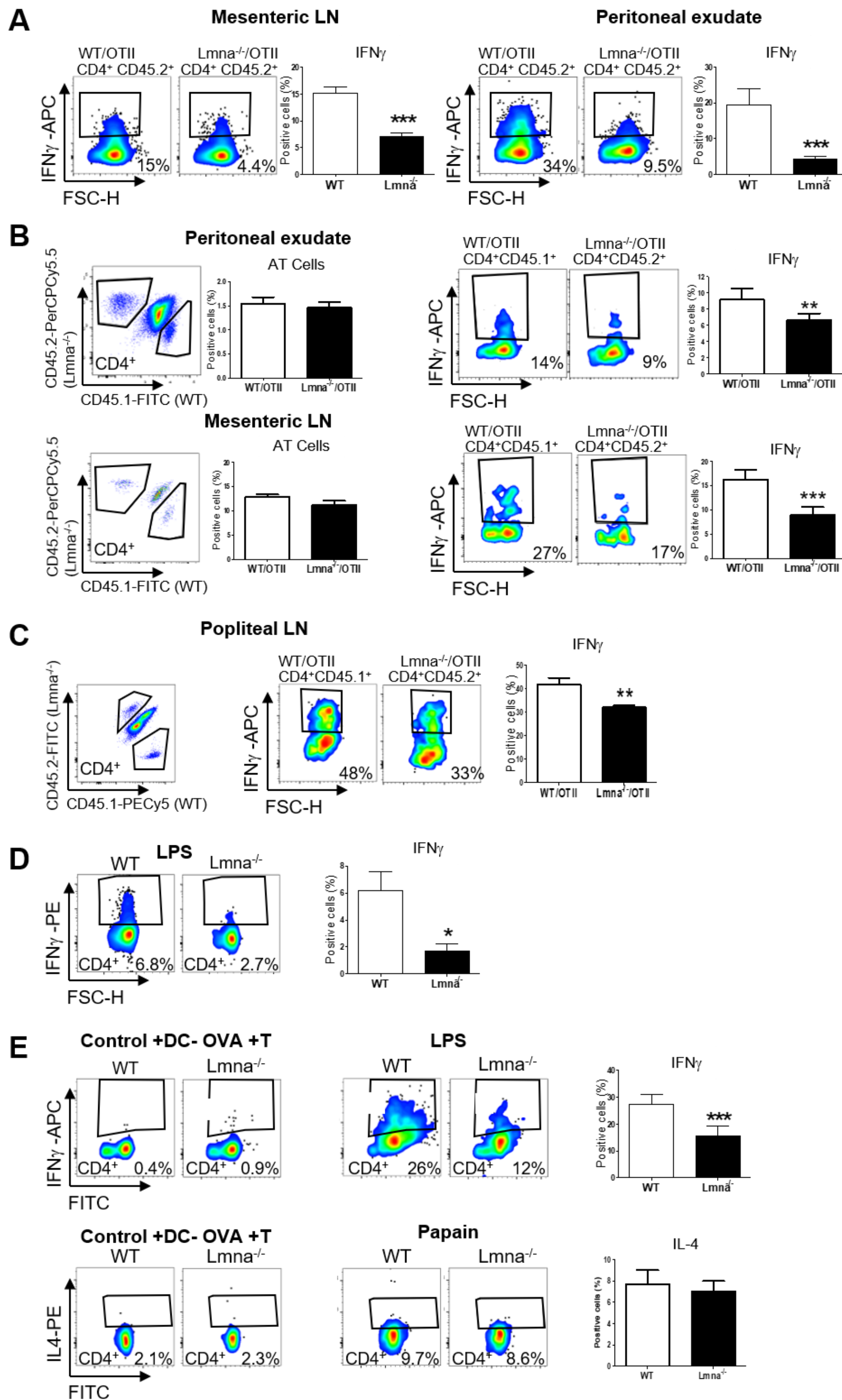

Figure 19. Compromised *in vivo* Th1 differentiation of naïve CD4$^+$ T-cells from *Lmna*$^{-/-}$ mice. Percentage of IFNγ$^+$ CD4$^+$ T-cells. (**A**) Recipient CD45.1$^+$ WT mice were adoptively transferred with CD45.2$^+$ WT/OTII or CD45.2$^+$ *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells. Five days after intraperitoneal VACV-OVA infection, mesenteric lymph nodes and the peritoneal exudate were analyzed (n=10 WT and 9 *Lmna*$^{-/-}$ mice from two independent experiments). Data are means ± SEM analyzed by unpaired Student's *t*-test. **(B)** CD45.1$^+$/CD45.2$^+$ WT mice were adoptively transferred with a mix of CD45.1$^+$ WT/OTII and CD45.2$^+$ *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells and infected intraperitoneally with VACV-OVA. After 5 days, peritoneal exudate and mesenteric lymph nodes were analyzed (n=11 mice from two independent experiments). Data are means ± SEM analyzed by paired Student's *t*-test. (**C**) CD45.1$^+$/CD45.2$^+$ WT mice were adoptively transferred as in B, and after 5 days of intradermal infection with VACV-OVA the popliteal lymph nodes were analyzed (n=6 mice from two independent experiments). Data are means ± SEM analyzed by paired Student's *t*-test. **(D)** OVA-loaded LPS-matured BMDCs were adoptively transferred to WT recipient mice. After 18h, recipients received intravenous injections of a mix of CD45.1$^+$ WT and CD45.2$^+$ *Lmna*$^{-/-}$*/OTII* CD4$^+$ naïve T-cells. Four days later, the popliteal lymph nodes were analyzed (n=3 mice from one representative experiment out of two). Data are means ± SEM analyzed by unpaired Student's *t*-test. (**E**) Isolated splenic CD11c$^+$ cells were incubated with OVA and LPS or with OVA and papain for 4h, and then adoptively transferred to WT recipients. Control mice were transferred with CD11c$^+$ cells in the absence of OVA (DC-OVA). After 18h, recipients received intravenous injections of a mix of CD45.1$^+$ WT and CD45.2$^+$ *Lmna*$^{-/-}$ CD4$^+$ naïve T-cells. Four days later, the popliteal lymph nodes were analyzed. The percentage of IL4$^+$ CD4$^+$ T-cells was also investigated (n=4 mice from two independent experiments). Data are means ± SEM analyzed by unpaired Student's *t*-test. *P< 0.05; **P<0.01; ***P<0.001.

6.3.3. Lamin A/C enhances T-bet expression.

The master regulator of Th1 differentiation is T-bet. Analysis of T-bet expression in CD4$^+$ T-cells revealed that the absence of A-type lamins reduced both the percentage of T-bet$^+$ cells and the T-bet expression level in anti-CD3/CD28-stimulated CD4$^+$ T-cells (**Figure 20A and B**). In agreement with the effect on Th1 differentiation, lamin A/C deficiency also diminished the percentage of IFNγ$^+$ CD4$^+$ T-cells (**Figure 20A**). Similar results were obtained when cells were stimulated with anti-CD3/CD28 antibodies in the presence of Th1-differentiation cytokines (**Figure 20C**). Moreover, lamin A/C expression was also important for T-bet expression *in vivo* after Th1 differentiation triggered by VACV infection (**Figure 20D**). Low T-bet protein expression in *Lmna*$^{-/-}$ T-cells was accompanied by lower mRNA expression *in vitro* (**Figure 20E**), suggesting regulation at the level of mRNA synthesis.

A-type lamins regulate TCR clustering and subsequent downstream signaling upon T-cell activation (González-Granado J.M. *et al*, 2014). However, altered Th1 differentiation in the absence of lamin A/C was not exclusively related to differences in TCR clustering, since the percentage of IFNγ$^+$ and T-bet$^+$ *Lmna*$^{-/-}$ CD4$^+$ T-cells was also lower after TCR-independent T-cell stimulation with PMA and ionomycin (**Figure 20A**).

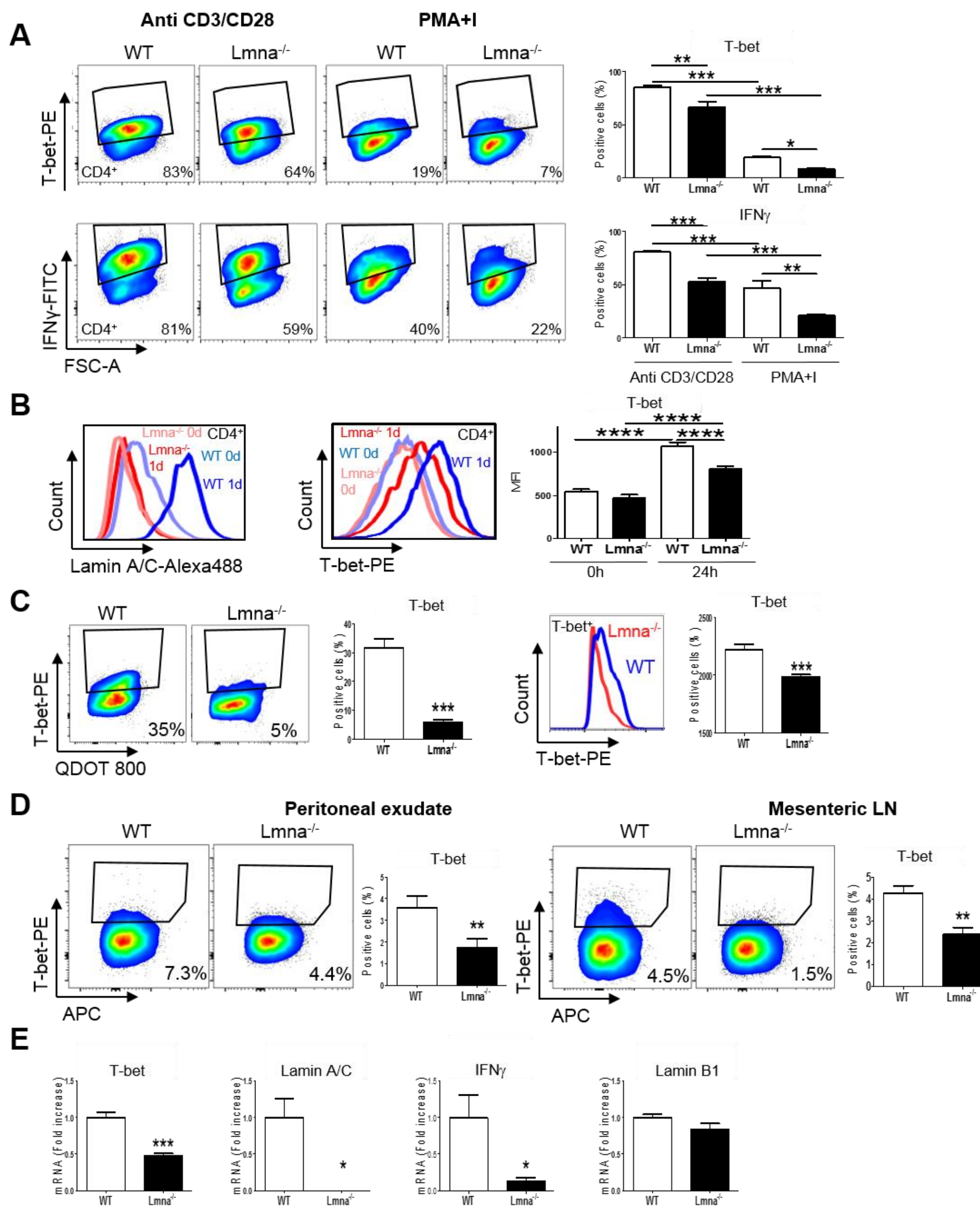

Figure 20. ***Lmna***$^{-/-}$ **CD4**$^{+}$ **T-cells show impaired *in vitro* and *in vivo* T-bet expression.** **(A)** Percentage of IFNγ^{+} and T-bet^{+} cells after stimulation of CD4^{+} T-cells from WT and *Lmna*$^{-/-}$ mice with anti-CD3/CD28 antibodies, or PMA plus ionomycin for 4 days in the presence of IL-2. **(B)** T-bet and lamin A/C expression in WT and *Lmna*$^{-/-}$ CD4^{+} T-cells 48 h after stimulation with anti-CD3/CD28 antibodies (n=12 mice). **C)** Percentage of T-bet^{+} cells after stimulation of WT and *Lmna*$^{-/-}$ CD4^{+} T cells with anti-CD3/CD28 for 7 days in the presence of Th1 polarizing antibodies and cytokines (n=6 mice from two independent experiments). **(D)** Percentage of T-bet^{+} CD4^{+} T cells in peritoneal exudates and mesenteric LN of WT recipient mice reconstituted with a 1:1 mix of WT and *Lmna*$^{-/-}$ bone marrow and intraperitoneally infected with VACV for 6 days (n=4 mice from two independent experiments). **(E)** RT-qPCR analysis of the indicated genes in WT and *Lmna*$^{-/-}$ CD4^{+} T-cells stimulated *in vitro* with anti-CD3/CD28 antibodies for 48 h (n=3 pools of 3 mice). Data are means ± SEM analyzed by one-way ANOVA with Bonferroni's multiple comparison test (A and B), or unpaired Student's t-test (C, D and E) *P<0.05; **P<0.01; ***P<0.001.

6.3.4. Quantitative proteomic assays reveals that lamin A/C modifies the expression of several epigenetic enzymes.

To further address the mechanism by which lamin A/C might modify Th commitment, we performed a comparative proteomic analysis between 48-h activated CD4$^+$ T-cells from WT and *Lmna*$^{-/-}$mice. The analysis quantified about 7180 proteins, of which 103 showed significant differences (p-value ≤ 0.05) among WT and *Lmna*$^{-/-}$ CD4$^+$ T-cells based on proteins quantified at least with 2 peptides, and Zq values higher than ±2; of these, 51 were upregulated and 52 downregulated in *Lmna*$^{-/-}$ CD4$^+$ T-cells compared to WT. Within the 103 resulting proteins differentially expressed in *Lmna*$^{-/-}$ and WT T-cells, sixty-six proteins were categorized in six functional groups according to UniProt Database information: epigenetics, DNA machinery, immune system, metabolism, cytoskeleton, and vesicular transport (**Figure 21A**). Interestingly, the categories with the highest number of differentially regulated proteins between *Lmna*$^{-/-}$ and WT CD4$^+$ T-cells were epigenetics and DNA machinery, with 16 and 15 proteins respectively (**Figure 21A**). This suggests that lamin A/C may directly or indirectly regulates the expression of several enzymes involved in epigenetic and transcription processes in CD4$^+$ T-cells. Within the group of enzymes responsible of epigenetic modifications we found: three histone

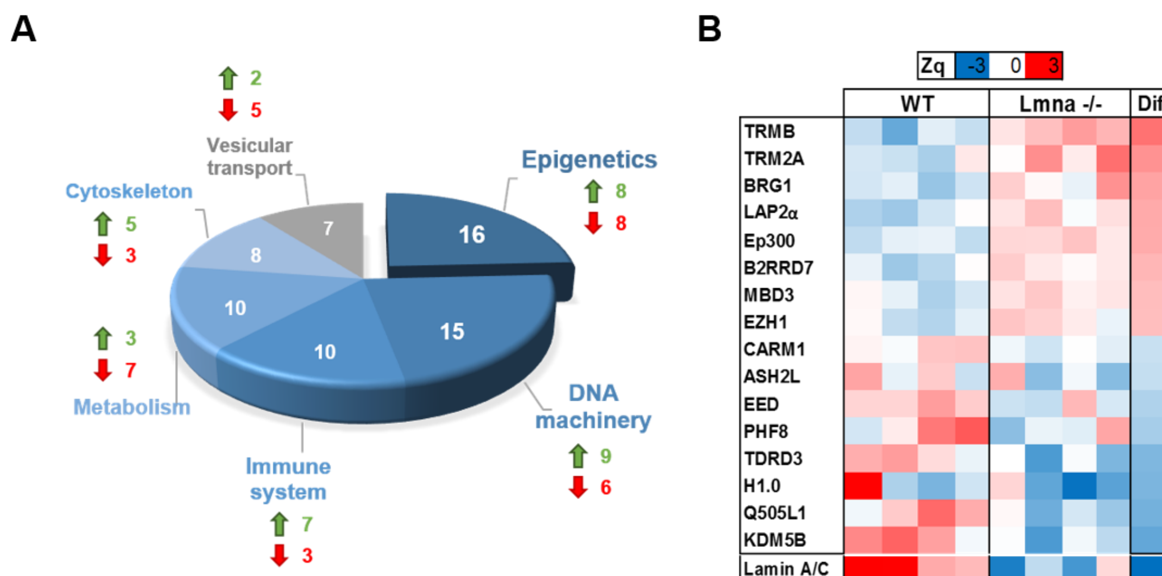

Figure 21. Lamin A/C regulates the expression of several epigenetic enzymes at protein level. CD4$^+$ T-cells were isolated from WT and *Lmna*$^{-/-}$ mice spleens and lymph nodes and stimulated with anti CD3/CD28 antibodies for 2 days. At this time, alive cells were selected by FACS to carry out high throughput quantitative proteomic analysis using isobaric labelling (TMT 10-Plex analysis). **(A)** Sixty-six proteins differentially expressed between *Lmna*$^{-/-}$ and WT CD4$^+$ T-cells are shown. They were categorized into six functional groups: epigenetics, DNA machinery, immune system, metabolism, cytoskeleton, and vesicular transport. The categories with the highest number of differentially regulated proteins were epigenetics and DNA machinery. Upregulated and downregulated proteins in *Lmna*$^{-/-}$ CD4$^+$ T-cells are indicated with a green arrow and a red arrow respectively for each category. **(B)** Heat-map representing protein abundance changes in proteins from epigenetics category. For each protein, gene name is displayed together with the corresponding Zq values (standardized log2 ratio) in a color scale (red means upregulated and blue downregulated). Average difference (Dif) between WT and *Lmna*$^{-/-}$ Zq values is also shown (n=4 pool of 5 mice). Data are means ± SEM analyzed by Student´s t-test. All the proteins represented showed at least a p-value <0.05 or lower.

methyltransferases (EZH1, CARM1 and ASH2L), two histone acetyltransferases (B2RRD7 and EP300), and two histone demethylases (PHF8 and KDM5B) (UniProt Database) (**Figure 21B**). In addition, MBD3, a protein which binds to methyl-CpG domains (UniProt Database), was also upregulated in *Lmna*$^{-/-}$ CD4^{+} T-cells, as well as LAP2α (**Figure 21B**).

6.3.5. Lamin A/C upregulates H3K4me1 epigenetic modification in *Cd69* and *Tbet* promoters.

Due to the results obtained by quantitative proteomic analysis, we postulated that lamin A/C could be an epigenetic regulator determining the Th fate commitment of CD4^{+} T-cells. To investigate this hypothesis, we performed chromatin immunoprecipitation-qPCR (ChIP-qPCR) assays with *in vitro* activated with anti-CD3/CD28 antibodies for 48 h *Lmna*$^{-/-}$ and WT CD4^{+} T-cells. We studied H3K4me3, H3K27me3, and H3K4me1 histone modifications in the following gene promoters: *Cd69, Cd25*, *Il2, Tbet,* and *Gata3* (**Figure 22**).

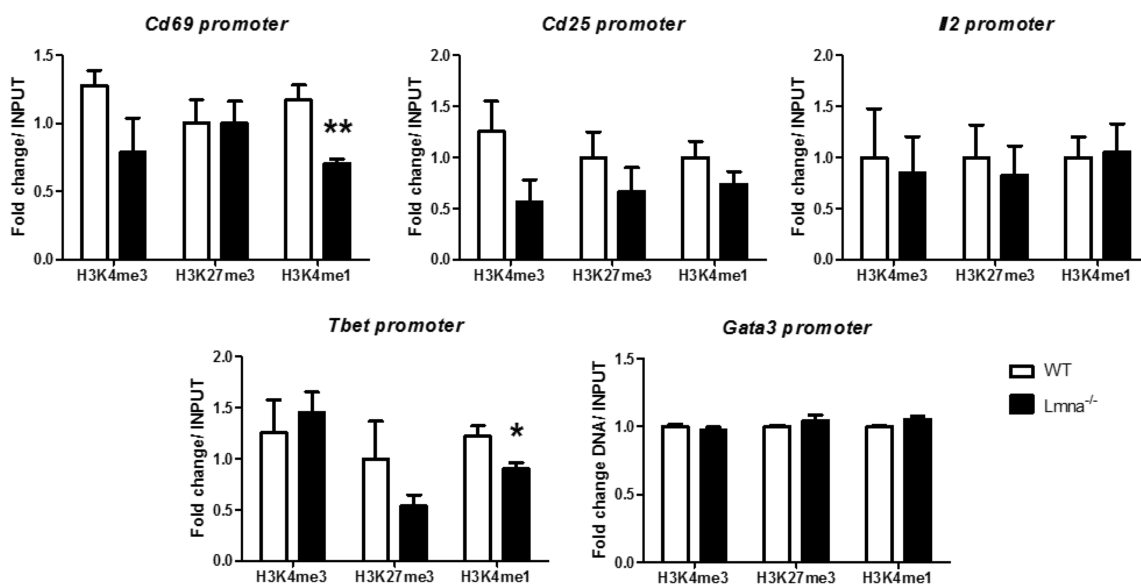

Figure 22. Lamin A/C regulates epigenetics through H3K4me1 modification in *Cd69* and *Tbet* genes. Naïve CD4^{+} T-cells were isolated from WT and Lmna$^{-/-}$ mice spleens and lymph nodes and stimulated with anti CD3/CD28 antibodies for 2 days. Alive cells were selected by FACS and crosslinked to develop ChIP-qPCR analysis. Representative graphs of ChIP-qPCR analysis for the indicated gene promoters. Data are shown as fold change relative to WT and normalized by the correspondent input (n=2-4 pool of 5 mice). Data are means ± SEM of 2-4 independent experiments from a total of 6 analyzed by Student's t-test.

No differences were detected between *Lmna*$^{-/-}$ and WT CD4^{+} T-cells in any of the epigenetic modifications studied for *Il2, and Gata3* gene promoters. So that, lamin A/C did not seem to regulate proliferation neither Th2 polarization by epigenetic changes. Likewise, H3K4me3 and H3K27me3 did not exhibit any distinct result in *Cd69* and *Tbet* gene promoters. However, H3K4me1 enhancer mark

was differentially present in *Cd69* and *Tbet*, being less abundant in *Lmna*$^{-/-}$ T-cells compared with WT. *Cd25* promoter showed less H3K4me3 modification on *Lmna*$^{-/-}$ T-cells compared with WT, although this variance was not significant (p= 0,13) (**Figure 22**).

6.3.6. Lamin A/C regulates Th1 immunity against pathogen infection.

T-bet and IFNγ play major roles during *in vivo* Th1 responses against pathogens (Sacks, D. & Noben-Trauth, N., 2002; Mougneau E. *et al*, 2011; Swain S.L. *et al*, 2012). The low expression of these factors in *Lmna*$^{-/-}$ CD4^{+} T-cells therefore prompted us to investigate the role of lamin A/C in Th1 mediated-pathogen clearance *in vivo*. For this, *Rag1*$^{-/-}$ mice lacking T and B-cells were adoptively transferred with naïve CD25^{-} CD4^{+} T-cells from WT or *Lmna*$^{-/-}$ mice. In our experiments, *Rag1*$^{-/-}$ mice adoptively transferred with CD25^{-} CD4^{+} *Lmna*$^{-/-}$ T-cells were more susceptible to primary VACV infection, as indicated by an elevated viral titer and a reduced Th1 response in the spleen (**Figure 23A and B**). Moreover, *Ifnγ* and *Tbet* mRNA expression was reduced in adoptively transferred mice, whereas the Th2 transcription factor *Gata3* was unaffected (**Figure 23C**). We also analyzed the importance of lamin A/C expression for Th1 responses against the intracellular parasite *Leishmania major* (Sacks, D. & Noben-Trauth, N., 2002). Mice on the C57BL/6 background control *L. major* infection by developing a protective Th1 response (Iborra S. *et al*, 2011). C57BL/6 mice lacking lamin A/C in the whole immune system (**Figure 23D-H**) or specifically in T-cells (**Figure 23I-K**) developed progressively larger and non-healing lesions (**Figure 23D, E, I**) after inoculation of the ear dermis with a low dose of *L. major* parasites. This effect correlated with a higher parasite burden in the infection site and in draining lymph nodes 3 weeks after infection (**Figure 23F, J**). At this time, *Lmna*$^{-/-}$ mice also showed reduced percentages of IFNγ-producing CD4^{+} T-cells (**Figure 23G, K**) and diminished IFNγ production by this cell population (**Figure 23H**). These results further support the role of lamin A/C in Th1 responses against pathogens.

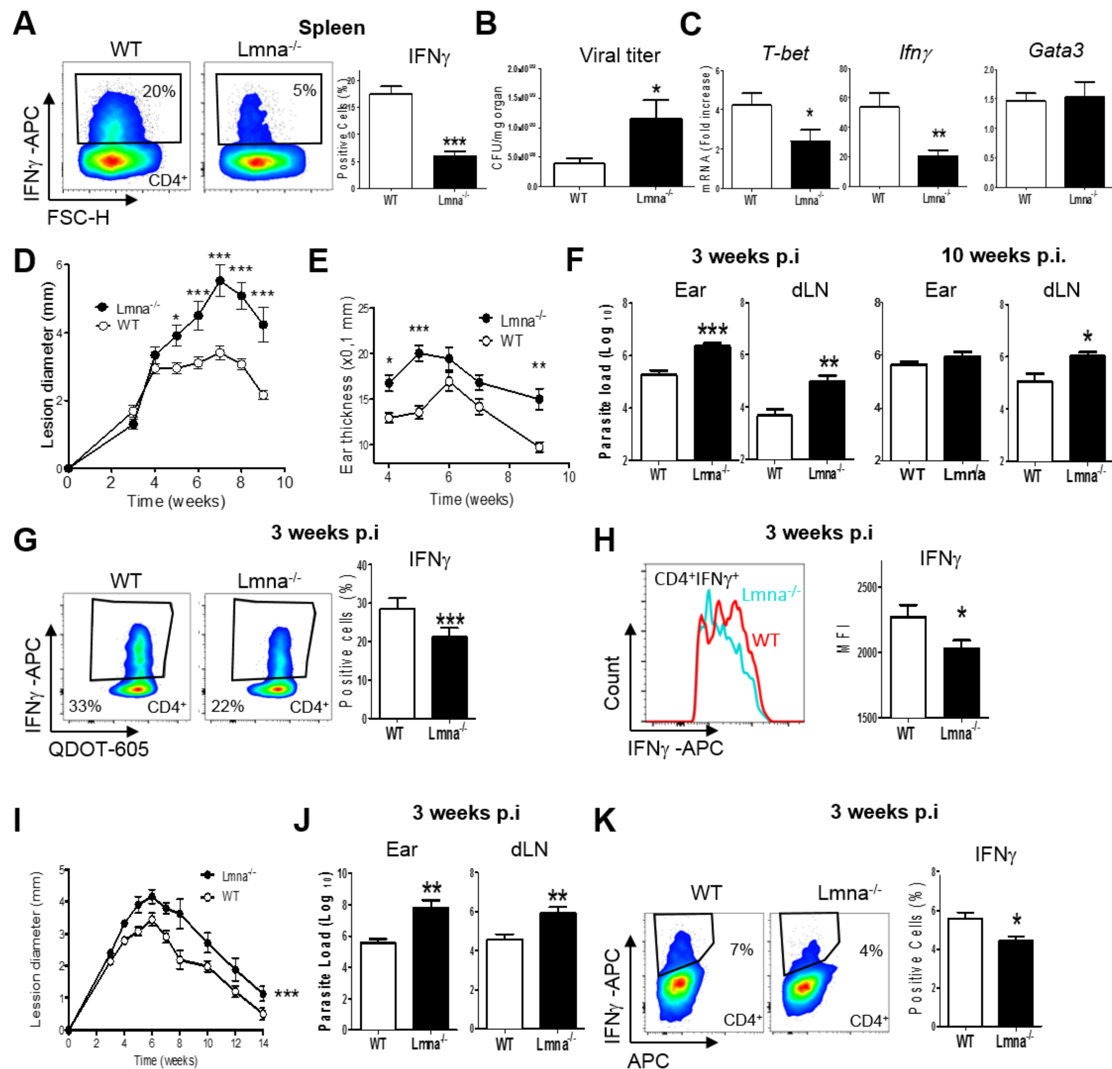

Figure 23. Lamin A/C deficiency enhances susceptibility to VACV and *Leishmania major*. (A-C) *Rag1*$^{-/-}$ mice were inoculated intravenously with naive WT or *Lmna*$^{-/-}$ CD4^{+} T-cells and infected with VACV by tail scarification. After 10 days, animals were analyzed to determine **(A)** the percentage of IFNγ^{+} CD4^{+} T-cells in the spleen (n=5 mice), **(B)** the viral titers in the tail (n=5 mice), and **(C)** mRNA levels of T-bet (Tbx21), IFNγ, and Gata-3 in the gut (n= 7 and 8 mice). **(D-H)** WT mice were adoptively transferred with WT or *Lmna*$^{-/-}$ bone marrow. Two months after reconstitution, *Leishmania major* promastigotes were injected intradermally in the ears (n=8-9 mice). Lesion size **(D)** and ear thickness **(E)** were quantified at the indicated times. **(F)** Parasite burden in ears and draining lymph nodes was measured 3 and 10 weeks after infection. **(G-H)** The percentages of **(G)** IFNγ^{+} CD4^{+} T-cells and **(H)** the CD4^{+} T-cell IFNγ content was quantified in the ears 3 weeks after infection. **(I-K)** WT and CD4-CRE *Lmna*$^{flox/flox}$ mice were injected intradermally in the ears with *Leishmania major* promastigotes (n=9). **(I)** Lesion size at the indicated times. **(J)** Parasite burden in the ears and draining lymph nodes at 3 weeks post-infection. **(K)** Percentage of IFNγ^{+} CD4^{+} T-cells in the ears. Data analysis in A-C, F-H, J-K: unpaired Student's t-test; D-E, I: two-way ANOVA with Bonferroni's post-hoc multiple comparison test. *P<0.05; **P<0.01; ***P<0.001.

6.3.7. Lamin A/C enhances cytotoxic capacity of $CD4^+$ T-cells.

Viral clearance requires the efficient coordination of multiple immune effector mechanisms. In accordance with our studies, the role of lamin A/C in viral clearance seems to be $CD4^+$ T-cell-dependent, since only $CD4^+$ T-cells were adoptively transferred to T and B-cell deficient *Rag1*$^{-/-}$ mice before mouse infection with VACV. Effector $CD4^+$ T-cells have been shown to respond to viral pathogens through 2 mechanisms: cytokine production (mostly IFNγ and TNFα), and a direct cytolytic activity mediated by perforin and FAS (also known as CD95) (Swain S.L. *et al*, 2012). It is known that the cytotoxic activity of $CD4^+$ T-cell effectors is dependent on the expression of the transcription factors T-bet, Eomesodermin (Eomes) and Blimp-1, which are crucial for the development of cytotoxic $CD4^+$ T-cells *in vivo* through the induction of Granzyme B (Gzmb) and Perforin 1 (Prf1) (Qui, H. Z. *et al*, 2011). In addition to increasing T-bet and IFNγ mRNA expression, we found that the presence of lamin A/C enhances the mRNA expression of *Eomes*, *Blimp1*, *Granzyme B* (*Gzmb*), *Perforin 1* (*Prf1*), but not *Granzyme A* (*Gzma*) in $CD4^+$ T-cells differentiated to Th1 *in vitro* (**Figure 24A**). Moreover, lamin A/C seems to play a role in $CD4^+$ T-cell cytotoxicity *in vivo* since VACV-OVA-generated *Lmna*$^{-/-}$ Th1 cells were deficient for OVA-loaded $B220^+$ $MHCII^+$ B-cell killing after the adoptive transfer of these cells to *Rag1*$^{-/-}$ mice (**Figure 24B**). In these experiments, *Rag1*$^{-/-}$ mice were inoculated intravenously with either naïve CD45.2/WT or CD45.2/*Lmna*$^{-/-}$ $CD4^+$/OTII T-cells and infected with VACV-OVA i.p. After 5 days, splenocytes from CD45.2/WT/dsRED and CD45.1/WT mice were isolated and loaded or not with OVA-OTII-peptide, respectively. A mix of 1:1 CD45.2/WT/dsRED and CD45.1/WT splenocytes were inoculated intravenously in the previously inoculated and infected *Rag1*$^{-/-}$ mice. After 16 hours, animals were analyzed to determine in the spleen by flow cytometry: **(B)** the killing capacity of CD4/OTII cells of each genotype by calculating the ratio between CD45.2/WT/dsRED/+OVA and CD45.1/WT/-OVA cells, and **(C)** the presence of CD4/OTII cells by the percentage of CD4/OTII T cells (**Figure 24B-C**).

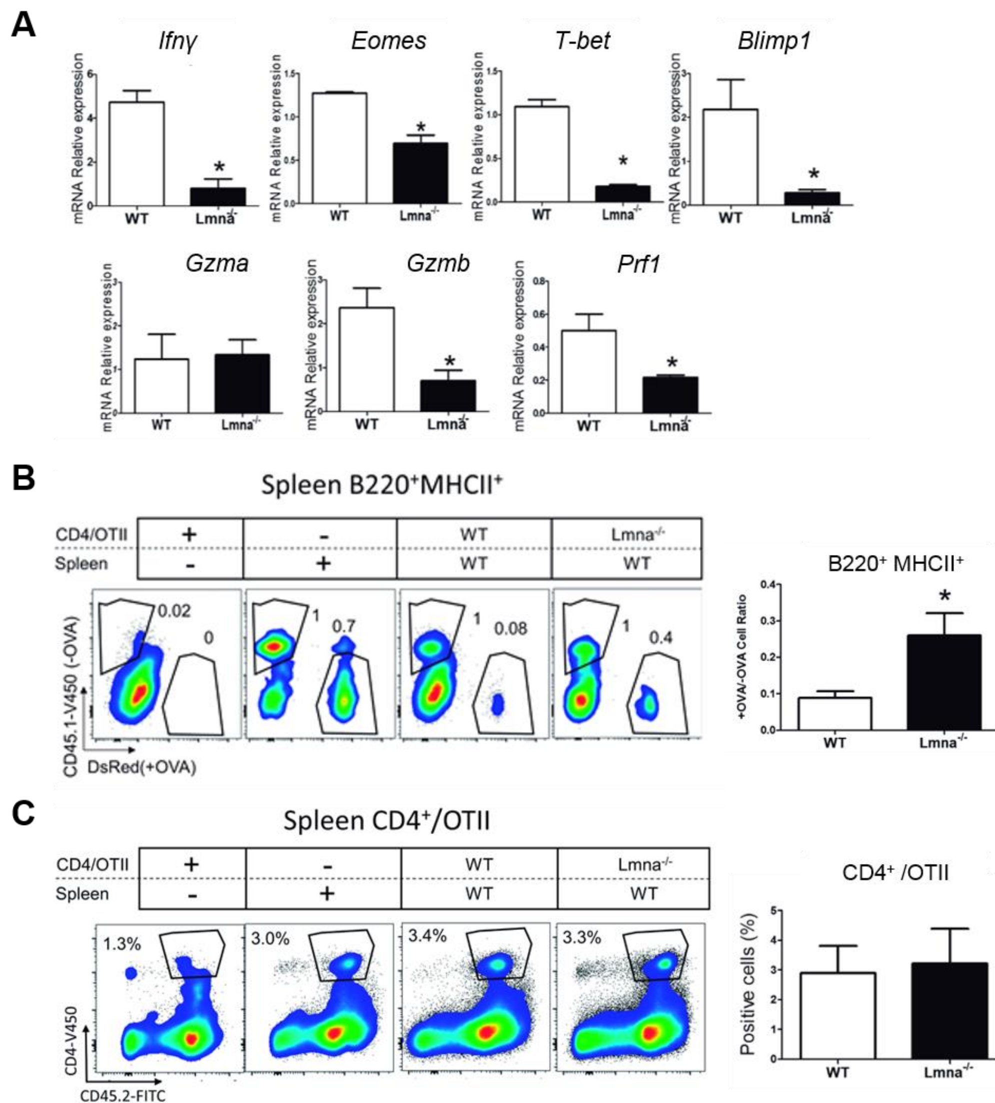

Figure 24. Lamin A/C enhances cytotoxic capacity of CD4^{+} T-cells *in vitro* and *in vivo*. (**A**) CD4^{+}CD25^{-} T-cells were isolated from spleens of WT and *Lmna*$^{-/-}$ mice and activated with anti-CD3/CD28 antibodies for 2 days. Some cells remained in culture with Th1 polarizing cytokines for additional 5 days. Graphs show the mRNA levels of the indicated genes quantified by RT-qPCR at 7 days. Data are means ± SEM of at least two independent experiments (n = 4 pools of 3 mice). **(B-C)** *Rag1*$^{-/-}$ mice were inoculated intravenously with either naïve CD45.2/WT or CD45.2/*Lmna*$^{-/-}$ CD4+/OTII T-cells and infected with VACV-OVA intraperitoneally. After 5 days, splenocytes from CD45.2/WT/dsRED and CD45.1/WT mice were isolated and loaded or not with OVA, respectively. A mix of approximately 1:1 CD45.2/WT/dsRED and CD45.1/WT splenocytes were inoculated intravenously in the previously inoculated and infected *Rag1*$^{-/-}$ mice. After 16 hours, animals were analyzed to determine in the spleen by flow cytometry: (**B**) The killing capacity of CD4/OTII cells of each genotype by calculating the ratio between CD45.2/WT/dsRED/+OVA and CD45.1/WT/-OVA cells and **(C)** the presence of CD4/OTII cells by the percentage of CD4/OTII T cells. Mice adoptively transferred with CD4/OTII cells but not splenocytes and, mice transferred with splenocytes without CD4/OTII were used as controls (n=8 from two independent experiments). Data are means ± SEM analyzed by unpaired Student’s t-test. * P<0.05.

6.4. Analysis of lamin A/C role in Treg polarization and suppressive function.

6.4.1. Lamin A/C deficiency in CD4$^+$ T-cells enhances Treg differentiation *in vitro*.

Since we discovered that lamin A/C controls Th1 differentiation (Toribio-Fernández R. *et al*, 2018), we decided to study if lamin A/C could regulate CD4$^+$ T-cell differentiation towards other Th phenotypes as Treg or Th17. To analyze that, naïve CD4$^+$ T-cells were isolated from spleen and LN of WT and *Lmna*$^{-/-}$ mice, and cultured *in vitro* with anti-CD3 and anti-CD28 antibodies, and Treg or Th17 polarizing cytokines. Interestingly, *Lmna*$^{-/-}$ CD4$^+$ T-cells produced significantly greater amount of iTreg-cells (CD25$^+$ Foxp3$^+$) than WT cells in all the experiments developed. Moreover, Foxp3 MFI analysis by flow cytometry showed that *Lmna*$^{-/-}$ CD4$^+$ T-cells express higher levels of Foxp3 than WT CD4$^+$ T-cells (**Figure 25A**). However, Th17 differentiation was very variable between experiments, and no significant differences were obtained between *Lmna*$^{-/-}$ and WT CD4$^+$ T-cells in cell percentage neither in IL-17 MFI (**Figure 25B**). These experiments demonstrate that lamin A/C impairs Treg differentiation *in vitro* by modifying Foxp3 expression levels, and validate that lamin A/C is an important regulator of T-cell differentiation following TCR stimulation.

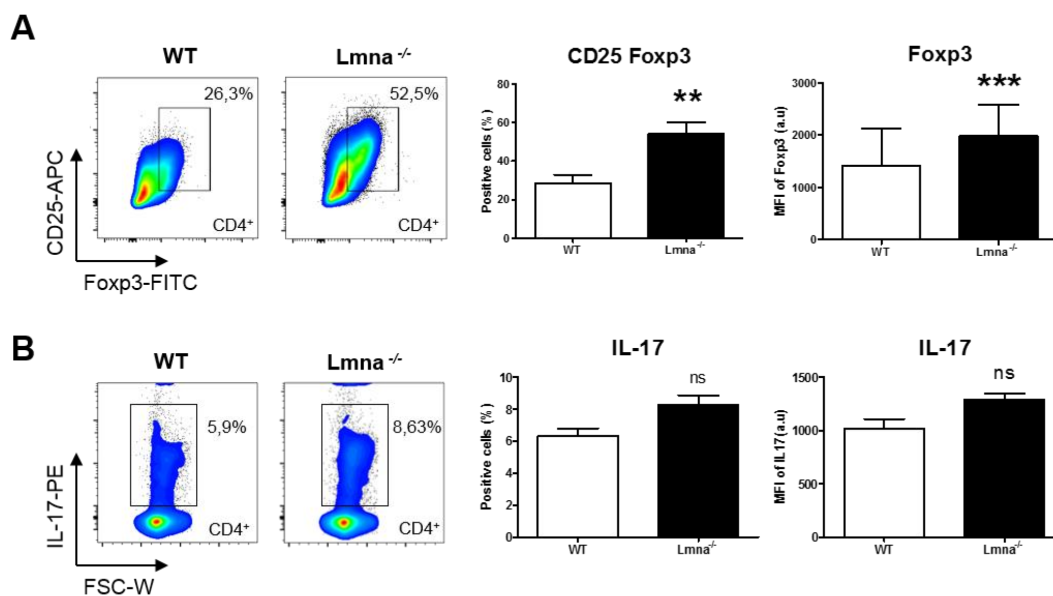

Figure 25. Enhanced *in vitro* Treg differentiation and Foxp3 expression in CD4$^+$ T-cells from *Lmna*$^{-/-}$ mice. Naïve CD4$^+$ T-cells were isolated from WT and *Lmna*$^{-/-}$ mice spleens and stimulated with antibodies anti CD3/CD28 for 5 days in the presence of cytokines to trigger **(A)** Treg differentiation and **(B)** Th17 differentiation. Plots and graphs show the percentage of **(A)** Treg and **(B)** Th17 differentiated cells of the total CD4$^+$ T-cells (n=3-6, pools of at least 2 mice); and **(A)** Foxp3 mean fluorescence intensity (MFI) of Foxp3$^+$ CD4$^+$ T-cells, and **(B)** IL-17 MFI of IL-17$^+$ CD4$^+$ T-cells T-cells (n=7 pools of 2 mice). Data are means ± SEM of at least three independent experiments analyzed by unpaired Student's t-test. $^{**}P<0.01$;$^{***}P<0.001$.

6.4.2. Lamin A/C overexpression in CD4$^+$ T-cells impairs Treg differentiation.

To demonstrate that lamin A/C impairs Treg differentiation, we decided to perform an *in vitro* overexpression of lamin A/C by the transfection with a GFP-lamin A/C retrovirus. To do that, we produced retrovirus *in vitro* with GFP-empty or GFP-lamin A/C vectors. In parallel, we isolated splenocytes from CD45.1 WT/OTTI and CD45.2 *Lmna*$^{-/-}$/OTII, and cultured them in the presence of

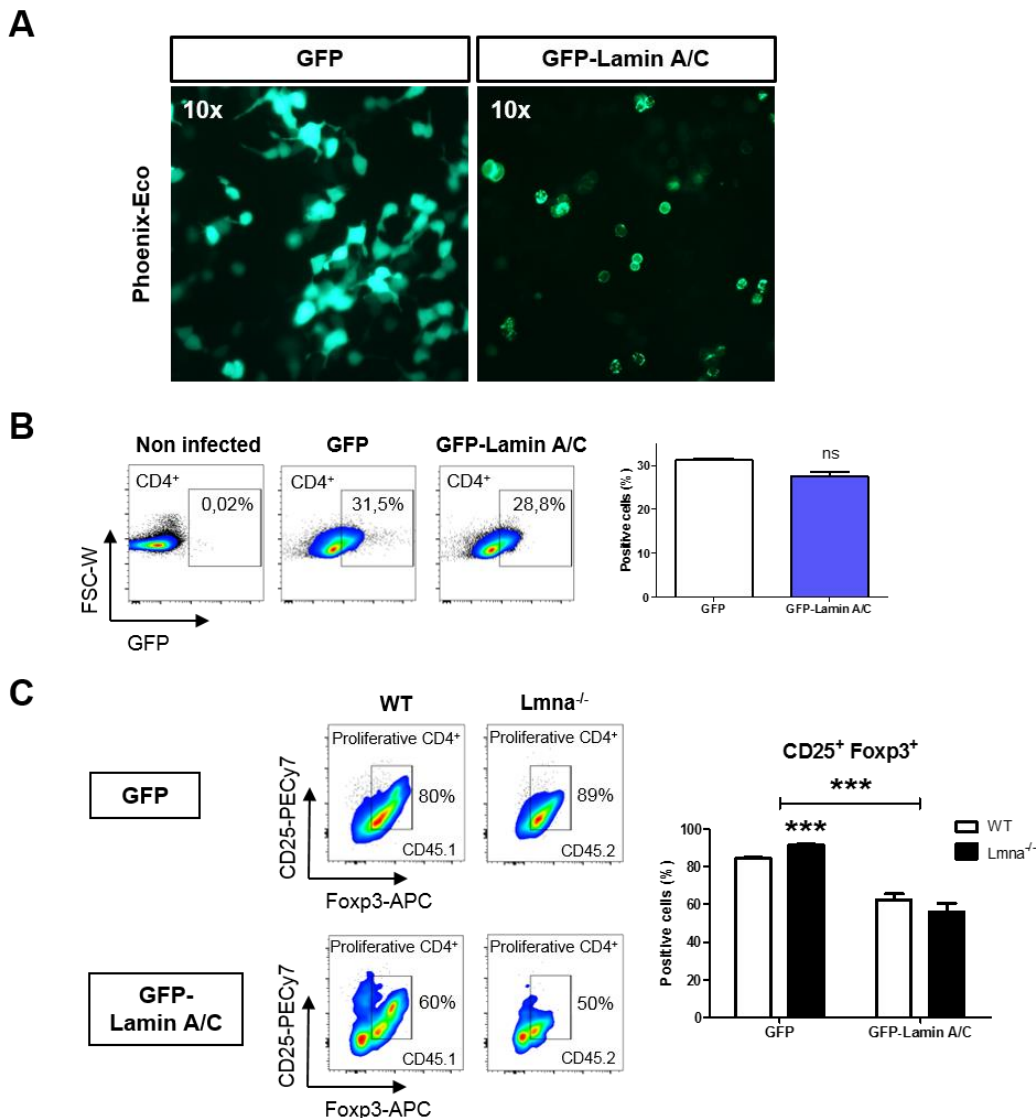

Figure 26. Lamin A/C overexpression impairs Treg differentiation. Splenocytes were isolated from spleen of CD45.1 WT/OTII mice and CD45.2 Lmna$^{-/-}$/OTII and *in vitro* cocultured in the presence of OVA-peptide for 48 h. At this time, splenocytes were transfected with GFP or GFP-lamin A/C retrovirus and *in vitro* cultured with cytokines to promote Treg differentiation **(A)** Representative micrographs of GFP or GFP-lamin A/C retrovirus Phoenix-Eco producer cells. 10x magnification. **(B)** Plots of GFP$^+$ transfected splenocytes and its quantification graph (n=3 mice). Data are means ± SEM of one representative experiment from three by Student's t-test. **(C)** Percentage of CD25$^+$ Foxp3$^+$ cells by flow cytometry analysis. Representative plots are shown (n=6 mice). Data are means ± SEM of two independent experiments by one-way ANOVA with Bonferroni's multiple comparison test. ns= not significant ; ***P<0.001.

OVA/OTII-peptide, which induced their activation. After 48 h, GFP expression was checked by fluorescence microscopy in Phoenix-Eco cells (**Figure 26A**). Then, we infected splenocytes by spinofection, and after 72 h, GFP expression levels was studied by flow cytometry in $CD4^+$ T-cells. The infection similarly worked with GFP-empty or GFP-lamin A/C viruses (**Figure 26B**). Additionally, cytokines to promote Treg differentiation were added to splenocytes after the spinofection protocol, and $CD25^+$ $Foxp3^+$ cell percentage was analyzed at day 5 of *in vitro* differentiation. GFP-lamin A/C expressing $CD4^+$ T-cells exhibited significantly lower Treg differentiation in comparison to GFP-empty vector expressing cells. Even though, in the case of GFP-empty vector expressing $CD4^+$ T-cells, *Lmna*$^{-/-}$/OTII showed significant upper Treg polarization than WT/OTII as was expected according our previous results (**Figure 26C**).

6.4.3. Lamin A/C controls Foxp3 expression but does not modulate it epigenetically.

To study the mechanism by which lamin A/C intervenes in Treg differentiation, we analyzed by RT-qPCR the mRNA expression levels of *Foxp3* in *in vitro* activated *Lmna*$^{-/-}$ and WT T-cells with anti-CD3/CD28 for 48 h. In parallel we also analyzed *Rorγt* expression to confirm that lamin A/C does not regulate Th17 differentiation. Thus, lamin A/C deficient T-cells exhibited an enhanced *Foxp3* expression at mRNA level, while *Rorγt* expression was the same in comparison whith WT T-cells (**Figure 27A**).

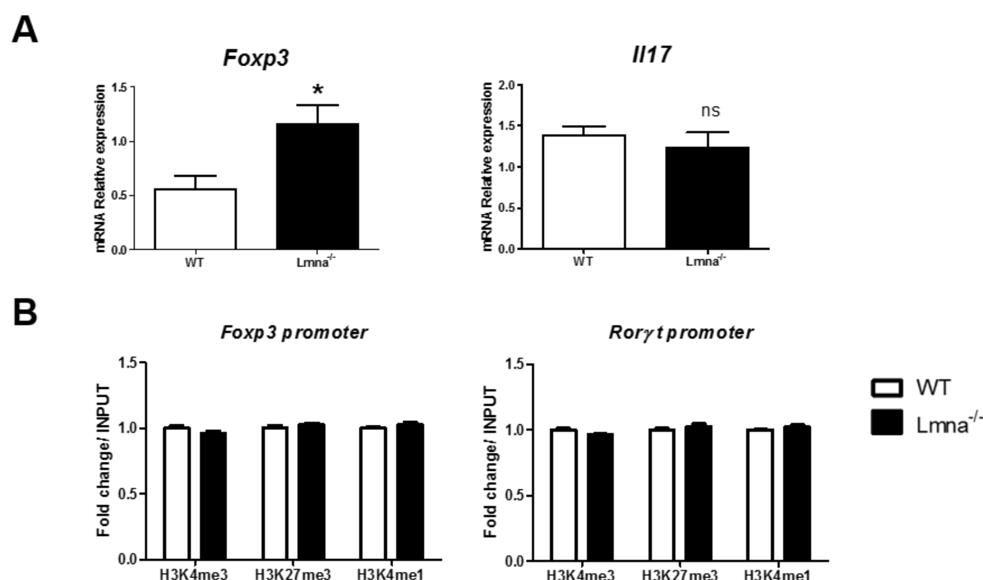

Figure 27. Lamin A/C controls *Foxp3* expression but does not promote any epigenetic modification in *Foxp3* promoter. $CD4^+$ T-cells were isolated and activated *in vitro* with anti-CD3/CD28 for 48 h. (**A**) mRNA expression of *Foxp3* and *Rorγt* genes was measured by RT-qPCR (n=6 mice). (**B**) ChIP-qPCR analysis of the indicated histone modifications in *Foxp3* and *Rorγt* promoters. Data is shown as fold change relative to WT and input sample (n=2 pool of 4 mice). Data are means ± SEM analyzed by unpaired Student's t-test. * $P<0.05$, ns= not significant.

Moreover, we decided to address if lamin A/C could modulate also epigenetic modifications in *Foxp3* promoter. So that, we developed ChIP-qPCR assays with *Lmna*$^{-/-}$ and WT CD4^{+} T-cells *in vitro* activated in the same conditions. Our results showed no changes between *Lmna*$^{-/-}$ and WT CD4^{+} T-cells in any of the epigenetic modifications studied (H3K4me3, H3K27me3, and H3K4m1) for *Foxp3* neither *Rorγt* promoter (**Figure 27B**).

6.4.4. Lamin A/C mediates Treg and Th1differentiation in IBD mice model.

In order to study in parallel the role of lamin A/C in Treg and Th1 differentiation and in an *in vivo* physiological condition, we decided to perform an IBD model by the adoptive transfer of *Lmna*$^{-/-}$ or WT naïve CD4^{+} CD25^{-} T-cells into a T- and B-cell immunodeficient *Rag1*$^{-/-}$mice, a well-stablished IBD mouse model (**Figure 28A**). As it was mentioned before, this

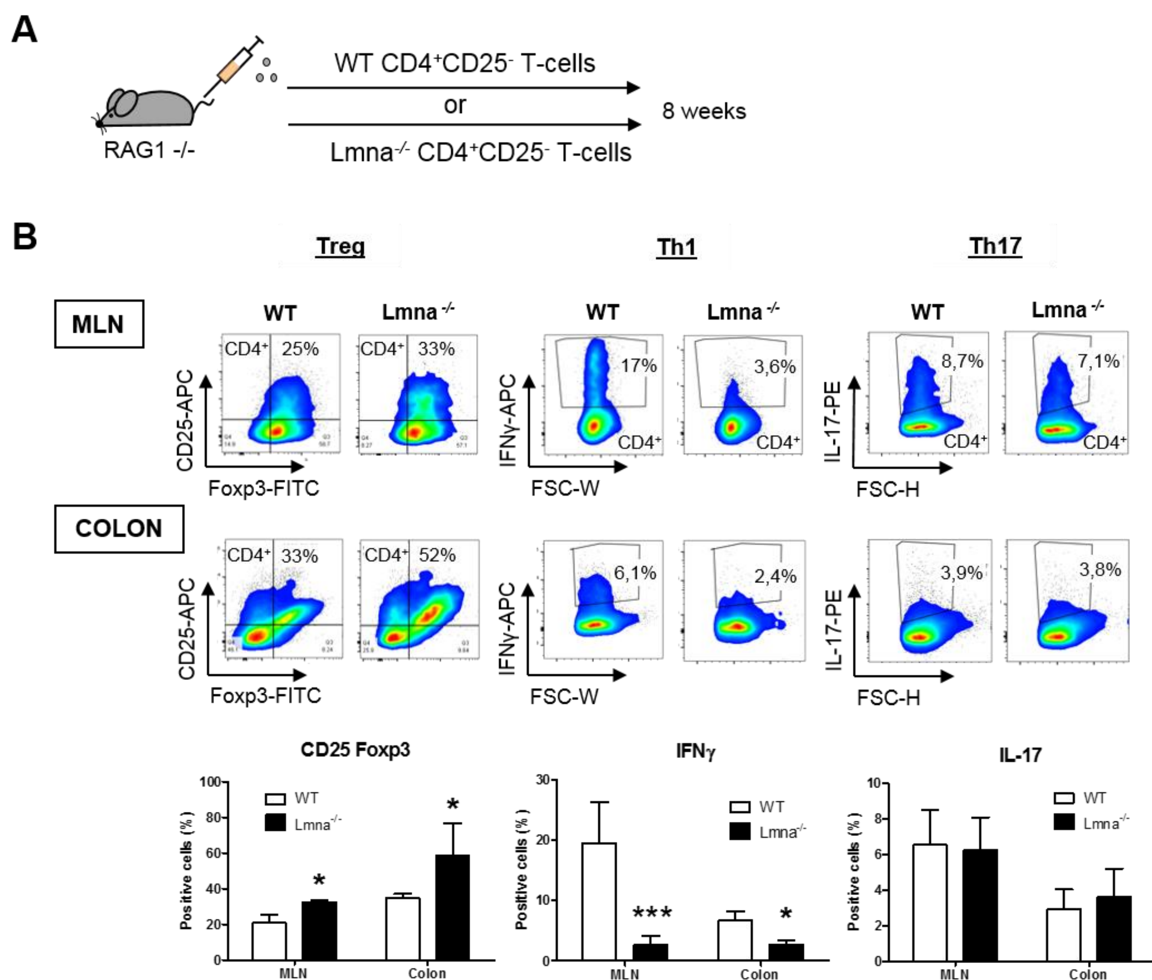

Figure 28. A-type lamins mediate Treg and Th1 differentiation in IBD mice model. **(A)** *Lmna*$^{-/-}$ and WT CD4^{+} CD25^{-} T-cell were adoptively transferred in *Rag1*$^{-/-}$ mice to induce IBD development during 8 weeks. **(B)** Quantification of the percentage of CD25^{+} Foxp3^{+}, IFNγ and IL-17 producer cells of the total CD4^{+} T-cells in mesentheric lymph nodes (MLN) and lamina propria of the colon (n=3, representative experiment from 5). Data are means ± SEM of 3 mice analyzed by unpaired Student's t-test. *P<0.05;***P<0.001.

model simulates human Crohn's disease in which Treg and Th1 are main players of the immune response (Powrie F. *et al*, 1993).

After 8 weeks of disease development, mice were sacrificed and Treg, Th1 and Th17 populations were analyzed in MLN and colon by flow cytometry. As in the preceding experiments, $CD25^+$ $Foxp3^+$ T-cells were significantly increased in *Lmna*$^{-/-}$ $CD4^+$ $CD25^-$ T-cells adoptively transferred mice, compared with WT $CD4^+$ $CD25^-$ T-cells adoptively transferred mice. As well, IFNγ levels revealed less Th1 differentiation when lamin A/C was absent in T-cells. In addition, IL-17 was also measured to analyzed Th17 differentiation, and no differences were found between *Lmna*$^{-/-}$ and WT $CD4^+$ T-cells in any of organs studied (**Figure 28B**).

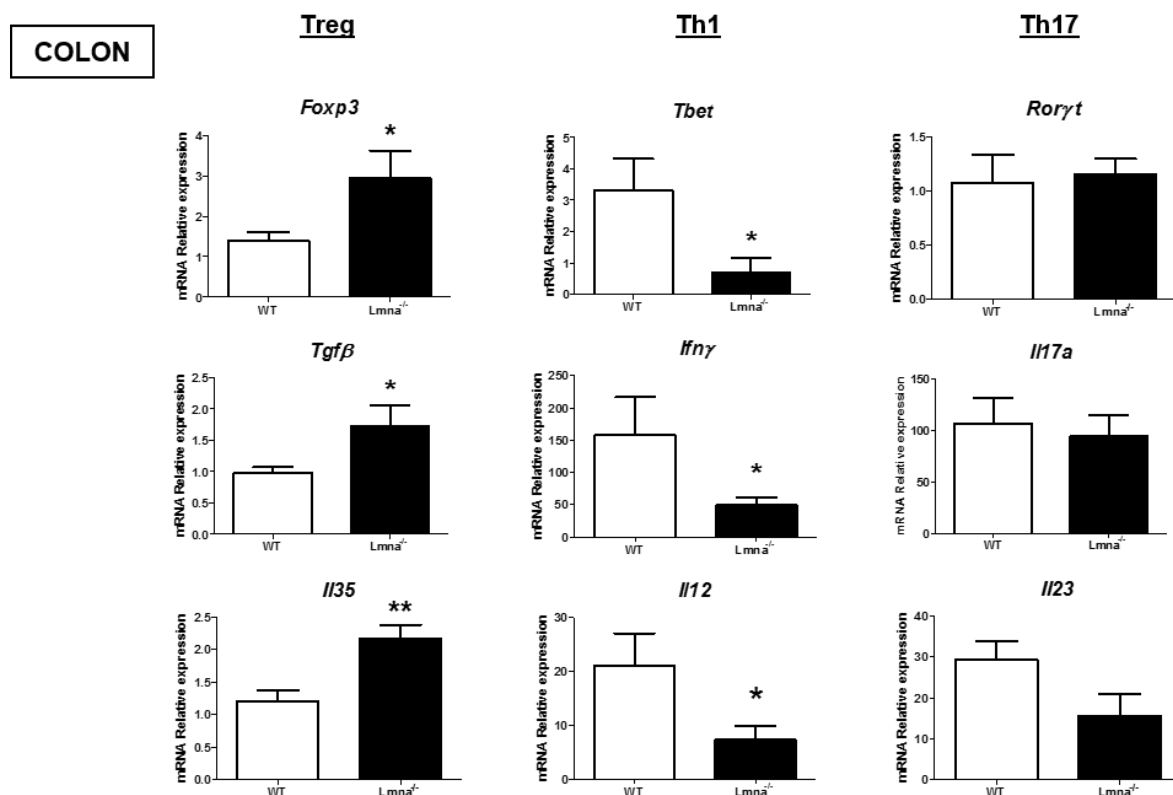

Figure 29. mRNA expression levels confirm the role of A-type lamins in Treg and Th1 differentiation. RT-qPCR analysis of indicated genes related with Treg (*Foxp3*, *Tgfβ*, and *Il-35*), Th1 (*T-bet*, *Ifnγ*, and *Il-12*) and Th17 (*Rorγt*, *Il-17a*, and *Il-23*) differentiation in colon (n=6 mice from a representative experiment from 5). Data are means ± SEM of 3 mice analyzed by unpaired Student's t-test. *P<0.05; **P<0.01.

In order to further study the Th populations and their functionality *in vivo* against the IBD, mRNA expression levels were also evaluated in colon. Thus, results showed the same tendency that we have observed before: an enhanced Treg differenciation, and on the contrary, a reduced Th1 differentiation when *Lmna*$^{-/-}$ $CD4^+$ $CD25^-$ T-cells were transferred to *Rag1*$^{-/-}$ mice. Consequently, *Foxp3*, *Tgfβ*, and *Il35* mRNA expression levels were augmented, while *Tbet*, *Ifnγ*, and *Il12* were diminished in *Lmna*$^{-/-}$ $CD4^+$ $CD25^-$ T-cells adoptively transferred mice, in comparison to WT. Again, no differences were observed

in expression levels of the genes related with Th17 cells, *Rorγt* and *Il17a*. However, *Il23* expression levels were reduced in *Lmna*$^{-/-}$ CD4^{+} CD25^{-} T-cells adoptively transferred mice, in comparison with WT, although the obtained results were not significantly different (**Figure 29**).

6.4.5. Lamin A/C deficiency in CD4^{+} T-cells protects against IBD development.

Besides the analysis of Th and Treg populations described before, during the IBD mouse model development, other measures were studied to define the inflammatory process expected for this colitis model. Weightloss and symptoms were monitored twice a week, and revealed that mice adoptively transferred with *Lmna*$^{-/-}$ CD4^{+} CD25^{-} T-cells, developed a mild disease, showing less weightloss and less symptomatology in comparison with WT CD4^{+} CD25^{-} T-cells adoptively transferred mice. (**Figure 30A**).

Additionally, colon length was measured. This is a common feature of colitis that reveals the inflammation and healing of the tissue (Lindebo Holm T. *et al*, 2012). *Lmna*$^{-/-}$ T-cells transferred mice exhibited less shortening of the colon, which means less colon inflammation, compared with WT (**Figure 30B**). To assess the anti-inflammatory phenotype of *Lmna*$^{-/-}$ CD4^{+} T-cells in IBD, IL-10, an anti-inflammatory cytokine widely studied in this disease (Schreiber S. *et al*, 1995; Szkaradkiewicz A. *et al*, 2009; Zhu L. *et al*, 2017), was quantified by flow cytometry in MLN (**Figure 30C**) and in colon by RT-qPCR (**Figure 30D**). As we expected, IL-10 expression levels were augmented in mice that were transferred with *Lmna*$^{-/-}$ T-cells, compared with mice transferred with WT T-cells. Other cytokines were also analyzed by RT-qPCR in the colon of these mice. In line with the results described above, expression levels of *Il6*, a pro-inflammatory cytokine (Szkaradkiewicz A *et al*, 2009), were diminished in *Lmna*$^{-/-}$ T-cells transferred mice; and on the contrary, mRNA expression of *Il22*, another anti-inflammatory cytokine (Sugimoto K *et al*, 2008; Leung J.M. *et al*, 2013; Mizoguchi A. *et al*, 2018), was increased (**Figure 30D**).

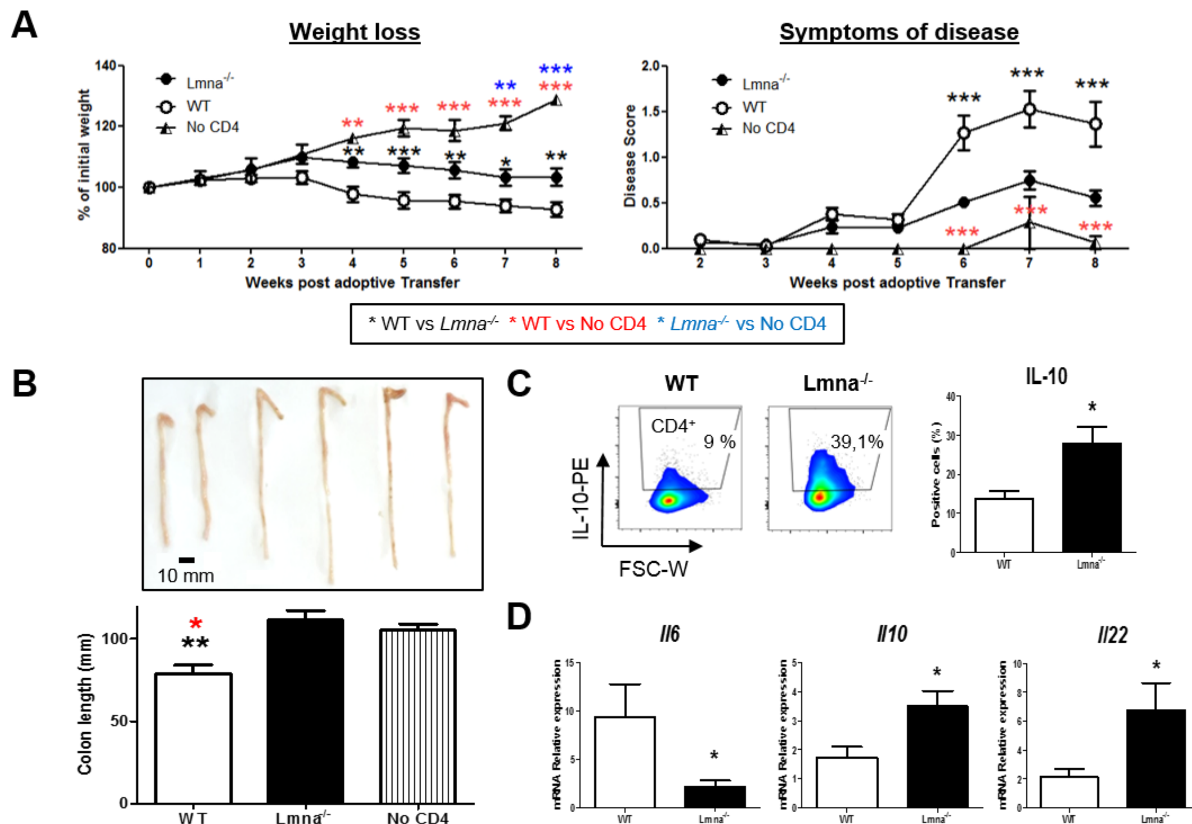

Figure 30. A-type lamins protect from IBD development in mouse. *Rag1*$^{-/-}$ mice were adoptively transferred with WT or *Lmna*$^{-/-}$ CD4^{+} CD25^{-} T-cells to generate IBD. Mice were sacrificed 8 weeks after T-cell transfer. **(A)** Evolution of body weightloss and colitis symptoms during IBD development (n=6-7, at least 6 mice from a representative experiment from 5). Data are means ± SEM of at least 6 mice analyzed by two-way ANOVA with Bonferroni's multiple comparison test. **(B)** Representative photograph of colons and measurement of colon length (mm) after 8 weeks of IBD development (n=6-7, at least 6 mice from a representative experiment from 5). Data are means ± SEM of at least 6 mice analyzed by one-way ANOVA with Bonferroni's multiple comparison test. **(C)** Quantification of IL-10 producer cells of the total CD4^{+} T-cells in mesenteric lymph nodes (MLN) after 8 weeks of IBD development **(D)** RT-qPCR analysis of indicated genes related with inflammation in the colon after 8 weeks of IBD development (n=7, at least 7 mice from a representative experiment from 5). Data are means ± SEM of at least 6 mice analyzed by unpaired Student's t-test. *P<0.05; **P<0.01; ***P<0.001.

Furthermore, histopathological analysis was performed in colon slices stained with hematoxylin-eosin to assess the severity of colitis. The following features were taken into account for disease score quantification: leukocyte infiltration, goblet cell depletion, epithelial hyperplasia, crypt damage, and submucosal inflammation. Ulceration was also considered when it was present. Representative micrographs clearly showed that mice transferred with *Lmna*$^{-/-}$ CD4^{+} T-cells, displayed a slight colitis compared with mice transferred with WT CD4^{+} T-cells, which exhibited a severe colon inflammation (**Figure 31**).

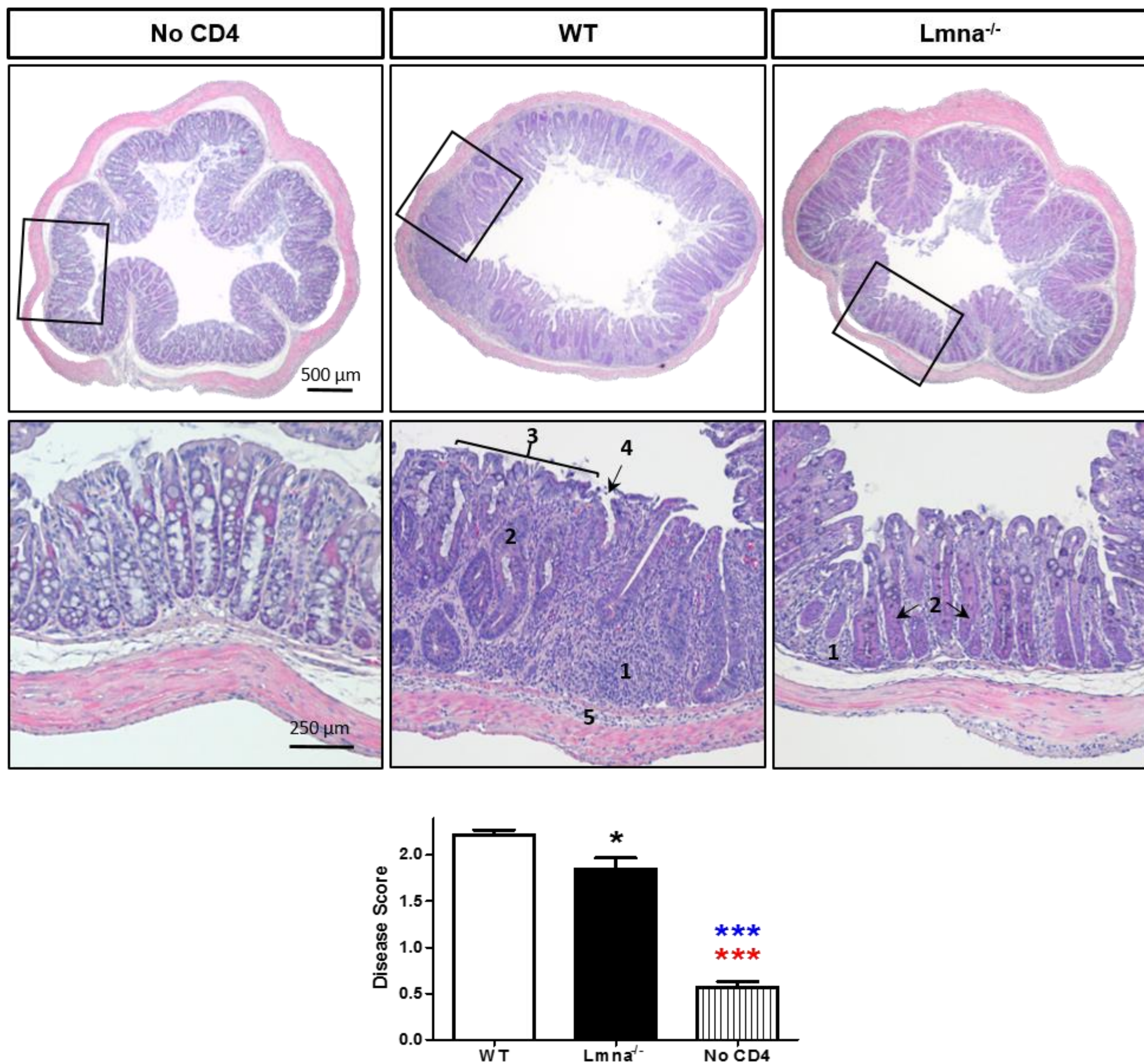

Figure 31. Histopathological examination of colon corroborates that lamin A/C deficiency in CD4+ T-cells protects against IBD development. Representative micrographs of hematoxylin-eosin stained colon sections (bars of 500 µm at 4x magnification, and 250 µm at 10x magnification). Graph shows disease score (0-3) recapitulating markers of intestinal inflammation quantification indicated as follows: leukocyte infiltration (1), goblet cell depletion (2), epithelial hyperplasia (3), crypt damage (4), and submucosal inflammation (5) (n=6-7, at least 6 mice from a representative experiment from 5). Data are means ± SEM analyzed by one-way ANOVA with Bonferroni's multiple comparison test. *P<0.05;**P<0.01;***P<0.001.

6.4.6. Lamin A/C deficiency in CD4$^+$ T-cells confers greater Treg suppressive function.

Next, we decided to study if lamin A/C deficiency not only enhances Treg differentiation, but also could improve Treg functionality. Naïve CD4$^+$ CD25$^-$ T-cells were isolated from *Lmna*$^{-/-}$ and WT mice and *in vitro* differentiated towards Treg phenotype. At 5 days of differentiation, CD25$^+$ iTregs were positively selected by antibody and magnetic bead binding (**Figure 32A**). The obtained iTregs were characterized by RT-qPCR, analyzing mRNA expression levels of cytokines that are commonly released by Treg cells (IL-10, TGFβ, and IL-35), Treg surface receptors of CD28 family (CTLA4, ICOS, PD-1), and other characteristic Treg surface molecules (CD25, GITR, LAG-3, and CD49b). *Il10, Tgfβ, Cd25*, *Gitr* and *Pd1* mRNA expression was significantly upgraded in Tregs derived from *Lmna*$^{-/-}$ T-cells (indicated as *Lmna*$^{-/-}$ Tregs), in comparison with those derived from WT T-cells (indicated as WT Tregs). Additionally, *Lag3* and *Cd49b* expression was also augmented in Tregs derived from *Lmna*$^{-/-}$ T-cells, although the difference with WT was not so significant (**Figure 32B**). Moreover, we performed an *in vitro* suppression assay in order to test the capacity of these Tregs to inhibit Th1 proliferation. This experiment showed that Tregs derived from *Lmna*$^{-/-}$ T-cells exhibit greater suppressive capacity against Th1 cells proliferation, than Tregs derived from WT T-cells (**Figure 32C**).

However, these experiments were performed with CD25$^+$ iTregs, so that, to corroborate this data with CD25$^+$ Foxp3$^+$ iTregs, we created a RFP-Foxp3 and *Lmna*$^{-/-}$ in CD4$^+$ T-cells mice model. RFP-Foxp3 permitted us to select by FACS the CD25$^+$ Foxp3$^+$ *in vitro* differentiated Tregs (iTregs) (**Figure 33A**), and perform again an *in vitro* suppressive assay over Th1 cells. Percentage of IFNγ$^+$ cells were measured by flow cytometry showing again that iTregs from *Lmna*$^{-/-}$ CD4$^+$ T-cells have better suppressive function than iTregs derived from WT (**Figure 33B**).

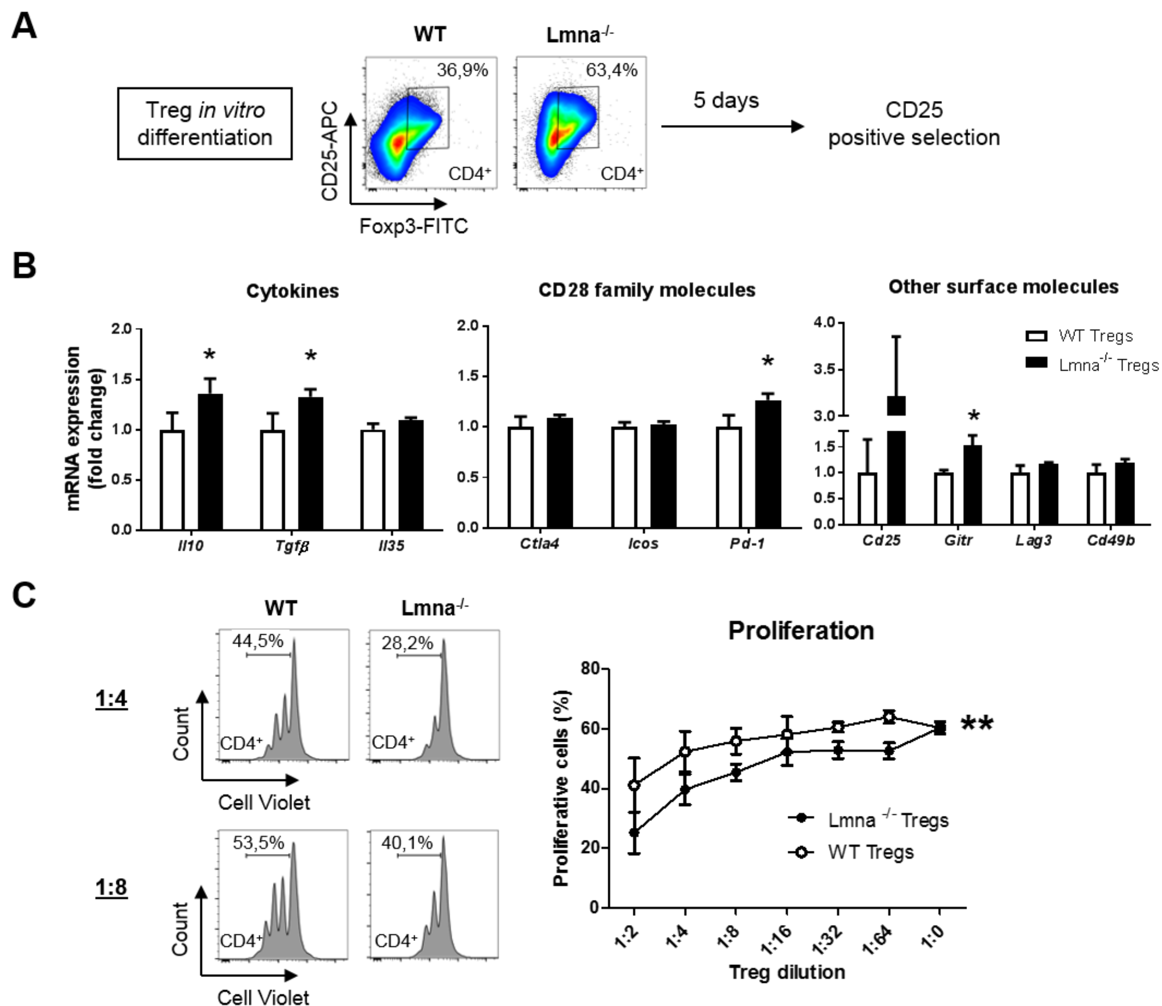

Figure 32. CD25⁺ Tregs derived from *Lmna*$^{-/-}$ CD4⁺ T-cells show better suppressive function than Tregs derived from WT CD4⁺ T-cells. Naïve CD4 ⁺ T-cells were isolated from WT and *Lmna*$^{-/-}$ mice spleens, stimulated with antibodies anti CD3/CD28, and polarized towards Treg in the presence of cytokines. **(A)** At day 5 of *in vitro* Treg differentiation, CD25⁺ Tregs were positively selected by magnetic columns. **(B)** RT-qPCR analysis of indicated Treg-related genes; mRNA expression is show as fold change *v.s* WT (n=6-9, at least 6 pool of 2 mice). Data are means ± SEM of 6 pool of 2 mice analyzed by Student's t-test. **(C)** Treg suppression assay with *in vitro* differentiated CD25⁺ Tregs from CD45.2 WT and *Lmna*$^{-/-}$ mice, and naïve CD4⁺ T-cells isolated from CD45.1 WT mice spleens. Tregs were cocultured in the indicated serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:0) *v.s* a constant naïve CD4⁺ T-cell concentration in the presence of IL-12, IL-2 and soluble anti-CD3/CD28 antibodies. Naïve CD4⁺ T-cells were stained with Cell Violet to quantify the Th1 proliferation rate. Representative plots of 1:4 and 1:8 Treg dilution are shown and percentage of proliferative cells is shown in the graph (n=4 mice of a representative experiment of two). Data are means ± SEM of 4 mice analyzed by two-way ANOVA with Bonferroni's multiple comparison test. *P<0.05;**P<0.01;***P<0.001.

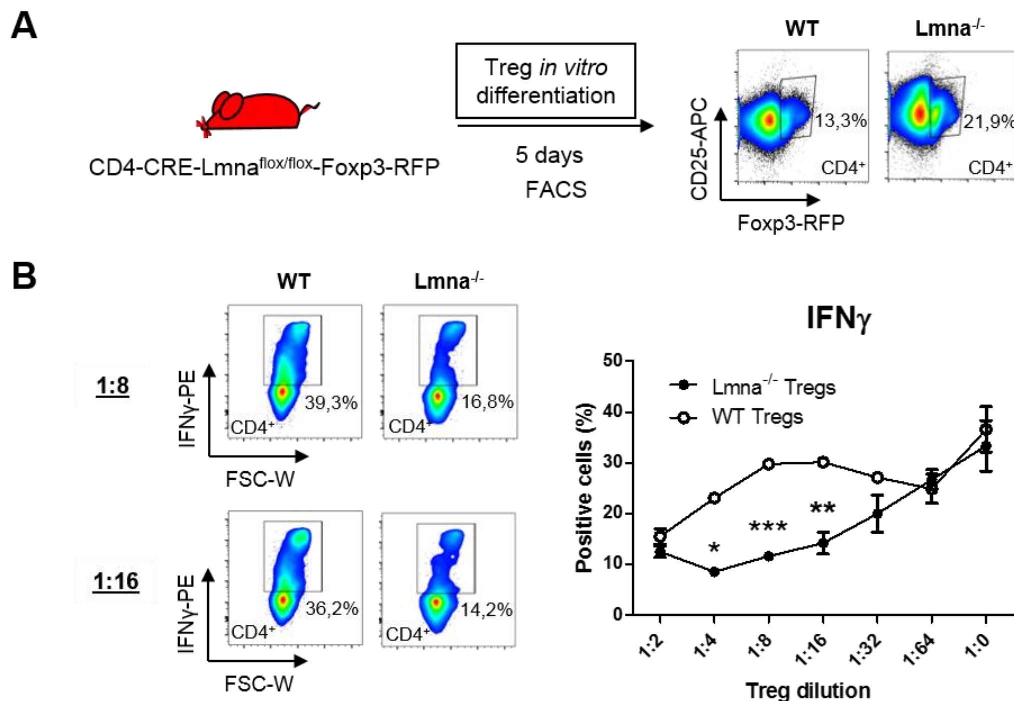

Figure 33. CD25⁺ Foxp3⁺ Tregs derived from *Lmna*$^{-/-}$ CD4⁺ T-cells display superior suppressive function than Tregs derived from WT CD4⁺ T-cells. **(A)** At day 5 of *in vitro* Treg differentiation, Foxp3-RFP⁺ Tregs from CD4-CRE-*Lmna*$^{flox/flox}$-Foxp3-RFP mice were selected by FACS. **(B)** These WT and *Lmna*$^{-/-}$ Foxp3-RFP⁺ Tregs were cocultured with naïve CD4⁺ T-cells isolated from CD45.1 WT in the presence of IL-12, IL-2 and soluble anti-CD3/CD28, to carry out the same suppression assay described before in Figure 28. Representative plots of 1:8 and 1:16 Treg dilution are shown, and percentage of IFNγ⁺ cells is shown in the graph (n=3 pool of 2 mice of a representative experiment of two). Data are means ± SEM analyzed by two-way ANOVA with Bonferroni's multiple comparison test. *P<0.05;**P<0.01;***P<0.001.

6.5. Study of the physiological mechanism that regulates lamin A/C expression in CD4⁺ T-cells upon antigen recognition.

6.5.1. Retinoic acid downregulates lamin A/C expression in CD4⁺ T-cells.

As it was mentioned before, it has been described that RA downregulates lamin A/C expression in leukocytes, and that *LMNA* gene has a response element for RA (Olins A.L. *et al*, 2001; Okumura K. *et al*, 2004). Therefore, we decided to analyzed if RA downregulates lamin A/C in CD4⁺ T-cells. To study that, we isolated naïve CD4⁺ T-cells from WT mice, and activated them *in vitro* in the presence or not of RA. We maintained RA treatment during 48 h, moment in which lamin A/C raises its maximum expression level. Precisely at that moment, we harvested and fixed the cells and analyzed lamin A/C expression by immunofluorescence. Our results corroborated that RA treatment *in vitro* downregulates lamin A/C expression in activated CD4⁺ T-cells. We observed less percentage of lamin A/C⁺ cells (**Figure 34A**), and less lamin A/C MFI per cell analyzed by Imaris software (**Figure 34B**).

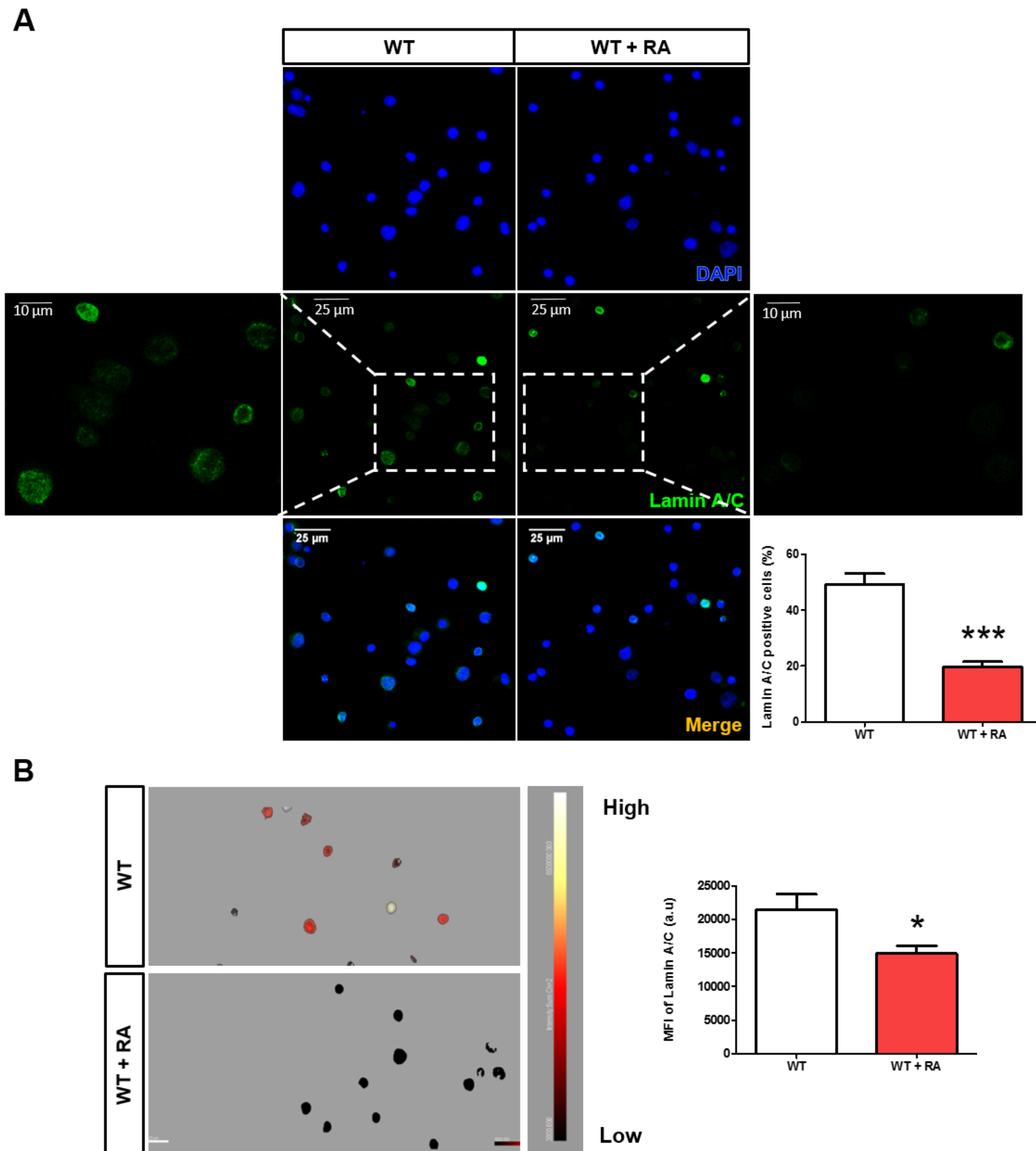

Figure 34. Retinoic acid (RA) downregulates lamin A/C expression levels in CD4⁺ T-cells. Naïve CD4⁺ T-cells were isolated from WT mice spleens and stimulated with anti CD3/CD28 antibodies for 2 days in the presence of retinoic acid (RA), or its solvent as a control to compare with. **(A)** Immunofluorescent labelling of the nuclear envelope protein lamin A/C (green) in 48 h-activated CD4⁺ T-cells counterstained with DAPI (blue). Merge of both colors is also showed. Scale bars of 25 µm. Graph shows the percentage of lamin A/C expressing cells quantification. **(B)** Representative figure of volume, intensity analysis with Imaris software of lamin A/C staining, and MFI analysis expressed in arbitrary units of a Z-stack maximal projection (n=6 samples from 3 pool of 2 mice). Data are means ± SEM analyzed by unpaired Student's t-test. *P<0.05;***P<0.001.

6.5.2. Retinoic acid-producing CD103^{+} DCs from mesenteric lymph nodes regulate physiologically lamin A/C expression in CD4^{+} T-cells.

To study if lamin A/C expression is physiologically regulated depending on the lymphoid organ, we analyzed lamin A/C protein expression by flow cytometry in spleen, MLN, and PLN, upon VACV i.p. infection (spleen and MLN analysis) or i.d infection in the footpad (PLN analysis) to promote CD4^{+} T-cell activation. Six days post infection we observed that lamin A/C expression was decreased in CD4^{+} T-cells from spleen and MLN, but it was higher in CD4^{+} T-cells from PLN (**Figure 35A**).

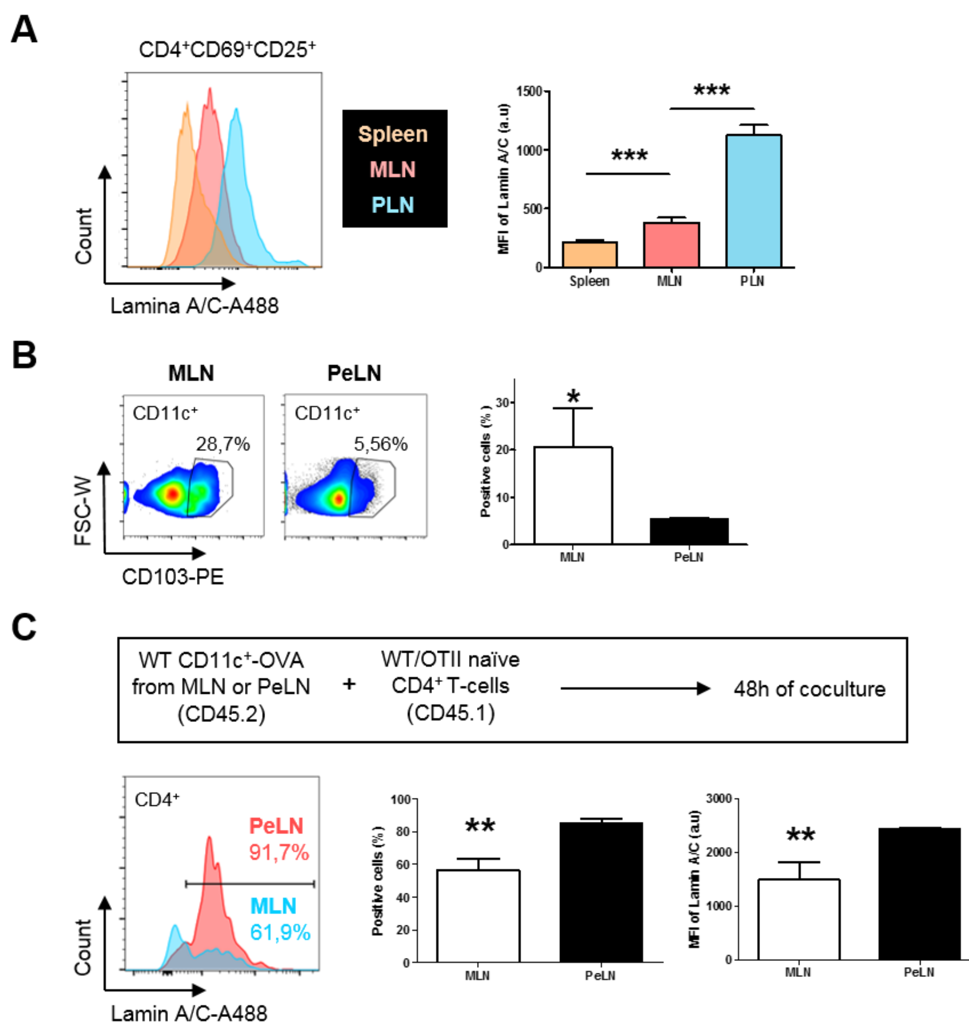

Figure 35. CD103^{+} DC population is mostly present in mesentheric lymph nodes (MLN), and downregulates lamin A/C expression in CD4^{+} T-cells. CD11c^{+} DCs were isolated from MLN or peripheral lymph nodes (PeLN) of CD45.2 WT mice. **(A)** Lamin A/C MFI analysis by flow cytometry of CD4^{+}CD69^{+} CD25^{+} T-cells upon intraperitoneal (spleen and MLN) and footpad (PLN) VACV infection (n=5 mice). **(B)** Representative plots show the enrichment of MLN in CD11c $^{+}$CD103 $^{+}$ DC *v.s* PeLN. Graph shows the percentage of CD11c $^{+}$ CD103 $^{+}$ DC in MLN and PeLN (n=4 mice). **(C)** Coculture of OVA-loaded CD11c^{+} isolated from MLN or PeLN of CD45.2 WT mice and CD45.1 WT/OTII naïve CD4^{+} T-cells from spleen. Plot and graphs show lamin A/C mean fluorescence intensity (MFI) and percentage of lamin A/C positive CD4^{+} T-cells at 48 h of activation. Data are means ± SEM of a representative experiment from three analyzed by one-way ANOVA (A) and unpaired Student's t-test (B, C) *P<0.05;**P<0.01;***P<0.001.

Additionally, it is known that CD103$^+$ DC population, mostly present in MLN, PP, and intestine, produce RA, while CD103$^-$ DCs, preferentially located in spleen and PeLN, do not (Iwata M. *et al*, 2004; Coombes J.L *et al*, 2007; Esterházy D. *et al*, 2016). To corroborate this, we isolated CD11c$^+$ DCs from MLN or PeLN, and analyzed the percentage of CD103$^+$ and CD103$^-$ DC populations. As we expected, CD103$^+$ DC were highly present in MLN compared with PeLN (**Figure 35B**). Then, in order to relate this information with our previous findings, we loaded CD11c$^+$ DCs (CD45.2) from MLN or PeLN with OVA, and cultured them with WT/OTII naïve CD4$^+$ T-cells from spleen (CD45.1) for 48 h. After this time, we checked lamin A/C levels in CD4$^+$ T-cells by flow cytometry. CD11c$^+$ DCs from MLN greatly downregulated lamin A/C expression in CD4$^+$ T-cells, compared with CD11c$^+$ DCs from PeLN (**Figure 35C**).

6.5.3. Retinoic acid and lamin A/C deficiency in CD4$^+$ T-cells enhance Treg differentiation *in vitro*.

It has been described that RA augments Treg differentiation (Mucida D. *et al*, 2007; Benson M.J. *et al*, 2007; Mucida D. *et al*, 2009). Likewise, we have demonstrated that lamin A/C deficiency in CD4$^+$ T-cells enhances Treg differentiation *in vitro* and *in vivo*. Furthermore, our data showed that RA

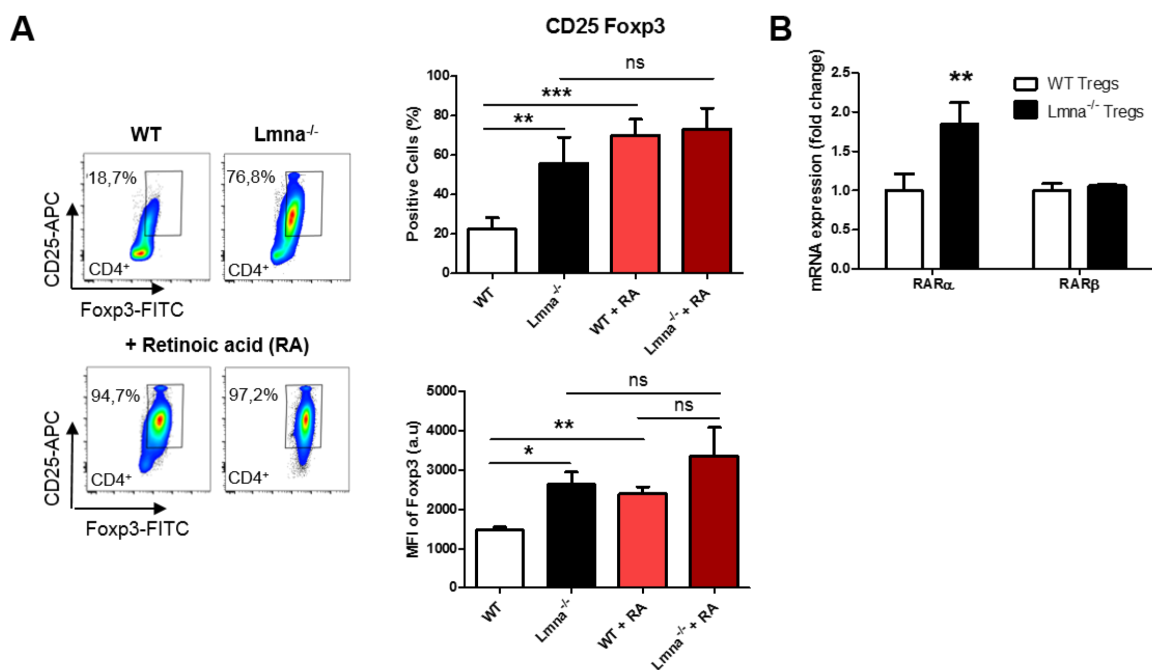

Figure 36. Retinoic acid (RA) augments Treg differentiation *in vitro* as well as lamin A/C deficiency. Naïve CD4$^+$ T-cells were isolated from WT and *Lmna*$^{-/-}$ mice spleens and stimulated with antibodies anti CD3/CD28 for 5 days in the presence of RA or its corresponding solvent for 48 h; moment in which RA was washed and Treg cytokines were added. **(A)** At day 5, percentage of CD25$^+$ Foxp3$^+$ Tregs and Foxp3 MFI were analyzed by flow cytometry. Representative plots and quantification data are shown (n=6 mice). Data are means ± SEM of one representative experiment of three analyzed by two-way ANOVA with Bonferroni's multiple comparison test. **(B)** RT-qPCR analysis of RA receptor genes. mRNA expression is show as fold change relative to WT (n=6-9, at least 6 pool of 2 mice). Data are means ± SEM analyzed by Student's t-test. *P<0.05; **P<0.01; ***P<0.001.

downregulates lamin A/C expression in CD4$^+$ T-cells. So that, we decided to address if lamin A/C was involved in the molecular mechanism of RA effect in Treg differentiation. Thereby, we performed *in vitro* Treg differentiation assays with WT and *Lmna*$^{-/-}$ CD4$^+$ T-cells, in the presence or not of RA during 48 h, and polarizing cytokines towards Treg fate added at 48 h till the end of the experiment. The obtained results corroborated that RA and the lack of lamin A/C separately enhances *in vitro* Treg differentiation. Thus, CD25$^+$ Foxp3$^+$ cell percentage of WT CD4$^+$ T-cells treated with RA was significantly higher than the obtained with WT CD4$^+$ T-cells (treated with the solvent of RA as control). Besides, CD25$^+$ Foxp3$^+$ cell percentage was again significantly greater in *Lmna*$^{-/-}$ CD4$^+$ T-cells than in WT (non treated cells); while *Lmna*$^{-/-}$ CD4$^+$ T-cells treated with RA exhibited non-significant differences in Treg percentage as non-treated *Lmna*$^{-/-}$ CD4$^+$ T-cells. Foxp3 MFI of Foxp3$^+$ CD4$^+$ T-cells was also measured showing the same tendency of results (**Figure 36A**). Moreover, to decipher lamin A/C role in RA mechanism related with Treg differentiation, we analyzed the expression of two RA receptors, *Rarα* and *Rarβ*, by RT-qPCR, in *in vitro* differentiated Tregs from *Lmna*$^{-/-}$ or WT CD4$^+$ T-cells. *Rarα* mRNA expression was significantly upregulated in Tregs derived from *Lmna*$^{-/-}$ CD4$^+$ T-cells, while *Rarβ* exhibited no differences compared with WT Tregs (**Figure 36B**).

6.5.4. Lamin A/C deficiency in CD4$^+$ T-cells enhances Treg differentiation and protects from IBD development in mouse at similar levels than retinoic acid treatment.

To corroborate the *in vitro* previous results, and because it has been described that RA treatment protects from IBD in mouse (Bai A. *et al*, 2009), we developed the same IBD mice model described before, in which naïve *Lmna*$^{-/-}$ or WT CD4$^+$ T-cells were adoptively transferred to *Rag1*$^{-/-}$ mice. During the first two weeks after the adoptive transfer, we treated the mice with RA by an i.p. injection once a day (**Figure 37A**). After 8 weeks, we sacrificed the mice and studied Treg cell percentage (CD25$^+$ Foxp3$^+$ population) and Foxp3 MFI of Foxp3$^+$cells in MLN of the mice (**Figure 37B**). These data corroborate the same results previously observed *in vitro*. Furthermore, we analyzed weightloss (**Figure 37C**) and colon shortening (**Figure 37D**) in order to assess the inflammation severity. Again, mice transferred with *Lmna*$^{-/-}$ T-cells were protected from the disease, exhibiting less weightloss and larger colon. Likewise, mice transferred with WT T-cells and treated with RA were similarly protected, presenting less weightloss and less colon shortening in comparison with non-treated WT T-cell adoptively transferred mice.

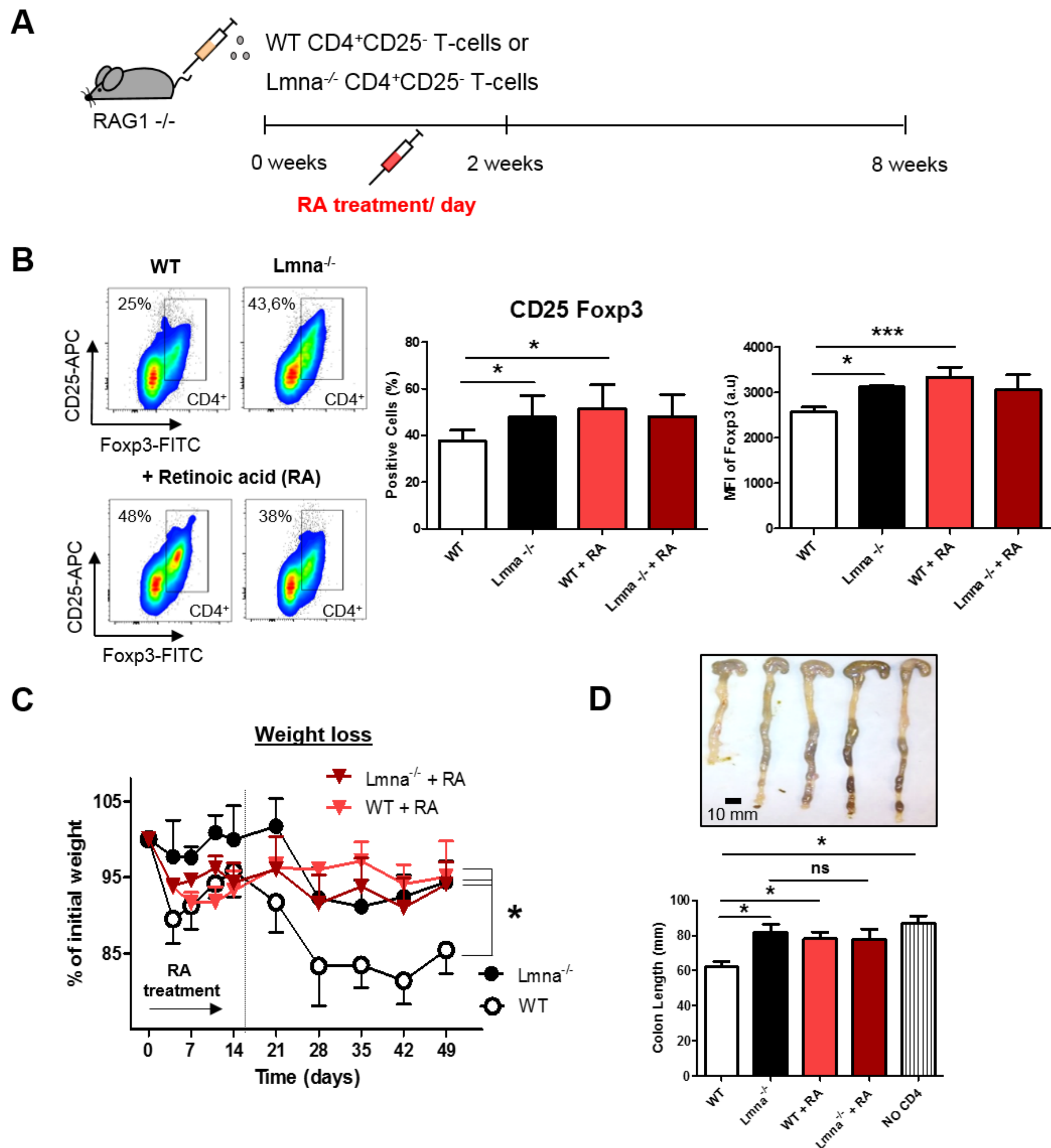

Figure 37. Retinoic acid (RA) augments Treg differentiation *in vivo* and protects from IBD as well as lamin A/C deficiency in CD4⁺ T-cells. **(A)** *Rag1*$^{-/-}$ mice were adoptively transferred with WT or *Lmna*$^{-/-}$ CD4^{+} CD25^{-} T-cells to induce IBD development. During the first two weeks, mice were intraperitoneally injected with RA once a day. Mice were sacrificed 8 weeks after the T-cell transfer. **(B)** Flow cytometry analysis of CD25^{+} Foxp3^{+} cells percentage of the total CD4^{+} T-cells, and Foxp3 MFI of CD25^{+} Foxp3^{+} T-cells in MLN is shown. **(C)** Weightloss evolution during IBD development. **(D)** Representative photograph of colons and measurement of colon length (mm) after 8 weeks of IBD development (n=5-9, at least 5 mice of a representative experiment of three). Data are means ± SEM analyzed by two-way ANOVA with Bonferroni's multiple comparison test. *P<0.05; ***P<0.001.

Discussion

7. DISCUSSION

7.1. Lamin A/C and T-cell development.

CD4-*Lmna*$^{-/-}$ mice were generated to knockdown lamin A/C specifically in CD4^{+} T-cells. Lamin A/C expression levels in CD4-*Lmna*$^{-/-}$ mice were significantly downregulated in comparison with WT. However, it seems that the CRE recombinase linked to the *Cd4* gen promoter, was not 100% efficient, since we observed some lamin A/C-expressing CD4^{+} T-cells coming from CD4-*Lmna*$^{-/-}$ mice. The incomplete gene excision of floxed loci is one of the limitations of the cre-loxP system that has been reported in other models (McLellan M.A. *et al*, 2017). This effect could be due to the fact that not all the loci recombine equally, and by virtue of the fact that the size of the targeted gene within the floxed loci can interfere in the gene excision efficiency (Zheng B. *et al*, 2000). Despite of our reduced efficiency, our results show that this mice model can be very useful to study lamin A/C functionality in CD4^{+} T-cells. It is important to take into account that CD4^{+} surface receptor is expressed in CD8^{+} T-cells during their development in the thymus (Germain R.N., 2002). Therefore, in the experiments performed with this CD4-CRE mice model, as the *Leishmania major in vivo* infections, the CD8^{+} T-cell population also lacked lamin A/C. Except for these assays, we have always performed *in vitro* and *in vivo* experiments with naïve CD4^{+} T-cells isolated by negative selection, including and anti-CD8 antibody, to avoid lamin A/C deficient CD8^{+} T-cell effect in our results.

As it was mentioned before, Hale J.S. *et al* described in 2010, that absence in lamin A/C in the immune system does not affect lymphocyte development. They reported that the *Lmna*$^{-/-}$ (full knockout) mice have an impairment in T and B-lymphocytes development only due to an indirect effect of lamin expression loss in non-lymphoid cells. They showed that WT hosts transplanted with bone marrow from *Lmna*$^{-/-}$ mice displayed a good engraftment of the thymus and a normal thymocyte development. Furthermore, we have corroborated that the reconstitution of WT recipient mice with *Lmna*$^{-/-}$ bone marrow drives normal T-cell development. Even so, we have generated the CD4-*Lmna*$^{-/-}$ mice and evaluated the basal levels of CD4^{+} and CD8^{+} T-cell populations in lymphoid organs (spleen, thymus and LN), body weight and lymphoid organ size. Our data demonstrate that lamin A/C downregulation in CD4^{+} and CD8^{+} T-cells does not affect to mouse body weight, lymphoid organ size, nor lymphocyte development in the thymus, raising the expected percentages for each population in spleen and LN.

7.2. Lamin A/C in T-cell activation and proliferation.

As it was mentioned previously, the first step in Th effector development is T-cell activation promoted by antigen recognition. Then, T-cells will proliferate and differentiate towards different T-helper (Th) phenotypes in the case of CD4^{+} T-lymphocytes, or towards cytotoxic phenotype in the case of CD8^{+} T-lymphocytes (Delves P.J & Roitt I.M., 2000). Thanks to our laboratory previous work, it is known that lamin A/C is expressed upon TCR stimulation, enhancing immune synapse formation and T-cell activation (González-Granado J.M. *et al*, 2015). Now we have corroborated its expression in

$CD4^+$ T-cells upon antigen recognition, and its function as a T-cell activation enhancer in mouse upon VACV infection. Thereby, here we have confirmed that lamin A/C is an important regulator of T-cell activation upregulating CD69 and CD25 surface receptors expression. CD69 is a receptor molecule induced by antigen recognition in T-cells, thus it has been widely used to determine the degree of T-cell activation (Rocha-Perugini V. *et al*, 2014; Cibrián D. & Sánchez-Madrid F., 2017). CD25, is also induced by TCR stimulation, and it is known for being the alpha subunit of the IL-2 receptor (Kisielow P & Von Boehmer H., 1995). IL-2 is a key growth factor promoting T-cell proliferation and survival (Van Parijs L. *et al*, 1999), thereby since we observed that lamin A/C upregulates CD25 expression, we decided to evaluate the role of lamin A/C in T-cell proliferation. However, our results show the contrary of what we expected, lamin A/C does not seem to have a role in T-cell proliferation upon antigen recognition, since no significant differences have been observed neither in *in vitro* nor *in vivo* assays. It has been described that IL-2 is merely an accessory signal to promote T-cell clonal expansion. The priming of naïve T-cells after the interaction with the APC, is enough to promote T-cell proliferation. Thereby, it has been demonstrated that IL-2 is required for *in vitro* T-cell expansion, while *in vivo*, IL-2 predominantly acts in Treg differentiation induction and maintenance (Reinhard O., 2015). So that, CD25 upregulation by lamin A/C in activated T-cells does not imply that WT T-cells have to proliferate more than lamin A/C deficient T-cells in our experiments. Moreover, T-cell expansion starts just after T-cell activation, more or less 24-48 h after TCR stimulation. Precisely, lamin A/C peak of expression in $CD4^+$ T-cells lasts approximately 48 h from TCR activation. So that, when T-cell proliferation is highly starting (at least 48 h after activation), lamin A/C levels are being decreased in the T-cell. Therefore, this is the main elucidation that supports lamin A/C does not intervene in $CD4^+$ T-cell proliferation although CD25 levels are increased by lamin A/C upon T cell activation.

There is evidence that lamin A/C deficiency is involved in the G1/S transition step of the cell cycle, and that lamin A/C deficiency lead to proliferative defects in fibroblasts and HeLa cells (Dechat T. *et al*, 2007).Moreover, lamin A/C expression has been usually related with differentiated and non-proliferating cells (Roeber R.A. *et al*, 1989), existing some previous investigations that have reported that the absence of lamin A/C promotes growth of tumor cells and aggravates cancer dissemination (Venables R.S. *et al*, 2001; Tilli C.M. *et al*, 2003). Fibroblasts are supposed to express lamin A/C constantly (Dechat T. *et al*, 2007) unlike T-cells, in which we have observed a transient expression, and tumor cells in which lamin A/C expression varies depending on the cancer subtype (Willis N.D. *et al*, 2008; Wang Y. *et al*, 2009; Capo-chichi C.D. *et al*, 2011; Maresca G. *et al*, 2012). This variability in lamin A/C expression indicates that lamin A/C does not have the same behavior of expression in all cell types. This could explain why we have not observed defects in proliferation of lamin A/C deficient T-cells.

Besides, it has been widely demonstrated that lamin A/C deficient cells migrate easily (Denais C.M. *et al*, 2016). This finding supports the idea that lamin A/C downregulation 48 h after TCR stimulation, gives to lymphocytes the opportunity to proliferate and migrate easily through lymphatic circulation towards the infected or/and inflamed tissues. It would be of great interest to confirm this hypothesis by *in vivo* microscopy techniques in future experiments. Furthermore we could further study if lamin A/C affect to T-cell proliferation and migration, promoting a steady overexpression of lamin A/C in $CD4^+$ T-cells. Moreover, it is still necessary to further investigate the role of A-type lamins in cell-migration and proliferation, since it seems that lamin A/C expression is very variable depending on the cell type, a fact that could have functional consequences in the behavior of the T cell.

7.3. Lamin A/C and Th1 response.

Interestingly, it is known that A-type lamins are expressed only in differentiated cells while B-type lamins are expressed ubiquitously, even in stem cells (Dechat T. *et al*, 2010). So that, the role of lamin A/C in cell differentiation has been widely studied in other non-immunological cell types as adipocytes (Lund E. & Collas P, 2013), osteoblasts (Akter R. *et al*, 2009; Rauner M. *et al*, 2009), and stem cells (Espada J. *et al*, 2008). Here, we have corroborated *in vivo* that lamin A/C expression in $CD4^+$ T-cells is transient, peaking 24 h-48 h after antigen recognition. However, we need to further study lamin A/C expression in Th differentiated $CD4^+$ T-cells. We have observed that lamin A/C expression in $CD4^+$ T-cells 6 days after *in vitro* activation, is lower than the peak of expression at 48 h after TCR activation. This observation suggests that lamin A/C expression is required some hours after antigen recognition to enhance T-cell activation, but afterwards lamin A/C is no longer required. Nevertheless, it would be interesting to deeply analyzed lamin A/C expression once T-lymphocytes are already polarized into different Th phenotypes. Maybe lamin A/C is differentially expressed depending on the Th-cell type or the degree of diferentiation/specialization. In line with this, memory T-cells display a second antigen recognition (MacLeod M.K.L. *et al*, 2010), thus lamin A/C could be expressed again to control this second round of T-cell activation. Therefore, it would be exciting to decipher if lamin A/C is expressed in memory T-cells, regulating its activation and effector function.

The Th fate of antigen-engaged T-cells depends on many factors, including the type of antigen encountered, the TCR signal intensity, the duration of antigen presentation, and the local cytokine milieu (Constant S.L. & Bottomly K., 1997; Szabo S.J. *et al*, 2000; O'Shea J.J. & Paul W.E., 2010; Zhu J. *et al*, 2010). Th1 differentiation is favored by stronger T-cell activation signals and longer T-DC cell-cell interactions (van Panhuys, N., 2014 and 2016). Lamin A/C enhances T-cell activation by increasing TCR signal strength and promoting T-DC interaction (González-Granado J.M. *et al*, 2015). Weaker TCR signal intensity in the absence of lamin A/C could also account for the observed reduction in $CD4^+$ T-cell activation triggered by VACV infection. Regarding T-cell differentiation driven by the cytokine milieu, IL-2, IL-12 and IFNγ are known to facilitate Th1 differentiation, whereas IL-4 favors Th2 differentiation. Moreover, some cytokines promote differentiation to a specific Th phenotype without

affecting other phenotypes. For example, IL-33 enhances Th1 but not Th2 polarization in the presence of IL-12 (Komai-Koma M. *et al*, 2016). Lamin A/C expression could also thus regulate the production and release of cytokines and the surface receptors expression that are important for the Th1 phenotype. In this regard, we found that lamin A/C deficiency reduces membrane expression of the alpha chain of the IL-2 receptor CD25 in $CD4^+$ T-cells activated by *in vivo* infection with VACV. Moreover, incubation of $CD4^+$ T-cells with IL-2 or Th1 polarizing cytokines during *in vitro* differentiation experiments did not rescue the impaired Th1 differentiation of *Lmna*$^{-/-}$ cells, indicating that the intracellular signals drive by the nuclear envelope protein lamin A/C are which directly regulate Th1 differentiation. Our *in vitro* and *in vivo* results clearly identify lamin A/C as a crucial driver of Th1 polarization enhancing T-bet expression. Strikingly, this effect is not counterbalanced by an increase in Th2 differentiation. Distinct signaling pathways can drive cell differentiation toward Th1 or Th2. In response to TCR activation and mTOR costimulation, Akt modulates transcription of *T-bet* but not *Gata3*, thus affecting Th1 but not Th2 differentiation. In contrast, PKC-θ activation yields the opposite result (Lee K. et al, 2010). Additionally, *Lmna*$^{-/-}$ mice exhibit an elevated mTOR (C1) signaling with a chronic Akt activation (Ramos F.J. *et al*, 2012). Therefore, it would be ideal to address mTOR and Akt regulation by lamin A/C in $CD4^+$ T-cells. Indeed, previous work of our laboratory demonstrated that lamin A/C binds to Erk1/2 and c-Fos, regulating AP-1 transcription-factor target genes in fibroblasts (Ivorra C. *et al*, 2006; González J.M. *et al*, 2008), and that lamin A/C controls $CD4^+$ T-cell activation through ERK1/2 signaling (González-Granado J.M. *et al*, 2014). Here, we have demonstrated again that lamin A/C regulates pERK 1/2 levels, therefore affecting T-cell activation. Interestingly, the formation of c-Fos/c-Jun dimers can potentiate Th1 differentiation (van Panhuys, N., 2016). So that, it would be interesting to check if lamin A/C potentiate the dimerization of c-Fos and c-Jun. Moreover, lamin A/C is known for its role in chromatin organization, interacting directly with DNA, forming what is called lamin associated domains (LADS) (van Steensel B. & Belmont A.S., 2017). This maps of lamin interactions are reorganized during cell differentiation, depending on the cell type (Peric-Hupkes D. *et al*, 2010). Because of that, we postulate that lamin A/C is capable of regulating specifically the transcription of genes that are related with Th1 but not with Th2 differentiation. Furthermore, it has been reported that lamin A/C gets to control cell-differentiation by modulating epigenetic changes (Oldenburg A. *et al*, 2017). Additionally, one key factor of T-cell differentiation commitment is the epigenetic regulation (Morinobu A. et al, 2004). There have been described several histone modifications in Th master regulator genes and lineage-specific cytokine genes, that determines the Th fate (Hirahara K. *et al*, 2011). In line with this, we have demonstrated that lamin A/C deficiency downregulates the expression of T-bet, diminishing H3K4me1 epigenetic modification in *T-bet* promoter, while in *Gata-3, Foxp3 and RorγT* promoters lamin A/C does not alter any of the studied epigenetic modifications (H3K4me3, H3K27me3, and H3K4me1). So that, one mechanism by which lamin A/C regulates specifically Th1 differentiation, consists in the upregulation of the enhancer mark H3K4me1 in *T-bet* gene promoter, what facilitates *T-bet* transcription and therefore Th1 differentiation. The role of lamin A/C in Th

differentiation epigenetics was, until now, completely unknown, thus, our work reveals that lamin A/C is a key epigenetic modulator of Th1 differentiation. Additionally, there are already known many of the responsible enzymes of chromatin histone modifications (Dimitrova E. *et al*, 2015). Interestingly, the histone demethylase JmjD3 interacts with T-bet transcription factor affecting Th1 differentiation and cytokine expression (Li Q. *et al*, 2014). This demethylase was one of our candidates to be modulated by lamin A/C, however our high throughput proteomic study has not showed significant differences at protein level for this enzyme. Nevertheless, our results showed other lysine demethylases as PHF8 and KDM5B that were significantly downregulated in lamin A/C deficient $CD4^+$ T-cells. Remarkably, KDM5B demethylates Lys-4 of histone H3, being responsible of the H3K4me1 modification, which is downregulated in *T-bet* promoter of *Lmna*$^{-/-}$ $CD4^+$ T-cells according to our studies. Our data also expose significant changes in methyltransferases as EZH1, CARM1, and ASH2L, and acetyltransferases as B2RRD7, and Ep300. Therefore, it would be of interest to study the histone modifications written by these enzymes in *Lmna*$^{-/-}$ $CD4^+$ T-cells compared to WT. EED PcG protein downregulation in *Lmna*$^{-/-}$ $CD4^+$ T-cells also stands out among the quantitative proteomic results. This could be related with the fact that lamin A/C interacts with PcG proteins (Cesarini E. et al, 2015), which are epigenetic repressors that control a wide number of genes during differentiation processes (Prezioso C. & Orlando V., 2011). But PcG proteins and lamin A/C have not yet been jointly related with Th differentiation. Hence, our findings invite to explore further this research path in the future, being extremely interesting to perform ChIP-seq assays for H3K4me1 and other histone modifications, according to our proteomic results.

Furthermore, it has been described that the concentration of histone modifications enzymes at the cytoplasm and nucleus is dynamically and tightly controlled at protein level in response to environmental changes during pathophysiological processes (Zou C. & Mallampalli R.K., 2014). Accordingly, we have seen that lamin A/C expression in $CD4^+$ T-cells is also very dynamic depending on the environment in which they are confined. Moreover, we have demonstrated that lamin A/C seems to be an important regulator of histone modifications enzymes at protein level.

Apart from the role of lamin A/C in $CD4^+$ T-cell epigenetics, our quantitative proteomic analysis has also revealed that lamin A/C regulates the expression of several proteins related with DNA machinery, immune system, metabolism, cytoskeleton and vesicular transport. The role of lamin A/C in gene expression has been already reported (Brohnstein L. *et al*, 2018). As well, it is known the physical connection between A-type lamins and cytoskeleton through nesprins and SUN domain proteins (Schrimer E.C. & Foisner R., 2007). Moreover, our laboratory has previously described lamin A/C relation with cytoskeleton dynamics, reporting that lamin A/C increases the polymerization of actin filaments and the translocation of MTOC, thus enhancing T-cell activation (González-Granado J.M., et al 2015). Thereby, our proteomic data confirms these previous findings. Besides, all this project demonstrates a key function of lamin A/C in $CD4^+$ T-cells activation and differentiation, however it would be interesting to study if lamin A/C expression is also important for other immune cell types. The

role of lamin A/C in T-cell metabolism has not been explored yet. What is known is that metabolism is another factor closely related with T-cell proliferation and differentiation. Once a T-cell is activated, a metabolic reprogramming starts after TCR stimulation accompanied by signals from co-stimulatory molecules as CD28, or cytokines as IL2, which induce the activation of phosphatidylinositol 3'-kinase (PI3K)-Akt signaling pathway, leading to a rapid increase in the glucose uptake and glycolysis (Coe D.J. *et al*, 2014). Other molecules downstream of TCR/CD28, as P38 and ERK, are also linked with the induction of glycolysis after T-cell stimulation (Marko A.J. *et al*, 2010). Furthermore, it is known that effector Th cells as Th1 uses glycolysis and glutamine energy sources, while Treg, memory and naïve T-cells rely on fatty acids oxidation to generate ATP (Almeida L. *et al*, 2016). Therefore, it would be really interesting to explore if lamin A/C deficient CD4$^+$ T-cells exhibit a diminished glycolysis and an augmented fatty acids oxidation, which would fit with our results in T-cell activation, and Th1 and Treg differentiation. Vesicular transport also seems to be regulated by lamin A/C in CD4$^+$ T-cells according to our proteomic results. It has been reported that vesicular trafficking during IS formation is extremely important for surface receptors recycling in T-cells, to can recognize properly the APC (Onnis A. *et al*, 2016). Besides, exosomes secretion is a key communication channel between T-cell and APC, affecting to T-cell activation dynamics (Bustos-Morán E. *et al*, 2017; Torralba D. *et al*, 2018). Thereby, this could be another mechanism regulated by lamin A/C to be studied in the future.

Viral clearance requires the efficient coordination of multiple immune effector mechanisms (Swain S.L. *et al*, 2012). Our work shows that lamin A/C deficiency in CD4$^+$ T-cells results in a defective T-cell differentiation towards Th1 phenotype, impairing the response against VACV and delaying viral clearance. These results suggest that lamin A/C is an essential component of the immune response against cytopathic viruses. Remarkably, lamin A/C expression in CD4$^+$ T-cells seem to be enough to regulate the viral clearance, since only CD4$^+$ T-cells were adoptively transferred to T- and B-cell deficient *Rag1*$^{-/-}$ mice before mouse infection with VACV in our experiments. Accordingly, in humans, CD4$^+$ T-cells are critical for anti-viral immunity and the control of VACV replication (Munier C.M. *et al*, 2016). CD4$^+$ T-cells can also regulate innate immune responses that mediate viral clearance (Swain S.L. *et al*, 2012). This viral clearance is independent on B-cells and CD8$^+$ T-cells, and might be important for appropriate CD4$^+$ T-cell dependent responses to VACV (Mota B.E.F. *et al*, 2011). Effector CD4$^+$ T-cells have been shown to respond to viral pathogens through two mechanisms: cytokine production (mostly IFNγ and TNFα) and a direct cytolytic activity mediated by perforin and FAS (also known as CD95) (Swain S.L. *et al*, 2012). The cytotoxic activity of CD4$^+$ T-cell effectors does not require Th1 cell polarization or T-bet expression but is dependent on the expression of the transcription factor Eomesodermin (Eomes), which is crucial for the development of cytotoxic CD4$^+$ T-cells *in vivo* through the induction of granzyme B (Qui H.Z. *et al*, 2011). In addition to increasing T-bet and *Ifnγ* mRNA expression, we find that the presence of lamin A/C enhances the expression of *Eomes, Blimp-1, Prf1, and granzyme B* mRNA in CD4$^+$ T-cells *in vitro* differentiated to Th1. Thereby, we have

demonstrated that lamin A/C is a key regulator of Th1 effector response directly affecting to IFNγ production, and $CD4^+$ lymphocytes cytotoxic activity. This finding could be applied for improving the immune response of immunocompromised individuals against pathogen infections. Thus, lamin A/C is a potential therapeutic target for infectious and immune diseases.

As it was mentioned before, the protozoan parasite *Leishmania major* cause cutaneus leishmaniasis which consists in skin lesions in humans that self-heal; however, the parasite can cause prolonged non-healing skin ulcers with extensive tissue destruction and become systemically distributed (Reithinger R. *et al*, 2007). C57BL/6 mice develop a protective immune response against *L. major*. We show that WT recipient C57BL/6 mice reconstituted with *Lmna*$^{-/-}$ bone marrow develop poorer Th1 responses against *L. major* infection, with worse clearance of the pathogen than recipients of WT cells. The immune response against *L. major* involves multiple cell types, and *Lmna*$^{-/-}$-reconstituted mice lack lamin A/C throughout the immune system. Therefore, to define the defective response to *L. major* to the lack of lamin A/C specifically in T-cells, we inoculated the ears of WT and *Lmna*$^{-/-}$-CD4-CRE mice. Healing was impaired in the ears of this mice lacking lamin A/C in T-cells. C57BL/6 mice develop a Th1-type response to *L. major*, and lesions heal in the absence of $CD8^+$ T-cells following a high *L. major* dose (Belkaid Y. *et al*, 2002; Uzonna J.E. *et al*, 2004). Moreover, cytolytic $CD8^+$ T-cells are pathogenic when recruited to *Leishmania* lesions in large numbers (Novais F.O. *et al*, 2013). Therefore, the deficient response observed in *Lmna*$^{-/-}$-CD4-CRE mice is likely associated with the lack of lamin A/C in $CD4^+$T-cells. The early immune response determines whether *L. major* skin infection will be self-healing or chronic. C57BL/6 mice develop $CD4^+$ Th1 cell-mediated resistance upon *L. major* infection, whereas other mouse strains develop a $CD4^+$ Th2-type response and are extremely susceptible to infection (Scott P. & Novais, F.O., 2016). Therefore $CD4^+$ Th1 cells are crucial for reducing *Leishmani*a infection producing IFNγ, IL-2, and TNFα locally at the cutaneous lesion, and leading to macrophage activation and parasite elimination (Iborra S. *et al*, 2016). Our experiments show a diminished percentage of IFNγ-producing $CD4^+$ T-cells and reduced IFNγ production in *Lmna*$^{-/-}$ mice. IFNγ secretion by $CD4^+$ T-cells induces macrophages to produce nitric-oxide (NO) and tumor necrosis factor (TNF), thus controlling *L. major* infection (Scott P. *et al*, 1988; Scott P. & Novais, F.O., 2016). Downregulated IFNγ production in *Lmna*$^{-/-}$ $CD4^+$ T-cells could thus lead to impaired macrophage NO production, explaining the diminished pathogen clearance observed in the absence of lamin A/C. Importantly, we show that lamin A/C in $CD4^+$ T-cell is an important mediator of the immune control against this pathogen. Our study reveals a novel role for lamin A/C as a regulator of T-cell differentiation, controlling the maintenance of Th1 populations in response to pathogen infections. These data contribute to the understanding of the molecular mechanisms driving $CD4^+$ T-cell responses and suggest strategies to modulate T-cell function, which could provide a route toward therapeutic immunization and long-lasting protection.

7.4. Lamin A/C and Treg response.

According to our *in vitro* and *in vivo* results, lamin A/C not only regulates Th1 differentiation, but also modulates Treg polarization. Lamin A/C deficient T-cells upregulate Foxp3 expression, which correlates with an enhanced Treg differentiation *in vitro*. Therefore, lamin A/C impairs Treg polarization directly diminishing Foxp3 expression. Besides, our experiments with retroviruses promoting lamin A/C overexpression in $CD4^+$ T-cells, clearly supports that lamin A/C regulates Foxp3 expression and Treg differentiation. However, as it has been discussed before, our data indicates that the mechanism through which lamin A/C modulates Foxp3 is not based on epigenetics, since we did not observed any significant difference in H3K4me3, H3K27me3 and H3K4me1 modifications in *Foxp3* promoter. It is certain that we have only studied three histone modifications, so more research is needed to assure that this is not the mechanism through which lamin A/C controls Foxp3 expression. It is known that Foxp3 expression in naïve peripheral T-cells in *in vitro* and *in vivo* systems, depends on TGFβ signaling (Chen W. *et al*, 2003). Interestingly, A-type lamins have been shown to interact with the receptor regulated SMAD (rSMAD) at the INM antagonizing TGFβ signaling (Lin F. *et al*, 2001; Van Berlo J.H. *et al*, 2005). In line with this, we have observed by high throughput proteomics analysis that lamin A/C deficient $CD4^+$ T-cells display TGFβ signaling upregulation compared to WT. We have not included this result on this work, because we need to further validate it by western blot. Foxp3 expression can be also induced by TCR signals and IL-2. TCR activation promote the binding of TFs as NFAT, CREB, AP-1 and ATF promoter (Merkenschlager M. & von Boehmer H., 2010). We have observed that lamin A/C is also induced by TCR activation, and some studies have demonstrated that lamin A/C can interact with TFs modulating gene expression and even affecting cell differentiation (Dreuillet C. *et al*, 2002; Lloyd D.J. *et al*, 2002). Moreover, the molecular connection between lamin A/C and these TFs has not been studied yet, therefore it is an interesting question to address. Thus, lamin A/C could negatively modulate the expression of TGFβ and other genes related with Treg differentiation. Regarding IL-2, we have not observed a connexion between lamin A/C and IL-2 expression, although CD25 (or alpha chain of IL-2 receptor) is clearly upregulated by lamin A/C in $CD4^+$ T-cells 48 h after antigen recognition. CD25 expression is maintained by Treg cells after T-cell activation, so that CD25 is a surface marker commonly used to study Tregs (Curotto de Lafaille M.A *et al*, 2004). For this reason, we have used not only Foxp3 but also CD25 to gate Treg cells by flow cytometry. Nevertheless, we have observed higher percentage of $CD25^+$ $Foxp3^+$ population in *Lmna*$^{-/-}$ $CD4^+$ T-cells. Moreover, we have found that lamin A/C epigenetically regulates CD25 expression after 48 h of T-cell activation, enhancing its expression as well as CD69. CD69 is also expressed by a specific population of Treg cells, which is characterized by the expression of GITR, CTLA-4, ICOS and CD38 (Cortés J.R. et al, 2014). Although we have not analyzed CD69 in *Lmna*$^{-/-}$ Tregs, we have checked *Gitr, Ctla-4*, and *Icos* mRNA levels, and the expression of *Gitr* was significantly increased in *Lmna*$^{-/-}$ Tregs. For this reason, it would be interesting to analyzed also $CD69^+$ Treg population derived from *Lmna*$^{-/-}$ $CD4^+$ T-cells. So that, it seems that lamin A/C dynamically regulates CD25. This is not a trivial explanation due to the high plasticity of epigenetic

changes depending on the signals coming from the environment (Ho S. *et al*, 2012). However, we need to better characterize by time-course experiments the lamin A/C modulation of CD25 and CD69 expression in CD4$^+$ T-cells. Interestingly, all these molecules, CD25, CD69, TGFβ and IL-2 are part of PI3K-Akt-mTOR signaling (Merkenschlager M. & von Boehmer H., 2010), thus Treg and Th1 differentiation processes share this molecular pathway. Furthermore, it is known that mTOR is an important regulator of CD4$^+$ T-cell differentiation that promotes Th differentiation, since mTOR deficient mice showed a severely impairment in Th1, Th2 and Th17 polarization. Furthermore, it has been reported that the mTOR suppression in CD4$^+$ T-cells promotes Treg differentiation (Delgoffe G.M. *et al*, 2009). Moreover, mTOR altered signaling has been associated with *LMNA* mutations (Liao C.Y. *et al*, 2016). Therefore, we postulate that lamin A/C could also affect mTOR expression, being this an interesting hypothesis to study in the future. The mTOR upregulation by lamin A/C expression after TCR stimulation would be a plausible mechanism to explain how lamin A/C has an opposite dual role in Th1 and Treg differentiation. Nevertheless, this hypothesis would not explain our observations in Th2 and Th17 differentiation therefore, alternative mechanisms may be accounting for these results. The T-cell transfer model of IBD has permitted us to corroborate our previous *in vitro* results regarding naïve CD4 T cell differentiation. In fact, with this model of colitis, we have been able to observe, an enhancement of Treg and an impairment of Th1 polarization due to the absence of lamin A/C in naïve CD4$^+$ T-cells. Th17 cells are also main players in IBD promoting inflammation (Ueno A. *et al*, 2018). However, the model of naïve CD4$^+$ CD25$^-$ T-cells transfer into immunodeficient mice, which we have performed, leads to a Th1-mediated colitis (Coombes J.L. *et al*, 2005). Maybe, this is the reason why we have not obtained any significant difference in Th17-expressing cells percentages related with the lack of lamin A/C in CD4$^+$ T-cells. It would be interesting to check if we can reproduce these observations in alternative mouse model of colitis, for example in the Dextran Sodium Sulfate (DSS) induced chronic colitis mouse model. Nevertheless, our *in vitro* differentiation assays towards Th17 phenotype, shows the same appreciation; lamin A/C does not modulate Th17 polarization. Thus, lamin A/C seems to control specifically Th1 and Treg responses, and not Th2 nor Th17. These findings are also very interesting because almost all the literature related with Th populations, is always based on Th1/Th2 paradigm against infections (Romagnani S., 1997), and Th17/Treg balance in autoimmune diseases (Noack M. & Miossec P., 2014). For this reason, our work introduces a novel concept, the functional Th1/Treg balance in IBD. This work shows how Th1 and Treg populations are functionally counter-regulated by different mechanims. This balance between Th1 and Treg cells, has not been described in dept yet, although Crohn's disease has been associated with Th1 cytokine profile, and Tregs are the cells in charge of controlling this Th imbalance to solve colon inflammation (Brand S., 2009). In line with this, our results show how *Lmna*$^{-/-}$ CD4$^+$ T-cells protect against IBD development in mouse, by the upregulation of Treg population and detriment of Th1. Thus, the transfer of naïve *Lmna*$^{-/-}$ CD4$^+$ T-cells remarkably provokes less inflammation in the colon compared with WT CD4$^+$ T-cells, at least, in Crohn's disease mouse model. In contrast with Crohn's disease, ulcerative colitis is characterized by

a Th2 cytokine profile (Brand S., 2009). Since we know that lamin A/C does not affect to Th2 differentiation, it would be interesting to investigate how can affect lamin A/C deficiency in CD4$^+$ T-cells in the development of ulcerative colitis in mouse. It is necessary to confirm if our findings are fulfilled in all IBD scenarios, in order to strengthen the lamin A/C as a therapeutic target against these autoimmune pathologies.

The IBD is a multifactorial disease that have been related with environmental factors, genetic susceptibility, and changes in the microbiota populations (Vanhove W. *et al*, 2016; Young C.E., 2016). The microbiota is an indispensable factor in the colitis development (Guarner F., 2008; Sartor R.B., 2008). Prove of that is the finding that IL-10 knockout mice, a well-known mouse model of IBD, cohoused under germ-free conditions do not develop colon inflammation at all (Kuhl A.A. *et al*, 2007). In addition, it has been clinically observed that some patients respond to antibiotic treatment (Khan K.J. *et al*, 2011). Therefore, in order to avoid microbiota heterogeneity, we have always performed our IBD mouse experiments under cohousing conditions (Laukens D. *et al*, 2016). Thus, our results cannot be attributed to the microbiota component of mice.

The protection from IBD development in lamin A/C deficient CD4$^+$ T-cells transferred *Rag1*$^{-/-}$ mice could be due to an increase in Treg differentiation or to a Treg better immunosuppressive response. Thereby, we decided to study the suppressive activity of Tregs coming from *Lmna*$^{-/-}$ CD4$^+$ T-cells (*Lmna*$^{-/-}$ Tregs from now on) in comparison to WT (WT Tregs from now on). Additionally, it is known that Tregs must express CD25 at some point of their life to display their suppressive activity (Curotto de Lafaille MA *et al*, 2004). Hence, we have analyzed the expression of surface molecules that have been related with Treg activity in CD25$^+$ Tregs *in vitro* differentiated from *Lmna*$^{-/-}$ and WT CD4$^+$ T-cells. Our results show that *Lmna*$^{-/-}$ Tregs express higher levels of IL-10 and TGFβ and upregulate PD-1 and CD25 markers compared to WT Tregs. These data mean that *Lmna*$^{-/-}$ Tregs have better suppressive function compared to WT, since it is known that IL-10 and TGFβ are immunosuppressive cytokines released by Tregs (Schmidt A. *et al*, 2012) and CD25 and PD-1 expression is needed to exert the Treg functionality (Curotto de Lafaille MA *et al*, 2004;). In addition, GITR has been reported to be a marker of active Tregs (Ronchetti S. *et al*, 2015), and we have also observed higher levels of *Gitr* expression in *Lmna*$^{-/-}$ Tregs compared to WT. The surface molecule CTLA-4 has been suggested to intervene in Treg suppressive activity *in vivo*, due to the finding that CTLA-4 deficient or knockout mice exhibit spontaneous autoimmunity with a Treg amelioration (Bachmann M.F. *et al*, 1999; Takahashi T. *et al*, 2000). Regarding this information, it is true that we have not obtained significant results that show that *Lmna*$^{-/-}$ Tregs express higher levels of CTLA-4. However, it has been described that CTLA-4 deficient Tregs could display the immunosuppressive activity through compensatory mechanisms related with IL-10 and TGFβ (Tang Q. *et al*, 2004; Read S. *et al*, 2006). So that, it seems that IL-10 and TGFβ are the key players of Treg immunosuppressive activity. Furthermore, we have functionally demonstrated that *Lmna*$^{-/-}$ Tregs have higher suppressive power than WT Tregs, carrying out Treg *in vitro* suppressive

assays against Th1 proliferation. Hence, we have robustly confirmed that lamin A/C deficiency in $CD4^+$ T-cells not only improves Treg differentiation also enhances the Treg immunosuppressive function, being these the explanation why we have observed less IBD development in lamin A/C deficient $CD4^+$ T-cells transferred mice compared to WT transferred mice and have better suppressive power against Th1 proliferation. Although CD25 is a surface marker that has been commonly used to study Tregs, not all the Treg populations express CD25 (Curotto de Lafaille MA *et al*, 2004). However, the TF Foxp3, is constitutively expressed by Tregs and is considered to confer their suppressive ability to block colitis and other autoimmune diseases. Because of that, Foxp3 has been used as a more specific marker for Tregs (Hori S. & Sakaguchi S., 2004; Zheng Y. *et al*, 2010). For these reasons, we have studied the suppressive function of $CD25^+$ $Foxp3^+$ Treg cells differentiated from naïve *Lmna*$^{-/-}$ $CD4^+$ T-cells in comparison to WT. Hence, we have observed that like $CD25^+$ Tregs$CD25^+$ $Foxp3^+$ Tregs derived from lamin A/C deficient $CD4^+$ T-cells have better suppressive function against Th1 differentiation compared to $CD25^+$ $Foxp3^+$ Tregs derived from WT $CD4^+$ T-cells. In line with these observations, it has been demonstrated that the transfer of $CD4^+$ $CD25^+$ Tregs can not only prevent the IBD, but can also revert the established inflammation in mouse (Uhlig H.H. *et al*, 2006). So that, our work suggests that the transfer of *Lmna*$^{-/-}$ $CD25^+$ or *Lmna*$^{-/-}$ $CD25^+$ $Foxp3^+$ Treg cells could be a promising cell therapy against IBD, because it would suppress in a more efficient manner the intestine inflammation provoked by an upregulation of Th1 and Th17 populations.

Supporting our data, it has been previously described that lamin A/C is a risk biomarker in colorectal cancer (Willis N.D. *et al*, 2008). Moreover, it is known that patients with Crohn's disease have a higher risk to suffer gastrointestinal tract and hematopoietic system cancers, compared with the general population. The presence of IBD, especially if it is long standing, promotes the development of malignancies (Stidham R.W. & Higgins P.D.R., 2018). Thereby, it seems clear that lamin A/C regulation has an important role in the intestine health. Hence, it would be interesting to analyze lamin A/C expression levels in human colonic samples from IBD patients, to address if lamin A/C could be a risk biomarker not only in colorectal cancer, but also in IBD. Additionally, we could investigate in the future, if lamin A/C deficiency in $CD4^+$ T-cells protects from colorectal cancer development in mouse models.

7.5. Physiological regulation of lamin A/C by retinoic acid.

As it was previously mentioned, there is almost no information about the regulation of A-type lamins expression in immune cells. It has been described that there is a RA responsive element in the LMNA promoter (Okumura K. et al, 2004), and that RA downregulates lamin A/C expression in leukocytes (Olins A.L. *et al*, 2001). Now, we have demonstrated *in vitro* that RA downregulates antigen-recognition-induced lamin A/C expression in $CD4^+$ T-cells. Additionally, we have observed that lamin A/C expression in $CD4^+$ T-cells depends on the environment in which T-cells are confined *in vivo*. Thus, lamin A/C is highly expressed in activated $CD4^+$ T-cells that are localized in PLN, while in MLN and spleen, activated $CD4^+$ T-cells express significantly lower levels of lamin A/C. Therefore, it seems that

some molecule differentially released or expressed in membrane depending on the lymphoid organ, is capable of modulating lamin A/C expression. In accordance to this, it is known that RA is present at different levels depending on the tissue microenvironment. Tissues such as intestine, liver, and eyes, present high levels of RA, while in blood, RA is present at low quantity (Pino-Lagos K. *et al*, 2008). RA is a key molecule in the intestine due to its role promoting gut-homing (Iwata *et al*, 2004). In the intestine, RA is produce by epithelial cells, DCs and macrophages. DCs can release RA for T-cells undergoing activation, and RA released by intestinal epithelial cells can also affect T-cells in the intraepithelial compartment (Coombes J.L et al, 2007). It has been described the specific DCs populations in charge of RA release. $CD103^+$ DCs are known to be responsible of this RA production in the gut, MLN and PP, whereas $CD103^-$ DCs do not produce RA. Hence, $CD103^+$ DCs are preferentially located in intestinal regions, while $CD103^-$ DCs are more abundant in PeLN and spleen (Esterházy D. et al, 2016). Accordingly, our data confirm that MLN contain higher quantity of $CD103^+$ DCs compared with PeLN. In addition, we have shown how $CD11C^+$ DCs from MLN downregulates significantly lamin A/C expression levels in $CD4^+$ T-cells, in comparison to $CD11C^+$ DCs from PeLN, which do not greatly downregulate lamin A/C. Regarding that $CD103^+$ are the DC population which predominantly produces RA, our results clearly indicate that RA is one of the molecules that physiologically regulates lamin A/C expression in T-cells, depending on the immunological compartment in the organism.

RA levels can also change the phenotype of DCs and macrophages to indirectly affect T-cell activation, differentiation and function. Thus, in general, RA at high concentrations generate DCs with a low T-cell activation capacity, inducing $Foxp3^+$ Tregs; while low levels of RA are required for optimal activation of T-cells and induction of effector T-cells (Kim C.H., 2013). Thereby, RA may have a double effect on lamin A/C expression: a direct one on the *LMNA* promoter, and another one that may affects DCs indirectly changing lamin A/C expression upon antigen-recognition. Additionally, RA has an s effect on Th1/Th2 polarization. In vitamin A deficient mice, Th1 cells are increased (Cantorna M.T. *et al*, 1995), and in the "pinkie" mice where RA receptors function is insufficient, Th1 response is augmented (Spilianakis C.G. *et al*, 2005). However, RA is not a direct polarizing agent, it acts on other cells to indirectly regulate the Th response (Kim C.H., 2013). Thereby, until now, the mechanism for RA effects in Th differentiation was unclear. Interestingly, all this information perfectly fits with our findings about lamin A/C role in T-cell activation and differentiation, and taking into account our data that confirms lamin A/C downregulation by RA. Furthermore, several studies have reported the RA capacity to induce Treg differentiation from naïve T-cells. Hence, RA induces Foxp3 expression when is added during naïve $CD4^+$ T-cell activation (Sun C.M. *et al*, 2007). It is known that this process can be dependent on TGFβ expression (Coombes J.L. *et al*, 2007; Mucida D. *et al*, 2010); nevertheless, how RA affect Foxp3 gene expression is not clearly understood yet (Benson M.J. *et al*, 2007; Mucida D. *et al*, 2007). We have corroborated these data, observing as well that RA augments naïve Treg

differentiation. Similarly, we have observed that lamin A/C downregulation during CD4$^+$ T-cell activation has the same effect, an enhancement of Treg polarization. Moreover, it has been described that the expression of RA receptors alfa-type (RARα) is enhanced in T-cells by the presence of RA (Halevy O. et al, 1994). Accordingly, our data shows that Tregs polarized from lamin A/C deficient CD4$^+$ T-cells upregulate *RARα* mRNA expression. However, the role of RA in Th17 differentiation is not clear. Some authors point out that RA promotes Treg and Th17 polarization reciprocally (Mucida D. *et al*, 2007), while others defend that RA inhibits Th17 differentiation (Schambach F. *et al*, 2007; Elias K.M. *et al*, 2008). Again, this discrepancy coincide with our results, as we have not obtained clear results about the effect of lamin A/C on Th17 differentiation.

In addition, as it was briefly mentioned in the introduction, RA or vitamin A have been used in mouse models to ameliorate colon inflammation (Bai A. et al, 2009). We have corroborated this effect in parallel to the same appreciation for lamin A/C deficiency in CD4$^+$ T-cells. Therefore, our results point out that lamin A/C downregulation in CD4$^+$ T-cells ameliorates the symptoms derived from IBD as well as RA treatment, through the enhancement of Treg differentiation and impairment of Th1 differentiation. However, RA effect is a multi-target treatment for IBD, while lamin A/C downregulation in CD4$^+$ T-cells can specifically solve the Th population misbalance characteristic of the IBD. Furthermore, the use of retinoids to treat various types of dermatoses as psoriasis, has brought to light its multiple side effects. Systemic administration of these compounds has been frequently associated with liver toxicity, mucocutaneous adverse effects, and abnormalities of serum lipid profiles, which might be related to an increased risk of coronary heart disease. Even, retinoids have been classified as teratogenic, and long-term treatments have shown also skeletal abnormalities, so they cannot been prescribe for pregnant women or children (David M. *et al*, 1988). For these reasons, lamin A/C downregulation may be a potential and specific therapeutic approach to take into account for IBD. To give greater relevance to this idea, we want to develop, in the near future, molecular techniques as lentivirus or CRISPR CAS9 technology, to downregulate lamin A/C specifically in CD4$^+$ T-cells, and thus treat Crohn's disease in addition or not to other treatments.

7.6. Proposed model.

All together our results suggest that antigen recognition-induced lamin A/C expression in CD4$^+$ T-cells is physiologically regulated by APC's RA release at the moment of IS formation by microenvironmental factors. TCR activation induces lamin A/C expression which enhances T-cell activation by the upregulation of CD69 and CD25 surface markers among other mechanisms 48 h after T-cell activation. Likewise, H3K4me1 epigenetic modification is upregulated in *T-bet* gene promoter by lamin A/C expression, which augments T-bet TF expression and determines Th1 fate commitment. Furthermore, thanks to lamin A/C expression, this Th1 differentiated cells display better effector function against infections, due to their greater cytotoxicity and quantity (**Figure 38A**).

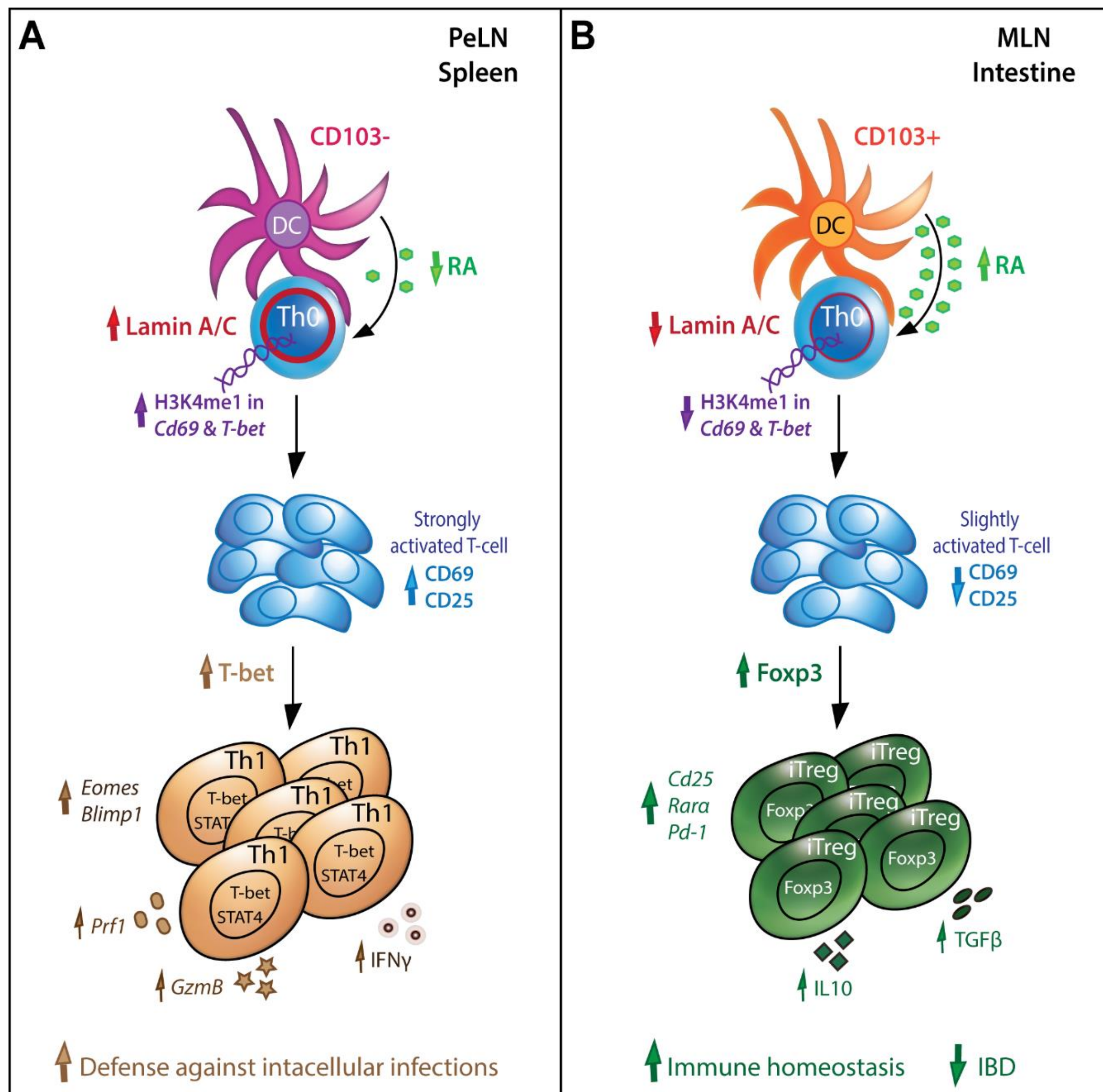

Figure 38. Schematic representation of lamin A/C role in Th differentiation and adaptive immune response. (A) Peripheral lymph nodes (PeLN) and spleen are enriched in CD103^{-} DCs that produce low amounts of retinoic acid (RA) among other molecules. This low amount of RA permits high lamin A/C expression levels which is induce by TCR activation after antigen recognition. Lamin A/C epigenetically enhances CD69 and T-bet expression by the upregulation of H3K4me1 modification in their gene promoters. Likewise, lamin A/C augments CD25 expression, thus improving T-cell activation. The upregulation of the Th1 master regulator T-bet determines that CD4^{+} T-cells polarize towards Th1 phenotype. Moreover, lamin A/C determines that these Th1 cells display a more efficient immune response against intracellular infections, improving their cytotoxicity through the upregulation of Interferon γ (IFNγ), Perforin 1 (*Prf1*), Granzyme B (*GzmB*), and the transcription factors Eomes and Blimp-1. **(B)** On the contrary, in mesenteric lymph nodes (MLN) and intestine, CD103^{+} is the main DC population which produce high amounts of RA. RA downregulates lamin A/C expression in CD4^{+} T-cells during the interaction DC-T-cell. Lamin A/C downregulation determines a mild T-cell activation through the downregulation of CD69 and CD25. However, lamin A/C downregulation does not affect T-cell proliferation, but modifies the Th fate, downregulating T-bet and upregulating the master regulator of Treg cells Foxp3, and other Treg surface markers as *Cd25*, *Rarα*, and *Pd-1*. Therefore, lamin A/C downregulation enhances Treg differentiation, and improves Treg suppressive function also by the upregulation of TGFβ, IL-10, implying and advantage against inflammatory chronic diseases as IBD.

However, if lamin A/C is downregulated due to RA release by the APCs, T-cell activation will not be so strong, and *Foxp3* expression will be enhanced leading T-cell differentiation towards Treg phenotype. As well, lamin A/C downregulation determines that these Treg cells have greater suppressive function, being an advantage against IBD development (**Figure 38B**).

In conclusion, this work set A-type lamins as key regulators of Th1 and Treg differentiation, being for this reason a potential therapeutic target for infectious diseases and IBD.

7.7. Final recapitulation and future perspectives.

The immune system is one of the most dynamics systems of our organism, having to deal with a great variety of infections, chronic inflammatory diseases, and tumors. Because of that, immune homeostasis supposes a constant challenge for immune cells. This feature determines the complexity of immune mechanisms (Huntington N.D. & Gray D.H.D., 2018). Many of the specific mechanisms exert by immune cells are already known, however, from my point of view, there is a lack of information about how immune response integrates with other systems of the body, and about immune response regulation depending on the environment. In line with this, it has been investigated the relationship between psychological stress and physical vulnerability with the adaptive immune response (Segerstrom S.C. & Miller G.E., 2004; Dhabhar F.S., 2014). Furthermore, it has been known for a long time the increased susceptibility of aged people to infections, but it is not known the signaling pathways responsible of this connection (Gardner I.D., 1980). Additionally, the effectiveness of vaccines, overcoat the ones against influenza viruses, have a reduced efficacy and depends on the individual (Osterholm M.T. *et al*, 2012). Interestingly, it has been described that lamin A/C expression is gradually lost with aging in osteoblasts and cardiomyocytes (Duque G. & Rivas D., 2006; Afilalo J. et al, 2007). Accordingly, Xin L. and collaborators described in 2015 that accelerated aging *Lmna*^{Dhe} mice mimicked the infection susceptibility to pathogens, and exhibited a systemic expansion of suppressive Foxp3^{+} Tregs. Our works robustly demonstrates that lamin A/C is regulated by micro environmental immune changes in CD4^{+} T-cells, being a key regulator of T-cell activation and Th differentiation, augmenting Th1 and blocking Treg, through epigenetic and/or transcription changes of T-cell key genes. Thus, we suggest that lamin A/C upregulation could be a specific therapy to improve the immune response against different pathologic situations. Besides, we have shown how a nuclear protein can be responsible of the adaptive immune response depending on micro-environmental changes.

The importance of the immune system in the development or origin of several non-immune diseases has been underestimated, and in the last years, the immune diseases are spreading out in the population. For this reason, immune system is currently becoming more important, and the immunotherapy is living a golden age. For example the well-known anti-TNFα antibody treatments are being used for many autoimmune diseases as rheumatoid arthritis, IBD, psoriasis and ankylosing spondylitis (Monaco C. *et al*, 2015). The immunotherapy against cancer is taking also relevance as anti-PD-1 antibody treatments

(Topalian S.L. *et al*, 2012). However, these treatments are not specific and are far from getting a 100% effectiveness in all the diagnosed patients. Therefore, it is necessary to find new signaling molecules that regulate the adaptive immune response. Our results point out that lamin A/C is an important regulator of Th1/Treg adaptive immunity protecting from Crohn's Disease development when it is downregulated in $CD4^+$ T-cells. Moreover, our data demonstrate that lamin A/C downregulation in $CD4^+$ T-cells improves directly Treg suppressive activity. Therefore, a cell therapy with Tregs derived from *Lmna*$^{-/-}$ would target specifically the immune misbalance characteristic of IBD, being a plausible treatment with no expected side effects. This therapy could replace or improve the current treatments for IBD. Moreover, this work brings to light the important role of lamin A/C in T-cell mediated immune not only in IBD, but also in infectious scenarios. So, in conclusion, lamin A/C could be a new therapeutic target not only for IBD and autoimmune pathologies, but also for other diseases caused by an immune dysregulation as infectious diseases, HIV, cancer, or atherosclerosis.

Conclusions

8. CONCLUSIONS

The obtained conclusions from this project are listed as follows:

1) Lamin A/C deficiency does not affect CD4$^+$ T-cell development.
2) Lamin A/C participates in CD4$^+$ T-cell activation by upregulating CD69 and CD25 through epigenetic mechanisms, but does not affect T-cell proliferation.
3) Lamin A/C favors Th1 differentiation of CD4$^+$ T-cells, without affecting Th2 differentiation, through H3K4me1 epigenetic modification of *T-bet* gene promoter, thus facilitating T-bet transcription factor expression.
4) Lamin A/C controls CD4$^+$ T cell cytotoxic capacity by the enhanced transcription of *Ifnγ*, *GzmB*, *Prf1*, *Eomes*, and *Blimp-1*, and regulates Th1 effector response against vaccinia virus and *Leishmania major* infections.
5) Lamin A/C depletion in CD4$^+$ T-cells facilitates Treg differentiation by upregulation of Foxp3 transcription factor expression, without affecting Th17 differentiation.
6) Lamin A/C deficiency in CD4$^+$ T-cells protects from IBD development in mice through increased Treg differentiation and reduced Th1 differentiation.
7) Lamin A/C deficiency in CD4$^+$ T-cells increases Treg suppressive function by upregulating *Tgfβ*, and *Il10* transcription levels.
8) Retinoic acid released by CD103$^+$ DCs in mesenteric lymph nodes, downregulates lamin A/C expression in CD4$^+$ T-cells upon antigen recognition.

CONCLUSIONES

Las conclusiones obtenidas de este trabajo se encuentran enumeradas a continuación:

1) La falta de expresión de lamina A/C no afecta al desarrollo de células T $CD4^+$.
2) La lamina A/C participa en la activación de la célula T $CD4^+$, subiendo la expresión de CD69 y CD25 mediante mecanismos epigenéticos, sin afectar a la proliferación de la célula T.
3) La lamina A/C favorece la diferenciación a Th1 de las células T $CD4^+$, sin afectar a la diferenciación Th2, mediante la modificación epigenética H3K4me1 en el promotor del gen *T-bet*, facilitando así, la expresión del factor de transcripción T-bet.
4) La lamina A/C controla la capacidad citotóxica de las células T $CD4^+$ facilitando la transcripción de de *Ifnγ*, *GzmB*, *Prf1*, *Eomes*, y *Blimp-1*, y regula la respuesta efectora Th1 frente a las infecciones del virus vacuna y *Leishmania major*.
5) La depleción de la expresión de lamina A/C en las células T $CD4^+$, facilita la diferenciación a células T reguladoras mediante el aumento en la expresión del factor de transcripción Foxp3, sin afectar a la diferenciación Th17.
6) La falta de expresión de lamina A/C en las células T $CD4^+$, protege frente al desarrollo de enfermedad inflamatoria intestinal en ratón, mediante el aumento de la diferenciación T reguladora y la disminución de la diferenciación Th1.
7) La falta de expresión de lamina A/C en las células T $CD4^+$, aumenta la función supresora T reguladora, subiendo los niveles de transcripción de *Tgfβ* y *Il10* en estas células.
8) El ácido retinoico liberado por las células dendríticas $CD103^+$ en los ganglios mesentéricos, disminuye la expresión de la lamina A/C en las células T $CD4^+$ que se produce tras el reconocimiento antigénico.

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9. REFERENCES

9.1. Journal articles.

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Annexes

10. ANNEXES.

- **Publications derived from this Thesis:**

Toribio-Fernández, R., Zorita, V., Rocha-perugini, V., Iborra, S., Martínez, G., Chevre, R., …González-Granado, J. M. (2018). Lamin A / C augments Th1 differentiation and response against vaccinia virus and *Leishmania major*. *Cell Death and Disease, 9(9),* 1–15.

Toribio-Fernandez, R., Zorita, V., Herrero-Fernandez, B., & Gonzalez-Granado, J. M. (2018). An *In Vivo* Mouse Model to Measure Naïve CD4 T Cell Activation, Proliferation and Th1 Differentiation Induced by Bone Marrow-derived Dendritic Cells. *Journal of Visualized Experiments.*

Toribio-Fernández et al. Cell Death and Disease 20189:9
DOI 10.1038/s41419-017-0007-6

Cell Death & Disease

ARTICLE Open Access

Lamin A/C augments Th1 differentiation and response against vaccinia virus and *Leishmania major*

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Abstract

Differentiation of naive CD4$^+$ T-cells into functionally distinct T helper (Th) subsets is critical to immunity against pathogen infection. Little is known about the role of signals emanating from the nuclear envelope for T-cell differentiation. The nuclear envelope protein lamin A/C is induced in naive CD4$^+$ T-cells upon antigen recognition and acts as a link between the nucleus and the plasma membrane during T-cell activation. Here we demonstrate that the absence of lamin A/C in naive T-cell reduces Th1 differentiation without affecting Th2 differentiation in vitro and in vivo. Moreover, *Rag1*$^{-/-}$ mice reconstituted with *Lmna*$^{-/-}$CD4$^+$CD25$^-$ T-cells and infected with vaccinia virus show weaker Th1 responses and viral removal than mice reconstituted with wild-type T-cells. Th1 responses and pathogen clearance upon *Leishmania major* infection were similarly diminished in mice lacking lamin A/C in the complete immune system or selectively in T-cells. Lamin A/C mediates Th1 polarization by a mechanism involving T-bet and IFNγ production. Our results reveal a novel role for lamin A/C as key regulator of Th1 differentiation in response to viral and intracellular parasite infections.

Introduction

The nuclear envelope is composed of nuclear pore complexes, the outer and inner nuclear membranes, and the nuclear lamina. The nuclear lamina is a filamentous protein layer mainly composed of A- and B-type lamins and provide mechanical stability to the inner nuclear membrane, regulating nucleus positioning, chromatin structure, nuclear pore complex organization, nuclear envelope breakdown and reassembly during mitosis, DNA replication, DNA damage responses, cell-cycle progression, cell differentiation, cell polarization during cell migration, and transcription[1,2]. We have previously shown that lamin A expression is triggered in naive T-cells upon antigen recognition and enhances T-cell activation by coupling actin dynamics and immunological synapse formation[3].

T-cells orchestrate the protection against microbial pathogens[4]. In peripheral lymphoid organs, antigen-presenting cells (APCs) stimulate cognate CD4$^+$ T-cells, which proliferate and undergo differentiation into distinct specialized effector T helper (Th) cells that are essential for the development of adaptive immune responses[5]. Tight control of naive T-cell differentiation is crucial for eliciting an appropriate host defense, triggering immune-mediated inflammation without deleterious tissue damage. Th subsets are defined by the differential expression of surface markers, transcription factors, and effector cytokines and play essential and distinct roles in mediating or directing the nature of the response to pathogens, commensals, and vaccines. T-cell differentiation in diverse Th subsets depends on the type of antigen encountered, the TCR signal intensity, and the local

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cytokine milieu[4,6–8]. Indeed, Th1 differentiation, which is required for host defense against intracellular pathogens, involves interferon-γ (IFNγ) production in an interleukin (IL)-2-dependent manner by the transcription factor T-bet[6]. Th2 differentiation is triggered by extracellular pathogens or allergens through the induction of GATA-3 and the activation of IL-4-dependent signal transducer and activator of transcription factor 6 (Stat-6)[9]. Signals emanating from the nuclear interior may also condition naive T-cell polarization. Here we show that lamin A/C expression augments $CD4^+$ T-cell Th1 differentiation in response to pathogen infection by regulating T-bet transcription factor expression and IFNγ production.

Results

Lamin A/C regulates Th1 differentiation

To analyze the role of A-type lamins in antigen-dependent T-cell differentiation, *Lmna*$^{-/-}$ and wild-type (WT) mice were back-crossed with OTII mice, which express a TCR (T-cell receptor) specific for ovalbumin (OVA) peptide. Naive $CD4^+$ T-cells were isolated from *Lmna*$^{-/-}$/OTII or WT/OTII mice and co-cultured with OVA-loaded WT APCs in the absence of polarizing cytokines. Compared with WT $CD4^+$ T-cells, fewer *Lmna*$^{-/-}$ $CD4^+$ T-cells were $IFN\gamma^+$, indicating the importance of lamin A/C for antigen-dependent Th1 differentiation (Fig. 1a). This difference was not abolished by addition of IL-2 (Fig. 1b). We next directed Th1 or Th2 differentiation in vitro by incubating WT and *Lmna*$^{-/-}$ $CD4^+$ T-cells with anti-CD3 and anti-CD28 antibodies and Th1 or Th2 polarizing cytokines. Interestingly, *Lmna*$^{-/-}$ $CD4^+$ T-cells produced fewer Th1 cells than WT cells but similar numbers of Th2 cells (Fig. 1c). Th1 differentiation triggered by co-culture with OVA-loaded WT APCs in the presence of Th1 polarizing cytokines was also lower in $CD4^+$ T-cells from *Lmna*$^{-/-}$/OTII mice than in WT/OTII T-cells (Fig. 1d). These experiments suggest that lamin A/C is an important intrinsic regulator of T-cell differentiation following TCR stimulation.

Lamin A/C enhances Th1 responses in vivo

It has been previously shown that intrinsic lamin A/C does not affect T-cell development[10]. Moreover, we did not observe any role for lamin A/C in very early T-cell activation. Indeed, TCR stimulation triggered similar levels of extracellular signal–regulated kinase 1/2 (ERK1/2) phosphorylation in WT and *Lmna*$^{-/-}$ CD4 T-cells (Figure S1a, day 0), indicating that lamin A/C is not involved in T-cell development and early TCR activation. We have previously shown that lamin A/C is transiently expressed in $CD4^+$ T-cells upon antigen recognition[3]. Confirming our previous observation, levels of pERK1/2 were increased in WT lamin A/C-expressing cells but not in *Lmna*$^{-/-}$ CD4 T-cells after a second TCR stimulation, when lamin A/C is already expressed in WT CD4 T-cells (ref. 3; and Figure S1b), (Figure S1a, day 1).

To investigate the role of lamin A/C in Th1 differentiation in vivo, mice were infected with vaccinia virus (VACV), which provokes a robust Th1 immune response in mice[11,12]. VACV infection induces transient expression of lamin A/C, peaking at 1 day after infection in draining lymph nodes (Figure S2). At 3 days after intraperitoneal VACV infection, the frequency of $IFN\gamma^+CD4^+$ T-cells in mesenteric lymph nodes and peritoneal exudate was lower in *Lmna*$^{-/-}$ mice than in WT mice (Fig. 2a, b). To study the role of lamin A/C specifically in the immune system, we reconstituted lethally irradiated WT CD45.1 mice with WT or *Lmna*$^{-/-}$ CD45.2 bone marrow for 2 months. Confirmation of bone marrow reconstitution with anti CD45.1 and CD45.2 antibodies revealed no significant differences between genotypes. After intraperitoneal infection with VACV, we analyzed the frequency of $IFN\gamma^+$ and IL-4^+ in $CD4^+$ T-cells. *Lmna*$^{-/-}$-reconstituted mice had proportionately fewer $IFN\gamma^+$ T-cells, and similar numbers of $IL4^+$ T-cells with respect to WT-reconstituted mice, in both spleen and peritoneal exudate (Fig. 2c). To trigger antigen-specific Th1 responses in vivo, we reconstituted WT mice with either WT/OTII or *Lmna*$^{-/-}$/OTII bone marrow and then infected the mice intraperitoneally with VACV-OVA. As before, the *Lmna*$^{-/-}$/OTII-reconstituted $CD4^+$ population included a lower proportion of $IFN\gamma^+$ cells than did WT/OTII-derived $CD4^+$ cells (Fig. 2d). Moreover, reconstitution with a mix of bone marrow from WT/OTII and *Lmna*$^{-/-}$/OTII mice produced a lower percentage of $IFN\gamma^+CD4^+$ T-cells in *Lmna*$^{-/-}$/OTII than in WT/OTII cells (Fig. 2e). Our results indicate that lamin A/C depletion in the hematopoietic compartment impairs Th1 differentiation in vitro and in vivo. To directly assess the importance for Th1 differentiation of lamin A/C in $CD4^+$ T-cells, we adoptively transferred WT mice with $CD4^+$ T-cells from WT/OTII or *Lmna*$^{-/-}$/OTII mice. Th1 differentiation from *Lmna*$^{-/-}$ $CD4^+$ T-cells was weaker than from their WT counterparts, producing a lower percentage of $IFN\gamma^+CD4^+$ T-cells (Fig. 3a). Similar results were obtained when WT recipient mice were transferred with a mix of WT/OTII and *Lmna*$^{-/-}$/OTII $CD4^+$ T-cells followed by VACV-OVA inoculation, either intraperitoneally (Fig. 3b) or subcutaneously in the footpad (Fig. 3c). These results indicate that lamin A/C expression specifically regulates $CD4^+$ T-cell differentiation toward Th1 cells in vivo.

We next investigated whether the action of lamin A/C in $CD4^+$ T-cell polarization was related to antigen presentation by dendritic cells (DC). WT mice received subcutaneous injections of lipopolysaccharide (LPS)-matured OVA-loaded bone marrow-derived DCs (BMDCs), which trigger Th1 differentiation[13], and 18 h

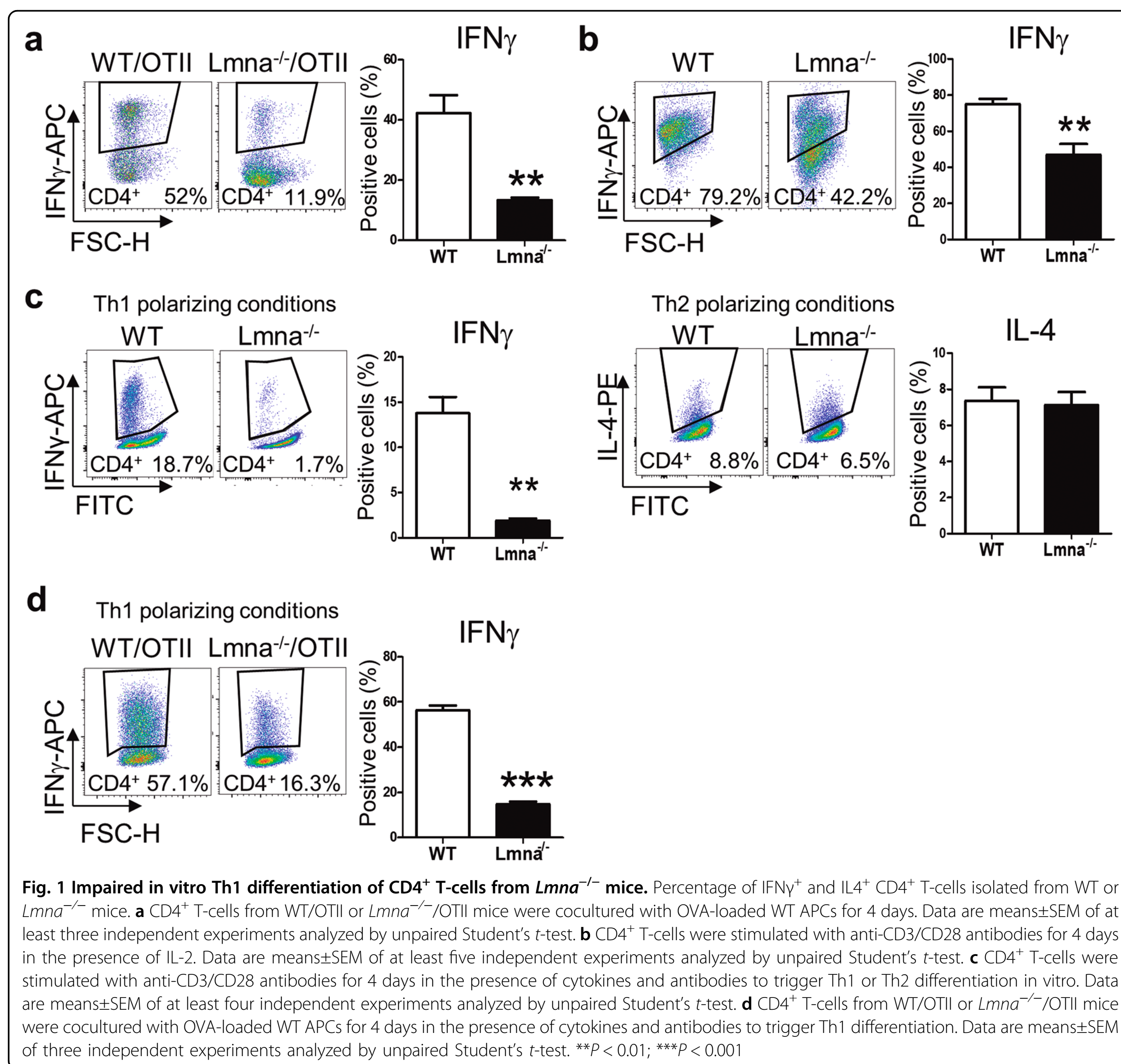

Fig. 1 Impaired in vitro Th1 differentiation of CD4$^+$ T-cells from *Lmna*$^{-/-}$ mice. Percentage of IFNγ$^+$ and IL4$^+$ CD4$^+$ T-cells isolated from WT or *Lmna*$^{-/-}$ mice. **a** CD4$^+$ T-cells from WT/OTII or *Lmna*$^{-/-}$/OTII mice were cocultured with OVA-loaded WT APCs for 4 days. Data are means±SEM of at least three independent experiments analyzed by unpaired Student's *t*-test. **b** CD4$^+$ T-cells were stimulated with anti-CD3/CD28 antibodies for 4 days in the presence of IL-2. Data are means±SEM of at least five independent experiments analyzed by unpaired Student's *t*-test. **c** CD4$^+$ T-cells were stimulated with anti-CD3/CD28 antibodies for 4 days in the presence of cytokines and antibodies to trigger Th1 or Th2 differentiation in vitro. Data are means±SEM of at least four independent experiments analyzed by unpaired Student's *t*-test. **d** CD4$^+$ T-cells from WT/OTII or *Lmna*$^{-/-}$/OTII mice were cocultured with OVA-loaded WT APCs for 4 days in the presence of cytokines and antibodies to trigger Th1 differentiation. Data are means±SEM of three independent experiments analyzed by unpaired Student's *t*-test. $^{**}P < 0.01$; $^{***}P < 0.001$

later received intravenous injections of WT/OTII or *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells. After 7 days, Th1 differentiation of adoptively transferred T-cells was analyzed in draining lymph nodes. *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells produced fewer IFNγ$^+$CD4$^+$ T-cells in the presence of LPS-treated BMDCs than WT/OTII cells (Fig. 3d). In other experiments, WT mice received subcutaneous injections of splenic CD11c$^+$ DCs incubated 4 h in medium containing LPS or papain to potentiate antigen-dependent T-cell differentiation to Th1 or Th2, respectively[13,14]. As before, 18 h later the mice received intravenous injections of WT/OTII or *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells, and Th1 and Th2 differentiation was analyzed in draining lymph nodes after 7 days. *Lmna*$^{-/-}$ CD4$^+$ T-cells produced fewer IFNγ$^+$CD4$^+$ T-cells in the presence of LPS-treated BMDCs than their WT counterparts; in contrast, in the presence of papain-treated BMDCs the proportion of IL4$^+$ cells was similar in WT and *Lmna*$^{-/-}$ CD4$^+$ T-cells (Fig. 3e). These results indicate that lamin A/C expression specifically regulates CD4$^+$ T-cell differentiation toward Th1 cells in vivo.

Lamin A/C enhances T-cell activation but is not essential for T-cell proliferation

Reduced Th1 cell polarization from *Lmna*$^{-/-}$ CD4$^+$ T-cells could be due to impaired T-cell activation, which is enhanced by lamin A in vitro[3]. We therefore analyzed CD4$^+$ T-cell activation in response to VACV infection in the absence of lamin A/C expression. A 1:1 mix of naive CD4$^+$ T-cells from *Lmna*$^{-/-}$/OTII and WT/OTII mice

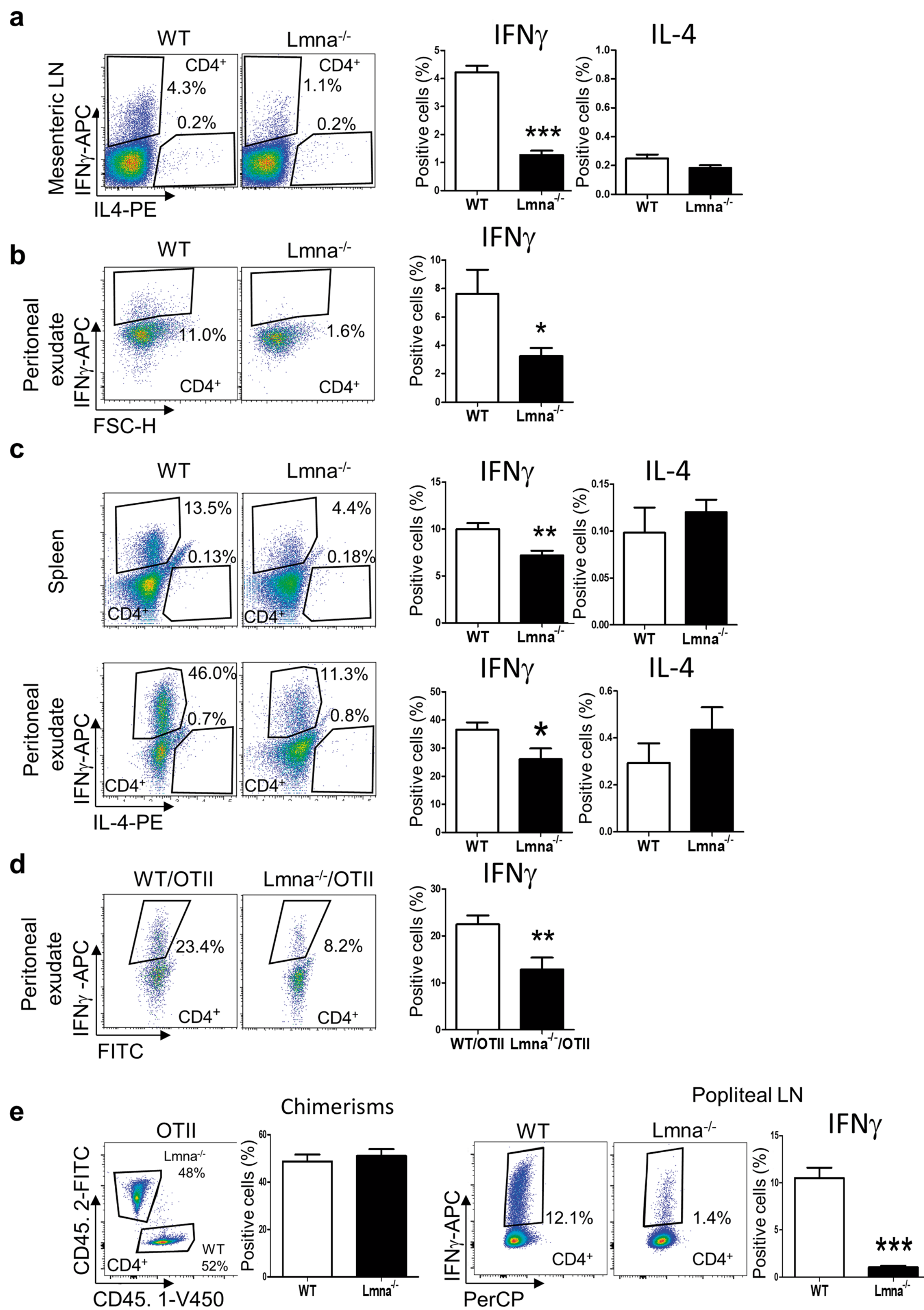

Fig. 2 (See legend on next page.)

was adoptively transferred into WT recipient mice before intradermal infection with VACV-OVA. Surface expression of the T-cell activation markers CD25 and CD69 was measured 48 h later (Fig. 4a, b). Accordingly, compared with WT cells, *Lmna*$^{-/-}$ CD4$^+$ T-cells in draining lymph nodes expressed less CD25 and CD69.

Next, we investigated whether lamin A/C could regulate T-cell proliferation, which occurs after antigen recognition and activation. However, proliferation after in vitro activation with anti-CD3 and anti-CD28 was similar in *Lmna*$^{-/-}$ and WT CD4$^+$ T-cells (Fig. 4c). Similarly, after VACV infection, WT and *Lmna*$^{-/-}$ mice had similar levels of proliferating CD4$^+$ T-cells, as shown by Ki67 expression (Fig. 4d), proliferating cell nuclear antigen (PCNA) expression (Fig. 4e), and 5-bromo-2-deoxyuridine (BrdU) incorporation (Fig. 4f–g). These experiments show that A-type lamins regulate T-cell activation without affecting cell proliferation.

Lamin A/C enhances T-bet levels

The master regulator of Th1 differentiation is T-bet. Analysis of T-bet expression in CD4$^+$ T-cells revealed that deficiency in A-type lamins reduced both the percentage of T-bet$^+$ cells and T-bet expression levels in anti-CD3/CD28-stimulated CD4$^+$ T-cells (Fig. 5a, b). In agreement with the effect on Th1 differentiation, lamin A/C deficiency also diminished the percentage of IFNγ$^+$CD4$^+$ T-cells (Fig. 5a). Similar results were obtained after stimulation with anti-CD3/CD28 antibodies in the presence of Th1-differentiation cytokines (Fig. 5c). Moreover, lamin A/C expression was also important for T-bet expression in vivo after Th1 differentiation triggered by VACV infection (Fig. 5d). Low T-bet protein expression in *Lmna*$^{-/-}$ T-cells was accompanied by lower mRNA expression in vitro (Fig. 5e), suggesting regulation at the level of mRNA synthesis.

A-type lamins regulate TCR clustering and subsequent downstream signaling upon T-cell activation[3]. However, altered Th1 differentiation in the absence of lamin A/C was not exclusively related to differences in TCR clustering, since the percentage of IFNγ$^+$ and T-bet$^+$ *Lmna*$^{-/-}$ CD4$^+$ T-cells was also lower after TCR-independent T-cell stimulation with phorbol myristate acetate (PMA) and ionomycin (Fig. 5a).

Lamin A/C regulates Th1 immunity against pathogen infection

T-bet and IFNγ play major roles during in vivo Th1 responses against pathogens[11,15,16]. The low expression of these factors in *Lmna*$^{-/-}$ CD4$^+$ T-cells prompted us to investigate the role of lamin A/C in Th1-mediated pathogen clearance in vivo. *Rag1*$^{-/-}$mice lacking T- and B-cells were adoptively transferred with naive CD25$^-$CD4$^+$ T-cells from WT or *Lmna*$^{-/-}$ mice. Mice were infected with VACV by tail scarification. Without CD4$^+$ T-cell adoptive transfer, 100% of *Rag1*$^{-/-}$ mice die 12 days after VACV infection; CD4$^+$ T-cell adoptive transfer extends lifespan by accelerating viral clearance at least in part through a mechanism mediated by Th1 cells[17]. In our experiments, *Rag1*$^{-/-}$ mice adoptively transferred with CD25$^-$CD4$^+$ *Lmna*$^{-/-}$ T-cells were more susceptible to primary VACV infection, as indicated by an elevated viral titer and a reduced Th1 response in the spleen (Fig. 6a, b). Moreover, IFNγ and T-bet mRNA expression was reduced in adoptively transferred mice, whereas the Th2 transcription factor GATA3 was unaffected (Fig. 6c). Viral clearance requires the efficient coordination of multiple immune effector mechanisms[11]. The role of lamin A/C in viral clearance seems to be CD4$^+$ T-cell dependent, since only CD4$^+$ T-cells were adoptively transferred to *Rag1*$^{-/-}$ mice before VACV infection. Effector CD4$^+$ T-cells have been shown to respond to viral pathogens through two mechanisms: cytokine production (mostly IFNγ and tumor necrosis factor-α (TNFα)) and a direct cytolytic activity mediated by perforin and FAS (also known as CD95)[11]. CD4$^+$ T-cell cytotoxic activity does not require Th1 cell polarization but is dependent on the expression of the transcription factors T-bet, eomesodermin (Eomes), and Blimp-1, which are crucial for their development in vivo through the induction of granzyme B (GzmB) and perforin 1 (Prf1)[18,19]. Interestingly, *Rag1*$^{-/-}$ mice adoptively transferred with *Lmna*$^{-/-}$ CD4$^+$ T-cells and scarified with VACV had lower levels of IFNγ mRNA in the large

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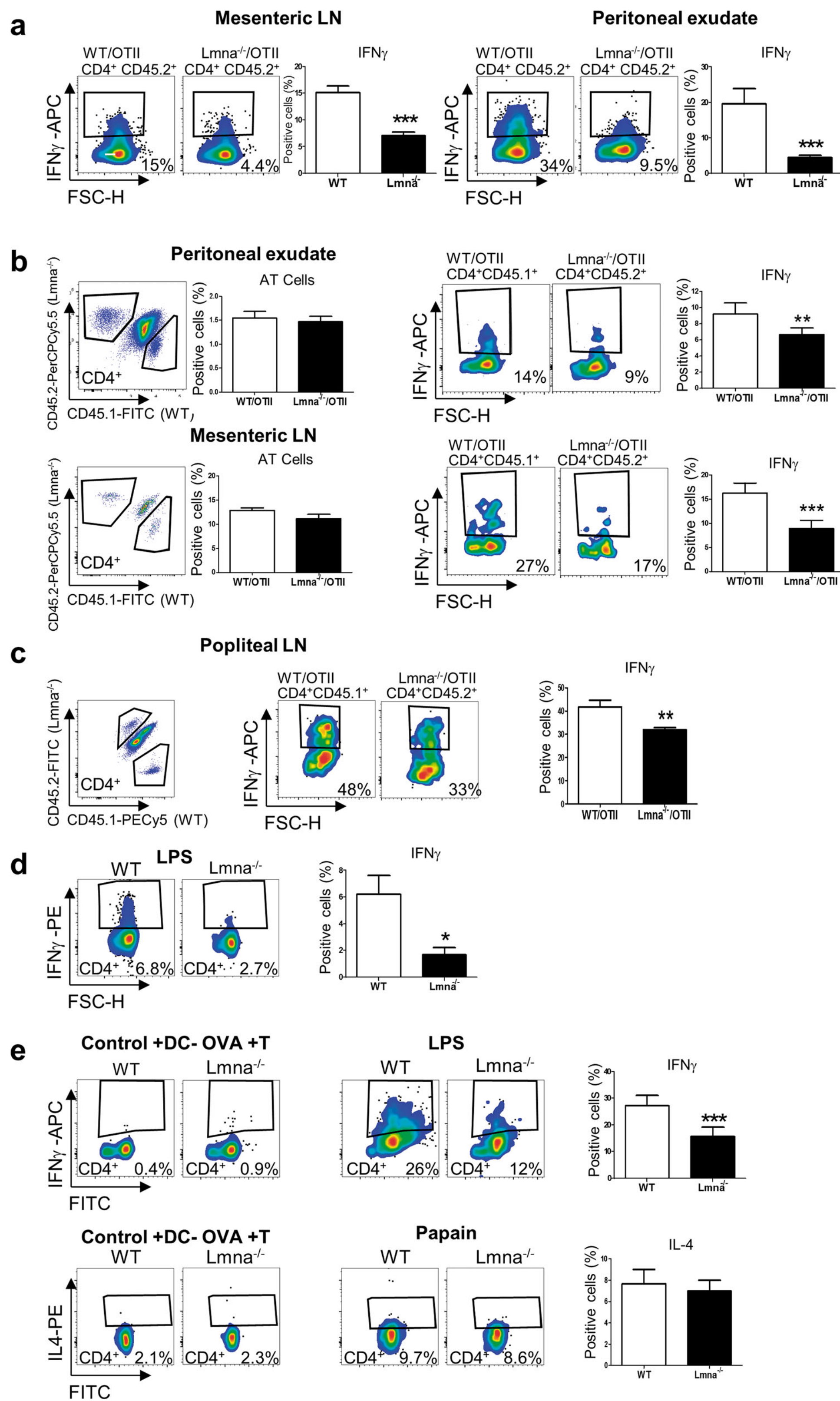
Fig. 2 Impaired in vivo Th1 differentiation of naive CD4$^+$ T-cells from *Lmna*$^{-/-}$ mice. Percentage of IFNγ$^+$ or IL4$^+$ CD4$^+$ T-cells upon VACV infection. **a**, **b** WT and *Lmna*$^{-/-}$ mice were intraperitoneally infected with VACV, and after 3 days mesenteric lymph nodes **a** and peritoneal exudate **b** were analyzed, ($n = 11$ WT and 7 *Lmna*$^{-/-}$ mice). Data are means±SEM analyzed by unpaired Student's *t*-test. **c** Irradiated CD45.1$^+$ WT mice were reconstituted with CD45.2$^+$ WT or CD45.2$^+$ *Lmna*$^{-/-}$ bone marrow and infected intraperitoneally with VACV. After 5 days, spleens and the peritoneal exudate were analyzed by flow cytometry ($n = 7$ and 8–11 mice from two independent experiments). Data are means±SEM analyzed by unpaired Student's *t*-test **d** Irradiated CD45.1$^+$ WT mice were reconstituted with CD45.2$^+$ WT/OTII or CD45.2$^+$ *Lmna*$^{-/-}$/OTII bone marrow and infected intraperitoneally with VACV-OVA. After 5 days, peritoneal exudate was analyzed ($n = 9$ and 6 mice from 2 independent experiments). Data are means ±SEM analyzed by unpaired Student's *t*-test. **e** Irradiated CD45.1$^+$/CD45.2$^+$ WT mice were reconstituted with a mix of CD45.1$^+$ WT/OTII with CD45.2$^+$ *Lmna*$^{-/-}$/OTII bone marrow. Five days after VACV-OVA intradermal infection in the footpad, popliteal lymph nodes were analyzed ($n = 9$ and 9 mice from 2 independent experiments). Data are means±SEM analyzed by paired Student's *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



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intestine than mice receiving WT CD4$^+$ T-cells (Fig. 6c). In addition, we found that lamin A/C expression in CD4$^+$ T-cells differentiated to Th1 in vitro positively regulates the mRNA expression of eomes, Blimp1, GzmB, and Prf1 but not granzyme A (GzmA) (Figure S3). Lamin A/C also regulates CD4$^+$ T-cell cytoxicity in vivo, since VACV-OVA-generated *Lmna*$^{-/-}$ Th1 cells were less efficient to kill OVA-loaded B220$^+$MHCII$^+$ B cells, after being adoptively transferred to *Rag1*$^{-/-}$ mice (Figure S4).

We also analyzed the importance of lamin A/C expression for Th1 responses against the intracellular parasite *L. major*[16]. Mice on the C57BL/6 background control *L. major* infection by developing a protective Th1 response[20]. C57BL/6 mice lacking lamin A/C in the whole immune system (Fig. 6d–h) or specifically in T-cells (Fig. 6i–k) developed progressively larger and non-healing lesions (Fig. 6d, e, i) after inoculation of the ear dermis with a low dose of *L. major* parasites. This effect correlated with a higher parasite burden in the infection site and in draining lymph nodes after 3 weeks (Fig. 6f, j). At this time, *Lmna*$^{-/-}$ mice showed a reduced percentage of IFNγ$^+$ CD4$^+$ T-cells (Fig. 6g, k), with less IFNγ production (Fig. 6h). These results further support the role of lamin A/C in Th1 responses against pathogens.

Discussion

The role of nuclear envelope proteins in the regulation of immune homeostasis remains largely unknown[21]. Similarly, little is known about the molecular signals that switch immune function from protective to tissue damaging. Adaptive immunity is finely regulated by the interplay between immune regulatory mechanisms and effector T-cell responses. T-cells play central roles in orchestrating the protection against diverse microbial pathogens[4]. Many studies have investigated membrane mediators important for T-cell fate decision, while others have assessed the role of cytoplasmic proteins; however, the importance of nuclear envelope proteins for naive T-cell differentiation remained overlooked. Despite studies characterizing signaling pathways from the membrane to the nucleus, there is a lack of information about signals arising in the nuclear interior and the role of nuclear proteins in mediating the transmission of information to the cytoplasm. It has been previously shown that intrinsic lamin A/C deficiency does not affect T- and B-cell development, driving the generation of functional and self-MHC-restricted CD4$^+$ and CD8$^+$ T-cells[10]. We previously demonstrated that transient lamin A/C expression in CD4$^+$ T-cells after antigen recognition contributes to T-cell activation[3]. In the present study, we have identified the nuclear envelope protein lamin A/C as a crucial driver of Th1 polarization, both in vitro and in vivo.

Lamin A/C is expressed upon T-cell activation, and its deficiency downregulates the transcription factor T-bet, thereby impairing naive CD4$^+$ T-cell differentiation toward the Th1 phenotype. Strikingly, this effect is not counterbalanced by an increase in Th2 differentiation. Distinct signaling pathways can drive T-cell differentiation[22]. In response to TCR activation and mTOR costimulation, Akt modulates transcription of T-bet but not Gata3, thus affecting Th1 but not Th2 differentiation. In contrast, PKC-θ activation yields the opposite result[22]. Lamin A/C is an important regulator of gene transcription during adipose tissue differentiation[23] and controls the phosphorylation of signaling molecules in other cellular contexts[1,24]. Indeed, we previously showed that lamin A/C binds to ERK1/2 and c-Fos, regulating AP-1 transcription factor target genes[24,25] and that lamin A/C controls CD4$^+$ T-cell activation through ERK1/2 signaling[3]. Interestingly, the formation of c-Fos/c-Jun dimers can potentiate Th1 differentiation[26]. Lamin A/C thus seems to specifically regulate pathways involved in Th1 differentiation, directly modulating the transcription of Th1-related genes. This process occurs in a TCR-dependent manner, although lamin A/C can also regulate signaling downstream the TCR, as observed after stimulation with PMA plus ionomycin.

The Th fate of antigen-engaged T-cells depends on many factors, including the type of antigen encountered,

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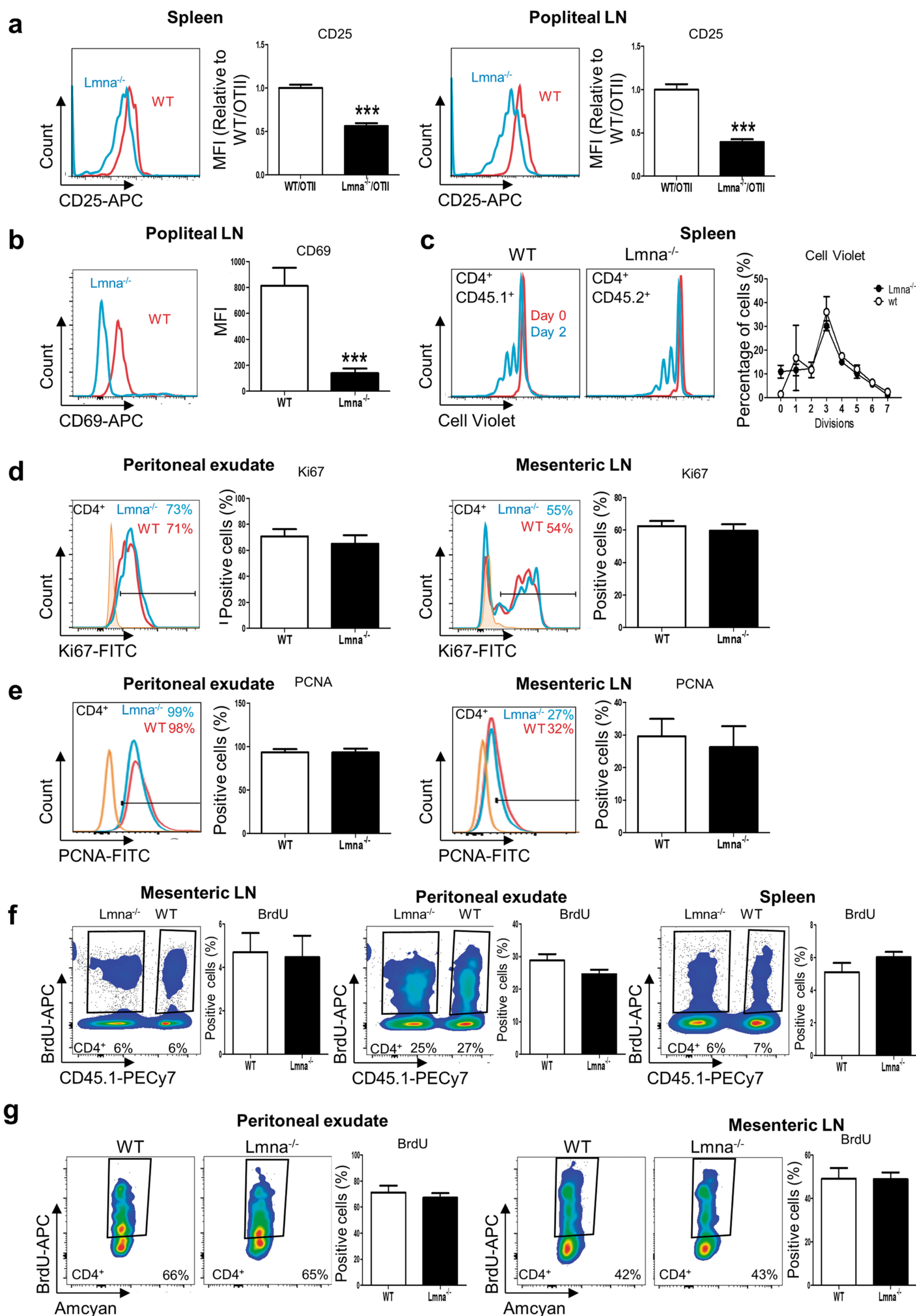
Fig. 3 Compromised in vivo Th1 differentiation of naive CD4$^+$ T-cells from *Lmna*$^{-/-}$ mice. Percentage of IFNγ$^+$ CD4$^+$ T-cells. **a** Recipient CD45.1$^+$ WT mice were adoptively transferred with CD45.2$^+$ WT/OTII or CD45.2$^+$ *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells. Five days after intraperitoneal VACV-OVA infection, mesenteric lymph nodes and the peritoneal exudate were analyzed ($n = 10$ WT and 9 *Lmna*$^{-/-}$ mice from 2 independent experiments). Data are means±SEM analyzed by unpaired Student's *t*-test. **b** CD45.1$^+$/CD45.2$^+$ WT mice were adoptively transferred (AT) with a mix of CD45.1$^+$ WT/OTII and CD45.2$^+$ *Lmna*$^{-/-}$/OTII CD4$^+$ T-cells and infected intraperitoneally with VACV-OVA. After 5 days, peritoneal exudate and mesenteric lymph nodes were analyzed ($n = 11$ and 11 mice from 2 independent experiments) Data are means±SEM analyzed by paired Student's *t*-test. **c** CD45.1$^+$/CD45.2$^+$ WT mice were AT as in **b**, and after 5 days of intradermal infection with VACV-OVA the popliteal lymph nodes were analyzed ($n = 6$ and 6 mice from 2 independent experiments). Data are means±SEM analyzed by paired Student's *t*-test **d** OVA-loaded LPS-matured BMDCs were AT to WT recipient mice. After 18 h, recipients received intravenous injections of a mix of CD45.1$^+$ WT and CD45.2$^+$ *Lmna*$^{-/-}$/*OTII* CD4$^+$ naive T-cells. Four days later, the popliteal lymph nodes were analyzed ($n = 3$ and 3 mice from 1 representative experiment out of 2). Data are means±SEM analyzed by unpaired Student's *t*-test **e** Isolated splenic CD11c$^+$ cells were incubated with OVA and LPS or with OVA and papain for 4 h and then AT to WT recipients. Control mice were transferred with CD11c$^+$ cells in the absence of OVA (DC-OVA). After 18 h, recipients received intravenous injections of a mix of CD45.1$^+$ WT and CD45.2$^+$ *Lmna*$^{-/-}$ CD4$^+$ naive T-cells. Four days later, the popliteal lymph nodes were analyzed. The percentage of IL4$^+$ CD4$^+$ T-cells was also investigated ($n = 4$ and 4 mice from 2 independent experiments). Data are means±SEM analyzed by unpaired Student's *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



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the TCR signal intensity, the duration of antigen presentation, and the local cytokine milieu[4,6–8,26,27]. Th1 differentiation is favored by stronger T-cell activation signals and longer T–DC cell–cell interactions[8,26]. Lamin A/C enhances T-cell activation by increasing TCR signal strength and promoting T–DC interaction[3]. Weaker TCR signal intensity in the absence of lamin A/C could account for the observed reduction in $CD4^+$ T-cell activation triggered by VACV infection. However, this cannot fully explain the differences in Th1 vs. Th2 differentiation in lamin A/C-deficient T-cells, indicating that additional factors are involved. Regarding the cytokine milieu, IL-2, IL-12, and IFNγ are known to facilitate Th1 differentiation, whereas IL-4 favors Th2 differentiation. Moreover, some cytokines promote differentiation to a specific Th phenotype without affecting others. For example, IL-33 enhances Th1 but not Th2 polarization in the presence of IL-12[28]. Lamin A/C expression could regulate the production and release of cytokines important for Th1 phenotype. In this regard, we find that lamin A/C deficiency reduces membrane expression of the IL-2 receptor CD25 in $CD4^+$ T-cells activated by VACV infection in vivo. Incubation of $CD4^+$ T-cells with IL-2 or Th1 polarizing cytokines during in vitro differentiation did not rescue the impaired *Lmna*$^{-/-}$ Th1 differentiation, indicating that intracellular signals driven by the nuclear envelope protein lamin A/C directly regulate Th1 differentiation.

Viral clearance requires the efficient coordination of multiple immune effector mechanisms[11]. We show that lamin A/C deficiency in $CD4^+$ T-cells results in a defective Th1 differentiation, impairing the response against VACV and delaying viral clearance. These results suggest that lamin A/C is an essential component of the immune response against cytopathic viruses. Remarkably, the role of lamin A/C in viral clearance seems to be $CD4^+$ T-cell dependent, since only $CD4^+$ T-cells were adoptively transferred to T- and B-cell-deficient *Rag1*$^{-/-}$ mice before mouse infection with VACV. Accordingly, in humans $CD4^+$ T-cells are critical for antiviral immunity and control of VACV replication[12]. $CD4^+$ T-cells can also regulate innate immune responses that mediate viral clearance[11], which is independent on B-cells and $CD8^+$ T-cells, and might be important for appropriate $CD4^+$ T-cell responses against VACV[17]. Interestingly, *Lmna*$^{-/-}$ $CD4^+$ T-cells show reduced cytotoxic activity in vivo and impaired in vitro mRNA expression of transcription factors involved in the regulation of $CD4^+$ T-cell cytotoxicity, like eomes, Blimp1, GzmB, and Prf1.

The protozoan parasite *L. major* cause cutaneous leishmaniasis[29], usually provoking local self-healing skin lesions in humans. However, the parasite can cause prolonged non-healing skin ulcers with extensive tissue destruction and become systemically distributed[29]. C57BL/6 mice develop protective immune responses against *L. major*. We show that WT recipient C57BL/6 mice reconstituted with *Lmna*$^{-/-}$ bone marrow develop poorer Th1 responses against this parasite, with worse pathogen clearance than recipients of WT cells. The immune response against *L. major* involves multiple cell types. Importantly, our results clearly demonstrate that lack of lamin A/C specifically in T-cells impaired healing of ear lesions. C57BL/6 mice develop Th1-dependent responses to *L. major*, and following a high *L. major* dose the lesions heal in the absence of $CD8^+$ T-cells[30,31]. Moreover, cytolytic $CD8^+$ T-cells are pathogenic when recruited to *Leishmania* lesions in large numbers[32]. Therefore, impaired responses observed in $Lmna^{flox/flox}$×CD4-CRE mice likely associate with lamin A/C-deficient $CD4^+$ T-cells. The early immune response determines whether *L. major* skin lesions will be self-healing or chronic. C57BL/6 mice develop $CD4^+$ Th1 cell-mediated resistance, whereas other mouse strains develop $CD4^+$ Th2 responses and are extremely susceptible to infection[33]. Therefore, $CD4^+$ Th1 cells are crucial for reducing *Leishmani*a infection[34], producing IFNγ, IL-2, and TNFα locally and leading to macrophage activation and parasite elimination. Our experiments show a diminished percentage of IFNγ-producing $CD4^+$ T-cells

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Fig. 4 *Lmna*$^{-/-}$ $CD4^+$ T-cells show impaired in vivo and in vitro activation in response to VACV infection. a, **b** WT $CD45.1^+/CD45.2^+$ mice were adoptively transferred with a mix of $CD4^+$ T-cells from $CD45.1^+$/OTII WT and $CD45.2^+$/OTII *Lmna*$^{-/-}$ mice and then intradermally infected in the footpad with VACV-OVA for 2 days. Spleen and popliteal lymph nodes were analyzed, and the expression of **a** CD25 and **b** CD69 was quantified (n = 6 mice). Data are means±SEM analyzed by unpaired Student's *t*-test **c** Splenocytes from $CD45.1^+$ WT/OTII and $CD45.2^+$ *Lmna*$^{-/-}$/OTII mice were mixed, stained with cell violet, and cultured in the presence of OVA for 2 days. $CD4^+$ T-cell proliferation was quantified and the number of cell divisions is shown in the graph. Data are means±SEM of six independent experiments analyzed by two-way ANOVA with Bonferroni's multiple comparison test. **d**, **e** $CD4^+$ T-cells from $CD45.1^+$ WT and $CD45.2^+$ *Lmna*$^{-/-}$ mice were mixed 1:1 and adoptively transferred to CD45.1/CD45.2 WT mice. Recipient mice were then intraperitoneally infected with VACV for 7 days. The peritoneal exudates and mesenteric lymph nodes were analyzed, and the percentage of **d** $Ki67^+$ or **e** $PCNA^+$ $CD4^+$ T-cells was quantified (n = 5 mice from 2 independent experiments) Data are means±SEM analyzed by unpaired Student's *t*-test. **f** $CD45.1^+$ WT recipient mice were transplanted with a 1:1 mix $CD45.1^+$ WT and $CD45.2^+$ *Lmna*$^{-/-}$ bone marrow and infected intraperitoneally with VACV. Mice were injected with BrdU, and 24 h later the percentage of $BrdU^+$ $CD4^+$ T-cells was quantified in mesenteric lymph nodes, peritoneal exudate, and spleen (n = 3 mice from 1 representative experiment) Data are means±SEM analyzed by paired Student's *t*-test. **g** $CD4^+$ T-cells from $CD45.2^+$ WT or $CD45.2^+$ *Lmna*$^{-/-}$ mice were mixed 1:1 and adoptively transferred to $CD45.1^+/CD45.2^+$ WT mice. Recipient mice were infected intraperitoneally with VACV for 6 days and injected with BrdU. After 24 h, the percentage of $BrdU^+$ $CD4^+$ T-cells was quantified in the peritoneal exudate and mesenteric lymph nodes (n = 5 and 5 mice). Data are means±SEM analyzed by paired Student's *t*-test. ***P < 0.001

and reduced IFNγ production in *Lmna*$^{-/-}$ mice. IFNγ secretion by CD4^{+} T-cells induces macrophages to produce nitric oxide and TNF, thus controlling *L. major* infection[33,35]. This mechanism could explain the diminished pathogen clearance observed in the absence of lamin A/C. Importantly, we show that lamin A/C in CD4^{+}

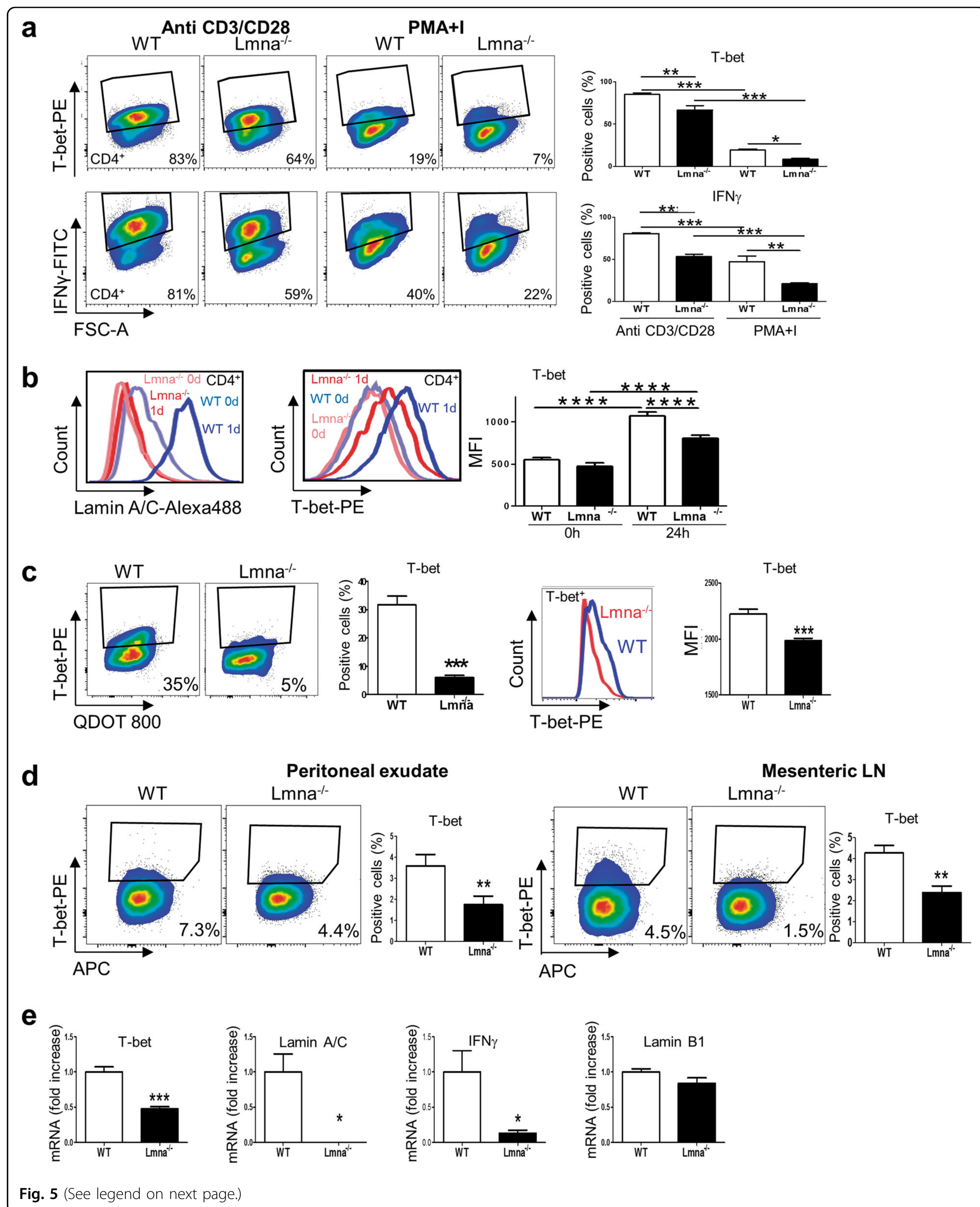

Fig. 5 (See legend on next page.)

T-cell is an important mediator of the immune control against this pathogen.

Our study reveals a novel role for lamin A/C as a regulator of T-cell differentiation, controlling the maintenance of Th1 populations in response to pathogen infections. These data contribute to the understanding of the molecular mechanisms driving $CD4^+$ T-cell responses and suggest that strategies to modulate T-cell function could provide a route toward therapeutic immunization and long-lasting protection.

Materials and methods

Mice

Lmna$^{-/-}$ mice have been described previously[36]. C57BL/6-Tg (TcraTcrb)425Cbn/J mice (OTII mice) express a TCR specific for the OVA peptide (amino acid residues 323–339) in the context of I-Ab and the CD4-CRE and were obtained from the Jackson Laboratory (stock number 004194 and 017336, respectively). C57BL/6-CD45.2$^+$*Lmna*$^{-/-}$ OTII mice were generated by crossing C57BL/6-CD45.2$^+$ OTII mice with C57BL/6-CD45.2$^+$*Lmna*$^{-/-}$ mice. C57BL/6-CD45.1$^+$CD45.2$^+$ WT mice were generated by crossing C57BL/6-CD45.2$^+$ mice with C57BL/6-CD45.1$^+$ mice. C57BL/6-CD45.1$^+$ and C57BL/6-CD45.1$^+$CD45.2$^+$ WT mice were used as recipients for adoptive transfer. C57BL/6-CD45.1$^+$CD45.2$^+$/OTII WT mice were generated by crossing C57BL/6-CD45.2$^+$/OTII mice with C57BL/6-CD45.1$^+$/OTII mice. *Lmna*$^{\text{flox/flox}}$ mice were kindly provided by Y. Zheng[37]. C57BL/6*Lmna*$^{\text{flox/flox}}$CD4-CRE mice were generated by crossing C57BL/6-CD4-CRE with C57BL/6-*Lmna*$^{\text{flox/flox}}$ mice. C57BL/6 transgenic β-actin DsRed mice (Tg (ACTB-DsRed*MST)1Nagy/J; The Jackson Laboratory) was also used. All mice were bred at the CNIC in specific pathogen-free conditions.

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.

Antibodies and reagents

Anti-lamin-A/C (n-18), anti-p-ERK1/2, anti-PCNA, and PE-conjugated anti-lamin A/C were obtained from Santa Cruz Biotechnology. Alexa Fluor 488-conjugated anti-lamin-A/C was obtained from Cell Signaling. Anti-CD3, anti-CD28, fluorescein isothiocyanate (FITC)-conjugated-CD45.1, V450-conjugated anti-CD45.1, PerCPCy5.5-conjugated anti-CD45.1, PerCPCy5.5-conjugated anti-CD45.2, APC-conjugated anti-CD45.1, v450-conjugated anti-CD45.2, FITC-conjugated anti-CD45.2, APC-conjugated anti-IFNγ, and V450-conjugated anti-CD4 were from Tonbo Bioscience. PECy5-conjugated anti-CD45.1, PECy7-conjugated anti-CD45.1, PE-conjugated anti-Tbet, FITC-conjugate anti-CD8, APC-conjugated anti-CD69, APC-conjugated anti-CD25, PE-conjugated anti-T-bet, FITC-conjugated anti-Ki67, PE-conjugated anti-IL-4, and biotinylated antibodies against B220, CD19, MHCII, CD11c, CD11b, CD44, CD49b, IgM CD25, and CD8α were from BD Biosciences.

T-cell stimulation and polarization

$CD4^+CD25^-$ T-cells from spleens were purified by negative selection on separation columns (Miltenyi Biotec) after labeling the cells with a cocktail of biotinylated antibodies against B220, CD19, MHCII, CD11c, CD11b, CD44, CD25, CD49b, IgM, and CD8α and a solution containing streptavidin-bound magnetic microbeads (Miltenyi Biotec). For polarizing experiments, $CD4^+CD25^-$ T-cells from WT/OTII and *Lmna*$^{-/-}$/OTII mice were stimulated with irradiated autologous WT APCs previously incubated with OVA peptide (10 μg/ml) for 30 min at 37 °C. $CD4^+CD25^-$ T-cells from WT and *Lmna*$^{-/-}$ mice were stimulated with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 antibodies (2 μg/ml).

Polarizing conditions were as follows: for Th1 polarization: IL-12 (10 ng/ml) and anti-IL-4 (4 μg/ml), with only IL-2 (10 ng/ml) during the rest period; for Th2 polarization: IL-4 (10 ng/ml) and anti-IFNγ (4 μg/ml), with only IL-2 (10 ng/ml) during the rest period. Antibodies and cytokines were from BD bioscience and Tonbo, respectively. Cells were cultured in RPMI-1640 medium (GIBCO) for the indicated times.

(see figure on previous page)

Fig. 5 *Lmna*$^{-/-}$ $CD4^+$ T-cells show impaired in vitro and in vivo T-bet expression. a Percentage of IFNγ$^+$ and T-bet$^+$ cells after stimulation of $CD4^+$ T-cells from WT and *Lmna*$^{-/-}$ mice with anti-CD3/CD28 antibodies or PMA plus ionomycin for 4 days in the presence of IL-2 (Data are means ±SEM of at least 2 independent experiments analyzed by one-way ANOVA with Bonferroni's multiple comparison test. **b** T-bet and lamin A/C expression in WT and *Lmna*$^{-/-}$ $CD4^+$ T-cells 48 h after stimulation with anti-CD3/CD28 antibodies ($n = 12$ and 12 mice). Data are means±SEM analyzed by one-way ANOVA with Bonferroni's multiple comparison test **c** Percentage of T-bet$^+$ cells after stimulation of WT and *Lmna*$^{-/-}$ $CD4^+$ T-cells with anti-CD3/CD28 for 7 days in the presence of Th1 polarizing antibodies and cytokines ($n = 5$ and 6 mice from 2 independent experiments). Data are means±SEM analyzed by unpaired Student's *t*-test **d** Percentage of T-bet$^+$ $CD4^+$ T-cells in peritoneal exudates and mesenteric lymph nodes of WT recipient mice reconstituted with a 1:1 mix of WT and *Lmna*$^{-/-}$ bone marrow and intraperitoneally infected with VACV for 6 days ($n = 4$ and 4 mice from 2 independent experiments). Data are means±SEM analyzed by paired Student's *t*-test **e** RT-qPCR analysis of the indicated genes in WT and *Lmna*$^{-/-}$ $CD4^+$ T-cells stimulated in vitro with anti-CD3/CD28 antibodies (n = pools of 3 mice). Data are means±SEM from 3 independent experiments analyzed by unpaired Student's *t*-test $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

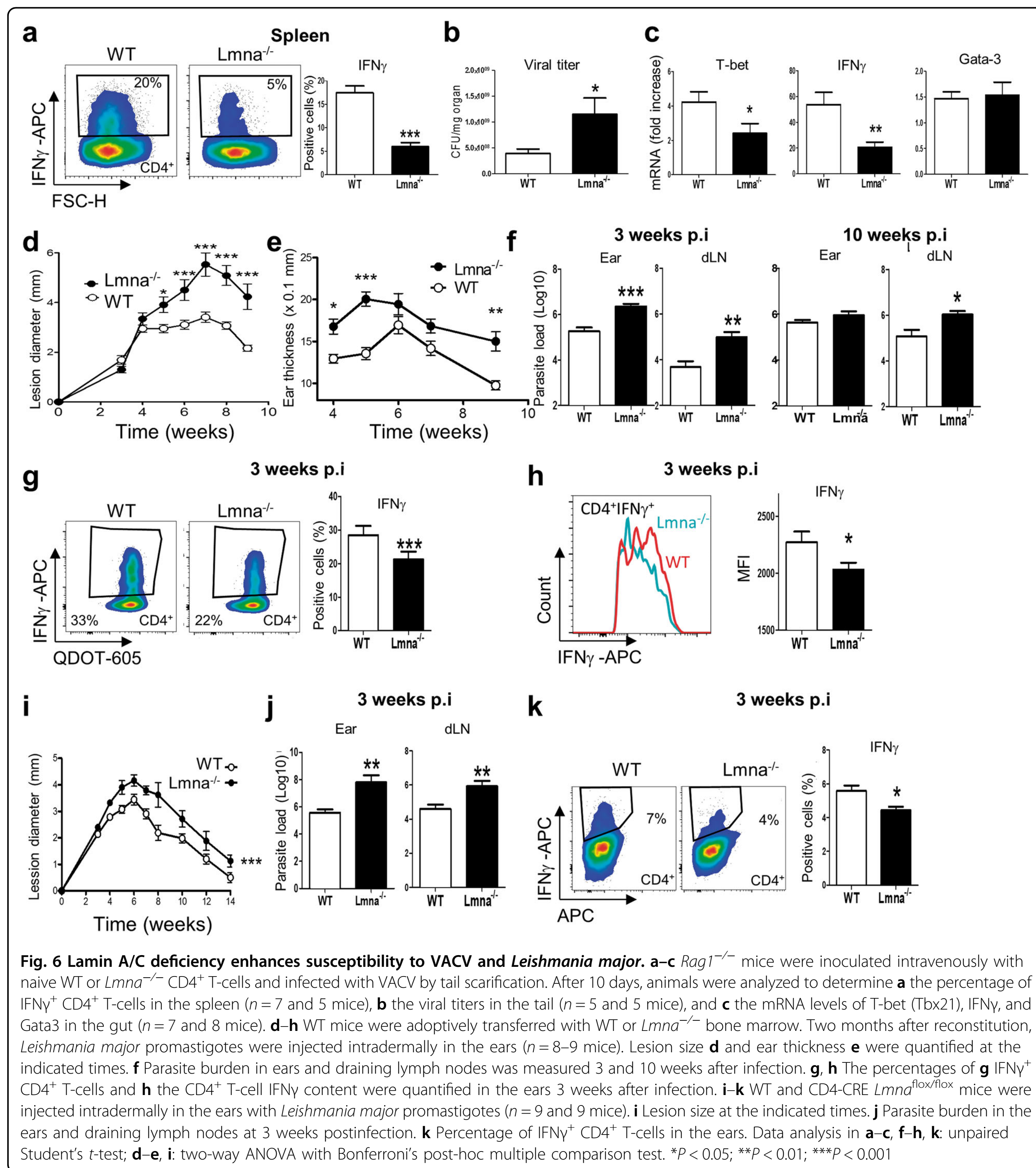

Fig. 6 Lamin A/C deficiency enhances susceptibility to VACV and *Leishmania major*. **a–c** *Rag1*$^{-/-}$ mice were inoculated intravenously with naive WT or *Lmna*$^{-/-}$ CD4$^+$ T-cells and infected with VACV by tail scarification. After 10 days, animals were analyzed to determine **a** the percentage of IFNγ$^+$ CD4$^+$ T-cells in the spleen ($n = 7$ and 5 mice), **b** the viral titers in the tail ($n = 5$ and 5 mice), and **c** the mRNA levels of T-bet (Tbx21), IFNγ, and Gata3 in the gut ($n = 7$ and 8 mice). **d**–**h** WT mice were adoptively transferred with WT or *Lmna*$^{-/-}$ bone marrow. Two months after reconstitution, *Leishmania major* promastigotes were injected intradermally in the ears ($n = 8$–9 mice). Lesion size **d** and ear thickness **e** were quantified at the indicated times. **f** Parasite burden in ears and draining lymph nodes was measured 3 and 10 weeks after infection. **g**, **h** The percentages of **g** IFNγ$^+$ CD4$^+$ T-cells and **h** the CD4$^+$ T-cell IFNγ content were quantified in the ears 3 weeks after infection. **i**–**k** WT and CD4-CRE *Lmna*$^{flox/flox}$ mice were injected intradermally in the ears with *Leishmania major* promastigotes ($n = 9$ and 9 mice). **i** Lesion size at the indicated times. **j** Parasite burden in the ears and draining lymph nodes at 3 weeks postinfection. **k** Percentage of IFNγ$^+$ CD4$^+$ T-cells in the ears. Data analysis in **a**–**c**, **f**–**h**, **k**: unpaired Student's *t*-test; **d**–**e**, **i**: two-way ANOVA with Bonferroni's post-hoc multiple comparison test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Adoptive transfer

Splenic CD4$^+$CD25$^-$ T-cells from CD45.2$^+$ *Lmna*$^{-/-}$, CD45.1$^+$CD45.2$^+$ WT, or CD45.2$^+$ WT mice were isolated by negative selection on MACS separation columns (Miltenyi Biotec) after labeling the cells with a cocktail of biotinylated antibodies against B220, CD19, MHCII, CD11c, CD11b, CD44, CD25, CD49b, IgM, and CD8α, as well as with a solution containing streptavidin-bound magnetic microbeads (Miltenyi Biotec). Adoptive transfer experiments were performed by inoculating CD45.1$^+$ or CD45.1$^+$CD45.2$^+$ WT recipient mice with 1×10^6 isolated CD4$^+$ T-cells. In some experiments, CD45.1$^+$CD45.2$^+$ WT recipient mice were inoculated with a 1:1 mix of 2×10^6 CD25−CD4$^+$ T-cells obtained from the spleens of

CD45.2$^+$ *Lmna*$^{-/-}$ and CD45.1$^+$ WT mice. In VACV experiments, adoptive cell transfer was performed 24 h before inoculation with the VACV virus[38]. Similar adoptive transfers were performed with CD4$^+$ T-cells isolated from CD45.1$^+$CD45.2$^+$ WT/OTII, CD45.1$^+$ WT/OTII, or CD45.2$^+$ Lmna$^{-/-}$ mice.

Generation of BMDCs

BMDCs were generated as described in ref. 39. BMDCs were obtained from bone marrow cell suspensions after culture on non-treated cell culture dishes in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-ME, and 20 ng/ml GM-CSF (PeproTech, London, UK). Cells were collected at day 9 and BMDCs were isolated as CD11c+ MHC-II+ Ly6G− cells. Maturation was induced after overnight incubation with LPS from *Escherichia coli* O111:B4 (1 µg/ml; Sigma-Aldrich).

Vaccinia virus, scarification assays, and viral titration in organs

The VACV strain Western Reserve (WR, ATCC number VR-1354) was a gift from Jonathan W. Yewdell and Jack R. Bennink (NIH, Bethesda, Maryland, USA). VACV was propagated in BSC-40 cells and purified by sucrose gradient ultracentrifugation. WT or OVA-expressing VACV (1×10^6 plaque-forming units) were administered by intraperitoneal injection or intradermal injection in the footpad[38].

Animals were anesthetized with an intraperitoneal injection of Ketamine (100 mg/kg body weight) and Xylazine (10 mg/kg) diluted in sterile phosphate-buffered saline (PBS). Thirty 1 cm scarifications were made with a 26 G syringe along the base of the tail, avoiding bleeding[40]. Then 10 ml of PBS containing 10^7 VACV plaque-forming units (p.f.u.) were added to the area and allowed to air dry. For mock infection only, PBS was added.

Animals were euthanized and the tail aseptically removed, weighed, frozen at −80 °C, and stored at −80 °C until use. The samples were homogenized with a Tissue Ruptor (QIAGEN, USA) in 0.5 ml Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin and 100 µg/ml streptomycin. The homogenates were sonicated for 3 min at 40% amplitude, freeze-thawed twice (−80 °C/37 °C), sonicated again under the same conditions, and then serially diluted in DMEM without FBS. To quantify p.f.u., the dilutions were added to monolayers of CV-1 cells seeded on 24-well plates. The cells were preincubated for 1 day at 37 °C and 5% CO_2, the dilutions (0.2 ml) were added to each well, and the cells were further incubated under the same conditions for 1 h. After this, 0.5 ml DMEM containing 0.5% FBS were added to each well. After 24 h, the cells were stained for 5 min with crystal violet solution (0.5% crystal violet, 10% ethanol, and 1% paraformaldehyde) and washed again. Viral plaques were counted and plaque number was multiplied by the reciprocal of sample dilution and converted to p.f.u./g.[17]

Leishmania parasites, preparation, inoculation, and quantification

In vivo experiments were carried out using *L. major* Friedlin strain FV1 (MHOM/IL/80/Friedlin), generously provided by Dr. D. Sacks (NIH)[20]. For *Leishmania* challenge, parasites were kept in a virulent state by passage in mice. Parasites were cultured and differentiated as described[41]. Mice were infected by i.d. injection of 10^3 or 5×10^4 metacyclic *L. major* promastigotes into the dermis of both ears. Lesion size in the ear was determined with digital callipers (Duratool)[20]. The limiting dilution assay was used to determine parasite number[41]. Parasite load was expressed as the number of parasites in the whole organ.

Bone marrow transplantation

WT recipient mice received 13 Gy of total body irradiation administered in two treatments from a ^{137}Cs source. Bone marrow cells from CD45.2$^+$ WT or CD45.2$^+$ *Lmna*$^{-/-}$ mice were transplanted into CD45.1$^+$ WT recipients by injection into the retro-orbital sinus immediately after irradiation. A mix 1:1 of bone marrow cells from CD45.1$^+$ WT and CD45.2$^+$ *Lmna*$^{-/-}$ mice were transplanted into CD45.1$^+$CD45.2$^+$ WT recipients by injection into the retro-orbital sinus immediately after irradiation. Approximately 8 weeks after transplantation, the chimeric condition of the mice was assessed by flow cytometric analysis of blood cells stained with a combination of fluorescently labeled anti-CD45.1 and anti-CD45.2 antibodies to detect T-cells from donor and from recipient mice, which confirmed that >90% of the cells analyzed were derived from the transplanted bone marrow cells

Flow cytometry

CD4$^+$ T-cells were stimulated with PMA (20 ng/ml) (Sigma Aldrich) plus ionomycin (1 µg/ml) (Sigma Aldrich) for 6 h. Brefeldin A (Sigma Aldrich) was added for the last 2 h to allow intracellular cytokine accumulation. Surface antigens were stained with antibodies, then fixed and made permeable with either a Cytofix/Cytoperm kit (BD Pharmingen), then intracellular cytokines and transcription factors in cells were stained. Data were acquired on FACSCantoII or LSRFortessa flow cytometers (BD Biosciences) and analyzed with the BD FACSDIVA (BD Biosciences) or FlowJo (Treestar Inc) software.

Reverse transcription–quantitative PCR

Total RNA was isolated with Qiazol Lysis Reagent (Qiagen) and isopropanol precipitation or with the

RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA concentration and purity were assessed from the ratio of absorbances at 260 and 280 nm. Complementary DNA (cDNA) was synthesized from total RNA (0.1–1 μg per reaction) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers and RNase Inhibitor, according to the manufacturer's protocol. Quantitative PCR was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using the PCR Power SYBR Green PCR Master Mix (Applied Biosystems), with technical triplicates.

The sequences of RT-qPCR primers were as follows: T-Bet-Forward 5′-GAAAGGCAGAAGGCAGCAT-3′; T-Bet-Reverse 5′-GAGCTTTAGCTTCCCAAATGAA-3′; Hprt1-Forward 5′-CCTAAGATGAGCGCAAGTTGAA-3′ and Hprt1-Reverse 5′-CCACAGGACTAGAACACCT GCTAA-3′; *GzmA*-Forward 5′-GACTGCTGCCCACTG-TAACG-3′; *GzmA*-Reverse 5′-TCAATATCTGTTGT TCTGGCTCCTTA-3′; *GzmB*-Forward 5′-TGTCTCTGG CCTCCAGGACAA-3′; *GzmB*-Reverse 5′- CTCAGGC TGCTGATCCTTGATCGA-3′; *Prf1*-Forward 5′- GCGT CTCCAGTGAATACAAAG-3′; *Prf1*-Reverse 5′- TACTT CGACGTGACGCT-3′; *Eomes*-Forward 5′-GCCTAC-CAAAACACGGATA-3′; *Eomes*-Reverse 5′-TCTGTT GGGGTGAGAGGAG-3′; *Ifng*-Forward 5′- TGGCTCT GCAGGATTTTCATG-3′; *Ifng*-Reverse 5′- TCAAGTGG CATAGATGTGGAAGAA-3′; *Blimp-1*-Forward 5′-ACACACAGGAGAGAAGCCACATGA-3′; *Blimp-1*-Reverse 5′- TCGAAGGTGGGTCTTGAGATTGCT-3′; *gapdh*-Forward 5′-CTACACTGAGGACCAGGTTGTC-3′; *gapdh*-Reverse 5′-GGTCTGGGATGGAAATTGTG-3′. *Lmnb1*-Forward 5′-CAACTGACCTCATCTGGAA-GAAC 3′; *Lmnb1*-Reverse 5′-TAAGACTGTGCTTC TCTGAGC-3′; *Lmna*-Forward-5′-TGAGTACAACCTG CGCTCAC-3′; and *Lmna*-Reverse: 5′-TGACTAGG TTGTCCCCGAAG-3′. The extent of expression of a gene of interest was analyzed by the comparative Ct method with the Biogazelle qBasePLUS software using as internal control the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HPRT1 (hypoxanthine phosphoribosyltransferase 1). Results are represented as fold change relative to control conditions.

Proliferation assays

Twenty-four hours before the sacrifice of the mice, a single dose of 1 mg BrdU (BD Pharmingen) was injected intraperitoneally (i.p.). To assess BrdU incorporation, mesenteric lymph nodes, peritoneal exudate, and spleen were stained for CD4, fixed, and permeabilized using the BD BrdU Flow Kit (BD Pharmigen) according to the manufacturer's instructions. Cells were incubated at 37 °C for 60 min in 30 μg of DNase, followed by staining with anti-BrdU-FITC for 40 min, washed, and analyzed by flow cytometry. Peritoneal exudate and mesenteric lymph node cells were fixed and permeabilized using Foxp3 fixation/permeabilization buffer (BD Pharmingen) and stained for PCNA-FITC and Ki67-FITC.

In vivo cytotoxicity assays

Splenic CD45.2$^+$WT or CD45.2$^+$*Lmna*$^{-/-}$ CD4$^+$/OTII T-cells were isolated as described in the adoptive transfer section. Recipient *Rag1*$^{-/-}$ mice were inoculated intravenously with 2×10^6 of either naive CD45.2/WT or CD45.2/*Lmna*$^{-/-}$ CD4$^+$/OTII T-cells and infected with VACV-OVA i.p. After 5 days, splenocytes from CD45.2/WT/dsRED and CD45.1/WT mice were isolated and loaded or not with OVA OTII peptide, respectively. An approximately 1:1 mix of 5×10^6 CD45.2/WT/dsRED and CD45.1/WT splenocytes was inoculated intravenously in the recipient *Rag1*$^{-/-}$ mice, which were previously inoculated and vaccinia infected. After 16 h, animals were analyzed to determine the killing capacity of CD4/OTII cells in the spleen by flow cytometry. Killing capacity was determined as the ratio between CD45.2/WT/dsRED/+OVA and CD45.1/WT/-OVA cells and the percentage of CD4/OTII T-cells. Mice adoptively transferred with CD4/OTII cells only and mice transferred with splenocytes in the absence of CD4/OTII cells were used as controls.

Statistical analysis

Statistical analyses were performed with Prism GraphPad or Microsoft Office Excel. Unless otherwise stated, statistical significance was calculated by two-tailed Student's *t*-test. When specified, one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni's post-hoc multiple comparison test was used. Significance of differences was calculated as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and **** $P > 0.0001$.

Acknowledgements
The authors thank J. Mateos, I. Fernández-López, and M.J. Andrés-Manzano for technical assistance and S. Bartlett for English editing. This study was supported by grants to J.-M.G.-G. from Instituto de Salud Carlos III (ISCIII) (PI14/00526, PI17/01395, CP11/00145, CPII16/00022), the Miguel Servet Program, and Fundación Ramón Aréces; to V.A. (RD12/0042/0028 SAF2013-46663-R, SAF2016-79490-R); to F.S.-M. (SAF2014-55579-R, INDISNET-S2011/BMD-2332, ERC-2011-AdG 294340-GENTRIS, and PIE13/00041); and to D.S. (SAF-2013-42920R, SAF2016-79040-R, and Fondation ACTERIA) with co-funding from the Fondo Europeo de Desarrollo Regional (FEDER). The CNIC is supported by the Ministry of Economy, Industry and Competitiveness (MEIC) and the Pro CNIC Foundation and is a Severo Ochoa Center of Excellence (SEV-2015-0505). S.I. is funded by grant SAF2015-74561-JIN, R.T.-F. by the Fundación Ramón Aréces, V. Z. by ISCIII, and J.-M.G.-G. by the ISCIII Miguel Servet Program and the Instituto de Investigación Sanitaria Hospital 12 de Octubre (imas12).

Author contributions
R.T.-F., V.Z., S.I., V.R.-P., and J.-M.G.-G. conceived and designed research. R.T.-F., V. Z., and S.I. performed experimental work. G.M.d.H., R.C., B.D., D.S., F.S.-M., and V. A. provided scientific input. D.S., F.S.-M., V.A., and J.-M.G.-G. acquired funding. V. R.-P. and J.-M.G.-G. wrote the manuscript. J.-M.G.-G. supervised the project. All authors revised the manuscript and approved its final version

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Conflict of interest
The authors declare that they have no competing interests.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information
The online version of this article (https://doi.org/10.1038/s41419-017-0007-6) contains supplementary material.

Received: 2 July 2017 Revised: 18 September 2017 Accepted: 19 September 2017
Published online: 08 January 2018

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