PROGRAMA DE DOCTORADO EN CIENCIAS DE LA VISIÓN

TESIS DOCTORAL

ALTERNATIVA TERAPÉUTICA PARA EL TRATAMIENO DE LA INFLAMACIÓN CRÓNICA DE LA SUPERFICIE OCULAR ASOCIADA A LA REDUCCIÓN DE LA EXPRESIÓN DE LA TROMBOSPONDINA-1

ALTERNATIVE THERAPY FOR THE TREATMENT OF CHRONIC OCULAR SURFACE INFLAMMATION ASSOCIATED WITH DECREASED THROMBOSPONDIN-1 EXPRESSION

Presentada por **Laura Soriano Romaní** para optar al grado de Doctor/a por la Universidad de Valladolid

Dirigida por: Dra. Yolanda Diebold Luque

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AUTORIZACIÓN DEL DIRECTOR/A DE TESIS

(Art. 7.2 de la Normativa para la presentación y defensa de la Tesis Doctoral en la UVa)

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como Directora de la Tesis Doctoral titulada " Alternativa terapéutica para el tratamiento de la inflamación crónica de la superficie ocular asociada a la reducción de la expresión de la trombospondina-1", realizada por Dª. Laura Soriano Romaní, alumna del Programa de Doctorado en Ciencias de la Visión, impartido por el Instituto de Oftalmobiología Aplicada, autoriza su presentación, considerando que es APTA para su defensa.

Valladolid, 18 de abril de 2017

La Directora de la Tesis,

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de mi hogar, si pasara cualquier cosa tenía gente a la que acudir, es algo que ni siquiera sé si merezco, dado la/lo boquita/bocazas que tengo/soy. Ellos me acogieron como una más (y a Juan), cosa que no pueden decir todos en sus comienzos… Empezando por la incombustible MªPaz, la primera persona que vi cuando llegué aquí y me facilitó el primer día y, por qué no decirlo, todos los que vendrían después. A Carmen, gran mami confidente. A los que ya no están: el isleño, el capo de la familia Abengózar, el gran José Pinto (espero canción y lo sabes, sin presión), Esther y su arte, Anita, Amar y su bucle… A los más "nuevos": Mª Luz (GSO), Elenita, Amanda, Cris y Tito. Y a los no tan nuevos: Charly, Nieves, las Dras. Marina y Sarita (Saret, gracias… ¡Siempre nos quedará la "vaina"!) y el Dr. López con sus sabios consejos. Al gran grupo del comunio, ¡volveré a por esas chuletitas de lechazo en Ciguñuela como ganadora! Mención especial requieren los actuales y ex miembros del ilustre Nanoteam. Los grandes (también literalmente) Crespo y Jesús, ¡parece mentira lo que hemos hablado de ciencia entre tanto fichaje y oferta de mercado! Las sabias Dras. Contreras, García e Isabel. ¡Qué complicado ha sido seguirles el ritmo! Lo he hecho lo mejor que he podido, dadas las circunstancias. En concreto la Dra. Contreras me ha tenido que aguantar en mis estancias en Boston y me introdujo en esa gran familia que tienen allí. No sé si podré asumir semejante gasto de los favores que le debo… pero lo intentaré. Además puede que alguno más caiga por el camino, ¡el *n* siempre hay que aumentarlo! Y el Sheriff Anthony (porque "Sheriff Tonet" no es tan mediático, ¿no?) siempre facilitando cualquier procedimiento por complicado que sea. Me encanta tu estilo, lo tengo que decir. Y el de tu hermana, sí señor. A todos vosotros, muchas gracias por esos cafés y comidas juntos cuando el día no prometía mucho, y por esas cenas o casas rurales memorables. Gracias a todos por todo… No lo puedo expresar de otra manera.

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Y no me quiero aLARGAr más. Gracias a todo el que me ha ayudado de cualquier modo en la tesis, que no sois pocos, dado lo que me gusta preguntar. Por los saludos mañaneros, los consejos, las risas, los bailes, las correcciones… Seguro se me escapa algún nombre, no me lo tengáis en cuenta… Disculpadme, y ¡muchas gracias a todos!

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ÍNDICE/*TABLE OF CONTENTS*

PRÓLOGO/*PROLOGUE*

BIOGRAFÍA BREVE/*BIOSKETCH*

Me licencié en Biotecnología en mi ciudad natal, Valencia, en la Universidad Politécnica de Valencia en 2011. Una beca ERAMUS me ofreció la oportunidad de formar parte de un grupo de investigación perteneciente a la *Universidade do Minho* (Braga, Portugal) y decidí que quería seguir formándome como investigadora. Con esta idea me marché a Valladolid, al Instituto de Oftalmobiología Aplicada de la Universidad de Valladolid (IOBA-UVa), donde realicé el Máster en Investigación en Ciencias de la Visión durante el curso 2011-2012, bajo la tutela de la Dra. Yolanda Diebold Luque. Tras ello, comencé la Tesis Doctoral en el Programa de Doctorado en Ciencias de la Visión formando parte del Grupo de Superficie Ocular del IOBA-UVa, centrando mi investigación en la búsqueda de nuevas terapias para patologías de base inflamatoria.

Durante el doctorado he realizado distintas estancias en el extranjero: en el Departamento de Ciencias de la Visión en la Universidad de Liverpool (Liverpool, Reino Unido), en el Departamento de Oftalmología de la Universidad de Boston (Boston, Massachusetts, EE.UU.) y en la empresa RenaSci Ltd. (Nottingham, Reino Unido). También he colaborado en tareas docentes en el Grado en Óptica y Optometría y en el Máster en Investigación en Ciencias de la Visión. Con el trabajo realizado durante la Tesis Doctoral, y con la ayuda de todo el equipo investigador y los colaboradores, se han obtenido publicaciones científicas y participaciones en distintos congresos nacionales e internacionales.

I graduated with a Bachelor´s Degree in Biotechnology in my hometown, Valencia, from the Polytechnic University of Valencia in 2011. The Erasmus Scholarship Program offered me the opportunity to be part of a research group belonging to the University of Minho (Braga, Portugal). After this experience I decided to continue my training as a researcher. With this in mind, I moved to Valladolid, to the Institute of Applied Ophthamo-Biology of the University of Valladolid (IOBA-UVa) where I obtained a Master´s Degree in Visual Science Research in 2012, under the supervision of Dr. Yolanda Diebold. After this, I

enrolled in the Ph.D. Program in Visual Sciences being part of the Ocular Surface Group at the IOBA-UVa and focusing my research on the development of novel therapies for inflammation-based diseases.

During my Ph.D. I have done several internships abroad: in the Department of Eye and Vision Science at the University of Liverpool (Liverpool, UK), in the Department of Ophthalmology at Boston University (Boston, Massachusetts, USA) and in the company RenaSci Ltd. (Nottingham, UK). Also, I have served as a teaching assistant in the Optics and Optometry Degree and in the Master Degree in Visual Science Research. With the work achieved during my Ph.D and with the support of the research group and other colleagues, we have published papers and presented communications at National and International Research Meetings.

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El trabajo de investigación realizado ha sido posible gracias a la financiación de los siguientes proyectos de investigación/*research projects*:

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- **Soriano-Romaní L**, Vicario-de-la-Torre M, Crespo-Moral M, López-García A, Herrero-Vanrell R, Molina-Martínez IT, Diebold Y. Novel anti-inflammatory liposomal formulation for the pre-ocular tear film: *in vitro* and *ex vivo* functionality studies in corneal epithelial cells. *Exp Eye Res*. 2017 Jan; 154:79-87. Índice de Impacto (JCR 2015): 2.998. Posición de la revista y área: 13/56 Ophthalmology (**Q1**).

- **Soriano-Romaní L**, Contreras-Ruiz L, García-Posadas L, López-García A, Masli S, Diebold Y. Inflammatory Cytokine-Mediated Regulation of Thrombospondin-1 and CD36 in Conjunctival Cells. *J Ocul Pharmacol Th.* 2015 Sep; 31(7):419-28. Índice de Impacto (JCR 2015): 1.754. Posición de la revista y área: 28/56 Ophthalmology (**Q2**); 169/255 Pharmacology & Pharmacy (**Q3**).

- **Soriano-Romaní L**, García-Posadas L, López-García A, Paraoan L, Diebold Y. Thrombospondin-1 induces differential response in human corneal and conjunctival epithelial cells lines under *in vitro* inflammatory and apoptotic conditions. *Exp Eye Res*. 2015 May; 134:1-14. Índice de Impacto (JCR 2015): 2.998. Posición de la revista y área: 13/56 Ophthalmology (**Q1**).

Además, el trabajo realizado se ha presentado en los siguientes congresos nacionales o internacionales/*contributions in national and international meetings*:

- **I Congreso Nacional de jóvenes investigadores en Biomedicina.** Valencia, España. Del 28 y 29 de noviembre de 2016.
  Laura Soriano-Romaní, Marta Vicario-de-la-Torre, Mario Crespo-Moral, Antonio López-García, Rocío Herrero-Vanrell, Irene T. Molina-Martínez, Yolanda Diebold. Novel anti-inflammatory liposomal formulation mimicking the pre-ocular tear film: functionality studies in corneal epithelial cells.

- **Association for Research in Vision and Ophthalmology (ARVO) 2016 Annual meeting.** Seattle, Washington, EE.UU. Del 1 al 5 de mayo de 2016.
  Laura Soriano-Romaní, Marta Vicario-de-la-Torre, Mario Crespo-Moral, Antonio López-García, Rocío Herrero-Vanrell, Irene T. Molina-Martínez, Yolanda Diebold. Functionality of a liposome-based anti-inflammatory formulation in an *in vitro* corneal inflammation model.

- **Association for Research in Vision and Ophthalmology (ARVO) 2015 Annual meeting.** Denver, Colorado, EE.UU. Del 3 al 7 de mayo de 2015.
  Laura Soriano-Romaní, Laura contreras-Ruiz, Laura García-Posadas, Antonio López-García, Sharmila Masli, Yolanda Diebold. Comparison of the expression of TGF-β2 activating molecules in conjunctival inflammation.

- **120th German Ophthalmological Society (DOG) Congress.** Leipzig, Germany. Del 25 al 28 de septiembre de 2014.
  Marta Vicario-de-la-Torre, María Caballo-González, José Manuel Benítez-del-Castillo, Beatriz de Las Heras, Manuel Guzmán, **Laura Soriano-Romaní**, Yolanda Diebold, Rocío Herrero-Vanrell, Irene T. Molina-Martínez. Novel Ophthalmic Formulations of Liposomes Loaded with Anti-inflammatory Drugs and Omega-3 Fatty Acids for Dry Eye Treatment.

- **11th International Symposium on Ocular Pharmacology and Therapeutics (ISOPT) Clinical.** Reykjavik, Iceland. Del 19 al 22 de junio de 2014.
  Rocío Herrero-Vanrell, Marta Vicario-de-la-Torre, María Caballo-González, Pedro Arriola, **Laura Soriano-Romaní**, Yolanda Diebold, Beatriz de Las Heras, José M. Benítez-del-Castillo, Irene T. Molina-Martínez. Novel ophthalmic formulations of liposomes loaded with anti-inflammatory drugs and omega-3 fatty acids for dry eye treatment.

- **11th International Symposium on Ocular Pharmacology and Therapeutics (ISOPT) Clinical.** Reykjavik, Iceland. Del 19 al 22 de junio de 2014
  Laura Soriano-Romaní, Marta Vicario-de-la-Torre, Antonio López-García, Rocío Herrero-Vanrell, Irene T. Molina-Martínez, Yolanda Diebold. *In vitro* activation of steroid receptors in corneal epithelial cells using a novel anti-inflammatory liposomal formulation.

- **Association for Research in Vision and Ophthalmology (ARVO) 2014 Annual meeting.** Orlando, Florida, EE.UU. Del 4 al 8 de mayo de 2014.
  Laura Soriano-Romaní, Antonio López-García, Laura García-Posadas, Luminita Paraoan, Yolanda Diebold. *In vitro* effect of thrombospondin-1 in ocular surface epithelial cell lines.

- **SIRCOVA International Meeting 2013.** Valencia, España. Del 6 al 8 de junio de 2013.
  Laura Soriano-Romaní, Marta Vicario-De-La-Torre, Antonio López-García, Rocío Herrero-Vanrell, Irene T. Molina-Martínez, Yolanda Diebold. Activation of steroid receptors using a novel liposomal anti-inflammatory formulation.

- **11th Scientific Meeting of the Association for Ocular Pharmacology and Therapeutics (AOPT).** Alicante, España. Del 7 al 10 de febrero 2013.
  Marta Vicario-de-la-Torre, **Laura Soriano-Romaní**, José Manuel Benítez-del-Castillo, Eva Vico Ruiz, Laura Morales, María Caballo-González, Manuel Guzmán, Beatriz de Las Heras, Yolanda Diebold, Rocío Herrero-Vanrell and Irene T. Molina-Martínez. Novel formulation for dry eye treatment based on liposomes and bioadhesive polymers.

ABREVIATURAS/*ABBREVIATIONS*

AA/AB	Azul alcián	*Alcian blue*
Ab	Anticuerpo	*Antibody*
ADNc/cDNA	Ácido desoxiribonucleico complementario	*Complementary desoxyribonicleic acid*
ARNm/mRNA	Ácido ribonucleico mensajero	*Messenger ribonucleic acid*
BAC	Cloruro de benzalconio	*Benzalkonium chloride*
BCA	Ácido bicinconínico	*Bicinchoninic acid*
bp	Pares de bases	*Base pairs*
C-terminal	Carboxilo-terminal	*Carboxyl-terminal domain*
C6	Cumarina-6	*Coumarin-6*
CALT	Tejido linfoide asociado a la conjuntiva	*Conjunctival-associate lymphoid tissue*
DED	Síndrome de Ojo Seco	*Dry Eye disease*
DMEM F12	Medio de cultivo Eagle modificado de Dulbecco con F12	*Dulbecco´s Modified Eagle Medium + F12 medium mixture*
EE	Eficiencia de encapsulación	*Encapsulation efficiency*
EGF	Factor de crecimiento epidérmico	*Epidermic growth factor*
ELISA	Ensayo por inmunoabsorción ligado a enzimas	*Enzyme-linked immunosorbent assay*
FBS	Suero fetal bovino	*Fetal bovine serum*
FC/PC	Fosfatidilcolina	*Phosphatidylcholine*
FITC	Isotiocianato de fluoresceína	*Fluorescein isothiocyanate*
GAPDH	Gliceraldehído-3-fosfato deshidrogenasa	*Glyceraldehyde-3-phosphate dehydrogenase*
H&E	Hematoxilina y eosina	*Hematoxylin and eosin*
HCE	Línea celular de epitelio corneal humano	*Human corneal epitelial cell line*
hCrF	Fibroblastos corneales humanos primarios	*Human corneal fibroblasts*

hCrF-CM	Medio condicionado de fibroblastos corneales humanos	*Human corneal fibroblasts conditioned medium*
HEPES	ácido N-2-Hidroxietilpiperazina-N'-2 etanosulfónico	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
HPLC	Cromatografía líquida de alto rendimiento	*High performance liquid chromatography*
HRGP	Glicoproteína rica en histidina	*Histidine-rich glycoprotein*
HSPG	Proteoglicanos heparán sulfato	*Heparan sulfate proteoglycans*
IF	Inmunofluorescencia	*Immunofluorescence*
IFN-γ	Interferon-γ	*Interferon-γ*
IL	Interleuquina	*interleukin*
IOBA-NHC	Línea celular de epitelio de conjuntiva humana normal	*Normal human conjunctiva cell line*
KRFK	Lisina-arginina-fenilalanina-lisina	*Lysine-arginine-phenylalanine-lysine*
LAP	Péptido asociado a la latencia	*Latency-associated peptide*
LP	Liposoma	*Liposome*
LRP	Proteína relacionada con el receptor de lipoproteína	*Lipoprotein receptor related protein*
LSKL	Leucina-serina-lisina-leucina	*Leucine-serine-lysine-leucine*
MHC	Complejo mayor de histocompatibilidad	*Major histocompatibility complex*
MPA	Acetato de medroxiprogesterona	*Medroxyprogesterone acetate*
N-terminal	Dominio amino-terminal	*Amino-terminal domain*
OCT	*Optimal cutting temperature*	*Optimal cutting temperature*
PAS	Ácido periódico de Schiff	*Periodic acid-Schiff*
PBS	Tampón fosfato salino	*Phosphate buffered saline*
Q-PCR o real time RT-PCR	Reacción en cadena de la polimerasa con retro-transcripción a tiempo real	*Real time reverse transcription polymerase chain reaction*

RG/GR	Receptor de glucocorticoides	*Glucocorticoid receptor*
RGD	Arginina-glicina-aspartato	*Arginine–glycine-aspartic acid*
RIPA	Tampón del ensayo de radioinmunoprecipitación	*Radioimmunoprecipitation assay buffer*
PR/RP	Receptor de progesterona	*Progesterone receptor*
RT-PCR	Reacción en cadena de la polimerasa con retrotranscripción	*Reverse transcription polymerase chain reaction*
rTSP-1	Trombospondina-1 humana recombinante	*Human recombinant thrombospondin-1*
SBE-SEAP	Elementos de unión a smad acoplados a un gen reportero de fosfatasa alcalina	*Smad-binding elements coupled to a secreted alkaline phosphatase reporter gene*
SEM	Error estándar de la media	*Standard error of the mean*
SMA	Actina de músculo liso	*Smooth muscle actin*
SNP	Polimorfismo de nucleótido único o simple	*Single nucleotide polymorphism*
RT/TA	Temperatura ambiente	*Room temperature*
TBS-T	Tampón Tris salino con Tween 20	*Tris-buffered saline with Tween20*
TEER	Resistencia eléctrica transepitelial	*Transepithelial electrical resistance*
TGF	Factor de crecimiento transformante	*Transforming growth factor*
THBS1	Gen de la trombospondina-1 humana	*Human thrombospondin-1 gene*
TNF	Factor de necrosis tumoral	*Tumor necrosis factor*
Tris	2-Amino-2-hidroximetil-propano-1,3-diol	*2-Amino-2-hydroxymethyl-propane-1,3-diol*
TSP-1	Trombospondina-1	*Thrombospondin-1*
TSR	Repeticiones de trombospondina de tipo 1	*Thrombospondin type 1 repeats*

WB	Western *blotting*	*Western blotting*
XTT	2,3-bis(2-metoxi-4-nitro-5-sulfonil)-2H-tetrazolio-5-carboxianilida	*2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide*
λ	Longitud de onda	*Wavelength*

RESUMEN EN ESPAÑOL

SÍNTESIS DEL TRABAJO

Las enfermedades inflamatorias de la superficie ocular ponen en riesgo la visión y afectan a la calidad de vida de los pacientes que las sufren y son, además, cada vez más comunes en nuestra sociedad. Las múltiples causas que provocan reacciones inflamatorias en la superficie ocular involucran distintos factores humorales y celulares, lo que puede ocasionar fracasos al emplear tratamientos terapéuticos genéricos. El estudio de la fisiopatología de estas enfermedades puede ayudar en la búsqueda de tratamientos personalizados que ofrezcan una solución terapéutica más efectiva. En este contexto se ha demostrado la relación que existe entre el desarrollo de una inflamación ocular crónica y los niveles reducidos de la proteína matricelular trombospondina-1 (**TSP-1**). Una de las causas de estos niveles de expresión "patológicos" es un polimorfismo en el gen de esta proteína. Este polimorfismo podría emplearse como un marcador genético para el diagnóstico temprano de una inflamación ocular, permitiendo tratamientos preventivos para evitar la condición crónica. Por todo ello, el objetivo principal de esta tesis es el desarrollo de una alternativa terapéutica para prevenir o tratar la inflamación de la superficie ocular ligada a niveles reducidos de la TSP-1.

Por una parte y para estudiar la función de la TSP-1 en condiciones experimentales de inflamación ocular, se emplearon líneas celulares humanas de epitelio de córnea y de conjuntiva, así como cultivos primarios de conjuntiva humana y modelos *in vivo* en ratones. Estos estudios compararon los niveles de la TSP-1 y de moléculas relacionadas con la activación del factor transformante de crecimiento (**TGF**)-β2, como el receptor CD36, en condiciones basales y tras inducir inflamación. Se seleccionó la isoforma TGF-β2 ya que es la isoforma predominante en la mucosa ocular y se ha propuesto a la TSP-1 como su principal activador. La metodología empleada incluye técnicas de análisis molecular como el Western *blotting*, la inmunofluorescencia, el ensayo por inmunoabsorción ligado a enzimas (**ELISA**) o la reacción en cadena de la polimerasa con retrotranscripción (**RT-PCR**). Los resultados obtenidos muestran

que, en condiciones de inflamación, los niveles de expresión de la TSP-1 o del CD36 disminuyen y no se activan los niveles de TGF-β2 esperados, indicando la posible contribución de estas moléculas a la regulación de los procesos inflamatorios. Por todo ello, se seleccionó el péptido KRFK derivado de la TSP-1, que activa todas las isoformas del TGF-β, como potencial molécula terapéutica para estas patologías. Para probar la eficacia del péptido aplicado de manera tópica ocular, se emplearon ratones modificados genéticamente que no expresan la TSP-1 y que desarrollan una inflamación en la superficie ocular. Los resultados demuestran que el péptido derivado de la TSP-1 previene la aparición de signos inflamatorios en la superficie ocular, modulando el fenotipo de las células dendríticas oculares en estos ratones.

Por otra parte, ya que la aplicación tópica y local es la preferida para la administración de fármacos oculares, se propuso el desarrollo de un sistema de liberación de fármacos que encapsulara y protegiera al péptido seleccionado hasta el tejido donde ejerce su función. Se consideró el empleo de una formulación liposomal como posible sistema de encapsulación. Previamente a la encapsulación del péptido, se estudió la funcionalidad de la formulación como sistema de liberación empleando una molécula anti-inflamatoria control, el acetato de medroxiprogesterona (**MPA**). Se observó cómo las células epiteliales corneales interaccionaban con el sistema, causando cambios en los receptores del MPA encapsulado, siendo posible observar un efecto *in vitro* del mismo.

Tras los resultados obtenidos en los estudios anteriores, se evaluó si el péptido KRFK, derivado de la TSP-1 y que activa el TGF-β, puede ser encapsulado en la formulación liposomal mejorando la penetración del péptido en la superficie ocular. Los resultados demuestran que el péptido puede ser encapsulado de modo eficiente, lo que mejora su biodisponibilidad y penetración en tejidos corneales. Este novedoso sistema desarrollado puede resultar de gran interés para las patologías inflamatorias crónicas de la superficie ocular asociadas a niveles disminuidos de la expresión de la TSP-1.

ORGANIZACIÓN DE LA TESIS DOCTORAL

La tesis se presenta en la modalidad de "compendio de publicaciones" y opta a la mención de "doctorado internacional"; en consecuencia, se organiza siguiendo los requisitos de la Universidad de Valladolid para ambas modalidades. Por su carácter internacional y la inclusión de los artículos científicos como cuerpo de la misma, está redactada mayoritariamente en inglés. Sin embargo, también se incluye un resumen en español que contiene los objetivos, la metodología, los principales resultados y las conclusiones, siguiendo con la normativa de la Universidad de Valladolid. Tanto el resumen en español como el redactado en inglés contienen una extensa introducción para facilitar la comprensión y la relación temática de los estudios. El cuerpo de la tesis está formado por seis estudios, tres de ellos (los estudios 1, 2 y 5) son artículos ya publicados en revistas indexadas, cumpliendo con los requisitos de la modalidad de la tesis. Todos los estudios se realizaron en colaboración con distintos centros nacionales e internacionales; en algunos de ellos la doctoranda ha realizado estancias de investigación. En concreto, los estudios 2, 3 y 4 se han llevado a cabo en colaboración con la Universidad de Boston (*Boston University*, Boston, MA, EE.UU.), donde la doctoranda ha realizado dos estancias de aproximadamente dos meses cada una, cumpliendo con los requisitos para optar a la mención de "doctorado internacional".

La organización de la tesis está determinada por el objetivo de la misma: desarrollar una formulación novedosa para el tratamiento de la inflamación de la superficie ocular. Para cumplir con el objetivo principal, no solo se buscó estudiar las causas de esta inflamación con el fin de encontrar una molécula terapéutica novedosa, sino también un sistema de encapsulación óptimo para su aplicación tópica ocular. Por ello, la tesis se ha organizado como un sumatorio de la búsqueda de una molécula inmunomoduladora y la búsqueda de un sistema de encapsulación adecuado (**figura 1**).

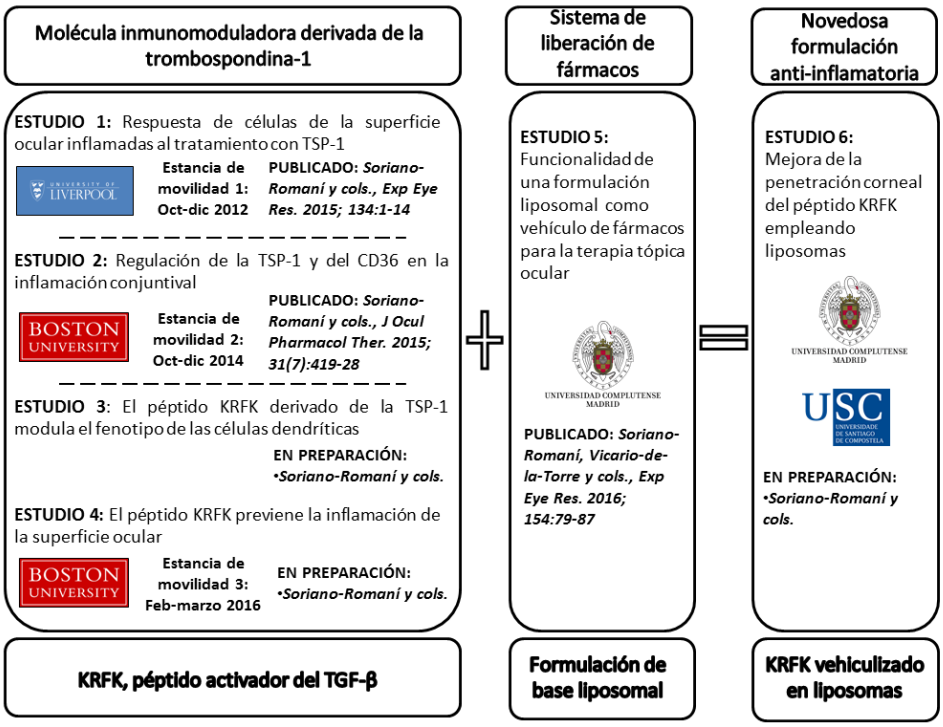

Figura 1. Esquema de la organización de los seis estudios realizados en la Tesis Doctoral.

Una gran parte de la tesis se centró en el estudio del papel de la TSP-1 en la inflamación ocular, con la finalidad de analizar y entender su papel inmunomodulador e identificar posibles dianas terapéuticas (los estudios 1, 2, 3 y 4). En el primer estudio de la tesis se analizó la respuesta *in vitro* al tratamiento con TSP-1 de líneas celulares humanas epiteliales de la superficie ocular en condiciones inflamatorias y apoptóticas. Se estudiaron los cambios en marcadores de inflamación y apoptosis, así como en la expresión de los receptores de TSP-1: CD36 y CD47. Todos estos resultados se agruparon en el **Estudio 1** y se recogen en la publicación: ***Thrombospondin-1 Induces Differential Response in Human Corneal and Conjunctival Epithelial Cell Lines Under In Vitro Inflammatory and Apoptotic Conditions*** (Soriano-Romaní y cols., *Experimental Eye Research. 2015 May; 134:1-14*). En este trabajo demuestra cómo tras la adición de TSP-1 al medio de cultivo se reducen los niveles de TGF-β2 total en la línea celular de conjuntiva "inflamada" y la posible implicación del

CD36 en esta función. A continuación se desarrolló el **Estudio 2** donde se analizaron los cambios en la expresión de la TSP-1 y del CD36, moléculas implicadas en la activación del TGF-β2, en condiciones de inflamación conjuntival. En este estudio se utilizaron células primarias de conjuntiva, tanto epiteliales como estromales, y dos modelos experimentales de conjuntivitis en ratón. Este **Estudio 2** también ha dado lugar a una publicación: ***Inflammatory Cytokine-Mediated Regulation of Thrombospondin-1 and CD36 in Conjunctival Cells*** (Soriano-Romaní y cols., *Journal of Ocular Pharmacology and Therapeutics.* 2015 Sep; 31(7):419-28). Los resultados obtenidos en estos trabajos motivaron la selección de un péptido derivado de la TSP-1 y activador del TGF-β, el péptido KRFK, como una molécula con posible potencial terapéutico para tratar la inflamación ocular asociada a niveles reducidos en la expresión de la TSP-1. Para analizar este posible potencial terapéutico se emplearon ratones deficientes en TSP-1 que desarrollan una inflamación en la superficie ocular y muestran un desequilibrio entre las poblaciones de linfocitos T efectores y reguladores. En el **Estudio 3** se demuestra la funcionalidad *in vitro* de este péptido, su penetración a través del epitelio conjuntival, así como su eficacia *in vivo* para modular las poblaciones de linfocitos T periféricos en estos ratones. **Este estudio está en preparación para su publicación**. Tras estos resultados se estudió el efecto del péptido KRFK tanto para la prevención como para el tratamiento de la enfermedad de la superficie ocular que desarrolla dicho modelo animal. Estos experimentos mostraron los cambios tanto en la córnea y en la conjuntiva, como en la glándula lagrimal tras la aplicación tópica del péptido. Los resultados, que se recogen en el **Estudio 4**, son muy positivos cuando se trata de prevenir la inflamación, obteniéndose una mejora de los signos inflamatorios en todas las estructuras analizadas de la superficie ocular. **Este estudio también está en preparación para su publicación**. De estos cuatro estudios se puede concluir que el péptido KRFK, derivado de la TSP-1 y que activa el TGF-β, tiene potencial terapéutico para prevenir la inflamación ocular de los pacientes que muestren niveles de TSP-1 disminuidos.

Paralelamente al estudio del papel de la TSP-1 en la inflamación ocular se inició el **Estudio 5.** En él se analizó la funcionalidad de una formulación liposomal como sistema de liberación de moléculas terapéuticas. La prueba de concepto se llevó a cabo encapsulando el acetato de medroxiprogesterona, un anti-inflamatorio empleado en la práctica clínica como corticoide "suave". Este **Estudio 5** se ha publicado con el título: ***Novel Anti-inflammatory Liposomal Formulation for the Pre-ocular Tear Film: In vitro and Ex vivo Functionality Studies in Corneal Epithelial Cells*** (Soriano-Romaní y cols., *Experimental Eye Research. 2017 Jan; 154:79-87*). En este trabajo se optimizaron modelos *in vitro* y *ex vivo* para contrastar la funcionalidad y la penetración del fármaco en la superficie ocular. Tras los prometedores resultados obtenidos, se decidió emplear esta formulación para desarrollar un sistema de liberación que proteja el péptido derivado de la TSP-1 tras su administración tópica ocular.

Por todo ello, en el último estudio se preparó un protocolo para la encapsulación del péptido derivado de la TSP-1 en esta formulación liposomal. En el **Estudio 6** se optimizó el protocolo para encapsular el péptido en la formulación liposomal y, tras una caracterización fisicoquímica de la formulación resultante en términos de tamaño, pH y osmolaridad, se evaluó la penetración del péptido en distintos modelos *in vitro*. Los resultados indican que el péptido encapsulado tiene una mejor penetración corneal frente al péptido libre en el modelo empleado. **Estos resultados se han presentado para su publicación como una comunicación corta en *Experimental Eye Research*.**

INTRODUCCIÓN

1. La superficie ocular y la unidad funcional lagrimal

La superficie ocular es la parte anterior y más externa del ojo e incluye la película lagrimal, la córnea, el limbo, la conjuntiva, la glándula de Meibomio y la glándula lagrimal principal y las accesorias. La integridad de todos sus componentes y en concreto la de la córnea, por situarse en el eje óptico y tener que mantener su transparencia para la refracción y transmisión de la luz, es esencial para permitir la visión. Por ello la superficie ocular forma parte de la unidad funcional lagrimal, definida en 1998 (Stern y cols., 1998), de la que también forman parte los mecanismos de comunicación y regulación en los que participan no sólo el sistema nervioso sino también el hormonal, el vascular y el inmune (**figura 2**). Todos los componentes de esta unidad funcional están comunicados y mantienen la homeostasis de la superficie ocular.

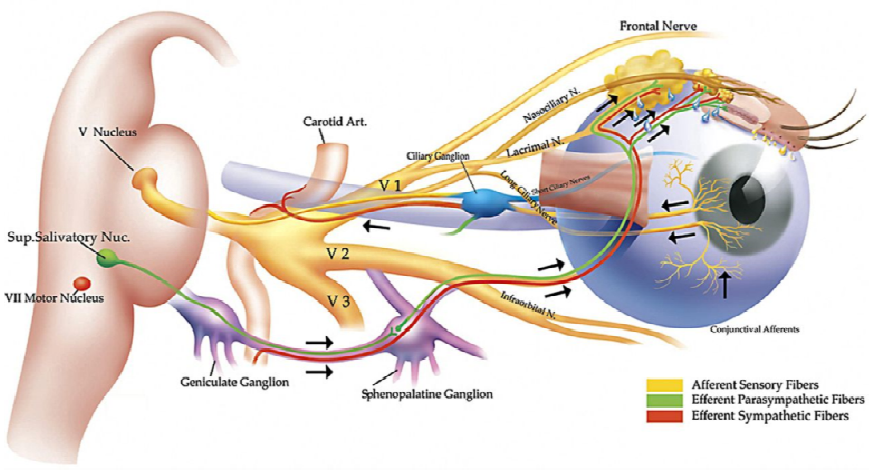

Figura 2. Representación de la unidad funcional lagrimal. Imagen de Pflugfelder, Beuerman y Stern. Marchel Dekker, Inc., New York, 2004, 11–39.

1.1. Inflamación de la unidad funcional lagrimal

Si cualquier componente de esta unidad funcional lagrimal se ve comprometido, se impide el correcto funcionamiento de la superficie ocular y se desarrolla una inflamación local. Si esta inflamación no se resuelve, puede dar

lugar a un estado de inflamación crónico (Stern y cols., 2004). Un ejemplo de patología inflamatoria crónica es el síndrome de ojo seco, cuyos efectos primarios son la pérdida de agudeza visual y de calidad de vida, y que puede verse acompañado de dolor crónico. Este ejemplo ilustra la gravedad de estas enfermedades dentro del campo de la oftalmología, ya que afectan entre el 5-35% de la población (Bron y cols., 2014; Paulsen y cols., 2014) y no hay, actualmente, tratamientos efectivos para la recuperación funcional de los tejidos dañados en estos pacientes.

Los componentes de la superficie ocular que participan en las respuestas inflamatorias incluyen (i) las barreras de protección epiteliales (Mantelli y cols., 2013), (ii) las células del sistema inmunitario presentes en el epitelio y en el estroma corneolimbar (Hamrah y cols., 2003) y (iii) el tejido linfoide asociado a la mucosa conjuntival (**CALT**), que tiene todos los componentes para llevar a cabo una respuesta inmune completa (Knop & Knop, 2000). Normalmente las células más externas de la superficie ocular, las células epiteliales, son las primeras en reaccionar ante el estímulo inflamatorio. Su respuesta inicial va a provocar la presencia de moléculas que modularán la consecutiva respuesta inflamatoria (Stevenson y cols., 2012).

Independientemente del estímulo que provoque una reacción de inflamación aguda, esta se llevará a cabo en las primeras 24–48 horas. Esta respuesta inicial dará lugar a infiltrados de linfocitos polimorfonucleares en el lugar de la inflamación. Estos linfocitos son el tipo celular propio en este estado de inflamación aunque también podemos encontrar células *natural killer*, macrófagos o, dependiendo del estímulo inicial, linfocitos T (McDermott y cols., 2005).

La inflamación aguda termina cuando el estímulo inicial se ha eliminado o los mediadores inflamatorios están inactivados. Sin embargo, si el estímulo no se elimina y/o las células inflamatorias o los mediadores solubles persisten, se lleva a cabo una respuesta inflamatoria tardía, que puede perpetuarse y hacerse crónica. En este caso, los linfocitos T activados y los anticuerpos secretados por

los linfocitos B, propios de la respuesta inmune adaptativa, son los protagonistas. El objetivo de ambos es proporcionar factores celulares y humorales especializados en evitar o combatir el estímulo inicial. Esta respuesta tardía comienza con la activación de macrófagos y de células dendríticas (**DCs**) en la zona inflamada. Debido a las citoquinas inflamatorias presentes en esta zona ambos tipos celulares se activan y maduran a células presentadoras de antígeno (**APCs**). Estas APCs, tras haber procesado el antígeno, migran tanto a los nódulos linfáticos locales como al bazo, presentando el antígeno a los linfocitos T inmaduros para su diferenciación. Estos linfocitos T inmaduros se diferencian a células CD4+, tanto a linfocitos T efectores o *helper* (**Th**) como a linfocitos T reguladores (**Tregs**), que migran a la zona de inflamación y regulan la respuesta inmune (**figura 3**).

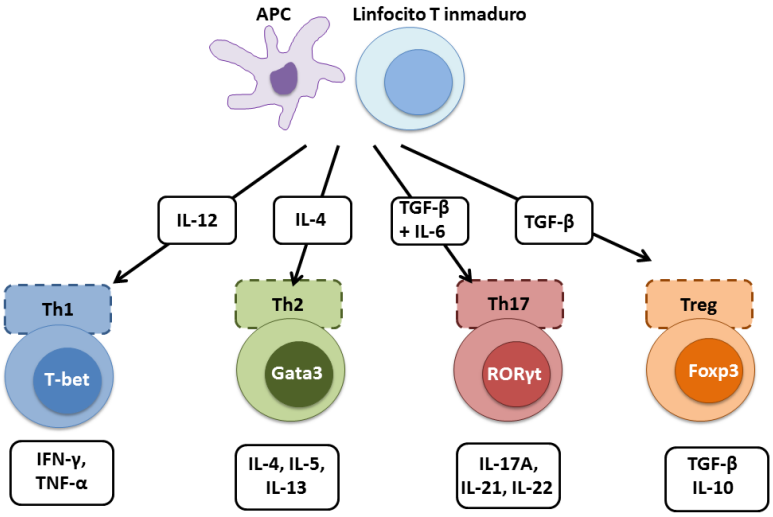

Figura 3. Esquema que representa la diferenciación de los linfocitos T CD4+ inmaduros tras su contacto con las APCs. Los diferentes subtipos de linfocitos T efectores o reguladores maduros se caracterizan por la expresión de factores transcripción específicos (T-bet, Gata3, RORγt o Foxp3) y la secreción de ciertas citoquinas. Adaptado de Lora y Macpherson, *Nature Reviews Immunology,* 2010.

De este modo, muchas de las manifestaciones clínicas son comunes entre los pacientes con distintas patologías de base inflamatoria. Cabe destacar que los niveles de ciertas citoquinas inflamatorias están incrementados en la lágrima de

pacientes con síndrome de ojo seco. Entre estas citoquinas se encuentran la interleuquina (IL)-6, la IL-8 o la IL-1β (Massingale y cols., 2009; Boehm y cols., 2011; Na y cols., 2012; Contreras-Ruiz y cols., 2014; Yoon y cols., 2007; Lam y cols., 2009). También se pueden distinguir ciertos signos inflamatorios específicos o biomarcadores que pueden derivar de la causa inicial que ha desencadenado la inflamación.

Entre las causas de una inflamación ocular se pueden encontrar tanto factores ambientales como genéticos. Algunos de los factores ambientales implicados pueden ser el uso de lentillas, una cirugía, una alergia o una infección, entre otros. Recientemente se ha propuesto un marcador genético que podría identificar pacientes con mayor susceptibilidad a desarrollar una inflamación crónica en la superficie ocular. El marcador, un polimorfismo de un solo nucleótido (**SNP**) en el gen de la trombospondina-1 (**TSP-1**), el gen ***THBS1***, se identificó en pacientes que se habían sometido a una cirugía refractiva (Contreras-Ruiz y cols., 2014). Este tipo de cirugías generan una inflamación local que suele desaparecer a los pocos días. Sin embargo, los sujetos con este SNP no son capaces de resolver esta inflamación local y generan una patología crónica. Este SNP causa una disminución en la expresión proteica de la TSP-1, ya que afecta a la región promotora del gen. Este factor genético podría permitir la prevención de una inflamación crónica de la superficie ocular mediante tratamientos específicos.

2. La trombospondina-1 (TSP-1)

La TSP-1 es una proteína matricelular de 450 kDa, compuesta por 3 monómeros de 150 kDa cada uno, perteneciente a una familia de proteínas relacionadas estructuralmente llamadas trombospondinas (Adams & Lawler, 2004). Esta glicoproteína modula funciones celulares tales como la migración, la proliferación o la apoptosis celular, ya que facilita las interacciones célula-célula y célula-matriz extracelular (Chen y cols., 2000). La **figura 4** ilustra la estructura de la TSP-1 con los distintos dominios que la componen, los receptores celulares

y ligandos a los que se une cada dominio y las principales funciones que llevan a cabo.

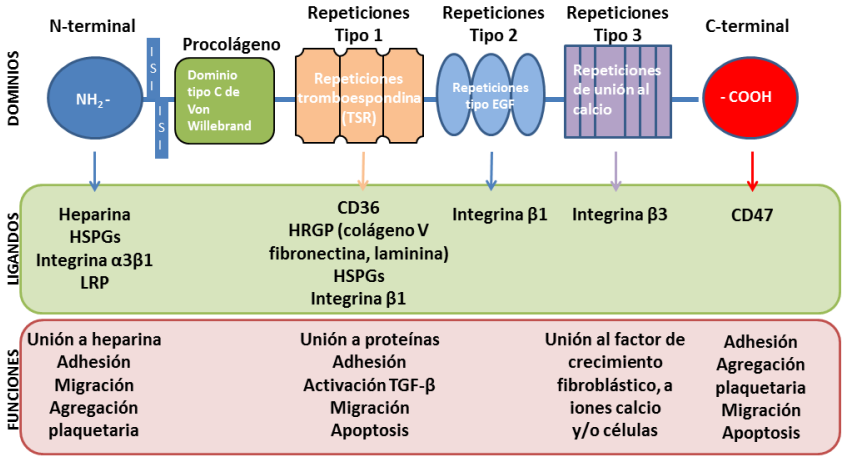

Figura 4. Estructura de un monómero de la TSP-1. HSPGs: proteoglicanos de heparán sulfato; HRGP: glicoproteína rica en histidina; LRP: proteína relacionada con el receptor de lipoproteína.

Cada monómero consiste en distintos dominios rodeados por los dominios globulares carboxilo (**C-**) y amino (**N-**) terminal. Estos dominios incluyen un dominio homólogo al procolágeno, también denominado dominio tipo C de von Willebrand, y los dominios con las repeticiones de tipo I, II y III. El dominio con repeticiones tipo I o de trombospondina (**TSR**) incluye regiones que se unen al receptor CD36 y al factor de crecimiento transformante (**TGF**)-β latente, activándolo. Este dominio también se puede unir a otros ligandos de la matriz extracelular como distintos colágenos, fibronectina, laminina, heparina, elastasa y algunas metaloproteasas de matriz. El dominio con repeticiones tipo III o de unión a iones calcio, contiene 13 sitios de unión al calcio para estabilizar la estructura globular de la molécula. Estos sitios también están distribuidos entre los dominios de tipo II y C-terminal. La presencia de una secuencia RGD dentro de repeticiones tipo III permite la unión a las integrinas β3, mientras que los sitios de unión a las integrinas β1 se distribuyen entre las repeticiones del tipo I, tipo II y en el dominio N-terminal. En el otro extremo, el C-terminal, hay una región que se une a la proteína asociada a integrina, el receptor CD47.

2.1. Localización de la TSP-1 en la superficie ocular

La TSP-1 se localiza en distintas estructuras oculares. En concreto, en la superficie ocular se ha detectado tanto el ARN mensajero (**ARNm**) como la proteína en el epitelio, el estroma y el endotelio corneal humano (Sekiyama y cols., 2006). Sin embargo, la localización de la TSP-1 en la conjuntiva humana no es tan clara. Dependiendo de la técnica empleada, algunos autores no observan expresión en la conjuntiva (Sekiyama y cols., 2006; Chen y cols., 2012) mientras que, cuando está presente, se describe una expresión leve y focal (Aspiotis y cols., 2007). Un estudio realizado en ratones localizó esta glicoproteína en el epitelio conjuntival indicando la importancia de la misma para la homeostasis de la mucosa ocular (Contreras-Ruiz & Masli, 2015).

2.2. Funciones principales de la TSP-1 en la superficie ocular

Las principales funciones de la TSP-1 en la superficie ocular descritas hasta el momento son la inhibición de la angiogénesis corneal, la inducción de la cicatrización de heridas corneales y la inmunoregulación de la mucosa ocular (Schöllhorn y cols., 2015; Hiscott y cols., 2006; Masli y cols., 2014).

La primera función que se le atribuyó a la TSP-1 fue la de inhibidor de la angiogénesis (Good y cols., 1990). Posteriormente se caracterizó que inhibe la angiogénesis a través de un efecto directo en la migración y la supervivencia de las células endoteliales (Jimenez y cols., 2000). En la córnea, también se ha observado que modula el crecimiento de vasos, tanto sanguíneos como linfáticos. Sin embargo, los ratones deficientes en TSP-1 no evidencian un crecimiento de vasos sanguíneos en este tejido de modo espontáneo, lo que indica que la avascularidad corneal está regulada de modo redundante (Cursiefen y cols., 2004).

También se ha descrito la participación de la TSP-1 en la cicatrización de heridas en tejidos avasculares, como la córnea. Para estudiar estos procesos se han realizado distintas queratotomías penetrantes en modelos experimentales observando un claro incremento de la producción de TSP-1 tras provocar la

herida (Uno y cols., 2004; Matsuba y cols., 2011; Blanco-Mezquita y cols., 2013). Además, si se bloquea la expresión de la TSP-1 aumenta el tiempo de cierre de la herida e incluso, en las heridas más penetrantes, nunca llega a cicatrizar (Blanco-Mezquita y cols., 2013; Uno y cols., 2004). Una de las causas de la inducción de la expresión de la TSP-1 es que ayuda a activar el TGF-β circundante, y éste es clave en la diferenciación de los queratocitos a miofibroblastos (Nor y cols., 2005). Los miofibroblastos son un tipo celular con gran capacidad de migración y que regula los procesos de cicatrización. En estas heridas corneales, los miofibroblastos co-localizan con la TSP-1 evidenciando la importancia de su función como activador del TGF-β (Blanco-Mezquita y cols., 2013; Matsuba y cols., 2011).

Sin embargo, entre las principales funciones descritas de la TSP-1, la función inmunomoduladora parece la más relevante para la superficie ocular. Una evidencia de ello es que los ratones deficientes en TSP-1 desarrollan una inmunopatogénesis ocular de modo espontáneo (Turpie y cols., 2009). Además, un estudio reciente en humanos demuestra la susceptibilidad a padecer una inflamación crónica tras una cirugía ocular, debido a niveles reducidos de la TSP-1 (Contreras-Ruiz y cols., 2014). Se ha comprobado que la TSP-1, mediante su unión al receptor CD47 de las células dendríticas, induce un fenotipo tolerogénico en estas células (Doyen y cols., 2003; Demeure y cols., 2000). Recientemente, se ha confirmado la importancia de la unión de la TSP-1 al receptor CD47 para revertir los signos inflamatorios observados en los ratones deficientes en TSP-1 (Contreras Ruiz y cols., 2017). Este estudio demuestra que se revierten los signos inflamatorios tras inducir linfocitos Tregs e inhibir los Th17. Por lo tanto, confirma el papel de la TSP-1 en la homeostasis de la mucosa ocular mediante regulación de la respuesta inmune sistémica.

La TSP-1 también modula la capacidad inflamatoria de las APCs oculares a través de la activación de la isoforma TGF-β2 (Masli y cols., 2006; Mir y cols., 2015). La isoforma TGF-β2 es la isoforma predominante en la mucosa ocular (Contreras-Ruiz & Masli, 2015), como también lo es en otras mucosas (Chu y cols., 2004; Maheshwari y cols., 2011), y sus niveles están incrementados en

patologías inflamatorias de la superficie ocular (Benito y cols., 2013). Las distintas isoformas del TGF-β se producen unidas a un péptido asociado a la latencia (**LAP**), por lo que tienen que disociarse del complejo LAP/TGF-β, para liberar la molécula de TGF-β activo y funcional. Las integrinas, activadoras del TGF-β latente en la mayoría de las superficies mucosas, no activan la isoforma TGF-β2 debido a la ausencia de la secuencia RGD de unión a integrina en el LAP2 de esta isoforma (Munger y cols., 1999). Sin embargo, la TSP-1 activa cualquier isoforma del TGF-β a través de una secuencia específica situada entre las repeticiones de tipo I de su estructura (Schultz-Cherry y cols., 1994). Por todo ello este mecanismo de activación del TGF-β independiente de integrinas tiene gran relevancia en la mucosa ocular.

Recientemente se ha demostrado que las células caliciformes de la conjuntiva también participan en la modulación de la respuesta inflamatoria mediante la activación del TGF-β2 que llevan a cabo la TSP-1 y el receptor CD36 (**figura 5**) (Contreras-Ruiz & Masli, 2015). Aunque esta activación se puede llevar a cabo extracelularmente, ya se determinó la participación del CD36 en la misma (Yehualaeshet y cols., 1999). Por todo ello la TSP-1, el CD36 y el TGF-β2 participan en el mantenimiento de la homeostasis en la mucosa ocular, induciendo un fenotipo tolerogénico en las APCs oculares.

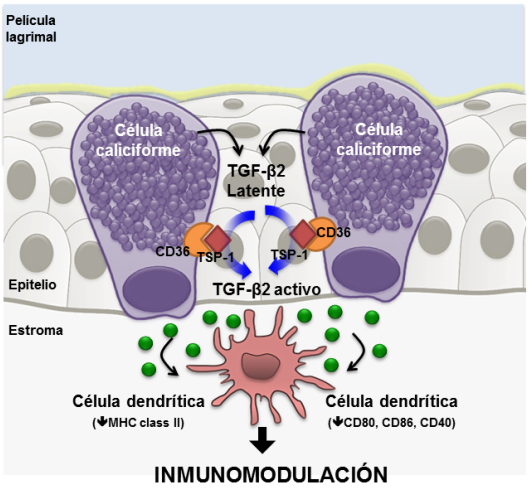

Figura 5. Esquema que representa la inmunomodulación de las células caliciformes en la mucosa ocular tras activar el TGF-β2. Adaptado de Contreras-Ruiz y cols., PLoS ONE, 2015.

2.3. Los ratones deficientes en TSP-1: un modelo de estudio de la inflamación crónica en la superficie ocular

Los ratones deficientes en la expresión de la TSP-1 (B6.129S2-Thbs1tm1Hyn/J) son homocigóticos para la mutación dirigida al gen *THBS1* (https://www.jax.org/strain/006141). Estos ratones muestran ciertos niveles de ARNm de TSP-1 anormal en múltiples tejidos y, sin embargo, no se detecta la TSP-1 a nivel proteico. En general, los ratones deficientes en TSP-1 muestran un aumento en el número de glóbulos blancos circulantes y una considerable hiperplasia en varios linajes de células epiteliales. Estos ratones modificados genéticamente se han empleado para estudiar procesos inflamatorios en los pulmones, los ojos y la piel, entre otras estructuras.

En concreto, el ratón deficiente en TSP-1 desarrolla de modo espontáneo una inflamación en la mucosa ocular similar a la que se observa en pacientes con síndrome de Sjögren, una enfermedad autoinmune que puede ir acompañada de un ojo seco severo y crónico (Turpie y cols., 2009). El grupo de la doctora Masli, en la Universidad de Boston (Boston, MA, EE.UU.), ha caracterizado las células y las moléculas más relevantes implicadas en esta inflamación, asociándolas a los signos inflamatorios que estos ratones desarrollan con la edad (**figura 6**).

El primer signo detectable es una deficiencia secretora, tanto en las células caliciformes de la conjuntiva, como en las células acinares de la glándula lagrimal, que comienza a partir de las 6-8 semanas de edad. Esto provoca una disminución en la calidad y la cantidad de lágrima, lo que afecta a las distintas estructuras de la superficie ocular. El defecto epitelial en la córnea se observa a partir de las 12 semanas de edad (Turpie y cols., 2009). Este signo de daño corneal está acompañado de una inflamación conjuntival, caracterizada por el aumento de vasos linfáticos y de la expresión de citoquinas inflamatorias, y la disminución del número de células caliciformes y de los niveles de mucinas (MUC5AC) en lágrima (Contreras-Ruiz y cols., 2013). También en la glándula lagrimal se detecta un aumento en la expresión de citoquinas inflamatorias (Turpie y cols., 2009; Shatos y cols., 2016). Todo ello provoca la fase de

inducción, donde las APCs presentes en la superficie ocular del ratón deficiente en TSP-1 migran desde la misma hasta los nódulos linfáticos locales y el bazo para activar a los linfocitos T inmaduros. En ambos órganos linfoides secundarios se observa una mayor población de linfocitos T efectores y una menor de Tregs, en comparación con ratones control. La activación y la migración de estos linfocitos a la superficie ocular desempeñan un papel central en la fase efectora que dará lugar a la inflamación crónica. En la glándula lagrimal se observan infiltrados inflamatorios de linfocitos entre las 12–24 semanas de edad (Turpie y cols., 2009; Shatos y cols., 2016), lo que evidencia que ya se ha llevado a cabo la fase efectora.

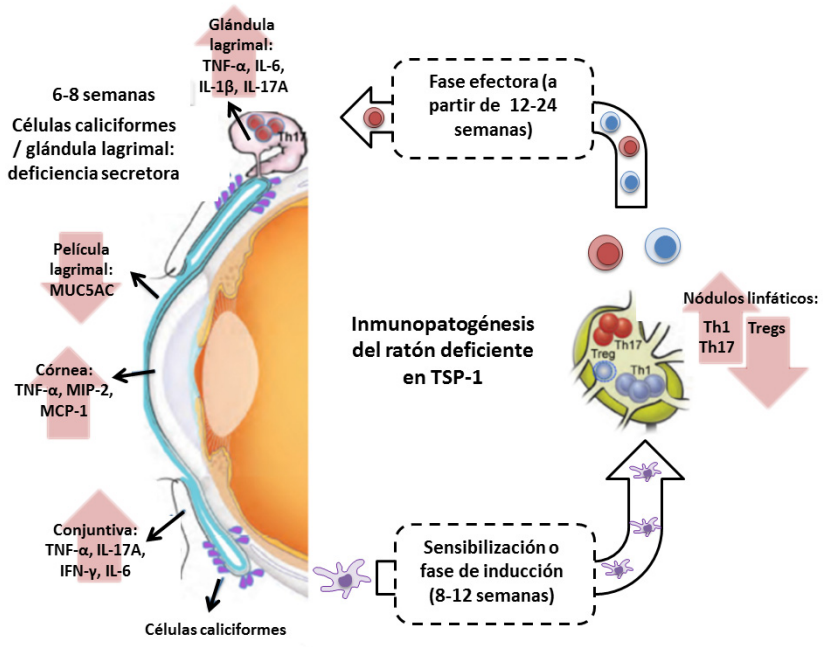

Figura 6. Esquema de la inmunopatogénesis que desarrolla el ratón deficiente en TSP-1 en las distintas estructuras de la unidad funcional lagrimal con el transcurso del tiempo. Adaptado de Masli y cols*., Current Eye Research,* 2014.

2.4. El péptido KRFK: derivado de la TSP-1 y activador del TGF-β

Tras descubrir que la secuencia RFK en la TSP-1 era suficiente para la activación del TGF-β (Schultz-Cherry y cols., 1995), se sintetizó el péptido KRFK, derivado de la secuencia de la TSP-1. Este péptido se ha empleado para profundizar en el mecanismo de activación del TGF-β y estudiar la importancia

de esta función concreta de la TSP-1. Este péptido es capaz de unirse a la secuencia LSKL localizada en el extremo N-terminal del LAP del TGF-β latente, liberando la molécula activa de TGF-β (**figura 7**). La secuencia LSKL es común a todas las LAP asociadas a las distintas isoformas del TGF-β, por lo que tanto la TSP-1 como solo esta secuencia derivada de la misma, pueden activar todas las isoformas del TGF-β.

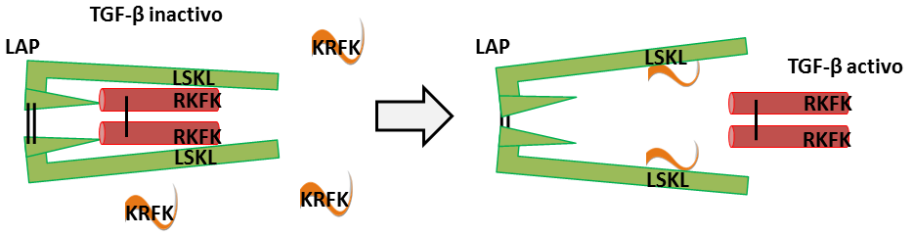

Figura 7. Esquema que muestra cómo el péptido KRFK, derivado de la secuencia de la TSP-1, interacciona con la secuencia LSKL localizada en el péptido asociado a la latencia (LAP) y libera el TGF-β activo.

El péptido KRFK también se ha empleado *in vivo* en distintos modelos experimentales, para estudiar la contribución de la activación del TGF-β que lleva a cabo la TSP-1 en distintos contextos patológicos. Por ejemplo, tras describir la importancia de la TSP-1 para la homeostasis pulmonar en ratones, Crawford y colaboradores (1998) evaluaron si el péptido KRFK podía revertir la neumonía que los ratones deficientes en TSP-1 desarrollan. Los resultados muestran que la inyección intraperitoneal del péptido KRFK revierte algunos de los signos de neumopatía que desarrollan estos ratones. Otro estudio lo empleó en ratones BALB/c inmunodeficientes, para estudiar la importancia de los niveles de TGF-β activo en el crecimiento de tumores (Yee y cols., 2004). Los hallazgos de ese estudio sugieren que la regulación del TGF-β activo que lleva a cabo la TSP-1 tiene un papel relevante en la inhibición del crecimiento y la angiogénesis tumoral. También se empleó de manera tópica en heridas cutáneas para solventar la cicatrización anormal características de los ratones deficientes en TSP-1 (Nor y cols., 2005).

3. Tratamientos anti-inflamatorios para la superficie ocular

Independientemente de la causa de la inflamación en la superficie ocular los fármacos empleados frente a este problema se suelen dividir entre esteroideos (corticoides o corticosteroides) y no esteroideos. Los corticosteroides han sido los fármacos empleados de rutina por oftalmólogos para el tratamiento de patologías de la superficie ocular de base inflamatoria. Son derivados del núcleo ciclopentanoperhidrofenantreno o esterano y difunden a través de la membrana celular, donde se encuentran los receptores de glucocorticoides. Estos receptores son nucleares: tras unirse a su ligando, dimerizan y se translocan al núcleo celular. Una vez allí, actúan a nivel genético, uniéndose a elementos de respuesta hormonal que provocan una disminución de la secreción de citoquinas, de quimiocinas y de moléculas de adhesión, entre otras moléculas pro-inflamatorias, estimulando la apoptosis linfocitaria (Barnes, 2009). Por ello, tras su aplicación disminuyen los signos inflamatorios en estos pacientes. Sin embargo, en su aplicación tópica oftálmica, se han descrito efectos secundarios relevantes, como son la aparición de glaucoma, el desarrollo de cataratas, la mayor susceptibilidad a padecer queratitis infecciosa o la interferencia con los procesos de cicatrización corneal. Por ello se prefiere el uso de corticosteroides denominados “suaves” (Bodor, 1994). Un ejemplo de estos corticosteroides suaves es el acetato de medroxiprogesterona (**MPA**), uno de los más empleados por los oftalmólogos para sustituir corticoides de larga duración que han podido crear cierta dependencia.

Los fármacos anti-inflamatorios no esteroideos (**NSAID**) pueden ser de origen natural o químico, e inhiben inespecíficamente la ruta de las ciclooxigenasas, inhibiendo la formación de prostaglandinas. Las prostaglandinas son proteínas con acción quimiocinética y actúan como mediadores de las respuestas inflamatorias celular y humoral. Sin embargo, una vez formadas, los NSAID no pueden inhibir su acción. Entre los más empleados se encuentran la indometacina, el diclofenaco y el ibuprofeno.

Cabe destacar que hay otros agentes, no incluidos en los grupos anteriores, pero que son cada vez más importantes en los tratamientos de las patologías inflamatorias como el síndrome de ojo seco (Pflugfelder, 2003). Especial mención merece el tratamiento tópico con ciclosporina A. Este péptido, derivado del hongo *Tolypocladium inflatum*, actúa a distintos niveles, principalmente inhibiendo la activación nuclear de factores de transcripción para la activación de linfocitos T. Esto genera una disminución en la producción de citoquinas inflamatorias y en los procesos apoptóticos que activan las células epiteliales de la superficie ocular, característica en estas patologías (Gao y cols., 1998).

Si bien todas estas opciones ayudan a los pacientes, todavía no se ha conseguido un tratamiento que revierta completamente la situación de inflamación crónica y recupere funcionalmente los tejidos afectados. Este hecho, junto con la baja calidad de vida de estos pacientes y el aumento en la prevalencia de estas enfermedades, despierta un interés que dirige la investigación hacia la búsqueda de nuevos fármacos que sean cada vez más eficientes. Por ello aparecen de forma continuada novedosos tratamientos para la superficie ocular que proponen nuevas dianas terapéuticas para contener o evitar este tipo de patologías oculares (Nebbioso y cols., 2016).

3.1. La vía tópica como vía de administración de fármacos oculares

Las principales vías de administración de fármacos para la superficie ocular son la tópica, que contacta directamente con la superficie ocular, y la subconjuntival, que requiere de una inyección en esta zona. La vía tópica es la preferida por ser no invasiva, de menor coste y la mejor aceptada por parte de los pacientes debido a su fácil aplicación.

No obstante esta vía tiene ciertas restricciones debidas a la anatomía y la fisiología ocular. La propia superficie ocular, y específicamente el epitelio corneal, es la principal barrera de defensa del ojo frente a una posible amenaza exterior, por lo que está muy especializada para no permitir el paso de cualquier sustancia al interior ocular. Desde el punto de vista biofarmacéutico, se pueden

identificar tres barreras principales para la administración tópica ocular: la película lagrimal, su rápido drenaje por el conducto naso-lagrimal y la impermeabilidad del epitelio corneal. La película lagrimal tiene entre sus componentes complejas glicoproteínas como son las mucinas, y enzimas o péptidos con actividad lítica. La función de estos componentes es proteger a la superficie ocular de cualquier agente extraño, incluidas moléculas terapéuticas. Además está en continua renovación, lo que provoca el drenaje de la misma y, con ella, de cualquier sustancia activa instilada. Finalmente, el fármaco que consigue llegar hasta la córnea se encuentra con un epitelio con uniones fuertes, como las uniones estrechas o los desmosomas, que evita el paso de sustancias de más de 500 Da (Hämäläinen y cols., 1997). En general, se acepta que solo el 1-5% del fármaco instilado es capaz de atravesar la barrera corneal (Davies, 2000; Urtti, 2006). Sin embargo, hay que tener en cuenta que la superficie ocular tiene mucho más epitelio conjuntival que corneal, y que este epitelio es de 15 a 25 veces más permeable que el corneal (Hämäläinen y cols., 1997). De la cantidad absorbida por la conjuntiva cierto porcentaje pasará a la circulación sistémica, reduciendo la cantidad de fármaco con acción local.

Las formulaciones de medicamentos que se pretendan aplicar de forma tópica en el ojo deben tener unas características fisicoquímicas determinadas que contribuyan a mejorar su biodisponibilidad. Entre ellas, cabe destacar que el tamaño nanométrico mejorará su paso a través de los epitelios, y que el pH y la osmolaridad deberán ser similares a los de la lágrima, para evitar irritación. Los valores de pH y de osmolaridad de la lágrima se encuentran entre 6.5–7.8 (Abelson y cols., 1981; Coles & Jaros, 1984) y 283-318 mOsm/Kg (Tomlinson y cols., 2006), respectivamente.

En resumen, hay una necesidad de mejorar la biodisponibilidad de las sustancias activas aplicadas de forma tópica ocular, pero se debe conseguir teniendo en cuenta ciertas características fisicoquímicas. Para ello, la tecnología farmacéutica ofrece diferentes estrategias, como son los sistemas de liberación controlada de fármacos.

3.2. Los liposomas como sistemas de liberación controlada de fármacos

Se han desarrollado diferentes estrategias para optimizar las condiciones de aplicación de fármacos destinados a la vía tópica ocular. Entre estas estrategias cabe destacar la tecnología desarrollada para transportar y liberar de forma controlada y a la zona deseada la sustancia activa, logrando un mejor efecto terapéutico. Entre los sistemas desarrollados con más presencia en el diseño de formulaciones oculares se encuentran las micropartículas, las nanosuspensiones, las nanopartículas, los liposomas, los dendrímeros o las ciclodextrinas (Sahoo y cols., 2008; Patel y cols., 2013).

Los liposomas, elaborados por primera vez en la década de los 60 (Bangham y cols., 1965), son todavía actuales por las ventajas que ofrecen para la aplicación de fármacos en la superficie ocular (Agarwal y cols., 2014; Mishra y cols., 2011; Lim y cols., 2015). Entre estas ventajas, cabe destacar su buena biocompatibilidad, su fácil preparación o la posibilidad de emplearlos para encapsular tanto sustancias activas hidrofílicas como hidrofóbicas. La principal desventaja de estos vehículos radica en su inestabilidad, que se solventa, en parte, mediante la adición de moléculas que estabilizan su estructura como el colesterol (Briuglia y cols., 2015). La naturaleza anfifílica de los fosfolípidos que los componen provoca la formación de una o más esferas concéntricas constituidas por bicapas lipídicas o lamelas, y cuyo diámetro puede oscilar desde 80 nm hasta 10 µm (**figura 8**) (Mishra y cols., 2011).

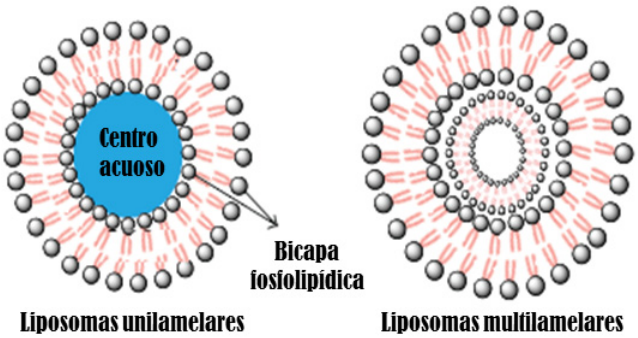

Figura 8: Representación de las diferentes estructuras de los liposomas. Adaptado de Mishra *y cols., Journal of Drug Delivery*, 2011.

Aplicados en la superficie ocular, los liposomas mejoran la penetración de fármacos a través de la córnea (Di Tommaso y cols., 2012), mostrando un efecto más duradero de la sustancia activa (Hathout y cols., 2007; Law y cols., 2000). Este hecho permite el uso de una menor concentración del fármaco, reduciendo la toxicidad asociada a elevadas dosis del mismo. En general, los liposomas interaccionan con la superficie celular y penetran al interior celular mediante endocitosis (Lee y cols., 1993). Este proceso depende de la composición de los liposomas, su carga electrostática y su tamaño. En la superficie ocular, dada la impermeabilidad de la córnea y la carga positiva del epitelio de la superficie ocular, se prefieren los liposomas aniónicos nano-métricos (Law y cols., 2000). La composición de las formulaciones liposomales puede modificarse con el uso de moléculas mucoadhesivas, capaces de establecer enlaces no covalentes con las mucinas de la película lagrimal. Estas moléculas, como el quitosano o el ácido hialurónico, mejoran la biodisponibilidad del fármaco ya que aumentan el tiempo de residencia pre-corneal. La mayoría de las formulaciones liposomales para administración tópica contienen fosfatidilcolina, colesterol y polímeros hidrófilos conjugados con lípidos como ingredientes principales.

JUSTIFICACIÓN DEL TRABAJO

Se ha demostrado que la base de importantes enfermedades de la superficie ocular es un proceso inflamatorio que afecta a todos los componentes de la unidad funcional lagrimal (Stern y cols., 2004). Actualmente, los esfuerzos para recuperar funcionalmente los tejidos dañados en esas situaciones no han dado los resultados esperados, posiblemente debido a la diferente etiología de la inflamación ocular. Por ello, consideramos que es necesario profundizar en los mecanismos fisiopatológicos que desencadenan la patología inflamatoria de la superficie ocular para desarrollar tratamientos especializados más efectivos.

En esta tesis se ha profundizado en el papel que juega la TSP-1 en la inflamación crónica ocular. Hay varias evidencias que apuntan hacia el potencial terapéutico de esta glicoproteína. En primer lugar, el ratón deficiente en la expresión de la TSP-1 desarrolla de modo espontáneo una patología inflamatoria crónica en la superficie ocular (Turpie y cols., 2009). En segundo lugar, la TSP-1 induce un fenotipo inactivo o tolerogénico en las células dendríticas (DCs) (Doyen y cols., 2003; Masli y cols., 2006), uno de los tipos celulares que inicia la respuesta inmunitaria específica. Y, en tercer lugar, un polimorfismo en la zona promotora del gen *THBS1*, que causa una menor expresión de la TSP-1, se relaciona con una mayor susceptibilidad a desarrollar una inflamación crónica ocular tras una cirugía refractiva (Contreras-Ruiz y cols., 2014). Este último estudio sugiere que se podría establecer un diagnóstico temprano sobre si un sujeto va a desarrollar una patología inflamatoria crónica tras un estímulo inicial mediante un genotipado en el gen *THBS1*. Por todo ello, los inicios de este trabajo se centraron en estudiar las funciones de la TSP-1 en la superficie ocular. Tras una extensa búsqueda bibliográfica, se contactó con distintos grupos de investigación internacionales para establecer colaboraciones, como el de la Dra. Luminita Paraoan de la Universidad de Liverpool (Reino Unido) y el de la Dra. Sharmila Masli de la Universidad de Boston (EE.UU.). Ambos grupos han estudiado la TSP-1 y su influencia en el contexto ocular. De este modo se pudo abordar el estudio de esta molécula en la inflamación ocular, con el objetivo

futuro de evaluar un posible tratamiento basado en ella. Se seleccionó el péptido KRFK derivado de la TSP-1 como molécula con potencial terapéutico para el tratamiento de patologías inflamatorias de la superficie ocular.

Además de identificar posibles moléculas terapéuticas, es necesario evaluar y seleccionar un sistema adecuado para su aplicación tópica ocular. Hay que considerar igualmente que las circunstancias impuestas por la anatomía y la fisiología del ojo dificultan la entrada de agentes terapéuticos por la vía tópica, la preferida para tratar enfermedades de la superficie ocular. Esto hace necesario dirigir la investigación hacia el desarrollo de formulaciones farmacéuticas que aumenten la biodisponibilidad de las sustancias activas. Nuestro grupo de investigación, el Grupo de Superficie Ocular (GSO) del Instituto de Oftalmobiología Aplicada (IOBA) de la Universidad de Valladolid, y en concreto el equipo investigador de la Dra. Yolanda Diebold Luque, directora de este trabajo, tienen una vasta experiencia en sistemas nanotecnológicos de liberación de fármacos oculares (Diebold & Calonge, 2010). Uno de los colaboradores dentro de este ámbito es el grupo de las Dras. Rocío Herrero Vanrell e Irene Molina Martínez del Departamento de Farmacia y Tecnología Farmacéutica de la Universidad Complutense de Madrid. Este grupo tiene experiencia en el diseño de nuevos sistemas de aplicación tópica y ha patentado una formulación liposomal desarrollada con características similares a las de la película lagrimal (patente ES 2 284 398). Se propuso emplear una de las formulaciones liposomales desarrolladas y caracterizadas (Vicario-de-la-Torre y cols., 2014) como sistema de encapsulación para el péptido derivado de la TSP-1.

Todo lo indicado hasta aquí justifica que se haya planteado la búsqueda de una alternativa terapéutica basada en la TSP-1, con la ayuda de la tecnología farmacéutica. La experiencia previa del GSO junto con la de los colaboradores, expertos en distintas disciplinas, ha garantizado que este trabajo haya sido multidisciplinar para intentar aunar la experiencia y visión de todos ellos, con el fin último de encontrar soluciones al importante problema clínico y social que genera la inflamación crónica ocular.

HIPÓTESIS Y OBJETIVOS

Hipótesis

Es posible desarrollar un tratamiento de aplicación tópica para prevenir y/o tratar los procesos inflamatorios crónicos de la superficie ocular basado en el papel inmunomodulador desempeñado por la TSP-1 en esa mucosa.

Objetivo general

Desarrollar una alternativa terapéutica eficaz para tratar la inflamación crónica de la superficie ocular asociada a la reducción de la expresión de la TSP-1.

Objetivos específicos

1. Caracterizar y comparar la expresión de la TSP-1 y de algunos de sus receptores, CD36 y/o CD47, en líneas celulares de epitelio corneal y conjuntival, y cultivos primarios de células epiteliales y estromales de la conjuntiva (**estudios 1 y 2**).
2. Determinar si se alteran los niveles de TSP-1 y de su receptor CD36, moléculas implicadas en la activación del TGF-β2, en distintos modelos experimentales de inflamación de la superficie ocular (**estudios 1 y 2**).
3. Evaluar *in vitro* si el péptido KRFK, derivado de la TSP-1 y activador de la citoquina TGF-β, puede atravesar el epitelio conjuntival y afectar al fenotipo de las células dendríticas deficientes en TSP-1 (**estudio 3**).
4. Evaluar *in vivo* la eficacia terapéutica del péptido KRFK usando un modelo animal de inflamación de la superficie ocular relacionado con una deficiencia en la TSP-1 (**estudios 3 y 4**).
5. Desarrollar, caracterizar y evaluar si una formulación liposomal diseñada para su aplicación tópica ocular puede ser empleada como vehículo de moléculas anti-inflamatorias, como el acetato de medroxiprogesterona o el péptido KRFK derivado de la TSP-1 (**estudios 5 y 6**).

METODOLOGÍA

Este apartado incluye un resumen de las muestras y de las técnicas empleadas en este trabajo de investigación indicadas en la **figura 9**.

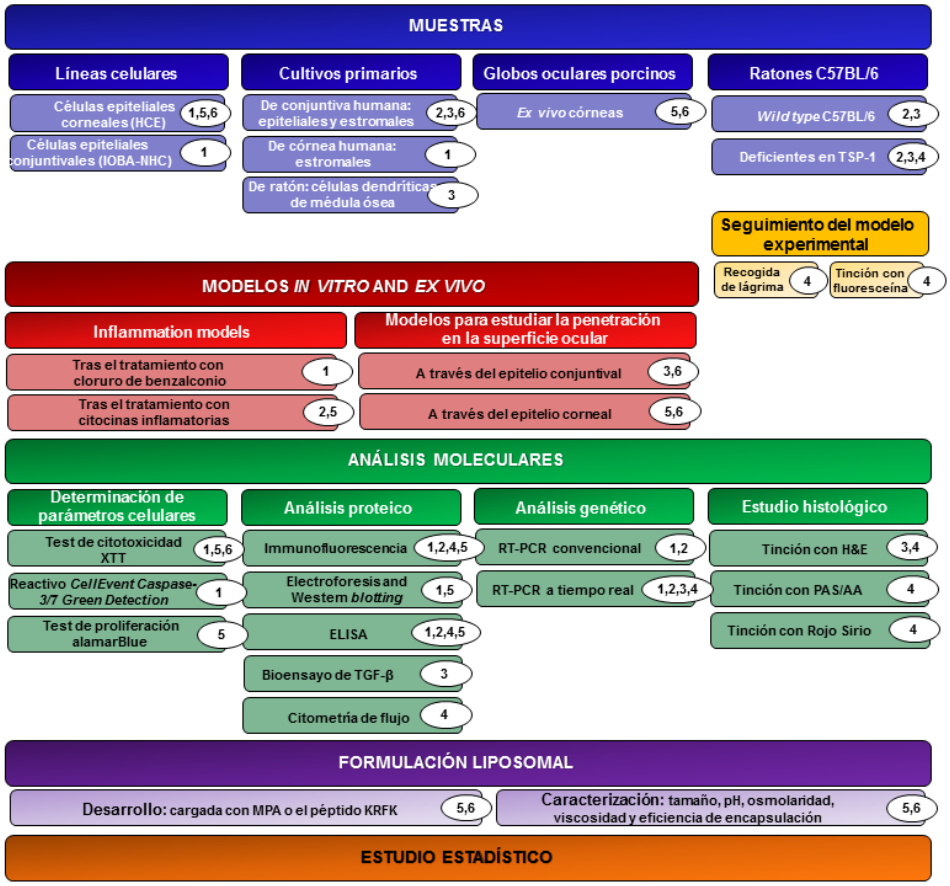

Figura 9. Esquema general de la metodología empleada en la Tesis Doctoral. En los círculos se indican el/los estudio/s en los que se ha empleado cada muestra o método. ELISA: ensayo por inmunoabsorción ligado a enzimas; RT-PCR: reacción en cadena de la polimerasa con retrotranscripción; H&E: hematoxilina y eosina; PAS/AA: ácido periódico de Schiff/azul alcián.

1. Muestras empleadas

Todas las muestras humanas recogidas y los experimentos realizados se han desarrollado siguiendo las directrices de la declaración de Helsinki.

1.1. Líneas celulares

Se emplearon las líneas celulares de origen humano de epitelio de córnea HCE (Araki-Sasaki y cols., 1995) y de epitelio de conjuntiva IOBA-NHC (Diebold y cols., 2003). Ambas se emplearon en el estudio 1 y, además, la línea de córnea en los estudios 5 y 6.

1.2. Cultivos primarios

Las muestras de tejidos oculares humanos empleados para preparar cultivos primarios fueron anillos corneosclerales que procedían de donante cadáver de la Clínica Barraquer (Barcelona, España), tras firmar acuerdos de colaboración. En el empleo de estas muestras se ha seguido la normativa del Real Decreto 1716/2011, por la que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano. De estos anillos corneosclerales, que habían sido descartados para trasplante de córnea, se obtuvieron diferentes tejidos. Aislando la conjuntiva bulbar, se obtuvieron células tanto epiteliales como estromales de conjuntiva a partir de la técnica de explantes, ya optimizada y caracterizada por nuestro grupo de investigación (García-Posadas y cols., 2013). Estos cultivos fueron empleados en los estudios 2, 3 y 6. Aislando la córnea, y tras su digestión empleando colagenasa, se obtuvieron cultivos primarios de fibroblastos corneales usados en el estudio 1.

Además, se generaron cultivos primarios a partir de muestras de tejido de ratón. Se obtuvieron células dendríticas (DCs) primarias generadas a partir de la médula ósea aislada de dos cepas de ratones diferentes, ratones *wild type* y deficientes en TSP-1, cuyas referencias se pueden encontrar en la sección 1.4. El protocolo de aislamiento se llevó a cabo según lo descrito por Madaan y colaboradores (2014). Estos cultivos se emplearon en el estudio 3.

1.3. Globos oculares porcinos

Los globos oculares de cerdo empleados provenían del matadero local Justino Gutiérrez SL (Laguna de Duero, Valladolid). Tras lavarlos con una mezcla de antibiótico y antimicótico y eliminar los tejidos adyacentes, se prepararon las córneas para su utilización *ex vivo* en los estudios 5 y 6.

1.4. Ratones C57BL/6

Todos los experimentos con animales se realizaron de acuerdo con la Declaración de ARVO para el Uso de Animales en la Investigación en Oftalmología (http://www.arvo.org) y todos los protocolos utilizados fueron aprobados por el Comité Institucional de Cuidado y Uso de Animales de la Facultad de Medicina de la Universidad de Boston, donde se realizaron estos experimentos. Todos los procedimientos se llevaron a cabo por personal acreditado para el manejo de animales de experimentación.

Se emplearon dos cepas distintas de ratones. Se utilizaron ratones *wild type* C57BL/6 (Charles River Laboratories, Wilmington, MA, EE.UU.) en el estudio 2 y 3; y deficientes en TSP-1 (The Jackson Laboratory, Bar Harbor, MN, EE.UU.) en los estudios 2, 3 y 4.

<u>Seguimiento de la patología ocular que desarrolla el ratón deficiente en TSP-1</u>

Los ratones deficientes en TSP-1 desarrollan una enfermedad inflamatoria crónica en la superficie ocular con la edad. Para evaluar el desarrollo de esta enfermedad se realizaron tinciones corneales con fluoresceína y se recogió la lágrima, previamente inducida tras la inyección intraperitoneal de pilocarpina, para analizar el contenido en MUC5AC, a distintas edades de estos ratones. Ambos procedimientos se realizaron según lo indicado por Turpie y colaboradores (2009). Estos procedimientos se llevaron a cabo en los ratones empleados en el estudio 4.

2. Modelos *in vitro* o *ex vivo*

2.1. Modelos de inflamación *in vitro*

Modelo basado en el tratamiento con cloruro de benzalconio (**BAC**)

Se caracterizó un modelo que causaba inflamación y muerte celular tras un tratamiento durante tiempos cortos con una solución de BAC al 0.001%. Se emplearon 5 y 15 minutos de tiempo de contacto con las células IOBA-NHC y HCE, respectivamente, generando un porcentaje de muerte celular similar en ambos tipos celulares. Estas células mostraron niveles elevados de marcadores de inflamación como la interleuquina (**IL**)-6, o de apoptosis como la caspasa-3/7 activada, durante las siguientes 24 horas en cultivo. Este modelo se realizó en el estudio 1.

Modelo basado en el tratamiento con citoquinas inflamatorias

Se emplearon distintos modelos de inflamación basados en uno ya publicado por nuestro grupo de investigación en células de epitelio conjuntival (Enriquez-de-Salamanca y cols., 2008). En el estudio 2, los fibroblastos conjuntivales se trataron con 10 ng/mL de IL-1β (PeproTech, London, UK) durante 24 horas para simular condiciones de inflamación. En el estudio 5 las células HCE se trataron con 25 ng/mL de factor de necrosis tumoral (**TNF**)α (PeproTech) durante 24 horas. Tras el tratamiento con TNFα la producción de IL-6 en las células HCE se mantuvo incrementada tras 24-48 horas en cultivo, lo que permitió emplear esta citoquina como marcador de inflamación y evaluar tratamientos anti-inflamatorios sobre estas células.

En ambos modelos las células se mantuvieron en medio de cultivo sin suplementos para evitar el enmascaramiento de los resultados debidos a la adición de suero u otro componente al medio de cultivo.

2.2. Modelos para el estudio de la penetración de sustancias activas en la superficie ocular

Modelo para el estudio de la penetración a través del epitelio conjuntival

Este modelo consiste en medir el paso de una sustancia activa previamente marcada con un fluoróforo a través de un cultivo de células epiteliales primarias, obtenidas según lo indicado en la sección 1.2. Estas células se sembraron en la parte superior de un inserto con un tamaño de poro de 0.4 µm (Nunc, Roskilde, Dinamarca). Se midió la resistencia eléctrica transepitelial (**TEER**) del cultivo celular en el inserto para controlar la integridad de la capa celular. Cada 15-30 minutos se recogió una muestra del sobrenadante de la parte de abajo del inserto y se cuantificó mediante fluorometría. Tras hacer una recta patrón con cantidades conocidas de la sustancia activa marcada, se calculó la cantidad en µg de sustancia activa que atravesaba el epitelio conjuntival. El esquema de este modelo se representa en la **figura 10**. Los resultados se muestran como porcentaje de la sustancia activa liberada en cada tiempo de análisis.

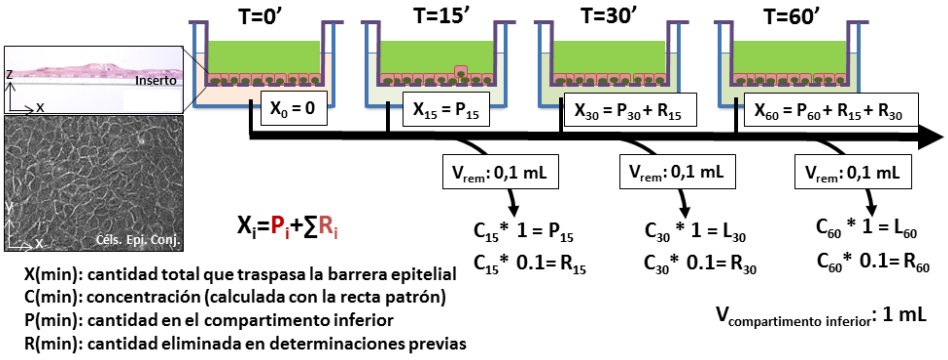

Figura 10. Esquema del modelo empleado para estudiar la penetración de una sustancia activa a través del epitelio conjuntival.

Modelo *ex vivo* para el estudio de la penetración a través del epitelio corneal

Para llevar a cabo este modelo se emplearon globos oculares porcinos obtenidos según lo indicado en la sección 1.3. Se colocó un anillo de silicona de 11 mm de diámetro en la córnea y se aplicó la formulación o sustancia activa de interés, que estaba marcada con un fluoróforo. Los tiempos de contacto

empleados fueron de 5 y 60 minutos. Posteriormente se lavaron los tejidos profusamente y se fijaron con paraformaldehído tamponado al 2.5%. La zona de la córnea central se incluyó en *Optimal Cutting Temperature* (**OCT**). Se realizaron cortes histológicos de 5 µm de espesor en frío, a -18ºC. Se observaron en el microscopio de fluorescencia, empleando el mismo tiempo de exposición, ganancia e intensidad de la cámara, y se determinó la penetración corneal en las distintas capas del epitelio. Este modelo se empleó en los estudios 5 y 6.

3. Análisis moleculares

3.1. Determinación de parámetros celulares

Estudio de la viabilidad celular empleando el test de citotoxicidad XTT

Se empleó el test colorimétrico de viabilidad celular XTT (Sigma-Aldrich, St. Louis, MO, EE.UU.), basado en la reducción de la sal de sodio 2,3-bis(2-metoxi-4-nitro-5-sulfonil)-2H-tetrazolio-5-carboxianilida (**XTT**) por las deshidrogenasas mitocondriales. Si se lleva a cabo esta reducción, se forman cristales de formazán con distintas propiedades de absorción de luz. Este hecho puede ser monitorizado espectrofotométricamente a una longitud de onda (**λ**) de 450 nm. En todos los casos se emplearon células sin ningún tratamiento como control negativo de toxicidad y células expuestas a 0.001% de BAC como control positivo de toxicidad. Los resultados se muestran como porcentaje de viabilidad, normalizado respecto al control negativo (100%). Este test se empleó en los estudios 1, 5 y 6.

Estudio de la apoptosis celular empleando el reactivo *CellEvent™ Caspase-3/7 Green Detection*

Se empleó el kit *CellEvent™ Caspase-3/7 Green Detection Reagent* (Thermo Fisher Scientific, Waltham, MA, EE.UU.) que contiene un sustrato que, al unirse a la caspasa-3/7 activada, es fluorogénico. Posteriormente, todos los núcleos celulares se tiñeron empleando el colorante Hoechst 33342 (Sigma-Aldrich). Se tomaron 3 fotos representativas de cada pocillo con un objetivo X20,

presentando los resultados como porcentaje de apoptosis. Esta medida se llevó a cabo en el estudio 1.

Estudio de la proliferación celular empleando el reactivo alamarBlue®

El reactivo alamarBlue® (Bio-Rad, Oxford, Reino Unido) se utilizó para medir cambios en la tasa de proliferación de las células en cultivo. Este reactivo se compone de resazurina, que penetra en las células viables donde es reducido a resorufin, un compuesto que emite fluorescencia ($\lambda_{excitación}$: 560 nm; $\lambda_{emisión}$: 590 nm). El reactivo no es tóxico, lo que permite determinar cómo proliferan las células a lo largo del tiempo si se realizan diferentes determinaciones a distintos tiempos. Los resultados se muestran como porcentaje de proliferación celular normalizado respecto a los valores obtenidos en la primera determinación realizada (tiempo 0). Este ensayo se empleó en el estudio 5.

3.2. Análisis de proteínas

Inmunofluorescencia

Para la detección de ciertas proteínas, así como su localización celular o tisular, se empleó esta técnica basada en la reacción antígeno-anticuerpo (**Ac**) y la detección de fluorescencia. Para ello se trataron las células o las secciones de tejido previamente de modo que: 1) se permeabilicen las membranas celulares si se busca detectar una proteína intracelular (empleando tritón al 0.3%), 2) se recuperen ciertos antígenos si previamente se ha fijado el tejido (empleando un tratamiento con tripsina al 0.01%) y, 3) se bloqueen las posibles uniones inespecíficas del Ac (normalmente, empleando un tratamiento con suero fetal bovino al 4-5%). Tras ello, se incubó el Ac primario empleando una concentración óptima para su detección (**tabla 1**). En general en este trabajo los Acs primarios se incubaron a 4°C durante toda la noche. Tras lavar las muestras para eliminar el exceso del Ac primario, se incubó con un Ac secundario que estaba ligado a un fluoróforo. Tras ello, se realizó una contratinción nuclear con ioduro de propidio (Thermo Fisher Scientific) o con Hoechst 33342. Los

resultados se analizaron empleando un microscopio de fluorescencia. Esta técnica se empleó en los estudios 1, 2, 4 y 5.

Tabla 1: Anticuerpos primarios empleados en las técnicas de inmunofluorescencia (IF) o Western *blotting* (WB).

Ac	[ ] para IF (µg/mL)	[ ] para WB (µg/mL)	Casa comercial	Estudio
Vimentina	1	-	Santa Cruz Biotechnology, Santa Cruz, CA, EE.UU.	1
α-SMA	2.5	-	Abcam, Cambridge, UK	1
TSP-1	4	1	Santa Cruz Biotechnology	1,2
CD36	10	5	Abcam	1,2
CD47	4	1	Santa Cruz Biotechnology	1
Caspasa-3	-	1	Abcam	1
TGF-β2	10		R&D systems, Minneapolis, MN, EE.UU.	2
RG	5	2.5	Abcam	5
RP	5	2.5	Abcam	5
GAPDH	-	0.5	Santa Cruz Biotechnology	1,5

SMA: actina de músculo liso; RG: receptor de glucocorticoides; RP: receptor de progesterona; GAPDH: gliceraldehído-3-fosfato deshidrogenasa.

Además en el estudio 2 se compararon niveles de expresión de una proteína entre distintas condiciones experimentales. Para ello, se tomaron las imágenes empleando siempre la misma intensidad, exposición y ganancia de la cámara. Estos parámetros se optimizaron observando los controles negativos y los experimentales en los que se esperaba mayor señal. Posteriormente estas imágenes se analizaron empleando el programa ImageJ (http://imagej.nih.gov/ij/, National Institutes of Health, Bethesda, MD, EE.UU.). También se llevó a cabo la cuantificación relativa de la localización nuclear en el estudio 5. Para delimitar la zona nuclear se emplearon las imágenes obtenidas con la tinción nuclear. Los resultados se muestran como porcentaje de localización nuclear.

Electroforesis y Western *blotting*

Esta técnica también emplea la reacción antígeno-Ac para la detección de proteínas. En primer lugar, para obtener los lisados celulares se empleó un tampón de lisis celular del ensayo de radioinmunoprecipitación (**RIPA**) y se cuantificó la cantidad de proteína de los lisados empleando el kit Pierce® BCA *Protein Assay Kit* (Thermo Fisher Scientific), midiendo la absorción colorimétrica en un multilector de placas (λ: 562 nm). En segundo lugar, tras desnaturalizar las proteínas obtenidas se realizó la electroforesis en gel de poliacrilamida con dodecil sulfato sódico, detergente que recubre los polipéptidos de cargas negativas. Las proteínas desnaturalizadas y con una densidad de carga uniforme se separaron según el peso molecular. Los parámetros empleados para la electroforesis fueron de 15 minutos a 70 V seguido de 1 hora a 120 V. En tercer lugar, se llevó a cabo la transferencia, empleando un amperaje de 350 mA durante 1 hora y 30 minutos. Y, por último, sobre esta membrana se llevó a cabo la inmunodetección como sigue: se incubó con un Ac primario (**tabla 1**) y, tras lavar la membrana, se incubó con un Ac secundario conjugado con la enzima peroxidasa. Estas membranas se revelaron y se observaron las bandas en el lugar de las proteínas, que se cuantificaron de forma relativa a una muestra control. Dichos valores se normalizaron a los de una proteína control, la GAPDH. Esta técnica se llevó a cabo en los estudios 1 y 5.

Técnica de inmunoabsorción ligada a enzima (ELISA)

La técnica ELISA cuantifica niveles de proteína secretada mediante el uso de una curva patrón que relaciona una concentración conocida de la proteína de interés con su absorbancia. Así, al añadir una muestra de valor desconocido y leer la absorbancia, se puede iterar la concentración de la proteína de interés. En esta tesis se emplearon distintos kits de ELISA para, siguiendo las instrucciones del fabricante, detectar distintas proteínas de interés. Se emplearon kits específicos para la detección de la TSP-1 (R&D systems) en los estudios 1 y 2; de la IL-6 (Diaclone, Besançon, Francia) en los estudios 1 y 5; del TGF-β2 (R&D

systems) en el estudio 2; de la IL-8 (Diaclone) en el estudio 5; y de la MUC5AC (TSZ ELISA, Waltham, MA, EE.UU.) en el estudio 4.

Bioensayo para determinar la cantidad de TGF-β

Esta técnica cuantifica la cantidad del TGF-β en los sobrenadantes celulares a partir de la respuesta producida por el cultivo de estos sobrenadantes en otras células, las MFB-F11 (Tesseur y cols., 2006). Las células MFB-F11 son fibroblastos obtenidos de ratones deficientes en TGF-β que han sido transfectados con un plásmido que consta de elementos de unión a los factores de transcripción Smad, proteínas intracelulares de transducción de las señales del TGF-β, acoplados al gen de la fosfatasa alcalina (**SBE-SEAP**). Este bioensayo permite cuantificar el TGF-β con mayor sensibilidad que el ELISA. Para la lectura de los resultados se empleó el *Great EscAPe SEAP Reporter system 3* (Clontech, Mountain View, CA, EE.UU.). Los resultados se muestran como porcentaje de TGF-β activado, ya que permite medir el TGF-β total y el activado. Este bioensayo se empleó en el estudio 3.

Citometría de flujo

Esta técnica obtiene información de poblaciones celulares mediante un estudio individualizado de las células que lo componen. Se obtiene información de distintos parámetros celulares, según las siguientes medidas realizadas sobre el rayo de luz láser que incide sobre cada célula:

- Dispersión delantera o de avance (*forward scatter*): mide la dispersión de la luz en la dirección del haz incidente, siendo proporcional al tamaño de la célula.
- Dispersión lateral (*side scatter*): mide la dispersión de la luz en dirección a 90 grados con respecto a la luz incidente, siendo proporcional a la complejidad estructural de la célula.
- Fluorescencia: detecta y analiza la luz fluorescente emitida por las células tras la incubación con Acs ligados a fluorocromos. Este análisis permite combinar

la detección de más de una proteína, dependiendo de los distintos filtros adaptados. Empleando distintos Ac (Thermo Fisher Scientific) se midieron los niveles intracelulares de interferón (**IFN**)-γ, IL-17A y de Foxp3.

Esta técnica se empleó en el estudio 3.

3.3. Análisis genético

RT-PCR convencional

Para analizar los niveles de ARNm de un gen de interés se extrajo el ARN total empleando el *Rneasy Mini kit* (Qiagen, Valencia, CA, EE.UU.) y se cuantificó mediante *Quant-iT™ RNA assay* (Thermo Fisher Scientific). Se añadió 1 µg para hacer la transcripción reversa (**RT**) y obtener el cDNA empleando *SuperScript® VILO™ cDNA synthesis Kit* (Thermo Fisher Scientific). Posteriormente, se amplificó mediante una reacción en cadena de la polimerasa (**PCR**), empleando cebadores concretos según el gen a amplificar, nucleótidos, tampón y Taq polimerasa (Biotools B&M Labs S.A., Madrid, España). El producto de esta reacción se analizó mediante una electroforesis en gel de agarosa, que incluye *Blue Juice™ Gel Loading Buffer* (Thermo Fisher Scientific), para observar las bandas de interés empleando luz ultravioleta. Los cebadores empleados para amplificar los genes de la TSP-1, el CD36 y el CD47 (Origene Technologies, Rockville, MD, EE.UU.) tienen de referencia HP206797, HP200058 y HP225827, respectivamente. Esta técnica se empleó en los estudios 1 y 2.

RT-PCR a tiempo real

También se puede analizar el ARNm de un gen a tiempo real para comparar los niveles de éste entre distintas condiciones. En este caso, para la reacción en cadena de la polimerasa se empleó el *SYBR Green PCR Master Mix* (Applied Biosystems, Carlsbad, CA, EE.UU.) al que se añadieron los cebadores (los empleados en la RT-PCR convencional y los incluidos en la **tabla 2**) y 5-10 ng de cDNA. Se realizaron hasta 40 ciclos de desnaturalización a 95°C y elongación a 60°C de las cadenas de ARN, midiendo la fluorescencia tras cada elongación. Los

datos se analizaron mediante una técnica semicuantitativa descrita por Livak & Schmittgen (2001). Esta técnica se empleó en los estudios 1, 2, 3 y 4.

Tabla 2: Cebadores empleados en los distintos estudios.

Gen	Secuencia cebador directo	Secuencia cebador reverso	Estudio
MHC clase II	5´-AGG GCA TTT CGT GTA CCA GTT-3´	5´-GTA CTC CTC CCG GTT GTA GAT-3´	3
CD80	5´-GAA TTA CCT GGC ATC AAT ACG-3´	5´-CTT AAT GGT GTG GTT GCG AGT C-3´	3
IFN-γ	5'-TCA GCA ACA ACA TAA GCG TCA T-3´	5'-GAC CTC AAA CTT GGC AAT ACT CAT-3´	3
IL-17	5'-AGT GAA GGC AGC AGC GAT CAT-3´	5'-CGC CAA GGG AGT TAA AG-3´	3
Foxp3	5'-GGA GAG GCA GAG GAC ACT CAA-3'	5'-GTG GTT TCT GAA GTA GGC GAA-3'	3
IL-6	5´-AGT CAA TTC CAG AAA CCG CTA TGA-3´	5´-TAG GGA AGG CCG TGG TTG T-3´	4
IL-1β	5´-TCT GAA GCA GCT ATG GCA ACT GTT-3´	5´-CAT CTT TTG GGG TCC GTC AAC T-3´	4
TNFα	5´-GGC CTC CCT CTC ATC AGT TCT ATG-3´	5´-GTT TGC TAC GAC GTG GGC TAC A-3´	4
IL-17A	5´-AGT GAA GGC AGC AGC GAT CAT-3´	5´-CGC CAA GGG AGT TAA AG-3´	4
GAPDH	5´-GAACGTGAAGGTCGGAGTCAAC-3´	5´-CGTGAAGATGGTGATGGGATTTC-3´	1,2,3,4

MHC: Complejo mayor de histocompatibilidad; TNF: Factor de necrosis tumoral.

3.4. Tinciones histológicas

Los tejidos oculares de los ratones deficientes en TSP-1 empleados en el estudio 4 se recogieron y se fijaron con paraformaldehído tamponado al 4%. Tanto los globos oculares como las glándulas lacrimales se incluyeron en parafina y se cortaron en secciones sagitales de 5 µm. Estas secciones se tiñeron con hematoxilina y eosina (**H&E**) para su evaluación morfológica, incluyendo la medida del grosor de los epitelios y la evaluación de posibles infiltrados inflamatorios. Las secciones de los globos oculares también se tiñeron con ácido periódico de Schiff y azul alcián (**PAS/AA**) para llevar a cabo el contaje de las células caliciformes en la conjuntiva tarsal y bulbar. Además, se realizó la tinción con Rojo Sirio para evaluar el contenido en colágeno y la posible evaluación de procesos fibróticos.

4. Desarrollo y caracterización de la formulación liposomal

4.1. Desarrollo de la formulación

Las formulaciones liposomales que se emplearon en los estudios 5 y 6 se prepararon en el Departamento de Farmacia y Tecnología Farmacéutica de la Universidad Complutense de Madrid, según lo descrito previamente por Vicario-de-la-Torre y colaboradores (2014). De forma resumida, la formulación se preparó con fosfatidilcolina, colesterol y vitamina E en una proporción 8:1:0.08, respectivamente. Para su elaboración se empleó la técnica de hidratación de la película lipídica, también conocida como método de Brangham (Bangham AD *y cols.,* 1965), empleando una solución acuosa hipotónica de ácido bórico (8.38%), borato sódico (0.755%) y trehalosa (16%). En el estudio 5, se añadió el acetato de medroxiprogesterona (**MPA**) como fármaco anti-inflamatorio, o bien la cumarina-6 (**C6**), de características fisicoquímicas similares al MPA, pero fluorescente. De este modo la C6 se empleó como un fármaco modelo para el estudio de la interacción del fármaco con las células en cultivo. En el estudio 6 se añadió el péptido KRFK derivado de la TSP-1 marcado covalentemente con isotiocianato de fluoresceína (**FITC**). Tras ello, los liposomas se sonicaron y se filtraron o extrusaron, obteniéndose liposomas con tamaños homogéneos, próximos a 200 nm.

4.2. Caracterización fisicoquímica

En los estudios 5 y 6 se llevó a cabo la caracterización de algunas de las propiedades fisicoquímicas de la formulación liposomal desarrollada. Ésta incluye el tamaño de la formulación y su distribución, empleando un analizador de tamaño de partícula; el pH, empleando un pH-metro; la osmolaridad, empleando un osmómetro; y la viscosidad, empleando un reómetro.

También se determinó la eficiencia de encapsulación de la molécula terapéutica a encapsular. En el estudio 5, tras centrifugar la formulación para separar los liposomas (49,263 g, 10°C, 60 minutos), se analizaron los sobrenadantes. Se midió el MPA por cromatografía líquida de alta eficacia

(**HPLC**) o la C6 empleando el espectrofotómetro ($\lambda_{excitación}$: 490 nm; $\lambda_{emisión}$: 520 nm). En el estudio 6 se emplearon tubos Eppendorf especiales (Millipore Corporation, Billerica, MA, EE.UU.) para separar los liposomas y, con espectrofotómetro ($\lambda_{excitación}$: 490 nm; $\lambda_{emisión}$: 525 nm), se analizó la cantidad de péptido KRFK-FITC que no era encapsulado y quedaba en el sobrenadante.

5. Tratamiento de datos y estudio estadístico

En todos los estudios realizados, los datos se presentan como el valor promedio ± el error estándar de la media (**SEM**). Para determinar las diferencias estadísticamente significativas entre dos muestras se empleó la prueba *t* de Student, llevando a cabo la corrección de Welch si las varianzas de las muestras no eran homogéneas (prueba F). Para las comparaciones entre más de dos muestras se llevó a cabo un ANOVA, empleando para las comparaciones a pares el test Turkey, o el test Games-Howell si las varianzas de las muestras no eran homogéneas (test de Levene). El nivel de significación se estableció en $p \leq 0.05$. Los análisis estadísticos se llevaron a cabo con el asesoramiento de la Unidad de Estadística del IOBA (Dra. Itziar Fernández Martínez).

PRINCIPALES RESULTADOS Y DISCUSIÓN

A continuación se presenta un resumen de los resultados y la discusión de los seis estudios en los que se divide esta memoria de tesis.

ESTUDIO 1. La trombospondina-1 induce una respuesta diferente en líneas celulares epiteliales de córnea y de conjuntiva humana en condiciones de inflamación y apoptosis *in vitro*.

En este estudio se emplearon las líneas celulares epiteliales de córnea y de conjuntiva, HCE e IOBA-NHC, respectivamente. En primer lugar, se analizó la expresión de la TSP-1 y de sus receptores CD36 y CD47 en condiciones normales. La expresión de estas moléculas se comparó entre ambas líneas celulares a nivel proteico, mediante Western *blotting*, inmunofluorescencia y ELISA; y a nivel de ARNm, mediante RT-PCR. En segundo lugar, se optimizó un modelo que inducía la expresión de marcadores de inflamación y de apoptosis en ambas líneas celulares empleando BAC al 0.001%. Los marcadores de inflamación utilizados fueron la producción de la citoquina pro-inflamatoria IL-6, empleada en modelos previos de inflamación en estas líneas celulares, y del TGF-β2, por estar directamente relacionada con la TSP-1. En cuanto a los marcadores de apoptosis se empleó la caspasa-3/7 activada. En tercer lugar, se realizó el tratamiento con TSP-1 de ambas líneas celulares pre-tratadas con BAC, y se analizaron posibles cambios en los marcadores de inflamación y apoptosis, así como en los receptores de la TSP-1.

Los resultados mostraron que las células epiteliales de córnea producen más TSP-1 que las de conjuntiva, lo que se sabe ocurre también *in vivo*, probablemente debido al carácter anti-angiogénico de la TSP-1. Sin embargo, ambos receptores estudiados se expresaron igual o más en la línea de conjuntiva que en la de córnea. En condiciones normales las células corneales, a diferencia de las conjuntivales, aumentaron la expresión del CD36 y CD47, así como del TGF-β2 activo, tras el tratamiento con TSP-1. Estos resultados no se observaron

si las células habían sido previamente inflamadas. En condiciones de inflamación el CD36 disminuyó en ambas líneas celulares, aunque esta reducción no era significativa en las células de conjuntiva si se trataban con TSP-1. La TSP-1 disminuyó el TGF-β2 latente, incrementado tras el tratamiento con BAC, en las células conjuntivales pero no en las corneales.

En conclusión, este estudio demuestra que ambas líneas celulares de epitelio de córnea y de conjuntiva humanas producen distintas cantidades de TSP-1 y expresan los receptores CD36 y CD47, por lo que pueden emplearse para profundizar en el estudio de la TSP-1. Las condiciones que inducen la expresión de los marcadores de inflamación y de apoptosis empleando BAC también afectan a la expresión del CD36 en ambas líneas celulares. En estas condiciones de inflamación, el efecto más relevante tras la adición de TSP-1 exógena fue la reducción de los niveles de TGF-β2 total y latente en la línea celular de epitelio conjuntival.

ESTUDIO 2. Regulación de la expresión de la trombospondina-1 y del CD36 mediada por citoquinas inflamatorias en células conjuntivales

La hipótesis del estudio 2 fue que modelos experimentales de conjuntivitis tendrían alterados los niveles de expresión de la TSP-1, del receptor CD36 o del TGF-β2, evidenciando el papel de estas moléculas en la modulación de los procesos inflamatorios. Para probar dicha hipótesis se trabajó con 2 modelos experimentales en ratones que desarrollan inflamación conjuntival. El primero de ellos es un modelo de ojo seco inducido mediante su tratamiento con escopolamina y flujo de aire, mientras que el segundo modelo es espontáneo y aparece en ratones modificados genéticamente que, precisamente, carecen de TSP-1. Se comparó la intensidad de la inmunodetección de la TSP-1, el CD36 y el TGF-β2 total en secciones histológicas de conjuntiva de ambos modelos, empleando el programa ImageJ. También se obtuvieron cultivos primarios de conjuntiva, tanto fibroblastos como células epiteliales y, como en el estudio 1, se caracterizó y comparó la expresión de la TSP-1 y del CD36 en estos cultivos. Esta

caracterización se realizó a nivel proteico mediante técnicas de inmunofluorescencia e ELISA, y de ARNm mediante RT-PCR. Ya que los fibroblastos mostraron una mayor expresión de la TSP-1, se trataron con la citoquina inflamatoria IL-1β y se estudiaron los cambios en la expresión de las distintas moléculas de interés (la TSP-1, el CD36 y el TGF-β2) empleando las mismas técnicas de análisis molecular.

Los resultados obtenidos empleando los modelos experimentales de conjuntivitis mostraron que ambos modelos tenían niveles superiores de TGF-β2 total en la conjuntiva en comparación con los ratones control. Los ratones con una inflamación inducida en la superficie ocular mostraron niveles reducidos de CD36 en la conjuntiva, mientras que los ratones que carecen de TSP-1 y son un modelo espontáneo de inflamación, tienen la expresión de este receptor aumentada. Por otra parte, los fibroblastos conjuntivales tratados con IL-1β disminuyeron la expresión de la TSP-1 y del CD36 mostrando valores de TGF-β2 activado por debajo del límite de detección.

En conclusión, los análisis realizados en los distintos modelos experimentales de inflamación muestran que los procesos inflamatorios provocan cambios en los niveles de la TSP-1 y/o del CD36. Además estos cambios están acompañados por un incremento de los niveles de TGF-β2 total, sin detectar TGF-β2 activado. Esto corrobora la importancia de la activación del TGF-β2 llevada a cabo por la TSP-1 y el CD36 para el mantenimiento de la homeostasis de la mucosa conjuntival.

ESTUDIO 3. El péptido KRFK derivado de la trombospondina-1 modula el fenotipo de las células dendríticas *in vitro* e *in vivo.*

En este estudio se empleó el péptido KRFK, derivado de la secuencia peptídica de la TSP-1 y que activa el TGF-β, para estudiar si esta función de la TSP-1 puede alterar el fenotipo de las células dendríticas (DCs), y las posibles consecuencias de esta función *in vivo.* Para ello, se evaluó la funcionalidad *in vitro* del péptido, empleando DCs deficientes en TSP-1. Posteriormente se

analizó si este péptido aplicado de modo tópico ocular puede atravesar el epitelio conjuntival, alterando el desequilibrio periférico en las poblaciones de linfocitos T inflamatorios de estos ratones.

Con este objetivo, se sintetizó el péptido KRFK y un péptido control inactivo (KQFK). Se aislaron y cultivaron DCs de la médula ósea de ratón, y se trataron con ambos péptidos. Los resultados mostraron que el péptido KRFK era funcional *in vitro* ya que activó el TGF-β latente producido por las DCs deficientes en TSP-1 y disminuyó los niveles de ciertos marcadores de maduración de estas células, como el MHC de clase II y el CD80.

Aunque el péptido ya había sido probado *in vivo*, en ninguno de los trabajos publicados se había aplicado de manera tópica ocular. Por ello, el péptido marcado con FITC se empleó en un modelo *in vitro* de conjuntiva humana para determinar su penetración a través del epitelio conjuntival. Además, basados en previos estudios empleando péptidos de manera tópica, se aplicaron 5µL/ojo a una concentración de 1 µg/mL de manera tópica en el ojo de ratones control para determinar su localización tras 1 y 3 horas de su administración. Los resultados mostraron que el péptido podía atravesar la barrera epitelial de la conjuntiva de manera constante. Además, a pesar de las conocidas barreras biofarmacéuticas de la aplicación tópica, tras 1 hora de la instilación del péptido se localizaban acúmulos del mismo en distintas estructuras de la superficie ocular. Estos resultados indicaban que el péptido podía ser un buen candidato para tratar la inflamación de la superficie ocular.

En la inflamación que desarrolla el modelo de ratón deficiente en TSP-1, como ocurre en las patologías inflamatorias de la superficie ocular, tras la activación de las DCs oculares, éstas migran a los nódulos linfáticos locales, los cervicales, donde presentan el antígeno generando linfocitos T activados inflamatorios. Por ello estos ratones, junto con los signos inflamatorios de la superficie ocular, muestras un desequilibrio periférico entre los linfocitos T inflamatorios y reguladores. Tras los resultados obtenidos *in vitro*, se aplicó el péptido KRFK en ratones deficientes en TSP-1 para analizar si podía alterar la

respuesta inflamatoria sistémica. Se analizaron las poblaciones Th1 (CD4+ IFN-g+), Th17 (CD4+ IL-17+) y los Tregs (CD4+ Foxp3+) de los nódulos linfáticos cervicales mediante citometría de flujo y RT-PCR a tiempo real. Estos análisis mostraron que el péptido derivado de la TSP-1 disminuye las poblaciones de linfocitos efectores Th1 y Th17 aumentando la de los reguladores Treg, lo que es consistente con el efecto del péptido observado *in vitro* en las DCs deficientes en TSP-1.

En conclusión, este trabajo muestra que el péptido KRFK, derivado de la TSP-1, modula el fenotipo de las DCs hacia un estado inmaduro y tolerogénico *in vitro*. Estos efectos también se detectan *in vivo*, ya que el péptido aplicado tópicamente atraviesa el epitelio conjuntival e inhibe el desarrollo de linfocitos T efectores y promueve el de los linfocitos T reguladores en los nódulos linfáticos cervicales. Estos resultados demuestran el potencial de la aplicación tópica del péptido KRFK derivado de la TSP-1 que, alterando el fenotipo de las DC y la respuesta inmune sistémica, podría aliviar los signos inflamatorios asociados en la superficie ocular.

ESTUDIO 4. El péptido KRFK previene la aparición de signos inflamatorios en el modelo experimental de inflamación crónica de la superficie ocular deficiente en trombospondina-1.

El objetivo de este estudio fue analizar cambios en los signos inflamatorios de la superficie ocular en ratones deficientes de TSP-1 tras instilar el péptido KRFK empleado en el estudio 3. Estos ratones, además de mostrar un desequilibrio en las poblaciones de linfocitos T, desarrollan una inflamación bien caracterizada a las 12 semanas de edad en las distintas estructuras de la superficie ocular.

Se realizaron dos aproximaciones para estudiar el potencial terapéutico del péptido: un estudio de prevención y otro de tratamiento. En el estudio de prevención se trataron ratones que todavía no tenían signos clínicos, de 8 semanas de edad, para analizar si el péptido prevenía la aparición de los signos inflamatorios que se observan a las 12 semanas de edad. En el estudio de

tratamiento se emplearon ratones con una superficie ocular inflamada, de 16 semanas de edad, para analizar si este péptido reducía o limitaba los signos inflamatorios presentes. En ambas aproximaciones se instilaron 10 μg/ratón diariamente, del péptido derivado de la TSP-1 y del péptido control inactivo, durante 2 semanas. Antes de comenzar y al finalizar el tratamiento se recogió lágrima para medir niveles de MUC5AC y se midió la tinción corneal en los animales para el seguimiento del desarrollo de la patología ocular. Entre los signos inflamatorios evaluados además se incluyeron los niveles de citoquinas inflamatorias en la conjuntiva y en la glándula lagrimal junto con un estudio histopatológico de estos tejidos. También se evaluó la presencia de posibles signos de fibrosis, un proceso regulado por el TGF-β y que, por lo tanto, podría ser un efecto secundario no deseado del tratamiento con el péptido.

En el estudio de prevención, los resultados mostraron que el péptido derivado de la TSP-1 previene el aumento del defecto epitelial corneal y la disminución en el nivel de MUC5AC en la lágrima. Además, el péptido disminuyó la expresión de citoquinas inflamatorias como la IL-6, el TNFα o la IL-1β en la conjuntiva y en la glándula lagrimal, estructuras que no mostraban alteraciones morfológicas. Por otra parte, en el estudio de tratamiento, la administración del péptido aumentó significativamente los niveles de MUC5AC en lágrima. Sin embargo, no se observaron otros efectos anti-inflamatorios en el resto de tejidos evaluados. En ninguno de los estudios se observaron indicios de procesos fibróticos derivados del tratamiento con el péptido.

Este trabajo demuestra que el péptido KRFK, derivado de la TSP-1 y que activa el TGF-β, es eficaz en la prevención de los signos clínicos inflamatorios que los ratones deficientes en TSP-1 desarrollan con la edad, sin observar signos acompañantes de fibrosis. Este péptido podría ser una alternativa terapéutica para el tratamiento de la inflamación crónica ocular en sujetos con niveles de TSP-1 reducidos.

ESTUDIO 5. Una nueva formulación liposomal anti-inflamatoria para la película lagrimal: estudios de funcionalidad *in vitro* y *ex vivo* en células epiteliales corneales.

En este estudio se empleó una formulación liposomal con características similares a la película lagrimal desarrollada por el Departamento de Farmacia y Tecnología Farmacéutica de la Universidad Complutense de Madrid. La hipótesis inicial fue que esta formulación se puede emplear como vehículo para moléculas anti-inflamatorias adicionando así una función terapéutica a la misma.

Para probar esta hipótesis se encapsuló como fármaco anti-inflamatorio modelo el acetato de medroxiprogesterona (**MPA**) y para el estudio de la interacción celular la cumarina-6 (**C6**) fluorescente. Ambas formulaciones fueron caracterizadas fisicoquímicamente, mostrando unas propiedades adecuadas para ser empleadas como colirios. Además se emplearon como controles la formulación liposomal sin fármaco y una formulación comercial de MPA que no incluía ningún vehículo. Se evaluó la interacción y la funcionalidad de las formulaciones tras 1 hora de contacto con la línea celular HCE. También se empleó un modelo *ex vivo* porcino para estudiar la penetración del fármaco vehiculizado a través del epitelio corneal tras 5 y 60 minutos de contacto con la formulación. Tras 60 minutos de tiempo de contacto la viabilidad celular de las células HCE no se veía afectada e interaccionaban con los liposomas. El modelo *ex vivo* empleado mostró cómo la C6 encapsulada en estos liposomas penetraba en todas las capas del epitelio corneal tras 5 minutos de contacto.

Para determinar si el fármaco vehiculizado en esta formulación permanecía funcional en las células diana, se estudiaron cambios en la expresión de los receptores que mayor afinidad tienen por el MPA: el receptor de progesterona y el de glucocorticoides. Se estudiaron los cambios tanto en la expresión proteica, mediante Western *blotting*, como en la localización del receptor como medida de su activación, mediante inmunofluorescencia. Los resultados obtenidos mostraron que el receptor de progesterona se activaba e incrementaba su expresión tras el tratamiento con la formulación que encapsulaba el MPA. Sin

embargo, ninguno de estos cambios se observó con el resto de formulaciones estudiadas.

Para finalizar, se estudiaron cambios funcionales en las células corneales como efecto del tratamiento con las formulaciones. Se estudiaron cambios tanto en la proliferación celular, mediante el reactivo alamarBlue®, como en la producción de IL-6 e IL-8 tras haber "inflamado" *in vitro* las células con la citoquina inflamatoria TNFα, mediante ELISA. Ambos procesos, la proliferación y la producción de citoquinas, se reducen tras el tratamiento con corticoides como el MPA en cultivos *in vitro*. La formulación liposomal que vehiculizaba el MPA disminuyó la proliferación de las células corneales tras 48 horas del tiempo de contacto. Tras este tiempo la formulación liposomal con MPA también disminuyó la producción de ambas citoquinas inflamatorias. Sin embargo, la formulación comercial de MPA que no incluye el vehículo liposomal solo disminuyó la producción de IL-6 de manera significativa.

En conclusión, tras el tratamiento de las células corneales con la formulación con MPA se observa la incorporación de la formulación a las células, la activación del receptor de progesterona y una disminución en la producción de marcadores de inflamación en estas células. Además estos efectos observados *in vitro* son mayores si el MPA está vehiculizado en la formulación liposomal frente al MPA sin vehículo. Por lo tanto, la formulación preparada puede ser empleada como un sistema eficaz de encapsulación para moléculas anti-inflamatorias para mejorar la terapia tópica ocular.

ESTUDIO 6. Mejora de la penetración corneal *in vitro* de un péptido derivado de la trombospondina-1 tras su encapsulación en una formulación liposomal.

Este trabajo se llevó a cabo como una prueba de concepto para evaluar si el péptido KRFK derivado de la TSP-1 puede ser encapsulado en el sistema liposomal empleado en el estudio 5, mejorando la penetración del péptido en distintos epitelios de la superficie ocular.

Para ello se empleó el péptido KRFK marcado covalentemente con FITC. El Departamento de Farmacia y Tecnología Farmacéutica de la Universidad Complutense de Madrid elaboró la formulación liposomal y la liofilizó. Se reconstituyó con el péptido para facilitar su encapsulación. Se emplearon distintos tiempos de sonicación en la preparación de la formulación para estudiar la influencia que este parámetro puede tener en eficacia de encapsulación (**EE**) del péptido. La formulación con mayor EE (≈90%) se caracterizó mostrando un tamaño de 230 nm y un pH y una osmolaridad adecuados. Además, se comprobó la biocompatibilidad de la nueva formulación tras 1 hora de contacto con las células HCE.

Tras ello se analizó la penetración del péptido vehiculizado en la formulación liposomal y se comparó con la del péptido desnudo, empleando distintos modelos experimentales. Se estudió la penetración *in vitro* a través del epitelio conjuntival empleando insertos con células epiteliales conjuntivales primarias y la penetración a través del epitelio corneal empleando córneas porcinas *ex vivo.* Los resultados mostraron que el vehículo no mejoraba significativamente la penetración del péptido en el epitelio conjuntival pero sí en el epitelio corneal, con el que parece interaccionar incrementando su biodisponibilidad y mejorando su penetración.

En conclusión, este estudio proporciona la prueba de concepto de que el péptido KRFK, derivado de la TSP-1, puede ser vehiculizado en la formulación liposomal con características de película lagrimal. Tras su aplicación tópica, los resultados obtenidos sugieren que esta formulación protegerá el péptido de su degradación e incrementará el tiempo de retención del mismo en el epitelio corneal mejorando su efecto terapéutico.

CONCLUSIONES

Tras la realización de este trabajo, se presentan las siguientes conclusiones:

1. Se ha demostrado la expresión diferencial de la TSP-1 y de sus receptores CD36 y/o CD47 entre las líneas celulares de epitelio de córnea y de conjuntiva humana y entre cultivos primarios de células del epitelio y del estroma conjuntival. Esto evidencia las diferencias en la expresión de la TSP-1 entre los distintos tipos celulares oculares *in vitro* e indica que estos tipos celulares pueden ser empleados para profundizar en el estudio de los mecanismos moleculares de la TSP-1.
2. La inducción en modelos experimentales de procesos inflamatorios de la supericie ocular afecta a la expresión de las moléculas implicadas en la activación del TGF-β2 dependiente de la TSP-1. Esto indica la importancia de esta función de la TSP-1 para el mantenimiento de la inmunoregulación de la superficie ocular.
3. Se ha demostrado que el péptido KRFK, derivado de la TSP-1 y que activa el TGF-β, penetra a través de células del epitelio conjuntival e induce un fenotipo tolerogénico en células dendríticas deficientes en TSP-1 *in vitro.* Esto demuestra el potencial inmunosupresor del péptido para la terapia tópica ocular.
4. La administración tópica del péptido KRFK en ratones deficientes en TSP-1 altera las poblaciones de linfocitos T en los nódulos linfáticos locales, disminuyendo los linfocitos T efectores y aumentando los T reguladores. Además, el péptido previene la aparición de los signos inflamatorios que estos ratones desarrollan con la edad en la córnea, la conjuntiva y la glándula lagrimal. Este péptido se podría aplicar de forma eficiente para prevenir la inflamación ocular inducida por niveles reducidos en la expresión de la TSP-1.
5. Se ha desarrollado y caracterizado una nueva formulación liposomal que encapsula de modo eficiente moléculas anti-inflamatorias como el acetato de medroxiprogesterona o el péptido KRFK derivado de la TSP-1, manteniendo las propiedades fisicoquímicas adecuadas para los colirios oculares. Además,

esta formulación mejora la penetración de la molécula activa en tejidos con gran impermeabilidad como la córnea, sin causar alteraciones.

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ENGLISH SUMMARY

ABSTRACT

Inflammatory diseases of the ocular surface are important because they threaten vision and reduce patient quality of life. Furthermore, the prevalence of inflammatory diseases is increasing in the aging population. The multiple etiologies of ocular surface inflammation involve different humoral and cellular factors. Some of these factors are not effectively targeted by generic therapeutic treatments, thus causing failures in effectiveness. The pathophysiological study of inflammatory diseases could help to find novel therapeutic targets. Personalized treatments that target specific molecules would offer a more effective therapeutic solution.

Recently, a genetic polymorphism that causes reduced expression of thrombospondin-1 (**TSP-1**) has been associated with a susceptibility to develop chronic ocular surface inflammation after a refractive surgery. This polymorphism could be used as a genetic marker for the early diagnosis of ocular inflammation, allowing preventive treatments to avoid the chronic condition. Therefore, the main objective of this thesis was the development of a novel alternative therapy to prevent or treat chronic inflammation of the ocular surface associated with decreased TSP-1 expression.

We first studied the role of TSP-1 in inflammatory experimental conditions using human corneal and conjunctival epithelial cell lines, primary cell cultures of human conjunctiva, and *in vivo* mouse disease models. These studies aimed to associate the levels of TSP-1 and related molecules, such as the TSP-1 receptor CD36, with transforming growth factor (**TGF**)-β2 activation. The isoform TGF-β2 was selected as is the predominant isoform in ocular surface tissues and TSP-1 has been proposed as its main activator. The methodologies included molecular analysis techniques such as Western blotting, immunofluorescence, enzyme-linked immunosorbent assay (**ELISA**), and retrotranscription polymerase chain reaction (**RT-PCR**). The results showed that reduced expression of either TSP-1 or CD36, both of which are activators of TGF-β2, is determined by the experimental

inflammatory conditions. This supports the important roles for these three molecules in ocular surface inflammatory processes.

Based on this information, the peptide KRFK derivative of TSP-1, which can activate all isoforms of TGF-β, was selected as a potential therapeutic molecule for immune-based ocular pathologies. To assess the possible therapeutic efficacy of topically administered TSP-1-derived peptide KRFK, we used TSP-1-deficient mice, a model of chronic ocular surface inflammation. The results showed that KRFK prevented the development of inflammatory symptoms in the ocular surface by modulating the dendritic cell phenotype.

Because topical and local administration is the preferred route for the delivery of ocular drugs, the development of a system to encapsulate and protect the TSP-1-derived peptide KRFK was proposed. A liposomal formulation constructed with natural components of the tear film was evaluated as a possible delivery system. Prior to the peptide encapsulation, the functionality of the formulation was tested with the anti-inflammatory drug medroxyprogesterone acetate. The results showed uptake of the drug by the corneal epithelial cells, changes in drug-binding cellular receptors, and *in vitro* anti-inflammatory effects.

Based on the preceding studies that documented the roles of TSP-1, TSP-1-derived peptide KRFK, CD36, and TFG-β2 in regulating ocular surface inflammation, and based on the successful development and use of the novel liposomal formulation as a drug delivery system, we then determined if KRFK can be loaded in the liposomal formulation and its delivery improved to ocular surface structures. We found that KRFK was efficiently encapsulated in the liposomal formulation and the delivery to the corneal epithelium improved. This novel system may be of great interest for treating ocular surface inflammatory pathologies induced by decreased TSP-1 expression.

THESIS REPORT STRUCTURE

This Doctoral Thesis report is presented as a "compendium of publications" and applies for the International-awarded Doctoral Thesis Degree. Consequently, this thesis has been organized to accomplish the regulations of the International Doctorate Committee at the University of Valladolid, Valladolid, Spain. One of these regulations is that a Spanish summary containing the objectives, methodology, and the main results and conclusions should be included if the thesis is written mainly in a foreign language. Also, the summaries in Spanish and English include an introduction to justify the thematic unit of the work and to facilitate its understanding. In this thesis, six different studies are included. Five are original research articles, three of which (Studies 1, 2, and 5) are published papers in scientific journals within the Journal of Citation Report, fulfilling the requirements of the "compendium of publications". All studies were performed in collaboration with different national and international research centers. Particularly, some of the experiments from Studies 2, 3, and 4 were performed during my stay at Boston University (Boston, MA, USA). I have been at Boston University twice, for 2 months each time, fulfilling the requirements to apply for the International-awarded Doctoral Thesis Degree.

The organization of the thesis is defined by its main objective: the development of a novel therapeutic formulation for the treatment of a specific form of chronic ocular surface inflammation. To satisfy this aim, we studied not only the causes of this chronic inflammation, but also a delivery system for the topical ocular administration of a novel therapy. Therefore, the thesis has been organized additively as depicted in **Figure 1**.

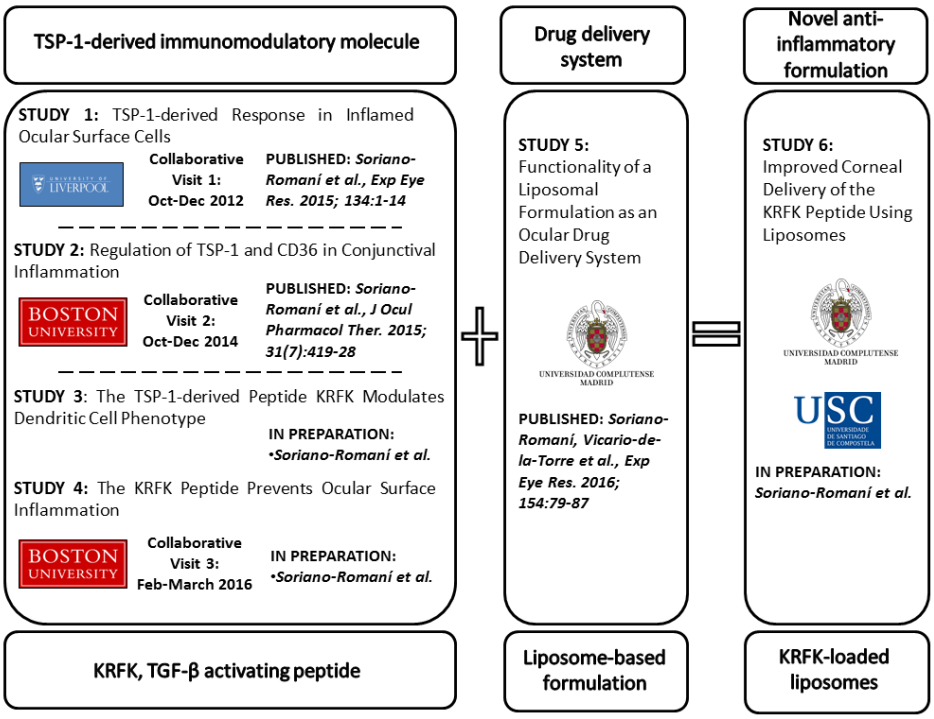

Figure 1. Thesis organization with the six studies.

To identify possible therapeutic targets of ocular surface inflammation, we decided to focus the work on the role of thrombospondin-1 (**TSP-1**). Therefore, the main part of the thesis investigates TSP-1 as a key player of ocular surface immunomodulation (Studies 1, 2, 3, and 4). In **Study 1,** the *in vitro* response to TSP-1 treatment of inflamed corneal and conjunctival epithelial cell lines was analyzed. For that, changes in the expression of inflammatory and apoptotic markers, as well as in the TSP-1 receptors CD36 and CD47, were assessed. These results were published together in ***Thrombospondin-1 Induces Differential Response in Human Corneal and Conjunctival Epithelial Cell Lines Under In vitro Inflammatory and Apoptotic Conditions*** (Soriano-Romaní *et al*., Experimental Eye Research. 2015 May; 134: 1-14). This work showed that TSP-1 treatment downregulated the increased levels of total TGF-β2 in the "inflamed" conjunctival cells and the possible involvement of CD36 in this function. Afterwards, **Study 2** analyzed changes in TSP-1 and CD36 expression, both of which are TGF-β2-activating molecules, under conjunctival inflammatory

conditions. Two experimental mouse models of conjunctivitis and primary epithelial and stromal conjunctival cells were used in this study. Study 2 was published as ***Inflammatory Cytokine-Mediated Regulation of Thrombospondin-1 and CD36 in Conjunctival Cells*** (Soriano-Romani *et al*., Journal of Ocular Pharmacology and Therapeutics. 2015 Sep; 31 (7): 419-28). In this work, the absence or reduced expression of either of the molecules involved in TGF-β2 activation supported their roles in the conjunctival pro-inflammatory conditions. After these results, the TGF-β-activating peptide KRFK, a TSP-1-derived peptide, was selected as a possible molecule with therapeutic potential for chronic ocular surface inflammation associated with decreased TSP-1 expression. To assess its therapeutic potential, TSP-1-deficient mice were used as an experimental model of chronic ocular surface inflammation. **Study 3** demonstrated the *in vitro* functionality of the TSP-1-derived peptide KRFK, its penetration through the conjunctival epithelium, as well as its *in vivo* efficacy to modulate the imbalanced T cell populations in the local lymph nodes observed in these mice. **This study is currently in preparation for publication**. After these findings, the effect of topically administered TSP-1-derived peptide in ocular surface tissues was analyzed in **Study 4**. The effect of topically administered TSP-1-derived peptide KRFK was assessed before and after the disease onset in these mice, in prevention and treatment studies, respectively. Inflammatory symptoms in the cornea, conjunctiva, and the main lacrimal gland after the administration of the TSP-1-derived peptide in both studies were evaluated. We concluded that topically administered TSP-1-derived peptide prevents inflammatory manifestations in the ocular surface. **This study is currently in preparation for publication**. From these four studies, we concluded that the TSP-1-derived peptide KRFK may have therapeutic potential to prevent chronic ocular surface inflammation in patients with decreased TSP-1 levels.

In parallel with the first four studies that explored TSP-1 and related molecules in chronic ocular surface inflammatory disease, the second part of the thesis was started with the aim of finding a suitable delivery system for the

peptide selected. In **Study 5,** the functionality of a liposomal formulation as an anti-inflammatory drug release system was analyzed. The proof of concept was performed by loading medroxyprogesterone acetate, an anti-inflammatory drug used in clinical practice as a "soft" corticoid, in the liposomal formulation. This study was published as ***Novel Anti-inflammatory Liposomal Formulation for the Pre-ocular Tear Film: In vitro and Ex vivo Functionality Studies in Corneal Epithelial Cells*** (Soriano-Romaní *et al*., Experimental Eye Research. 2017 Jan; 154: 79-87). In this study, *in vitro* and *ex vivo* models were studied to analyze the functionality and penetration of the encapsulated drug in corneal cells. We concluded that this formulation could be a useful delivery system to protect the TSP-1-derived peptide after topical ocular administration.

Therefore in **Study 6,** a protocol for TSP-1-dervied peptide encapsulation in the liposomal formulation was designed. Additionally, penetration was assessed in different *in vitro* models of the ocular surface. The results suggested that this liposomal formulation loaded with the TSP-1-derived peptide KRFK could be an improved delivery system to achieve therapeutic levels of the peptide when applied topically to the eye. **This study has been submitted as short communication to Experimental Eye Research.**

INTRODUCTION

1. The ocular surface and the lacrimal functional unit

The ocular surface is the anterior and outermost part of the eye, and it includes the tear film, cornea, limbus, conjunctiva, Meibomian glands, and the main and accessory lacrimal glands. The integrity of all components is essential to proper vision. The integrity of the cornea is of special interest because it is localized on the optical axis and must be transparent to allow the refraction and transmission of the light. The ocular surface is part of the lacrimal functional unit as defined by Stern *et al*. (1998). This pathophysiological functional unit also includes the network of communication and regulation involving not only the nervous but also the hormonal, vascular, and immune systems (**Figure 2**). All components of the functional unit are interrelated to maintain the homeostasis of the ocular surface.

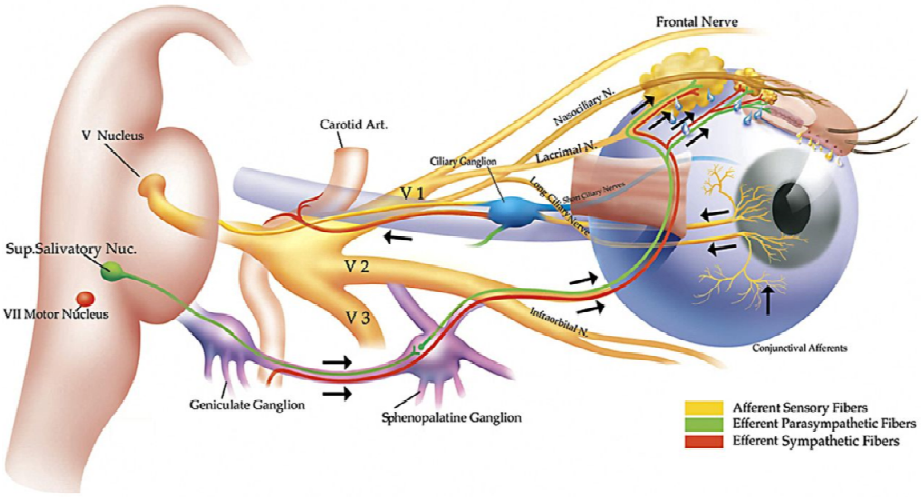

Figure 2. Integrated lacrimal functional unit. Image taken from Pflugfelder, Beuerman, and Stern. Marchel Dekker, Inc., New York, 2004, 11–39.

1.1. Inflammation of the lacrimal functional unit

If any of the components of the lacrimal functional unit is compromised, the ocular surface homeostasis could be lost and local inflammation developed. If this inflammation is not resolved, it can lead to a chronic inflammation (Stern *et*

al., 2004). The hallmark of chronic ocular surface inflammatory pathologies is dry eye disease (**DED**). The primary effects are loss of visual acuity and quality of life and may be accompanied by chronic pain. This example illustrates the importance of these inflammatory diseases in the field of ophthalmology because it affects 5% to 35% of the population and the prevalence increases with age (Bron *et al*., 2014; Paulsen *et al*., 2014). Currently, there are no effective treatments to achieve a complete functional recovery of the damaged tissues in these patients.

The ocular surface components involved in inflammatory responses include (i) epithelial protection barriers (Mantelli *et al*., 2013), (ii) immune cells present in the epithelium and corneolimbal stroma (Hamrah *et al*., 2003), and (iii) conjunctiva-associated lymphoid tissue (**CALT**) that has all the components for a complete immune response (Knop & Knop, 2000). Usually, the outermost cells of the ocular surface, the epithelial cells, are the first element to respond to external inflammatory stimuli. The initial response elicits the presence of molecules that will in turn modulate the subsequent inflammatory response (Stevenson *et al*., 2012).

Regardless of the triggering stimulus, an acute inflammatory reaction takes place within the first 24-48 hours. This initial response results in the infiltration of polymorphonuclear lymphocytes at the inflamed area. These lymphocytes are the characteristic cell type in this inflamed state although natural killer cells, macrophages, or even T cells can also be found (McDermott *et al*., 2005).

Acute inflammation ends when the initial stimulus is removed or the inflammatory mediators are inactivated. However, if the stimulus is not removed and/or the inflammatory cells or soluble mediators persist, a late inflammatory response occurs. In this case, the activated T cells and the antibodies secreted by B cells are the main players in the adaptive immune response. These cells and molecular mediators are specialized cellular and humoral factors to fight and eliminate the initial stimulus. These responses begin with the activation of macrophages and dendritic cells in the affected area. Both cell types become

differentiated to mature antigen-presenting cells (**APCs**) as a result of the inflammatory environment in the area. These APCs process the initiating antigen, migrate to the local lymph nodes and spleen, and present the antigen to immature T cells for differentiation into CD4+ T effector, helper (**Th**), or regulatory T cells (**Treg**) (**Figure 3**). In turn, the newly differentiated cells migrate to the inflamed area and regulate the following immune response.

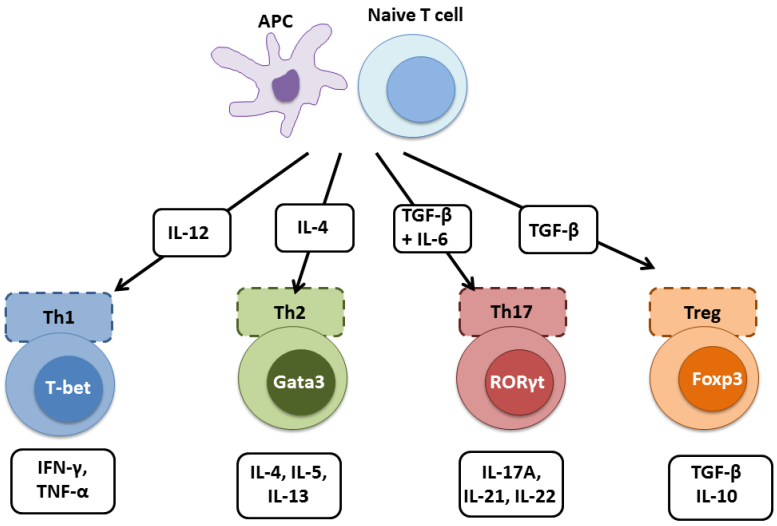

Figure 3. Differentiation of the main CD4+ T cell subtypes after interaction of naive T cells with APCs. The different subtypes of mature effector or regulatory T cells are characterized by the expression of specific transcription factors (T-bet, Gata3, ROR or Foxp3) and the secretion of certain cytokines. Adapted from Lora and Macpherson, *Nature Reviews Immunology,* 2010.

Many of the clinical manifestations of ocular surface disease are common among patients with immune-based pathologies. For instance, the levels of certain inflammatory cytokines, such as interleukin (**IL**)-6, IL-8, or IL-1β, are increased in the tears of DED patients (Massingale *et al.*, 2009; Boehm *et al.*, 2011; Na *et al.*, 2012; Contreras-Ruiz *et al.*, 2014; Yoon *et al.*, 2007; Lam *et al.*, 2009). It is also possible to distinguish specific inflammatory molecules defined by the initial stimulus.

This initial stimulus can include environmental and genetic factors. Some of the environmental factors include the use of contact lenses, allergy or infection,

and ocular surgery, among others. Recently, a genetic marker has been proposed that could identify patients with higher susceptibility to develop chronic ocular surface inflammation. The marker, a single nucleotide polymorphism (**SNP**) in the human thrombospondin (**TSP**)-1 gene (***THBS1***), was identified in patients undergoing refractive surgery (Contreras-Ruiz *et al*., 2014). This surgery induces damage in the corneal nerves and generates local inflammation, which usually is resolved after a few days. However, subjects with this SNP are not able to resolve this local inflammation and develop a chronic pathology. The SNP decreases the expression of TSP-1 because it affects the promoter region of the gene. This genetic factor could be used for the early diagnosis of these subjects, thus allowing the prevention of the chronic condition through specific treatments.

2. Thrombospondin-1 (TSP-1)

TSP-1 is a member of the structurally interrelated thrombospondin protein family (Adams & Lawler, 2004). It is a 450 kDa matricellular glycoprotein, composed of 3 monomers of 150 kDa each. It modulates cellular functions such as cell migration, proliferation, and apoptosis as it facilitates cell-cell or extracellular cell-matrix interactions (Chen *et al*., 2000). **Figure 4** illustrates the TSP-1 structure with its different domains, the cellular receptors, and ligands to which each domain binds, and the main functions.

Each monomer consists of different domains between the carboxyl- (C-) and amino- (N-) terminal globular domains. These domains include a procollagen homologous domain, also called von Willebrand type C domain, and three types (type I, II, and III) of repeated sequences. The type I repeats (**TSR**) include regions that bind to the receptor CD36 and latent transforming growth factor (TGF)-β. In addition, TSR repeats bind other extracellular matrix proteins, including collagen, fibronectin, laminin, heparin, elastase, and some matrix metalloproteases. The type III domain contains 13 contiguous calcium binding sites to stabilize the globular structure of the molecule. These sites are also distributed throughout

the TSP-1 structure, in the type II and C-terminal domains. The presence of an RGD sequence within type III repeats allows binding to β3 integrins, whereas β1 integrin binding sites are distributed between the type I, type II, and N-terminal domains. Additionally, the C-terminus binds integrin-associated protein (CD47).

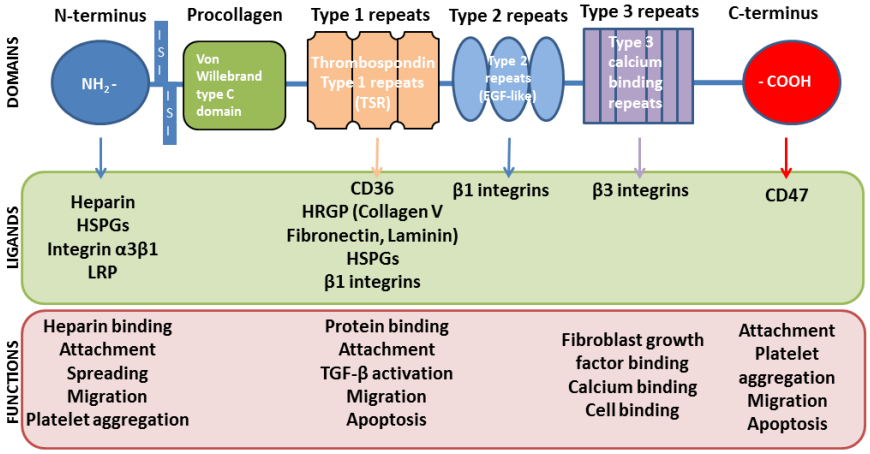

Figure 4. Structure of the TSP-1 monomer. HSPGs, heparan sulfate proteoglycans; HRGP, histidine-rich glycoprotein; LRP, lipoprotein receptor related protein.

2.1. Location of TSP-1 on the ocular surface

TSP-1 is located in different ocular structures. Specifically, the messenger RNA (**mRNA**) and the protein have been detected in the human corneal epithelium, stroma, and endothelium (Sekiyama *et al.*, 2006). However, the localization of TSP-1 in the human conjunctiva is not so clear. Depending on the technique performed, most authors do not find TSP-1 expression in the conjunctiva (Sekiyama *et al.*, 2006; Chen *et al.*, 2012), but when it is present, the expression is mild and focal (Aspiotis *et al.*, 2007). A recent study performed in mice localized TSP-1 in the conjunctival epithelium, highlighting its relevance for ocular mucosa homeostasis (Contreras-Ruiz & Masli, 2015).

2.2. Main functions of TSP-1 in the ocular surface

The main functions of TSP-1 in the ocular surface described so far are the inhibition of corneal angiogenesis, promotion of corneal wound healing, and

ocular mucosal immunomodulation (Schöllhorn *et al*., 2015; Hiscott *et al*., 2006; Masli *et al*., 2014).

The first function attributed to TSP-1 is the inhibition of angiogenesis (Good *et al*., 1990) that is mediated through a direct effect on endothelial cell migration and survival (Jimenez *et al*., 2000). In the cornea, TSP-1 inhibits the growth of both blood and lymphatic vessels. However, TSP-1-deficient mice do not show spontaneous corneal blood vessel growth, indicating that corneal avascularity is redundantly regulated (Cursiefen *et al*., 2004).

The involvement of TSP-1 in wound healing of avascular tissues, like the cornea, has also been described. To study this role, different penetrating keratotomy techniques have been performed in murine models. In these experimental models, the production of TSP-1 clearly increases after injury (Uno *et al*., 2004; Matsuba *et al*., 2011; Blanco-Mezquita *et al*., 2013). In addition, if TSP-1 expression is blocked, the wound closure time increases or the wound never heals in most penetrating keratotomies (Blanco-Mezquita *et al*., 2013; Uno *et al*., 2004). The induction of TSP-1 expression in the area helps to activate the surrounding TGF-β, which is the key player in the differentiation of keratocytes to myofibroblasts (Nor *et al*., 2005). Myofibroblasts are a more specialized cell type, with great migration capacity that regulates the healing process. In corneal wounds, myofibroblasts co-localize with TSP-1 expression, indicating the functional importance as a TGF-β activator (Blanco-Mezquita *et al*., 2013; Matsuba *et al*., 2011).

However, among the described functions of TSP-1, the immunomodulatory function seems to be the most relevant for the ocular surface. One of the supportive observations is that TSP-1-deficient mice spontaneously develop an ocular surface immunopathogenesis (Turpie *et al*., 2009). Additionally, a study performed with human subjects demonstrates a susceptibility to develop chronic ocular inflammation after surgery in subjects with reduced TSP-1 levels (Contreras-Ruiz *et al*., 2014). TSP-1 binds to CD47 on dendritic cells, which induces a tolerogenic phenotype (Grimbert *et al*., 2006; Doyen *et al*., 2003).

Moreover, TSP-1 binding to CD47 reverses ocular surface inflammatory signs in TSP-1-deficient mice by promoting Treg induction and inhibiting Th17 development (Contreras-Ruiz *et al*., 2017). This study confirms the TSP-1 role in ocular mucosal homeostasis by the regulation of the systemic immune response.

TSP-1 also modulates ocular the APC phenotype through activation of the TGF-β2 isoform (Masli *et al*., 2006; Mir *et al*., 2015). Without TSP-1, APCs exposed to latent TGF-β2 do not suppress the Th1-mediated inflammatory response (Masli *et al*., 2006). The TGF-β2 isoform is the predominant isoform in the ocular mucosa (Contreras-Ruiz & Masli, 2015), as it is in other mucosal surfaces (Chu *et al*., 2004; Maheshwari *et al*., 2011). Moreover, levels of TGF-β2 are upregulated in DED patients (Benito *et al*., 2013). The different TGF-β isoforms are secreted as a complex with a latency-associated peptide (**LAP**). This complex must be dissociated to release the active and mature TGF-β molecule. Integrins, which activate latent TGF-β on most mucosal surfaces, do not activate the TGF-β2 isoform due to the absence of the integrin-binding RGD sequence in the LAP2 of this isoform (Munger *et al*., 1999). However, TSP-1 mediates the activation of any TGF-β isoform through a specific sequence located in the type I repeats of its structure (Schultz-Cherry *et al*., 1994). These interactions demonstrate the importance of integrin-independent TGF-β activation in the ocular mucosa.

It has recently been proposed that conjunctival goblet cells participate in ocular mucosal immunomodulation by activating TGF-β2 in a TSP-1- and CD36-dependent manner (**Figure 5**) (Contreras-Ruiz & Masli, 2015). Although TGF-β2 can be extracellularly activated, CD36 may also be involved (Yehualaeshet *et al*., 1999). Therefore, TSP-1, the transmembrane receptor CD36, and the cytokine TGF-β2 have an immunomodulatory function in maintaining mucosal immune homeostasis at the ocular surface.

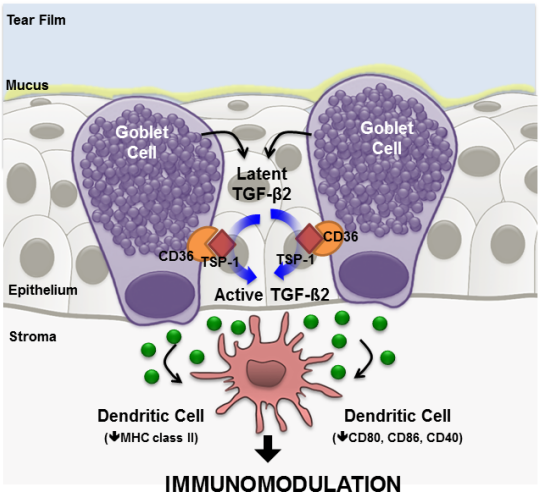

Figure 5. Schematic of the immunomodulatory function of conjunctival goblet cells through TGF-β2 activation. Adapted from Contreras-Ruiz *et al*., PLoS One, 2015.

2.3. TSP-1-deficient mice: a model of chronic ocular surface inflammation

Mice deficient in TSP-1 expression (B6.129S2-Thbs1tm1Hyn/J) are homozygous for this targeted mutation of the *THBS1* gene (https://www.jax.org/strain/006141). These mice show low levels of abnormal TSP-1 mRNA in multiple tissues; however, the TSP-1 protein is not detected. In general, TSP-1-deficient mice exhibit increased numbers of circulating white blood cells and considerable hyperplasia of various epithelial cell lineages. These mice have been used to study inflammatory responses in the lungs, eyes, and skin, among others.

TSP-1-deficient mice spontaneously develop inflammation in the ocular mucosa. This ocular surface inflammation is similar to that for patients with Sjögren's syndrome, an autoimmune disease that can be accompanied by a severe and chronic dry eye (Turpie *et al*., 2009). The research group led by Dr. Masli at Boston University has characterized cells and molecules related to the inflammatory signs that these mice develop over time in different structures of the lacrimal functional unit (**Figure 6**).

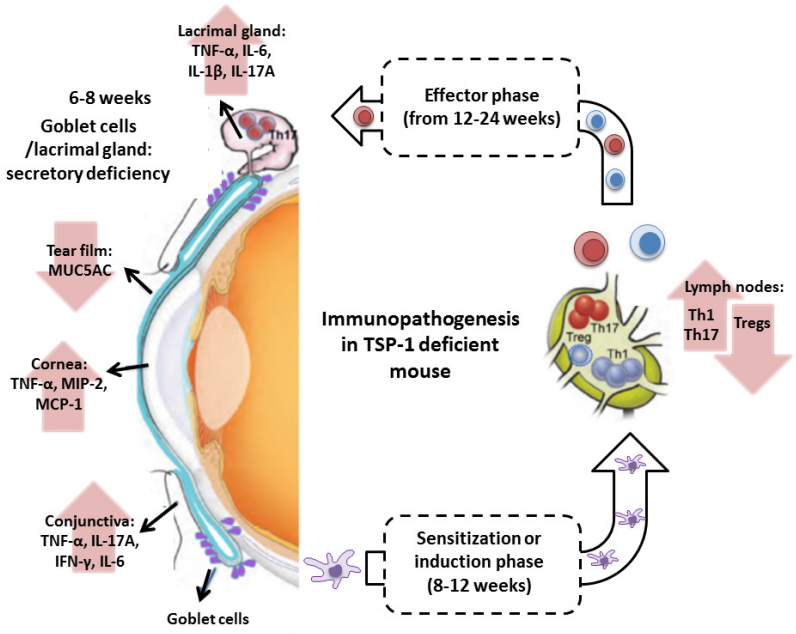

Figure 6. Overview of immunopathogenesis developed by TSP-1-deficient mice in different structures of the lacrimal functional unit and peripheral lymph nodes. Adapted from Masli *et al., Current Eye Research,* 2014.

The first detectable sign at 6-8 weeks of age is a secretory deficiency in conjunctival goblet cells and lacrimal gland acinar cells. This first manifestation leads to a decrease in the quality and quantity of tears, which affects the different structures of the ocular surface. For instance, corneal epithelial defects occur at 12 weeks of age (Turpie *et al*., 2009). This corneal damage is accompanied by conjunctival inflammation, characterized by increased lymphatic vessels and inflammatory cytokine expression, and decreases in the number of goblet cells and tear mucin (MUC5AC) levels (Contreras-Ruiz *et al*., 2013). Also at this age, increased inflammatory cytokine expression occurs in the main lacrimal gland (Turpie *et al*., 2009; Shatos *et al*., 2016). These changes trigger the sensitization or induction phase, in which ocular APCs migrate to local lymph nodes and spleen to activate immature T cells. In both secondary lymphoid organs, a larger population of effector T cells and a smaller number of Treg cells develop compared to control mice. The activation of these lymphocytes and their homing to the ocular surface play a central role in the

effector phase that leads to chronic inflammation. In the main lacrimal gland, inflammatory infiltrates are observed at 12-24 weeks of age (Turpie *et al*., 2009; Shatos *et al*., 2016), providing evidence of the effector phase.

2.4. The KRFK peptide: a TGF-β-activating peptide derived from TSP-1

Following the discovery that the RFK sequence in the TSR repeats was enough for TGF-β activation (Schultz-Cherry *et al*., 1995), the KRFK peptide, derived from the TSP-1 sequence, was synthesized. This peptide has been used to study in depth the mechanism of TGF-β activation and the importance of this specific TSP-1 function in several pathologies. Regarding the mechanism, this peptide binds to the LSKL sequence located at the N-terminus of the LAP, causing the active and mature TGF-β molecule to be released (**Figure 7**). The LSKL sequence is common to all LAPs associated with the different TGF-β isoforms, which means that both the KRFK peptide and TSP-1 molecule can activate any TGF-β isoform.

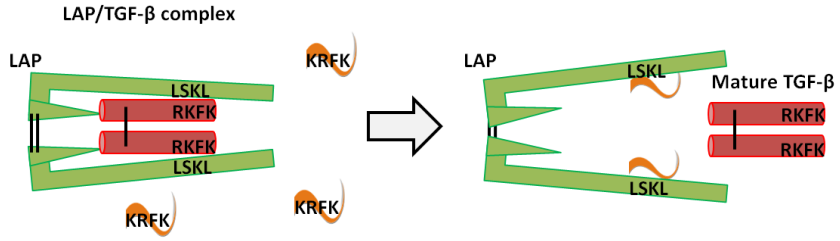

Figure 7. Diagram showing how the TSP-1-derived peptide KRFK interacts with the LSKL sequence localized in the LAP and release a mature and activated TGF-β.

This peptide has also been used in different experimental *in vivo* models to study the contribution of TSP-1-dependent TGF-β activation in pathological contexts. For example, after showing the importance of TSP-1 for lung homeostasis, Crawford *et al*. (1998) determined if the KRFK peptide could reverse the pneumonia that develops in TSP-1-deficient mice. The results from that study show that intraperitoneal injection of this TSP-1-derived peptide reverses some of the signs of pneumopathy that these mice develop. Another study aimed to reduce tumor growth in immunocompromised BALB/c mice by

inducing the expression of TSP-1-derived sequences with or without KRFK (Yee *et al*., 2004). The findings suggest that TSP-1-dependent TGF-β activation plays a role in the inhibition of angiogenesis and tumor growth. This TSP-1-derived peptide was also used topically in cutaneous wounds to alleviate abnormal scarring characteristic in TSP-1-deficient mice (Nor *et al*., 2005).

3. Anti-inflammatory treatments for the ocular surface

The anti-inflammatory drugs used to treat ocular surface inflammation are usually divided into steroids (corticosteroids or corticosteroids) and non-steroid drugs. Corticosteroids are routinely used by ophthalmologists to control inflammatory signs in ocular surface pathologies. They are derived from the cyclopentanoperhydrophenanthrene nucleus or sterane and diffuse through the cell membrane to the cytoplasm where the intracellular glucocorticoid receptors are located. After binding the ligand, the receptors dimerize and translocate to the cell nucleus. Once there, they act at the genetic level, downregulating the secretion of cytokines, chemokines, and adhesion molecules, among other pro-inflammatory molecules, and stimulating lymphocyte apoptosis (Barnes, 2009). As a result, the inflammatory symptoms are ameliorated. However, relevant side effects have been described, such as the appearance of glaucoma, cataracts, bacterial keratitis, and increased corneal scarring. Therefore, the use of "soft" corticosteroids is preferred (Bodor, 1994). For instance, medroxyprogesterone acetate (**MPA**) is one of the most often used soft corticosteroids prescribed by ophthalmologists to replace long-term treatment with stronger agents that can create some dependence.

Non-steroidal anti-inflammatory drugs (**NSAIDs**) have a natural or chemical origin and inhibit the cyclooxygenase pathway in a non-specific way. This pathway induces the formation of prostaglandins, proteins with chemokinetic action that act as mediators of cellular and humoral inflammatory responses. However, NSAIDs cannot inhibit prostaglandin action if the prostaglandins have

already been formed. The most commonly used NSAIDs include indomethacin, diclofenac, and ibuprofen.

There are other agents, not included in the two previous groups, but with increasing importance in ocular inflammatory pathologies (Pflugfelder, 2004). For example, cyclosporin A is used to treat DED. This peptide, derived from the fungus *Tolypocladium inflatum*, acts as inhibitor of the activation of T cell transcription factors. As a result, the production of inflammatory cytokines is reduced, which inhibits the apoptotic processes in ocular surface epithelial cells observed in these pathologies (Gao *et al*., 1998).

Although the above described therapeutic options help patients, a treatment that promotes the complete recovery of the affected tissues has not been achieved yet. This fact, together with the low quality of life of these patients and the increasing prevalence of these diseases, have prompted interest in research towards drug discovery and development. Therefore, novel treatments for ocular surface inflammation that propose new therapeutic targets are continuously emerging (Nebbioso *et al*., 2016).

3.1. Topical delivery of therapeutic drugs

There are two main routes for ocular surface drug administration: the topical and the subconjunctival routes. The topical route directly contacts the ocular surface, while the subconjunctival requires an injection in this area. Topical administration as eyedrops is preferred because it is non-invasive, has lower cost, and allows higher patient compliance due to the easy application.

However, this pathway has certain drawbacks due to the eye anatomy and physiology. The ocular surface is the first line of defense against possible external threats, therefore it is highly specialized to prevent the passage of most substances. From the biopharmaceutical point of view, three main barriers can be identified: the tear film, rapid drainage through the naso-lacrimal duct, and the relative impermeability of the corneal epithelium. Among the components of the tear film are complex proteins, including glycoproteins such as mucins and

lytic peptides or enzymes. These components function to prevent the entrance of most foreign agents into and through the ocular surface structures, and that includes instilled therapeutic molecules. The tear film is also in continuous renewal, which causes the dilution and drainage of instilled molecules. Finally, therapeutic molecules that reach the cornea encounter tight junctions in the corneal epithelium that prevent the passage of substances over 500 Da (Hämäläinen *et al*., 1997). In general, it is accepted that only 1% to 5% of the instilled drug crosses the corneal barrier (Davies, 2000; Urtti, 2006). However, it should be noted that the ocular surface has much more conjunctival than corneal epithelial surface, and the conjunctival epithelium is 15 to 25 times more permeable than is the corneal epithelium (Hämäläinen *et al*., 1997). A certain percentage of the therapeutic molecule absorbed by the conjunctiva will pass into the systemic circulation, reducing the advantage of the local action.

The therapeutic agents in eyedrops should have specific physicochemical characteristics. To increase the likelihood of passage through the ocular surface epithelia, the physical dimensions of agents should be in the range of nanometers or smaller. To avoid irritation, the carrier pH and osmolarity should be similar to those of the tear film, 6.5-7.8 (Abelson *et al*., 1981; Coles & Jaros, 1984) and 283-318 mOsm/kg (Tomlinson *et al*., 2006) respectively.

In summary, there is a need to improve the bioavailability of active molecules applied topically to the eye, taking into account certain physicochemical characteristics that must be met. To face this problem, pharmaceutical technology offers different strategies that include variations in drug delivery systems.

3.2. Liposomes as a drug delivery system

Different strategies have been developed to optimize bioavailability of topically administered drugs. Drug delivery systems transport and release the active molecule in a controlled way to the desired area, achieving an improved therapeutic effect. The most often studied systems developed for ocular

administration of therapeutic agents include microparticles, nanosuspensions, nanoparticles, liposomes, dendrimers and cyclodextrins (Sahoo *et al*., 2008; Patel *et al*., 2013).

Liposomes were first developed in the 1960s (Bangham *et al*., 1965), but they are still in use because of multiple advantages (Lim *et al*., 2015; Agarwal *et al*. 2014; Mishra *et al*., 2011). The main advantages of liposomes are good biocompatibility, easy preparation, and the ability to encapsulate both hydrophilic and hydrophobic drugs. The main disadvantage of these vehicles is instability, which is partially solved by the addition of stabilizing molecules such as cholesterol (Briuglia *et al*., 2015). The amphiphilic nature of phospholipids allows these molecules to form one or more concentric spheres composed of lipid bilayers or lamellae with diameters ranging from 80 nm to 10 µm (Mishra *et al*., 2011). Each lamella is separated by an aqueous compartment to form uni- or multilamellar liposomes (**Figure 8**).

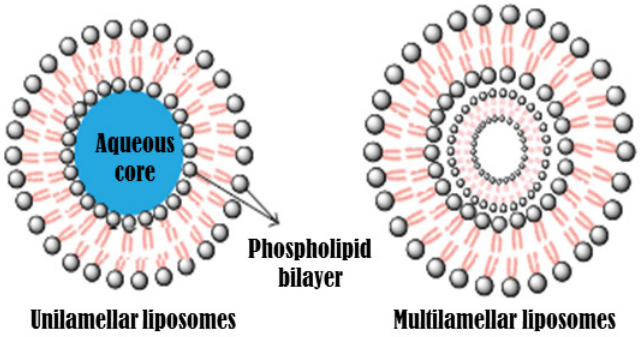

Figure 8. Graphic representation of different liposomal structures. Adapted from Mishra *et al., Journal of Drug Delivery*, 2011.

In the ocular surface, liposomes improve corneal drug penetration (Di Tommaso *et al*., 2012), and have long-term efficacy (Hathout *et al*., 2007; Law *et al*., 2000). This allows the use of lower concentration of the drug, and thus reducing toxicity associated with higher doses. In general, the cellular uptake of liposomes is mediated by high-affinity binding to the cell surface and subsequent endocytosis (Lee *et al*., 1993). This process is modulated by liposome

composition, electrostatic charge, and size. For the ocular surface, given the impermeability of the cornea and the positive charge of the ocular surface epithelia, nano-metric, anionic liposomes are preferred (Law *et al*., 2000). Liposomes constructed in this fashion establish intimate contact with the corneo-conjunctival surface. Moreover, the composition can be modified with the use of mucoadhesive molecules, capable of establishing non-covalent bonds with the mucins of the tear film. These molecules, such as chitosan or hyaluronic acid, improve the bioavailability of the drug as they increase pre-corneal residence time. The majority of liposomal formulations for topical administration contain phosphatidylcholine, cholesterol, and lipid-conjugated hydrophilic polymers as the main ingredients.

MOTIVATION

The basis of many important ocular surface diseases is an inflammatory process that affects all components of the lacrimal functional unit (Stern *et al*., 2004). Currently, the efforts to fully recover the functionality of damaged tissues in such situations have not provided the desired results. The shortcomings may be due to the different etiologies of the inflammatory processes. Therefore, we consider that it is necessary to investigate the pathophysiological mechanisms that trigger the inflammatory pathology of the ocular surface to develop more effective personalized treatments.

In this thesis, we have studied in depth the role of TSP-1 in ocular surface inflammation. There are several lines of evidence suggesting the therapeutic potential of this glycoprotein in the eye. First, TSP-1-deficient mice spontaneously develop an ocular surface inflammation (Turpie *et al*., 2009). Second, TSP-1 modulates the tolerogenic phenotype of ocular APCs, a cell type that initiates adaptive immune responses (Doyen *et al*., 2003; Masli *et al*., 2006). Third, a SNP in the promoter region of the *THBS1* gene that causes lower TSP-1 expression has been associated with a greater susceptibility to the development of chronic ocular surface inflammation after refractive surgery (Contreras-Ruiz *et al*., 2014). Thus, this polymorphism may serve as a potential genetic marker and, consequently, it may allow an early diagnosis to possibly avoid the chronic condition. As a result, we initially focused on the role of TSP-1 in ocular surface homeostasis. Following an extensive literature search, we contacted international research groups led by Dr. Luminita Paraoan at the University of Liverpool (UK) and by Dr. Sharmila Masli at Boston University (USA) for possible collaborative efforts. Both groups have studied TSP-1 and its influence in the ocular context. In collaboration with both groups, we planned to study the role of this molecule in ocular inflammation, with the aim of finding a TSP-1-based therapy for the treatment of chronic inflammatory pathologies. A TSP-1-derived peptide, the KRFK peptide, was selected as a molecule with therapeutic potential for the treatment of inflammatory pathologies of the ocular surface.

Beyond identifying the exact molecular targets that warranted investigation of their role in ocular surface inflammatory diseases, we needed to evaluate and select the most appropriate delivery system for any potential therapeutic agents that we might select or develop. The topical route is the preferred to treat ocular diseases. However, the anatomy and physiology of the eye hinder the entry of therapeutic agents. This fact directed the research towards the development of pharmaceutical formulations to increase the bioavailability of the drugs. Our research group, the Ocular Surface Group (**GSO**) from the Institute of Applied OphthalmoBiology (IOBA), University of Valladolid, Valladolid, Spain, and particularly the research team led by Dr. Yolanda Diebold have vast experience in nanotechnology systems for the release of anti-inflammatory ocular drugs (Diebold & Calonge, 2010). One of the collaborators in this area is the group led by Drs. Rocío Herrero-Vanrell and Irene Molina-Martínez, from the Department of Pharmacy and Pharmaceutical Technology at the Complutense University of Madrid, Madrid, Spain. This group has expertise in the design of new ocular drug delivery systems, and they patented a liposomal formulation with tear film characteristics (patent ES 2 284 398). They offered us the opportunity to use one of their recently developed and characterized liposomal formulations as a drug delivery system for the TSP-1-derived peptide selected (Vicario-de-la-Torre *et al*., 2014).

All this information justifies the approach of this research work to find an alternative therapy for chronic ocular surface inflammation based on TSP-1 and to use advanced pharmaceutical technology to deliver it. The previous experience of our research group together with that of our collaborators guarantees a multidisciplinary approach to this work. In the end, it all comes down to experts in different disciplines who combine their experience to find solutions to the clinical problems that generate ocular inflammation.

HYPOTHESIS AND OBJECTIVES

Hypothesis

This research work has the following hypothesis:

It is possible to develop a topically administered treatment to prevent and/or treat chronic inflammatory processes of the ocular surface based on the immunomodulatory role played by TSP-1 in this mucosal surface.

General objective

To develop an effective therapeutic alternative to existing treatments for chronic ocular surface inflammation induced by decreased TSP-1 expression.

Specific objectives

1. To characterize and compare the expression of TSP-1 and its receptors, CD36 and/or CD47, in corneal and conjunctival epithelial cell lines and primary cultures of conjunctival epithelial and stromal cells (**Studies 1 and 2**).
2. To determine changes in the expression of TSP-1 and CD36, both of which are TGF-β2-activating molecules, in different experimental models of ocular surface inflammation (**Studies 1 and 2**).
3. To evaluate the *in vitro* effect of the TGF-β-activating peptide KRFK, a TSP-1-derived peptide, on TSP-1-deficient dendritic cells and its ability to cross conjunctival epithelium (**Study 3**).
4. To evaluate *in vivo* the therapeutic efficacy of the KRFK peptide in a mouse model of ocular surface inflammation associated with TSP-1 deficiency (**Studies 3 and 4**).
5. To develop, characterize, and evaluate if a liposomal formulation designed for topical ocular application can be used as a vehicle for anti-inflammatory drugs, such as medroxyprogesterone acetate and the KRFK peptide (**Studies 5 and 6**).

METHODOLOGY

We have used the following methods (**Figure 9**) to carry out the objectives of this thesis. Detailed descriptions for the methodologies used in the experiments are included in each study.

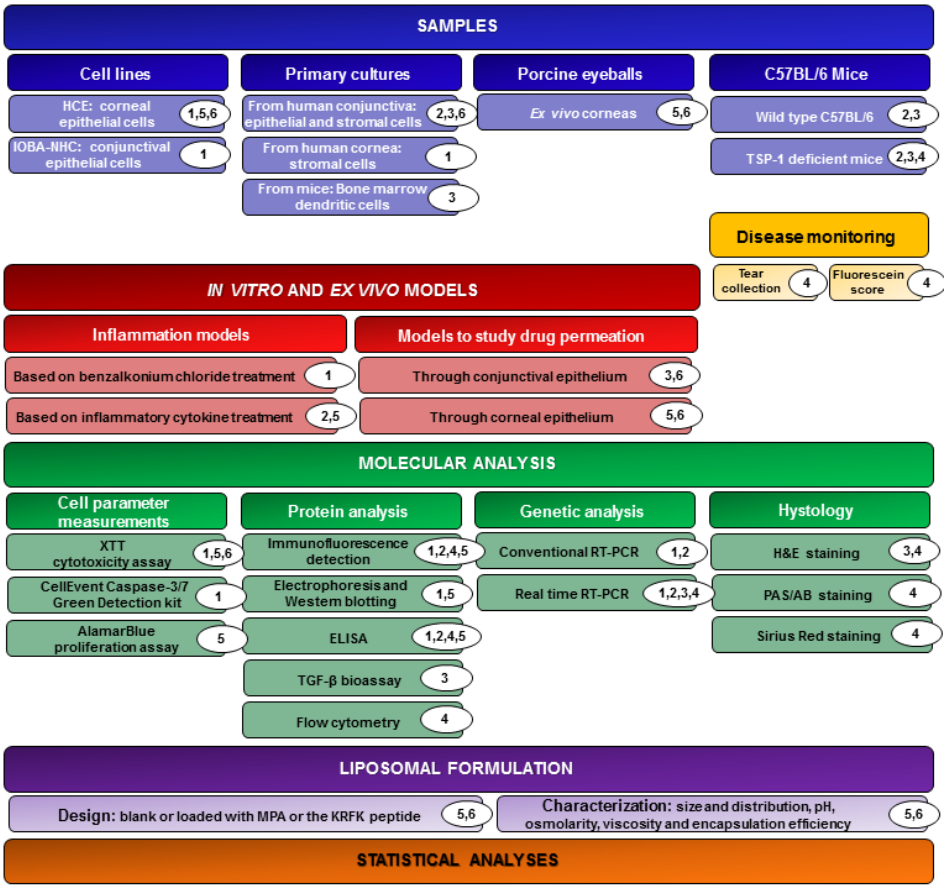

Figure 9. Schematic overview of the methodologies used. The study number in which each method was used is indicated in the white circles. ELISA: enzyme-linked immunosorbent assay; RT-PCR: reverse transcription polymerase chain reaction; H&E: hematoxylin and eosin; PAS/AB: periodic acid-Schiff/alcian blue.

SUMMARY OF RESULTS

Study 1

Cultured ocular surface epithelial cell lines can respond to TSP-1 via CD36 or CD47. Corneal epithelial cells, which produce a significant amount of the TSP-1 glycoprotein *in vitro*, up-regulated the expression of both receptors and active TGF-β2 levels after exogenous TSP-1 treatment. This up-regulation was not observed if cells were under *in vitro* simulated inflammation and apoptosis conditions. However, conjunctival epithelial cells, which produce very low TSP-1 *in vitro* at basal conditions, downregulated latent TGF-β2 levels after TSP-1 treatment under *in vitro* induction of inflammation and apoptosis conditions.

Study 2

TSP-1 and CD36, both activators of TGF-β2, are affected by the *in vitro* and *in vivo* inflammatory environment. Conjunctival fibroblasts responded to inflammatory mediators such as IL-1β in the tissue environment by down-regulating TSP-1 and CD36 expression. In the mouse models, increased conjunctival immunostaining of TGF-β2 and reduced CD36 was detected in experimental dry eye mouse model as compared with WT mice. Interestingly, increased TGF-β2 and CD36 conjunctival immunostaining was detected in TSP-1-deficient mouse model.

Study 3

The TSP-1-derived peptide KRFK *in vitro* activated TGF-β in TSP-1-deficient dendritic cell supernatants, modulating cell phenotype towards an immature state, as observed by the low expression of MHC class II and the co-stimulatory molecule CD80. After topical administration, the TSP-1-derived peptide traversed the conjunctival epithelium and altered the imbalanced T cell population in the lymph nodes of TSP-1-deficient mice *in vivo.* Particularly, the KRFK peptide inhibits Th1 and Th17 effector cells while promoting Treg development, which is consistent with the effect of KRFK peptide *in vitro*.

Study 4

The TSP-1-derived peptide KRFK prevented corneal barrier disruption along with a significant improvement in MUC5AC levels in tears in the TSP-1-deficient mouse model of ocular surface inflammation. Moreover, the topical administration of KRFK peptide reduced levels of inflammatory cytokines present in conjunctival and lacrimal gland tissues with no evident side effects.

Study 5

A liposomal formulation loaded with the anti-inflammatory agent MPA was developed with the required physicochemical properties for eyedrops. The MPA loaded in the liposomal formulation permeated *ex vivo* through corneal barriers and was efficiently delivered to corneal epithelial cells. Also, the MPA delivered *in vitro* activated specific drug receptors and showed anti-proliferative and anti-inflammatory effects in corneal epithelial cells.

Study 6

The KRFK peptide was efficiently encapsulated in the liposomal formulation used in Study 5. This formulation had the required physicochemical properties in terms of size, pH, and osmolarity for topical ocular formulations. Moreover, the encapsulated KRFK peptide had improved *in vitro* delivery to the corneal epithelium compared to free KRFK peptide.

CONCLUSIONS

After completing this work, the following **conclusions** are presented:

1. We demonstrated differential expression of TSP-1 and its receptors CD36 and/or CD47 in human corneal and conjunctival epithelial cell lines and in primary cultures of conjunctival epithelial and stromal cells. This corroborates the differences in TSP-1 expression among the ocular cell types *in vitro* and indicates that these cell cultures can be used to further study molecular mechanisms of TSP-1.
2. The induction of inflammatory processes in the ocular surface using experimental models affects the expression of molecules associated with the activation of TGF-β2 that are dependent on TSP-1. This indicates the importance of this TSP-1 function for the maintenance of ocular surface immunoregulation.
3. The TGF-β activating peptide KRFK, derived from TSP-1, penetrates through conjunctival epithelial cells and induces a tolerogenic phenotype in TSP-1-deficient dendritic cells *in vitro*, indicating the immunosuppressive potential of the peptide for ocular therapy.
4. Topically administered KRFK peptide in TSP-1-deficient mice modulates the T cell population in cervical lymph nodes. This may explain the prevention of inflammatory signs in the cornea, conjunctiva, and lacrimal gland observed in this mouse model of ocular inflammation after KRFK administration. This demonstrates the potential therapeutic efficacy of the peptide KRFK to prevent ocular inflammation in eyes with decreased TSP-1 expression.
5. We developed and characterized a novel liposomal formulation that efficiently encapsulates anti-inflammatory molecules such as medroxyprogesterone acetate and the KRFK peptide, maintaining suitable physicochemical properties for eyedrops. We demonstrated improved corneal permeation of the therapeutic molecules, showing the potential of the liposomal formulation as a drug delivery system.

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STUDY 1. Thrombospondin-1 Induces Differential Response in Human Corneal and Conjunctival Epithelial Cell Lines Under *In Vitro* Inflammatory and Apoptotic Conditions

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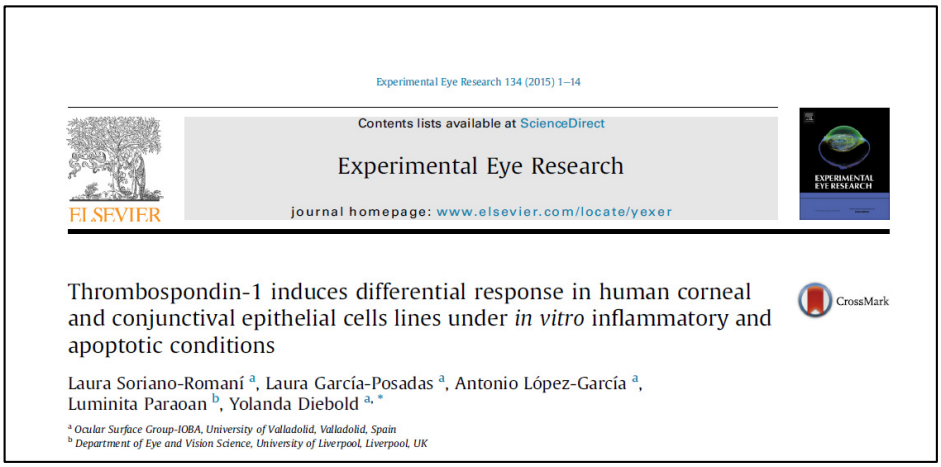

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Thrombospondin-1 induces differential response in human corneal and conjunctival epithelial cells lines under *in vitro* inflammatory and apoptotic conditions

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Thrombospondin-1 Induces Differential Response in Human Corneal and Conjunctival Epithelial Cell Lines Under *In Vitro* Inflammatory and Apoptotic Conditions

Highlights

- Ocular surface epithelial cells actively participate in TSP-1-regulated homeostasis.
- BAC exposure provides a suitable *in vitro* inflammation and apoptosis model.
- CD36 mRNA is down-regulated after BAC exposure in ocular surface epithelial cells.
- TSP-1 down-regulates BAC-induced latent TGF-β2 in conjunctival epithelial cells.
- BAC-induced changes affect CD36 and CD47 role in ocular surface epithelial cells.

Abstract

Recently, thrombospondin-1 (TSP-1) has been reported to be critical for maintaining a healthy ocular surface. The purpose of the study was to characterize the expression of TSP-1 and of its receptors CD36 and CD47 in corneal and conjunctival epithelial cells and determine the effect of exogenous TSP-1 treatment on these cells, following the induction of inflammation- and apoptosis-related changes. The expression of TSP-1, CD36 and CD47 by corneal and conjunctival cell lines was firstly characterized by ELISA, immunofluorescence analysis, Western blotting and reverse transcription polymerase chain reaction (RT-PCR). Benzalkonium chloride (BAC) exposure for 5 or 15 min was used as pro-inflammatory and pro-apoptotic stimulus for corneal or conjunctival epithelial cells, respectively. To analyze inflammation and apoptosis-related changes, IL-6 and TGF-β2 secretion determined by ELISA was used as inflammatory markers, while activated caspase-3/7 levels and cell viability, determined by CellEvent™ Caspase-3/7 Green Detection Reagent and

XTT cytotoxicity assay, respectively, were used as apoptotic markers. Changes in CD36 and CD47 mRNA expression were quantified by real time RT-PCR. Corneal epithelial cells secreted and expressed higher protein levels of TSP-1 than conjunctival epithelial cells, although TSP-1 mRNA expression levels were similar and had lower CD36 and CD47, both at protein and mRNA levels. Both cell lines responded to exogenous TSP-1 treatment increasing CD36 at protein and mRNA levels. Blocking experiments revealed a predominance of TSP-1/CD47 rather than TSP-1/CD36 interactions to up-regulate CD36 levels in conjunctival epithelial cells, but not in corneal epithelial cells. BAC exposure increased IL-6 secretion and caspase-3/7 levels and decreased cell viability in both, corneal and conjunctival epithelial cells. Moreover, BAC exposure increased latent TGF-β2 levels in conjunctival epithelial cells. Interestingly, CD36 mRNA expression was down-regulated after BAC exposure in both cell lines. Exogenous TSP-1 treatment reduced TGF-β2 up-regulated levels by BAC exposure in conjunctival epithelial cells and less pronounced reduced IL-6 in BAC-exposed corneal epithelial cells. The effect on CD36 and CD47 regulation was less pronounced or even opposite depending on the inflammation- and apoptosis-related markers tested. Our results show evidence of the capacity of corneal and conjunctival epithelial cells to respond to TSP-1 via CD36 or CD47. Experimental simulation of inflammation- and apoptosis-related conditions changed the effects differentially elicited by TSP-1 on corneal and conjunctival epithelial cells, suggesting an unexpected and relevant contribution of TSP-1 on ocular surface homeostasis regulation.

Keywords

Apoptosis; Conjunctiva; Cornea; Epithelial cell; Inflammation; Thrombospondin-1.

Abbreviations

Ab: antibody

BAC: benzalkonium chloride

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

EGF: epidermal growth factor

TBS: tris buffered saline

DMEM/F12: Dulbecco's Modified Eagle Medium + F12 medium mixture

HCE: corneal epithelial cell line

hCrF: human corneal fibroblasts

hCrF-CM: human corneal fibroblast-conditioned medium

IL: interleukin

IOBA-NHC: conjunctival epithelial cell line

Q-PCR: real time reverse transcription polymerase chain reaction

RT: room temperature

RT-PCR: reverse transcription polymerase chain reaction

rTSP-1: recombinant thrombospondin-1

SEM: standard error of the mean

TGF-β: transforming growth factor-β

TSP-1: thrombospondin-1

α-SMA: alpha smooth muscle actin

1. Introduction

Thrombospondin-1 (TSP-1) is a 450 kDa glycoprotein that was initially discovered in platelets and subsequently in a variety of cell types. This adhesive matricellular protein is the archetypal member of a family of five structurally related proteins known as thrombospondins (Adams and Lawler. 2004). It is one of the first natural angiogenesis inhibitors for which a signaling pathway has been outlined (Good *et al*. 1990) and its role as a major regulator of cellular processes is underlined by its ability to interact with cell receptors and extracellular molecules (Bornstein. 1995, Chen *et al*. 2000, Friedl *et al*. 2002, Lahav. 1993).

One of the most interesting and controversial roles of TSP-1 is as inflammatory modulator (Lopez-Dee *et al*. 2011) and as a possible mediator of apoptosis subsequent to chronic inflammation. CD36, a TSP-1 cell membrane receptor, is crucial in TSP-1-mediated inflammatory and apoptotic responses in some cells types such as endothelial or immune cells (Cursiefen *et al*. 2011, Jimenez *et al*. 2000, Li *et al*. 2003). A down-regulation of inflammation by CD36 has been described in lung epithelial cells (Sharif *et al*. 2013). Together with CD36, CD47 also modulates inflammatory and apoptotic response via TSP-1 in other cell types (Lamy *et al*. 2007).

In the eye, the properties of TSP-1 as a modulator in different inflammation- and apoptosis-related processes have been recently reviewed (Hiscott *et al*. 2006, Masli *et al*. 2014). Specifically at the ocular surface, the increased expression of TSP-1 during corneal wound healing process (Blanco-Mezquita *et al*. 2013, Matsuba *et al*. 2011, Uno *et al*. 2004) or the involvement in ocular surface inflammatory responses mainly via transforming growth factor-β (TGF-β) activation (Streilein *et al*. 2002) evidence the important role played by TSP-1 in different physiological conditions.

TSP-1 mRNA has been detected in corneal epithelium and stroma (Armstrong *et al*. 2002, Cursiefen *et al*. 2004) and the protein has been immuno-localized in corneal and limbal epithelia and in corneal endothelium (Cursiefen *et al*. 2004,

Hiscott *et al*. 1996, Sekiyama *et al*. 2006, Uno *et al*. 2004). However, less is known regarding TSP-1 expression in the conjunctiva. Some studies reported a mild and focal TSP-1 staining in normal conjunctival epithelium (Aspiotis *et al*. 2007, Gandhi *et al*. 2013). Given the ocular surface tissues expression of TSP-1, it is likely that CD36 and CD47 also play a significant role in ocular surface homeostasis.

CD36 has been reported to be expressed by ocular epithelial cells, particularly by corneal and limbal epithelial cells (Mwaikambo *et al*. 2006). Recently, CD36 knock-out mice have been described to develop spontaneous corneal defects that increased in frequency and severity with age (Klocke *et al*. 2011). However, to the best of our knowledge, CD36 expression in conjunctival tissues has not been described yet. There is even less information on CD47 and its role in ocular surface. This ubiquitous membrane receptor has been recently reviewed as a potential therapeutic protein (Mawby *et al*. 1994, Sick *et al*. 2012). Although TSP-1 ligation to CD47 regulates inflammation, apoptosis or cell migration (Graf *et al*. 2002, Lamy *et al*. 2007, Rebres *et al*. 2005), the role of CD47 in ocular surface epithelial cells has not been studied.

Although the inflammatory responses that occur in an inflamed ocular surface are mainly due to inflammatory cells, there is growing evidence that epithelial cells can secrete different cytokines and chemokines which act in inflammatory diseases (Enriquez-de-Salamanca *et al*. 2008). *In vitro* inflammation models in epithelial cells have been widely used to study the inflammation and its different signaling pathways induced by different conditions/treatments, including pro-inflammatory cytokines or ultraviolet light. For instance, benzalkonium chloride (BAC), one of the most commonly used preservatives in ophthalmic solutions, is also used to induce inflammation and apoptosis because of its well-known toxicity in corneal and conjunctival epithelial cells (Baudouin *et al*. 2010). Moreover, BAC is also used to induce ocular surface inflammation in animal models (Lin *et al*. 2011, Xiong *et al*. 2008).

Taking all this information into account, the aim of this study was to analyze inflammation- and apoptosis-related markers, including CD36 and CD47, in corneal and conjunctival epithelial cells exposed to TSP-1. The study used BAC as a pro-inflammatory and pro-apoptotic stimulus and also determined the basal TSP-1, CD36 and CD47 expression levels in corneal and conjunctival epithelial cell lines.

2. Materials and methods

2.1. Materials

All materials used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Dulbecco's Modified Eagle Medium (DMEM)/F-12, and some of its supplements, such as fetal bovine serum (FBS), penicillin, and streptomycin were from Invitrogen-GIBCO (Inchinnan, UK). Human epidermal growth factor (EGF) and bovine insulin were from Invitrogen (Eugene, OR). Human recombinant TSP-1 (rTSP-1) was from R&D Systems, Inc. (Minneapolis, MN). Cell culture plates and multichamber Permanox® or Glass® slides were from Nunc (Roskilde, Denmark). For Western blotting experiments, bicinchoninic acid (BCA) assay was from Pierce (Rockford, IL) and acrylamide and Tris buffered saline (TBS) were purchased from Bio-Rad Laboratories (Hercules, CA). Antibodies (Abs) used for immunocytochemistry or Western blotting are indicated in Table 1. For blocking experiments, anti-CD36 and anti-CD47 Abs were used, together with rabbit and mouse IgG isotype control Abs from Abcam and Santa Cruz Biotechnology, respectively. Fluoromount-G™ mounting media was from SouthernBiotech (Birmingham, AL). Human TSP-1,interleukin-6 (IL-6) and TGF-β2 enzyme-linked immunosorbent assay (ELISA) kits were from R&D systems (TSP-1 and TGF-β2), Inc. and Diaclone (Bensançon, France)(IL-6). CellEvent™ Caspase-3/7 Green Detection Reagent was from Invitrogen. Kits for RNA isolation were from Qiagen (Valencia, CA), and Quant-iT™ RNA assay and SuperScript VilocDNA kit were from Invitrogen. All reagents for reverse transcription polymerase chain reaction (RT-PCR), were from Biotools B&M Labs

S.A. (Madrid, Spain), except Blue Juice™ Gel Loading Buffer 10X (Invitrogen) and TSP-1, CD36, and CD47 primers (OriGene Technologies, Inc., Rockville, MD, USA). SYBR Green PCR Master Mix was from Applied Biosystems (Carlsbad, CA). The ChemiDoc® gel documentation system and the Quantity One software used to visualize the acrylamide and agarose gels and to analyze the resulting images were from Bio-Rad Laboratories. The microscope Leica DMI 6000B and the LAS AF Lite software used to visualize phase contrast or fluorescence images and analyze them were from Leica Microsystems (Wetzlar, Germany). The SpectraMAX® M5 multidetection microplate reader and the SoftMax Pro 4.8 software used to analyze cytotoxicity assay were from Molecular Devices (Sunnyvale, CA). The conventional reverse transcription polymerase chain reaction (RT)-PCR was done in MyGene™ L Series Peltier Thermal Cycler from LongGene®Scientific Instruments Co., Ltd (Tuen Mun, Hong Kong) and Real Time RT-PCR (Q-PCR) was done in 7500 Real-Time PCR System from Applied Biosystems. For statistical analyses, Statistical Procedures for the Social Sciences software (SPSS 20.0; SPSS Inc., Chicago, IL) was used.

2.2. Cell lines and culture conditions

Two cell lines were used. The Human Corneal Epithelial (HCE) cell line (Araki-Sasaki *et al*. 1995) from passages 29 to 43 was cultured in DMEM/F-12 medium supplemented with 10% FBS, 5000 U/mL penicillin/streptomycin, 10 ng/mL EGF, and 5 µg/mL insulin. The IOBA - Normal Human Conjunctiva (IOBA-NHC) epithelial cell line (Diebold *et al*. 2003) from passages 66 to 75 was cultured in DMEM/F-12 medium supplemented with 10% FBS, 5,000 U/mL penicillin/streptomycin, 2.5 µg/mL fungizone, 2 ng/mL EGF, 1 µg/mL insulin, and 0.5 µg/mL hydrocortisone.

Both cell lines were cultured at 37 °C in a 5% CO_2 – 95% air atmosphere. Medium was changed every other day and cells were observed daily by phase contrast microscopy. To measure basal amount of TSP-1, CD36 and CD47, cells were grown for 3 days in basal medium made of DMEM/F-12 medium

supplemented with 10% FBS, 5,000 U/mL penicillin/streptomycin, and 2.5 µg/mL fungizone (basal conditions).

2.3. Isolation and culture of primary corneal stromal cells from human tissue

Cadaveric non-diseased human corneoscleral buttons (n = 5) were obtained from the Barraquer Eye Bank (Barcelona, Spain) after being discarded for cornea transplant. This study was in strict accordance with the Tenets of the Declaration of Helsinki and Spanish Regulations concerning the use of human tissues for biomedical research, and had the approval of the Institutional Review Board of the University of Valladolid. In brief, a 7.5 mm trephine was used to isolate the cornea from the limbus, and the central button of the cornea was cut in small pieces. Then, corneal pieces were digested with 2 mg/mL collagenase type I overnight at 37 ˚C. After that, loosened cells were recovered with the help of a pipette and centrifuged at 156 g for 5 min. Finally, cells were cultured in basal medium. The purity of the cultures was assessed by two methods: evaluating positive staining for vimentin and negative for α-SMA, and using a trypsinization time of 2 min in order to only remove the spindle-shaped cells from the culture plate. Human corneal fibroblasts (hCrF) from passages 2 to 4 were used.

2.4. Treatment of corneal and conjunctival epithelial cells with TSP-1

Firstly, TSP-1 basal production of human corneal and conjunctival epithelial cells and corneal fibroblasts was measured by ELISA. Corneal and conjunctival epithelial cells were seeded in a 24-well plate and grown for 48 h. Then, cells were exposed to 1 µg/mL rTSP-1 or hCrF-conditioned medium (hCrF-CM) (which contained about 1 µg/mL TSP-1) for 24 h. Untreated cells were used as controls. The medium was changed to serum-free medium and maintained for an additional 24-hour period. At least 3 independent experiments in duplicate were done.

In order to determine if TSP-1 effect on corneal and conjunctival epithelial cells in terms of changes in CD36 expression were triggered through TSP-1/CD36

and/or TSP-1/CD47 interaction, blocking experiments were done. Cells were treated with 10 µg/mL anti-CD36 and anti-CD47 Abs (see Table 1) and exposed to 1 µg/mL rTSP-1. Corneal and conjunctival epithelial cell mRNA was isolated at this time point and after 24 hours in serum-free medium. Isotype control Abs-treated cells were used as controls. At least 3 independent experiments were done.

2.5. In vitro induction of inflammation and apoptosis in TSP-1-treated cells

Corneal and conjunctival epithelial cells were cultured for 48 h. Then, cells were maintained in serum-free medium with or without 1 µg/mL rTSP-1 for 24 h. At this time point, cells were exposed to 0.001% BAC for 15 (corneal cells) or 5 (conjunctival cells) min and then maintained in a serum-free medium with or without 1 µg/mL rTSP-1 for an additional 24-hour period. Cells unexposed to rTSP-1 were used as controls for each cell line. A scheme of the protocol used is depicted in Figure 1. At least 3 independent experiments in duplicate were done.

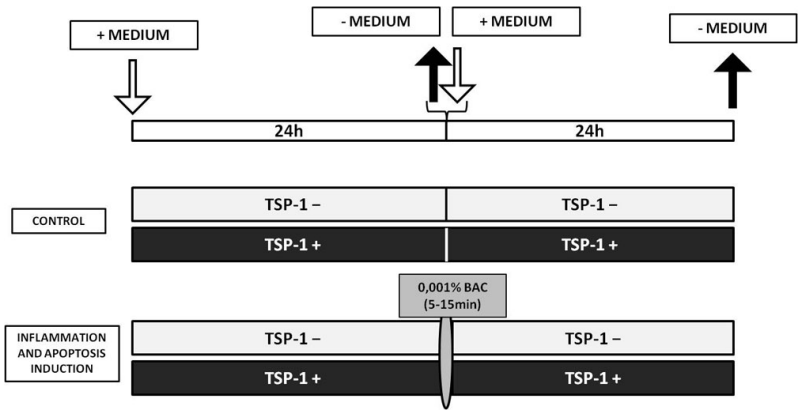

Figure 1: **Schematic showing the protocol used for the *in vitro* induction of inflammation and apoptosis in TSP-1-treated corneal and conjunctival epithelial cell lines**. White arrows indicate fresh culture medium additions and black arrows indicate a removal of conditioned media. BAC exposure time was 15 min for corneal epithelial cells or 5 min for conjunctival epithelial cells. TSP-1 – = DMEM/F-12; TSP-1+ = DMEM/F-12 with 1 µg/mL of rTSP-1.

To measure BAC effect on cell viability, the XTT (2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) cytotoxicity assay was used. Untreated and TSP-1-treated corneal and conjunctival epithelial cells were exposed to 0.001% BAC for 5, 15 or 30 min. After washing them, cells were maintained in serum-free medium for 24 h. Then, the XTT reagent was added according to manufacturer´s instructions. Absorbance of quadruplicate samples was read in a microplate reader. Cell viability was determined as the percentage of cells recovered after BAC exposure compared to control cells exposed to serum-free culture medium. Also, BAC-induced cell morphology changes were evaluated by recording a 30 min time-lapse video with the phase contrast microscope immediately after BAC exposure, under a dry X20 objective using phase contrast microscopy.

IL-6 and TGF-β2 production was quantified following BAC-induced inflammatory changes and compared to basal IL-6 and TGF-β2 production of corneal and conjunctival epithelial cells. For this purpose, human IL-6 and human TGF-β2 ELISA kits were used according to manufacturer´s instructions. For TGF-β2 measurements, both acidified (total TGF-β) or non-acidified (active TGF-β2) samples were assayed. The alamarBlue® assay was performed after supernatants collection of BAC-exposed cells, to calculate cell numbers and use this value to normalize concentration values.

To determine the induction of apoptosis following BAC exposure, levels of activated caspase-3/7 were measured. CellEvent™ Caspase-3/7 Green Detection Reagent was used according to manufacturer's instructions. Nuclei were counterstained with Hoechst 33342 for 5 min at room temperature (RT). Positive activated caspase-3/7 cells / total cell numbers (total number of nuclei detected) were counted in 3 independent fields per well.

2.6. Immunofluorescence analysis

Cells were fixed in ice-cold methanol and kept at -20 ˚C. Then, slides were incubated at RT for 1 h in blocking buffer composed of phosphate-buffered

saline with 4% donkey serum to block non-specific binding. They were then incubated with the primary Abs (Table 1) for 1 h at RT (CD47) or overnight at 4 °C (vimentin, α-SMA, TSP-1, CD36). Incubations with Alexa Fluor 488-conjugated secondary Abs (Table 1) were performed for 1 h at RT. Cell nuclei were counterstained with propidium iodide. Preparations were visualized under a dry X40 objective using fluorescence microscopy. Specificity of Abs had been previously tested. At least 3 independent experiments in duplicate were done.

Table 1. Antibody sources and concentrations. ICC = immunocytochemistry; WB = Western blotting.

	Antibody	Host species	Dilution for ICC	Dilution for WB	Source
Primary antibodies	Vimentin	Mouse	1:200	-	Santa Cruz Biotechnoloy, Santa Cruz, CA
	α-SMA	Mouse	1:100	-	Abcam, Cambridge, UK
	TSP-1	Goat	1:50	1:200	Santa Cruz Biotechnology
	CD36	Rabbit	1:50	1:500	Abcam
	CD47	Mouse	1:50	1:200	Santa Cruz Biotechnology
	Caspase-3	Rabbit	-	1:1000	Abcam
	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Mouse	-	1:2000	Santa Cruz Biotechnology
Secondary antibodies	AlexaFluor 488 anti-mouse IgG	Donkey	1:200	-	Invitrogen
	AlexaFluor 488 anti-rabbit IgG	Donkey	1:200	-	Invitrogen
	AlexaFluor 488 anti-goat IgG	Donkey	1:200	-	Invitrogen
	Peroxidase-conjugated anti-mouse IgG	Donkey	-	1:5000	Jackson Immuno-Research Laboratories, West Grove, PA
	Peroxidase-conjugated Anti-rabbit IgG	Goat	-	1:1000	Sigma-Aldrich
	Peroxidase-conjugated Anti-goat IgG	Rabbit	-	1:1000	Sigma-Aldrich

2.7. Electrophoresis and Western blotting

Cells were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer plus protease inhibitors. Total protein content was quantified using BCA assay. A total protein amount of 15 µg was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels, transferred to nitrocellulose membranes, and blocked for 1 h in TBS containing 0.05% Tween-

20 and 5% bovine serum albumin. Membranes were incubated with primary Abs (Table 1) overnight at 4 °C and with peroxidase-conjugated secondary Abs (Table 1) for 1 h at RT. GAPDH was used as loading control. Immunoreactive bands were visualized and analyzed by densitometry analysis using the ChemiDoc® gel documentation system and the Quantity One software. At least 3 independent experiments in duplicate were done.

2.8. Reverse transcription polymerase chain reaction (RT)-PCR and SYBR-Green real time RT-PCR (Q-PCR)

Isolation of total RNA from cells was done using the RNeasy Mini Kit. Contaminating DNA was eliminated by digestion with RNase-Free DNase Set. Total RNA concentration was measured with the QuantiT™ RNA assay. The extracted RNA was reverse-transcribed to cDNA with the SuperScript Vilo cDNA kit according to manufacturer's instructions. Primer pairs used were GAPDH (sense: 5´-GAACGTGAAGGTCGGAGTCAAC-3´; antisense: 5´-CGTGAAGATGGTGATGGGATTTC-3´), TSP-1 (Catalog No. HP206797), CD36 (Catalog No. HP200058) and CD47 (Catalog No. HP225827).

Each reaction of conventional RT-PCR contained 1 µL of primers, 50 ng of cDNA, 5 µL of PCR buffer 10X, 1 µL dNTPs and 1 µL Taq polymerase in a final volume of 50 µL. The cycle profile was 95˚C for 2 min, 39 cycles of 95˚C for 20 s, 60˚C for 30 s, 72˚C for 40 s and 72˚C for 10 min. RT-PCR products were mixed with 2 µL of loading buffer and were resolved on 4% agarose gels.

The Q-PCR reaction was performed with 10 ng cDNA, 1 µL primers and 10 µL SYBR Green PCR Master Mix in a final volume of 20 µL. The PCR conditions were: denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 60 s, and a final cycle of 95˚C for 90 s. All reactions were performed in duplicate. To assure the specificity of the PCR products, a melting curve analysis was performed. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen. 2001) using the GAPDH signal for normalization and control conditions as reference value.

For all reactions, levels of GAPDH for each sample were used as endogenous controls and negative controls with omission of cDNA were included. At least 3 independent experiments in duplicate were done.

2.9. Statistical Analyses

Data were expressed as means ± standard error of the mean (SEM). To compare 2 groups, a Student's *t*-test was done. For more than 2 groups, a one-way ANOVA followed by pairwise comparisons (Tukey test) or a Brown-Forsythe test followed by pairwise comparisons (Games-Howell test) were performed, depending on Levene's test for homogeneity of variances. Statistical significance ($p \leq 0.05$) or tendency ($p \leq 0.1$) were considered.

3. Results

3.1. Expression of TSP-1 in corneal and conjunctival epithelial cells at basal conditions

Expression of TSP-1 was investigated in corneal and conjunctival epithelial cells using corneal fibroblasts as positive controls. TSP-1 was not detected in fresh basal medium by ELISA; however, a 180 kDa immunoreactive band corresponding to TSP-1 was detected by Western blotting (data not shown). Corneal epithelial cells secreted significantly higher levels of TSP-1 (mean concentration of 954.32 ± 77.42 ng/mL) than conjunctival epithelial cells (mean concentration of 22.77 ± 2.83 ng/mL) (Figure 2A). TSP-1 was immunolocalized in both cell types and was mainly distributed in the perinuclear area (Figure 2B). Intracellular TSP-1 protein levels (normalized to respective GAPDH levels) were also higher in corneal epithelial cells compared to conjunctival epithelial cells, as determined by Western blotting. Corneal fibroblasts had significantly higher levels of intracellular TSP-1 than conjunctival epithelial cells, but not corneal epithelial cells (Figure 2C). Normalized levels of TSP-1 mRNA expression were similar in corneal and conjunctival epithelial cellls. Both were lower than the TSP-1 mRNA expression levels of corneal fibroblasts, being significantly different

in conjunctival epithelial cells, but not in corneal epithelial cells (p=0.07) (Figure 2D).

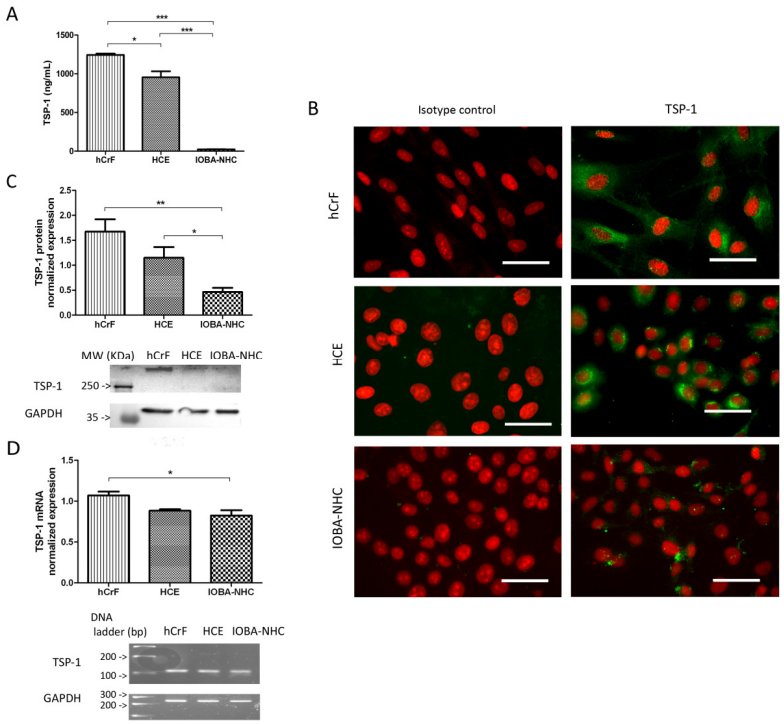

Figure 2: **TSP-1 expression in corneal (HCE) and conjunctival (IOBA-NHC) epithelial cell lines, using corneal fibroblasts (hCrF) as positive controls, at basal conditions. A.** TSP-1 production after 3 days in culture determined by ELISA. **B.** Immunolocalization of TSP-1 (green). Nuclei of cells were stained in red, with propidium iodide. Isotype controls included the omission of primary antibodies. Scale bar: 50 µm. **C.** Intracellular protein expression, normalized to GAPDH levels, determined by Western blotting. **D.** TSP-1 mRNA expression, normalized to GAPDH levels, determined by conventional RT-PCR. Bars represent the mean value ± SEM from, at least, 3 independent experiments. Statistically significant differences between samples are indicated with asterisks (Tukey test; *$p \leq 0.05$, ***$p \leq 0.001$).

3.2. Expression of CD36 and CD47 in corneal and conjunctival epithelial cells at basal conditions

CD36 and CD47 were immunolocalized in plasma membranes of both cell types (Figure 3A). Intracellular protein levels of both receptors appeared slightly higher in conjunctival epithelial cells than in corneal epithelial cells, as

determined by Western blotting; however, the difference was not significant for CD36 (p=0.06) or CD47 (p=0.12) (Figure 3B). However, RT-PCR revealed that mRNA levels of both receptors were significantly higher in conjunctival epithelial cells than in corneal epithelial cells (Figure 3C).

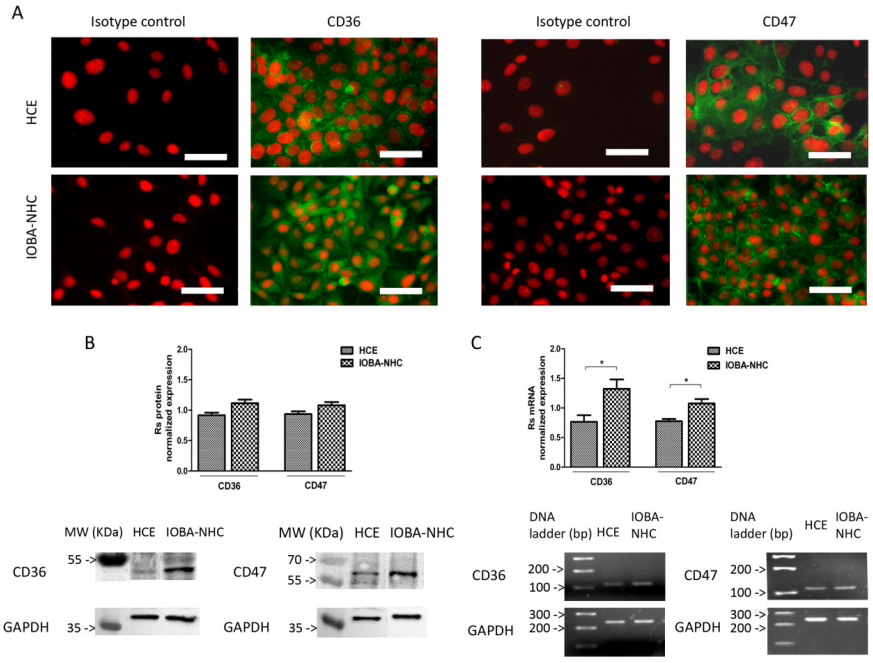

Figure 3: **Expression of CD36 and CD47 in corneal (HCE) and conjunctival (IOBA-NHC) epithelial cell lines at basal conditions. A.** Immunolocalization of CD36 and CD47, both in green. Nuclei of cells were stained in red, with propidium iodide. Isotype controls included the omission of primary antibodies. Scale bar: 50 µm. **B.** Intracellular protein expression, normalized to GAPDH levels, determined by Western blotting. **C.** CD36 and CD47 mRNA expression, normalized to GAPDH levels, determined by conventional RT-PCR. Bars represent the mean value ± SEM from, at least, 3 independent experiments. Statistically significant differences between samples are indicated with asterisks (Student t-test; *$p \leq 0.05$).

3.3. Exogenous TSP-1 up-regulated CD36 protein expression, but not CD47, IL-6, TGF-β2 or activated caspase-3, both in corneal and conjunctival epithelial cells

Changes in CD36, CD47 and caspase-3 protein expression or IL-6 and TGF-β2 production were determined after TSP-1 treatment by Western blotting or ELISA, respectively. In corneal epithelial cells, CD36 expression increased after treatment with rTSP-1 or in hCrF-CM-treated cells, although the latter did not

reach significance (p=0.1), compared to that of controls. However, after the serum-free period, the differences observed in CD36 protein levels were not significant. On the contrary, there were no differences between CD36 levels after rTSP-1 or hCrF-CM treatments in conjunctival epithelial cells. However, after the serum-free period, minimal decrease of CD36 protein levels was observed, except in rTSP-1-exposed cells, which CD36 protein levels were increased (Figure 4A).

Regarding CD47 protein expression, the differences following the treatments in corneal epithelial cells were not significant. However, after the serum-free period, CD47 protein levels were significantly decreased in hCrF-CM-treated conjunctival epithelial cells when compared to untreated control cells (Figure 4B).

Activated caspase-3 was not immunodetected at any time point or condition analyzed either in corneal or conjunctival epithelial cells. Similar expression levels of a 32 kDa immunoreactive band were found in all the samples corresponding to a non-cleavage (non-activated) form of caspase-3 (data not shown), indicating that cells were not apoptotic.

No differences were found in IL-6 production in any condition studied in corneal epithelial cells. However, IL-6 production in conjunctival epithelial cells after serum-free period was significantly higher in hCrF-CM-treated cells compared to untreated control cells or rTSP-1-treated cells (Figure 4C), which suggested a stimulatory effect of hCrF-CM on conjunctival epithelial cells.

No significant differences were found in TGF-β2 production after the serum-free period in any of the TSP-1-treated cells compared to that of control cells. However, there was an inverse pattern of TGF-β2 production by corneal and conjunctival epithelial cells after hCrF-CM treatment (Figure 4D). Thus, corneal cells tended (p=0.09) to increase total TGF-β2 levels secreted in hCrF-CM-treated cells compare to control cells, while conjunctival cells tended to decrease total (p=0.08) and latent (p=0.09) TGF-β2 levels in hCrF-CM-treated cells compared to control cells.

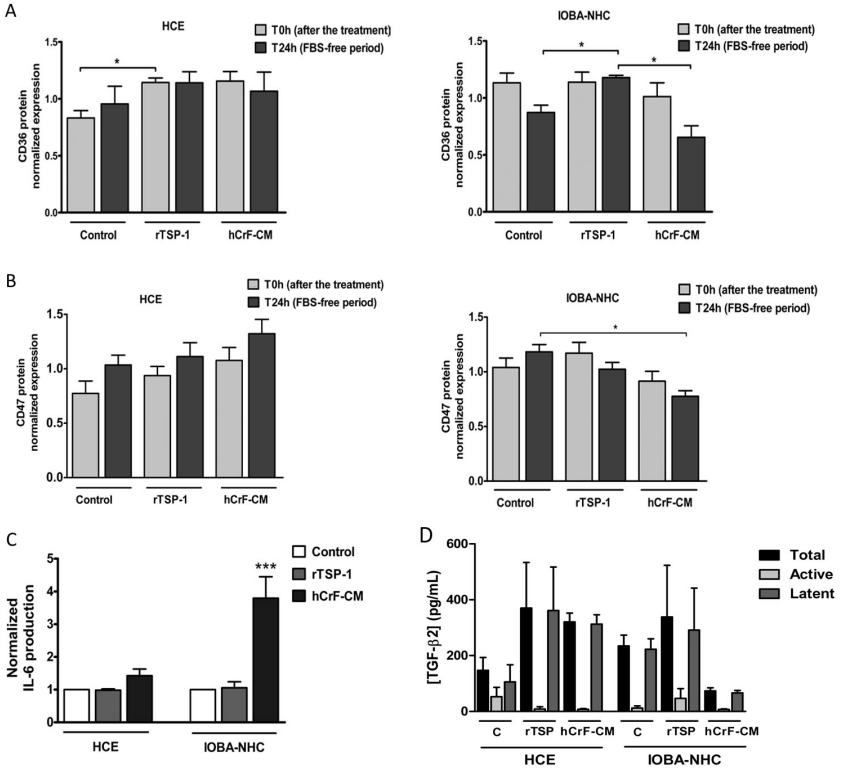

Figure 4: **Changes in CD36 and CD47 protein expression or IL-6 and TGF-β2 production induced by TSP-1 treatment of corneal (HCE, left panel) and conjunctival (IOBA-NHC, right panel) epithelial cell lines**. Changes in **A.** CD36 or **B.** CD47 protein expression, normalized to GAPDH levels, were evaluated immediately after the 24-hour treatment (T 0 h) and 24 h after the treatment (T 24 h) by Western blotting. **C.** IL-6 production, normalized to control values for each cell line,at T 24 h determined by ELISA. **D**. Total, active, and latent TGF-β2 levels at T 24 h determined by ELISA. Treatments included basal medium (control), 1 μg/mL of rTSP-1 added to the basal medium (rTSP-1) or corneal fibroblast-conditioned medium (hCrF-CM). Values are expressed as mean ± SEM from 3 independent experiments in duplicate. Statistically significant differences between samples at same time points are indicated with asterisks (Tukey test or Games-Howell test; $*p \leq 0.05$).

3.4. Blocking TSP-1/CD36 or TSP-1/CD47 pathways changed TSP-1 effect on CD36 expression.

To determine whether the increase in CD36 levels was due to TSP-1/CD36 or TSP-1/CD47 interactions, CD36 and CD47 receptors were blocked using abs against TSP-1 binding site. Cells were treated with rTSP-1 and CD36 mRNA analysed at different time points. Results showed an up-regulation of CD36

mRNA levels after TSP-1 treatment, consistently with protein levels in corneal epithelial cells (Figure 4A). However, after blocking CD36 or CD47, no changes were observed in any condition studied (Figure 5A). Interestingly, in conjunctival epithelial cells, at the time point in which CD36 protein levels were increased (24 h after TSP-1 treatment), no significant increase was observed at message level (Figure 5B). However, mRNA CD36 levels tended (p=0.1) to increase after TSP-1 treatment in control and anti-CD36-treated cells, but not in anti-CD47-treated cells. Moreover, after TSP-1 treatment, anti-CD36-treated cells tended (p=0.08) to have higher CD36 mRNA levels than anti-CD47-treated cells (Figure 5B).

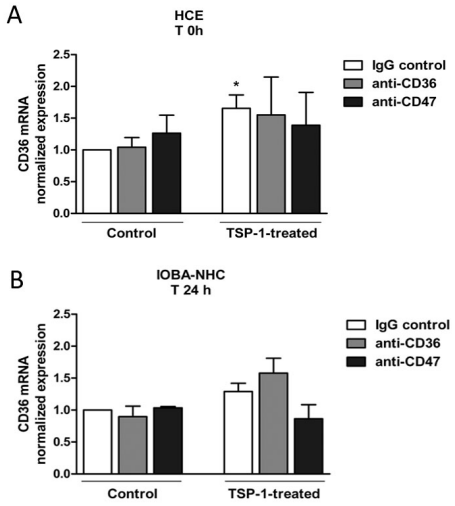

Figure 5: **Changes in CD36 mRNA levels after blocking CD36 and CD47 with abs against the TSP-1 binding site in corneal (HCE) and conjunctival (IOBA-NHC) epithelial cell lines .**. Changes in CD36 mRNA levels, normalized to GAPDH levels were evaluated **A.** immediately after 24-hour treatment (T 0 h) for HCE cell line and **B.** 24 h after the treatment (T 24 h) for IOBA-NHC cell line. Values are expressed as mean ± SEM from 3 independent experiments. Statistically significant differences, when compared to IgG isotype control-treated cells, are indicated with asterisks (*$p \leq 0.05$).

3.5. BAC exposure induced cytotoxicity in a time-dependent manner and increased IL-6 production and caspase-3/7 levels in corneal and conjunctival epithelial cells

Different exposure times to 0.001% BAC followed by a serum-free 24-hour period were used to induce cytotoxicity in corneal and conjunctival epithelial

cells. Also, unexposed cells were maintained in a serum-free medium (controls) or 10 % serum-supplemented medium to rule out a protective effect induced by serum. BAC exposure affected cell viability in a different time-dependent manner. In corneal epithelial cells, 15 min significantly reduced cell viability compared to controls, while 5 min were enough to have similar effect on conjunctival epithelial cells. Moreover, the lack of serum in the culture medium significantly affected conjunctival epithelial cells, but not corneal epithelial cells (Figure 6A). The respective exposure times determined above were used in further experiments as a similar decrease in cell viability was obtained in both cell lines.

Cell morphology was modified in BAC-exposed cells, as determined by time-lapse video microscopy. BAC exposure induced a cell-shape change, a progressive cytoplasm shrinkage, cell-cell junctions breakage, and intense cell blebbing that increased with BAC exposure time (Figure 6B). However, these changes were more noticeable in conjunctival epithelial cells than in corneal epithelial cells, indicating a different tolerance to BAC-induced alterations in the specific epithelial cell lines investigated.

To determine whether BAC exposure induced inflammatory and apoptotic changes in corneal and conjunctival epithelial cells at these exposure times, IL-6 and TGF-β2 production and changes in activated caspase-3/7 levels, respectively, were determined. BAC exposure significantly increased both IL-6 production and caspase-3/7 activation in corneal and conjunctival epithelial cells compared to control unexposed cells. Also, BAC-exposed conjunctival epithelial cells significantly increased total and latent TGF-β2 levels, and tended (p=0.09) to increase levels of the active molecule (Figure 6C, D and E).

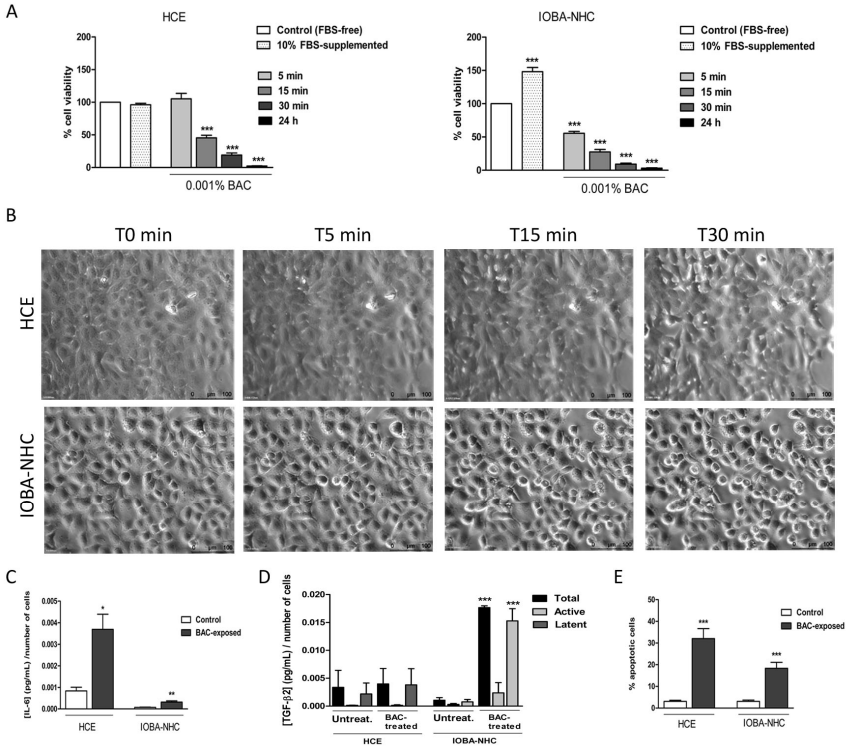

Figure 6: **Changes in inflammation- and apoptosis–related markers induced by BAC exposure in corneal (HCE) and conjunctival (IOBA-NHC) epithelial cell lines. A.** Percentage of cell viability after 5, 15, and 30 min of BAC exposure followed by a 24-hour period of serum-free medium using the XTT cytotoxicity assay. **B.** Representative pictures immediately after (T0 min), 5, 15, or 30 min of 0.001% BAC exposure under a dry X20 objective using phase contrast microscopy. **C.** Production of IL-6 or **D.** total, active or latent TGF-β2 in BAC-exposed or unexposed cells determined by ELISA. Concentration values were normalized to cell numbers determined by alamarBlue. **E**. Percentage of apoptotic cells in BAC-exposed or unexposed cells, quantified through caspase-3/7 positive stained cells / total number of cells. Values are expressed as mean ± SEM from, at least, 3 independent experiments in duplicate. Statistically significant differences respect to control cells are indicated with asterisks (Tukey test or Games-Howell test; *$p \leq 0.05$, ***$p \leq 0.001$).

3.6. BAC exposure reduced CD36 mRNA levels, but not CD47 mRNA levels or endogenous TSP-1 secretion, in corneal and conjunctival epithelial cells

To determine possible expression changes in TSP-1 or its receptors induced by BAC exposure, CD36 and CD47 mRNA levels and TSP-1 production were quantified. BAC exposure led to reduced CD36 mRNA levels in both corneal and

conjunctival epithelial cells, while CD47 mRNA levels did not change (Figure 8A and B, untreated cells).

Corneal epithelial cells did not alter TSP-1 secretion in any condition studied. Mean TSP-1 concentrations of corneal epithelial cells were 465,14 ± 50.09 ng/mL after 24 h in 10% serum-supplemented medium; 408.15 ± 26.31 ng/mL after 24 h in serum-free medium; or 282.14 ± 66.49 ng/mL after BAC exposure. However, after normalizing these concentration to cell numbers, there were no statistical differences. Conjunctival epithelial cells secreted lower levels of TSP-1 (mean concentration and SEM of 28.96 ± 3.49 ng/mL) after 24 h in 10% serum-supplemented medium, being undetectable if cells were grown in serum-free medium, either exposed or unexposed to BAC (data not shown).

3.7. TSP-1 treatment induced different changes in inflammation- and apoptosis-related markers in BAC-exposed corneal and conjunctival epithelial cells

To determine whether TSP-1 changed inflammation- and apoptosis-related markers, BAC-exposed and unexposed cells were treated with TSP-1, IL-6 and TGF-β2 production, activated caspase-3/7 and cell viability were analyzed.

Regarding IL-6 production, the increased levels observed after BAC exposure compared to unexposed control cells were not significantly different in corneal epithelial cells treated with TSP-1, although the values tended (p=0.09) to be decreased. In conjunctival epithelial cells, which produced less IL-6 than corneal epithelial cells, IL-6 production did not change after TSP-1 treatment (Figure 7A). The second inflammatory marker analyzed, TGF-β2, did not change after BAC exposure in corneal epithelial cells, although active TGF-β2 increased after TSP-1 treatment in control cells (Figure 7B). Interestingly, TSP-1 treatment significantly decreased total and latent TGF-β2 increased in BAC-exposed conjunctival epithelial cells (Figure 7B). The increased levels of activated caspase-3/7 measured after BAC exposure did not change significantly compared to unexposed cells after TSP-1 treatment in either corneal or conjunctival epithelial

cells (Figure 7C). TSP-1 tended (p=0.1) to reduce activated caspase-3/7 levels of unexposed corneal epithelial cells. Cell viability changed due to TSP-1 treatment in corneal epithelial cells. TSP-1 treatment increased cell viability of unexposed cells or tended to (p=0.1) increase cell viability of BAC-exposed corneal epithelial cells. However, TSP-1 treatment did not change cell viability in conjunctival epithelial cells (Figure 7D).

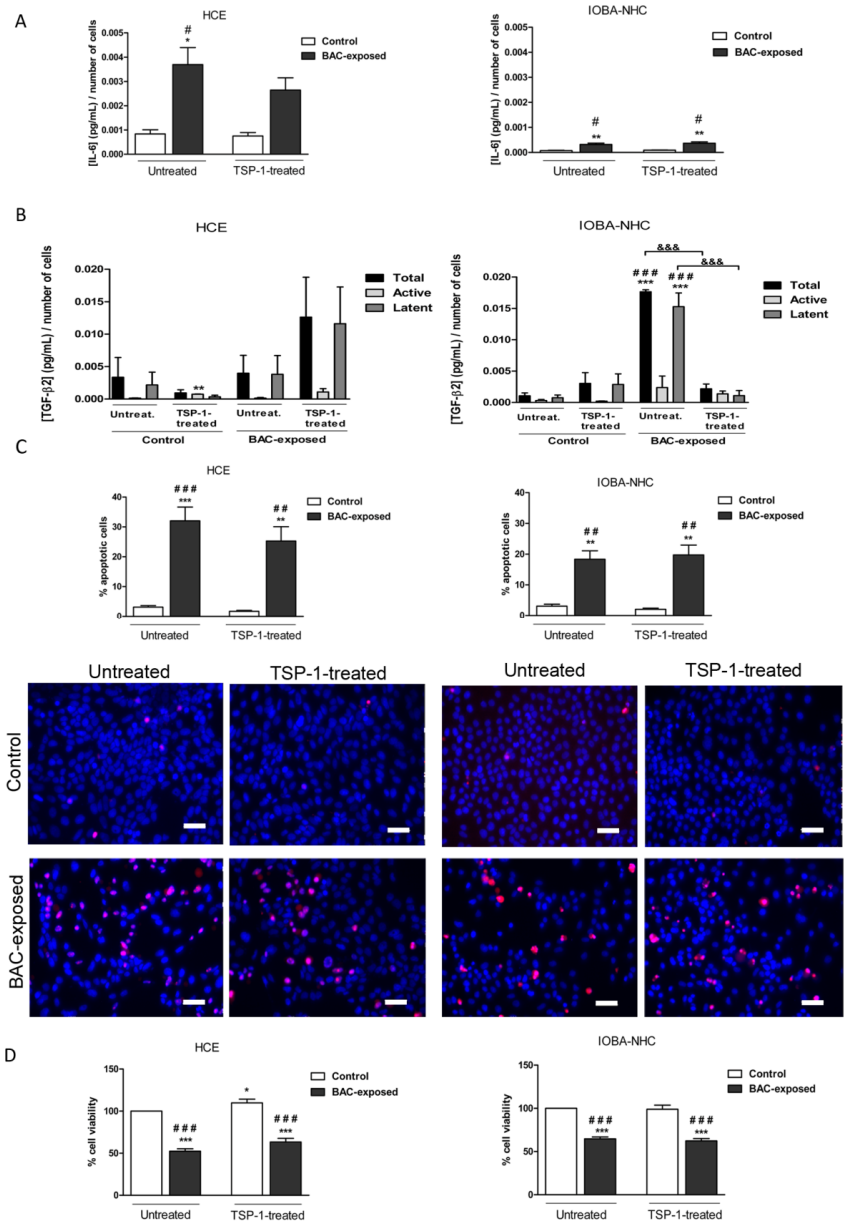

Figure 7: Caption on next page

Figure 7: **Changes in inflammation- and apoptosis-related markers induced by TSP-1 treatment in BAC-exposed corneal (HCE, left panel) and conjunctival (IOBA-NHC, right panel) epithelial cell lines. A.** Production of IL-6 or **B.** total, active and latent TGF-β2 determined by ELISA. Concentration values were normalized to cell numbers determined by alamarBlue. **C.** Percentage of apoptotic cells, quantified through caspase-3/7 positive stained cells / total number of cells, and representative micrographs of cells in the different conditions studied. Nuclei of all cells are stained in blue (Hoechst), while nuclei of apoptotic cells are stained in red (caspase-3/7). Scale bar: 50 μm. **D.** Percentage of cell viability determined by the XTT cytotoxicity assay. Values are expressed as mean ± SEM from, at least, 3 independent experiments in duplicate. Statistically significant differences, when compared to untreated control cells, are indicated with asterisks (Turkey test or Games-Howell test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$), and when compared to control TSP-1-treated cells, are indicated by hashes (Turkey test or Games-Howell test; #$p \leq 0.05$, ##$p \leq 0.01$, or ###$p \leq 0.001$).

3.8. TSP-1 treatment induced differential regulation of CD36 and CD47 levels in corneal and conjunctival epithelial cells under inflammatory and apoptotic conditions

Changes in CD36 and CD47 mRNA levels were analyzed after TSP-1 treatment in corneal and conjunctival epithelial cells (Figure 8). TSP-1 treatment up-regulated CD36 mRNA levels in unexposed corneal epithelial cells; however, it did not change the reduced CD36 mRNA levels in BAC-exposed cells. In conjunctival epithelial cells, TSP-1 treatment did not change CD36 mRNA levels in unexposed cells; however, the decreased CD36 mRNA levels obtained after BAC exposure returned to control values after TSP-1 treatment (Figure 8A).

On the other hand, CD47 mRNA levels also changed after TSP-1 treatment in corneal epithelial cells. TSP-1 treatment up-regulated CD47 mRNA levels in unexposed corneal epithelial cells. However, this up-regulation was not observed in BAC-exposed cells. On the contrary, TSP-1 treatment did not change CD47 mRNA levels in conjunctival epithelial cells (Figure 8B).

A summary of results obtained after exogenous TSP-1 treatment in all conditions studied using the corneal and conjunctival epithelial cell lines is shown in Table 2.

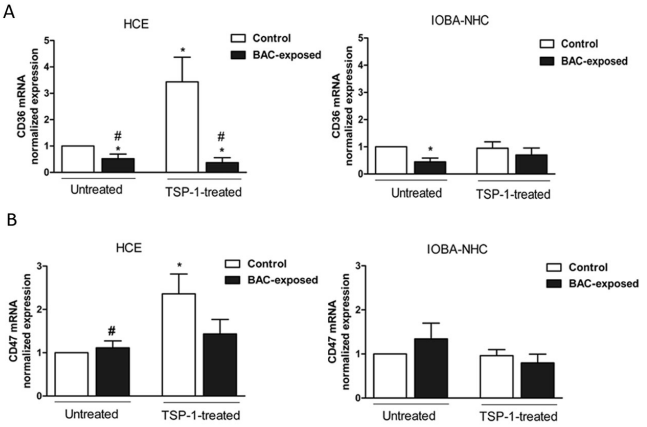

Figure 8: **Changes in CD36 and CD47 mRNA levels induced by TSP-1 treatment in BAC-exposed corneal (HCE, left panel) and conjunctival (IOBA-NHC, right panel) epithelial cell lines. A.** CD36 and **B.** CD47 mRNA levels normalized to GAPDH levels determined by Q-PCR. Values are expressed as mean ± SEM from, at least, 3 independent experiments in duplicate. Statistically significant differences, when compared to untreated control cells, are indicated with asterisks (Tukey test or Games-Howell test; *$p \leq 0.05$), and when compared to TSP-1-treated control cells, are indicated by hashes (Games-Howell test; #$p \leq 0.05$).

Table 2. Summary of results obtained in all conditions studied, indicating changes over basal control in corneal and conjunctival epithelial cell lines. * Cells unexposed to BAC and untreated with TSP-1 were used as control conditions. ↑↓ indicates differences over control conditions (↑ or ↓ = $p \leq 0.05$; ↑ ↑ or ↓ ↓ = $p \leq 0.01$; ↑ ↑ ↑ or ↓ ↓ ↓ = $p \leq 0.001$).

	Corneal cells				**Conjunctival cells**			
	Unexposed		**BAC-exposed**		**Unexposed**		**BAC-exposed**	
	- TSP-1	+ TSP-1	- TSP-1	+ TSP-1	- TSP-1	+ TSP-1	- TSP-1	+ TSP-1
IL-6 production	*	=	↑	=	*	=	↑↑	↑↑
Active TGF-β2	*	↑	=	=	*	=	=	=
Latent TGF-β2	*	=	=	=	*	=	↑↑↑	=
Activated caspase-3/7	*	=	↑↑↑	↑↑	*	=	↑↑	↑↑
Cell viability	*	↑	↓↓↓	↓↓↓	*	=	↓↓↓	↓↓↓
CD36 mRNA	*	↑	↓	↓	*	=	↓	=
CD47 mRNA	*	↑	=	=	*	=	=	=

4. Discussion

TSP-1 was firstly studied as an anti-angiogenic factor, although current knowledge supports its involvement in a variety of molecular processes that are

modulated by this glycoprotein. For instance, TSP-1 role in ocular surface homeostasis was recently demonstrated, as TSP-1 deficient mice develop chronic eye inflammation with age (Contreras-Ruiz *et al*. 2013b, Turpie *et al*. 2009). TSP-1 deficient mice had increased levels of inflammatory cytokines in corneal and conjunctival tissues before inflammatory infiltrates were detectable. These studies give evidence for the role of epithelial cells in inflammatory diseases underpinned by expression and secretion of molecules involved in the inflammatory cascade. Apoptosis pathway is closely linked with inflammation through their common mediators and transduction signals. Both inflammation and apoptosis are key processes linked with TSP-1 through CD36 and CD47 receptors in other cell types or inflammatory contexts. Also, these processes are involved in many severe ocular surface diseases, hence the need to enhance the knowledge of expression and regulation of TSP-1, CD36, and CD47 in corneal and conjunctival epithelial cells in order to understand the mechanisms of their action in corneal and conjunctival impairing diseases.

In this study we induced inflammation and apoptosis in ocular surface epithelial cells *in vitro* using BAC, a well-known preservative used in many ocular surface toxicity studies (Baudouin *et al*. 2010). We characterized the effect of TSP-1 on these processes and related it with the data on basal expression of TSP-1, CD36 and CD47 in human corneal and conjunctival cells. The ability of ocular surface epithelial cells to respond to TSP-1 treatment had not been studied yet.

For characterizing TSP-1 expression we used corneal fibroblasts as positive control, as these cells have a well-documented ability to produce TSP-1 which in turn simulates corneal stroma repair (Hiscott *et al* 1996). In fact, immunofluorescent detection of TSP-1 showed a similar distribution pattern in corneal and conjunctival epithelial cells to that reported for corneal stromal cells. Our results indicate that epithelial and stromal corneal cells produce high levels of TSP-1. This is in agreement with other studies in which TSP-1 was reported as responsible for the integrity and non-vascular repair of corneal injury (Hiscott *et al*. 1996; Uno *et al*. 2004), acting as a local source of TSP-1.

According to our results, corneal stromal cells produced more TSP-1 than epithelial cells, while both corneal cell types produced much more than conjunctival epithelial cells. As the conjunctiva is a vascular tissue, it is a potential site of platelet activation in which the release of TSP-1 can increase within seconds (Legrand *et al*. 1997). This fact could explain the low production of the anti-angiogenic TSP-1 by conjunctival epithelial cells. Our results are consistent with findings from studies using primary cells, in which no differences were obtained at the protein level between corneal and conjunctival tissues (Ma *et al*. 2006). Others authors reported higher TSP-1 mRNA expression in corneal cells than in conjunctival cells extracted directly from human tissues (Sekiyama *et al*. 2006).

The expression of CD36 and CD47 in ocular surface tissues is less well characterized. CD36 protein and mRNA were reported to be expressed in mouse cornea (Cursiefen *et al*. 2004). However, to the best of our knowledge, CD36 has not been studied in human corneal and conjunctival tissues yet. Regarding CD47, its role has not been either described yet in human corneal and conjunctival epithelial cells. In our study, both human corneal and conjunctival epithelial cells expressed protein and mRNA of CD36 and CD47 receptors. Both receptors were immunolocalized in the plasma membrane and showed slightly higher protein expression in conjunctival epithelial cells compared to corneal epithelial cells. Moreover, mRNA levels of TSP-1 receptors were significantly higher in conjunctival epithelial cells than in cornea epithelial cells. Thus, corneal and conjunctival epithelial cells may have a differential response to TSP-1 via these receptors.

To determine the effect of TSP-1 in different *in vitro* microenvironments, cells were treated with rTSP-1 or hCrF-CM. The TSP-1 involvement in inflammation and apoptosis processes in epithelial cells was studied in relation to well-established inflammatory and apoptotic markers. The inflammatory marker IL-6 is one of the increased cytokines in ocular surface inflammatory diseases (Enriquez-de-Salamanca *et al*. 2010, Turner *et al*. 2000) and the ability of both

cell lines used to express IL-6 *in vitro* was previously characterized by our group (Arranz-Valsero *et al*. 2013, Enriquez-de-Salamanca *et al*. 2008). Also, TGF-β2 was analysed, as TSP-1 is one of the master physiologic activators of TGF-β (Murphy-Ullrich and Poczatek. 2000). As early apoptotic marker, we used activated caspase-3 to indicate commitment to apoptosis, while cell viability was determined as a measure of the effects of apoptosis. Corneal and conjunctival epithelial cells were treated with exogenously added TSP-1 and changes in inflammatory or apoptotic markers, together with CD36 and CD47, were studied. Interestingly, we found a different time-dependent effect on the CD36 protein expression between corneal and conjunctival epithelial cells after the TSP-1 treatment at protein level. Corneal epithelial cells increased CD36 protein expression immediately after TSP-1 treatment. However, 24 h after treatment, there were no differences in CD36 protein expression levels. In conjunctival epithelial cells, the increase in CD36 expression was detected 24 h after the TSP-1 treatment. As corneal epithelial cells basally secreted high levels of TSP-1, the effect of exogenously added TSP-1 may not be as clearly seen as in conjunctival epithelial cells. These experiments provided evidence of a regulatory effect of TSP-1 on CD36 in corneal and conjunctival epithelial cells. According to our results, there was no difference in the effect of hCrF-CM or rTSP-1 treatments on corneal epithelial cells. However, in conjunctival epithelial cells, the use of hCrF-CM shows how the effect exerted by the same concentration of TSP-1 changes depending of cell microenvironment. HCrF-CM increased IL-6 production and reduced CD47 expression levels or tended to reduce latent and total TGF-β2 secreted levels compared to control. Also, although CD36 expression levels increased after rTSP-1 treatment, hCrF-CM did not increase CD36 levels. Therefore, conjunctival epithelial cells react to fibroblast-epithelial cell interactions in terms of IL-6, TGF-β2, CD36 and CD47 expression. Different molecules, aside from the increased IL-6, may also be involved in the CD36 and CD47 down-regulation, and further experiments are required to study this effect.

To determine whether changes in CD36 protein levels were due to TSP-1/CD36 and/or TSP-1/CD47 interactions, CD36 and CD47 were blocked using specific abs against the TSP-1 binding site, and CD36 mRNA analysed at different time points. In corneal epithelial cells, which also increased CD36 mRNA after TSP-1 treatment, there were no differences after blocking CD36 or CD47 suggesting the role of both receptors in the up-regulation of CD36 mRNA and protein levels. However, in conjunctival epithelial cells, the tendency to up-regulate CD36 mRNA levels, observed after TSP-1 treatment, was also observed after blocking CD36, but not after blocking CD47. This observation suggests a predominance of the interaction TSP-1/CD47 compared to TSP-1/CD36 in conjunctival epithelial cells.

Inflammation- and apoptosis-related changes induced by BAC exposure were studied. Firstly, to ensure significant reduction of cell viability in corneal and conjunctival epithelial cells, different BAC exposure time points were analyzed. Corneal epithelial cells needed more BAC exposure time than conjunctival epithelial cells to show similar reduction on cell viability. This observation is in agreement with previous reports that found higher reduction of cell viability in BAC-exposed conjunctival epithelial cells than in BAC-exposed corneal epithelial cells (Ayaki *et al*. 2008, Dutot *et al*. 2008). Corneal epithelial cells have a critical role for ensuring optic quality of the eye and an intact corneal epithelium is therefore essential to maintain ocular surface homeostasis. This required role of corneal epithelial cells makes them more resilient and therefore less susceptible to unfavorable conditions, such as BAC exposure or the lack of serum, than conjunctival cells. In addition, BAC exposure highly affected cell-cell junctions, as determined by time-lapse microphotography, which has been already characterized in different *in vitro* induced inflammatory conditions by our group (Contreras-Ruiz *et al*. 2012).

Both IL-6 and activated caspase-3/7 increased in BAC-exposed corneal and conjunctival epithelial cells. Our results confirmed the ability of BAC to activate IL-6 production in epithelial cells, as previously assessed in cultured corneal

epithelial cells (Paimela *et al*. 2012) and further supported by increased expression of IL-6 described in conjunctival epithelial cells from long-term treated glaucoma patients, in which BAC-preserved eyedrops were proposed as possible cause (Bensoussan *et al*. 2003). The apoptosis-related characteristics exhibited by corneal and conjunctival epithelial cells after BAC exposure were also in line with other published results (Guenoun *et al*. 2005), although in another report using corneal epithelial cells an increase in activated caspase-3 was not detected (Paimela *et al*. 2012). In that work, serum-supplemented medium was used after BAC exposure in contrast to our experimental setup. For that reason, we believe that the removal of serum in the culture medium is an issue of great importance to accurately determine the extent of BAC-induced apoptotic changes *in vitro*. Our results also showed an up-regulation of TGF-β2 after BAC exposure in conjunctival epithelial cells, which is consistent with a recent work showing BAC-induced TGF-β signaling pathway-related molecules in conjunctival fibroblasts (Huang *et al*. 2014).

As far as we know, changes in TSP-1, CD36, and CD47 in BAC-exposed human ocular surface epithelial cells had not been previously reported. Although no changes were found in TSP-1 production or CD47 mRNA levels, CD36 mRNA levels did decrease after BAC exposure in both corneal and conjunctival epithelial cells. This fact indicates an involvement of CD36 in inflammatory and apoptotic processes in both epithelial cell types. The CD36 receptor has emerged as a pivotal player in corneal homeostasis. For instance, spontaneous bacterial keratitis is developed in CD36 knockout mice (Klocke *et al*. 2011). It has been documented that hypoxia or inflammatory corneal neovascularization up-regulated CD36 in the mouse cornea (Mwaikambo *et al*. 2006, Mwaikambo *et al*. 2009). Our results provide evidence that CD36 could be a key element not only in corneal tissues, but also in the conjunctival epithelium.

Following the characterization of changes induced by BAC exposure, the ocular epithelial cells response to TSP-1 treatment was further studied in BAC-exposed and unexposed cells under serum-free conditions. TSP-1 induced a

differential response in corneal and conjunctival epithelial cells under inflammatory and apoptotic conditions. Interestingly, TSP-1 was able to reduce IL-6 up-regulated levels by BAC exposure in corneal epithelial cells. On the other hand, although TSP-1 had been shown to induce some changes in apoptosis-related markers and CD36 and CD47 mRNA levels in unexposed corneal epithelial cells, its effects on BAC-exposed cells were less pronounced or even opposite. Interestingly, these cells showed the ability to activate TGF-β2 after TSP-1 treatment in control cells, which had been previously showed after TNFα treatment (Benito *et al*. 2013). The activation of TGF-β though TSP-1/CD36 interaction has been previously showed (Yehualaeshet *et al*. 1999) and may explain the up-regulation of both molecules, TGF-β2 and CD36, after TSP-1 treatment. On the contrary, BAC-exposed conjunctival epithelial cells responded to the TSP-1 treatment down-regulating latent TGF-β2 levels without any effect on CD36 mRNA levels, which had been down-regulated by BAC exposure. Although no differences in active TGF-β2 were observed, the decrease in latent TGF-β2 after TSP-1 treatment suggests the involvement of TSP-1 on TGF-β2 activation also in conjunctival epithelial cells. The ability to activate TGF-β2 by conjunctival cells had been previously reported as well (Benito *et al*. 2013). These results support the importance of TSP-1 as a major TGF-β2 activator in ocular surface tissues, and suggest the influence of inflammatory and apoptotic conditions on this TSP-1 function. This is in agreement with several data reporting that TSP-1 deficient mice exposed to desiccating stress had lower expression of inflammation- and apoptosis-related markers compared to wild type animals (Gandhi *et al*. 2013). Desiccating stress is a well-known cause of inflammation and apoptosis in the ocular surface of mice (Luo *et al*. 2004, Yeh *et al*. 2003), which means that under inflammatory- and apoptotic-induced conditions, other activated mediators could regulate TSP-1 function. Taking all this into account our *in vitro* results highlight a differential response to TSP-1 of ocular surface epithelial cells depending on the physiological conditions,

although caution should be employed in a simple extrapolation to the *in vivo* conditions.

Some limitations of the study may be considered. It must be taken into account that the different endogenous TSP-1 production between corneal and conjunctival epithelial cells caused differences in the final TSP-1 concentration in the culture medium. A concentration-dependent role of TSP-1, even showing opposite results in other processes analyzed, has been reported (Taraboletti *et al*. 1990, Tolsma *et al*. 1993, Yamauchi *et al*. 2002). For this reason, a potential response of conjunctival epithelial cells to TSP-1 should not be completely excluded. On the other hand, *in vitro* conditions do not reproduce or include all active mediators in inflammation or apoptosis regulation present in inflammatory cells (Vallejo *et al*. 2000) or goblet cells (Contreras-Ruiz *et al*. 2013a). However, *in vitro* studies are widely used and necessary for initial molecular studies. In our study, corneal and conjunctival epithelial cell lines were used instead of primary cells due to their availability and as a quantifiable reduction in cell viability was required. There are however differences reported between cell lines and primary cells (De Saint Jean *et al*. 2004, Garcia-Posadas *et al*. 2012, Tong *et al*. 2009).

In conclusion, cultured ocular surface epithelial cells can respond to TSP-1 via CD36 or CD47. We have induced inflammation- and apoptosis-related changes using different BAC exposure times in corneal and conjunctival epithelial cells to further study the effect of TSP-1 under these conditions. Corneal epithelial cells, which produce a significant amount of the TSP-1 glycoprotein *in vitro*, up-regulated the expression of both receptors and active TGF-β2 levels after exogenous TSP-1 treatment. This up-regulation was not observed if cells were under simulated *in vitro* inflammation and apoptosis conditions induced by BAC exposure. However, conjunctival epithelial cells, which produce very low TSP-1 *in vitro*, do not seem to be the main source of TSP-1 in the conjunctiva under inflammatory and apoptotic conditions. However, conjunctival epithelial cells showed the ability to down-regulate BAC-induced latent TGF-β2 levels after TSP-

1 treatment. Nevertheless, caution needs to be exerted in extrapolating these *in vitro* results to the actual *in vivo* situation.

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STUDY 2. Inflammatory Cytokine-Mediated Regulation of Thrombospondin-1 and CD36 in Conjunctival Cells

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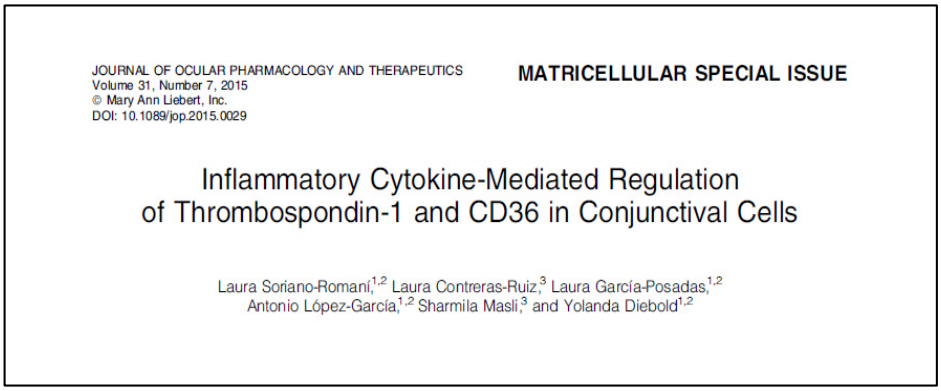

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MATRICELLULAR SPECIAL ISSUE

Inflammatory Cytokine-Mediated Regulation of Thrombospondin-1 and CD36 in Conjunctival Cells

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Inflammatory Cytokine-Mediated Regulation of Thrombospondin-1 and CD36 in Conjunctival Cells

Abstract

Purpose: Increased expression of TGF-β2 is reported in the conjunctiva of dry eye patients with no increase of anti-inflammatory activity of TGF-β2. Our aim was to compare the expression of molecules involved in TGF-β2 activation, TSP-1 and CD36, during murine and human conjunctival inflammation.

Methods: Human conjunctival tissue from cadaveric donors, human conjunctival epithelial primary cells and fibroblasts and murine conjunctivas were immunostained for TSP-1, CD36 or TGF-β2. Inflamed conjunctival tissues were obtained from C57BL/6 wild type (WT) mice induced to develop Experimental Dry Eye (EDE) with 10 days of desiccating conditions and scopolamine injections and TSP-1 deficient (TSP1-/-) mice, which spontaneously develop Sjögren's syndrome-associated conjunctival inflammation with age. Immunostaining intensities were compared using ImageJ software. Cultures of human conjunctival fibroblasts were stimulated with IL-1β and both secreted protein and message levels of TSP-1, CD36 and TGF-β2 were analyzed.

Results: TSP-1 and CD36 were detectable in human and murine conjunctival tissues as well as primary conjunctival epithelial cells and fibroblasts. Increased conjunctival immunostaining of TGF-β2 and reduced CD36 was detected in EDE mice as compared with WT mice. Interestingly, increased TGF-β2 and CD36 conjunctival immunostaining was detected in TSP1-/- mice. The expression of TSP-1 and CD36 was down-regulated in IL-1β-stimulated conjunctival fibroblasts, at both the protein and message level, while active TGF-β2 was undetected.

Conclusions: The absence or reduced expression of either of the molecules involved in TGF-β2 activation supports pro-inflammatory conditions in the conjunctiva. Changes in TSP-1 and CD36 may serve as potential biomarkers of conjunctival inflammation.

Keywords: Cytokine, Dry eye, Fibroblast, Inflammation, Molecular Biology.

Introduction

Conjunctiva is the mucosal tissue of the ocular surface and like other mucosa in the body, contains an associated lymphoid tissue, namely the conjunctival-associated lymphoid tissue (CALT). CALT contains all of the components necessary for a complete immune response[1]. In many cases, diseases of the ocular surface are associated with inflammation and the ocular mucosa plays an imperative role in modulating and resolving inflammation[2,3].

In the mucosal surfaces of the intestine and lung, transforming growth factor-β2 (TGF-β2), is the predominant isoform of TGF-β and plays an important role during inflammatory diseases[4-6]. Recently, TGF-β2 has also been described as the predominant isoform in mouse conjunctiva[7]. Moreover, increased levels of TGF-β2 in the conjunctiva in inflammatory diseases such as dry eye (DE) or vernal keratoconjunctivitis have been reported[8-10], suggesting ineffectiveness of its anti-inflammatory activity. TGF-β2 is secreted in a complex with its propeptide, latency-associated protein 2 (LAP2). In order to become biologically active this complex, LAP2/TGF-β2, must be dissociated during physiologic or pathologic events in a process termed latent TGF-β activation. Appropriate levels of active TGF-β2 are essential to avoid immunological and inflammatory disturbances due to its importance in mediating immune privilege, creating an immunomodulatory environment when activated[11, 12]. Integrins, which are up-regulated during conjunctival inflammation[13], cannot activate TGF-β2 due to the absence of the integrin binding arginine-glycine-aspartic acid (RGD) sequence in the LAP isoform associated with TGF-β2[14, 15].

Thrombospondin-1 (TSP-1) is a matricellular protein reported to efficiently activate TGF-β2[16] via ligation of its receptor CD36[17]. The role of TSP-1 in the ocular surface as a modulator in avascular repair and immunoregulation have been reviewed[18, 19]. On the other hand, CD36 is involved in diverse processes in the ocular surface including angiogenesis, inflammation, and oxidative stress scavenging, depending on the ligands with which CD36 can interact[20, 21].

The notion that both TSP-1 and CD36 are critical for ocular surface homeostasis has recently arisen, as TSP-1 and CD36 deficient goblet cells have been reported as incapable of activating their endogenous TGF-β2[7]. Moreover, TSP-1 deficient mice spontaneously develop conjunctival inflammation with age[22]. Regarding TSP-1 expression in human conjunctiva, some studies reported a mild and focal TSP-1 staining in normal human conjunctival epithelium[23], while others reported detection of the protein in corneal but not in conjunctival epithelia[24, 25] suggesting a pivotal role of TSP-1 in preventing corneal angiogenesis. A reported correlation between a polymorphism in the TSP-1 gene (*THBS1*) and chronic ocular surface inflammation in humans further supports the importance of TSP-1 in immunomodulation[26]. In addition, TSP-1 has been co-localized with CD36 in murine conjunctival goblet cells, and simultaneous TGF-β2 activation was detected, which points to a relevant role of TSP-1-dependent TGF-β2 activation in regulating mucosal immunity[7].

Based on these findings, we hypothesize that TSP-1 and CD36, both TGF-β2 activating factors, are affected by the surrounding inflammatory microenvironment, which would explain the lack of TGF-β2 activation in an inflamed conjunctiva. Thus, our aim was to study changes in TSP-1, CD36 and TGF-β2 in different models of conjunctival inflammation. Murine models of ocular surface inflammatory diseases and *in vitro* stimulation of conjunctival fibroblasts with inflammatory cytokines were used. We conclude that the absence or reduced expression of either of the molecules involved in TGF-β2 activation supports pro-inflammatory conditions in the conjunctiva, suggesting the use of these molecules as potential inflammatory biomarkers.

Methods

Materials

All materials used were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated. Dulbecco's Modified Eagle Medium (DMEM)/F-12 and some of its supplements, such as fetal bovine serum (FBS) and

penicillin/streptomycin, were from Invitrogen-GIBCO (Inchinnan, UK). Human serum (HS) was from Lonza Group Ltd (Basel, Switzerland) and human epidermal growth factor (EGF) and bovine insulin were from Invitrogen (Eugene, OR). Cell culture plates and multichamber Permanox® or glass slides were from Nunc (Roskilde, Denmark).

Primary antibodies (Abs) used for immunofluorescence were goat anti-TSP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-CD36 (Abcam, Cambridge, UK) and rat anti-TGF-β2 (R&D systems, Abingdon, UK). Secondary Abs were Alexa Fluor-conjungated Abs from Invitrogen. Fluoromount-G™ mounting media was from SouthernBiotech (Birmingham, AL). To display fluorescence micrographs a Leica DMI 6000B microscope and the LAS AF Lite software from Leica Microsystems (Wetzlar, Germany) were used.

Enzyme-linked immunosorbent assay (ELISA) kits for human TSP-1 and TGF-β2 were from R&D systems, and for human CD36 from Biorbyt (Cambridge, UK). The alamarBlue® colorimetric indicator assay used to calculate numbers of cells was from AbD Serotec (Oxford, UK). The SpectraMAX® M5 multidetection microplate reader and the SoftMax Pro 4.8 software used to analyze ELISA results were from Molecular Devices (Sunnyvale, CA).

The kit for RNA isolation, RNeasy® Mini Kit, was from Qiagen (Valencia, CA), and kits for RNA quantification (Quant-iT™ RNA assay) and cDNA synthesis (SuperScript® VILO™ cDNA kit) were from Invitrogen. Reagents for reverse transcription polymerase chain reaction (RT-PCR) were from Biotools B&M Labs S.A. (Madrid, Spain), except Blue Juice™ Gel Loading Buffer 10X (Invitrogen) and TSP-1 and CD36 primers (OriGene Technologies, Rockville, MD). SYBR Green PCR Master Mix was from Applied Biosystems (Carlsbad, CA). To visualize agarose gels the ChemiDoc® gel documentation system and Quantity One software from Bio-Rad Laboratories (Hercules, CA) were used. The conventional RT-PCR was done in a MyGene™ L Series Peltier Thermal Cycler from LongGene® Scientific Instruments Co., Ltd (Tuen Mun, Hong Kong), and Real Time RT-PCR (qPCR) was done in a 7500 Real-Time PCR System from Applied Biosystems.

For statistical analyses, Statistical Procedures for the Social Sciences software (SPSS 20.0; SPSS Inc., Chicago, IL) was used.

Mice and Murine Experimental Dry Eye (EDE) model

Wild type C57BL/6 (H-2b) mice (WT) between 6 and 12 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and Charles River Laboratories (Wilmington, MA). TSP-1 deficient mice of C57BL/6 background (TSP1-/-) were purchased from The Jackson Laboratory. These mice were subsequently bred in-house in a pathogen-free facility at Boston University, Boston, MA and were used at 10 weeks of age. C57BL/6 and TSP-1 deficient mice were euthanized by CO_2 inhalation and their eyeballs with attached eyelids were excised. All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University School of Medicine.

Tissue sections of eyeballs with attached eyelids from C57BL/6 mice to which experimental dry eye (EDE) had been induced as previously described[27], were kindly provided by Dr. Michael E. Stern, from Allergan Inc. (Irvine, CA). Briefly, EDE was induced by injecting scopolamine (0.5 mg in 0.2 ml) subcutaneously three times a day in alternate flanks for 10 days. Mouse cages were placed in front of a fan directed to blow air through the wired screen side of the cage 24 h per day with controlled humidity (less than 40%). After 10 days under desiccating conditions and scopolamine injections, eye tissues were excised.

Human tissues

Healthy human conjunctival tissues were obtained from corneoscleral buttons from cadaveric donors (n = 8; mean age ± standard error of the mean: 77.57 ± 4.02 years). Corneoscleral buttons were obtained with informed research consent from Barraquer Eye Bank (Barcelona, Spain). This study was in

strict accordance with the Tenets of the Declaration of Helsinki and Spanish Regulations concerning the use of Human Tissues for Biomedical Research, and had the approval of the Ethics Committee of the University of Valladolid.

Isolation and culture of epithelial and stromal primary cells from human conjunctival tissues

Human bulbar conjunctiva was carefully isolated, and epithelial and stromal (fibroblasts) cells were obtained using the explant technique as previously described[28] and optimized[29]. In brief, conjunctiva was carefully cut into small pieces and plated in 12-well plates using the epithelial culture media described below. Explants were fed by superficial tension until cell growth was observed. After that, more culture medium was added. The epithelial culture medium comprised DMEM/F12 supplemented with 2.5 µg/mL fungizone, 5,000 units/mL penicillin/streptomycin, 1 µg/mL insulin, 0.5 µg/mL hydrocortisone, 2 ng/mL EGF, and 10% HS.

Cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C and the medium was changed every other day. To ensure the purity of the cultures differential trypsinization with 0.25% trypsin/EDTA was used. Different trypsinization times were used to obtain fibroblasts (2 min) or epithelial cells (5 min). Cell morphology was evaluated by phase contrast microscopy, showing the common polygonal or spindle-shaped cells for epithelial or fibroblast cell types, respectively. Polygonal cells were fed with the epithelial culture medium described above, and fibroblasts with culture medium comprising DMEM/F12 supplemented with 2.5 µg/mL fungizone, 5,000 units/mL penicillin/streptomycin, and 10% FBS.

Epithelial cells from passages 0-1 or conjunctival fibroblasts from passages 2-4 were used. Previously, the purity of epithelial and fibroblast cultures had been assessed by specific linage markers using previously tested Abs[29].

In vitro induction of inflammation to human conjunctival primary cells

Conjunctival fibroblasts were cultured for 48 h in a 24-well plate. Prior to treatments, the cells were maintained in serum-free medium for 1 h. Then the cells were stimulated with 10 ng/mL IL-1β in 10% serum-supplemented or serum-free culture medium for 24 h. Untreated cells were used as controls. After treatment the supernatants were collected and RNA extracted. The alamarBlue® assay was performed after supernatant collection to calculate the number of cells. At least 3 independent experiments were performed.

TSP-1, CD36 and TGF-β2 detection by immunofluorescence

TSP-1, CD36, and TGF-β2 were immunodetected in murine and human conjunctival tissues, and in cultured conjunctival cells. Murine and human ocular tissues were rinsed with 15% sucrose in PBS, and then placed in 30% sucrose at 4˚C overnight. Tissues were then embedded in optimal cutting temperature compound and frozen. Cryostat sections (7 µm) were collected on poly-L-lysine-treated slides, fixed with acetone for 10 min and kept at -80˚C during shipment and until use.

Epithelial cells and fibroblasts were seeded onto 8-well multichamber Permanox® or glass slides respectively, and grown for 48 h. Then the cells were fixed in ice-cold methanol and kept at -20˚C until use.

Tissue cryosections and fixed cells were washed in phosphate-buffered saline (PBS) and incubated at room temperature (RT) for 2 h with blocking buffer composed of 2% bovine serum albumin and 0.3% Triton X-100 in PBS to block non-specific binding and allow permeability of the cell membrane. Afterwards, primary Abs against TSP-1 and CD36 (5 µg/mL), and TGF-β2 (10 µg/mL) were incubated overnight at 4°C in blocking buffer. 10 µg/mL of Alexa Fluor-conjugated secondary Abs were incubated for 1 h at RT. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI) or Hoechst. Negative controls included omission of primary Abs. Cells were observed under a dry objective and same exposure time, gain, and intensity were used for all the

pictures taken. To quantify TSP-1, CD36 or TGF-β2 immunofluorescence in murine conjunctivas, the two-channel micrographs were analyzed using ImageJ software v. 1.49 (http://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD). Then, micrographs were split into different channels and each channel thresholded. To subtract background in the red channel (corresponding to the protein of interest), negative controls were used to set threshold and the same value was used in all micrographs being analyzed from the same experiment, at the same time. The threshold for the blue channel, corresponding to the nuclei area, was set according to the area stained. Mean grey value was measured redirecting the measurement to the corresponding channel. The results shown are the ratio of the mean grey value for the red channel to that obtained from the blue channel from 2 independent animals, in triplicate.

TSP-1, CD36, and TGF-β2 measurement by enzyme-linked immunosorbent assay (ELISA)

An instant ELISA was used to determine the levels of TSP-1, CD36 and TGF-β2 in cell culture supernatants of conjunctival primary cells. The assays were performed according to the manufacturers' protocols. For TGF-β2 measurements, non-acidified (active TGF-β2) was assayed. The minimum detectable dose (MDD) for the human TSP-1, CD36, and TGF-β2 ELISAs were 355, 10 and 7 pg/mL, respectively, according to each ELISA kit's instructions.

The obtained concentration values were normalized against cell numbers obtained using the alamarBlue® assay. A standard curve representing the relationship between fluorescence intensity (560 nm excitation / 590 nm emission) and number of cells seeded had been done previously for each cell type, to normalize quantified concentrations. The results shown are the mean of concentration / number of cells calculated.

Quantification of TSP-1 and CD36 mRNA expression levels by reverse transcription polymerase chain reaction (RT-PCR) and SYBR-Green real time RT-PCR (qPCR)

Cell lysates were prepared from human conjunctival tissues, conjunctival epithelial cells and fibroblasts. Isolation and purification of total RNA was done using the RNeasy® Mini Kit following the manufacturer's instructions. Samples were then incubated with DNase for 10 min at RT, to remove any contaminating genomic DNA. Total RNA was quantified using the Quant-it™ RNA Assay Kit, with the Qubit fluorometer. Then cDNA was synthesized using the SuperScript® VILO™ cDNA synthesis Kit with 1 µg of RNA. For RT-PCR, 1 µL of primers, 50 ng of cDNA, 5 µL of PCR buffer 10X, 1 µL dNTPs and 1 µL Taq polymerase were mixed in a final volume of 50 µL. The thermal profile used was: 95˚C for 120 s, 39 cycles of 95˚C for 20 s, 60˚C for 30 s, 72˚C for 40 s and 72˚C for 600 s. RT-PCR products were resolved on 4% agarose gels.

For qPCR, 1 µL primers, 10 ng cDNA, and 10 µL SYBR Green PCR Master Mix were mixed in a final volume of 20 µL, using a thermal profile of: 50˚C for 120 s, 95˚C for 600 s, followed by 40 cycles of 95˚C for 15 s and 60 ˚C for 60 s, and a final cycle of 95˚C for 90 s. All reactions were performed in duplicate. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method[30], using the endogenous GAPDH signal for normalization. A negative, no-template control was included. Also, to ensure the specificity of the PCR products a melting curve analysis was performed. At least three independent experiments were performed.

The primer pairs used for both RT-PCR and qPCR, were GAPDH (sense: 5´-GAACGTGAAGGTCGGAGTCAAC-3; antisense: 5´CGTGAAGATGGTGATGGGATTTC-3´), TSP-1 (Catalog No. HP206797), and CD36 (Catalog No. HP200058).

Statistical Analyses

Data are expressed as the mean ± standard error of the mean (SEM). A Student´s *t*-test was used to compare groups. If groups had variances

significantly different (F test), Welch´s correction was done. Statistical significance of $p \leq 0.05$ was considered.

Results

Altered conjunctival expression of TGF-β2 and CD36 in experimental mouse models with conjunctivitis

To determine the expression of TSP-1, CD36 and TGF-β2 in conjunctival inflammation mouse models with reported conjunctivitis were used. In the EDE model exposure to a desiccating stress environment is known to induce conjunctival inflammation, whereas it is reported to develop spontaneously in TSP-1 deficient mice. Frozen sections of conjunctiva tissues harvested from WT, EDE and TSP-1 deficient mice were immunostained for CD36 and TGF-β2 while WT and EDE tissues were also immunostained for TSP-1.

The staining pattern for all the three molecules were evenly distributed predominantly in the epithelial layer of the mouse conjunctiva. The signal quantification revealed significant differences between mice with normal and inflamed conjunctiva. Significantly increased TGF-β*2* conjunctival staining was detected in both EDE and TSP-1 deficient mice as compared to normal WT mice (Figures 1 & 2). No difference was detectable in TSP-1 immunofluorescence, while staining for CD36 was significantly reduced in EDE conjunctival tissues compared to control conjunctival tissues (Figure 1). However, immunofluorescence of CD36 was significantly increased in TSP-1 deficient conjunctiva compared to normal control conjunctiva tissue (Figure 2).

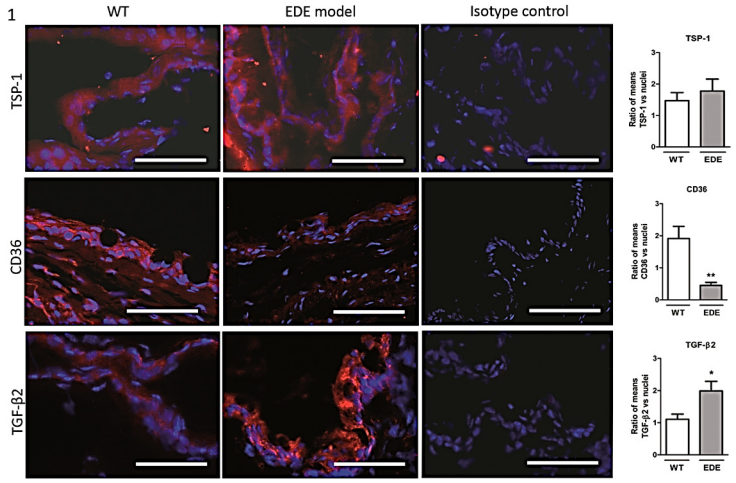

Figure 1: **Changes in TGF-β2 and CD36 conjunctival immunostaining in Experimental Dry Eye (EDE) mice compared to wild type (WT) mice.** Representative micrographs of TSP-1, CD36 and TGF-β2 immunostaining in WT and EDE model conjunctivas. Nuclei were stained in blue with Hoechst dye. Isotype controls include omission of primary antibodies. Scale bar: 50 µm. Immunofluorescence intensity analysis of images is presented in bar graphs as the mean ratio between protein of interest/nuclei ± SEM. Statistically significant differences between samples are indicated with asterisks (*t*-test; **p* ≤ 0.05, ***p* ≤ 0.01).

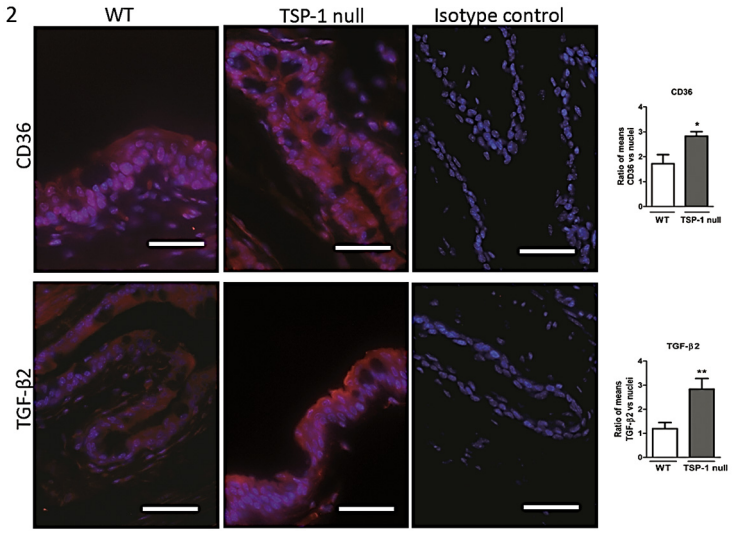

Figure 2: **Changes in TGF-β2 and CD36 conjunctival immunostaining in TSP-1 deficient (TSP-1 -/-) mice compared to wild type (WT) mice.** Representative micrographs of CD36 and TGF-β2 immunostaining in conjunctivas of WT and TSP-1 deficient mice. Nuclei were stained in blue with DAPI. Isotype controls include omission of primary antibodies. Scale bar: 50 µm. Immunofluorescence intensity analysis of images is presented in bar graphs as the mean ratio between protein of interest/nuclei ± SEM. Statistically significant differences between samples are indicated with asterisks (*t*-test; **p* ≤ 0.05, ***p* ≤ 0.01).

Expression of TSP-1 and CD36 in human conjunctival tissue and primary cells

To determine if TSP-1 and CD36 are detectable in human conjunctiva their expression was analyzed in human conjunctival tissues and primary cultures of conjunctival epithelial cells and fibroblasts. Both TSP-1 and CD36 were immunolocalized in human bulbar conjunctival tissue as well as in primary epithelial cells and fibroblasts (Figure 3A). In conjunctival tissue, TSP-1 and CD36 were mainly localized in conjunctival epithelium, although some staining was detectable in the stroma. In epithelial cells and fibroblasts TSP-1 appeared distributed in the cultured cell, showing higher intensity in the perinuclear area. CD36, an integral membrane receptor, was evenly distributed in the epithelial and fibroblast cell membrane.

The levels of TSP-1 were assessed in culture supernatants collected from confluent monolayers of primary conjunctival cells cultured for 3 days in the presence of 10% serum. Significantly increased basal levels of TSP-1 were detected in supernatants derived from conjunctival fibroblast cell cultures as compared to those derived from conjunctival epithelial cell cultures (Figure 3B). CD36 was undetectable in conjunctival cell supernatants. Message for TSP-1 and CD36 was detected in conjunctival tissue as well as primary conjunctival cells (Figure 3C).

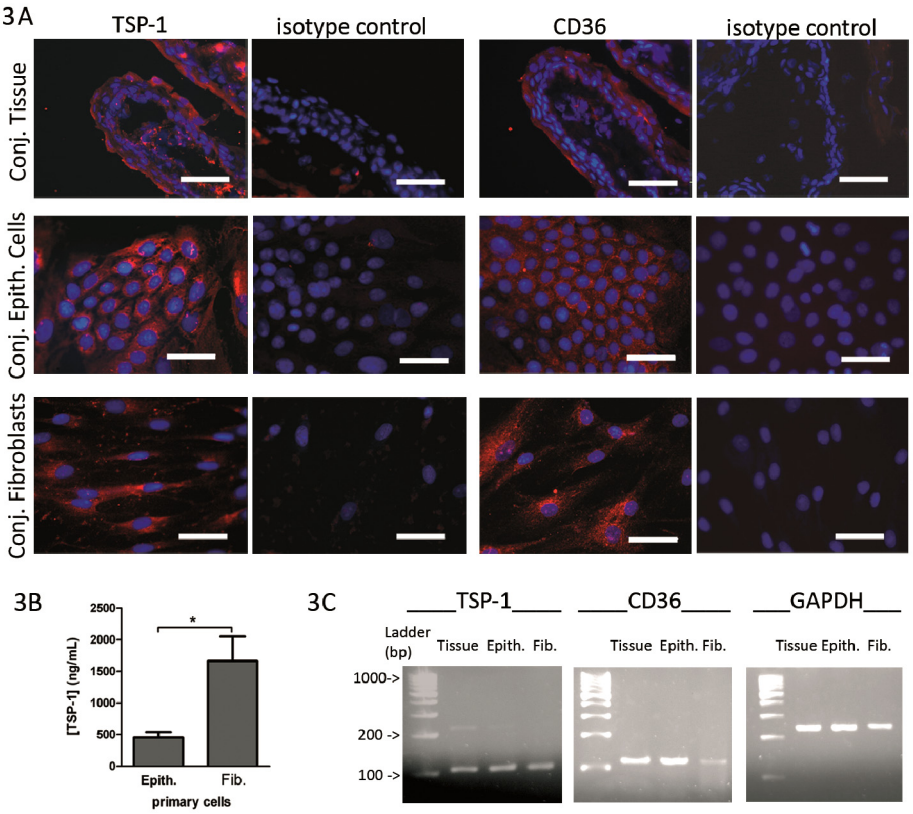

Figure 3: **The expression of TSP-1 and CD36 in human conjunctival tissue and primary cultures. A.** Immunofluorescence micrographs of TSP-1 and CD36 (in red) of conjunctival tissue (Conj. Tissue), conjunctival epithelial cells (Conj. Epith. Cells), and fibroblasts (Conj. Fibroblasts). Nuclei of cells were stained in blue with Hoechst dye. Isotype controls include omission of primary antibodies. Scale bar: 50 µm. **B.** Detection of TSP-1 in culture supernatants of confluent epithelial cells (Epith.) and fibroblasts (Fib.) after 3 days in serum-supplemented culture by ELISA. Bars represent the mean TSP-1 concentration (ng/mL) ± SEM. Statistically significant differences between samples are indicated with asterisks (*t*-test; $*p \leq 0.05$, $**p \leq 0.01$). **C.** Agarose gels showing mRNA expression of TSP-1, CD36 and GAPDH, determined by RT-PCR of conjunctival tissue (tissue), conjunctival epithelial cells (Epith.) and fibroblasts (Fib.).

Changes in TSP-1, CD36, and TGF-β2 expression in IL-1β-stimulated conjunctival fibroblasts

The pro-inflammatory cytokine IL-1β is detectable in human ocular surface tissue during dry eye and is considered a consistent marker of this condition[31-33]. Therefore primary cultures of conjunctival stromal cells, fibroblasts, were

stimulated with IL-1β and changes in their expression of TSP-1 and CD36 were assessed in the context of their ability to secrete active TGF-β. First, the toxicity of IL-1β was determined by assessing cell numbers using the alamarBlue® assay. Both serum containing (10% FBS) and serum free culture conditions were tested. No significant cell loss was detected in either culture condition in IL-1β stimulated cells compared to untreated control cells (data not shown). Levels of TSP-1 and active TGF-β2 protein secreted in culture supernatants were determined by ELISA. While no significant change in TSP-1 levels were detected in serum-containing cultures after IL-1β stimulation, in serum-free conditions TSP-1 levels were significantly reduced after IL-1β stimulation compared to untreated control cultures (Figure 4A). However, qPCR analysis indicated significant reduction in TSP-1 mRNA in IL-1β-stimulated cells in both culture conditions (Figure 4B).

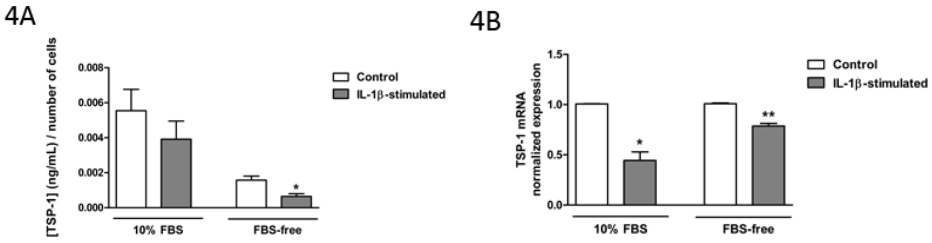

Figure 4: **Changes in TSP-1 expression in conjunctival fibroblasts mediated by IL-1β-stimulation. A**. Detection of TSP-1 in culture supernatants as determined by ELISA. **B.** TSP-1 mRNA levels normalized to GAPDH levels and referenced to control conditions, determined by qPCR. 10% serum-supplemented conditions are indicated by 10% FBS and serum-free conditions by FBS-free. Values are expressed as mean ± SEM from 3 independent experiments. Statistically significant differences, when compared to untreated control cells, are indicated with asterisks (*t*-test; *$p \leq 0.05$, **$p \leq 0.01$).

Active TGF-β2 levels were undetectable in culture supernatants collected from IL-1β-stimulated cells as compared to untreated cells (Figure 5A). These results are consistent with significantly reduced CD36 mRNA levels in IL-1β-stimulated conjunctival fibroblasts cultured under both serum-containing and serum-free conditions (Figure 5B).

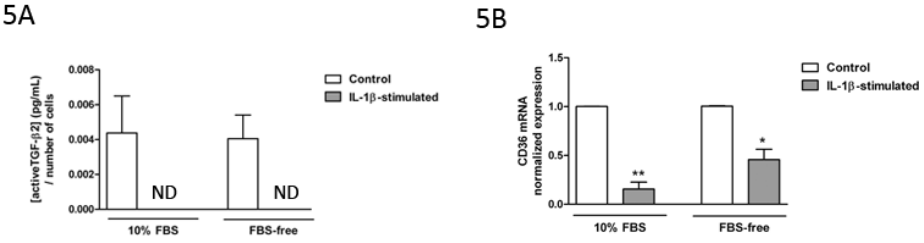

Figure 5: **Changes in active TGF-β2 levels and CD36 mRNA levels in conjunctival fibroblasts stimulated with IL-1β. A**. Levels of active TGF-β2 secreted by conjunctival fibroblasts in serum-free conditions determined by ELISA. Concentration values were normalized to cell numbers determined by the alamarBlue® assay. **B.** Levels of CD36 mRNA normalized to GAPDH levels and referenced to control conditions, determined by qPCR. 10% serum-supplemented conditions are indicated by 10% FBS and serum-free conditions by FBS-free. Values are expressed as mean ± SEM from, at least 3 independent experiments. Statistically significant differences, when compared to untreated control cells, are indicated with asterisks (*t*-test; *$p \leq 0.05$, **$p \leq 0.01$). ND: Not detected.

Discussion

In this study we used not only inflammatory murine models, but also cytokine-induced inflammation in human conjunctival fibroblasts to analyze changes in TSP-1, CD36 and/or TGF-β2. Murine models included an EDE model[34] induced in C57BL/6 mice and a spontaneous model in TSP-1 deficient mice that develop a well-characterized chronic dry eye[22,35]. The induced EDE model has been characterized showing a reduction in goblet cell number and increased inflammatory cytokines in the conjunctiva[34]. Also, in TSP-1 deficient mice a damaged and inflamed conjunctival epithelial barrier develops spontaneously with age accompanied by increased apoptosis in the lacrimal gland and reduced goblet cell density and tear mucin secretion. Pro-inflammatory cytokines such as IL-6 and TNFα, among others, are increased in the conjunctiva of 6-week old TSP-1 deficient mice[22]. Our results showed higher expression of TGF-β2 in conjunctival tissues of EDE and TSP-1 deficient mice compared to those tissues of WT mice, as determined by immunofluorescence detection. It has been previously reported that no TGF-β2 increase is detectable at message or protein levels in mice exposed to desiccating stress for 5 or 10 days[8,36]. In those studies, mice were exposed to the airflow acutely during 16 or 18 h, while our model

included 24 h of airflow exposure. This fact, together with different techniques used to compare TGF-β2 expression may explain the different results obtained. Our results obtained in murine models are in agreement with previously reported results in human conjunctival cells harvested from DE patients with respect to increased levels of TGF-β2[9].

In addition, our results indicate unaltered TSP-1 expression in EDE conjunctiva with significantly reduced CD36 expression in contrast to the increased CD36 expression in TSP-1 deficient conjunctiva. Several *in vitro* studies have reported expression of both pro-inflammatory[37,38] and anti-inflammatory[39-41] cytokines in macrophages resulting from the ligation of CD36 by ligands like apoptotic cells, oxidized low density lipoprotein (oxLDL) and TSP-1, but not on epithelial cells. However, *in vivo* studies appear to predominantly support anti-inflammatory role of CD36[41-43]. In particular, reduced CD36 expression in respiratory as well as intestinal mucosa was associated with the development of inflammation[42,43]. In conjunctival epithelial cells, TSP-1:CD36 interaction is reported to activate immunoregulatory cytokine TGF-β[7]. Therefore it is conceivable that in conjunctival mucosa CD36 down-modulates inflammatory immune responses via binding TSP-1 and activating TGF-β. On the one hand such a function of CD36 may explain its reduced expression in EDE, while on the other hand the absence of TSP-1 may allow increased detection of unoccupied CD36 as noted in TSP-1 deficient mice. Thus together our findings corroborate other studies supporting anti-inflammatory role of CD36 and suggest that the absence or reduced expression of either CD36 or TSP-1, molecules involved in TGF-β2 activation, may result in inflammatory conditions in the conjunctiva.

We evaluated TSP-1 and CD36 expression in human conjunctival tissue from cadaveric donors and in human conjunctival epithelial cells and fibroblasts. To our knowledge, characterization of TSP-1 and CD36 had not been previously reported in human conjunctival primary cells. Expression of TSP-1 and CD36 in murine goblet cell primary cultures has been recently described[7]. We detected the expression of both TSP-1 and CD36 at the protein and message level in

conjunctival epithelial cells and fibroblasts. Therefore, both conjunctival epithelial cells and fibroblasts can activate TGF-β2 locally in the conjunctiva. Since both showed a similar expression pattern but fibroblasts expressed higher levels of TSP-1 than epithelial cells, this cell type was chosen to study further the role of TSP-1 in an inflammatory environment. The activation of fibroblasts during keratoconjunctivitis further supports the use of this cell type[44].

In addition, in inflammation-based diseases such as DE, loss of TGF-β2-expressing goblet cells in the ocular surface[45] suggests an involvement of other cell types in the immunomodulatory process. Recent research indicates that fibroblasts can contribute to the inflammatory reaction by releasing inflammatory modulators in response to different cytokines. In particular, IL-1β has been previously identified in the tears and conjunctival epithelium of patients with ocular inflammatory disease[31-33]. Moreover, IL-1β has been previously used to study the conjunctival inflammatory processes as increased release of inflammatory cytokines and chemokines, such as IL-6 or IL-8, has been observed in IL-1β-stimulated conjunctival cells[46,47]. Therefore we chose to activate our conjunctival fibroblast cultures with the inflammatory cytokine IL-1β in 10% serum-supplemented or serum-free culture medium, and changes in TSP-1, CD36 and TGF-β2 were analyzed. It is also important to take into account IL-1β mediated regulation of the fibroblast cell proliferation rate as reported previously [48,49]. For this reason, cell number was measured after stimulation and used to normalize secreted protein levels. Consistent with previous reports, we detected increased proliferation after IL-1β stimulation of conjunctival fibroblasts in serum-free conditions (data not shown).

We observed that conjunctival fibroblasts respond to an *in vitro* IL-1β-induced inflammatory stimulation by altering their TSP-1, CD36 and TGF-β2 expression. Reduced expression of TSP-1 was detected at both the protein and message level in IL-1β-stimulated conjunctival fibroblasts. This result agreed with a previously reported association between reduced levels of TSP-1 with increased IL-1β in ocular surface epithelial cells derived from individuals with

chronic dry eye[26]. Although consistent with previously reported increased TGF-β2 message in human conjunctiva[9] we detected increased TGF-β2 protein in mouse conjunctiva during dry eye, interestingly, biologically active TGF-β2 was undetectable in IL-1β-stimulated cells. This result was consistent with their down-regulated CD36 and TSP-1 message. These findings further support the importance of TSP-1 and CD36 in TGF-β2 activation.

Detection of changes in TSP-1 and CD36 expression by conjunctival fibroblasts in serum-free culture condition but not in serum-supplemented cultures can be explained by the high TSP-1 content of the serum. Clearly the changes induced by IL-1β stimulation in cells were masked in the presence of serum-derived exogenous TSP-1. The clear differences noted in our results in the presence and absence of serum may also help explain the differences in observations often encountered with the use of cell lines and primary cells that differ in their serum supplementation needs for growth in culture.

To conclude, in this study we show that TSP-1 and CD36 are affected by the inflammatory environment both *in vitro* and *in vivo*. Conjunctival fibroblasts are able to respond to inflammatory mediators such as IL-1β in the tissue environment by down-regulating TSP-1 and CD36 expression. This suggests a lower availability of these molecules for TGF-β2 activation and would consequently allow progression of the inflammation. How this scenario would affect epithelial cell response remains to be elucidated and warrants further studies. Our results suggest that TSP-1 or CD36 may serve as potential biomarkers and conjunctival fibroblasts a potential screening tool to detect ocular surface inflammation.

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Author Disclosure Statement

No competing financial interests exist to disclose

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STUDY 3. Thrombospondin-1-Derived Peptide KRFK Modulates Dendritic Cell Phenotype *In Vitro* and *In Vivo*

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Thrombospondin-1-Derived Peptide KRFK Modulates Dendritic Cell Phenotype *In Vitro* and *In Vivo*

Abstract

Chronic ocular surface inflammatory disorders can lead to an impaired vision with a negative impact in the quality of life of subjects. The understanding of the molecular mechanisms involved in the chronic inflammatory response may be of a great value to identify novel molecular targets, as current treatments can lead to several complications. Recently, thrombospondin-1 (TSP-1) has been proposed as key element in ocular surface homeostasis. In this study, we assessed the potential of a TSP-1-derived peptide that activates transforming growth factor (TGF)-β, the KRFK peptide, as a topically applicable therapeutic in the treatment of ocular surface inflammation. The objective was to determine the ability of the peptide to activate dendritic cell (DC)-derived latent TGF-β and to influence their phenotype *in vitro*. Additionally, we determined if topically administered TSP-1-derived peptide KRFK can cross the ocular surface barriers and lead to changes in systemic immunity *in vivo*. Our *in vitro* results confirmed that the chosen peptide increases activated TGF-β derived from TSP-1-deficient DC in culture. In addition, such increase in activated TGF-β correlated with an anti-inflammatory tolerogenic DC phenotype, as observed by a low expression of major histocompatibility complex (MHC) class II and the co-stimulatory molecule CD80. The ability of a fluorescence-conjugated KRFK peptide to penetrate ocular surface epithelia after topical instillation was assessed. The results indicate that part of the peptide successfully crossed the conjunctival epithelium up to 1 h post-instillation, when the instilled peptide was detectable in different ocular surface structures. Such topical application of the peptide in a pre-clinical mouse model of chronic ocular surface inflammation (TSP-1-deficient mice) led to a significant reduction in pathogenic T helper type 1 (Th1: CD4+ IFN-γ+) and type 17 (Th17: CD4+ IL-17+) with an increase in regulatory T cells (Treg: CD4+ Foxp3+) in cervical lymph nodes. These systemic changes correlate with the ability of the peptide to support tolerogenic phenotype of DCs. In conclusion, this work

suggests a strong therapeutic potential of topically administered TSP-1-derived peptide KRFK in the treatment of chronic inflammatory disease that affects the ocular surface. The systemic effects of such topical application may improve the clinical signs of the ocular surface disease in TSP-1-deficient mouse model.

Keywords

Conjunctiva; Dendritic Cell; KRFK peptide; Thrombospondin-1; Transforming growth factor-β.

Abbreviations

APC: antigen-presenting cell
BFA: brefeldin A
BMDC: bone marrow derived dendritic cell
DC: dendritic cell
DMEM: Dulbecco's modified Eagle medium
Iono: ionomycin
ELISA: enzyme-linked immunosorbent assay
FITC: fluorescein isothiocyanate
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GM-CSF: granulocyte-macrophage colony stimulating factor
H&E: hematoxylin and eosin
IFN: interferon
IL: interleukin
LAP: latency-associated peptide
MHC: major histocompatibility complex
PMA: phorbol 12-myristate 13-acetate
RT: room temperature
RT-PCR: reverse transcription polymerase chain reaction
SEM: standard error of the mean
TEER: transepithelial electrical resistance
TGF: transforming growth factor
Th: T helper
TNF: tumor necrosis factor
Treg: regulatory T cell
TSP-1: thrombospondin-1
WT: wild type.

1. Introduction

Thrombospondin-1 (TSP-1) is an important contributor to mucosal immune homeostasis at the ocular surface. It was described as the major activator of transforming growth factor (TGF)-β *in vivo* (Crawford et al. 1998). In the ocular mucosa its significance is related to its ability to activate the predominant isoform: TGF-β2 (Contreras-Ruiz & Masli 2015). While integrins, activators of latent TGF-β at most mucosal surfaces, fails to activate this isoform due to an absence of the integrin binding RGD sequence in the latency-associated peptide (LAP)-2 of the TGF-β2 isoform (Annes et al. 2003; Munger et al. 1999), TSP-1, however, can activate this isoform (Ribeiro et al. 1999). At the ocular surface, the expression of both, TSP-1 and TGF-β2 defines the phenotype of neighboring antigen-presenting cell (APC) populations towards an immature state, highlighting this integrin-independent mechanism to activate TGF-β in ocular mucosal immunity (Contreras-Ruiz & Masli 2015).

These results explain the spontaneous development of chronic inflammatory disease of the ocular surface in mice with TSP-1 deficiency (Contreras-Ruiz et al. 2013; Turpie et al. 2009). This chronic disease is also associated with a peripheral imbalance between inflammatory and regulatory T cells. In humans, a polymorphism in the gene encoding TSP-1 that results in reduced TSP-1 protein expression is related with a susceptibility to chronic ocular surface inflammatory disease (Contreras-Ruiz et al. 2014). Collectively, these findings support the importance of TSP-1 in maintaining ocular mucosal immune homeostasis.

Considering that the ocular mucosa depends on TSP-1 to activate its endogenous TGF-β2 in the tissue microenvironment and to regulate local APC phenotype critical for maintaining peripheral immune balance, one therapeutic approach to treat ocular surface inflammation may be the use of peptides derived from TSP-1 that are capable of activating TGF-β. For ocular surface disorders topical instillation is the most usual and preferred non-invasive method for drug administration. Nevertheless, the ocular surface is the first defensive barrier against external threats and is very specialized to prevent the

passage of any substance. From the pharmacological point of view, an instilled drug has to overcome 3 main ocular barriers: the tear film, rapid drainage through the naso-lacrimal duct, and the impermeability of the corneal epithelium (Davies 2000; Urtti 2006). The conjunctival epithelium, however, with greater tissue surface area for drug contact is also 15 to 25 times more permeable than corneal epithelium (Hämäläinen et al. 1997).

A unique amino acid sequence, RFK, localized between the first and the second type 1 TSR repeats in the TSP-1 molecule is the minimal sequence required to activate TGF-β (Schultz-Cherry et al. 1995). The TSP-1-derived peptide KRFK was effective *in vivo* when administered intraperitoneally in young TSP-1-deficient mice in successfully reverting the airway epithelial hyperplasia, pancreatic islet hyperplasia and the acinar hypoplasia developed in these mice (Crawford et al. 1998). Also, KRFK peptide administration reverted the abnormal healing in skin wounds characteristic also in TSP-1-deficient mice (Nor et al. 2005).

This study tested the hypothesis that if a TSP-1-derived peptide that activates TGF-β, the KRFK peptide, overcomes ocular surface pharmacological barriers, it would help to activate TGF-β in the ocular mucosal tissue and facilitate modulation of local DC phenotype thereby restoring peripheral immune regulation.

2. Methods

2.1. Reagents and equipment

All materials used were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated. RPMI-1640 medium, penicillin and streptomycin, nonessential amino acid mixture, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, sodium pyruvate, fetal bovine serum and human serum were all from Lonza (Basel, Switzerland). Dulbecco's Modified Eagle Medium (DMEM)/F-12, human epidermal growth factor, bovine insulin were from Thermo Fisher Scientific (Waltham, MA). Murine recombinant granulocyte-

macrophage colony stimulating factor (GM-CSF) was from Biolegend (San Diego, CA). TGF-β activating peptides, both TSP-1-derived peptide KRFK and fluorescein isothiocyanate (FITC)-conjugated KRFK peptide, and a control peptide (KQFK) peptide were synthesized by Bionova (Madrid, Spain). Cell culture plates and inserts were from Nunc (Roskilde, Denmark). For the TGF-β bioassay, TGF-β2 was from R&D Systems (Minneapolis, MN), the Great EscAPe SEAP Reporter system 3 was from Clontech (Mountain View, CA), and Synergy H1 microplate reader from Biotek (Winooski, VT). For RNA analysis, TRIzol Reagent, the SuperScript® VILO™ cDNA synthesis Kit, SYBR Green PCR Master Mix and the 7200 Real Time System were from Thermo Fisher Scientific. For lymph node cell treatment, ionomycin (Iono) was from EMD Millipore. For flow cytometry analysis, eFluor 780-conjugated Fixable Viability Dye was from eBioscience (San Diego, CA), and all antibodies used (Fc-block, IgG1-FITC, IgG2a-PE, IgG2a-PE-Cy5, CD4-FITC, CD4-PE, CD4-PE-Cy5, interferon (IFN)-ɣ-FITC, IL-17A-PE, Foxp3-PE-Cy5 primary antibodies and Alexa Fluor-conjugated secondary antibodies were from Thermo Fisher Scientific. BD LSRII Flow Cytometer was from BD Bioscience (San Jose, CA). Further analysis of the data was performed using FlowJo v9.4.10 software from Tree Star, Inc. (Ashland, OR).

2.2. Mice

All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and all the protocols used were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

C57BL/6 (H-2b) female mice between 6 to 9 weeks old were purchased from Charles River Laboratories (Wilmington, MA) and TSP-1-deficient mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice were subsequently bred in-house in a pathogen-free facility at Boston University, Boston, MA.

2.3. Isolation, culture, and treatment of murine bone marrow-derived dendritic cells

Bone marrow-derived DCs (BMDCs) were generated from WT and TSP-1-deficient mice. Briefly, bone marrow cells were collected by flushing out each of the bone shafts and cultured in complete RPMI-1640 medium (100 U/mL penicillin and 100 mg/mL streptomycin, 0.1 mM nonessential amino acid mixture, 10 mM HEPES buffer, 1 mM sodium pyruvate, 10% fetal bovine serum) with murine recombinant GM-CSF (20 ng/mL) for 6 days.

TSP-1-derived (KRFK) or control (KQFK) peptides were used at 50 μM in serum-free RPMI-1640. The dose was chosen based on a previous study using TSP-1-deficient dermal fibroblasts (Crawford et al. 1998). After 24 h of treatment, supernatants and cells were collected for TGF-β bioassay and RNA analysis, respectively.

2.4. Isolation and culture of human conjunctival epithelial cells

Corneoscleral buttons from cadaveric donors were obtained with informed research consent from Barraquer Eye Bank in Barcelona, Spain. This study was in strict accordance with the Tenets of the Declaration of Helsinki and Spanish Regulations concerning the use of Human Tissues for Biomedical Research, and had the approval of the Institutional Review Board of the University of Valladolid.

Human bulbar conjunctiva was carefully isolated from corneoscleral buttons and cultures of conjunctival epithelial cells were obtained using the explant technique previously reported (García-Posadas et al. 2013). Briefly, conjunctival explants were plated and fed adding culture medium each day for 5 days. Then, culture media was replaced every other day for 10 days. The epithelial culture medium was DMEM/F12 supplemented with 2.5 μg/mL fungizone, 5,000 units/mL penicillin/streptomycin, 1 μg/mL insulin, 0.5 μg/mL hydrocortisone, 2 ng/mL epidermal growth factor, and 10% human serum.

2.5. TGF-β bioassay

The amount of total and active TGF-β in BMDC supernatants was measured using MFB-F11 cells, a fibroblasts cell line derived from TGF-β–knockout mice and stably transfected with the SBE-SEAP reporter (Tesseur et al. 2006). MFB-F11 cells were seeded in complete DMEM into flat-bottom 96-well plates. After overnight incubation, cells were washed and incubated in serum-free DMEM for 24 h before supernatants from BMDCs were added. Dilutions of TGF-β2 were used as standards. For measurement of total TGF-β, supernatants were acidified with 1 N HCl for 10 min and then neutralized with 1 N NaOH right before application. Active TGF-β was measured from untreated supernatants. After 24 h, culture supernatants were tested for SEAP activity using Great EscAPe SEAP Reporter system 3 according to the manufacturer's instructions. Samples from independent experiments were measured in triplicate.

2.6. Cumulative TSP-1-derived peptide KRFK release across conjunctival epithelial cell culture

The permeability of the peptide was assessed by the passage of FITC-conjugated KRFK peptide through conjunctival epithelial cells. Human conjunctival epithelial cells from passage 1 were cultured on top of an insert in a 24-well plate. The culture time and the number of cells seeded were optimized to allow the cells to reach confluence and stratify 1 to 4 epithelial layers in the insert, which mimics the *in vivo* situation. The transepithelial electrical resistance (TEER) was measured to determine cell layer integrity. The lower compartment was filled with fresh conjunctival epithelial cell medium, and the upper compartment was filled with same medium containing 50 µM of FITC-conjugated KRFK peptide. Inserts without cells were used as control. A volume of 100 µL of medium was removed from the lower compartment at predetermined time points up to 3 h (15, 30, 45, 60, 90, 120, 150, and 180 min), and replaced with fresh medium immediately. The medium collected from the lower compartment were analyzed at 490 nm and 520 nm excitation and emission wavelengths,

respectively. Serial dilutions of the peptide were done to establish a standard curve. The quantity of peptide released was calculated using the following equation:

$$Xi = Li + \sum Ri$$

Where X is a total quantity (μg) released at i time (min), L is the quantity released to the lower compartment at i time, and R is the quantity removed from the lower compartment during previous determinations. Results showed the percentage of the peptide released in the lower compartment after its passage through the insert with a confluent conjunctival epithelial culture at each time point of the analysis. At least 4 independent experiments in duplicate were done.

To assess the number of conjunctival epithelial layers in the insert, cells were fixed in 4% paraformaldehyde buffered solution, embedded in paraffin and sectioned (5 μm). Sections were stained with hematoxylin and eosin (H&E) and number of layers counted.

2.7. Topical FITC-conjugated TSP-1-derived peptide KRFK administration and localization in vivo

To assess the retention time of the peptide in the murine ocular surface after topical administration, 5 μL of FITC-conjugated KRFK peptide (1 μg/μL) was topically administered to WT mouse eyes. One and 3 h after administration, eyeballs were collected, embedded in optimal cutting temperature (OCT) compound, and sectioned at thickness of 5 μm. Cell nuclei were stained with Hoechst 33342 dye, and representative micrographs taken under a dry X20 objective.

2.8. Topical TSP-1-derived peptide KRFK administration to TSP-1-deficient mice

The mouse model of ocular surface inflammation characterized in TSP-1-deficient mice was used. Female mice (n=6) at 8 weeks of age were treated

topically (5 µL/eye) with TSP-1-derived (KRFK) or control (KQFK) peptide (10 µg/mouse) peptide, once a day for 2 weeks. Cervical lymph nodes were collected at 12 weeks of age, when these mice showed a well-established ocular inflammation (Turpie et al. 2009; Contreras-Ruiz et al. 2013).

2.9. In vitro stimulation of cervical lymph node cells

Cervical lymph nodes were collected from TSP-1-derived (KRFK) or control (KQFK) peptide-treated TSP-1-deficient mice and isolated cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and Iono (2 µg/mL) for 4 h. To inhibit protein transport, Brefeldin A (BFA, 1X) was added to the culture medium after 1h. Cells were harvested and analyzed by flow cytometry and real time reverse transcription polymerase chain reaction (real time RT-PCR).

2.10. Flow cytometry

Cervical lymph node cells stimulated with PMA/Iono/BFA were analyzed by flow cytometry to detect intracellular IFN-ɣ, IL-17 and Foxp3 in CD4+ cells. Cells were first incubated with eFluor 780-conjugated Fixable Viability Dye for 30 min at 4˚C. Then, intracellular expression of CD4, IFN-ɣ, IL-17A and Foxp3 were assessed by using primary antibodies and Alexa Fluor-conjugated secondary antibodies. Their respective isotype-matched antibodies served as negative controls. Fluorescent labeled cells were analyzed using BD LSRII Flow Cytometer. Further analysis of the data was performed using FlowJo v9.4.10 software.

2.11. Real time RT-PCR

Total RNA was isolated from peptide-treated TSP-1-deficient BMDCs or cervical lymph nodes collected from peptide-treated TSP-1-deficient mice using TRIzol Reagent. cDNA was synthesized using the SuperScript® VILO™ cDNA synthesis Kit and real time RT-PCR was performed using SYBR Green PCR Master Mix. All reactions were performed in triplicate using a thermal profile of 95°C for 180 s, 40 cycles at 95°C for 20 s, 53°C for 30 s, and 72°C for 40 s. A no-template

control was included. To ensure the specificity of the PCR products a melting curve analysis was performed at the end of each reaction. Relative mRNA expression levels were determined using the endogenous glyceraldehydes-3-phosphate dehydrogenase (GAPDH) signal for normalization. Pooled samples were measured in triplicates.

The primer pairs used in BMDC lysates were: major histocompatibility complex (MHC) class II (forward primer 5´-AGG GCA TTT CGT GTA CCA GTT-3´ and reverse primer 5´-GTA CTC CTC CCG GTT GTA GAT-3´), CD80 (forward primer 5´-GAA TTA CCT GGC ATC AAT ACG-3´ and reverse primer 5´-CTT AAT GGT GTG GTT GCG AGT C-3´), and GAPDH (forward primer 5´-CGA GAA TGG GAA GCT TGT CA-3´ and reverse primer 5´-AGA CAC CAG TAG ACT CCA CGA-3´).

The primers pairs used in cervical lymph node cell lysates were: IFN-γ (forward primer 5'-TCA GCA ACA ACA TAA GCG TCA T-3´ and reverse primer 5'-GAC CTC AAA CTT GGC AAT ACT CAT-3´), IL-17 (forward primer 5'-AGT GAA GGC AGC AGC GAT CAT-3´ and reverse primer 5'-CGC CAA GGG AGT TAA AG-3´) and Foxp3 (forward primer 5'-GGA GAG GCA GAG GAC ACT CAA-3' and reverse primer 5'-GTG GTT TCT GAA GTA GGC GAA-3').

2.12. Statistical Analyses

Data are expressed as the mean ± standard error of the mean (SEM). To compare two groups, a Student´s *t*-test was used, applying Welch´s correction in groups with different variances (F test). For more than 2 groups, a one-way analysis of variance followed by pairwise comparisons (Tukey´s test) or a Brown-Forsythe test followed by pairwise comparisons (Games-Howell test), were performed depending on Levene´s test. Statistical significance of $p \leq 0.05$ was considered.

3. Results

3.1. TSP-1-derived peptide KRFK activates secreted TGF-β in TSP-1-deficient BMDCs in vitro

The sequence RFK in TSP-1 molecule is sufficient to activate latent TGF-β. We tested the ability of KRFK peptide to activate latent TGF-β produced by TSP-1-deficient BMDCs as they are incapable of activating their endogenously produced TGF-β (Mir et al. 2015). We used untreated WT BMDCs as control. Cells from TSP-1-deficient mice were cultured in the presence of KRFK or inactive control (KQFK) peptides at 50 µM for a period of 24 h. Cultured supernatants were then analyzed using a bioassay for the levels of total and inactive TGF-β. Proportion of active TGF-β as compared to total TGF-β was determined as shown in Figure 1. Culture supernatants collected from WT BMDCs contained high proportion of active TGF-β as expected, whereas significantly reduced proportion of TGF-β was active in culture supernatants of TSP-1-deficient BMDCs. However, in the presence of KRFK peptide this proportion significantly increased as compared to that seen in TSP-1-deficient BMDCs treated with inactive control peptide. These results support the ability of the KRFK peptide to activate endogenously produced latent TGF-β by BMDCs.

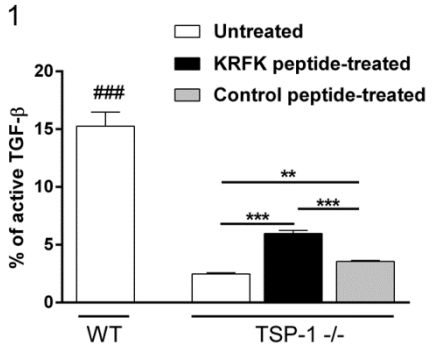

Figure 1**: TSP-1-derived peptide KRFK activates latent TGF-β produced by TSP-1-deficient BMDCs *in vitro***. WT and TSP-1-deficient BMDCs were cultured and treated with KRFK and control peptide (KQFK). The percentage of active TGF-β in the supernatants was calculated. Statistically significant differences between samples are indicated with asterisks (ANOVA, ** $p \leq 0.01$, *** $p \leq 0.001$). WT values are significantly different compared to all other samples, as indicated by hashes (ANOVA, ### $p \leq 0.001$). WT: C57BL/6 BMDCs supernatants, TSP-1 -/-: TSP-1-deficient BMDCs supernatants. Control peptide: KQFK peptide.

3.2. Expression of DC maturation markers is reduced in TSP-1-deficient BMDCs treated with TSP-1-derived peptide KRFK in vitro

One of the immunoregulatory activities of TGF-β includes signaling in DCs to maintain their immature and tolerogenic state characterized by low expression of MHC class II and co-stimulatory molecules. Considering the ability of the KRFK peptide to activate endogenously produced latent TGF-β by BMDCs, we evaluated its ability to modulate BMDC phenotype. As described previously, TSP-1-deficient BMDCs were left either untreated or cultured in the presence of KRFK or control (KQFK) peptides for a period of 24 h. Cells were lysed to harvest RNA that was subjected to real-time RT-PCR analysis to assess expression levels of MHC class II and the co-stimulatory molecule CD80. As shown in Figure 2, both MHC class II and CD80 message levels were down-regulated in BMDCs treated with KRFK as compared to untreated controls. While MHC class II expression was also down-regulated in BMDCs treated with control peptide, no such effect was noted in the expression of CD80. Expression of CD80 in KRFK-treated BMDCs was significantly reduced compared to those treated with control peptide.

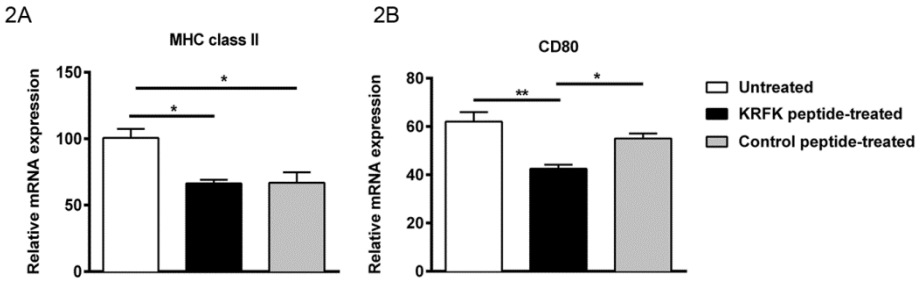

Figure 2: **TSP-1-derived peptide KRFK-activated TGF-β modulates TSP-1-deficient BMDC phenotype *in vitro***. The effect of KRFK peptide on TSP-1-deficient BMDC phenotype was analyzed by assessing MHC class II and CD80 mRNA expression. A) MHC class II and B) the co-stimulatory molecule CD80 mRNA levels normalized to GAPDH levels, determined by RT-PCR. Statistically significant differences between samples are indicated with asterisks (ANOVA, *$p \leq 0.05$, **$p \leq 0.01$). Control peptide: KQFK peptide.

3.3. TSP-1-derived peptide KRFK crosses conjunctival epithelium in vitro

To investigate the passage of the KRFK through conjunctival epithelial barrier, FITC-conjugated KRFK peptide release through conjunctival epithelial culture was

assessed using a cell insert. To ensure cell epithelial layer integrity, TEER was measured and, after conducting the experiment, cells were fixed and stained with H&E to determine number of stratified epithelial layers. The schematic in Figure 3A shows the experimental set up. Conjunctival epithelial cells obtained from cadaveric donors showed normal epithelial cell morphology (Figure 3B). Conjunctival epithelial cells showed TEER values of 229.00 ± 4.30 $\Omega.cm^2$ over the control (insert without cells), indicating the integrity of the cell layer. Moreover, after 3-5 days of culture, these cells were stratified from 1 to 4 epithelial layers, as determined by the H&E stained section (Figure 3C). The measurements of FITC-KRFK cumulative transport across the conjunctival epithelial culture showed steady passage of KRFK across conjunctival epithelium up to 180 min (Figure 3D). These results support the ability of KRFK to cross conjunctival epithelium.

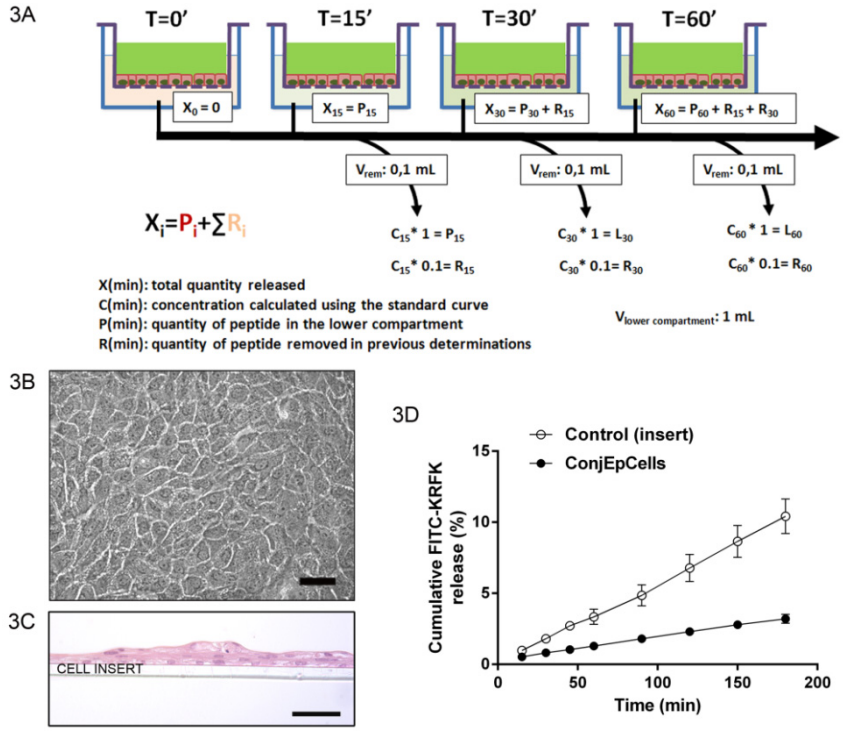

Figure 3: **Cumulative FITC-KRFK peptide transport across human conjunctival epithelial cells**. A) Schematic of the *in vitro* model of KRFK peptide transport across epithelial cell culture using inserts. B) Representative micrograph of conjunctival epithelial cells cultured onto inserts. C) H&E staining of the cross section of conjunctival epithelial cells seeded onto an insert. D) Percentage of cumulative transport of FITC-KRFK across the conjunctiva epithelial cell layers. ConjEpCells: cell insert with human conjunctival epithelial cells. Scale bar: 50 µm.

3.4. Topically administered TSP-1-derived peptide KRFK is retained in the ocular surface tissues up to one hour

To determine the dosing schedule of the KRFK *in vivo*, approximate retention time in the ocular surface has to be established. One hour after topical administration of FITC-conjugated KRFK peptide, ocular surface tissue was harvested, embedded in OCT compound and 5 µm-sections were examined after staining nuclei with DAPI. As shown in Figure 4A, an intense FITC fluorescence was detected in some areas of the cornea and the conjunctiva, indicating that peptide was still within the ocular surface tissues. Agglomeration of the peptide was noted in the conjunctival fornix area. In the cornea, the fluorescence was localized only in the outermost epithelial layers, while it was detected in the conjunctival stroma. These results provide the evidence that topically applied TSP-1-derived peptide KRFK is able to cross the conjunctival epithelial barrier. No significant fluorescent signal was observed in either of the ocular surface tissues 3 h after peptide administration (Figure 4B). All together these results indicate retention of topically applied KRFK peptide in the ocular surface tissues at least for a period of 1 h.

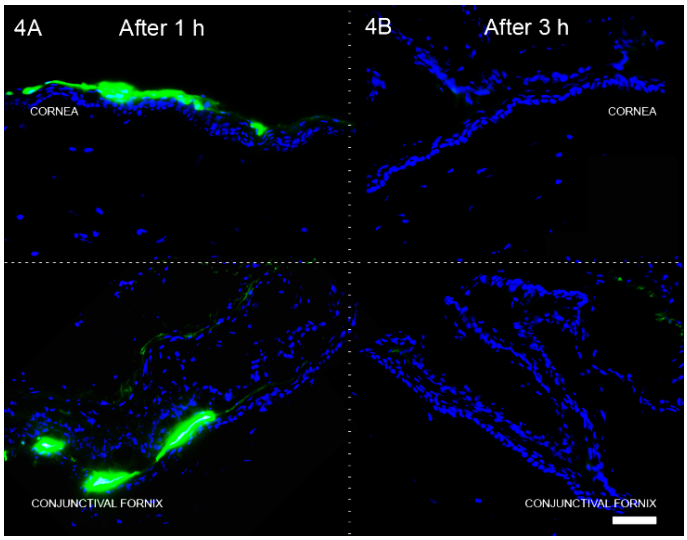

Figure 4: **Localization of FITC-KRFK peptide in the mouse cornea and conjunctiva after topical administration *in vivo***. Representative micrographs of the mouse cornea (up) and conjunctiva (down) after A) 1 and B) 3 h of topical administration of FITC-KRFK peptide (green). Nuclei were stained with DAPI (blue). Scale bar: 50 µm.

3.5. Topically administered TSP-1-derived peptide KRFK to TSP-1-deficient mice alters peripheral balance of CD4+ inflammatory effectors.

The ability of TSP-1-derived peptide KRFK to activate latent TGF-β and modulate DC phenotype, together with its potential to cross conjunctival epithelial barrier, make KRFK peptide a candidate to topically treat ocular surface inflammation.

Resident APCs, such as DCs, upon activation/maturation are known to migrate to the local draining lymph nodes where they present antigens to generate activated effector T cells. In the TSP-1-deficient mice, DCs in the conjunctiva are known to migrate to cervical lymph node where inflammatory effectors are detected (Contreras-Ruiz et al. 2013). To assess if topically applied KRFK can influence systemic inflammatory immune response, 8-week-old TSP-1-deficient mice were treated daily with KRFK or inactive control KQFK peptides for a period of 2 weeks. At the end of the study period cervical lymph nodes from both groups were collected and analyzed by flow cytometry to assess frequency of inflammatory Th1 (CD4+ IFN-γ+), Th17 (CD4+ IL-17+), regulatory Treg (CD4+ Foxp3+) subsets. As shown in Figure 5A reduced frequency of Th1 and Th17 effectors along with an increase in frequency in Treg cells was detected in KRFK-treated TSP-1-deficient mice compared to control peptide-treated group of mice.

These observations were confirmed by detection of significantly reduced message of IFN-γ as detected by real-time PCR analysis in KRFK-treated group compared to the control group of TSP-1-deficient mice (Figure 5B). No significant change was detected in message for IL-17A.

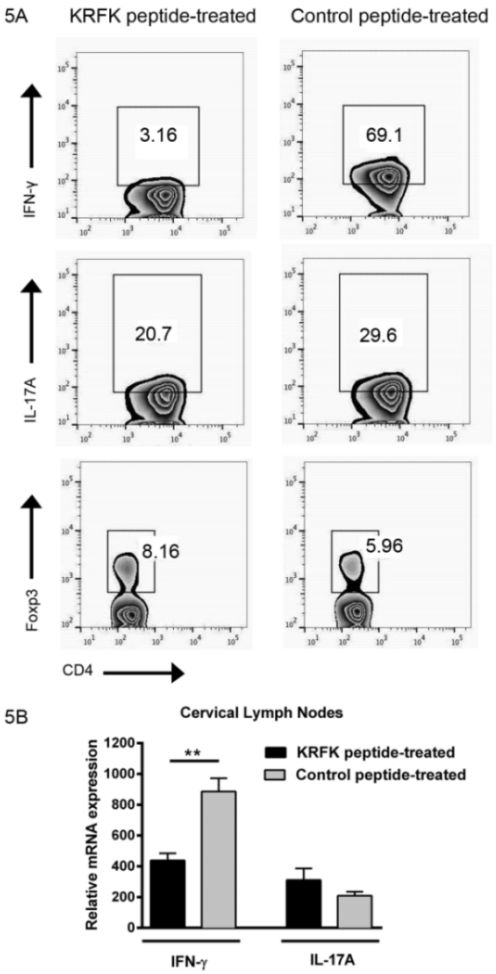

Figure 5: **TSP-1-derived peptide KRFK modulates lymph node T cell populations in topically treated TSP-1-deficient mice**. Lymph node cells were collected from KRFK peptide and control peptide (KQFK)-treated TSP-1-deficient mice and *in vitro* stimulated (see section 2.9). A) Representative flow cytometry plots of IFN-γ, IL-17A and Foxp3 staining together with CD4 of viable gated cells. B) IFN-γ and IL-17A mRNA levels normalized to GAPDH levels, determined by RT-PCR. Statistically significant differences between samples are indicated with asterisks (*t*-test, $^{**}p \leq 0.01$). Control peptide: KQFK peptide.

4. Discussion

In this study we show that TSP-1-derived peptide KRFK *in vitro* activates TGF-β and modulates DC phenotype towards an immature state. *In vivo*, topically administered TSP-1-derived peptide KRFK traverses ocular surface barriers and alters peripheral balance of immune effectors by reducing inflammatory effectors while increasing regulatory T cells. Therefore, our results strongly

support the therapeutic potential of the KRFK peptide in the treatment of chronic ocular surface inflammation.

The expression levels of TSP-1 at the ocular surface, and its ability to activate TGF-β, determine the maturation state of DCs and the development of regulatory or inflammatory immune responses (Contreras-Ruiz & Masli 2015; Mir et al. 2015). The TGF-β-activating sequence in KRFK peptide-derived from TSP-1, effectively activated latent TGF-β produced by TSP-1-deficient BMDCs. Although no activity of control peptide (KQFK) was observed in previous studies (Crawford et al. 1998; Nor et al. 2005), in our experiments we detected active TGF-β in BMDCs treated with the control peptide. However, these levels of activated TGF-β were significantly lower compared to those detected in KRFK peptide-treated TSP-1-deficient BMDCs. Furthermore, significantly increased active TGF-β levels in KRFK-treated BMDCs led to significantly reduced expression of co-stimulatory molecules but similar change in MHC class II was not detected. It is possible that changes in MHC class II expression require longer exposure or concentration of KRFK in cultures. Overall, our results indicate a strong potential of KRFK peptide to alter DC phenotype towards a tolerogenic type.

These *in vitro* findings suggest that TSP-1-derived peptide KRFK could be used to contribute to the development of regulatory immunity modulating ocular APCs if applied topically in the eye. However, ocular surface barriers and the physicochemical properties of the peptide need to be considered to determine the dosing schedule. Usually, poor drug absorption is related to a high (>500 g/mol) molecular weight of the drug, the presence of more than 5 hydrogen bonds and more than 10 hydrogen bond acceptors in the drug structure, and a high lipophilicity. The TSP-1-derived peptide KRFK has none of these properties; although due to its small length, a low stability and short half-life is expected (Ibraheem et al. 2014). Its functionality was successfully tested earlier without structural modification (Crawford et al. 1998; Yee et al. 2004; Nor et al. 2005), but it has never been administered as eyedrops. Therefore, we performed

studies to determine ocular penetration and ocular surface residence time using a FITC-conjugated KRFK peptide. We first assessed KRFK permeability *in vitro* across conjunctival epithelial culture, as conjunctiva is the largest surface area and the most permeable epithelium at the ocular surface (Hämäläinen et al. 1997). This assay allowed detecting a steady passage of KRFK peptide across conjunctival epithelium during the evaluated period of time (15 min to 3 h). *In vivo*, a lower bioavailability of the peptide should be considered due to additional biopharmaceutical barriers such as tear drainage and proteolytic activity of the tear film (Urtti 2006). To further evaluate KRFK ocular surface residence time to determine approximate dosing schedule, we performed *in vivo* studies using topical application of the FITC-conjugated KRFK peptide. Our results indicate that KRFK peptide was retained in the cornea and the fornicial area of the conjunctiva up to 1 h, being detected also at the conjunctival stroma 1 h post-instillation. Together these results indicate that topically applied KRFK peptide may be a suitable candidate to treat ocular surface inflammation.

Based on *in vitro* results that supported potential changes in DC phenotype and reasonable bioavailability of the peptide which steady passage through conjunctival epithelium, we tested its efficacy in altering peripheral inflammatory immune responses in TSP-1-deficient mice after topical administration. The observed decline in effector Th1 and Th17 populations in combination with an increase in regulatory Treg population in TSP-1-deficient lymph nodes is consistent with the effect of KRFK peptide on TSP-1-deficient BMDCs *in vitro*. By activating TGF-β in the conjunctival tissue environment, it is likely that KRFK helps maintain tolerogenic phenotype of local DCs and prevents generation of inflammatory effectors while promoting development of Tregs. Although the peptide remained at the ocular surface for 1 h, the ability of TGF-β2-exposed DCs to increase the endogenous expression of TGF-β2 (Mir et al. 2015) would enhance KRFK effect *in vivo*, and result in a more lasting effect. Our findings in this study confirm the ability of TSP-1-derived peptide to restore peripheral balance of regulatory ocular immunity.

Overall, this work presents evidence of the potential anti-inflammatory therapeutic role of topically administered TSP-1-derived peptide KRFK that activates TGF-β. The different etiology of ocular surface inflammatory disorders causes that currently available therapeutic options are common and often ineffective and accompanied with several undesirable side effects. This fact led us to focus on novel and personalized treatments to selective ligands in order to avoid such side effects and offer a singular option depending on the molecules involved in the disease. Here, we present a TSP-1-derived peptide, the KRFK peptide, as a promising alternative for the treatment of chronic ocular surface inflammation.

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STUDY 4. Topical KRFK Peptide Prevents Inflammatory Manifestations in the Thrombospondin-1-Deficient Mouse Model of Chronic Ocular Surface Inflammation

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Manuscript in preparation

Topical KRFK Peptide Prevents Inflammatory Manifestations in the Thrombospondin-1-Deficient Mouse Model of Chronic Ocular Surface Inflammation

ABSTRACT

Ocular surface inflammatory disorders are among the leading causes of vision impairment worldwide and its prevalence is expected to increase with the aging population. Despite significant progress in the clinical treatments, this kind of disease is not fully alleviated in many patients. This fact may be explained by the different etiology of the inflammation, which modulates de consequent inflammatory response. A personalized treatment would help to avoid the failures using general treatments. Thrombospondin-1 (TSP-1) plays a critical regulatory role in the ocular mucosa and as such TSP-1-deficient mice spontaneously develop chronic ocular surface inflammation. In humans, a polymorphism in the gene encoding TSP-1 that results in decreased protein expression has been associated with chronic ocular surface inflammation. In this work, we proposed an alternative therapeutic approach to treat ocular inflammation associated with decreased TSP-1 expression: the use of the KRFK peptide, a TGF-β-activating TSP-1-derived peptide. To study the potential of the peptide, TSP-1-deficient mice before (prevention study) and after (treatment study) the onset of the disease were topically treated with KRFK or control peptides for 2 weeks. The disease was monitored by assessing corneal fluorescein score and levels of MUC5AC in tears. At the end of each study, the number of conjunctival goblet cell was assessed and a histopathology study of corneal, conjunctival and lacrimal gland tissues performed. In the prevention study further analysis of the expression of different inflammatory cytokines was assessed by real-time RT-PCR. In this study, we demonstrate that topical administration of TSP-1-derived peptide KRFK to TSP-1-deficient mice, prior to the disease onset, prevents corneal barrier disruption, improves MUC5AC levels in tears, and reduces levels of inflammatory cytokines in conjunctival and lacrimal gland tissues. The TSP-1-derived peptide KRFK administered in TSP-1-

deficient mice with an established ocular surface inflammation also improves MUC5AC levels in tears. We conclude that our data identify the KRFK peptide as a novel therapeutic option to efficiently prevent chronic ocular surface inflammation associated with deficient or decreased TSP-1 expression.

Keywords

Inflammation; KRFK peptide; Ocular Surface; Thrombospondin-1; Transforming growth factor-β.

Abbreviations

AB: alcian blue

APC: antigen-presenting cell

ELISA: enzyme-linked immunosorbent assay

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

H&E: hematoxilin and eosin

IL: interleukin

PAS: periodic acid-Schiff

PBS: phosphate buffered saline

RT: room temperature

RT-PCR: reverse transcription polymerase chain reaction

SMA: smooth muscle actin

SEM: standard error of the mean

TGF: transforming growth factor

Th: T helper

THBS1: thrombospondin-1 gene

TNF: tumor necrosis factor

Treg: regulatory T cell

TSP-1: thrombospondin-1

1. INTRODUCTION

Ocular surface inflammatory disorders are among the leading causes of vision impairment worldwide. The multifactorial etiology of these diseases can include environmental, such as an allergy, infection, surgery or injury; and genetic factors. If the initiating stimulus is not removed and/or the inflammatory cells or soluble mediators persist,a late inflammatory response occurs (McDermott et al. 2005). This late and chronic response is modulated by different cellular and humoral components in the surrounding microenvironment (Stevenson et al. 2012), which should be taken into account in treatments. These modulators may explain the unexpected failure to current prescriptions observed in some patients affected by immune-based ocular surface disorders. Moreover, general treatments normally include long-term use of immunosuppressive drugs with undesirable side effects. Personalized treatments that target specific molecules would offer a more effective therapeutic solution to these chronic inflammatory diseases.

Thrombospondin-1 (TSP-1) is an important contributor to immune homeostasis at the ocular surface. Recently, a polymorphism in the gene encoding thrombospondin-1 (*THBS1*) that results in decreased expression of the encoded TSP-1 protein in ocular surface epithelial cells has been associated with a susceptibility to develop chronic ocular surface inflammation after a refractive surgery (Contreras-Ruiz et al. 2014). Based on this study, *THBS1* polymorphism has been postulated as a potential genetic marker for the early diagnosis of ocular surface inflammation, allowing preventive treatment strategy to possibly avoid the chronic condition. Deficiency in TSP-1 in mice results in the spontaneous development of chronic ocular surface inflammation (Turpie et al. 2009; Contreras-Ruiz, Regenfuss, et al. 2013). Despite appearing normal at birth, these mice develop a chronic ocular surface inflammation with age, with earliest signs detectable in the conjunctiva by 8 weeks and a fully established ocular inflammation by 12 weeks of age. The disease is characterized by disruption of corneal epithelial barrier integrity, secretory dysfunction and inflammation of

conjunctiva and lacrimal gland, and a peripheral imbalance in regulatory T cells (Treg) and pathogenic T helper type 17 (Th17) effectors. The homing of these lymphocytes to ocular surface plays a central role in the effector phase of the chronic condition.

One of the main functions of TSP-1 associated with the modulation of ocular inflammatory responses is the activation of transforming growth factor (TGF)-β and the subsequent effect in antigen-presenting cells (APCs) (Contreras-Ruiz & Masli 2015). Several evidences demonstrate the relevance of the TSP-1-dependent TGF-β activation in the ocular mucosa: (i) TSP-1 can activate the predominant isoform TGF-β2 at this mucosa while other mechanisms, such integrins, cannot (Annes et al. 2003), (ii) TSP-1-deficient ocular cells, including goblet cells and dendritic cells, fails to activate its endogenous TGF-β *in vitro,* (Contreras-Ruiz & Masli 2015; Mir et al. 2015; Masli et al. 2006), (iii) our recent observation of increased total TGF-β2 in the conjunctiva of TSP-1-deficient mouse model of chronic ocular surface inflammation (Soriano-Romani et al. 2015).

All these evidences led us to study a TSP-1-derived peptide that activates TGF-β, the KRFK peptide (Schultz-Cherry et al. 1995), as potential therapeutic molecule to prevent or treat ocular surface inflammation associated with decreased TSP-1 expression. In previous studies, we demonstrated that instilled TSP-1-derived peptide KRFK crosses conjunctival epithelium and modulates local lymph node cell populations (manuscript submitted). In this work, we wanted to know if topically administered KRFK peptide can prevent or reverse inflammatory signs in the ocular surface tissues of TSP-1-deficient mice, including cornea, conjunctiva, and lacrimal gland.

2. MATERIAL AND METHODS

2.1. Mice

A breeding pair of TSP-1-deficient mice (C57BL/6 background) was purchased from Jackson Laboratory (Bar Harbor, MI). These mice were bred subsequently

in-house in a pathogen-free facility at Boston University, Boston, MA. All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the protocols used had the approval of the Institutional Animal Care and Use Committee at Boston University School of Medicine (Boston, MA).

2.2. TSP-1-derived peptides

The TGF-β-activating peptide KRFK, derived from the TSP-1 sequence, and the inactive peptide KQFK (control peptide) were synthesized by Bionova (Madrid, Spain). Both peptides were reconstituted in phosphate buffered saline (PBS) at a final concentration of 1 μg/μL. Both peptides were topically applied in both eyes of TSP-1-deficient mice (5 μL per eye) once a day during 2 weeks.

2.3. Prevention and treatment experimental set up and disease monitoring

A mouse model of ocular surface inflammation characterized in TSP-1-deficient mice was used (Turpie et al. 2009). We used two different approaches to study the therapeutic potential of the KRFK peptide. TSP-1-deficient mice (n=6-8/study group) at the age of 8 (prior to the onset of ocular surface inflammation – Prevention Study) or 16 weeks (after disease onset – Treatment Study) were treated topically with TSP-1-derived (KRFK) or control (KQFK) peptides in both eyes once a day for 2 weeks.

Disease progression was monitored in both studies by corneal fluorescein staining and tear MUC5AC content following the experimental set up depicted in Figure 1. Corneal fluorescein staining and pilocarpine-induced tear collection were performed as described previously (Turpie et al. 2009). For corneal fluorescein staining, 1% sodium fluorescein (Sigma-Aldrich, St. Louis, MO) was applied onto the mouse cornea under anesthesia. After washing the eyes with PBS, corneal staining was evaluated with a slit lamp microscope using a cobalt blue light. Punctate staining was recorded using a standardized National Eye

Institute Grading System of 0 to 3 for each of the five areas of the cornea (Lemp 1995). Pilocarpine-induced tears were collected and analyzed in quadruplicate to determine MUC5AC content using enzyme-linked immunosorbent assay (ELISA) kit (TSZ ELISA, Waltham, MA). Results showed the amount of MUC5AC (ng/mL) per tear volume. Mice were euthanized and the eye tissues collected at the end of each study period for cellular and molecular analyses.

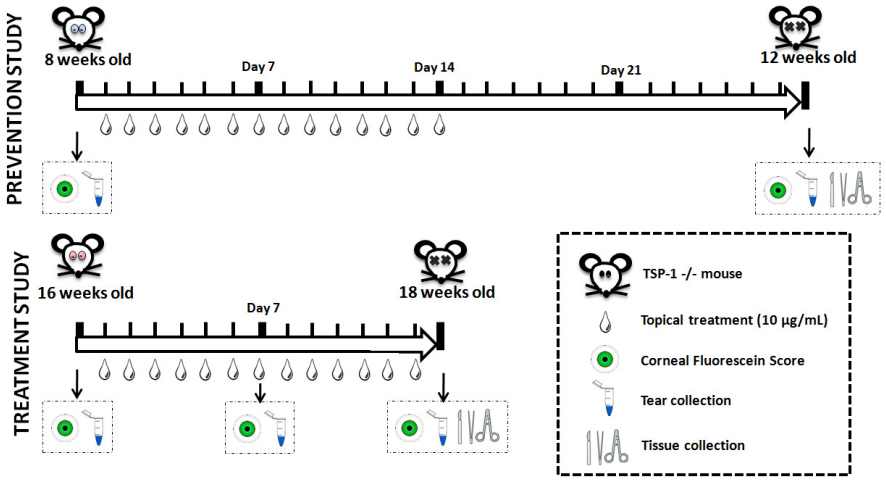

Figure 1: **Schematic showing the experimental set up used for the *in vivo* prevention and treatment studies**. In both approaches, TSP-1-deficient mice daily received in both eyes 5 µL/eye of TSP-1-derived peptide KRFK or control peptide KQFK during 14 days. Corneal fluorescein staining and tear collection to measure MUC5AC were done before starting the peptide administration (baseline) and at the completion of the experimental time course in the prevention study, or after 1 and 2 weeks of treatment in the treatment study. Eye tissues were collected at the completion of both experimental time courses for cellular and molecular analyses. TSP-1 -/-: TSP-1-deficient mouse.

2.4. Tissue processing

Lacrimal glands and eyeballs with attached eyelids were dissected and fixed in 4% paraformaldehyde buffered solution. Tissues were processed, embedded in paraffin, and sectioned in sagittal 5 µm sections (n≥3 per experimental group).

2.4.1. Histopathology study

Hematoxylin and eosin (H&E) staining was used for ocular surface tissue morphology and histopathology evaluation. Conjunctival and corneal epithelial

thicknesses were measured and evaluated. Inflammation in the lacrimal glands was graded on a 0–3 ordinal scale based on the inflammatory infiltrates as follows: Grade 0-no inflammation; Grade 1-mild inflammation (slight increase in lymphocytes); Grade 2-moderate inflammation (dense but focal lymphocytes); Grade 3-severe inflammation (dense and diffuse lymphocytes). Representative micrographs were taken using X10 objective.

Periodic acid–Schiff and alcian blue (PAS/AB) staining was used for goblet cell counting. Conjunctival goblet cells were counted in a masked fashion by two independent trained observers. Sagittal sections from the middle of the eye were evaluated as representative of whole conjunctiva to control variations in goblet cell density over the ocular surface. Results showed number of goblet cells counted in the superior and inferior bulbar and tarsal conjunctivas for each eyeball analyzed. Representative micrographs were taken using X10 objective.

Sirius red staining was used for collagen content and fibrosis evaluation. Briefly, the slides were stained with a saturated aqueous solution of picric acid containing 0.1% Sirius red (Sigma-Aldrich) for 1 h. Tissue sections were evaluated under a light microscope to grade the collagen deposition. Micrographs were taken using X10 objective and analyzed using ImageJ software v. 1.49 (http://imagej.nih.gov/ij/;National Institutes of Health, Bethesda, MD). Results showed percentage of Sirius red staining. Representative micrographs under polarized light were also taken using X40 objective.

2.4.2. Immunofluorescence analysis

Alpha-smooth muscle actin (α-SMA) was immunodetected in paraffin-embedded tissue sections of mouse eyeballs. Tissue sections were incubated with 0.01% trypsin and 0.3% Triton X-100 at room temperature (RT) for 10 min, for antigen retrieval and tissue permeation, respectively. Then, tissue sections were incubated with blocking buffer composed of 4% goat serum at RT for 1 h. Afterwards, primary antibody against α-SMA (2.5 μg/mL - Abcam, Cambridge, UK) was incubated at 4°C overnight in blocking buffer. Then, Alexa Fluor-

conjugated secondary antibody (10 µg/mL - Thermo Fisher Scientific, Waltham, MA) was incubated at RT for 1 h. Nuclei were counterstained using Hoechst dye. Negative controls included omission of primary antibodies. Representative micrographs were taken using same exposure time, gain, and intensity of the camera. The specificity of the antibody had been previously tested in our laboratory using control and TGF-β1-treated conjunctival fibroblasts.

2.5. Real time RT-PCR

RNA was isolated from conjunctival and lacrimal gland tissues using the TRIzol reagent (Thermo Fisher Scientific) and cDNA synthesized using the SuperScript® VILO™ cDNA synthesis Kit (Thermo Fisher Scientific) with 1 µg of RNA. For RT-PCR the thermal profile used was: 95˚C for 120 s, 39 cycles of 95˚C for 20 s, 60˚C for 30 s, 72˚C for 40 s and 72˚C for 600 s.

For real time RT-PCR, 1 µL primers, 10 ng cDNA, and 10 µL SYBR Green PCR Master Mix (Thermo Fisher Scientific) were mixed in a final volume of 20 µL, using a thermal profile of: 50˚C for 120 s, 95˚C for 600 s, followed by 40 cycles of 95˚C for 15 s and 60˚C for 60 s, and a final cycle of 95˚C for 90 s. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal was used for normalization. All reactions were performed in triplicate and included a no-template control. The primer pairs used are indicated in Table 1.

Table 1. Sequences of the primer pairs used.

Gen	Sequence for forward primer	Sequence for reverse primer
IL-6	5´-AGT CAA TTC CAG AAA CCG CTA TGA-3´	5´-TAG GGA AGG CCG TGG TTG T-3´
IL-1β	5´-TCT GAA GCA GCT ATG GCA ACT GTT-3´	5´-CAT CTT TTG GGG TCC GTC AAC T-3´
TNFα	5´-GGC CTC CCT CTC ATC AGT TCT ATG-3´	5´-GTT TGC TAC GAC GTG GGC TAC A-3´
IL-17A	5´-AGT GAA GGC AGC AGC GAT CAT-3´	5´-CGC CAA GGG AGT TAA AG-3´
GAPDH	5´-GAACGTGAAGGTCGGAGTCAAC-3´	5´-CGTGAAGATGGTGATGGGATTTC-3´

IL: interleukin; TNFα: tumor necrosis factor-α; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

2.6. Statistical Analysis

Data are expressed as the mean ± the standard error of the mean (SEM). A Student´s *t*-test was used to compare 2 groups. If groups had variances significantly different (F test), the Welch´s correction was performed. For more than 2 groups, a one-way ANOVA followed by pairwise comparisons with Tukey test was performed. If groups had variances significantly different (Levene´s test), a robust test (Brown-Forsythe test) was performed, followed by pairwise comparisons with Games-Howell test. Statistical significance was considered from $p \leq 0.05$.

3. RESULTS

3.1. Prevention of ocular surface disease: Effects of topical KRFK peptide in 8-week-old (prior to onset of inflammation) TSP-1-deficient mice

TSP-1-deficient mice show a well-established ocular surface inflammation with disruption of corneal epithelial barrier integrity, reduced MUC5AC content in tears, and increased conjunctival and lacrimal gland inflammation at 12 weeks of age (Turpie et al. 2009; Contreras-Ruiz, Regenfuss, et al. 2013). Considering that mice deficient in TSP-1 remain healthy until the age of 8 weeks, we first evaluated the ability of TSP-1-derived peptide KRFK to prevent or attenuate the progression of the spontaneous surface disease. The inactive control peptide KQFK were topical administered to the control group of mice.

Corneal barrier integrity was assessed by scoring fluorescein corneal staining before (baseline – 8 weeks of age) and after the treatment at the end of the prevention study (2 weeks of treatment + 2 weeks of rest – 12 weeks of age). As shown in Figure 2A and 2B, KRFK peptide administration resulted in a significant decline in corneal staining scores as compared to control peptide-treated group. The evaluation of H&E-stained corneal tissue at the end of the study showed normal basal, wing, and squamous epithelial cells, from 3 to 7 epithelial layers with no differences in the epithelial corneal thicknesses between both

experimental groups, in both, TSP-1-derived and control peptide-treated groups (Figure 2C).

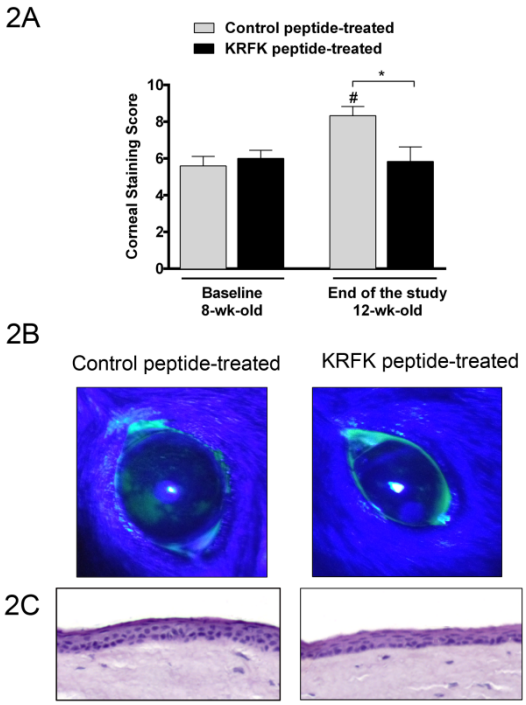

Figure 2: **Topically administered TSP-1-derived peptide KRFK effectively prevents corneal disruption in 12-week-old TSP-1-deficient mice.** A) Corneal staining scores at the baseline and at the end of the prevention study. B) Representative images of fluorescein staining at the end of the prevention study. C) Representative micrographs of H&E-stained corneal epithelium at the end of the prevention study. Statistically significant differences compared to the baseline are indicated with hashes, and between samples are indicated with an asterisks (Tukey test; *ó #$p \leq 0.05$).

The effect of topical KRFK peptide to prevent inflammation in the conjunctiva was evaluated by MUC5AC levels in tears, the expression of inflammatory cytokines and histological stainings, including H&E and PAS/AB stainings. TSP-1-derived KRFK peptide administration reversed the progressive decrease in tear MUC5AC levels showed in control peptide-treated TSP-1-deficient mice (Figure 3A). The improvement of tear MUC5AC secretion was accompanied by a reduction in IL-17A and TNFα mRNA expression after KRFK peptide administration (Figure 3B). H&E-stained conjunctivas from both experimental groups showed healthy folded conjunctival tissue, with normal epithelium

showing plump goblet cells interspersed amongst epithelial cells and some granulocytes in the connective tissue underneath. Relevant differences were not observed between experimental groups. Bulbar and tarsal goblet cells were counted in PAS/AB-stained conjunctivas. Although KRFK peptide administration increased the number of goblet cells in average, it did not reach significance compared to control peptide (KQFK)-treated conjunctivas (Figure 3C).

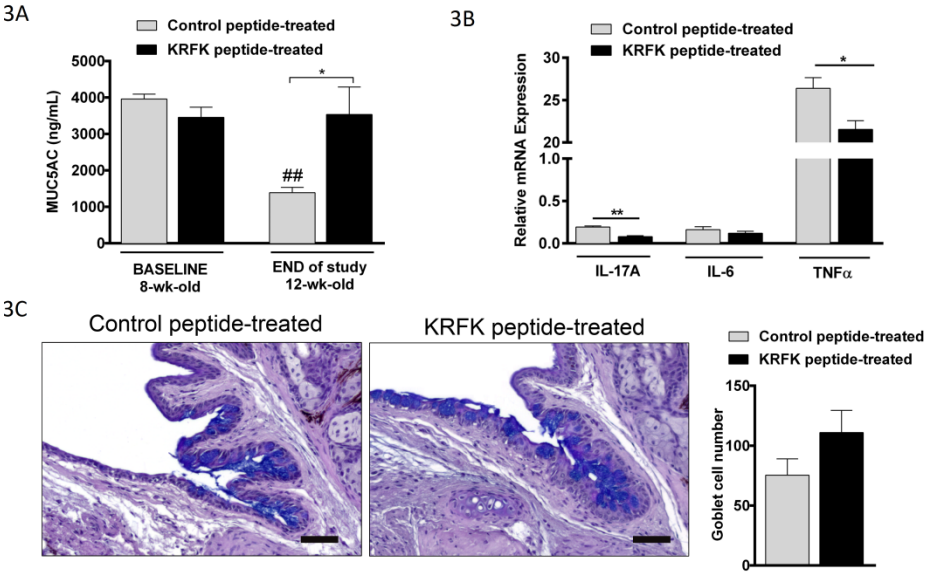

Figure 3: **Topically administered TSP-1-derived peptide KRFK prevents conjunctival inflammatory signs in 12-week-old TSP-1-deficient mice**. A) MUC5AC levels per volume of tears determined by ELISA at the baseline and at the end of the prevention study. B) IL-17A, IL-6 and TNFα mRNA expression, normalized to GAPDH levels, determined by real time RT-PCR at the end of the prevention study. C) Representative micrographs of PAS/AB-stained conjunctival tissue sections and goblet cell numbers at the end of the prevention study. Statistically significant differences compared to baseline levels are indicated with hashes, and between samples are indicated with an asterisks (Tukey test or *t* test; *$p \leq 0.05$; ** ó ##$p \leq 0.01$). Scale bar: 100 µm.

Possible changes in the main lacrimal gland were also evaluated at the end of the prevention study. The expression of inflammatory cytokines was assessed by real time RT-PCR and lacrimal gland morphology was evaluated by H&E staining. Results showed that the expression of IL-1β and IL-6 was significantly reduced after topical administration of TSP-1-derived peptide KRFK in comparison to

control peptide-treated group of mice (Figure 4A). The histological evaluation of the lacrimal glands showed foamy appearance of intracellular content in acinar cells. Ducts ranged from simple cuboidal to stratified epithelia, as expected, with no inflammatory infiltrations in periductal areas. Relevant morphological differences were not observed between experimental groups (Figure 4B).

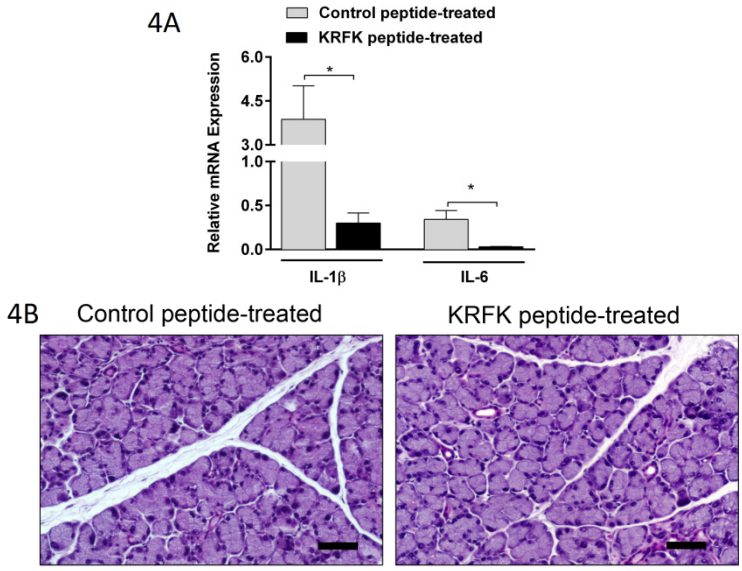

Figure 4: **Topically administered TSP-1-derived peptide KRFK reduces the expression of inflammatory cytokines in 12-week-old TSP-1-deficient lacrimal glands.** A) IL-6 and IL-1β mRNA expression, normalized to GAPDH levels, determined by real time RT-PCR at the end of the prevention study. B) Representative micrographs of H&E-stained lacrimal gland tissue sections at the end of the prevention study. Statistically significant differences between samples are indicated with asterisks (*t* test; **p*≤0.05). Scale bar: 100 µm.

3.2. Treatment of established ocular surface disease: Effects of topical KRFK in 16-week-old (after disease onset) TSP-1-deficient mice

Twelve-week-old TSP-1-deficient mice show a well-established ocular surface inflammation that is exacerbated with age. In the treatment study, 16-week-old TSP-1-deficient mice were topically treated with TSP-1-derived peptide KRFK or with the control peptide (KQFK) and changes in corneal, conjunctival, and lacrimal gland tissues were evaluated.

Corneal barrier integrity evaluation after KRFK peptide administration is showed in Figure 5A. Fluorescein corneal staining score was determined before (baseline - 16 weeks of age), and after 1 and 2 weeks of treatment. At the end of the treatment study, corneal staining scores improved from the baseline regardless of the treatment received. The histological evaluation of the H&E-stained corneal tissue sections did not show either significant differences in corneal thickness or morphology between experimental groups.

To assess inflammatory signs in the conjunctiva, MUC5AC levels in the tears, conjunctival morphology, and goblet cell numbers were evaluated. As shown in Figure 5B, KRFK peptide administration improved the declined tear MUC5AC levels with age in these mice. H&E-stained conjunctival tissue sections from both experimental groups showed a thick stratified epithelium with lower number of goblet cells intermixed with epithelial cells, evidencing some of the inflammatory signs reported in TSP-1-deficient mice in comparison to age-matched WT mice (Contreras-Ruiz, Regenfuss, et al. 2013). Despite the significant improvement in MUC5AC levels, relevant histological differences were not observed after KRFK peptide administration. The number of goblet cells counted in PAS/AB-stained conjunctivas treated with KRFK peptide was 52.66 ± 3.29, while control peptide (KQFK)-treated group showed 35.83 ± 10.52 goblet cells. Although topical KRFK increased in average the number of goblet cells, it was not significantly different between the experimental groups.

Lacrimal gland morphology and possible inflammatory infiltrates were also evaluated after KRFK or control peptide administration in 18-week-old TSP-1-deficient mice (Figure 5C). Normal acinar cells with pale and foamy intracellular content were observed in H&E-stained lacrimal gland tissue sections from both experimental groups. In contrast to younger TSP-1-deficient mice, 18-week-old mice from both experimental groups showed mild inflammatory infiltrates in periductal areas.

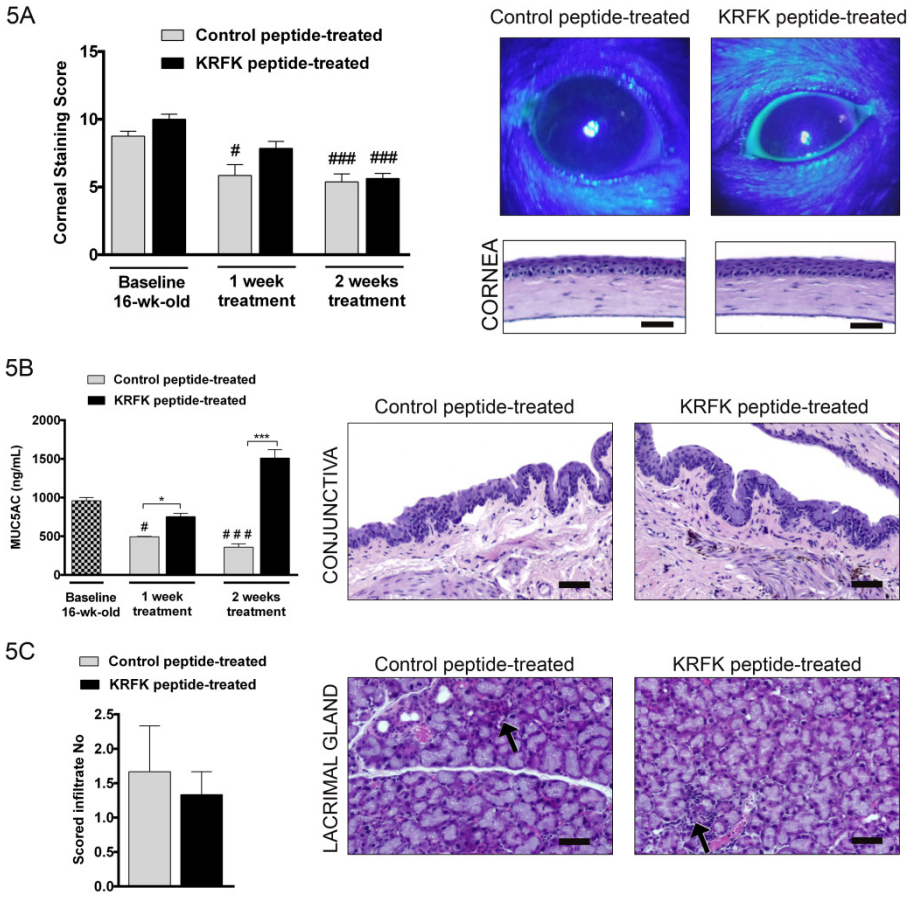

Figure 5: **Effects of topically administered TSP-1-derived peptide KRFK in 18-week-old TSP-1-deficient mice with an established ocular surface inflammation.** A) Corneal staining scores obtained at baseline and at the end of each week of treatment. Representative pictures of fluorescein staining and H&E-stained corneal tissues sections at the end of the treatment study. B) MUC5AC levels per volume of tears determined by ELISA at the baseline and at the end of each week of treatment. Representative micrographs of H&E-stained conjunctival tissue sections at the end of the treatment study. C) Representative micrographs of H&E-stained lacrimal gland tissue sections at the end of the treatment study. Arrows indicate inflammatory infiltrates in periductal areas. Statistically significant differences compared to baseline levels are indicated with hashes, and between samples are indicated with an asterisks (Tukey test; * ó # $p \leq 0.05$; ** $p \leq 0.01$; *** ó ### $p \leq 0.001$). Scale bar: 100 µm.

3.3. Topically administered TSP-1-derived peptide KRFK does not induce expression of fibrotic markers in the ocular surface of TSP-1-deficient mice

As the RFK sequence activates latent TGF-β expression (Schultz-Cherry et al. 1995), and activated TGF-β mediates wound repair (Nor et al. 2005) and fibrotic processes under pathological environment (Yamanaka et al. 2010), expression of fibrotic markers were analyzed after KRFK peptide administration at the end of the experimental time course in both prevention and treatment studies. Increased collagen deposition and myofibroblast cell type are common in fibrotic areas. Sirius Red staining for collagen content evaluation together with α-SMA expression as myofibroblast marker in ocular tissues were assessed. Figure 6 shows the evaluation of Sirius red and α-SMA stainings obtained at the end of the prevention study. Representative micrographs of Sirius red-stained tissue sections observed under white light or polarized light did not show significant differences between experimental groups, as confirmed by measuring collagen content identified as the pink-red staining (Figure 6A).

Alpha-SMA-expressing cells are localized in the smooth muscle areas and in tissue repair processes, as a marker of myofibroblast (Hinz et al. 2007). Representative micrographs of α-SMA immunostaining showed positive α-SMA matched with muscular fibers identified as pale yellow staining observed in Sirius red-stained tissue sections. Similar α-SMA staining area underlying conjunctival stroma in both TSP-1-deficient experimental groups and age-matched WT mice were observed, evidencing typical α-SMA staining in ocular tissues (Figure 6B).

Same evaluation was performed in KRFK-treated TSP-1-deficient mice from the treatment study. No signs of fibrosis were observed in KRFK-treated 18-week-old TSP-1-deficient mice in comparison to control peptide-treated group of mice (data not shown). These data evidence no fibrotic processes after KRFK peptide administration in both, prevention and treatment, studies.

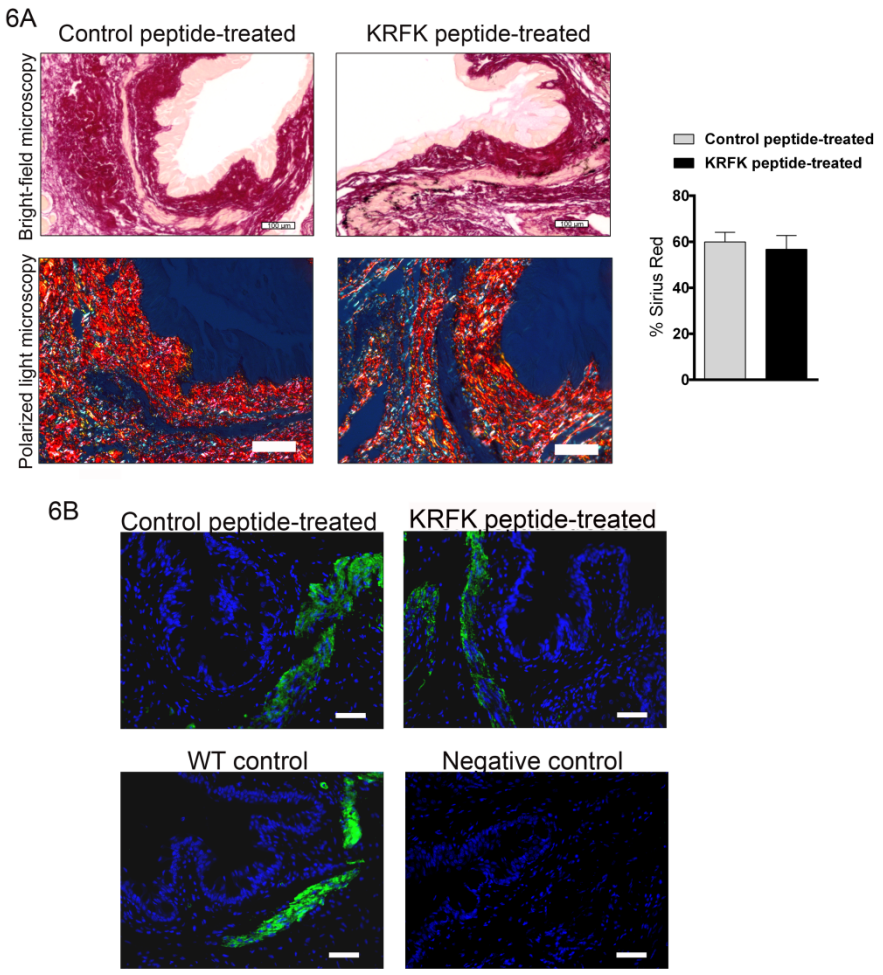

Figure 6: **Topically administered TSP-1-derived peptide KRFK does not induce local fibrotic processes in 12-week-old TSP-1-deficient mice.** A) Representative micrographs of Sirius red-stained ocular tissue sections obtained by bright-field and polarized light microscopy at the end of the prevention study. Graph shows percentage of Sirius red staining. B) Representative micrographs of the immunolocalization of α-smooth muscle actin (αSMA, green) in TSP-1-deficient at the end of the prevention study and in age-matched WT control ocular tissue sections. Nuclei were stained with Hoechst dye (blue). Negative control includes omission of primary antibody. Scale bar: 50 µm, otherwise indicated.

4. DISCUSSION

The development of an ocular immunopathogenesis in TSP-1-deficient mice was first described by Masli and colleagues (Turpie et al. 2009). This mouse model of ocular inflammation has a great value to enhance the knowledge of TSP-1 dependence in ocular surface homeostasis. In this work, we studied the

potential of a TSP-1-derived peptide that activates TGF-β, the KRFK peptide, to prevent or ameliorate inflammatory manifestations observed in this mouse model. The data presented here show that topical administration of the TSP-1-derived peptide KRFK prevents the development of ocular surface inflammatory signs known to appear in TSP-1-deficient mice with age. Moreover, the KRFK peptide administration reverses the compromised tear MUC5AC levels present in TSP-1-deficient mice with well-established ocular surface inflammation.

The observation that instilled KRFK peptide reduced effector and increased regulatory T cells in TSP-1-deficient cervical lymph node cell populations before the disease onset (unpublished observation) favored our choice to perform the preventive approach. In this study, we topically administered TSP-1-derived peptide KRFK to 8-week-old TSP-1-deficient mice during 2 weeks and inflammatory symptoms were evaluated at 12 weeks of age. Topical KRFK peptide administration successfully prevented the corneal barrier disruption described in TSP-1-deficient mice from 8 to 12 weeks of age (Turpie et al. 2009). This corneal change was also accompanied by others in the conjunctiva or the main lacrimal gland.

Although MUC5AC secretion is not pivotal for corneal integrity (Marko et al. 2014), inflammation-based diseases are associated with decreased levels of MUC5AC in tears, which correlates to the symptomatic severity of the diseases (Zhang et al. 2013). In the prevention study, TSP-1-derived KRFK peptide reversed the reduction in tear MUC5AC levels described in TSP-1-deficient mice with age (Contreras-Ruiz, Regenfuss, et al. 2013). In line with this improvement, KRFK peptide administration also decreased TNFα and IL-17 expression in the conjunctiva. These changes in the expression of inflammatory cytokines may affect goblet cell function, as TNFα causes decrease of MUC5AC secretion and goblet cell apoptosis *in vitro* and *in vivo* (Contreras-Ruiz, Ghosh-Mitra, et al. 2013; Ji et al. 2013). Therefore, reduction of goblet cell density is a feature of ocular surface inflammatory experimental models, including TSP-1-deficient mice (Dursun et al. 2002; Turpie et al. 2009), and chronic dry eye patients

(Argüeso et al. 2002). In our work, KRFK peptide administration decreased TNFα expression in the conjunctiva with no significant increase in goblet cell numbers, although these cells increased MUC5AC secretion indicating enhanced secretory function of these cells. All these findings corroborated that topical TSP-1-derived peptide KRFK prevents conjunctival inflammation.

In the prevention study, TSP-1-derived KRFK peptide administration also decreased the expression of inflammatory cytokines, such as IL-1β and IL-6, in the lacrimal gland. Higher levels of both cytokines are detected in the tears of patients with inflammation-based diseases of the ocular surface in comparison to control subjects (Lam et al. 2009; Massingale et al. 2009; Boehm et al. 2011; Na et al. 2012; Contreras-Ruiz et al. 2014). Particularly IL-1β is a key player of corneal epithelium-related pathologies. Previous reports showed that IL-1β upregulates marker expression of squamous metaplasia and alters corneal tight junction expression leading to a disruption of its barrier function (Kimura et al. 2009; Li et al. 2010; Chen et al. 2010). In our work, KRFK peptide administration reduced IL-1β expression which correlates with the reversal of compromised corneal barrier integrity. Overall, these data from corneal, conjunctival and lacrimal gland tissues indicate a clear efficacy of TSP-1-derived peptide KRFK to prevent inflammatory signs observed in the ocular surface of TSP-1-deficient mouse model.

The potential of TSP-1-derived peptide KRFK to ameliorate inflammatory signs observed in adult TSP-1-deficient mice was also addressed after the promising results obtained in the prevention study. We topically administered KRFK peptide to 16-week-old TSP-1-deficient mice with a well-established ocular inflammation during 2 weeks. Both control (KQFK) and KRFK peptides reduced corneal staining score. This unexpected result may be explained by the ability of the eyedrop instilled to wash and dilute inflammatory cytokines in the tear film, improving corneal environment and, consequently, corneal barrier. However, only KRFK peptide administration increased tears MUC5AC levels. This result showed a modulation of KRFK peptide-induced activated TGF-β in MUC5AC

levels under inflammatory condition, correlating with previous results in other mucosal surfaces showing TGF-β-induced MUC5AC expression (Chu et al., 2004; Jonckheere et al., 2004). Despite tear MUC5AC levels improvement, histological evaluation of H&E-stained corneal, conjunctival, or lacrimal gland tissue sections did not show significant differences after KRFK peptide administration. The potential of the TSP-1-derived peptide KRFK to treat established ocular surface inflammation may need more frequent administration or longer treatment period than what was shown to be efficient in the prevention study.

One of the most studied effects of active TGF-β is the modulation of wound healing processes, causing fibrosis if overexpressed (Tandon et al. 2010). As TSP-1-derived peptide KRFK has the ability to activate TGF-β (Schultz-Cherry et al. 1995), Sirius red and α-SMA stainings were used as fibrotic tissue markers to evaluate a potential pro-fibrotic side effect of the KRFK peptide. The Sirius red molecule intercalates into the tertiary groove of collagen molecules, including collagen types I, III, and IV. As expected, in control ocular tissue sections the corneal and conjunctival stroma exhibited a substantial collagen component. The amount of collagen, identified by pink-red color in Sirius red-stained tissue sections, showed no differences after KRFK peptide administration in both prevention and treatment studies. On the other hand, α-SMA is expressed by smooth muscle cells or myofibroblasts. Keratocytes are differentiated to myofibroblasts under tissue repair conditions, but also can represent the pathological tissue deformations in fibrotic processes (Hinz et al. 2007). The widely used myofibroblast marker α-SMA showed same staining pattern in all the ocular tissues analyzed. These data evidence no fibrotic processes after KRFK peptide administration in both studies. The predominant isoform of TGF-β in the murine ocular mucosa is the TGF-β2 (Contreras-Ruiz & Masli 2015), and therefore, it will be the most activated isoform by KRFK peptide. Some reports did not find this isoform as a key element in fibrotic processes (Tandon et al. 2010; Carrington et al. 2006). Moreover, recent reports doubts about the direct role of TGF-β in the complex processes associated with scarring reconstruction

of connective tissue, which needs to be further elucidated (Tsujino et al. 2017; Walraven et al. 2016).

In conclusion, our data support topical administration of the TSP-1-derived peptide KRFK as potential treatment for ocular surface inflammatory disorders associated with TSP-1 deficiency or reduced levels. The local and topical administration of KRFK peptide can offer the advantage over systemic administration of reduced systemic undesirable effects. Taken together, our results underline the role that TSP-1 exerts in the orchestration of inflammation via TGF-β activation and demonstrate that early administration of the TSP-1-derived peptide KRFK successfully prevents clinical manifestations of ocular inflammation advocating for personalized treatments in this type of pathologies.

Acknowledgments

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STUDY 5. Novel Anti-inflammatory Liposomal Formulation for the Pre-ocular Tear Film: *In vitro* and *Ex vivo* Functionality Studies in Corneal Epithelial Cells

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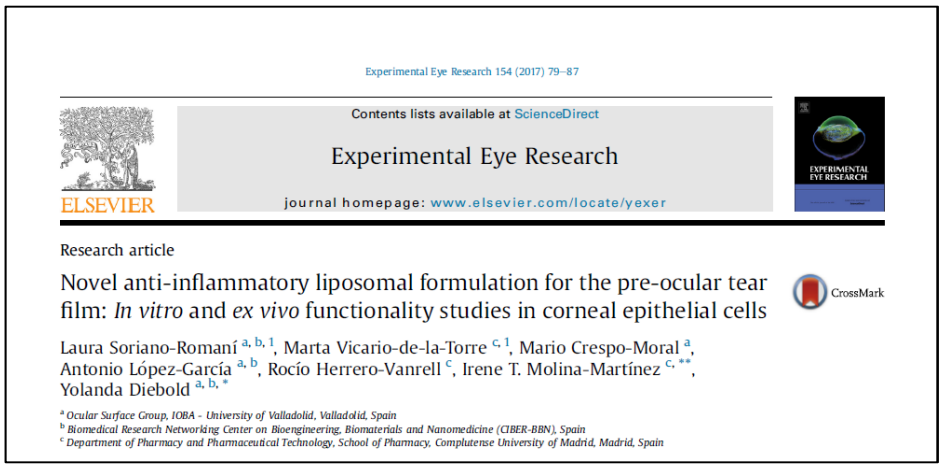

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Research article

Novel anti-inflammatory liposomal formulation for the pre-ocular tear film: *In vitro* and *ex vivo* functionality studies in corneal epithelial cells

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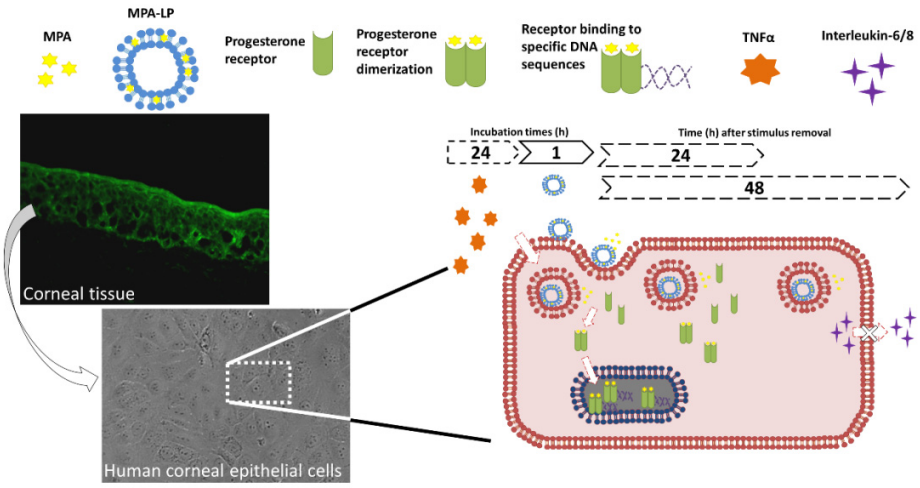
Novel Anti-inflammatory Liposomal Formulation for the Pre-ocular Tear Film: *In vitro* and *Ex vivo* Functionality Studies in Corneal Epithelial Cells

Highlights

- Medroxyprogesterone was incorporated into a liposomal artificial tear formulation.
- Encapsulated drug readily penetrates corneal tissue in an *ex vivo* porcine model.
- Encapsulated drug exerts anti-inflammatory effects in human corneal cells *in vitro*.
- The novel liposomal artificial tears enhance the effect of the drug *in vitro*.

Graphical abstract



Abstract

In ocular surface inflammatory diseases, such as dry eye disease, long-term symptom relief requires targeting the inflammation itself rather than treating only the surface-associated dryness with artificial tears. Therefore, we included an anti-inflammatory agent in an unpreserved liposome-based (LP) formulation used as artificial tears. Our aim was to characterize and study its *in vitro* and *ex vivo* cell uptake and functionality. Human corneal epithelial (HCE) cells were used to study MPA-LP-induced effects after 60 min of exposure, using blank LP and non-LP MPA formulations as controls. A fluorescent labeled LP formulation was used to determine uptake by HCE cells and localization in *ex vivo* porcine corneas. The LP formulation complied with the required physicochemical properties and had no cytotoxicity on HCE cells after 60 min of exposure. HCE cells showed LP-associated fluorescence at 24, 48, and 72 h after 60 min of exposure, and the LP-associated fluorescence was uniformly distributed throughout the porcine corneal epithelium immediately after 5 min of exposure. MPA-LP increased protein expression and nuclear translocation of progesterone receptor in comparison with controls as determined by Western blotting and immunofluorescence. Moreover, MPA-LP significantly reduced the cell proliferation rate and IL-6 and IL-8 production 48 h after the exposure period, as determined by the alamarBlue assay and ELISA, respectively. None of these effects were evident in blank LP-exposed cells and non-LP MPA formulation reduced only IL-6 production. Our results suggest that the LP-based formulation, used to replenish the lipids of the tear film, can be loaded with anti-inflammatory agents that can be delivered into the cells and activate specific drug receptors. These agents can reduce inflammatory cytokine production and may be effective in the treatment of inflammatory processes associated with ocular surface diseases.

Keywords

Corneal epithelial cells; Drug delivery; Glucocorticoid receptor; Inflammation; Liposome; Progesterone receptor.

Abbreviations

BAC: benzalkonium chloride

C6: coumarin-6

DED: dry eye disease

DMEM/F12: Dulbecco's Modified Eagle Medium + F12 medium mixture

EE: encapsulation efficiency

ELISA: enzyme-linked immunosorbent assay

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HCE: human corneal epithelial

HPLC: high performance liquid chromatography

IL: interleukin

LP: liposome

MPA: medroxyprogesterone acetate

PBS: phosphate buffered saline

PC: phosphatidylcholine

SEM: standard error of the mean

TNFα: tumor necrosis factor-alpha

XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

1. Introduction

Eye tissues are affected by diverse inflammation-based diseases that can be devastating as eyesight is impaired. A paradigm of such inflammatory disorders is dry eye disease (DED), a common age-related ocular pathology that results from dysfunction of the ocular surface and/or tissues that produce the tears. The primary effects of patients with DED are impaired functional visual acuity and low quality of life. At the molecular level, the main reason of these effects is an underlying inflammation process that profoundly alters the ocular surface tissues and the tear film. Recent research has led to innovations in the pharmacological treatment of DED. These advances have focused on tear replacement or tear preservation with novel artificial tears, topical and systemic anti-inflammatory and immunosuppressive therapy, and even the use of medical devices in the more severe grades of the disease (Pflugfelder *et al*. 2000). Despite significant progress in these treatments, the disease in many patients is not fully alleviated. Although current treatments with artificial tears are the first step for symptomatic relief, the ongoing inflammation is not addressed. The use of drug delivery systems is of great interest to improve the pharmacological management of DED because they can encapsulate active agents, direct them to the target tissue/cells, and release the cargo in a controlled fashion (Diebold and Calonge. 2010, Patel *et al*. 2013). In topically administered drugs, these devices overcome drawbacks such as low bioavailability due to the tear film barrier, rapid drainage, and poor corneal permeation.

Among drug delivery systems, liposomes (LPs), which are vesicles containing an aqueous core and delimited by a membrane-like lipid bilayer, ensure good biological compatibility and reduced toxicity. The existence of an aqueous core and the lipid bilayer confers the ability to encapsulate both hydrophilic and lipophilic compounds with improved drug stability. LPs have been employed to improve drug transport across the cornea (Di Tommaso *et al*. 2012) with prolonged drug effect (Hathout *et al*. 2007, Law *et al*. 2000). The idea of using LPs as carriers for topically administered anti-inflammatory drugs was first

reported in 1982 (Schaeffer and Krohn. 1982) and has been continuously revised (Lim *et al*. 2015). Corticosteroids are potent anti-inflammatory agents, poorly soluble in aqueous solution, which makes them good candidates for being encapsulated in drug delivery systems. However, with prolonged treatment, the potent activity of these drugs often causes adverse effects, such as increased intraocular pressure or cataract formation. For this reason, synthetic "soft" anti-inflammatory steroids, like medroxyprogesterone acetate (MPA), are frequently prescribed for topical administration in the eye.

Taking all of this information into account, our aim was to prepare and characterize a LP-based formulation loaded with the anti-inflammatory agent MPA that could be used as topically instilled artificial tears. Prior to proceeding with *in vivo* studies, it was necessary to determine by *in vitro* studies how corneal epithelial cells take up the drug delivery system and the encapsulated drug. Then, we asked whether or not the encapsulated drug was delivered into the cells and activated glucocorticoid and progesterone receptors, eliciting an anti-inflammatory effect in an *in vitro* inflammation model of human corneal epithelial cells.

2. Materials and methods

2.1. Materials and equipment

All materials used in this study were purchased from Sigma-Aldrich (St. Louis, MO) except as follows: Phospholipon 90G, >95% of phosphatidylcholine (PC), was purchased from Lipoid GmbH (Cologne, Germany) and trehalose was acquired from Cymit Química S.L. (Barcelona, Spain). The MPA-commercial, non-LP formulation or reference formulation Colircusi Medrivas® was from Alcon Cusi S.A. (Barcelona, Spain). Dulbecco's Modified Eagle Medium (DMEM)/F12 and some of its supplements, such as fetal bovine serum, penicillin, and streptomycin were from Invitrogen-GIBCO (Inchinnan, UK). Human epidermal growth factor and bovine insulin were from Invitrogen (Eugene, OR). Cell culture plates and multichamber Permanox® or Glass® slides were from Nunc (Roskilde,

Denmark). For Western blot experiments, the bicinchoninic acid assay was from Pierce (Rockford, IL) and acrylamide and Tris-buffered saline were purchased from Bio-Rad Laboratories (Hercules, CA). For immunocytochemistry and Western blotting, mouse monoclonal primary antibodies for glucocorticoid and progesterone receptors were from Abcam (Cambridge, UK), and the antibody for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Santa Cruz Biotechnology (Santa Cruz, CA). Fluoromount-G™ mounting media was from SouthernBiotech (Birmingham, AL). The alamarBlue® colorimetric indicator assay was from AbD Serotec (Oxford, UK). Tumor necrosis factor alpha (TNFα) was from PeproTech (London, UK). Human interleukin-6 (IL-6) and IL-8 enzyme-linked immunosorbent assay (ELISA) kits were from Diaclone (Bensançon, France). The Lipex Extruder® was from Lipex Biomembrane™ (Vancouver, Canada) and the particle size analyzer Zetatrac® was from Microtrac-Europe GmbH (Meerbusch, Germany). The high performance liquid chromatography (HPLC) Waters 600 E, binary HPLC pump, 717 Plus autosampler, in-line degasser, and 486 λ absorbance detector was from Waters (Barcelona, Spain) and the Mediterranean Sea® C18 100 Å 5-µm column (150 mm by 4 mm) from Teknokroma, S.A. (Barcelona, Spain). The osmometer K-7000 was from Knauer (Berlin, Germany) and the thermostatically controlled rheometer Rheostress RS1 was from Haake Technik GmbH (Düsseldorf, Germany). The SpectraMAX® M5 multidetection microplate reader and the SoftMax Pro 4.8 software used for absorbance and fluorescence measurements were from Molecular Devices (Sunnyvale, CA). The Leica DMI 6000B microscope and the LAS AF Lite software and ImageJ software v. 1.49 (http://imagej.nih.gov/ij/) used to visualize and analyze fluorescence micrographs, were from Leica Microsystems (Wetzlar, Germany) and from National Institutes of Health (Bethesda, MD), respectively. Final fluorescence micrographs were minimally adjusted in terms of brightness and contrast using Adobe Photoshop version 8.0.1. The ChemiDoc XRS system and the Quantity One software used to visualize the acrylamide gel and to analyze the resulting

images were from Bio-Rad Laboratories. The IBM SPSS Statistics version 23.0 software used for statistical analyses was from IBM Corp. (Armonk, NY).

2.2. Liposomal formulation preparation and fluorescent labeling

LP formulation composed of PC, cholesterol, vitamin E, and MPA at a molar ratio 13:3:0.2:0.3 was prepared by the solvent evaporation technique as previously described (Vicario-de-la-Torre *et al*. 2014). The film formed was hydrated with a dispersion solution comprised of borate buffer solution (135.5 mM) containing trehalose (42.5 mM). The MPA-LP formulations were extruded through a size-controlled 0.22-µm pore size polycarbonate membrane for ten cycles under nitrogen pressure (≤200 psi) to obtain lipid vesicles with a unimodal size distribution. Final PC and MPA concentrations in the resultant dispersion were 20 mg/mL and 200 µg/mL, respectively.

To obtain fluorescently-labeled LP formulation, the lipid mixture containing coumarin-6 (C6) instead of MPA was dissolved in chloroform, and the C6-LP formulation was obtained following the protocol described above. Fluorescent C6-LP at a final C6 concentration of 20 µg/mL was used in human corneal epithelial (HCE) cells and *ex vivo* porcine corneas (see sub-section 2.6 below). To determine LP-associated fluorescence, a suspension of C6 (not in liposomes) obtained from the supernatant after C6-LP centrifugation (49,263 g for 60 min at 10°C) was used as the control.

2.3. Characterization of liposomal formulation

2.3.1. Particle size distribution and encapsulation efficiency

Particle size distribution of blank, MPA-, and C6-LP formulations were measured by dynamic light scattering using a particle size analyzer at room temperature.

To determine the encapsulation efficiency, the LP formulation was centrifuged (49,263 g for 60 min at 10°C), and the supernatant was then analyzed by HPLC at 40°C. Isocratic MPA analysis was performed with a mobile

phase of acetonitrile/tetrahydrofurane/ultrapure milliQ water (45/10/45), a flow rate of 1 mL min^{-1}, and detection at 254 nm. The retention time was 12.5 min. This analytical procedure, described in Pharmacopeia (Shabir. 2003), was previously validated in our laboratory with regard to linearity, precision, and accuracy parameters. Encapsulation efficiency (*EE*) was calculated as follows:

$$EE = \frac{C0 - C1}{C0} * 100$$

Where *C0* and *C1* were the concentration of the drug in the LP dispersion and in the supernatant after centrifugation.

2.3.2. pH, osmolarity, and viscosity measurements

The pH of the MPA- and C6-LP formulations was measured using a pH meter in triplicate at room temperature.

Osmolarity was analyzed by a vapor pressure osmometer and performed in triplicate at 33°C, equivalent to the ocular surface temperature (Purslow and Wolffsohn. 2005).

Viscosity was measured using a thermostatically controlled rheometer. Viscosity was measured when the steady state was reached with shear rates increasing from 0 to 1000 s^{-1}. All determinations were made in triplicate at 33°C.

2.4. Culture conditions and liposomal formulation exposure

The HCE cell line (Araki-Sasaki *et al*. 1995) was cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum, 5,000 U/mL penicillin/streptomycin, 10 ng/mL human epidermal growth factor, and 5 µg/mL insulin. Cells from passages 32 to 38 were used.

Cells were grown until confluence and washed in supplement-free culture medium. Then, the LP formulations were diluted in supplement-free culture medium to get a final PC concentration of 5 mg/mL and added to the cultured cells for 60 min. A MPA-commercial formulation was used as anti-inflammatory non-LP reference formulation (Medrivas®), and unexposed cells or cells exposed

to the dispersion solution and blank LP formulation were used as controls. After the exposure period, the cells were washed 3 times in phosphate buffered saline (PBS) and fresh serum-free culture medium was added.

2.5. Cytotoxicity assay

To measure potential formulation toxicity, the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cytotoxicity assay was used. After the exposure period, the XTT reagent was added according to manufacturer´s instructions and absorbance read. At least 3 independent experiments in triplicates were performed. Benzalkonium chloride (BAC) at 0.001% was used as positive cytotoxicity control. The percentage of viable cells relative to control cells was determined.

2.6. Liposomal formulation uptake by HCE cells and localization in ex vivo porcine corneas

The C6-LP was quantified and localized by fluorometry and fluorescence microscopy, respectively. To ensure that the fluorescence originated from C6-LP, a C6 suspension was used as the control. For fluorometry determinations, HCE cells were plated in black 96-well plate and exposed to C6-LP or C6 suspension (not in liposomes) for 60 min. Then, cells were washed with PBS until the fluorescent signal of the PBS used for washing cells was the same that fresh PBS. The fluorescent signal (excitation wavelength: 490 nm; emission wavelength: 520 nm) of the bottom of each well was measured at 0, 24, 48, and 72 h after the exposure period. Unlabeled LP formulation in PBS was used as a blank. For fluorescence microscopy, HCE cells were plated in multichambered coverslip and exposed to C6-LP or C6 suspension for 60 min and over washed. Representative micrographs were taken at each time point using same optimized exposure time, gain, and intensity of the camera. Also, cell uptake was further characterized immediately after the exposure period. Cell nuclei were counterstained with

Hoechst dye and vertical spatial images were generated by Z-scans. At least 3 independent experiments in triplicates were performed.

In addition, *ex vivo* porcine corneas were used to localize the LP formulation in the stratified corneal epithelium and stroma. Porcine eyeballs (n=3), obtained from a local slaughterhouse, were washed with povidone iodine solution and PBS. Then, a 12-mm diameter silicon ring was placed on the central cornea, and a C6-LP or C6 suspension (not in liposomes) was applied for 5 and 60 min. Unlabeled blank LP formulation at the same exposure period was used as control. After the exposure, the eyeballs were washed with PBS and placed in 2.5% buffered paraformaldehyde. The central cornea was isolated and embedded in optimal cutting temperature compound and frozen. Representative micrographs of cryostat sections (5 µm) were taken using the same optimized exposure time, gain, and intensity for each group of samples. At least 3 independent experiments were performed.

2.7. Protein expression analysis by electrophoresis and Western blotting

Cells were homogenized in ice-cold radioimmunoprecipitation assay buffer plus protease inhibitors, and the protein content was quantified. Total protein was heated at 100˚C, and 15 µg of protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were subsequently transferred to nitrocellulose membranes, and incubated with a blocking solution composed of tris-buffered saline containing 0.05% Tween-20, 5% powdered milk, and 2.5% bovine serum albumin for 60 min. The membranes were incubated with 2.5 µg/mL of primary antibodies in blocking solution overnight at 4°C and with peroxidase-conjugated secondary antibodies for 60 min. Receptor protein expression was normalized to GAPDH expression. At least 3 independent experiments in duplicates were performed.

2.8. Glucocorticoid receptor and progesterone receptor localization by immunofluorescence analysis

Some HCE cultures were fixed in ice-cold methanol immediately after exposure to the liposomal formulations and kept at -20˚C while others were fixed 24 h after LP exposure. Slides were washed in PBS and incubated with 0.3% Triton X-100 for 10 min. The slides were incubated at room temperature for 60 min in PBS with 4% donkey serum. They were then incubated with 5 µg/mL of primary antibodies against glucocorticoid and progesterone receptors overnight at 4°C. Alexa Fluor 488-conjugated secondary antibody was incubated for 60 min at room temperature. Cell nuclei were counterstained with propidium iodide. Negative controls omitted the primary antibodies (isotype control). Micrographs were taken using the same optimized exposure time, gain, and intensity of the camera. To calculate percentage of nuclear receptor fluorescence, green (receptor) and red (nuclei) channels were thresholded. The percentage of nuclear translocation was calculated using the following formula:

$$\%\ \text{nuclear fluorescence} = \frac{\text{receptor fluorescence localized in nuclear area}}{\text{total receptor fluorescence}} x\ 100$$

Where the receptor fluorescence (green) localized in the nuclear area (demarcated by red) were the mean grey value of the thresholded green channel under the thresholded red channel and the total receptor fluorescence was the mean grey value of the thresholded green channel. The values were obtained from 4 independent experiments.

2.9. Cell proliferation assay

To determine possible changes in cell proliferation rate by MPA-LP exposure, the alamarBlue® cell proliferation assay was performed at 24, 48, and 72 h after the exposure period. Fluorescence was read according to manufacturer´s instructions. At least 3 independent experiments in duplicates were performed. The percentage of proliferation in treated cultures compared with values for untreated control cells at 24 h was determined.

2.10. In vitro inflammation model

To determine if MPA-LP induced an anti-inflammatory response in HCE cells, an *in vitro* inflammation model was used as previously described (Enriquez-de-Salamanca *et al*. 2008). Briefly, HCE cells were stimulated with 25 ng/mL TNFα for 24 h after a washing period with supplement-free culture medium. The cells were then exposed to blank LP, MPA-LP, and the reference formulation (Medrivas®). The cells were washed with PBS and fresh medium was added. Cell supernatants were collected 48 h after the exposure period, and IL-6 and IL-8 production quantified with human IL-6 and IL-8 ELISA kits according to manufacturer´s instructions. At least 3 independent experiments in duplicates were performed. The ratio of each interleukin concentration to number of cells was calculated by the alamarBlue® assay.

2.11. Statistical Analyses

Data were expressed as means ± standard errors of the mean (SEM). Student´s *t*-test was used to compare two groups. For groups with significantly different variances (F-test), Welch´s correction was done. For more than 2 groups, we performed either a one-way analysis of variance followed by pairwise comparisons (Tukey´s test) or a Brown-Forsythe test followed by pairwise comparisons (Games-Howell test), depending on Levene's test for homogeneity of variances. Differences were considered to be significant when $p \leq 0.05$.

3. Results

3.1. Liposomal formulation characterization and biocompatibility

MPA or C6 addition to the LP composition did not change the mean diameter of liposomes (Fig. 1A, Table 1). MPA and C6 were included in the initial lipid mixture and passively loaded into the lipid bilayer of the LPs. The encapsulation efficiency was close to 94% and 89%, respectively. The mean diameter, pH, osmolarity, and viscosity of blank, MPA- and C6-LP is shown in Table 1. The LP-based formulations did not show statistically significant differences in pH,

osmolarity, and viscosity compared with the borate-trehalose-buffered LP-free solution (data not shown).

Table 1: Liposomal formulation diameter, pH, osmolarity, and viscosity. LP: liposome-based formulation; C6: coumarin-6; MPA-LP: liposome-based formulation loaded with medroxyprogesterone acetate; C6-LP: liposome-based formulation loaded with C6.

	Blank LP	**MPA-LP**	**C6-LP**
Diameter (nm)	191.6 ± 2.4	185.5 ± 3.5	188.1 ± 2.0
pH	7.4 ± 0.1	7.4 ± 0.2	7.5 ± 0.3
Osmolarity (mOsm)	202.4 ± 0.1	206.0 ± 0.2	206.7 ± 0.3
Viscosity (mPas)	3.1 ± 1.1	3.0 ± 0.6	3.1 ± 1.0

The percentage of viable HCE cells did not differ significantly from untreated cells after 60 min of exposure with either blank, MPA-, or C6-LP (Fig. 1B). The reference formulation (Medrivas®) was well tolerated while the BAC solution caused significant cell death, as expected.

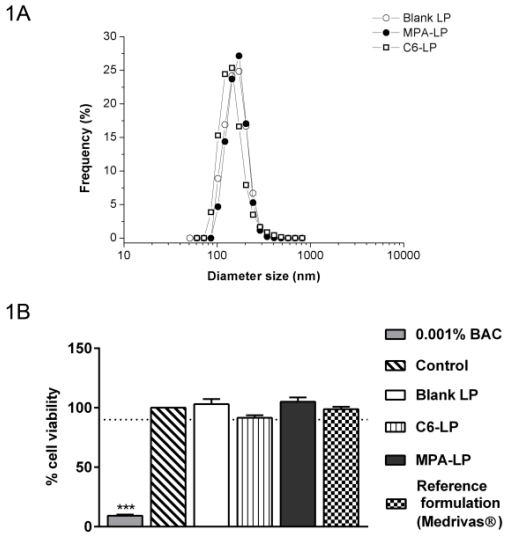


Figure 1: **Diameter size distribution and cytotoxicity**. (A) The diameter size distribution of the liposomal formulation did not change with the addition of MPA or C6 as determined by dynamic light scattering. (B) The percentage of viable cells did not differ from untreated cells with either blank, MPA- or C6- LP formulations after 60 min of exposure in HCE cells as measured by the XTT assay. ***$P \leq 0.001$ compared with control. LP: liposome-based formulation; C6-LP: liposome-based formulation loaded with coumarin-6; MPA-LP: liposome-based formulation loaded with medroxyprogesterone acetate; BAC: benzalkonium chloride.

3.2. Uptake and localization of LP formulation in vitro by HCE cells and epithelial localization in ex vivo porcine corneas

The cellular fluorescent signal was significantly higher after exposure to the C6-LP than after exposure to the C6 suspension at all times of analysis (Fig. 2A). However, C6 suspension can also interact with plasmatic membranes and some fluorescence was detected after sequential cell wash. Z-axis micrographs showed that after C6-LP exposure for 60 min, the fluorescence was located not only in the cell membrane but also intracellularly (Fig. 2B).

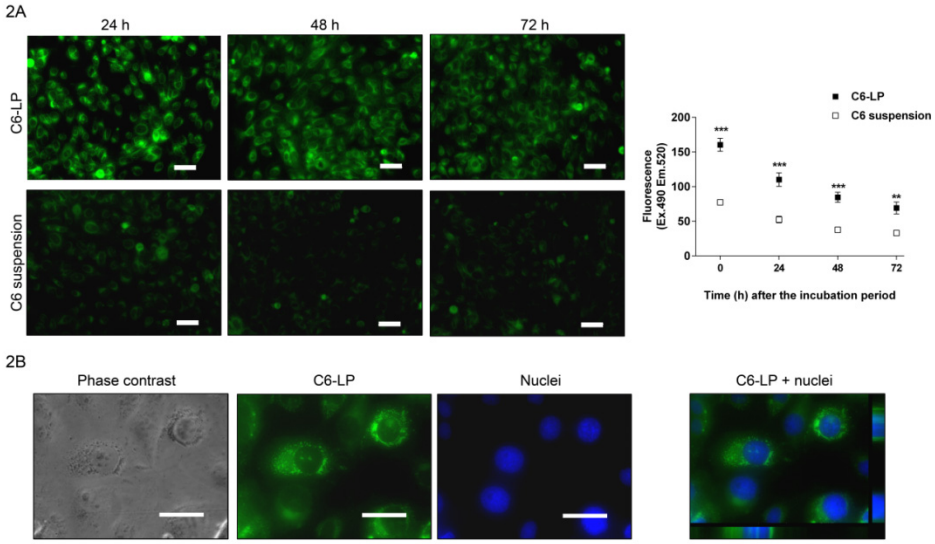


Figure 2: **Uptake and distribution in human corneal epithelial cells**. (A) At all times analyzed, the cellular fluorescent signal was significantly higher after C6-LP exposure than after C6 suspension exposure in HCE cells as determined by fluorescence microscopy (left) and fluorometry (right). (B) After the exposure time, C6-LP was taken up by HCE cells as Z-scans showed intracellular C6-LP localization. C6 stained in *green* and nuclei were stained in *blue* with Hoechst dye. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, compared at same time points. LP: liposome-based formulation; C6: coumarin-6; C6-LP: liposome-based formulation loaded with C6. Scale bar: 50 µm.

After 5 min of C6-LP exposure, the *ex vivo* porcine corneas showed fluorescence uniformly distributed throughout the corneal epithelium (Fig. 3). In contrast, penetration of the C6 suspension was largely limited to the outermost epithelial cells. After 60 min of exposure, the corneal stroma also showed

fluorescent signal. However, the C6 suspension-associated fluorescence was weaker and localized only in the outermost corneal epithelial layers, supporting the permeation of the LP formulation through the cornea. There was no fluorescent signal associated with unlabeled LP formulation at any time analyzed.

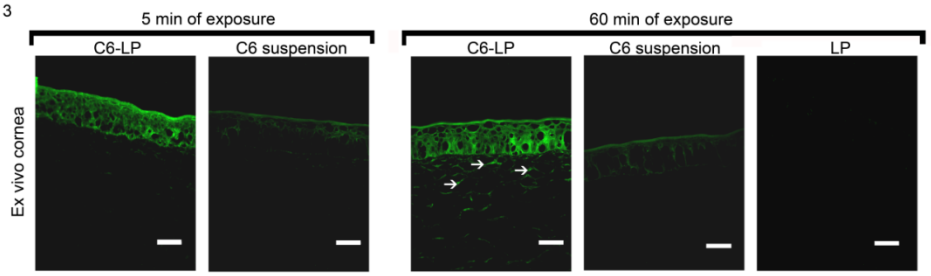


Figure 3: **Localization in *ex vivo* porcine corneas**. C6-LP was uniformly distributed throughout the corneal epithelium in *ex vivo* corneas after 5 min and 60 min of exposure. Arrows show stromal localization of C6-LP after 60 min of exposure in *ex vivo* porcine corneas. LP: liposome-based formulation; C6: coumarin-6; C6-LP: liposome-based formulation loaded with C6. Scale bar: 50 µm.

3.3. Effect of MPA-LP and reference formulation (Medrivas®) on glucocorticoid and progesterone receptor protein expression in HCE cells

Glucocorticoid and progesterone receptor protein expression did not differ immediately after exposure for 60 min (Fig. 4A). However, 24 h after exposure, progesterone receptor expression was increased after MPA-LP exposure compared with the control, blank LP-, and reference formulation-exposed cells (Fig. 4B). At that time point, glucocorticoid receptor expression was decreased in reference formulation-exposed cells compared with control (p=0.067), blank LP- (p=0.072), and MPA-LP-exposed cells, although only the latter reached statistical significance ($p \leq 0.05$).

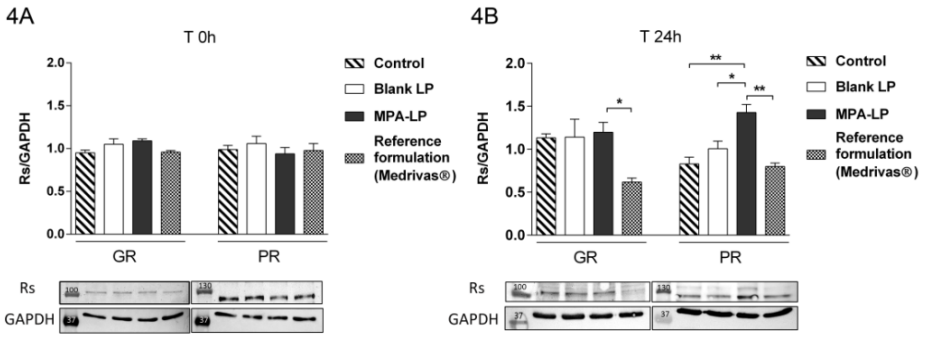


Figure 4: **Changes in the expression of glucocorticoid and progesterone receptors in human corneal epithelial cells**. Although no changes were shown immediately after (T 0 h) the exposure period (A), MPA-LP-exposed cells increased progesterone receptor expression compared with control, blank LP, and reference formulation-exposed cells after 24 h (T 24 h) the exposure period (B), as determined by electrophoresis and Western blotting. **P* ≤ 0.05; ***P* ≤ 0.01. LP: liposome-based formulation; MPA-LP: liposome-based formulation loaded with medroxyprogesterone acetate; GR: glucocorticoid receptor; PR: progesterone receptor; Rs: receptors; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

3.4. Effect of MPA-LP on nuclear translocation of glucocorticoid and progesterone receptors in HCE cells

There was no measurable nuclear translocation of the glucocorticoid receptor after exposure for 60 min for any of the LP or reference formulation treatments (Fig. 5A). However, immediately after the exposure period, nuclear translocation of the progesterone receptor was higher in MPA-LP-exposed cells than in blank LP-exposed and untreated cells (Fig. 5B). Twenty-four hours after the exposure, neither the glucocorticoid receptor nor the progesterone receptor translocation to the nucleus after MPA-LP exposure was evident (data not shown).

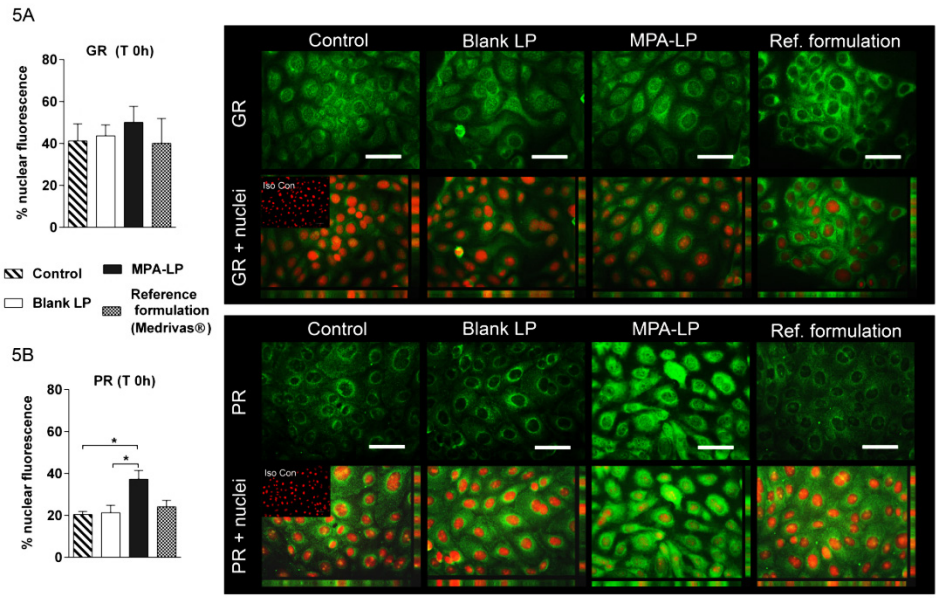


Figure 5: **Nuclear translocation (activation) of glucocorticoid and progesterone receptors in human corneal epithelial cells.** Although no changes were shown in glucocorticoid receptor nuclear translocation (A), MPA-LP-exposed cells increased nuclear translocation of progesterone receptor compared with control and blank LP-exposed cells (B) determined by fluorescence microscopy. Receptors stained in *green* and nuclei were stained in *red* with propidium iodide. Graph shows the percentage of nuclear receptor fluorescence calculated by measuring the receptor staining in the nuclear area to the total receptor staining. *$P \leq 0.05$. LP: liposome-based formulation; MPA-LP: liposome-based formulation loaded with medroxyprogesterone acetate; GR: glucocorticoid receptor; PR: progesterone receptor; Iso Con: isotype controls including omission of primary antibodies. Scale bar: 50 µm.

3.5. Time-dependent effect of MPA-LP on cell proliferation of HCE cells

At 24 h after the exposure period, cell proliferation in MPA-LP-exposed cells was significantly decreased compared with untreated control cells. At 48 h, the proliferation of MPA-LP-exposed cells was significantly lower than in blank LP-exposed cells (Fig. 6). Differences in cell proliferation disappeared 72 h after the exposure period.

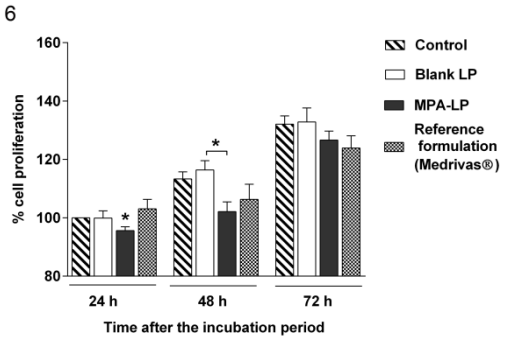


Figure 6: **Changes in corneal epithelial cell proliferation**. MPA-LP-exposed cells reduced cell proliferation compared with blank LP-exposed cells 48 h after the exposure period as determined by the alamarBlue® assay. *$P \leq 0.05$, compared with control, if not otherwise indicated by straddle bars. LP: liposome-based formulation; MPA-LP: liposome-based formulation loaded with medroxyprogesterone acetate.

3.6. Effect of MPA-LP on IL-6 and IL-8 production by inflamed HCE cells

Prior to determining the anti-inflammatory effect of MPA-LP, changes in glucocorticoid and progesterone receptors after TNFα stimulation were analyzed by electrophoresis and Western blotting, and immunofluorescence. Cells stimulated with TNFα did not show changes in protein expression or translocation of glucocorticoid and progesterone receptors compared with unstimulated cells (data not shown).

In response to TNFα stimulation for 24 h, the HCE cells increased IL-6 but not IL-8 production 48 h after inflammatory stimulus removal. MPA-LP exposure significantly reduced both basal and TNFα-induced IL-6 production, reaching unstimulated control levels (Fig. 7A). The reference formulation (Medrivas®) also reduced IL-6 production in basal and TNFα-stimulated cells. MPA-LP exposure also significantly reduced IL-8 production compared with the blank LP and reference formulations in TNFα-stimulated HCE cells (Fig. 7B). Interestingly, blank LP formulation reduced IL-8 production more than did the reference formulation.

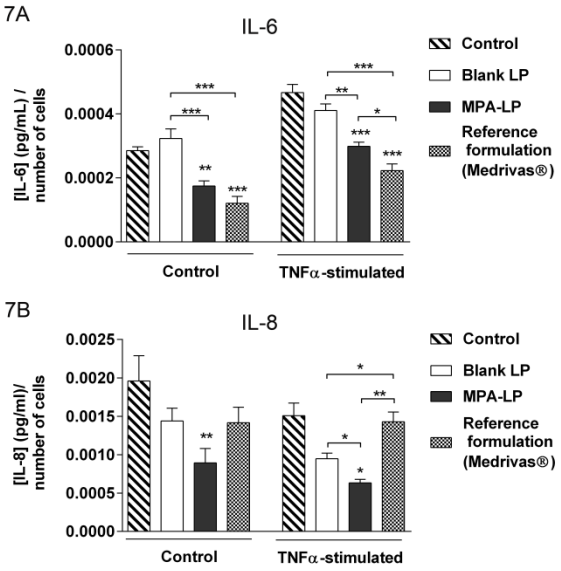


Figure 7: **Changes in inflammatory cytokine production in inflamed human corneal epithelial cells**. MPA-LP-exposed cells reduced IL-6 and IL-8 production compared with control and blank LP-exposed cells 48 h after the exposure period as determined by ELISA. Note the difference in scales for the y-axes of panels A and B. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001, compared with control, if not otherwise indicated by straddle bars. LP: liposome-based formulation; MPA-LP: liposome-based formulation loaded with medroxyprogesterone acetate; IL: interleukin; TNFα: tumor necrosis factor-alpha.

4. Discussion

In this work we report that a LP formulation, loaded with an anti-inflammatory drug, efficiently permeated through corneal epithelial barriers, activated specific drug receptors, and reduced the production of inflammatory cytokines IL-6 and IL-8 in cultured HCE cells. The scarcity of anti-inflammatory drugs in the market drove us to include an anti-inflammatory agent in a LP formulation designed as artificial tears to treat DED (Vicario-de-la-Torre *et al*. 2014). We selected the anti-inflammatory hormone MPA whose properties make it a good steroid-sparing agent, often used to decrease the dependency of a long-term therapy with steroids in ocular surface inflammatory diseases.

Taking into account the special requirements for topical ophthalmic formulations, i.e., pH = 6-9, osmolarity = 150-320 mOsm, viscosity < 20 mPas (Ali and Lehmussaari. 2006, Ammar *et al*. 2009), the values of our LP-based

formulations (Table 1) met all the requirements to be used as eyedrops. The liposomal formulation was comprised of components not only similar to those present in the natural tears, such as PC and cholesterol, but also antioxidants (vitamin E) and molecules able to provide special benefits for the ocular surface (trehalose) (Li *et al*. 2012, Pinto-Bonilla *et al*. 2015). Also, our liposomal formulation does not contain any preservative, thus avoiding the well-known harmful side effects associated with them (Baudouin *et al*. 2010).

We labeled the LP formulation with C6 to localize it in HCE cells and in *ex vivo* corneas after 5 and 60 min of exposure. Although the encapsulation efficiency for C6 was always around 90%, free C6 can penetrate through cell membranes. Consequently, we used a C6 suspension as control to distinguish LP-associated fluorescence from that caused by C6 in suspension. How the LPs bind to and are endocytosed by cultured cells has been widely reviewed (Duzgune&scedil *et al*. 1999, Lee *et al*. 1993). LP composition, charge, and size, as well as the types of cells to which they are exposed, influence the cellular uptake. Therefore, we analyzed cellular uptake of the LP formulation by HCE cells. It is important to point out that serum was avoided in all experiments as it partly inhibits the uptake of LPs (Duzgune&scedil *et al*. 1999). Results showed LP-associated fluorescence in HCE cells immediately, 24, 48, and 72 h after 60 min of exposure. Immediately after the exposure period, the cells had nanometer-sized dots, indicating that the LP did not aggregate in the formulation. Moreover, these dots corresponding to LPs are within the cell membranes and in the cytosol, as determined by Z-axis micrographs. This documents the efficient interaction of the LPs with the cultured cells and confirmed fast uptake by HCE cells *in vitro*.

To mimic actual *in vivo* exposure times, C6-LP was in contact with corneal tissues for only 5 min (Davies. 2000). After C6-LP exposure, the corneas showed fluorescence through the stratified epithelium, including the basal layers. After 60 min of C6-LP exposure, the corneal stroma was also stained. We therefore demonstrated that LP formulation was taken up by all epithelial layers and reached the corneal stroma after longer exposure times.

MPA binds to progesterone, glucocorticoid, androgen, mineralocorticoid, and estrogen receptors (Sitruk-Ware. 2004). For this study, we analyzed changes in protein expression and nuclear translocation of the glucocorticoid and progesterone receptors, which have greater affinity for the drug (Sitruk-Ware. 2004). Glucocorticoid and progesterone receptors are closely related members of the steroid receptor family, which act as ligand-activated transcription factors with well-characterized mechanisms of action. After binding a specific ligand, the receptors dimerize, and the dimers are then translocated to the nucleus, allowing the ligand-receptor complex to target specific DNA sequences (hormone response elements), affecting the inflammatory signaling cascade (Mangelsdorf *et al*. 1995). We calculated the percentage of glucocorticoid and progesterone receptors translocated to the nucleus in immunofluorescence micrographs as an indicator of receptor activation. Our results showed that while changes in nuclear translocation were observed immediately after the exposure time, the protein expression did not change at this time point and the effect of the drug was observed after 24 h of the exposure time. The progesterone receptor was activated after MPA-LP exposure, suggesting that MPA was released into cells. The translocation and the increased expression of the progesterone receptor, but not the glucocorticoid receptor, after MPA-LP exposure agrees with a study reporting a higher affinity of MPA for the progesterone receptor than for the glucocorticoid receptor (Sitruk-Ware. 2004). Unexpectedly, the protein expression of glucocorticoid receptor was decreased 24 h after reference formulation exposure. This effect may be due to the vasoconstrictor or the different excipients included in this formulation which interacts with the glucocorticoid receptor and can modify the expression of this receptor. It also could explain the lesser effect observed in proliferation or inflammation experiments. However, the analysis of the effect caused by those excipients is out of the scope of this study and more experiments need to be done to clarify this effect.

Corticosteroids affect *in vitro* cell proliferation; therefore, we analyzed changes in HCE cell proliferation using a standard proliferation assay. In previous studies, MPA reduced breast cancer epithelial cell proliferation (Cops *et al*. 2008, Sutherland *et al*. 1988), but did not affect corneal fibroblast proliferation (Zhou *et al*. 2012). Our findings showed a decrease in the proliferation rate of HCE cells 48 h after MPA exposure, which further supports efficient MPA transport by the LP formulation to the cells.

To determine the anti-inflammatory effect of MPA-LP in terms of reduction of IL-6 and/or IL-8 production, we optimized a cytokine-induced inflammation model using HCE cells. TNFα was used as an inflammatory stimulus, and the cytokine IL-6 and the chemokine IL-8 were used as inflammatory markers. The production of both inflammatory markers increased after TNFα stimulation in conjunctival epithelial cell line (Enriquez-de-Salamanca *et al*. 2008), and both are increased in the tears of patients with inflammatory diseases (Lam *et al*. 2009, Massingale *et al*. 2009). The production of both cytokines was analyzed 48 h after the exposure of TNFα. This time point was chosen because changes in the cell proliferation rate emerged 48 h after the MPA-LP exposure period, with C6-LP-associated fluorescence in the cells. Furthermore, a previous report using corneal fibroblasts showed MPA-induced collagen degradation after 36 h of exposure period (Zhou *et al*. 2012). To rule out a pro-inflammatory effect induced by components of the liposomal formulation (Joffre *et al*. 2007, Rodriguez and Larrayoz. 2010), blank LP formulation with different cholesterol concentrations were tested, and no inflammation-associated effects were present (data not shown). According to our data, the exposure of MPA-LP and the reference formulation (Medrivas®) reduced IL-6 production by HCE cells. However, only MPA-LP reduced IL-8 production. Interestingly, blank LP formulation also reduced IL-8 production in comparison to the reference formulation-treated cells and control cells, although the latter did not reach significance. This result confirms the importance of a vehicle in drug administration, not only to protect the drug and increase the residence time in

the ocular surface, but also to enhance therapeutic effect of the drug. This is in agreement with previous data reporting that components of the blank LP formulation, such as PC, trehalose, and vitamin E down-regulate IL-8 production of different epithelial cells *in vitro* (Cejkova *et al*. 2011, Ekstrand-Hammarstrom *et al*. 2007, Elisia and Kitts. 2015, Treede *et al*. 2009).

Although the *in vitro* and *ex vivo* results obtained with MPA-LP are promising, only *in vivo* experiments can evaluate the effect of physiological mechanisms such as the stress generated by eyelid wiping and tear flow on the liposomal formulation. In addition, the reference formulation (Medrivas®) contains not only the preservative BAC but also a vasoconstrictor, tetryzoline chlorhydrate, which might have effects that could not be assessed with our current *in vitro* approach.

5. Conclusions

To conclude, our findings show that an anti-inflammatory agent such as MPA loaded in the LP formulation permeated *ex vivo* through corneal barriers and was efficiently delivered to corneal epithelial cells. Also, the delivered MPA *in vitro* activated specific drug receptors and showed anti-proliferative and anti-inflammatory effects in HCE cells. Consequently, when applied topically as eyedrops, MPA-LP may attach to the hydrophobic corneal epithelium and continuously release the encapsulated drug, improving its biodistribution. In addition, the liposomal formulation does not contain preservatives or other bioactive agents required in the reference formulation, thus avoiding harmful side effects. In this sense, anti-inflammatory drugs included in unpreserved artificial tears can be useful for the treatment of inflammatory processes associated with DED or other ocular surface disorders that involve any inflammatory surface reactions.

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STUDY 6. Improved *In Vitro* Corneal Delivery of a Thrombospondin-1-Derived Peptide Using a Liposomal Formulation

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Improved *In Vitro* Corneal Delivery of a Thrombospondin-1-Derived Peptide Using a Liposomal Formulation

Abstract

Peptide delivery to and through ocular sites is a growing field of research interest. However, several barriers restrict the permeation and bioavailability of these molecules to target tissues. The main pharmacological barriers of topical administration are the tear film, fast drainage of the tear film, and poor corneal permeation. If the administered molecule is a peptide, instability and enzymatic degradation can be significant. Novel approaches such as the design and development of nanocarriers to overcome these drawbacks have been investigated with promising results. Therefore, in continuation of our previous study using a liposome-based (LP) formulation as topical drug delivery system, the aim of this work was to efficiently encapsulate a thrombospondin-1-derived peptide, KRFK, in this formulation and to assess peptide permeability through different ocular surface epithelia. LPs were prepared by the solvent evaporation technique and the labeled peptide FITC-KRFK was incorporated in the aqueous core. Different sonication times were used to optimize encapsulation efficiency. The selected formulation was further analyzed in terms of size, pH, osmolarity, and corneal epithelial cytotoxicity. The permeabilities of the LP-encapsulated and free KRFK peptides were assessed with *in vitro* models of conjunctival and corneal epithelia. Our results provide a proof of concept that the LP formulation efficiently encapsulates the KRFK peptide and improves corneal permeation. Data reported in this study strongly support that this formulation could be a more effective therapeutic approach than free peptide instillation and warrant further analysis using experimental *in vivo* models.

Keywords: Cornea; KRFK peptide; Liposome; Permeation; Thrombospondin-1

Main body

Topical delivery is considered the ideal route of drug administration for the treatment of diseases affecting the anterior segment of the eye. This route is not only the most patient compliant, but also easier and faster than traditional injection routes. However, efficient delivery of any pharmaceutical agent to the eye is restricted by several barriers, such as the tear film, rapid physiological drainage of the tear film, and the corneal epithelium that is impermeable to molecules above 500 Da (Hämäläinen et al. 1997). With recent advances in recombinant protein technology, the therapeutic role of peptides has received much attention. However, low peptide bioavailability due to a short half-life and *in vivo* instability can reduce efficacy, and ocular surface tissues contain proteases and aminopeptidases that can degrade administered peptides (Kashi & Lee 1986). To improve peptide efficacy, various strategies have been developed such as permeability enhancement, enzyme inhibition, protein structure modification, and protection by encapsulation (Ibraheem *et al*. 2014). Peptide protection in reservoirs includes the encapsulation of the active agent in liposomes (LPs) or particles. These constructs protect the encapsulated peptide against the effect of tear enzymes and release the cargo in a sustained manner (Patel *et al*. 2013; Tan *et al*. 2010).

Due to structural simplicity and biocompatibility, LP pharmaceutical formulations can be used not only as drug carriers, but also as components of artificial tears (Lim *et al*. 2015). As carriers, LPs improve drug transport across the cornea, providing a prolonged drug effect (Di Tommaso *et al*. 2012; Davis *et al*. 2014; Wang *et al*. 2011; Law *et al*. 2000; Hathout *et al*. 2007). The LPs are composed of natural components of the tear film, and therefore have surfactant properties that provide the linkage between the lipid and aqueous components of the tear film, reducing water evaporation and improving tear film stability (Simmons *et al*. 2015).

Recently, our research group studied the potential of a thrombospondin (TSP)-1-derived peptide, the KRFK sequence, to prevent chronic ocular surface

inflammation in an experimental mouse model (manuscript in preparation). In general, inflammatory diseases that affect the ocular surface are usually accompanied by tear film instability. These considerations led us to prepare a LP-based formulation that encapsulates the TSP-1-derived peptide. In addition to all of the mentioned advantages as a peptide delivery system, our LP formulation is capable of promoting the replenishment of the lipids in the tear film (Vicario-de-la-Torre *et al*. 2014). After ensuring proper encapsulation of the peptide, we used *in vitro* studies to assess how the encapsulated peptide permeates the ocular surface epithelia in comparison to the free peptide. In addition, we analyzed the size, pH, osmolarity, and biocompatibility of the KRFK peptide-loaded LP formulation (KRFK-LP formulation).

For this study, the following materials and methodologies were used. All materials were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. The LP formulation was composed of phosphatidylcholine (Lipoid GmbH, Cologne, Germany), cholesterol, and vitamin E as previously described (Soriano-Romaní *et al*. 2016). The peptide KRFK covalently conjugated with fluorescein isothiocyanate (FITC-KRFK) (Bionova, Madrid, Spain) was added to the lipid mixture to obtain the KRFK-LP formulation. Final phosphatidylcholine and FITC-labeled KRFK concentrations in the resultant dispersion were 28.5 mg/mL and 250 µg/mL, respectively. Also, a blank LP formulation, without loaded peptide, was prepared. The KRFK-LP formulation was sonicated at 21 W and 55 kHz for 0, 10, 20, or 30 min.

To determine the encapsulation efficiency (EE), the KRFK-LP formulation was centrifuged using Amicon Ultra-0.5 Centrifugal Filter Units (Millipore Corporation, Billerica, MA, USA). The supernatant was then analyzed by spectroscopy. The EE was calculated taking into consideration total concentration of the KRFK peptide in the LP dispersion and in the supernatant after centrifugation. The sonication time that showed the highest EE was characterized for mean size, osmolarity, pH, and biocompatibility.

The mean particle size and the size distribution of the KRFK-LP formulation were determined by photon correlation spectroscopy using a Zetasizer® 3000HS (Malvern Instruments Ltd., Malvern, UK). The pH and osmolarity of the KRFK-LP formulation were measured by a micropH 2001 (Crison Instruments SA, Barcelona, Spain) and by a Fiske 210 micro-sample osmometer (Fiske associates, Norwood, MA, USA), respectively. The biocompatibility of the KRFK-LP formulation was assessed in human corneal epithelial (HCE) cells after 60 min of exposure. The XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cytotoxicity assay (Thermo Fisher Scientific, Waltham, MA, USA) was performed using untreated HCE cells as controls.

We used conjunctival and corneal *in vitro* models to analyze the KRFK peptide permeability. For the conjunctival *in vitro* model, human bulbar conjunctiva was obtained from cadaveric donors (Barraquer Eye Bank in Barcelona, Spain). This study was in strict accordance with the Tenets of the Declaration of Helsinki and Spanish Regulations concerning the use of Human Tissues for Biomedical Research, and had the approval of the Institutional Review Board of the University of Valladolid. Conjunctival epithelial primary cultures were generated by the explant technique as previously described (García-Posadas *et al*. 2013). The human conjunctival epithelial cells obtained (passage 1) were cultured on top of a Transwell insert (Nunc, Rosklide, Denmark). The transepithelial electrical resistance (TEER) was measured to determine cell layer integrity. The lower compartment was filled with fresh culture medium, while the upper compartment was filled with culture medium containing 30 µg/mL of LP encapsulated or free KRFK peptide. The percentage of the KRFK peptide cumulative release from the upper to the lower compartment was determined every 30 min up to 180 min.

Due to the scarcity of human corneas discarded for transplantation, we used porcine corneas from a local slaughterhouse as the *ex vivo* model to study peptide delivery in this tissue. Localization of the KRFK peptide in porcine corneas was done as previously described (Soriano-Romaní *et al*. 2016). In brief,

corneal tissues were exposed to both the KRFK-LP formulation and the free KRFK peptide for 5 or 60 min. Fluorescent images of the corneal tissues exposed to KRFK-LP and free KRFK peptide were evaluated using the same camera exposure times, gains, and intensities. The measured fluorescent intensity was equivalent to the amount of the KRFK peptide in the tissue.

Data were expressed as the mean of at least three independent experiments ± standard error of the mean. To compare the KRFK-LP and free KRFK groups, Student´s *t*-tests were performed. If the groups had significantly different variances (F-test), Welch´s correction was done. Differences were considered to be significant with $p \leq 0.05$.

We first determined if the KRFK peptide was efficiently encapsulated by the LP formulation.The EEs after different sonication times were always higher than 80% (Figure 1A). After 10 min of sonication, the highest EE, 88.81 ± 1.23%, was obtained. This result, together with the susceptibility of the peptide to degradation when exposed to mechanical stress, favored our choice for 10 min of sonication for further analysis of the LP formulation.

The selected formulation showed a mean size of 230.43 ± 10.80 nm, a pH of 7.25 ± 0.04, and an osmolarity of 163.43 ± 15.94 mOsm, achieving essential requirements for ophthalmic formulations. The cytotoxic assay showed that the viability of HCE cells did not significantly differ from untreated cells after 60 min of exposure to either blank or KRFK-LP formulation (Figure 1B).

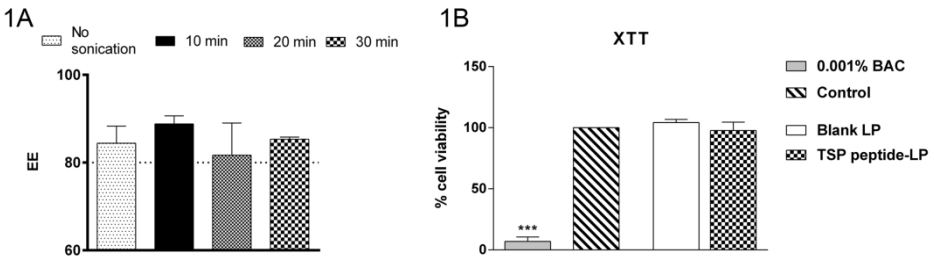

Figure 1. **KRFK peptide was efficiently encapsulated in the LP formulation and induced no signs of corneal cytotoxicity**. A) Encapsulation efficiency (EE) of KRFK peptide into the LP formulation after 0 (no sonication), 10, 20, or 30 min of sonication. B) Percentage of HCE cell viability when exposed to 10 min-sonicated KRFK-LP formulation. BAC, benzalkonium chloride; Blank LP, blank liposomal formulation; KRFK-LP, KRFK peptide loaded in the liposomal formulation. ***$p \leq 0.001$.

To determine if the encapsulation of KRFK peptide in the LP formulation improves passage through conjunctival epithelium, their cumulative release across epithelial conjunctival cells was assessed using inserts. Conjunctival epithelial cells seeded on top of the insert showed TEER values of 292.13 ± 34.02 $\Omega.cm^2$ over the control (insert without cells), evidencing cell layer integrity. The results did not show higher release if the peptide was encapsulated (Figure 2A). This result could be explained by the structure of the KRFK peptide, as it has less than 5 hydrogen bonds and less than 10 hydrogen bond acceptors expecting an acceptable absorption. Additionally, the conjunctival epithelium is 15 to 25 times more permeable than corneal epithelium that facilitates KRFK peptide passage (Hämäläinen *et al*. 1997). However, the protective role of the encapsulation has to be considered in an *in vivo* situation with the tear fluid.

We next determined the encapsulation of KRFK improved delivery of the peptide to the cornea. After 5 min of *ex vivo* corneal exposure, the fluorescence was largely limited to the outermost cornea epithelial cells, whether or not the KRFK peptide was encapsulated (Figure 2B). However, the signal of free KRFK peptide was weaker than that obtained with KRFK-LP formulation exposure, indicating higher peptide corneal retention of the LP encapsulated KRFK peptide. After 60 min of exposure, the staining patterns was similar to that at 5 min where it as distributed in the outermost corneal epithelial cells. However, after this exposure time, the inner layers of the corneal epithelium exposed to KRFK-LP showed a faint fluorescent signal. These findings support a higher bioavailability and permeation through corneal tissues of the KRFK peptide if it was encapsulated in the LP formulation.

Encouraging preliminary data are presented that support the efficacy of an LP formulation to protect a TSP-1-derived peptide, KRFK, for topical ocular administration. The LP-based formulation, which includes components similar to the aqueous and lipid layer of the tear film, is easy to formulate and biocompatible (Vicario-de-la-Torre *et al*. 2014).

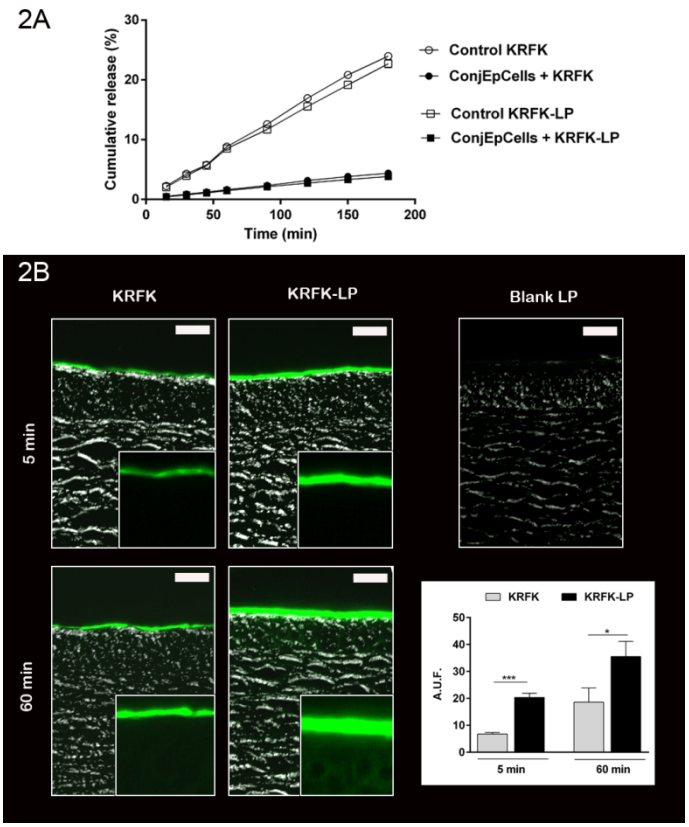

Figure 2. **KRFK peptide permeation through conjunctival and corneal epithelia of *in vitro* models**. A) Cumulative release of encapsulated (KRFK-LP) and free (KRFK) peptide through primary cultures of conjunctival epithelial cells (ConjEpCells). B) Representative micrographs after 5 and 60 min exposure of KRFK-LP or free KRFK peptide to *ex vivo* corneal tissues. Arbitrary units of fluorescence were measured in the corneal epithelium of each condition. Blank LP, blank liposomal formulation; KRFK-LP, KRFK peptide loaded in the liposomal formulation. **p*≤0.05; ****p*≤0.001. Scale bar: 50 µm.

We previously evaluated the ability of this LP formulation to encapsulate and release the corticoid medroxypreogesterone acetate, eliciting enhanced effects in corneal cells compared to the corticoid without vehicle (Soriano-Romaní *et al*. 2016). In this study, we wanted to know if the KRFK peptide could also be encapsulated in this LP formulation. We used the LP formulation with the aim of potentially improving peptide stability and protection from ocular surface enzymes (Ibraheem *et al*. 2014).

Overall, our results show efficient encapsulation and improved corneal permeation of the encapsulated KRFK peptide. We conclude that this

formulation could be a more effective therapeutic approach than free peptide instillation. Although our results are promising, these data warrant additional *in vivo* efficacy studies.

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EPÍLOGO/*EPILOGUE*

FORTALEZAS Y LIMITACIONES/*STRENGTH AND LIMITATIONS*

Este trabajo ha permitido avanzar en el conocimiento sobre la expresión de la TSP-1 en células de la superficie ocular y su papel en la inflamación ocular. Además, presenta una alternativa terapéutica para el tratamiento de patologías inflamatorias asociadas a la TSP-1 que presenta ciertas ventajas:

- Se propone esta alternativa para pacientes que tengan niveles de expresión de la TSP-1 reducidos. Por lo tanto, se espera que sea más eficiente que otros tratamientos ya que le aportará al paciente una molécula capaz de llevar a cabo una función que tiene limitada.
- Se ha desarrollado una formulación liposomal que mejorará la biodisponibilidad del péptido encapsulado tras su aplicación tópica.

Por otra parte, profesionales de diferentes disciplinas de la medicina han colaborado durante la realización de este trabajo de tesis lo que es, sin duda, una de las mayores fortalezas del mismo. Se ha contado con la experiencia de oftalmólogos y de ópticos-optometristas para conocer las necesidades en la clínica de nuevas terapias anti-inflamatorias y de la importancia de su aplicación tópica; de tecnólogos farmacéuticos para diseñar la formulación liposomal; de biólogos, bioquímicos y biotecnólogos, que han determinado la mejor manera de evaluar *in vitro* e *in vivo* la funcionalidad del péptido en cultivos celulares o animales de experimentación; y también de inmunólogos para aportar su punto de vista sobre los posibles efectos en células inmunes del péptido aplicado.

Sin embargo, este trabajo también presenta ciertas **limitaciones**:

- Se deben realizar pruebas de estabilidad de la formulación liposomal con el péptido KRFK de hasta 180 días.
- Se ha evaluado la acción terapéutica de una única concentración del péptido KRFK. Se deberían realizar estudios empleando distintas concentraciones del péptido, también encapsulado en la formulación liposomal.
- Se deben hacer estudios *in vivo* a mayor largo plazo para conocer los posibles efectos secundarios del péptido KRFK tras su aplicación tópica.

This thesis work allowed us to better understand the differential expression of TSP-1 by ocular surface cells and the TSP-1 role in ocular inflammation. In addition, it presents an alternative therapy for the treatment of inflammatory pathologies. This treatment presents some advantages:

- *It is proposed for patients with reduced TSP-1 expression. Therefore, it is expected to be more efficient than existing therapies because it will provide a molecule capable of performing a function that is reduced in the patient.*
- *We developed a liposomal formulation loaded with the KRFK peptide. This nanoconstruct will improve peptide bioavailability after topical administration.*

Professionals from different disciplines of biomedicine have collaborated during the completion of this thesis work. This collaboration is, undoubtedly, one of its greatest strengths. Experienced ophthalmologists and optometrists have advised on the needs in the clinic of new anti-inflammatory therapies and the importance of topical administration. Pharmaceutical technologists have designed the liposomal formulation with the necessary parameters and excipients. Biologists, biochemists, and biotechnologists have determined how to evaluate in vitro and in vivo the functionality of the KRFK peptide in cell cultures and in experimental animal models. Immunologists have provided their point of view of the possible effects of the peptide on immune cells.

Despite the progress made with the proposed therapy, this work has several limitations:

- *Stability tests for up to 180 days should be done for the liposomal formulation loaded with KRFK peptide.*
- *Just one concentration of the KRFK peptide was tested. Different concentration of the KRFK-liposomal formulation should be tested to better understand the dependence of the treatment on peptide concentration.*
- *In vivo longer-term studies are required to determine the possible side effects of KRFK.*

CONTINUACIÓN DEL TRABAJO/*ONGOING WORK*

Este trabajo muestra los primeros resultados al profundizar en el estudio de la contribución de la TSP-1 como activador del TGF-β, en la inflamación de la superficie ocular. Sin embargo, todavía queda mucho por hacer. Tras observar, durante el desarrollo de esta Tesis Doctoral, que el contexto inflamatorio puede afectar a los niveles de expresión de la TSP-1, el CD36 o el TGF-β2 (estudios 1 y 2), se planificó analizar los niveles de estas moléculas en pacientes con una inflamación ocular crónica y compararlos con los niveles observados en sujetos control. La hipótesis es que alguna de estas moléculas podría emplearse como bioindicador de una inflamación ocular crónica. Por ello, se preparó el protocolo y el consentimiento informado del estudio "Trombospondina-1 y moléculas relacionadas como indicadores de cambios inflamatorios en la superficie ocular". Estos documentos fueron aprobados en diciembre de 2015 por el Comité Ético de Investigación Clínica del Área de Salud de Valladolid-Este y la Comisión de Investigación del IOBA. Se están reclutando pacientes de Síndrome de Sjögren desde enero de 2016. Como parte del estudio se están recogiendo muestras de saliva, de sangre, de lágrima y de células epiteliales conjuntivales mediante una citología de impresión conjuntival. También se están recogiendo el mismo tipo de muestras de sujetos sanos, pareados en sexo y edad, para comparar niveles de TSP-1, CD36 o TGF-β2 entre ellos.

Actualmente otro estudiante de doctorado del grupo de la Dra. Yolanda Diebold está optimizando otros sistemas de administración de fármacos para la aplicación ocular tópica. Se podrían emplear y comparar diferentes sistemas para determinar el vehículo más adecuado para la administración tópica del péptido. Además, se ha planeado una estancia predoctoral en el laboratorio de la Dra. Masli en la Universidad de Boston, para continuar probando diferentes sistemas y dosis para tratar la inflamación crónica que desarrolla el modelo de ratón con deficiencia en la expresión de la TSP-1.

This work shows the contribution of TSP-1-dependent TGF-β activation in inflammatory processes of the ocular surface. However, much more work is needed. After the observation during Studies 1 and 2 of this thesis that the inflammatory context affects the expression of TSP-1, CD36, and TGF-β2, a study to analyze possible changes in these molecules in patients with chronic ocular inflammation has been planned. We hypothesized that any of these molecules could be used as biomarkers of chronic ocular inflammation. Therefore, a protocol and the informed consent forms of the study "Thrombospondin-1 and related molecules as indicators of inflammatory changes in the ocular surface" were prepared and approved by The Clinical Research Ethics Committee of the University of Valladolid and the Research Committee of IOBA in December 2015. Currently, we are recruiting Sjögren's Syndrome patients for this study and collecting samples of saliva, blood, tears, and conjunctival cells by impression cytology. Also, the same types of samples from healthy subjects, age- and gender-matched with patients, are being collected. We aim to analyze and compare levels of TSP-1, CD36, and TGF-β2 between control subjects and patients.

Another PhD student is currently optimizing other drug delivery systems for topical ocular application. Different systems can be compared to determine the most suitable system for the topical administration of the peptide. In addition, a pre-doctoral internship has been planned in the laboratory of Dr. Masli at Boston University, to continue testing different systems and doses to treat the TSP-1-deficient mouse model.

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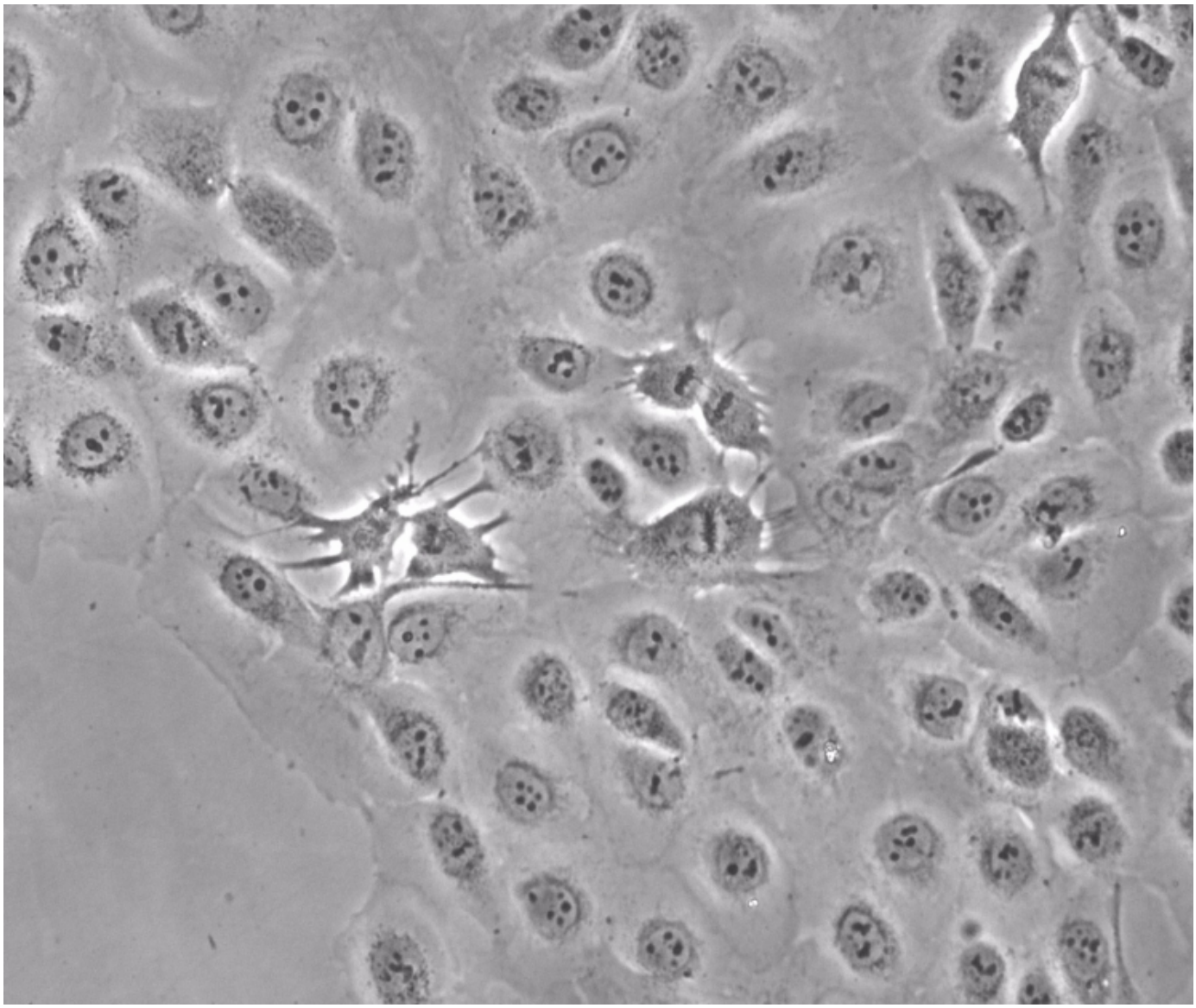

Sábado noche (marzo 2017)

Células humanas de epitelio corneal en cultivo, donde se pueden observar varias células en distintas fases de la mitosis celular. Autores: Laura Soriano-Romaní y Antonio López-García. IOBA, Universidad de Valladolid