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LA ISOFORMA GSK3α REGULA LA EXPRESIÓN DE IL-8 E IL-10 MEDIADA POR NF-κB Y CREB EN ENDOTELIO BOVINO INFECTADO CON *Staphylococcus aureus*.

Tesis que como requisito para obtener el grado de Doctor en Ciencias presenta:

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Morelia Michoacán Febrero de 2018

INDICE

1. Glosario

ADN	Ácido desoxirribonucleico
AMPc	Adenosin monofosfato cíclico
Akt	Proteína cinasa B
ARN	Ácido ribonucleico
AP1	Proteína activadora de la transcripción 1
CREB	Elemento de respuesta a unión con AMPc
CBP	Proteína de unión a CREB
GSK3	Glucógeno sintasa cinasa 3
IκB-α	Inhibidor de NF-κB-α
IKK	IκB-α cinasa
LTA	Ácido Lipoteicoico
LPS	Lipopolisacárido
MCP1	Proteína quimioatrayente de monocitos 1
mTORC1/2	Complejo de mamífero diana de rapamicina 1/2
NOD	Receptor tipo oligomerización de nucleótidos
PDK1	Cinasa dependiente de fosfoinositido 1
PGN	Péptidoglucano
PKA	Proteína cinasa A
PKB	Proteína cinasa B
PKC	Proteína cinasa C
PI3K	Fosfatidil-inositol 3-cinasa
PIP2	Fosfatidil inositol 2-fosfato
PIP3	Fosfatidil inositol 3-fosfato
PMAP	Patrón molecular asociado a patógeno
PRR	Receptor de reconocimiento de patógeno
PTEN	Fosfatidil inositol-3,4,5-trisfosfato 3-fosfatasa
RTK	Receptor con actividad tirosina cinasa
S6K	Proteína ribosomal S6 cinasa
STAT	Proteína traductora de señal y activadora de la transcripción
TIR	Dominio homólogo del receptor Toll/Interleucina-1
TLR	Receptor tipo Toll
NF-κB	Factor nuclear kappa B

2. Índice de tablas

3. Índice de figuras

4. Resumen

En mamíferos la enzima glucógeno sintasa cinasa 3 (GSK3) participa en diversos procesos celulares como la diferenciación, la apoptosis y la inflamación. Durante la inflamación producida por infecciones bacterianas, la enzima GSK3 se inhibe por fosforilación, lo que conduce a cambios en la expresión de genes que codifican para las interleucinas IL-8 (pro-inflamatoria) e IL-10 (anti-inflamatoria) mediante la activación de NF-κB y CREB, respectivamente. Se han identificado dos parálogos de la enzima GSK3 llamados GSK3α y GSK3β. Aunque la función de GSK3β en la producción de citocinas está bien establecida en modelos de monocitos estimulados con lipopolisacárido (LPS), no hay estudios que demuestren la participación de la isoforma GSK3α en este proceso. Previamente, nuestro equipo de trabajo observó que durante las infecciones de células de endotelio bovino (CEB) con el patógeno *Staphylococcus aureus* ocurre la inhibición preferencial de la isoforma GSK3α.

El objetivo de este trabajo fue demostrar que durante las infecciones de células endoteliales por *S. aureus*, la isoforma GSK3α controla la expresión de citocinas y como consecuencia de ello, la inflamación. En cursos temporales de infección, se observó una inhibición preferencial por fosforilación de la isoforma GSK3α, comparada con la inhibición de la isoforma GSK3β durante los primeros 40 min de infección se incrementó la–fosforilación de NF-κB en Ser536 (fosfo-NF-κB), su translocación al núcleo y la interacción con el co-activador CBP, lo que resultó en una mayor expresión de IL-8. Sin embargo, transcurridos los 40 min iniciales de infección, GSK3α pierde gradualmente su actividad constitutiva, incrementa la activación de CREB por fosforilación en Ser133 y su interacción con el co-activador CBP, lo que produjo un incremento en la expresión de IL-10. Simultáneamente, se redujo de manera gradual la abundancia relativa de fosfo-NF-κB y disminuyó también su presencia en el núcleo. Estos hallazgos fueron confirmados en modelos celulares por la técnica silenciamiento con ARN de interferencia (siRNA) específico dirigido a cada una de las isoformas de GSK3. El silenciamiento del gen de la isoforma GSK3α dio como resultado un incremento de la expresión de IL-10, mediado por el complejo CBP/CREB, y una reducción de la expresión de IL-8 por la disminución de la formación del complejo CBP/NF-κB. Los resultados indican que la isoforma GSK3α regula la expresión de las citocinas IL-8 e IL-10 en endotelio bovino infectado con *S. aureus*.

Palabras clave: Citocinas, célula endotelial, *Staphylococcus aureus,* GSK3, Inflamación.

5. Abstract

In mammals the enzyme glycogen synthase kinase 3 (GSK3) participates in various cellular processes such as differentiation, apoptosis and inflammation. During episodes of inflammation caused by bacterial infections, the GSK3 enzyme is inhibited by phosphorylation, which leads to changes in expression of IL-8 (pro-inflammatory) and IL-10 (anti-inflammatory) interleukins by activating NF-κB and CREB, respectively. Two GSK3 enzyme paralogs called GSK3α and GSK3β have been identified. Although the role of GSK3β as a regulator of cytokine production is well established in models of lipopolysaccharide stimulated monocytes (LPS), there are no studies demonstrating the involvement of GSK3α isoform in this process. Previously, our team observed that during bovine endothelial cell (BEC) infections with the pathogen *Staphylococcus aureus*, a preferential inhibition of the GSK3α isoform was detected.

The aim of this study was to assess if during endothelial cell infections with *S. aureus*, the GSK3α isoform controls cytokine expression and, as a consequence, inflammation. In temporary courses of infection, a preferential inhibition by phosphorylation of the GSK3α isoform was observed, compared to the inhibition of the GSK3β isoform, during the first 40 minutes of infection NF-κB phosphorylation was increased in Ser536 (phospho-NF-κB), its translocation to the nucleus and interaction with the CBP co-activator, which resulted in an induction of IL-8 expression. However, after the initial 40 minutes of infection, GSK3α gradually lost its constituent activity, CREB activation by phosphorylation increased in Ser133 and its interaction with the CBP co-activator, resulting in an increase in IL-10 expression. Simultaneously, the relative abundance of phospho-NF-κB was gradually reduced and its presence in the nucleus also decreased. These findings were confirmed in cellular models by the specific RNA interference silencing technique (siRNA) targeting each of the GSK3 isoforms. The silencing of the isoform GSK3α gene resulted in an increase in IL-10 expression, mediated by the CBP/CREB complex, and a reduction in IL-8 expression by decreasing the formation of the CBP/NF-κB complex. The results indicate that the GSK3α isoform regulates expression of IL-8 and IL-10 cytokines in *S. aureus* infected bovine endothelium.

6. Introducción

6.1. Generalidades de *Staphylococcus aureus.*

La bacteria *Staphylococcus aureus* pertenece a la familia microccocaceae, género *Staphylococcus*. Es un coco gram positivo no móvil que no forma esporas, tiende a crecer en forma de racimo, pero también puede encontrarse en pares y cadenas cortas. Es anaerobio facultativo y produce enzimas como coagulasa, catalasa y nucleasa (Thomer *et al.*, 2016). A esta esta bacteria se le considera uno de los mayores patógenos de animales y humanos. En el humano es capaz de colonizar e invadir cualquier tejido, debido en parte a la diversidad de factores de virulencia que expresa.

Se estima que alrededor del 30% de la población humana está colonizada por esta bacteria, lo que indica que puede vivir como comensal; sin embargo, puede producir enfermedades que van desde infecciones leves de la piel hasta infecciones potencialmente fatales como bacteriemia, endocarditis infecciosa, osteomielitis, meningitis, neumonía, etc (Tong *et al.*, 2015).

El estudio de las características de *S. aureus* ha recobrado importancia en los últimos años debido en parte al surgimiento de cepas resistentes a la acción de antibióticos. En este contexto, se ha reportado que esta bacteria carece del sistema de edición genética CRISPR/CAS (Marraffini 2013), lo que se ha asociado a un aumento de la capacidad de incorporación de material genético exógeno, incluidos los genes necesarios para desarrollar resistencia a una amplia variedad de antibióticos (Palmer y Gilmore 2010).

En el ambiente hospitalario *S. aureus* cobra particular interés por la presencia de cepas seleccionadas que presentan una alta resistencia a los antibióticos y a que es la principal causa de infecciones en tejidos blandos en pacientes canalizados o con dispositivos ortopédicos y en infecciones post-operatorias. Dada la versatilidad de infecciones que produce, el alto índice de personas colonizadas por esta bacteria y el incremento gradual de la resistencia a los antibióticos, *S. aureus* se considera actualmente un problema de salud mundial. Esta bacteria ha sido designada como el mejor ejemplo del desarrollo de la resistencia antimicrobiana (Chambers & DeLeo, 2009) y se han descrito ya varias cepas resistentes a todos los antibióticos disponibles excepto a la telavancina, un derivado semi-sintético de la vancomicina. No obstante, el uso de telavancina está altamente restringido porque ha inducido efectos teratogénicos en ratones (Bookstaver *et al.*, 2015).

6.2. Factores de virulencia

La patogénesis ligada a las infecciones por *S. aureus* está asociada a la evasión de la respuesta inmune y la pérdida de regulación de la respuesta inflamatoria. Se ha reportado que aunque la incidencia de infecciones producidas por la bacteria se incrementa en aquellos individuos pre-colonizados, ésta no es una característica indispensable. Además, las exposiciones previas no producen protección contra futuras infecciones (Giersing *et al.*, 2016), lo que indica el desarrollo de mecanismos por parte de la bacteria que impiden la instauración de una respuesta inmune adaptativa. Esta observación está apoyada también en la falla del desarrollo de vacunas efectivas en las que se han empleado proteínas capsulares o de pared celular sin efectividad clínica (Giersing *et al*., 2016).

Para lograr colonizar e invadir tejidos en el hospedero *S. aureus* produce una amplia variedad de proteínas que pueden estar unidas covalentemente a su pared celular o que son secretadas y que tienen un papel fundamental en la virulencia de la bacteria. En la Tabla 1 se resumen algunas de las proteínas asociadas a la virulencia de *S. aureus* y su efecto en el hospedero (Thomer *et al*., 2016).

Tabla 1. Factores de virulencia de *Staphylococcus aureus*

Nombre	Gen	Función propuesta	Defecto en la virulencia asociado a la pérdida del gen
Sintasa de adenosina	*adsA*	Síntesis de los inmunosupresores Ado,dADO	Supervivencia reducida en sangre
Nucleasa	*nuc*	Degradación de trampas extracelulares de neutrófilos	Supervivencia reducida en sangre
Factor de coagulación A	*clfA*	Coagulación	Incremento de la fagocitosis
Coagulasa	*coa* *vwb*	Coagulación	Incremento de la fagocitosis
Proteína A	*spa*	Unión al dominio Fcγ de Igs	Incremento de la opsonización y fagocitosis
Leucocidinas	*blgAB* *blgCB* *lukAB*	Lisis de leucocitos	Supervivencia reducida en sangre
Proteína repetida de unión a fibronectina A, B	*fnBPA* *fnBPB*	Unión a fibrinógeno y reconocimiento de la integrina $\alpha_5\beta_1$	Desconocida
Inhibidor del complemento estafilocócico	*scin* *scinB* *scinC*	Bloqueo de la convertasa C3	Desconocida
Proteína de unión extracelular del complemento	*ecb*	Bloqueo de la convertasa C3	Desconocida
Aureolisina	*aur*	Degradación de péptidos antimicrobianos	Reducción de la carga bacteriana en órganos
α-hemolisina	*hla*	Disrupción de barreras epiteliales y endoteliales	Reducción de la carga bacteriana en órganos
Proteína de repetidos Ser-Asp C,D	*sdrC* *sdrD*	Ligandos desconocidos	Reducción de la carga bacteriana en órganos

Determinantes de uso de hierro en superficie	*isdA* *isdB* *isdC*	Utilización del hierro del grupo hemo	Reducción de la carga bacteriana
Lipoproteína de transferencia diacil-glicérido	*lgt*	Lipidación de lipoproteínas	Hipervirulencia, abscesos sin células inmunes debido a la pérdida de ligandos de TLR-2
Proteína de adhesión extracelular	*eap*	Inhibición de la adherencia de neutrófilos al endotelio	Reducción de la carga bacteriana en órganos
Proteína de unión a fibrinógeno	*efb*	Unión a fibrinógeno	Reducción de la carga bacteriana en órganos
Modulinas solubles en fenol	*psmα*	Modulación de la producción de citocinas	Incremento de la carga bacteriana en órganos
Superantígenos estafilocócicos 5, 10	*ssl5* *ssl10*	Inhibidor de la quimiotaxis de neutrófilos	Desconocida
Proteína inhibidora de quimiotaxis	*chips*	Inhibidor de la quimiotaxis de neutrófilos	Desconocida
Proteína inhibidora del receptor de péptidos formilados	*flipr* *flipr-like*	Inhibidor de la quimiotaxis de neutrófilos	Desconocida

6.3. Fisiopatología de las infecciones por *Staphylococcus aureus*.

Una infección típica producida por *S. aureus* comienza con el ingreso de la bacteria a los tejidos blandos o al torrente sanguíneo a través de heridas en la piel (cortes superficiales) o el ingreso de cuerpos extraños (catéteres o tubos de respiración en hospitales). También se pueden producir infecciones en zonas sin heridas aparentes como los folículos capilares (Tong *et al*., 2015). Una vez en el interior del hospedero la bacteria comienza a expresar proteínas en la superficie de su pared celular que le facilitan la adherencia a las membranas celulares, por medio su interacción con receptores en las células del hospedero (Van Belkum *et al.*, 2002). Una característica de las infecciones producidas por *S. aureus* es la generación de infiltrados al sitio de la infección de células inmunitarias como neutrófilos en una primera etapa y de macrófagos en una segunda etapa. Sin embargo, la bacteria logra sobrevivir a la respuesta inmunitaria mediante distintos mecanismos y se mantiene viable en las lesiones denominadas abscesos en los que induce la apoptosis de los fagocitos y la formación de células "*foam*" (Kobayashi *et al.*, 2015). Se ha reportado que cuando *S. aureus* entra al torrente sanguíneo de ratones, se observa una disminución de la carga bacteriana en el transcurso de minutos hasta un par de horas y simultáneamente se detecta un incremento en la abundancia bacteriana en tejidos y órganos(Thomer *et a*l., 2016).

Aunque no se le considera un patógeno intracelular, se reconoce que *S. aureus* se internaliza en las células fagocíticas, epitelios y endotelios (Oviedo-Boyso *et al.*, 2011). Al inhibir la formación y acción de los fagolisosomas, la bacteria adquiere su propio nicho donde sobrevive y replica. Esto da como resultado la instauración de infecciones

recurrentes y de carácter crónico. En estas circunstancias, se ha reportado que la bacteria es capaz de modificar su fenotipo hacia el denominado "*variantes de colonia pequeña*" que muestra una capacidad inmunoreguladora disminuida en comparación con las bacterias de fenotipo normal (Tuchscherr *et al.*, 2010, Tuchscherr *et al.*, 2011). Estas variantes fenotípicas pueden permanecer en el interior de las células y sobrevivir durante el tiempo suficiente para producir infecciones recurrentes.

La capacidad de la bacteria para reclutar células inmunitarias en una primera etapa y de inhibir su función a través de la inducción de apoptosis o la inhibición de la formación de los fagolisosomas maduros radica en el potencial que tiene para manipular la respuesta inflamatoria, mediante la expresión de citocinas de carácter pro- o anti-inflamatorio como interleucina-8 (IL-8) o interleucina-10 (IL-10), respectivamente (Pietrocola *et al.*, 2017, Thammavongsa *et al.*, 2015). Estas características de la infección por *S. aureus* son explicadas por el modelo de infección "*Trojan horse*" (Figura 1), el cual establece que durante la primera etapa de la infección, la bacteria induce la expresión de citocinas quimioatrayentes y pro-inflamatorias con el fin de favorecer la infiltración de neutrófilos, mientras que en una segunda etapa las bacterias son fagocitadas por los neutrófilos y monocitos. Esto es sin duda un excelente modelo de co-adaptación porque las bacterias son capaces de sobrevivir durante días en las vacuolas de estas células, y pueden servir como vectores que ayudan a la extravasación y diseminación de la bacteria por el torrente sanguíneo y los tejidos sanos. Este modelo ha sido probado mediante la inoculación en ratones de neutrófilos viables con *S. aureus* internalizado, en los cuales se ha observado que los ratones que reciben los neutrófilos infectados desarrollan abscesos. La caracterización de estas lesiones muestra una predominancia de la población celular neutrofílica, lo que destaca su importancia en la instauración de una respuesta contra la bacteria, en parte porque son las células inmunitarias predominantes en el torrente sanguíneo y las más rápidas en acudir al sitio de la infección (Tan *et al.*, 2013).

6.4. Manipulación de la respuesta inflamatoria por *Staphylococcus aureus.*

Diversos estudios en modelos de experimentación en animales y cultivos celulares, han demostrado que la estimulación con *S. aureus* se caracteriza por una expresión alta de citocinas pro-inflamatorias, como IL-1, IL-6, IL-8 y factor de necrosis tumoral α (TNFα) tras el contacto inicial. Sin embargo, a medida que evoluciona la infección, la expresión de estas citocinas se abate y se estimula la expresión de citocinas anti-

inflamatorias en una segunda etapa como IL-4 e IL-10 (Tajima *et al.*, 2007) (Björkander *et al.*, 2016) (Ferreira *et al.*, 2012) (Günther *et al.*, 2011) (Günther *et al.*, 2017).

IL-8 es una citocina pro-inflamatoria y quimioatrayente de neutrófilos. Se le considera una de las primeras citocinas en liberarse tras el reconocimiento de la presencia de bacterias en el hospedero y juega un papel fundamental en la eliminación de *S. aureus* en modelos experimentales (Weitkamp *et al.*, 2002) (Venza *et al.*, 2007). Hay reportes aparentemente contradictorios acerca del papel de *S. aureus* en la inducción de esta citocina. Por ejemplo, se ha reportado que en células de endotelio estimuladas con *S. aureus* durante 30 min y posteriormente eliminado con lisostafina, se observa un incremento en la expresión de IL-8 a nivel de ARN mensajero y el péptido (Yao *et al.*, 1996). Otro estudio revela que la incubación de células de endotelio humano con cepas virulentas de *S. aureus* durante 60 min muestra niveles disminuidos de expresión de IL-8 en comparación a los niveles inducidos por cepas comensales (Tajima *et al*., 2007). Dichos efectos también han sido observados en cultivos celulares estimulados con el sobrenadante de cultivos de la bacteria (Al Alam *et al.*, 2010), por lo que se sospecha que las cepas más virulentas secretan factores al medio de cultivo que son los responsables de llevar a cabo la modulación en la expresión de IL-8 y por ende de la transmigración de neutrófilos (Tajima *et al.*, 2009). Además, debido a que la expresión de los factores de virulencia de *S. aureus* son dependientes de la densidad poblacional, los tiempos de incubación de la bacteria antes de la estimulación y el tiempo de contacto con las células es importante en el efecto final (Proctor *et al.*, 2014).

Por otra parte, la expresión de la síntesis de IL-10 induce la apoptosis prematura de neutrófilos, lo que contribuye a la sobrevivencia de la bacteria en los tejidos y se asocia a la formación de las capas de neutrófilos apoptóticos típicos de los abscesos provocados por *S. aureus* (Keel *et al.*, 1997) (Zurek *et al.*, 2015). La expresión de IL-10 inhibe la función y síntesis de las citocinas pro-inflamatorias y por ende la activación de la inflamación. Se ha especulado que la inducción de su expresión por *S. aureus* se asocia a la inhibición de la respuesta inmune innata para favorecer su sobrevivencia. Esta observación ha quedado de manifiesto en experimentos en ratones IL-10 KO los cuales presentan una elevada resistencia a las infecciones producidas por *S. aureus* durante episodios de infecciones localizadas. Sin embargo, en el aspecto fisiológico la función de IL-10 es de vital importancia para controlar procesos inflamatorios innecesarios o exacerbados, ya que la falta de su función resulta fatal durante episodios de infecciones agudas sistémicas (Leech *et al.*, 2017).

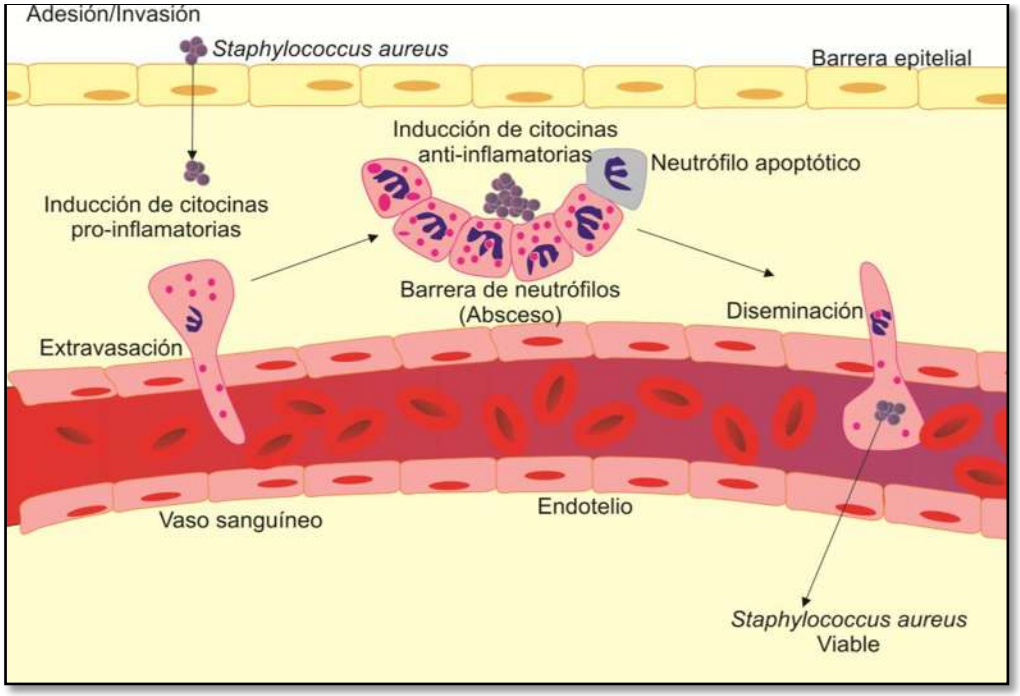

Figura 1. Diagrama del modelo de infección de *Staphylococcus aureus* "Trojan Horse". *S. aureus* es capaz de invadir los tejidos directamente o a través de heridas en la piel. Una vez dentro de los tejidos vivos, es reconocido por células epiteliales y endoteliales que producen citocinas de carácter pro-inflamatorio para favorecer la extravasación de neutrófilos principalmente. Los neutrófilos entonces se agrupan formando una barrera de células alrededor de las colonias de bacterias para impedir su diseminación (Absceso). En esta etapa, la bacteria, induce la expresión de citocinas anti-inflamatorias que reprimen las funciones bactericidas de los neutrófilos. Finalmente la bacteria fagocitada pero viable dentro de los neutrófilos es diseminada al torrente sanguíneo y otros órganos

Estos mecanismos que emplea *S. aureus* para producir en las células hospederas el cambio en la síntesis de citocinas de inmuno-activadoras en la primera etapa a inmunosupresoras en la segunda etapa se encuentra sin resolver hasta ahora. No obstante, se reconocen bien que diversas mutantes bacterianas en proteínas como las modulinas solubles en fenol no son capaces de inducir la expresión de IL-10 por las células dendríticas, lo que resulta en un incremento de la fagocitosis y eliminación de la bacteria (Armbruster *et al.*, 2016). Estas observaciones sugieren que estos mecanismos son responsabilidad más de la bacteria que una respuesta de la célula hospedera.

6.5. Reconocimiento de *Staphylococcus aureus* por las células del hospedero.

Se ha postulado que la patogenicidad de *S. aureus* depende de la expresión de factores de virulencia que secreta la bacteria o que están presentes en su pared celular (Lacey *et al.*, 2016). Sin embargo la función de estos factores en la estimulación del sistema inmune innato es incierta y existen reportes contradictorios (Fournier & Philpott, 2005). La respuesta celular a varios componentes de la pared

celular como son péptidoglucano (PGN) y el ácido lipoteicoico (LTA) ha sido ampliamente descrita (Kumar y Kumar, 2015).

LTA es el compuesto anfipático más abundante de la pared celular de *S. aureus*. Sus propiedades fisicoquímicas son similares a las del lipopolisacárido (LPS) de las bacterias Gram negativas (Su *et al.*, 2006). Se ha demostrado que LTA de *S. aureus* provoca la secreción de citocinas y quimiocinas (TNF-α, IL1-β, IL-10, IL-8, IL-12, leucotrienos y MCP1) de monocitos y macrófagos (Finney *et al.*, 2012) y que es similar en potencia al LPS para inducir respuestas inmunitarias en diversos cultivos celulares (Su *et al.*, 2006). Además, la inducción de la expresión de citocinas por LTA es similar a la que se observa con la bacteria completa cuando es administrada por vía intranasal, lo que desencadena una fuerte infiltración de neutrófilos y monocitos en pulmón (Leemans *et al.*, 2003). Se cree que TLR2 es importante en la respuesta inmune mediante el reconocimiento de LTA de *S. aureus*, debido a diversos estudios llevados a cabo en ratones $TLR2^{-/-}$ o por la reducción significativa de TNF-α e IL-6 en monocitos pre-tratados con anticuerpos anti-TLR2 y estimulados con LTA (Kielian *et al.*, 2005b) (de Oliviera Nascimento *et al.*, 2012).

PGN es un polímero constituido por cadenas de N-acetilglucosamina y ácido N-acetilmurámico interconectadas por anillos de pentaglicina, formando de esta manera el componente principal de la pared celular de *S. aureus*. Se ha reportado que PGN induce la producción de citocinas y quimiocinas como TNF-α, IL1-β, IL-6 e IL-8 en monocitos, macrófagos y otros tipos celulares (Tabla 2). Algunos datos experimentales han indicado que sólo una fracción de PGN es activa. Las cadenas solubles e insolubles de alto peso molecular de PGN son agonistas débiles y la hidrólisis de estas cadenas a azúcares y péptidos inhibe la reacción inflamatoria. En el caso particular de PGN de *S. aureus*, se considera que tres cadenas peptídicas entrecruzadas es la estructura mínima para ejercer un estímulo inflamatorio (Kielian *et al.*, 2005a) (Moreillon & Majcherczyk, 2003).

Tabla 2. Estimulación de la expresión de citocinas por *S. aureus* o por componentes de su pared celular.

Tipo celular	Componente bacteriano de *S. aureus.*	Proteínas que aumentan su expresión	Referencia
Macrófagos de ratón	*S. aureus* inactivado por calor	IL-10, TNF	Volz *et al*., 2010
Hepatocitos humanos	*S. aureus* completo	TLR2	Sweeney *et al*., 2011
Monocitos humanos	*S. aureus* inactivado por calor	TLR2	Esen *et al*., 2004
Monocitos, células T y células presentadoras de antígenos humanos	PGN, LTA	IL-10, IL-1β, IL-6, TNF, IL-12p40	Von-Aulock *et al*., 2003 Standiford *et al*., 1994
Queratinocitos humanos	PGN, LTA	IL-8, TNF	Mempel *et al*., 2003
Células de riñón embrionario humano	PGN	IL-8, TNF	Farhat *et al*., 2008
Microglía de ratón	*S. aureus* completo	TLR2, TNF	Kielian *et al*., 2005
Endotelio pulmonar de rata	LTA	eNOS	Mitchell *et al*., 2011

Se ha descrito que las células de microglia de ratones TLR2$^{-/-}$, estimuladas con PGN, expresan menos TNF-α e IL-10 que células de microglia derivadas de ratones normales (Fournier & Philpott, 2005). No obstante, si se estimulan con *S. aureus* inactivado por calor, las células de microglía TLR2$^{-/-}$ producen citocinas al igual que las células que expresan niveles normales de TLR2. Aparentemente, TLR2 es necesario para inducir la expresión de estas citocinas en células estimuladas con PGN, pero no es necesario si la estimulación se lleva a cabo con *S. aureus* inactivado por calor (Fournier, 2013). Diversos estudios han puesto en entredicho la especificidad de TLR2 en el reconocimiento de PGN de *S. aureus*. Esto es porque PGN altamente purificado o con deficiencias en la maduración de lipopéptidos es incapaz de estimular TLR2 humano, por lo que se ha propuesto que son precisamente estos lipopéptidos, dentro de la estructura del PGN y no el PGN en sí, los responsables de la activación de TLR2 (Travassos *et al.*, 2004). Un estudio posterior demostró que PGN purificado por HPLC es incapaz de estimular CD a través de TLR2; sin embargo, si las CD se pre-estimulan con PGN purificado y posteriormente con distintos ligandos de TLRs se induce un efecto sinérgico en la respuesta celular (Volz *et al.*, 2010, Ko & Lee, 2016).

Los macrófagos derivados de ratones TLR2$^{-/-}$, que no muestran cambios en la producción de TNF o IL-10 cuando se infectan con *S. aureus* inactivado por calor, muestran disminución de la producción de estas citocinas cuando se inhibe la internalización de la bacteria. De este trabajo se concluyó que además de TLR2 existen receptores intracelulares que modulan la respuesta inflamatoria contra *S. aureus* (Kielian *et al*., 2005a). Estos receptores posteriormente identificados como receptores de unión a dominios de oligomerización (NOD), al igual que los receptores

TLR, inducen la activación de vías de señalización celular como la vía del factor nuclear kappaB (NF-κB) y la vía del fosfatidilinositol trifosfato (PI3K) que constituyen vías moduladoras importantes de la producción de citocinas (Oviedo-Boyso *et al.*, 2014).

6.6. La vía de transducción NF-κB.

El factor de transcripción NF-κB pertenece a la familia Rel de factores transcripcionales, constituida por las proteínas NF-κB1 (p50 y su precursor p105), NF-κB2 (p52 y su precursor p100), RelA (p65), RelB y c-Rel (Hoesel & Schmid, 2013). Estas proteínas se caracterizan por la presencia de un dominio homólogo a Rel (RHD) que incluye una secuencia de localización nuclear (NLS), lo que les permite introducirse y permanecer en el núcleo (Oeckinghaus & Ghosh, 2009). Se les considera factores de transcripción pro-inflamatorios debido a que su actividad transcripcional es esencial para estimular la expresión de citocinas pro-inflamatorias como IL-1β, TNF-α, IL-8, e IL-6 (Lawrence, 2009).

Esta vía se puede activar cuando los receptores tipo TLR o NOD interaccionan con sus respectivos ligandos, lo que resulta en la fosforilación del complejo cinasa de IκB-α (IKK) formado por las proteínas IKKα e IKKβ. Esto a su vez estimula la fosforilación de la proteína inhibidora de NF-κB (IκB-α) que mantiene a NF-κB anclado en el citoplasma. Como resultado IκB-α es poli-ubiquitinada y degradada en el proteasoma 26S liberando así a NF-κB que es translocado al núcleo donde interacciona con co-factores como la acetiltransferasa CBP y con el ADN en el sitio consenso 5′-GGGRNYYYCC-3′ (R representa una purina, Y una pirimidina y N a cualquier nucleótido) (Oeckinghaus & Ghosh, 2009).

La estimulación de células tipo macrófagos, epitelio de córnea y endotelio con *S. aureus* induce la translocación de NF-κB al núcleo por la vía activada mediante TLR-2, dando como resultado el incremento en la expresión de citocinas pro-inflamatorias como IL-8 (Figura 2) (Kapetanovic *et al.*, 2007) (Yao *et al*., 1996) (Heimer *et al.*, 2010). Es importante destacar que este efecto se observa solo durante las etapas iniciales de estimulación con la bacteria pero en etapas posteriores esta actividad es inhibida (Tajima *et al*., 2007) (Tajima *et al*., 2009).

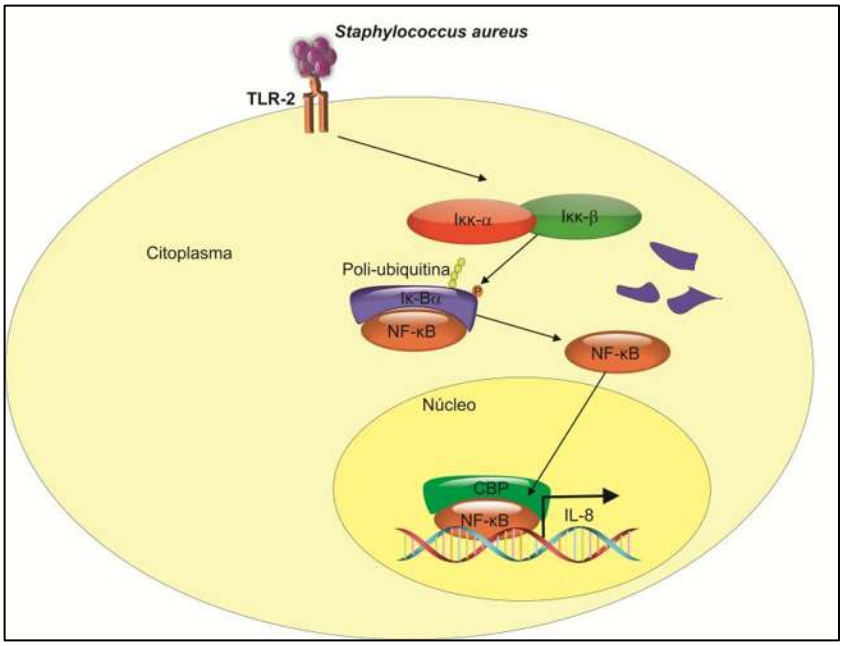

Figura 2. La vía de transducción NF-κB activada por *Staphylococcus aureus.* El PGN presente en la pared celular de la bacteria es reconocido por los receptores tipo Toll-2 (TLR-2) presente en la membrana celular. Esto conduce a la activación del complejo de cinasas IKK formado por las proteínas IKKα e IKKβ. Este complejo activado cataliza la fosforilación de IκB-α lo que estimula su ubiquitinación y posterior degradación en el proteasoma 26S. Como resultado NF-κB se libera del efecto represor de IκB-α y se transloca al núcleo, donde interacciona con la acetiltransferasa CBP y favorece la expresión de genes como IL-8.

6.7. La vía de transducción PI3K.

La enzima PI3K es un heterodímero formado por una subunidad catalítica de 110 kDa (p110) y una reguladora de 85 kDa (p85). Mediante la activación de receptores con actividad de tirosina cinasa (RTK), PI3K regula numerosas funciones fisiológicas, como la supervivencia y proliferación celular, la reorganización del citoesqueleto, la apoptosis, el metabolismo de la glucosa o la síntesis de citocinas (Fruman *et al.*, 2017). La activación de esta vía por TLRs se inicia cuando un estímulo promueve la fosforilación de residuos de Tyr en el dominio citosólico de los receptores. Estas fosfo-Tyr son reconocidas a su vez por el dominio SH2 (Src Homology 2 domain) de la subunidad p85 en el sitio consenso YXXM, lo que trae como consecuencia la activación de la subunidad catalítica p110. La subunidad p110 activada cataliza la síntesis de fosfatidilinositol-3,4,5-trifosfato (PIP3) por fosforilación del fostatidilinositol-4,5-bisfosfato (PIP2), cuya presencia en la cara citosólica de la membrana celular promueve el reclutamiento de proteínas con dominios homólogos de plekstrina como el caso de la enzima Akt (Figura 3) (Vanhaesebroeck *et al.*, 2010).

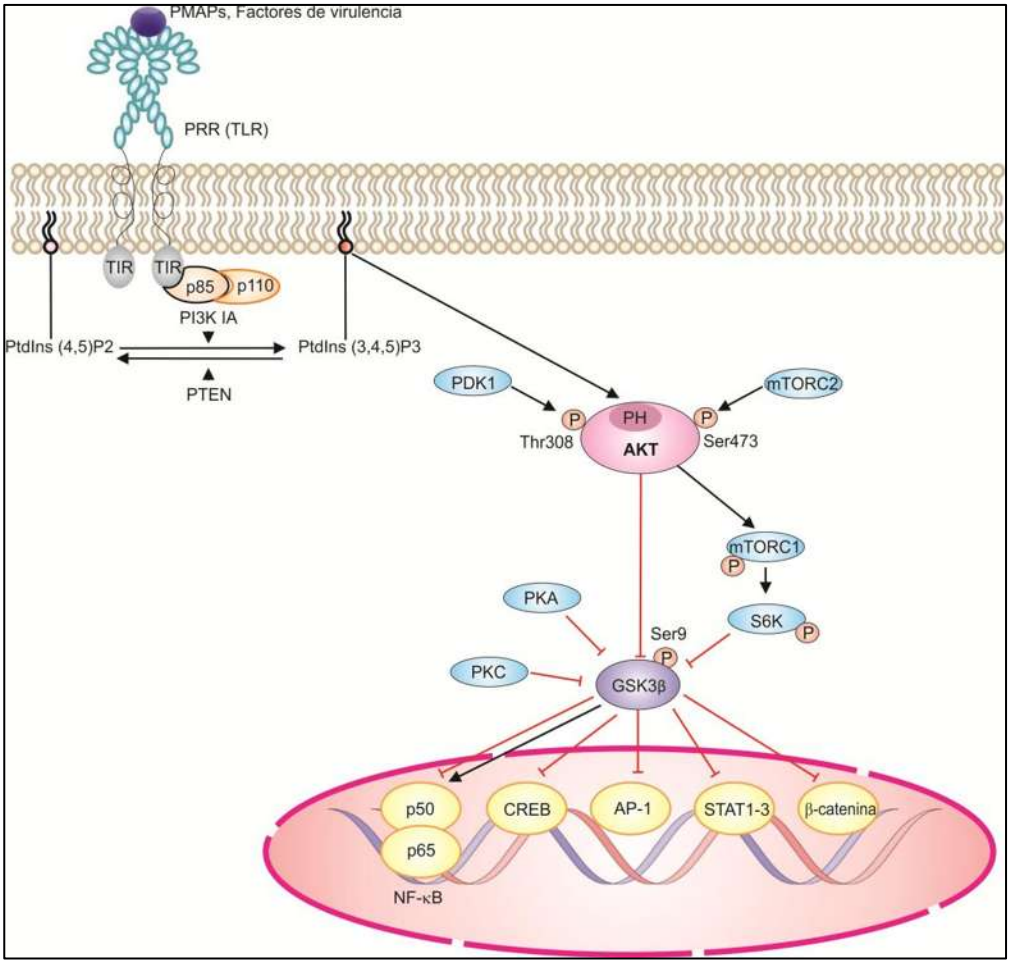

Figura 3. Esquema de la vía PI3K activada por receptores tipo TLR. Cuando el receptor TLR-2 se une a su ligando, se transduce la señal al interior de la célula y se induce la autofosforilación de los residuos de tirosina del receptor (TIR) lo que activa la cinasa PI3K formada por las subunidades p85 y p110 que catalizan la fosforilación del fosfolípido de membranan fosfatidil-inositol bifosfato (PIP2) a fosfatidil-inositol trifosfato (PIP3). El incremento en la abundancia de PIP3 en la membrana celular induce el reclutamiento de la proteína Akt a través de su dominio homólogo de Plekstrina (PH) la cual a su vez es fosforilada por las enzimas PDK1 en el residuo Thr308 y por mTORC2 en Ser473 lo que induce la completa activación de Akt. Akt activado entonces fosforila a GSK3β en el residuo Ser9 lo que resulta en la inactivación de GSK3β. La inactivación de GSK3β modifica la expresión de genes debido a la influencia que esta enzima tiene sobre varios factores transcripcionales.

La enzima Akt, también llamada proteína cinasa B (PKB) por su homología con las enzimas PKA y PKC, tiene 3 isoformas con 80% de homología entre ellas, aunque son codificadas por genes distintos (Gonzalez & McGraw, 2009). En su región N-terminal tienen un dominio homólogo de plekstrina que corresponde al dominio de unión a PIP3. Después de su reclutamiento a la cara interna de la membrana, Akt es fosforilada por la cinasa dependiente de 3'-fosfoinosítido (PDK1) en Thr308 y por

mTORC2 en Ser473. Estas fosforilaciónes de Akt resultan en su completa activación para que pueda fosforilar a sus proteínas sustratos, como la enzima glucógeno sintasa cinasa 3 (GSK3) (Fang *et al.*, 2000) que se ha identificado como uno de los principales reguladores de la producción de citocinas en células fagocíticas profesionales (leucocitos) y no profesionales (células endoteliales y epiteliales) (Cortés-Vieyra *et al.*, 2012).

6.8. La enzima GSK3.

La enzima GSK3 es una Ser/Thr cinasa que en mamíferos se encuentra en forma de 2 parálogos llamados GSK3α y GSK3β, que cumplen funciones diferentes aunque frecuentemente superpuestas. Estas isoformas son codificadas por genes diferentes y conservan un 98% de homología en el sitio catalítico (Beurel *et al.*, 2015). Se les puede encontrar formando parte de complejos en la membrana celular, el citoplasma y el núcleo.

Ambas isoformas pueden fosforilar a los mismos sustratos, aunque experimentos en ratones a los cuales se les ha borrado la copia de una u otra isoforma demuestra que en la mayoría de los casos la expresión de una isoforma puede compensar la falta de la otra con la excepción del hígado en el cual la eliminación de la isoforma beta induce la muerte embrionaria por malformaciones hepáticas (Hoeflich *et al.*, 2000). Recientemente, también se ha descrito que algunos sustratos presentan preferencia por una u otra isoforma lo que sugiere alta especificidad de sus funciones en distintos contextos (Soutar *et al.*, 2010).

GSK3 constituye una enzima particular en la célula por el hecho de que requiere que sus sustratos estén prefosforilados, es constitutivamente activa y es capaz de autofosforilarse en los residuos Tyr279 de GSK3α y Tyr216 de GSK3β durante su síntesis para estimular su propia actividad cinasa (Cole *et al.*, 2004). Esto significa que su actividad se inhibe por estimulación celular. En contraste a la mayoría de proteína cinasas que son específicas y tienen un promedio de 12 sustratos, se ha predicho por análisis computacional que GSK3β puede fosforilar 500 sustratos, de los cuales 100 han sido confirmados por experimentación *in vitro* (Linding *et al.*, 2007).

La regulación de la actividad de las isoformas de GSK3 se lleva a cabo por la fosforilación en los residuos Ser21 de GSK3α y Ser9 de GSK3β, lo que induce un cambio conformacional de la enzima que resulta en el bloqueo de su sitio catalítico por su propio amino terminal (Figura 4) (Beurel *et al*., 2015).

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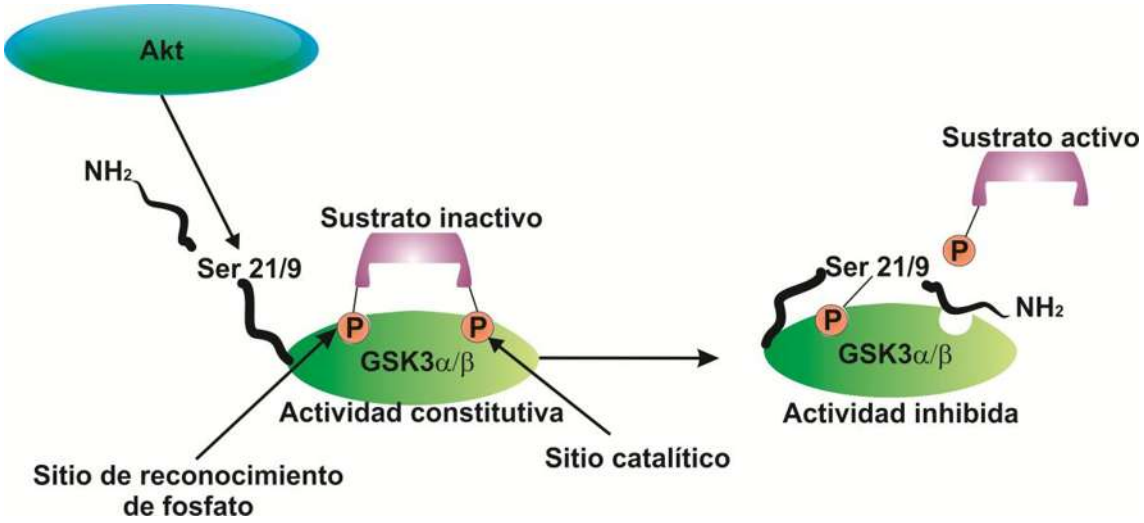

Figura 4. Modelo de fosforilación-inactivación de las isoformas de GSK3α/β en Ser21/9 por Akt. Después de fosforilar los residuos Ser21 y Ser9 presentes en GSK3αβ, el extremo amino-terminal de las enzimas se pliega sobre la misma enzima por constituir un pseudo-sustrato lo que resulta en el bloqueo físico de su sitio catalítico e impide el acceso de los sustratos de GSK3αβ lo que inhibe su función cinasa

GSK3β participa en la regulación de la síntesis de citocinas en respuesta a la infección por *Francisella tularensis*, *Mycobacterium tuberculosis* o *Helicobacter pylori* (Zhang *et al.*, 2009) (Chan *et al.*, 2009) (Nakayama *et al.*, 2009). Esta regulación está asociada a la vía PI3K-Akt en respuesta a estimulación de monocitos con LPS, lo que resulta en la pérdida de la regulación de la producción de citocinas pro- y anti-inflamatorias (Martin *et al.*, 2005).

6.9. Regulación de la inflamación por GSK3.

Los inhibidores farmacológicos de GSK3 como LiCl o el inhibidor específico SB216763 potencian la expresión de citocinas anti-inflamatorias como IL-10 y reprimen la expresión de citocinas pro-inflamatorias como IL-8 o IL-12p40 en distintos tipos celulares estimulados con ligandos de TLR (Deplanche *et al.*, 2016). Por otro lado, la expresión de mutantes puntuales constitutivamente activas de GSK3α (Ser21Ala) y GSK3β (Ser9Ala), favorecen la expresión de IL-8, TNF e IL-6 al mismo tiempo que abaten la expresión de IL-10 en modelos de infección bacteriana (Wang *et al.*, 2011).

El mecanismo de GSK3 que regula la síntesis de citocinas se estudió inicialmente en ratones GSK3β KO. Sin embargo, se observó que la eliminación de GSK3β produjo la muerte embrionaria de ratones, lo que constituye un fenotipo que es similar al observado en ratones NF-κB KO. El mismo estudio reportó que el tratamiento de fibroblastos con cloruro de litio (inhibidor de GSK3), inhibe la activación de NF-κB

inducido por TNFα. Estos datos, condujeron a la propuesta de que GSK3β constituye un regulador positivo de la actividad de NF-κB (Hoeflich *et al*., 2000), aunque GSK3β parece no fosforilar a NF-κB en los residuos Ser536 o Ser276, que son importantes para estimular la actividad de este factor transcripcional. Tampoco se han observado alteraciones en la estabilidad de IκB-α, la proteína inhibidora de NF-κB, como resultado de la actividad de GSK3 (Martin *et al*., 2005). En conjunto estos resultados sugieren que la regulación de GSK3 sobre NF-κB se lleva a cabo a nivel de complejos transcripcionales (Hoeflich *et al*., 2000). En contraste, se ha reportado que la actividad del factor transcripcional CREB y el co-factor β-catenina se favorece por la inhibición de GSK3. CREB es un factor transcripcional importante para la expresión de IL-10, en respuesta a estímulos inflamatorios. β-catenina es un co-factor que regula la diferenciación celular, pero también se ha involucrado en la regulación negativa de la vía de NF-κB en modelos de infección con *Salmonella typhimurium* (Silva-García *et al.*, 2014).

Aunque se ha demostrado que GSK3α puede regular la expresión de citocinas en contextos celulares diversos (Banko *et al.*, 2014, Silva-García *et al.*, 2018), esta isoforma, a diferencia de GSK3β, no ha sido relacionada con la regulación de la expresión de citocinas en el contexto de inflamación inducida por bacterias.

6.9.1. El mecanismo de competencia de NF-κB y CREB por CBP.

Los factores de transcripción NF-κB y CREB requieren como un paso esencial de su actividad transcripcional interaccionar con co-factores que tienen actividad de acetil-transferasa, como la proteína de unión a CREB (CBP). A pesar de que CBP es una proteína de expresión ubicua en las células, sus cantidades son limitadas y la formación de complejos transcripcionales depende de la abundancia de los factores transcripcionales activados (Vo & Goodman, 2001).

La competencia entre NF-κB y CREB por unirse a CBP fue demostrada en monocitos estimulados con LPS. Los datos demostraron que la actividad de GSK3β no estimuló directamente la actividad transcripcional de NF-κB, pero favoreció su función represora sobre el factor transcripcional CREB (Martin *et al*., 2005). En este modelo se propuso que cuando los monocitos son estimulados con LPS, las vías de activación de NF-κB y CREB son procesos que ocurren simultáneamente y de manera paralela. No obstante, la actividad constitutiva de GSK3 mantiene un efecto inhibidor sobre CREB al fosforilarlo en el residuo Ser129, con lo cual disminuye su afinidad por CBP y promueve la formación del complejo NF-κB/CBP. El tratamiento de los monocitos con

SB216763 o el silenciamiento de GSK3β favorecen la formación del complejo CREB/CBP, al mismo tiempo que se reduce la formación del complejo NF-κB/CBP.

Aunque este mecanismo está ampliamente aceptado para GSK3β, no ha sido probado si la isoforma GSK3α presenta las mismas características. Recientemente, se ha descrito que GSK3α tiene preferencia sobre GSK3β por la inhibición de CREB en células cerebrales de ratón (Liang & Chuang, 2006) e inhibe la función cinasa de Akt (su principal represor en la vía PI3K) lo que constituye una retro-regulación (Gulen *et al.*, 2012).

Es importante destacar que los mecanismos de regulación en la producción de citocinas por las isoformas de GSK3 no ha sido estudiado en el contexto de las infecciones producidas por el patógeno *S. aureus,* siendo la desregulación de la inflamación una de las características más significativas de las infecciones producidas por esta bacteria (Ver anexo I).

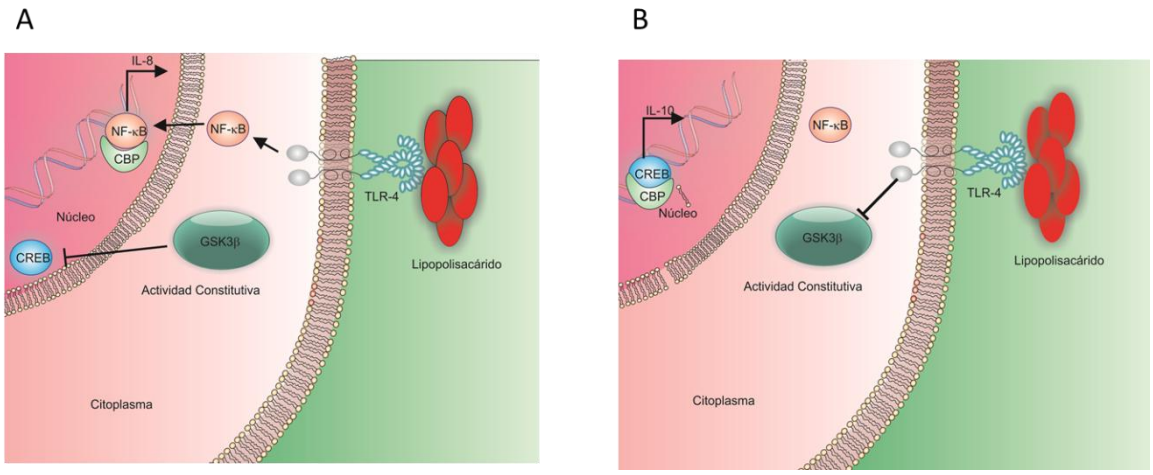

Figura 5. Modelo de competencia por CBP regulado por GSK3β.(A) Tras el reconocimiento inicial, los receptores TLR induce la activación de la vía de NF-κB lo que resulta en su translocación al núcleo y la inducción de la expresión de genes como IL-8, al mismo tiempo la actividad constitutiva de GSK3β reprime la activación del factor transcripcional CREB lo que impide en esta etapa la expresión de genes como IL-10. (B) Pasado el tiempo necesario, GSK3β pierde su actividad constitutiva, se libera el efecto represor sobre CREB, el cual ahora compite con NF-κB por interactuar con el co-activador CBP e induce la expresión de genes como IL-10 al mismo tiempo que reprime la expresión de genes dependientes de NF-κB como IL-8.

7. Antecedentes

En nuestro laboratorio se estudió previamente que las estimulaciones de células de endotelio bovino con una cepa de *S. aureus*, induce la activación de la vía PI3K-Akt lo que conduce a la inhibición-fosforilación de las isoformas de GSK3α/β con una mayor abundancia relativa de la isoforma GSK3α fosforilada (Figura 6). Esto resultó de particular interés debido a que en estudios previos de otros autores se ha mencionado que la isoforma GSK3α no se fosforila por estimulación con ligandos de TLR´s. Posteriormente, reportamos que la estimulación del endotelio bovino con PGN, procedente de *S. aureus*, indujo la fosforilación e inhibición preferentemente de la isoforma GSK3α.

Mediante la técnica de silenciamiento con ARN dirigido a las isoformas de GSK3, se observó que el silenciamiento de la isoforma GSK3α altera la expresión de IL-12p40 de manera diferencial con respecto a la isoforma GSK3β (ver **¡Error! No se encuentra el origen de la referencia.**). Sin embargo, no se ha profundizado en el mecanismo por el cual GSK3α pudiera estar efectuando la regulación de la producción de citocinas.

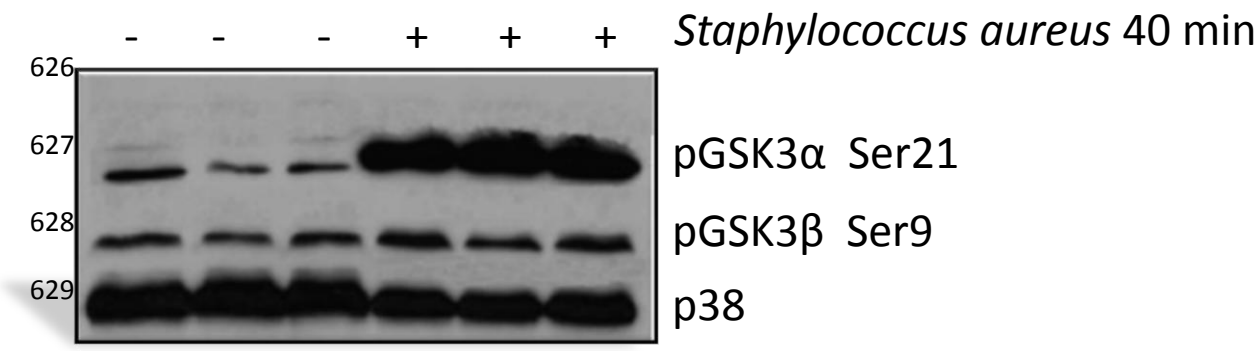

Figura 6. Efecto en la fosforilación de las isoformas de GSK3 en Células endoteliales de bovino estimuladas con *Staphylococcus aureus*. Se cultivaron células de endotelio bovino hasta en placas hasta confluencia, entonces se cultivaron en medio sin suero durante 4 h y se infectaron con 20 unidades formadoras de colonia (UFC) de *S. aureus* durante 40 min. Se obtuvieron los extractos de proteína total y se separaron mediante electroforesis desnaturalizante en geles de SDS al 10%. Se inmunodetectaron las proteínas pGSK3α Ser21 y pGSK3β Ser9. La proteína p38 se detectó para mostrar uniformidad de carga. Se muestran los resultados de 3 experimentos independientes.

8. Justificación

Staphylococcus aureus es una de las principales bacterias causante de infecciones en humanos y animales. Puede ocasionar infecciones severas y potencialmente fatales aun en individuos con sistemas inmunes competentes, y es altamente resistente a la terapia con antimicrobianos. La fisiopatología de las infecciones producidas por esta bacteria está asociada a la alteración de mecanismos inmunológicos relacionados con la inducción de inflamación a través de la producción de citocinas.

La respuesta inflamatoria provocada por *S. aureus* se caracteriza en la primera etapa por la expresión de citocinas pro-inflamatorias como IL-8, que además es quimio-atrayente de neutrófilos, seguida por la expresión de IL-10 que provoca la inactivación del sistema inmune e induce inmunotolerancia. Parece que el resultado de este cambio en la expresión del tipo de citocinas resulta en la sobrevivencia de la bacteria en el hospedero, debido a la incapacidad del sistema inmune para eliminarla. Se sabe que la producción de citocinas con actividad pro- o anti-inflamatoria en modelos de infección bacteriana está regulada por la actividad de la enzima GSK3 sobre factores transcripcionales como NF-κB y CREB.

Experimentos preliminares de nuestro laboratorio han demostrado que la infección de células de endotelio bovino con una cepa de *S. aureus* o con la molécula PGN induce la fosforilación-inhibición de GSK3α de manera preferencial. Esta inhibición preferencial está asociada a cambios en la expresión de la citocina IL-12p40. Sin embargo, hasta la fecha el mecanismo por el cual GSK3α regula la expresión de citocinas durante infecciones producidas por *S. aureus* no se ha caracterizado. El reconocimiento de los mecanismos moleculares por los cuales *S. aureus* manipula la respuesta inmunológica permitirá el diseño de estrategias efectivas para su control y erradicación.

9. Hipótesis

La expresión de citocinas pro- o anti-inflamatorias en células de endotelio bovino infectadas con *Staphylococcus aureus* depende de la actividad de GSK3α y su influencia sobre NF-κB y CREB.

10. Objetivo general

Determinar la participación de la enzima GSK3α en la expresión de citocinas que dependen de la actividad transcripcional de NF-κB y CREB.

11. Objetivos específicos

- Comparar los niveles de fosforilación de las isoformas de GSK3 inducidos por la infección de *S. aureus*.
- Analizar la activación de NF-κB y CREB, en función de la actividad de GSK3.
- Determinar la participación de GSK3α o GSK3β en la activación de NF-κB y CREB.
- Relacionar los niveles de expresión de IL-8 e IL-10 con la actividad de NF-κB y CREB.

12. Resultados

13. Capítulo I

Los antecedentes de nuestro equipo de trabajo señalan que *S. aureus* induce de manera preferencial la inhibición de la isoforma GSK3α en células endoteliales de bovino. Este tipo celular forma parte de las barreras físicas del sistema inmune y forman parte de los fagocitos no profesionales. Además son capaces de reconocer la presencia de microorganismos patógenos mediante los receptores tipo TLR y como resultado expresan citocinas que coordinan la respuesta inmunológica innata. La manipulación de la respuesta inflamatoria es un elemento clave de la patogénesis de *S. aureus* y se sabe que la enzima GSK3 regula la expresión de citocinas de carácter pro- o anti-inflamatorias en modelos de infección bacteriana. En este apartado se muestra evidencia experimental que señala a GSK3α como la principal isoforma responsable de la regulación de los factores de transcripción NF-κB y CREB y, como resultado de esta regulación, también la expresión de las citocinas pro-inflamatoria IL-8 y de la anti-inflamatoria IL-10.

frontiers in Immunology

ORIGINAL RESEARCH
published: 29 January 2018
doi: 10.3389/fimmu.2018.00092

Glycogen Synthase Kinase 3α Is the Main Isoform That Regulates the Transcription Factors Nuclear Factor-Kappa B and cAMP Response Element Binding in Bovine Endothelial Cells Infected with *Staphylococcus aureus*

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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: 26 October 2017
Accepted: 12 January 2018
Published: 29 January 2018

Citation:
Silva-García O, Rico-Mata R, Maldonado-Pichardo MC, Bravo-Patiño A, Valdez-Alarcón JJ, Aguirre-González J and Baizabal-Aguirre VM (2018) Glycogen Synthase Kinase 3α Is the Main Isoform That Regulates the Transcription Factors Nuclear Factor-Kappa B and cAMP Response Element Binding in Bovine Endothelial Cells Infected with Staphylococcus aureus. Front. Immunol. 9:92. doi: 10.3389/fimmu.2018.00092

Glycogen synthase kinase 3 (GSK3) is a constitutive enzyme implicated in the regulation of cytokine expression and the inflammatory response during bacterial infections. Mammals have two GSK3 isoforms named GSK3α and GSK3β that plays different but often overlapping functions. Although the role of GSK3β in cytokine regulation during the inflammatory response caused by bacteria is well described, GSK3α has not been found to participate in this process. Therefore, we tested if GSK3α may act as a regulatory isoform in the cytokine expression by bovine endothelial cells infected with *Staphylococcus aureus* because this bacterium is one of the major pathogens that cause tissue damage associated with inflammatory dysfunction. Interestingly, although both isoforms were phosphorylated–inactivated, we consistently observed a higher phosphorylation of GSK3α at Ser21 than that of GSK3β at Ser9 after bacterial challenge. During a temporal course of infection, we characterized a molecular switch from pro-inflammatory cytokine expression (IL-8), promoted by nuclear factor-kappa B (NF-κB), at an early stage (2 h) to an anti-inflammatory cytokine expression (IL-10), promoted by cAMP response element binding (CREB), at a later stage (6 h). We observed an indirect effect of GSK3α activity on NF-κB activation that resulted in a low phosphorylation of CREB at Ser133, a decreased interaction between CREB and the co-activator CREB-binding protein (CBP), and a lower expression level of IL-10. Gene silencing of GSK3α and GSK3β with siRNA indicated that GSK3α knockout promoted the interaction between CREB and CBP that, in turn, increased the expression of IL-10, reduced the interaction of NF-κB with CBP, and reduced the expression of IL-8. These results indicate that GSK3α functions as the primary isoform that regulates the expression of IL-10 in endothelial cells infected with *S. aureus*.

Keywords: inflammatory response, endothelial cells, *Staphylococcus aureus*, glycogen synthase kinase 3, nuclear factor-kappa B, cAMP response element binding, interleukin-8, interleukin-10

INTRODUCTION

Staphylococcus aureus is a Gram (+) bacterium that causes important infectious diseases among humans and animals. This bacterium expresses a broad range of virulence factors and cell-wall-associated structures (CWASs) that induce inflammation and are responsible for tissue damage (1). A hallmark of *S. aureus* is its ability to evade host innate immune response, which results in repeated infections and life-threatening diseases (2, 3). The molecular mechanisms that *S. aureus* employs to evade the host immune response are diverse, and some of them are poorly understood. One of these mechanisms involves an impairment in cell-signaling pathways leading to the suppression of interleukin-8 (IL-8) expression (4–7), a cytokine that shows pro-inflammatory and chemotactic activities in the first stages of *S. aureus* infections (8, 9). Moreover, IL-8 is essential to promote neutrophil survival and bacterial clearance because the inhibition of IL-8 expression in *S. aureus* infection models has been associated with cell death and bacterial survival (6). *S. aureus* infections also enhance an anti-inflammatory response through the induction of immune-suppressive and -tolerogenic cytokines expression (i.e., interleukin-10 (IL-10)) that contribute to bacterial persistence and immune tolerance (10, 11).

Host cells can recognize *S. aureus* CWASs through the plasma membrane TLR2 receptor. The binding of CWAS to TLR2 activates the phosphoinositide 3-kinase/Akt (PI3K/Akt)-signaling pathway that mediates a variety of cellular responses such as survival, proliferation, differentiation, apoptosis, and inflammation (12). The activation of PI3K leads to Akt phosphorylation at Ser473 and Thr308 by the constitutively active PDK1 and mTORC2 (13). In turn, the activated Akt regulates the activity of a wide range of substrates, among which glycogen synthase kinase 3 (GSK3) regulates the balance of the inflammatory response (14). GSK3 refers to the two mammalian paralogs GSK3α and GSK3β (15), which are unusually constitutively active and can be inactivated by phosphorylation at Ser21 (GSK3α) or Ser9 (GSK3β) by Akt upon bacterial stimulus (16). Since its discovery, GSK3β has been proposed as the principal GSK3 isoform involved in the regulation of many cellular functions, including the inflammatory response caused by bacterial infections. The other isoform, GSK3α, has not yet been associated with the regulation of the inflammatory response. This observation is important because the same stimulus can usually phosphorylate and inactivate both isoforms. The experimental evidence suggests that the physiological roles of GSK3 isoforms may be explained by different regulatory mechanisms, such as the formation of molecular complexes, subcellular compartmentation, and specific kinase modifications that are linked to specific stimuli and cellular contexts (17).

In a previous report, we demonstrated that the internalization of *S. aureus* by bovine endothelial cells (BECs) induced the activity of PI3K/Akt signaling and a preferential phosphorylation of GSK3α compared to GSK3β (18). More recently, we reported that peptidoglycan from *S. aureus* induced a higher phosphorylation of GSK3α than that of GSK3β, and this led to an increase in IL-12p40 expression (19). Several, studies in murine models have shown that both isoforms of GSK3 are not physiologically redundant (20). The deletion of GSK3α in myeloid cells attenuated atherosclerosis and promoted M2 macrophage phenotype and IL-10 expression (21). Interestingly, GSK3 isoforms are differentially expressed, depending on the tissue analyzed. For example, GSK3α is the main isoform expressed in the skeletal muscle, testis, and neutrophils but it is absent in birds (17). Regarding GSK3β, this isoform is predominant in a Th17 subtype of T cells compared to GSK3α, indicating that GSK3α and GSK3β expressions are tissue-specific (22). Also, GSK3α but not GSK3β promotes IL-10 expression in a mice model of high-fat diet-low-density lipoprotein in response to a variety of stimuli (20, 23), and it is the main isoform inactivated by the peptide neurotensin (24), which reduces inflammation after fibroblast stimulation with lipopolysaccharide (LPS) (25).

A molecular mechanism by which GSK3 regulates the balance in cytokines expression has been previously described in macrophages stimulated with LPS from *Escherichia coli*. It was observed that GSK3β regulated the pro- and anti-inflammatory cytokines expression through its influence on nuclear factor-kappa B (NF-κB) and cAMP response element-binding (CREB) activity (14). This mechanism involved the competition between CREB and NF-κB for the co-activator CREB-binding protein (CBP). According to this model, the activation of NF-κB is essential for IL-8 expression (26) and is positively favored by GSK3β (27). By contrast, GSK3β negatively regulates CREB by limiting its phosphorylation at Ser133 (14, 28), which results in a reduced CREB-DNA-binding activity (29) and subsequently low IL-10 expression (30). However, our experimental evidence indicates that this GSK3-dependent regulation of NF-κB and CREB activity in BEC infected with *S. aureus* primarily depends on GSK3α rather than on GSK3β.

Therefore, we propose that in BEC infected with *S. aureus*, the constitutive activity of GSK3α favors NF-κB activation and expression of IL-8 at initial stages. However, GSK3α activity inhibition at later stages of infection favors CREB activation that leads to IL-10 expression. Our data highlight the regulatory role of the GSK3α isoform on the inflammatory response in endothelial cells and may explain, in part, why *S. aureus* infections can evolve to a chronic state.

MATERIALS AND METHODS

Media and Chemicals

DMEM/F12 (F12), Trypsin-EDTA, Igepal CA-930, Akt-IV, and SB216763 were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Fetal calf serum (FCS) was acquired from Equitech-Bio, Inc. (Kerrville, TX, USA). A cocktail of sodium penicillin G, streptomycin sulfate, and amphotericin B was purchased from Gibco-BRL (Gaithersburg, MD, USA). Halt-TM Phosphatase inhibitor cocktail was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Protease inhibitor cocktail was acquired from GE Healthcare Biosciences (Little Chalfont, UK). Trizol reagent and EXPRESS One-Step SYBR GreenER Universal Kit were purchased from Invitrogen (Carlsbad, CA, USA). Bovine IL-10 and IL-8 TSZ ELISA kit were purchased from Biotang (Waltham, MA, USA). Plasmid pLKO.1-GSK3β-#1 was a gift from Alex Toker (Addgene plasmid #32497) and PLKO.1 plasmid was a gift

from Bob Weinberg (Addgene plasmid # 8453). pCF CREB M1 was a gift from Marc Montminy (Addgene plasmid # 22969). The generation of pCF empty vector was done by the enzyme digestion of pCF CREB M1 with SacI restriction enzyme, and then the purification and ligation of the 5-kb fragment were generated.

Antibodies

Antibodies against Laminin A/C, GAPDH, β-actin, CREB, CBP, and IgG-HRP-coupled antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phospho-glycogen synthase (Ser641), phospho-GSK3α (Ser21), phospho-GSK3α (Ser9), phospho-NF-κB p65 (Ser536), phospho-CREB (Ser133), GSK3β, GSK3α, and NF-κB p65 were purchased from Cell Signaling Technology (Boston, MA, USA).

Bacterial Strain, Cell Line, and Culture Conditions

The strain of *S. aureus* used in this study is an isolate from a clinical case of bovine mastitis and was obtained from the American-Type Culture Collection (27543). Bacteria were cultured overnight in 3 ml of Luria-Bertani (LB) medium at 37°C with continuous agitation. The inoculum for infection assays was prepared by adding 1 ml of this pre-culture to 49 ml of fresh LB medium and grown at 37°C. This inoculum was added to cells until the culture reached the initial-middle log phase at an optical density of 0.3 at 600 nm and washed with F12 medium.

Endothelial cells used in this study were obtained from bovine umbilical veins and immortalized by transfection with an expression vector containing the E6–E7 oncogenes of human papillomavirus 16 (BVE-E6–E7) (31). These BECs were grown and maintained in F12 supplemented with 10% FCS and a cocktail of sodium penicillin G, streptomycin sulfate and amphotericin B, unless otherwise noted. For pretreatment with inhibitors, BECs were pre-incubated with a medium alone or with 1 μM of Akt-IV, 10 μM of SB216763, or 20 μM of Parthenolide before infection with bacteria.

Infection Assay

Bovine endothelial cells were grown in six-well cell culture plates with F12 medium supplemented with FCS and antibiotics until 90–95% confluence. Then, cells were washed 2× with PBS and the medium was changed to F12 without serum and antibiotics. Cells were left in these conditions for at least 4 h. Bacteria were added at a multiplicity of infection (MOI) of 20 CFU/cell, and plates were centrifuged at 500 × *g* for 5 min and incubated in 5% CO_2 at 37°C for the times indicated.

BEC Transfection

For plasmids transfection containing siRNA against GSK3 isoforms, BECs were grown until 60–70% confluence in F12 medium without serum and antibiotics for 24 h. Then, transfection was performed with Lipofectamine 2000 from Thermo Fisher Scientific (Waltham, MA, USA); for siGSK3α, we added 3 μg of siRNA-GSK3α plasmid (Sigma–Aldrich) and 3 μg of PLKO.1; for siGSK3β, we added 3 μg of pLKO.1-GSK3β-#1 and 3 μg of PLKO.1; for siGSK3αβ, we added 3 μg of siRNA-GSK3α and 3 μg of pLKO.1-GSK3β-#1 plasmid; and for sicontrol, we added 6 μg of PLKO.1. After 24 h, the culture medium was changed to F12 with low serum (1%), and cells were incubated for 5 days. GSK3α and GSK3β gene-silenced cells were screened by Western blotting. For GSK3α, the targeting sequence was 5′-TACATCTGTTCTCGCTACTA-3′ (nucleotides 1535–1554, pLKO.1-GSK3α), and for GSK3β the targeting sequence was 5′-GAAGTCAGCTATACAGACACT-3′ (nucleotides 587–607, pLKO.1-GSK3β). To express the CREB-dominant-negative mutant, cells were cultured at 70–90% confluence in F12 medium without serum or antibiotics for 24 h; then, 350 ng of plasmid pCF-M1-CREB was transfected with Lipofectamine 2000 or 350 ng of pCF empty vector. pCF-M1-CREB plasmid encodes a CREB mutant in which Ser133 was replaced by Ala133. After 12 h, the medium was changed to fresh F12 and low serum, and cells were incubated for an extended period of 48 h. The medium was changed to F12 without serum or antibiotics, and cells were left for at least 4 h before stimulation with *S. aureus*. Transfection efficiency was monitored by detecting the FLAG tag present on the CREB-mutant construct in Western blot assays. The transfection efficiency of the empty pCF-M1 vector was monitored by the resistance of cells to Geneticin.

Protein Extraction, Subcellular Fractionation, and Western Blot Assay

To evaluate the relative abundance of phosphorylated and non-phosphorylated proteins, the total protein (cytosolic and nuclear) from control and treated cells was obtained by washing cells 2× with ice-cold PBS and lysing them with 80 μl of cold lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-930, 10 mM Na-pyrophosphate, 50 mM NaF, and 1 mM Na-orthovanadate) supplemented with 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail, which were added immediately before lysis. For subcellular protein fractionation, we followed the REAP method described by Suzuky et al. (32). Lysates were centrifuged at 16,000 × *g* for 20 min at 4°C, and the supernatant was recovered. Protein concentration was measured by Bradford using BSA as standard. Then, 30–40 μg of protein was separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels at 200 V for 1 h and electroblotted in a wet chamber onto 0.45 μm nitrocellulose membrane (Bio-Rad) at 250 mA for 1 h. Membranes were incubated in a blocking solution (5% non-fat milk solution) in TTBS (10 mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20); primary antibodies to target proteins were added and membranes incubated at 4°C overnight. Membranes washed 1× with PBS and 2× with TTBS were incubated with secondary antibodies. Proteins were detected with the Immobilon Western Chemiluminescent HRP substrate kit from Millipore (Billerica, MA, USA). Protein loading was verified by stripping the membranes with NaOH of 0.2 N for 5 min and washed 2× with TTBS; after a blocking step, the membranes were re-probed with antibodies against the indicated housekeeping proteins and detected in the same way as described. Data presented in **Figures 4** and **9C** were obtained by scanning the membranes using a C-DiGit blot scanner (LI-COR, Lincoln, NE, USA).

RNA Extraction and Reverse Transcription and Real-time Quantitative PCR (qRT-PCR)

After infection, BECs were washed 2× with ice-cold PBS, and the total RNA was extracted with Trizol reagent, following the isolation procedure described by the manufacturer. RNA was purified with RQ1 RNase-free DNase (Promega). One-step qRT-PCR was performed using the EXPRESS One-Step SYBR GreenER Universal Kit and the real-time StepOnePlus thermocycler from Applied Biosystems. Each reaction was performed with 100 ng of total RNA under the standard 20-µl reaction provided by Invitrogen. The one-step-cycling program conditions and oligonucleotide primers used were as described by Konnai et al. (2003) (33). Amplification of the expected single products was confirmed by single-peak analysis in temperature-melting curve and visualization on 1% agarose gels stained with ethidium bromide. Relative transcript levels of mRNA were calculated with the ΔΔCt method, using β-actin as the reference gene. Negative amplification of non-added RT enzyme reactions confirmed the absence of DNA in our assays.

Measurement of IL-8 and IL-10 Protein Levels

Bovine IL-8 and IL-10 proteins in culture supernatants were quantitated for the indicated times by sandwich ELISA, according to the manufacturer's instructions (Biotang, Waltham, MA, USA).

Co-IP Assays

Co-IP assays were performed with the Pierce Protein A/G Magnetic Beads kit (Thermo Fisher Scientific). Briefly, the control and infected cells (~1×10^7) were washed 2× with ice-cold PBS and lysed in washing/lysis buffer supplemented with 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail. Cell debris was collected by centrifugation at 18,000 × *g* for 10 min at 4°C. The supernatant was recovered and quantitated for protein concentration. Then, 5 µg of CBP IP antibody was added to 500 µg of protein extract; the volume was adjusted to 500 µl with lysis buffer and incubated with continuous agitation overnight at 4°C. Then, 25 µg of protein A/G magnetic beads was added and incubated for 1 h with continuous agitation at room temperature (~23°C); beads were washed 2× with a washing buffer and 1× with water. The target antigen was eluted with 1× lane marker buffer and 50 mM DTT in a final volume of 100 µl. Then, 30 µl of sample in elution buffer was loaded, and proteins were detected by Western blot as described earlier.

Statistical Analysis

The relative abundance of phosphorylated proteins was quantitated by densitometric analysis with the Image Processing and Analysis in Java Program ImageJ (http://rsbweb.nih.gov/ij). To calculate densitometric values, the intensity of the phosphorylated band was divided by the intensity of the non-phosphorylated one. These intensities were referred to a value of 1.0 that was arbitrarily assigned to the untreated control. The statistical significance of triplicate blots, IL-8 and IL-10 mRNA ΔΔCt value, and protein levels were evaluated with the Tukey HSD multiple-comparison test and one-way analysis of variance by using the JMP 6.0 program (SAS Institute). Differences between groups were considered significant at $P < 0.05$. All data are available on request.

RESULTS

Endothelial Cells Infected with *S. aureus* Express IL-8 and IL-10

The pro-inflammatory chemokine IL-8 is an early marker of bacterial infection (9). However, several authors have reported that *S. aureus* can induce or repress the IL-8 expression on different cellular physiological contexts, and this was correlated with the pathology produced by this bacterium (4–7). There is also evidence that *S. aureus* induces IL-10 expression, leading to immune tolerance (11). Therefore, we asked whether the expression of these cytokines depended upon the stage of infection. In order to evaluate the kinetics of IL-8 and IL-10 expressions, BECs were infected with *S. aureus* at a MOI of 20, which allows the survival of endothelial cells for 2, 4, and 6 h. Then, RNA was purified to analyze IL-8/IL-10 mRNA by qRT-PCR and their corresponding peptides in cell supernatants by ELISA. The expression of IL-8 mRNA increased by more than 2.5-fold in response to 2 h of *S. aureus* infection; however, at longer infection times of 4–6 h, IL-8 mRNA values returned to control levels (**Figure 1A**). Measurements of IL-8 peptide indicated an increase at 2 h post infection with similar values at 4 and 6 h (**Figure 1B**). In the case of IL-10 mRNA, an increase was observed at 4 h and remained without a statistical change for up to 6 h (**Figure 1C**) while its peptide reached a maximum at 4 h (**Figure 1D**). These data indicate that in BEC infected with *S. aureus*, the mechanism responsible for IL-8 expression precedes the mechanism leading to IL-10 expression.

S. aureus Infection Induces a Time-Dependent NF-κB/CREB Phosphorylation and NF-κB Nuclear Translocation

The transcription factors NF-κB and CREB induce IL-8 and IL-10 expressions, respectively (26, 34). The transcriptional activation of NF-κB, in response to bacterial ligands, involves the phosphorylation of p65 subunit at Ser536 and its translocation to the nucleus. These events usually take place in the first 15–40 min after cell stimulation with different ligands (8, 14, 32, 35, 36). We observed that the phosphorylation of p65 at Ser536 reached a maximum level at 40 min after bacterial challenge followed by a gradual reduction as compared to uninfected control (**Figure 2A**). Several authors have demonstrated that CREB is transcriptionally activated by phosphorylation at Ser133 in response to various stimuli (28, 37, 38). Accordingly, a significant increase in phospho-CREB at Ser133 at 60 min post infection was detected with a maximum observed at longer infection times of 90–120 min (**Figure 2B**). Interestingly, these longer infection times that we tested to induce an increase in phospho-CREB-Ser133 were the same as those required for NF-κB to become dephosphorylated and inactivated. These results allowed us to select 40 and 120 min as the infection

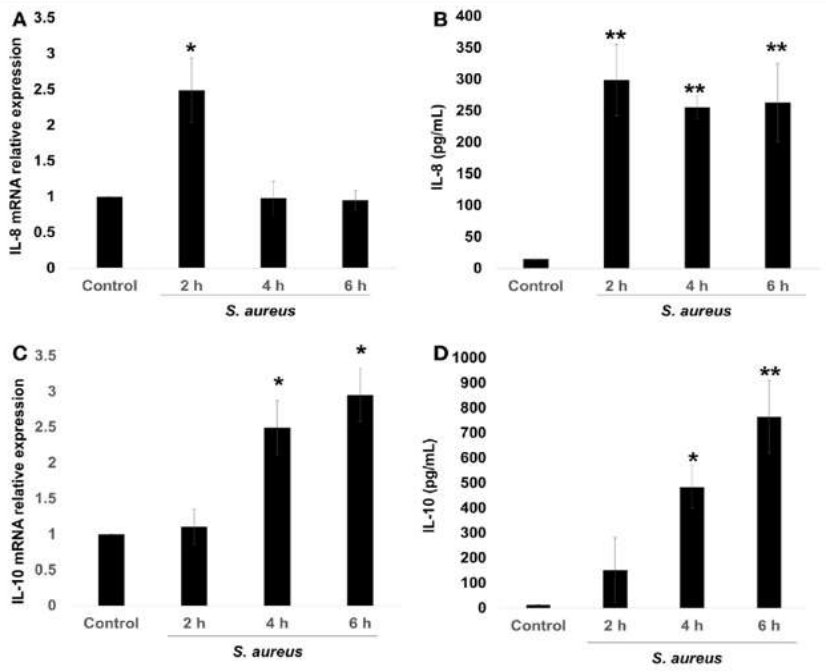

FIGURE 1 | *Staphylococcus aureus* infection induces interleukin-8 (IL-8) and interleukin-10 (IL-10) expressions in bovine endothelial cells (BECs). BEC controls were left uninfected or infected at a multiplicity of infection of 20 CFU/cell *S. aureus* for 2–6 h. Then, the total RNA was extracted and the relative expression of IL-8 mRNA **(A)** and IL-10 mRNA **(C)** was quantified by reverse transcription and real-time quantitative PCR using the ΔΔCt method. Data were normalized in reference to the expression of β-actin. Quantitation of IL-8 **(B)** and IL-10 **(D)** peptides was done by ELISA. Data are presented as the mean ± SEM of three independent experiments (SEM; $n = 3$ per experiment). $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

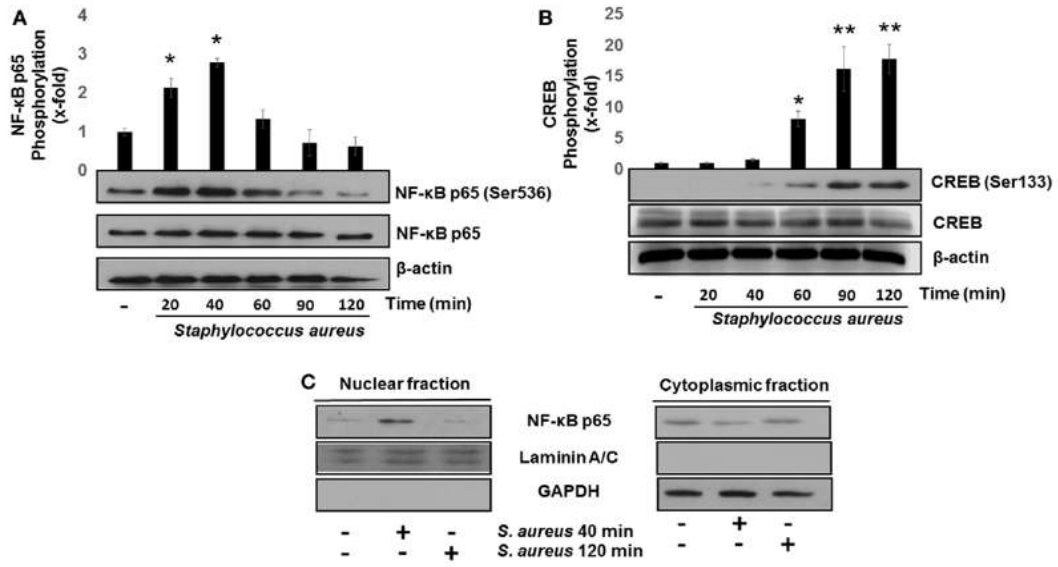

FIGURE 2 | *Staphylococcus aureus* activates nuclear factor-kappa B (NF-κB) and cAMP response element-binding (CREB) phosphorylation and induces NF-κB nuclear translocation. Bovine endothelial cells were left uninfected (–) or infected at a multiplicity of infection of 20 CFU/cell of *S. aureus* for 20–120 min. After infection, the total protein (nuclear + cytoplasmic) of **(A,B)** or nuclear- and cytoplasmic-enriched fractions **(C)** was analyzed by Western blotting to detect the relative abundance of phosphorylated NF-κB p65 at Ser536 **(A)**, phosphorylated CREB at Ser133 **(B),** or NF-κB p65 **(C)**. The detection of total unphosphorylated NF-κB p65 **(A)**, total unphosphorylated CREB **(B)**, and β-actin **(A,B)** was performed to ensure equal protein loading. GAPDH and Laminin A/C were detected as protein markers to ensure equal protein loading for cytoplasmic- and nuclear-enriched fractions, respectively **(C)**. Error bars in graphs A and B represent the mean ± SEM ($n = 3$) of densitometric values. $^{*}P < 0.05$, $^{**}P < 0.01$ referred to the uninfected control value.

times to explore the effects of *S. aureus* infection on NF-κB p65 and CREB, respectively.

The transcription of NF-κB-specific target genes involves its translocation from the cytoplasm to the nucleus. To evaluate NF-κB nuclear translocation, we infected BEC with *S. aureus* at 40 and 120 min and detected the relative abundance of NF-κB p65 subunit present in the nucleus or cytosol by Western blotting. The NF-κB p65 subunit was detected in the nuclear fraction after 40 min of infection, whereas at 120 min, we could not observe it (**Figure 2C**). These indicate that NF-κB phosphorylation and nuclear translocation are processes that take place in the initial phase of infection and precede CREB activation.

GSK3α/β Inhibition Favors CREB Phosphorylation at Ser133 in BEC Infected with *S. aureus*

GSK3α and GSK3β are constitutive active enzymes that regulate the activity of several transcription factors (15, 39). We have previously reported that PI3K/Akt-signaling pathway leads to the phosphorylation of GSK3α at Ser21 and GSK3β at Ser9 in *S. aureus* internalization by BEC (18). However, the role of each isoform in cytokine regulation was not evaluated. Therefore, we decided to investigate the contribution of GSK3α and GSK3β to the regulation of NF-κB and CREB activation in BEC infected with *S. aureus*. The time course of BEC infected with *S. aureus* showed that the phosphorylation of GSK3α (Ser21) and GSK3β (Ser9) increased significantly after 40 min of infection (**Figures 3A,B**), GSK3α phosphorylation (~8-fold compared with control) being higher than GSK3β (~2.5-fold compared with control). Interestingly, under basal conditions, BEC phosphorylation of GSK3α Ser21 is barely detected. Other authors have reported similar results about GSK3 relative phosphorylation under basal conditions in different cell types (40–43).

Next, we analyzed whether the increase in the relative abundance of phosphorylated GSK3α and GSK3β isoforms correlated with a decrease in the phosphorylation of glycogen synthase at Ser641 in BEC infected with *S. aureus*. A marked reduction of GSK3α/β kinase activity from 60 to 120 min post infection led to a gradual decrease in the relative abundance of phosphorylated glycogen synthase at Ser641 (**Figure 3C**). To further explore if GSK3α/β was associated with NF-κB p65 phosphorylation at Ser536, BECs were pretreated with SB to inhibit GSK3α/β activity before infection for 40 and 120 min. The inhibition of GSK3α/β with SB did not reduce the relative abundance of NF-κB p65 phosphorylated at Ser536 (**Figure 4A**, upper panel). In contrast to NF-κB, CREB phosphorylation was affected by the inhibition of GSK3α/β activity, because in *S. aureus*-infected cells, SB induced a substantial increase in the relative abundance of phospho-CREB-Ser133 compared to infected cells at 40 min (**Figure 4A**, middle panel). Interestingly, at 120 min, SB did not induce a greater increase in phospho-CREB-Ser133 compared to infected cells, indicating that the maximal increase of phospho-CREB-Ser133 in SB-pretreated cells peaked at 40 min

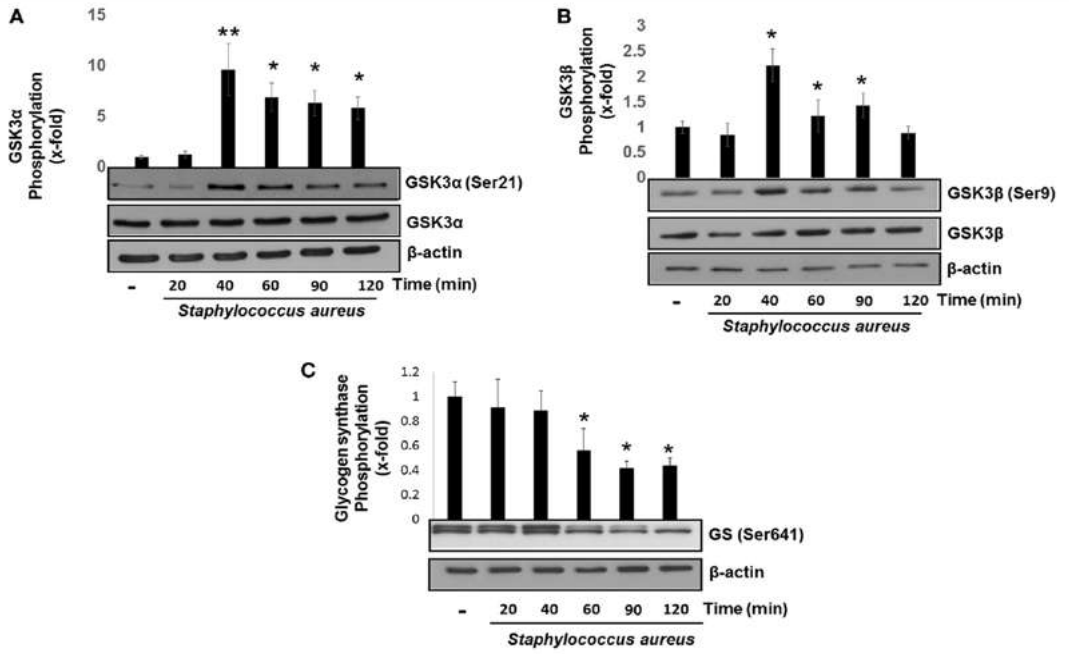

FIGURE 3 | *Staphylococcus aureus* inactivates glycogen synthase kinase 3 (GSK3α) and GSK3β by phosphorylation at Ser21 and Ser9. Bovine endothelial cells were left uninfected (–) or infected at a multiplicity of infection of 20 CFU/cell of *S. aureus* for 20–120 min **(A,B)** or 20–60 min **(C)**. The relative abundance of phosphorylated GSK3α at Ser21 **(A)**, GSK3β at Ser9 **(B)**, and glycogen synthase at Ser641 **(C)** was evaluated by Western blotting. The detection of total GSK3α, GSK3β, and β-actin was done to ensure equal protein loading. Error bars in graphs A–C are presented as the mean ± SEM of three independent experiments (SEM; $n = 3$ per experiment). $^*P < 0.05$, $^{**}P < 0.01$ as compared to control.

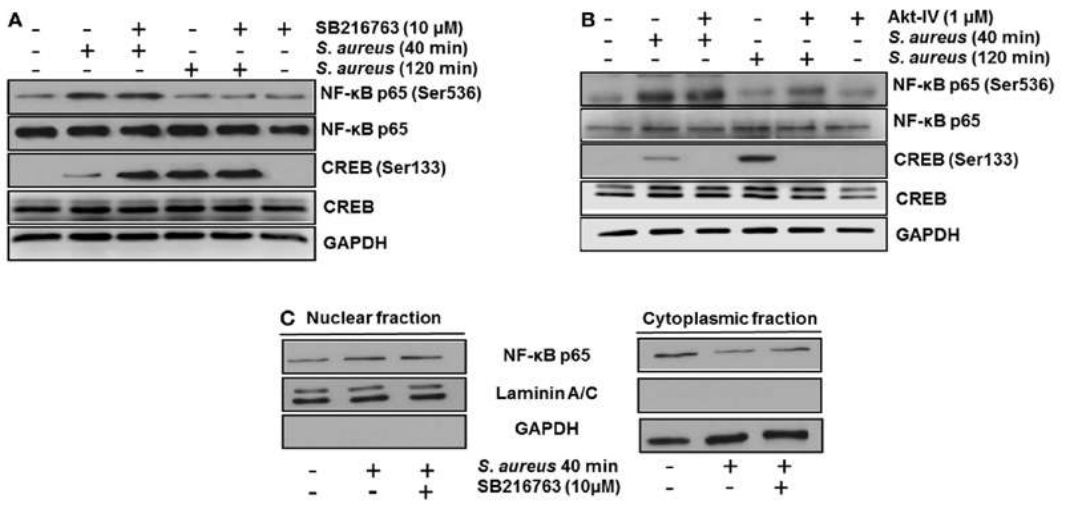

FIGURE 4 | The constitutive activity of glycogen synthase kinase 3 (GSK3α/β) isoforms regulates nuclear factor-kappa B (NF-κB) and cAMP response element-binding (CREB) activity. Bovine endothelial cells (BECs) were left untreated and uninfected (–) or pretreated with the GSK3α/β inhibitor SB216763 (10 μM) **(A)** or the Akt inhibitor Akt-IV (1 μM) **(B)** for 1 h before infection at a multiplicity of infection of 20 CFU/cell of *Staphylococcus aureus* for the indicated times. Controls pretreated with the inhibitors alone but not infected were also included. After infection, the relative abundance of phosphorylated forms of NF-κB p65 at Ser536 and CREB at Ser133 was detected by Western blotting. Unphosphorylated NF-κB p65 and CREB, and GAPDH were detected to ensure equal protein loading. **(C)** BECs were left untreated and uninfected (–) or pretreated with the GSK3α/β inhibitor SB216763 (10 μM) for 1 h before infection with 20 CFU/cell of *S. aureus* for the indicated time. After infection, nuclear- and cytoplasmic-enriched fractions were obtained, and unphosphorylated NF-κB p65 was detected. GAPDH and Laminin A/C were detected as protein markers to ensure equal protein loading for cytoplasmic- and nuclear-enriched fractions, respectively. Blots are representative of three independent experiments.

post infection. Because these observations showed that GSK3 inhibition accelerated the phosphorylation of CREB-Ser133, we tested the relative abundance of p65 and CREB phosphorylation in BEC treated with Akt-IV, an Akt inhibitor. We observed that Akt inhibition did not affect NF-κB p65 phosphorylation level at 40 min of infection; however, at 120 min, a slight increase in phospho-p65 was observed, suggesting that GSK3α/β activity promotes NF-κB phosphorylation at later stages of infection (**Figure 4B**, upper panel). Akt inhibition completely abolished CREB phosphorylation, indicating that the activated state of GSK3α/β inhibits phospho-CREB-Ser133 increase (**Figure 4B**, middle panel). Finally, the nuclear accumulation of NF-κB p65 was not affected by GSK3α/β inhibition with SB (**Figure 4C**).

To confirm that GSK3α/β activity interferes with CREB phosphorylation at Ser133, the genes of each GSK3 isoform were siRNA-silenced for 120 h. After this period, we infected BEC with *S. aureus* for 40 min, a time in which GSK3α/β is still active and CREB phosphorylation was barely higher than the uninfected control (**Figure 2B**). GSK3α/β gene silencing was about 60–70% for GSK3α and 50–60% for GSK3β (**Figure 5A**), demonstrating the efficiency of the siRNA interference technique. Under these conditions, phospho-CREB-Ser133 showed a marked increase in GSK3α/β-silenced cells, compared to non-transfected or control vector cells (**Figure 5B**). These data demonstrate that (1) NF-κB p65 phosphorylation at Ser536 and its nuclear translocation are not directly affected by GSK3α/β activity, (2) GSK3α/β activity directly affects CREB phosphorylation at Ser133, and (3) Akt plays a major role in CREB phosphorylation.

GSK3α Activity Regulates the Interaction between CBP and NF-κB or CREB

A critical step in the transcriptional regulation mediated by NF-κB or CREB is the interaction of each of these transcription factors with the co-activator CBP. NF-κB and CREB compete for the limiting amounts of CBP to form a complex in the nucleus (14, 44). To test whether GSK3α/β activity was necessary for NF-κB-CBP or CREB-CBP complex formation in BEC infected with *S. aureus*, we carried out co-IP assays. When BECs were pre-incubated with SB and infected with *S. aureus* for 40 min, IP of CBP showed a marked reduction of the NF-κB-CBP interaction and a strong increase of the CREB-CBP complex (**Figure 6A**). Accordingly, the inhibition of Akt with Akt-IV (GSK3α/β remains active under this condition) favored NF-κB, but not CREB, interaction with CBP. The inhibition of GSK3α/β with SB at 40 min or control without SB at 120 min led to an increase in CREB-CBP interaction (**Figure 6B**). Moreover, at 120 min post infection, the Akt inhibition favored the interaction of NF-κB with CBP (**Figure 6B**). These data suggest that an initial stage of infection GSK3α/β activity indirectly favors NF-κB-CBP complex formation but not CREB-CBP interaction, whereas at later stages of infection, when GSK3α/β loses its kinase activity, the opposite occurs.

To identify which GSK3 isoform was predominantly affecting CREB-CBP or NF-κB-CBP complex formation, we performed an siRNA-silencing assay to independently reduce the expression of each isoform (**Figure 6C**). After infection of BEC with *S. aureus* for 40 min, we immunoprecipitated CBP, and CREB or NF-κB

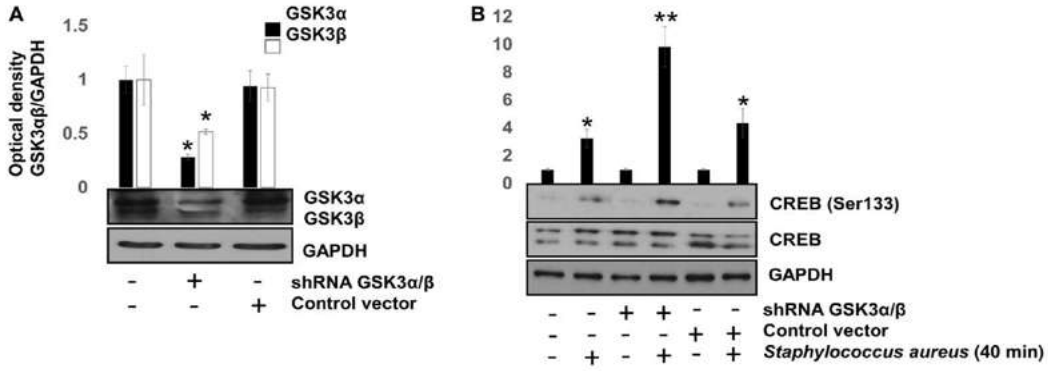

FIGURE 5 | The glycogen synthase kinase 3 (GSK3α/β) isoforms activity inhibits cAMP response element-binding (CREB) phosphorylation at Ser133. Bovine endothelial cells (BECs) were left with medium only or transfected for 120 h with PLKO.1 vector containing siRNA sequences to silence the expression of GSK3α and GSK3β genes or with PLKO.1 control vector. **(A)** Unphosphorylated GSK3α/β was detected to check for protein loading. Error bars in graph represent the mean ± SEM ($n = 3$) of three independent experiments. *$P < 0.05$, referred to the untransfected control. **(B)** BECs were infected with 20 CFU/cell of *Staphylococcus aureus* for 40 min. After infection, the relative abundance of phosphorylated CREB at Ser133 was analyzed by Western blotting. Unphosphorylated CREB and GAPDH were detected to ensure equal protein loading. Error bars in graph represent the mean ± SEM ($n = 3$) of three independent experiments. *$P < 0.05$, **$P < 0.001$ compared to control.

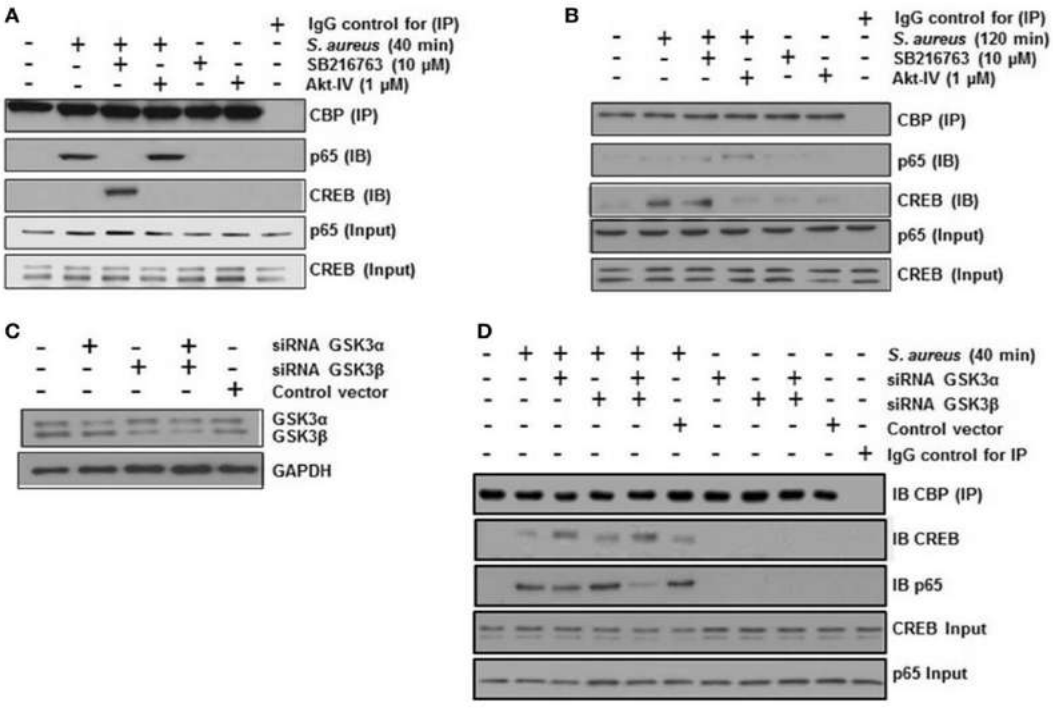

FIGURE 6 | The glycogen synthase kinase 3 (GSK3α/β) isoforms regulate the interaction of nuclear factor-kappa B (NF-κB) and cAMP response element binding (CREB) with the transcriptional co-activator CREB-binding protein (CBP). **(A,B)** Cells were left uninfected and untreated (–) or pretreated with the GSK3α/β inhibitor SB216763 (10 μM) or the Akt inhibitor Akt-IV (1 μM) for 1 h and infected with 20 CFU/cell of *Staphylococcus aureus* for 40 **(A)** or 120 min **(B)**. The total protein was extracted (input fraction) for immunoprecipitation (IP) of CBP. Eluates obtained were then used to detect (IB) NF-κB or CREB by Western blotting. A negative control with an iso-type IgG control was also included. NF-κB and CREB were detected in the input fractions to ensure equal protein loading. **(C,D)** Cells were transfected with plasmids containing siRNA sequences against GSK3α, GSK3β, or with control vector as indicated for 5 days. Then, siRNA of GSK3 isoforms was evaluated by Western blotting. **(D)** GSK3α/β-silenced cells were infected with *S. aureus* for 120 min, and then CBP protein was immunoprecipitated from cell lysates; NF-κB and CREB proteins were detected in the CBP immunoprecipitates by Western blotting. IgG control was included and the total CREB and NF-κB in input cell lysates are also shown.

was immunodetected in the precipitated fraction. In *S. aureus*-infected cells, GSK3α was the primary isoform that affected CREB-CBP interaction, because in either GSK3α or GSK3α/β-silenced cells, a stronger interaction between CREB and CBP was observed compared to the amount of CREB-CBP complex seen in GSK3β-silenced cells (**Figure 6D**). This preferential association found between CREB and CBP in GSK3α-silenced cells repressed NF-κB-CBP interaction because of the reduced amount of NF-κB-CBP complex in these cells compared to the infected controls or the GSK3β-silenced cells.

NF-κB and CREB Regulate IL-8 and IL-10 Expressions in BEC Infected with *S. aureus*

To evaluate the role of NF-κB on IL-8 and IL-10 expressions, we incubated BEC with 20 μM Parthenolide, an NF-κB inhibitor, for 1 h before infection. Under these conditions, a reduced IL-8 expression was observed at 2 h post infection (**Figure 7A**), whereas an increased IL-10 expression was detected at 6 h post infection (**Figure 7B**), indicating that NF-κB activity causes a slight inhibition of IL-10 expression in BEC infected with *S. aureus*. Then, we evaluated the role of CREB on IL-8 and IL-10 expressions by transfecting BEC with a plasmid containing a CREB-Ser133Ala-dominant-negative mutant (DN-CREB-Ser133Ala) (Figure S1 in Supplementary Material). At 2 h post infection, the DN-CREB-Ser133Ala-expressing cells showed an increased expression of IL-8 (**Figure 7C**). By contrast, at 6 h post infection, the DN-CREB-Ser133Ala-expressing cells showed a significant reduction of IL-10 expression (**Figure 7D**). These results confirm that NF-κB and CREB compete for binding CBP and that they are essential for IL-8 and IL-10 expressions in BEC infected with *S. aureus*.

GSK3α/β Activity Regulates IL-8 and IL-10 Expressions in BEC Infected with *S. aureus*

To investigate whether GSK3α/β activity influences NF-κB and CREB transcriptional activity and this, in turn, regulates IL-8 and IL-10 expressions, BECs were pre-incubated with SB or Akt-IV for 1 h. After infection with *S. aureus* for 6 h, the IL-8 and IL-10 peptides were quantitated by ELISA. We observed a reduction in IL-8 peptide compared to the infected control cells in the presence of SB, whereas in BEC treated with Akt-IV, a condition in which GSK3α/β isoforms are active, the IL-8 expression was higher after 6 h as compared to SB treated or infected control cells (**Figure 8A**). The expression of IL-10 increased when BECs were incubated with SB at 6 h post infection, whereas in the presence of Akt-IV, no change in IL-10 peptide compared to control was observed, indicating that active Akt (inactive GSK3α/β) was required to induce IL-10 synthesis (**Figure 8B**). These data suggest that GSK3α/β activity promotes IL-8 peptide synthesis during *S. aureus* infection and that the loss of GSK3α/β activity leads to a reduction of IL-8 and an increase in IL-10 expression.

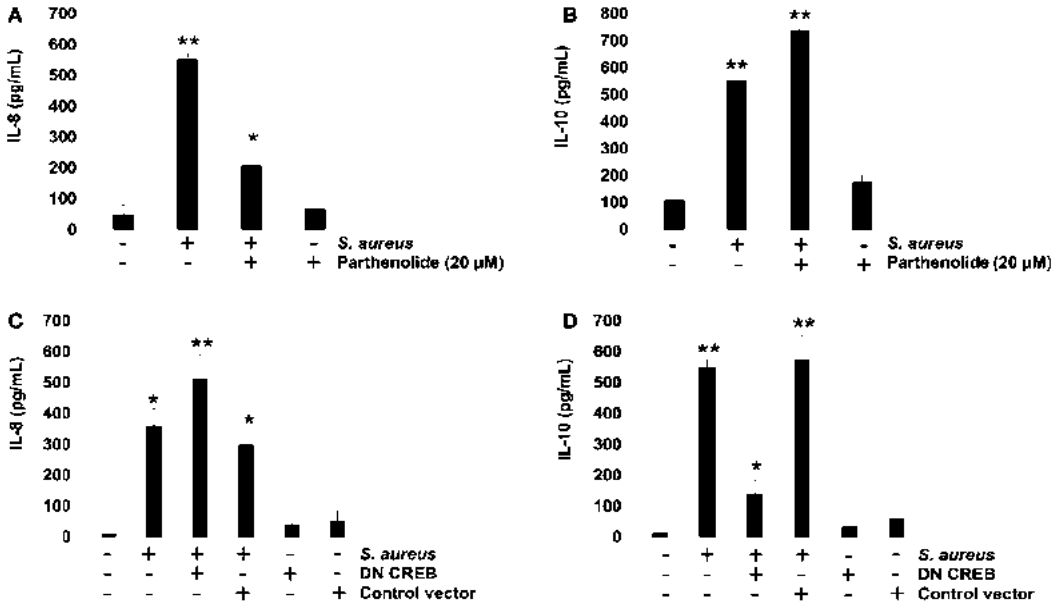

FIGURE 7 | *Staphylococcus aureus*-induced expression of interleukin-8 (IL-8) and interleukin-10 (IL-10) is regulated by nuclear factor-kappa B (NF-κB) and cAMP response element binding (CREB). **(A,B)** Bovine endothelial cells (BECs) were left uninfected and untreated (–) or pretreated with the NF κB inhibitor Parthenolide (20 μM) for 1 h and then infected with 20 CFU/cell of *S. aureus* for 6 h. A control group pretreated with 20 μM of Parthenolide was also included. **(C,D)** BECs were treated with a medium or transfected with a plasmid containing the dominant negative mutant of CREB S133A (DN CREB) or the control plasmid pCF (control vector) for 48 h and then infected with 20 CFU/cell of *S. aureus* for 6 h. Control-uninfected cells transfected with the DN-CREB or plasmid pCF were included. After infection, the conditioned media were collected in ice-cold tubes and kept frozen at −80°C. Quantitation of IL-8 and IL-10 was done by ELISA, according to the manufacturer's instructions. Data represent the mean ± SEM ($n = 3$) from two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ as compared to control.

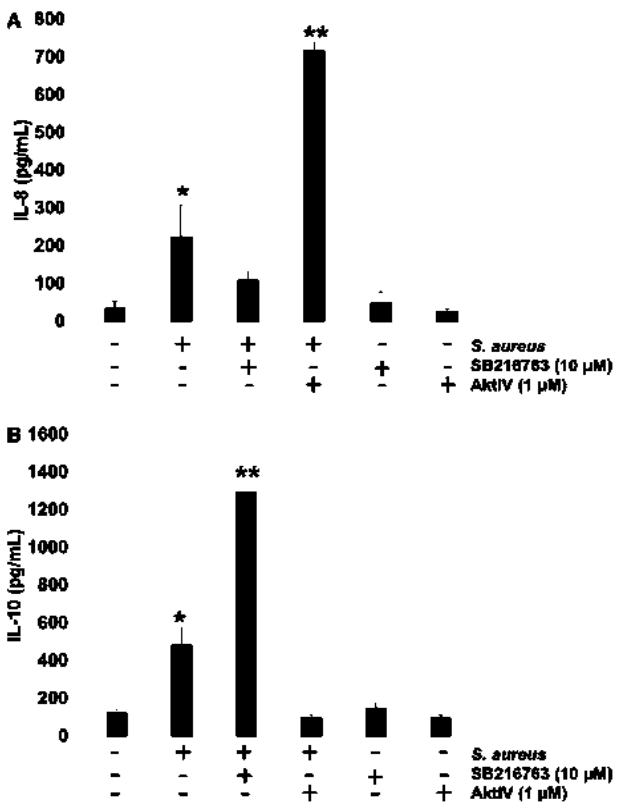

FIGURE 8 | The glycogen synthase kinase 3 (GSK3α/β) activity controls *Staphylococcus aureus*-induced expression of interleukin-8 (IL-8) and interleukin-10 (IL-10). **(A,B)** Bovine endothelial cells (BECs) were left uninfected and untreated (−) or pretreated with GSK3α/β inhibitor SB216763 (10 μM) or Akt inhibitor Akt-IV (1 μM) for 1 h and then infected with 20 CFU/cell of *S. aureus* for 6 h. A control pretreated with SB216763 (10 μM) or Akt-IV (1 μM) was also included. After infection, the conditioned media were collected in ice-cold tubes and kept frozen at −80°C. The quantitation of IL-8 and IL-10 was done by ELISA, according to the manufacturer's instructions. Data represent the mean ± SEM ($n = 3$) from two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared to control.

GSK3α Activity Is the Main Isoform That Regulates IL-8 and IL-10 Expressions in BEC Infected with *S. aureus*

We have presented evidence that in BEC infected with *S. aureus*, (1) GSK3α is highly phosphorylated, (2) GSK3α promotes CREB-CBP interaction, which is essential for IL-10 expression, and (3) GSK3α represses NF-κB-CBP interaction, which is critically indispensable for IL-8 expression. To confirm that GSK3α is the main isoform regulating these processes, we performed an siRNA-gene-silencing assay to reduce the expression of each GSK3 isoform in BEC infected with *S. aureus* for 6 h followed by the detection of IL-8 and IL-10 peptides by ELISA. We observed that GSK3α was the main isoform that regulates IL-8 and IL-10 expressions because GSK3α or GSK3α/β-silenced cells showed a stronger reduction of IL-8 expression and an increased IL-10 expression compared to GSK3β-silenced or control-infected cells (**Figures 9A,B**). Evidence of GSK3α and GSK3β gene-silencing efficiency is shown in **Figure 9C**. These data demonstrate that GSK3α is the primary isoform that regulates the expression of IL-8 and IL-10 in BEC infected with *S. aureus*.

DISCUSSION

Several reports have described an inhibition of IL-8 expression after a "*short-live*" temporary increase when *S. aureus* infects a variety of cells (4–7, 45). For example, in conjunctiva epithelial cells, a significant increase in IL-8 mRNA was detected at 3 h post infection and a marked reduction after 4 h, an effect attributed to phenol-soluble modulins from *S. aureus* (45). Also, bovine mammary epithelial cells infected with *S. aureus* expressed IL-8 at initial stages (8 h), but at longer infection times (24–48 h), no increase was detected (46). In neutrophils, *S. aureus* strain USA300 inhibited NF-κB-dependent IL-8 expression and promoted cell death (6). Interestingly, in this case, the inhibition of NF-κB was related to the *S. aureus* SaeR/S system regulation, which is important for phenol-soluble modulins expression (47). *S. aureus* infections also promoted IL-10 expression, a cytokine associated with bacterial survival and immune-tolerogenic response (10, 11, 48). Interestingly, as it is the case for IL-8, the phenol-soluble modulins were also involved in IL-10 expression by dendritic cells (49). The expression of IL-6 and TNF-α at initial stages of infection and IL-10 at longer stages was also detected in the serum of mice infected with *S. aureus* (50); however, in all these reports, a molecular mechanism was not described. In this work, we have presented experimental evidence indicating that BEC infected with *S. aureus* activates NF-κB and expresses IL-8 at initial stages of infection (2 h) and activates CREB and IL-10 expression at later stages (6 h). Apparently, this molecular switch from pro-inflammatory to an anti-inflammatory cytokine expression is mainly regulated by the activity state of GSK3α. We have also shown that GSK3α regulates the balance of cytokines expression by modulating the activity of NF-κB and CREB in a similar way as GSK3β (14).

For more than 10 years, GSK3β has been considered the main isoform responsible for the regulation of the inflammatory response (14, 51). Various studies have confirmed that GSK3β regulates the response to infections caused by Gram (−)/(+) bacteria like *Helicobacter pylori*, *Francisella tularensis*, and *Mycobacterium* sp. (52–54). Although several reports have provided evidence that GSK3α is the main isoform expressed in various cell types, shares the same substrates, and is also inactivated in similar ways as GSK3β (55–57), no correlation of GSK3α activity on the regulation of the inflammatory response after bacterial stimulation has been documented. This lack of information prompted us to explore whether GSK3α may be responsible for the regulation of pro- and anti-inflammatory cytokines expression in BEC infected with *S. aureus*. Data in this work indicate that the relative abundance of phospho-GSK3α was higher than that of phospho-GSK3β during the infection of BEC with viable *S. aureus* (**Figures 3A,B**), as we have previously reported (18). The predominance of phospho-GSK3α over phospho-GSK3β in our system suggests specific roles for each GSK3 isoform during the inflammatory response triggered by *S. aureus*. At initial

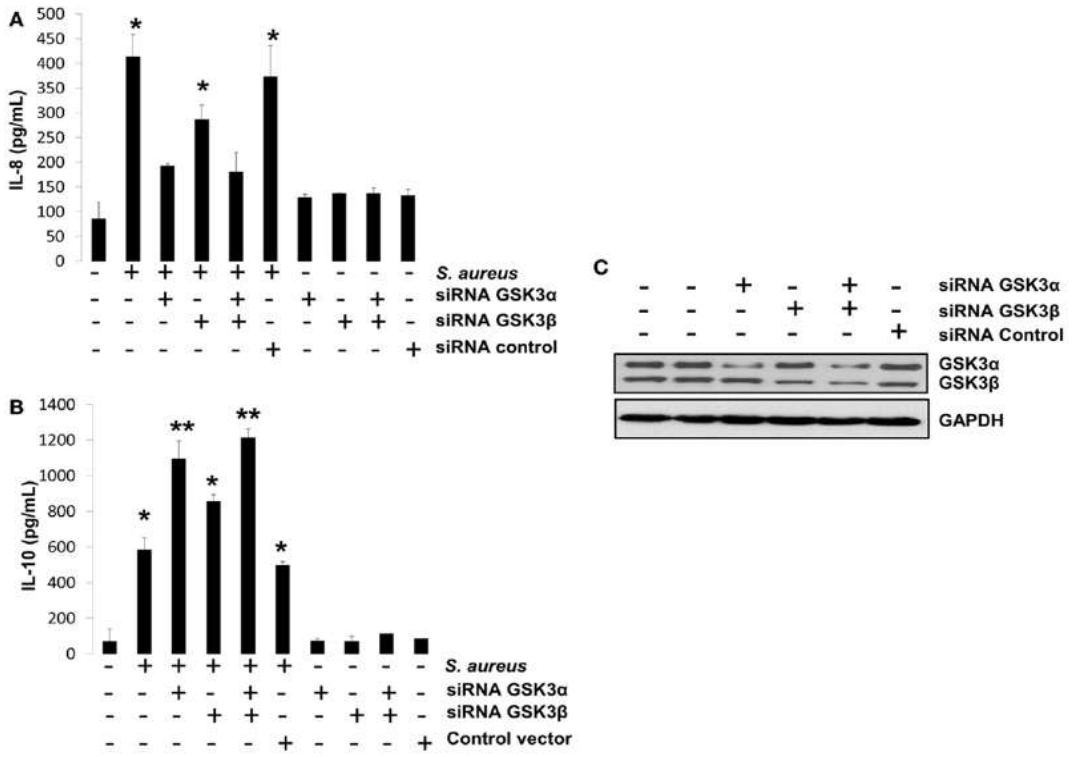

FIGURE 9 | The glycogen synthase kinase 3 (GSK3α) is the primary isoform that regulates interleukin-8 (IL-8) and interleukin-10 (IL-10) expression in bovine endothelial cell infected with *Staphylococcus aureus*. **(A–C)** Cells were left untransfected or transfected with plasmids containing siRNA against GSK3α or GSK3β or empty vector as indicated for 5 days. Then, cells were infected with *S. aureus* at a multiplicity of infection of 20 UFC/cell for 6 h or with a medium alone, and IL-8 **(A)** and IL-10 **(B)** peptide expressions were evaluated by ELISA technique. Controls uninfected/untransfected and transfected/uninfected were also included. **(C)** A Western blot to represent the effectiveness of silencing technique is also shown. Data represent mean ± SEM from results obtained in two independent experiments measured by duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

stages of infection, a gradually decreasing fraction of GSK3α and in minor proportion GSK3β remains activated, implying an indirect correlation between the amount of GSK3α in its active form and the expression of IL-8. In line with this thought, we found that complete GSK3α inhibition at 120 min post infection led to a substantial reduction of the NF-κB-CBP complex compared with the abundance of this complex observed at 40 min post infection (**Figure 6A**) and a high increase in CREB-CBP interaction (**Figure 6B**). Furthermore, the IL-8 expression increased and remained at high levels after 6 h when Akt was inhibited to avoid GSK3α inhibition (**Figure 8A**). These data indicate that GSK3α activity is required to maintain IL-8 expression at longer infection periods and that GSK3α inhibition was related to a loss of NF-κB transcriptional activity and the activation of CREB.

Another important issue derived from our results is how GSK3α represses the phosphorylation of CREB at Ser133 because GSK3 is unable to phosphorylate CREB in this residue. An intriguing possibility is that GSK3 constitutive activity controls an unidentified CREB kinase. In support of this notion, GSK3α was identified in rat cerebral cortical cells as the responsible isoform that represses CREB transcriptional activity (58) and reduces the affinity of Akt against different substrates by phosphorylating Akt at Thr312 (59). In addition, siRNA silencing of the GSK3α gene expression enhanced phospho-CREB-Ser133 and CRE transcriptional activity (59). In agreement, our evidence shows that Akt activity was critical to increase phospho-CREB-Ser133, which suggests that GSK3α inhibition promotes CREB phosphorylation at Ser133 by an Akt-dependent mechanism. Importantly, GSK3α is now known as a novel CREB-target gene that promotes the viability of cancer cells and NF-κB-dependent gene transcription of TERT (60), indicating a reciprocal regulation between CREB and GSK3α. Further investigation on the mechanisms of this reciprocal regulation deserves consideration in the context of the bacteria-activated inflammatory response.

In conclusion, during the first 60 min of BEC infection by *S. aureus,* GSK3α activity maintains its inhibitory influence on CREB phosphorylation at Ser133, limiting the formation of CREB-CBP complex and leaving CBP free to interact with NF-κB. This mechanism promotes a pro-inflammatory environment because of the IL-8 expression. If the stimulus persists for 120 min, GSK3α/β becomes fully phosphorylated by Akt, which indirectly inhibits NF-κB transcriptional activity. The inhibition of GSK3α, and in minor proportion GSK3β, causes an increase in phospho-CREB-Ser133, which is now able to compete for CBP to form the

transcriptionally active CREB-CBP complex. Such a molecular switch markedly reduces the expression of pro-inflammatory chemokine IL-8 and stimulates the expression of immune-suppressive and -tolerogenic cytokine IL-10. More experiments will be undoubtedly needed to clarify the specific mechanistic details of the differential actions of GSK3α on the regulation of the inflammatory response caused by pathogenic bacteria.

AUTHOR CONTRIBUTIONS

OS-G contributed with ideas of experimental design, performed 90% of the experiments, analyzed results, and wrote the first draft; RR-M performed 5% of the experiments; MM-P, performed 5% of the experiments; AB-P, JV-A, and JA-G critical review and corrections of this manuscript; VB-A central idea of the research, wrote this manuscript, and financially supported the research.

ACKNOWLEDGMENTS

This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT-México) grant number 152518 and Coordinación de la Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo, to VMB-A. OS-G is a recipient of a doctoral scholarship from CONACYT-México. RR-M was supported by a postdoctoral scholarship from CONACYT-México. MM-P was supported by a master scholarship from CONACYT-México.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00092/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

14. Capítulo II

En la sección anterior se mostró evidencia que indica que la estimulación de células de endotelio bovino con *S. aureus* induce la inhibición de las isoformas de GSK3α/β con una preferencia sobre la isoforma GSK3α. Sin embargo, GSK3 es una enzima particular en las células de mamífero por la cantidad de sustratos potenciales (aprox. 500) y confirmados (aprox 100) que posee. Esto significa que la regulación de su actividad tiene impacto en varios procesos celulares. Una de las proteínas reguladas por GSK3 es el co-activador β-catenina, el cual favorece su actividad cuando las isoformas de GSK3 se encuentran inactivas. β-catenina es el principal regulador de la vía de transducción canónica Wnt y su función forma parte de mecanismos de diferenciación y desarrollo celulares. De manera interesante la vía Wnt/β-catenina constituye un regulador negativo para la vía de NF-κB en modelos de infección con *Salmonella typhimurium*. La evidencia indica que la infección de las células endoteliales inhibe la actividad de la vía NF-κB en etapas tardías de la estimulación. Además, se sabe que las infecciones crónicas por *S. aureus* modifican la arquitectura celular y producen la diferenciación de macrófagos en células “foam”. La degeneración de los macrófagos en este tipo celular se ha asociado a la actividad de la vía Wnt en infecciones por *Mycobacterium tuberculosis*. En modelos de infección por *S. aureus* en el nemátodo *Caenorhabditis elegans* se ha observado que la proteína homóloga de β-catenina bar-1 regula la respuesta inmune. En esta sección se conjuntan y discuten datos acerca de que la inhibición de GSK3 inducida por *S. aureus* afecta la estabilidad de β-catenina, lo que puede derivar en la función de la vía Wnt. También se muestra la evidencia obtenida hasta el momento de la regulación de la vía Wnt por otros patógenos. Se incluye una revisión publicada en una revista internacional indizada en JCR.

14.1. Introducción

La vía de transducción de señales Wnt/β-catenina controla la expresión de genes que regulan diversas funciones celulares como la diferenciación y el ciclo celular (Silva *et al*., 2014). En células en reposo, los niveles relativos del co-activador β-catenina son controlados mediante su degradación en el proteasoma 26S. Para que este proceso se lleve a cabo, la proteína primero es fosforilada por las isoformas de la enzima GSK3α/β, lo que estimula la poli-ubiquitinación de β-catenina y la marca para ser degradada. Esto reprime la expresión de los genes que dependen de su actividad. Cuando las células son estimuladas con glicoproteínas de la familia Wnt, se induce la inactivación de la enzima GSK3α/β lo que reduce la ubiquitinación de β-catenina e induce un incremento de los niveles relativos de esta proteína en citoplasma y posteriormente en el núcleo. Como resultado se estimula la interacción de β-catenina con los factores transcripcionales de la familia Tissue Cell Factor/Leukocyte Enhancement Factor (TCF/LEF) que se encuentran unidos a la cromatina e inducen la expresión de genes involucrados en el desarrollo y diferenciación celular (Sun *et al*., 2011).

Se ha reportado que la estimulación de células epiteliales con diversas bacterias induce cambios en la actividad de la vía Wnt/β-catenina que conducen a cambios de la arquitectura celular y tisular en modelos de infección crónica (Liu *et al.*, 2010) (Villaseñor *et al.*, 2017). También se ha descrito que la activación de esta vía de transducción de señales reduce la expresión de citocinas pro-inflamatorias e inhibe la actividad de la vía NF-κB (Ma & Hottiger, 2016).

La estimulación de células de endotelio bovino con la bacteria *S. aureus* reduce la actividad de las isoformas de la enzima GSK3 que es la principal enzima que fosforila a β-catenina para que sea degradada. Además se ha observado que la actividad de la vía NF-κB se reduce en periodos mayores de 40 min de estimulación con la bacteria.

14.2. Materiales y métodos.

Se utilizaron células de endotelio bovino inmortalizadas y una cepa de *S. aureus* ATCC 27543. Los métodos de cultivo y estimulación fueron los reportados por Silva-García *et al*. (2018).

14.3. Resultados

14.3.1. *Staphylococcus aureus* induce la estabilización de β-catenina en células de endotelio bovino.

Para probar si la inactivación de las isoformas de GSK3 inducida por *S. aureus* (Capítulo I) tiene efecto en otras vías de transducción reguladas por esta enzima. Se infectaron las CEB con *S. aureus* durante 40-480 min, se obtuvieron los extractos proteicos y se evaluó la abundancia relativa de la proteína β-catenina. En la Figura 7A se observa que la abundancia relativa de β-catenina fue mayor en los extractos proteicos provenientes de las células infectadas con *S. aureus* un pico máximo alcanzado a los 120 min y que permanece en niveles similares hasta los 480 min. Para evaluar la participación de GSK3 en la estabilización de β-catenina inducida por *S. aureus*, las CEB se pre-trataron con el inhibidor de Akt AktIV (1 µM) con el objetivo de prevenir la inactivación de las isoformas de la enzima GSK3. Entonces se estimularon con la bacteria por 40-480 min y se obtuvieron los extractos proteicos. En la figura 6B se observa que el pre-tratamiento de las CEB con AktIV previene el aumento de los niveles relativos de β-catenina inducidos por la estimulación con *S. aureus*.

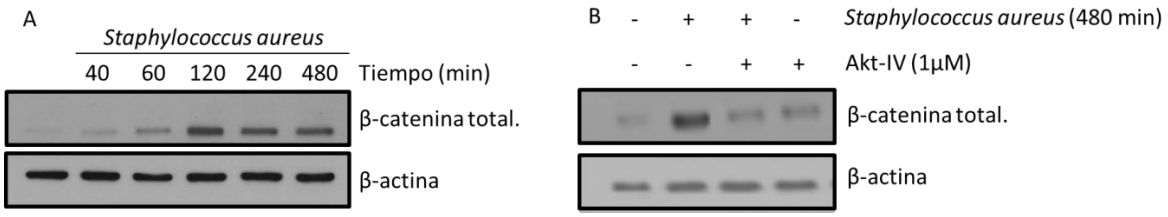

Figura 7. Efecto sobre el co-activador β-catenina en células de endotelio bovino (CEB) infectadas con *S. aureus*. Las CEB se incubaron durante 4h en ausencia de suero y se estimularon con una media de infección de 20 bacterias/célula durante 40-480 min (A) o se pretrataron con 1µM de Akt-IV durante 1h previa a la estimulación con la bacteria (B) y se evaluó la abundancia relativa de la proteína β-catenina.

14.4. Discusión.

La estimulación de la vía de transducción de señales Wnt/β-catenina por bacterias ya se ha reportado previamente, excepto para *S. aureus.* En este trabajo presentamos evidencia que sugiere que esta bacteria es capaz de inducir la estabilización del co-activador β-catenina y que este efecto involucra la actividad de las isoformas de la enzima GSK3, las cuales son inactivadas como resultado de la infección de las CEB con *S. aureus*. La estabilización de la proteína β-catenina tiene varios efectos en la célula; por ejemplo, inducir la expresión de los genes dependientes de la vía Wnt o la función de reprimir la actividad de la vía pro-inflamatoria NF-κB, lo que indica que la estabilización de β-catenina regula negativamente la producción de citocinas pro-inflamatorias. En este contexto se ha reportado que las infecciones por *S. aureus*

828 pueden producir degeneración del tejido infectado (García-Betancur *et al.*, 2017) y
829 también inactivan la vía de transducción NF-κB, por lo que la estabilización de β-
830 catenina es un efecto que podría estar relacionado. Más experimentos son necesarios
831 para corroborar esta hipótesis.

832

833 14.5. La vía Wnt/β-catenina estimulada por ligandos bacterianos.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 310183, 7 pages
http://dx.doi.org/10.1155/2014/310183

Review Article

The Wnt/β-Catenin Signaling Pathway Controls the Inflammatory Response in Infections Caused by Pathogenic Bacteria

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Received 14 March 2014; Accepted 27 June 2014; Published 17 July 2014

Academic Editor: Marisa I. Gómez

Innate immunity against pathogenic bacteria is critical to protect host cells from invasion and infection as well as to develop an appropriate adaptive immune response. During bacterial infection, different signaling transduction pathways control the expression of a wide range of genes that orchestrate a number of molecular and cellular events to eliminate the invading microorganisms and regulate inflammation. The inflammatory response must be tightly regulated because uncontrolled inflammation may lead to tissue injury. Among the many signaling pathways activated, the canonical Wnt/β-catenin has been recently shown to play an important role in the expression of several inflammatory molecules during bacterial infections. Our main goal in this review is to discuss the mechanism used by several pathogenic bacteria to modulate the inflammatory response through the Wnt/β-catenin signaling pathway. We think that a deep insight into the role of Wnt/β-catenin signaling in the inflammation may open new venues for biotechnological approaches designed to control bacterial infectious diseases.

1. The Wnt/β-Catenin Pathway

The protein β-catenin is a ubiquitously expressed main signal transducer of the canonical Wnt signaling pathway. It is also found in adherent junctions, forming a complex with E-cadherin, α-catenin, and actin filaments of the cytoskeleton [1]. The Wnt/β-catenin is an ancient and evolutionary conserved pathway present in lime molds, worms, and humans, with a prominent role in embryogenesis, cell differentiation, and stem cell maintenance self-renewal. Recently, mutations in genes encoding β-catenin or other Wnt pathway components have been identified in certain types of cancer, fibrosis, and inflammatory bowel disease [2].

β-Catenin is not detectable in the cytoplasm or nucleus in unstimulated cells because free β-catenin levels (the form not bound to the E-cadherin complex) is controlled by the *destruction complex*. This complex is composed mainly by the tumor suppressor adenomatous polyposis coli (APC) and Axin, which function as scaffolding proteins on which the Ser/Thr kinases casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β) phosphorylate β-catenin at the N-terminal residues Ser45, Ser33, Ser37, and Thr41 [3]. These phosphorylations label β-catenin to be polyubiquitinated by the Skp1-Cdc53-F-Box E3 ubiquitin ligase complex ($SCF^{\beta\text{-}TRCP}$) and degraded by the proteasome 26S (Figure 1, Wnt Off). GSK3α is also able to regulate β-catenin phosphorylation *in vitro* and *in vivo*, which indicates that both isoforms of GSK3 contribute to maintain low levels of β-catenin in basal conditions [4].

The Wnt/β-catenin pathway is activated when a secreted Wnt glycoprotein binds to the N-terminal extracellular cysteine rich domain of a Frizzled (Fzd) receptor (Figure 1, Wnt On). There are 19 Wnt proteins and 10 Fzd receptors identified in humans, whose function, regulation, and interaction in different cellular processes are currently an active area of research. Upon binding of Wnt to Fzd, the coreceptor low

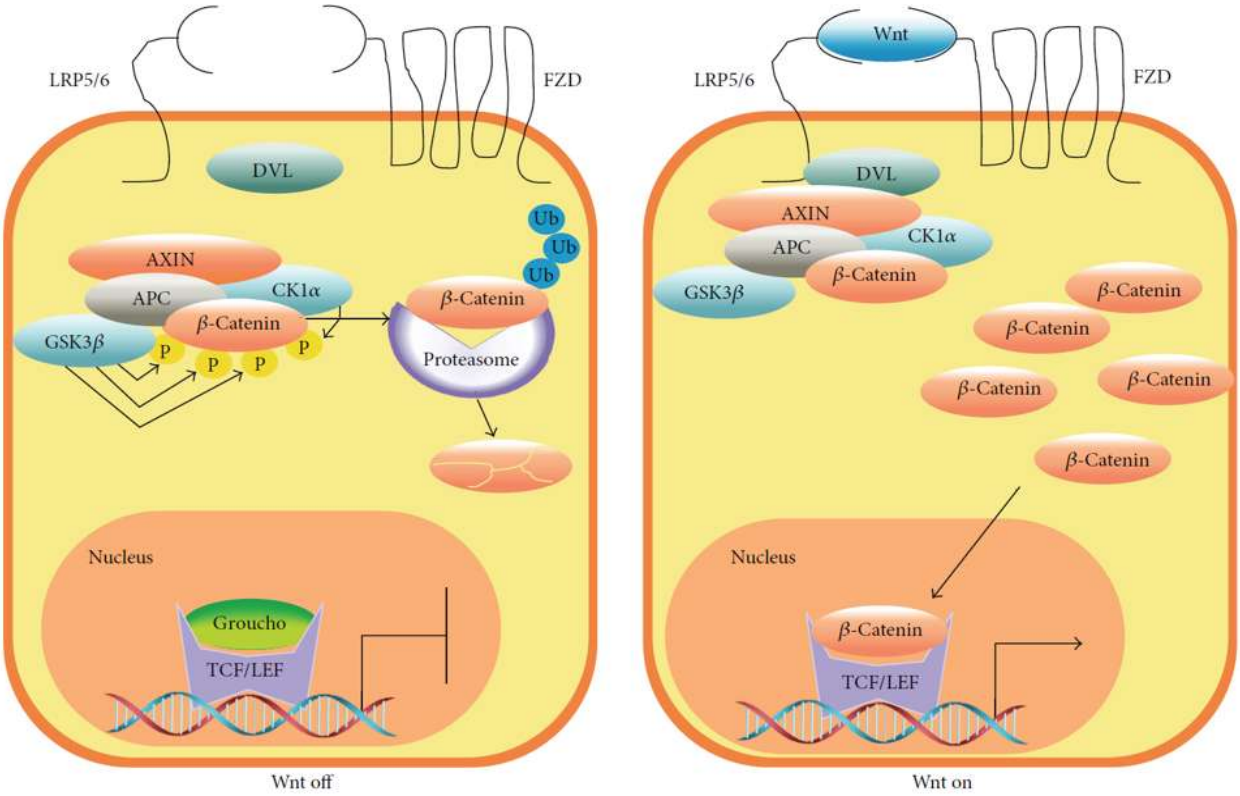

FIGURE 1: The Wnt/β-catenin pathway. In the absence of stimulus (Wnt off), β-catenin is constantly phosphorylated by CKIα and GSK3β. These phosphorylations constitute a signal for β-catenin polyubiquitination and hydrolysis by the proteasome 26S. In the presence of Wnt protein ligands (Wnt on), the *destruction complex* constituted by the proteins APC, Axin, CKIα, and GSK3 is inactivated and β-catenin, which is constantly synthetized, accumulates in the cytoplasm and nucleus where it interacts with TCF/LEF transcription factors to enhance expression of specific genes.

density lipoprotein 5/6 (LRP5/6) interacts with Fzd and dishevelled (DVL), leading to the recruitment of APC and Axin to the plasma membrane along with CK1α and GSK3β. In turn, CK1α and GSK3β phosphorylate LRP5/6 in the PPPSP motif, decreasing the GSK3β-dependent phosphorylation of β-catenin through a substrate competitive mechanism. As β-catenin is continuously synthetized, Wnt-induced inactivation of the *destruction complex* causes a transient stabilization and accumulation of β-catenin in the cytoplasm. Then β-catenin translocates to the nucleus and, by displacing the corepressor Groucho, enhances the expression of specific genes through the interaction with the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) proteins. Gene expression ends when the transcription complex is disassembled and nuclear APC exports β-catenin to the cytoplasm where it is degraded [2].

Approximately 400 genes involved in cell growth, differentiation, apoptosis, survival, and immune functions are regulated by the Wnt/β-catenin signaling pathway. A proinflammatory role of Wnt/β-catenin has been recently documented in 3T3-L1 preadipocytes stimulated with Wnt1 [5]. Activation of Wnt/β-catenin pathway with Wnt3a in mouse microglial cells leads to the expression and release of the proinflammatory cytokines interleukins (IL)-6, IL-12, and IFNγ [6]. In contrast, an anti-inflammatory role of Wnt/β-catenin pathway has also been demonstrated in mouse colon epithelial stem cells and macrophages infected with *Salmonella* [7] or *Mycobacterium* [8], which indicates that activation of the Wnt/β-catenin pathway downregulates the proinflammatory responses to certain bacterial infections [9, 10]. These apparently contradictory results when cells are stimulated with either Wnt proteins or pathogenic bacteria suggest that the anti- or proinflammatory role of Wnt/β-catenin may depend on the stimulus, the cell type, the activation context, and its crosstalk with other signaling pathways. In the next sections, we discuss experimental data demonstrating that Wnt/β-catenin is an important signaling pathway in the regulation of bacterial-induced inflammatory response.

2. *Salmonella typhimurium*: Crosstalk with the NF-κB Pathway

The role of Wnt/β-catenin in *Salmonella* infections has been investigated *in vitro* or in streptomycin pretreated mice

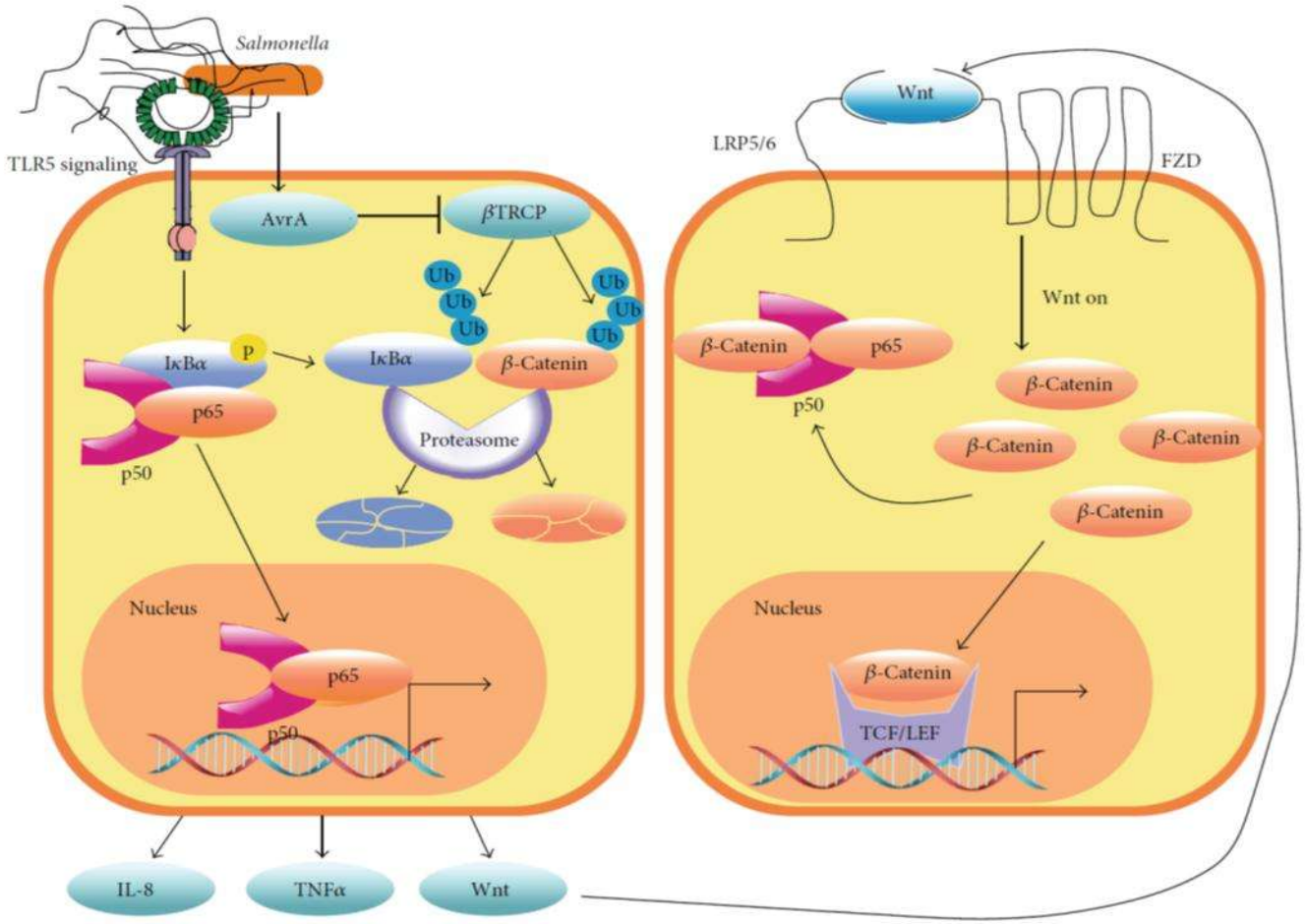

FIGURE 2: β-Catenin is an antagonist of the NF-κB pathway. In colon epithelial cells, *Salmonella* induces IL-8, TNFα, and Wnt expression via NF-κB activation. Moreover, the *Salmonella typhimurium* deubiquinating effector AvrA stabilizes IκBα and β-catenin. This step enhances β-catenin activity and at the same time inhibits NF-κB by stabilizing its inhibitor. The expression of Wnt in the first stage of infection stimulates Wnt/β-catenin pathway. The stabilized β-catenin then interacts with the NF-κB p50 subunit, decreasing its transcriptional activity. While NF-κB activity is inflammatory, its inhibition by β-catenin activation constitutes a mechanism to reduce inflammation.

models [7, 11–13]. From these studies it is known that infection of colon epithelial cells (CEC) with *Salmonella typhimurium* causes an increase in GSK3β-dependent β-catenin phosphorylation, leading to an upregulation of IL-6, IL-8, and Wnt2, via TLR5/NF-κB activation [10, 14]. These results were confirmed in HCT116 and T84 cell lines by expressing constitutively active β-catenin mutants that, upon interaction with the NF-κB p50 subunit, decreased the NF-κB DNA binding and transcriptional activities [10]. Similar results were obtained in cells pretreated with LiCl, an inhibitor of GSK3 activity, which mimics activation of Wnt/β-catenin [14]. It is worth to mention that infection with *Salmonella* induced a decrease in Axin1, which is a scaffolding protein controlling the levels of β-catenin [15]. These data indicate that induction of the NF-κB proinflammatory pathway by *Salmonella* is dependent upon β-catenin degradation, suggesting that β-catenin plays a negative regulatory role in the inflammatory response.

Infection of CEC with the avirulent *Salmonella* Phop^c strain induced an increase of β-catenin phosphorylation at Ser552, a site associated with enhanced transcriptional activity, indicating that a protein effector is responsible for β-catenin stabilization [7]. In fact, the *Salmonella* effector protein AvrA has been shown to affect β-catenin stability because infection of CEC with the *Salmonella* strain SL1344 Phop^c mutant that produces AvrA protein caused β-catenin stabilization, Wnt2/Wnt11 increased expression, and IL-6, IL-4, IFNγ, and TNFα decreased expression [11, 12]. A different *in vitro* and *in vivo* study has shown that *Salmonella* SL1344 strain activated NF-κB-dependent Wnt11/Wnt2 and IL-8 expression [10–12]. It is proposed that the deubiquitinase activity of AvrA inhibits βTRCP1-dependent ubiquitination of β-catenin and IκB, the cytoplasmic inhibitor of NF-κB. This AvrA inhibitory activity on ubiquitin ligases would make β-catenin more stable and the NF-κB transcriptional activity less effective (Figure 2). Furthermore, ectopic overexpression

of Wnt11 and Wnt2 decreased NF-κB and AP-1 transcriptional activity [11, 12]. This evidence indicates that Wnt11 and Wnt2 trigger a negative feedback mechanism that controls NF-κB activity through β-catenin activation.

3. *Citrobacter rodentium*: Crosstalk with the PI3K Pathway

Stabilization of β-catenin by enhanced phosphorylation at Ser552 has been observed in the intestinal epithelial cells from mice infected with the attaching/effacing (A/E) pathogen *C. rodentium* [16]. Mice treated with the PI3K inhibitor LY294002 decreased both the relative abundance of β-catenin phosphorylated at Ser552 and the expression of c-myc and cyclin D1. In contrast, IFNγ and TNFα expression in epithelial cells was increased after bacterial infection [17]. Interestingly, deletion of class IA PI3K gene in $IL10^{-/-}$ mice showed impaired Akt and β-catenin signaling [18].

GSK3β is a downstream effector of the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway as well as the Wnt/β-catenin *destruction complex* that negatively regulates β-catenin. The ability of GSK3β to promote or suppress the inflammatory response depends on the cell type and the stimulus [19]. In *C. rodentium* infections of CEC, Akt inhibits GSK3β by phosphorylation at Ser9 [20] with no phenotypic consequence reported so far. It is still not known yet whether this GSK3β phosphorylation depends on Fzd or TLR activation. Despite this lack of information, it is recognized that activation of the PI3K/Akt and Wnt/β-catenin pathways may cooperate to enhance β-catenin activity in *C. rodentium* infection mice models (Figure 3). In this context, it is also important to mention that in CEC infected with the Phop^c *Salmonella* strain, AvrA enhanced β-catenin phosphorylation at Ser552 by increasing Akt expression and Akt phosphorylation at Thr308 [21]. This evidence suggests that the PI3K/Akt signaling pathway cooperates to increase β-catenin activity in Phop^c *Salmonella* infections. However, whether AvrA directly alters PI3K activity, which is upstream of Akt, is not known. Future experiments should be designed to identify *C. rodentium* effector proteins responsible for β-catenin activation, which is also associated with CEC hyperplasia [20].

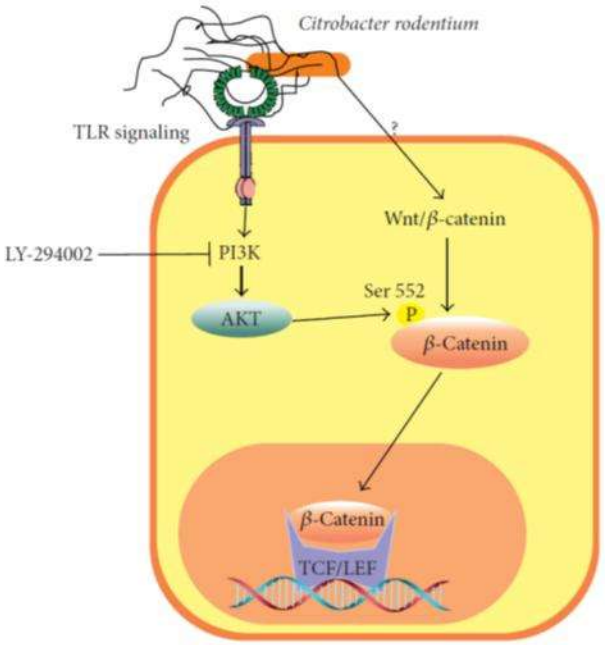

FIGURE 3: The PI3K/Akt signaling pathway enhances β-catenin activation. In colon epithelial cells infected with *Citrobacter rodentium*, Akt phosphorylates β-catenin at Ser552. The activation of β-catenin increases c-myc and reduces TNFα and IFNγ expression. An opposite effect is observed in cells incubated with the PI3K inhibitor LY294002.

4. *Mycobacterium tuberculosis*: The Roles of Wnt Ligands and Fzd Receptors

Mycobacterium tuberculosis is able to interfere with some components of the Wnt/β-catenin pathway such as Fzd1, which is upregulated by TNFα and synergistically enhanced by IFNγ [8]. Presence of Wnt3a in the lungs of mice infected with *M. tuberculosis* affects the ability of macrophages to produce TNFα by increasing β-catenin transcriptional activity [8]. It is proposed that the upregulation of Fzd1 constitutes a mechanism by which Wnt3a represses inflammation, leading to a negative loop of inflammatory control in mice macrophages during *M. tuberculosis* infection (Figure 4).

Macrophages infected with *Mycobacterium bovis* show a robust increase in Wnt5a and the Notch1-target genes cyclooxygenase 2 (COX2), prostaglandin E2 (PGE2), and suppressor of cytokine signaling-3 (SOCS-3), which are involved in downmodulation of the inflammatory response. Interestingly, the effect on genes controlled by Notch1 was dependent on β-catenin and Jagged1, a Notch1 ligand [22]. It was also observed that activation of Notch1 by iNOS/NO and TLR2 signaling depended on β-catenin. These data indicate that β-catenin acts at late stages of infection to repress inflammatory programs triggered by TLR2 and iNOS/NO.

Interestingly Fzd receptors 2, 7, and 8 and Wnt ligands 11 and 2 are upregulated in CEC infected with *S. typhimurium* [11, 12] but not in macrophages infected with *M. tuberculosis*, in which Wnt5a and Fzd4 are highly expressed [22]. These data point out that expression of specific set of Fzd receptors and Wnt ligands depend on the pathogenic bacteria and the cell type. This hypothesis has been confirmed by Blumenthal et al. (2006) who showed that Wnt5a and Fzd4 were constantly expressed in macrophages but not in lymphocytes infected with *M. bovis* [23]. The TLR-NF-κB pathway was responsible for Wnt5a expression and macrophages stimulated with Wnt5a produced IL-12p40 and IFNγ. Given that Wnt5a is able to activate β-catenin dependent and independent signaling, the role of β-catenin was not clearly established in this work. Recently, it was found that activation of the TLR-Myd88-NF-κB pathway in foamy macrophage-like cells obtained from granulomatous lesion of mice infected with *Mycobacterium tuberculosis* induced the expression of Wnt6 [24]. Interestingly, Wnt5a induced an inflammatory response while Wnt6 decreased TNFα

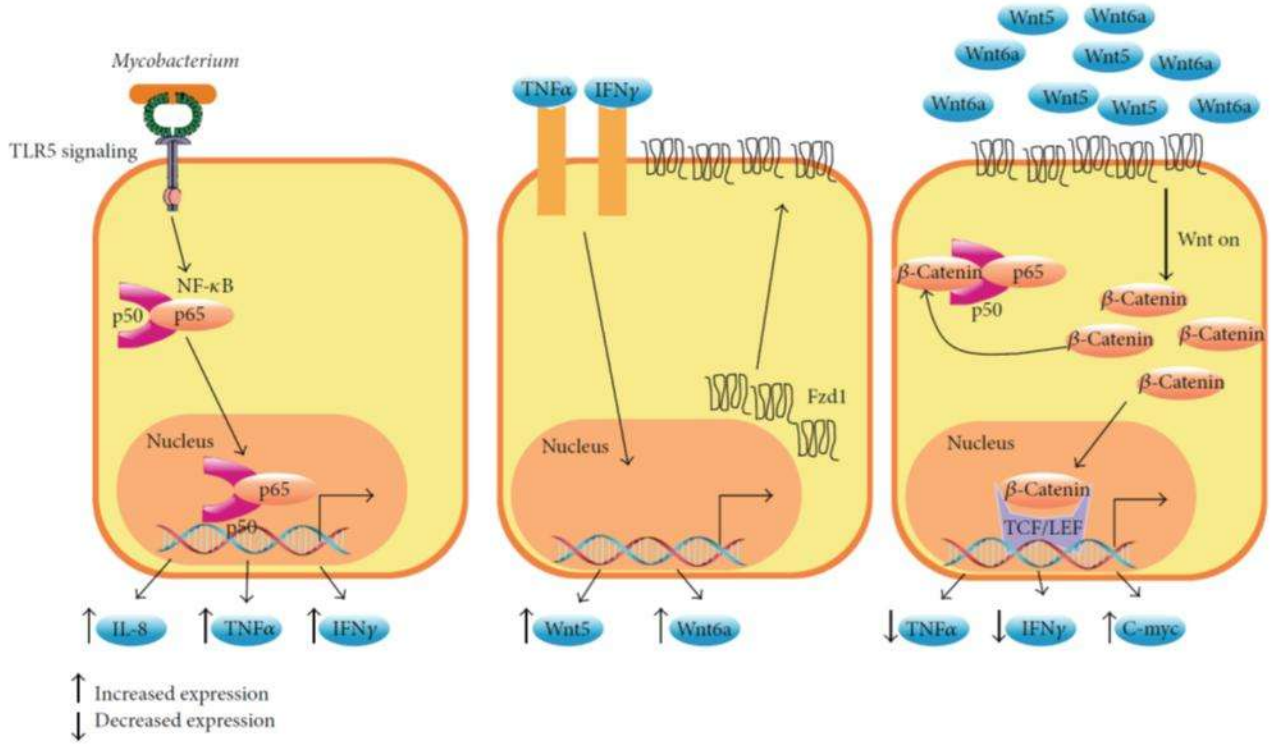

FIGURE 4: Frizzled receptors reduce inflammation. In epithelial cells and macrophages, *Mycobacterium tuberculosis* induces NF-κB-dependent expression of IL-8, TNFα, and IFNγ (left panel) through TLR signaling. In turn, TNFα and IFNγ stimulation increases Fzd1, Wnt6, and Wnt5a expression at the membrane cell surface (middle panel). Finally, the binding of Wnt3a, Wnt5a, or Wnt6 to Fzd1 (right panel) induces stabilization of β-catenin, inhibiting the NF-κB pathway. This interaction of β-catenin with NF-κB decreases the expression of proinflammatory molecules such as TNFα.

expression and triggered macrophage polarization toward an M2 phenotype [25, 26]. The fact that *Mycobacterium tuberculosis* induces β-catenin stabilization or Wnt5a expression may indicate a pathogenic mechanism whereby bacterial infection is successfully established. Other important and still unresolved issues refer to the *Mycobacterium* virulence factors responsible for regulating the expression of Wnt ligands and Fzd receptors and whether this regulation takes place in nonprofessional phagocytes.

5. *Pseudomonas aeruginosa*: Targeting the E-Cadherin Complex

Treatment of mouse corneal epithelial cells with LiCl, a compound that mimics Wnt/β-catenin pathway activation by inhibition of GSK3 activity, promotes host resistance against *Pseudomonas aeruginosa* infection [27]. Macrophages, neutrophils, and corneal epithelial cells infected with *P. aeruginosa* express proinflammatory cytokines IL-6, IL1-β, and TNFα, which are directly associated with the severity of the disease in a keratitis mouse model. In this model, β-catenin was degraded in a time-dependent mechanism. Furthermore, overexpression of β-catenin active mutants decreased proinflammatory cytokines, enhanced bacterial clearance, and reduced the severity of the disease caused by this bacterium [25, 26]. These data suggest that β-catenin degradation is required for higher expression of proinflammatory cytokines.

The quorum-sensing molecule acyl-homoserine lactone (AHL) from *P. aeruginosa* is able to disrupt the integrity of epithelial cell layer in Caco-2 cells by targeting β-catenin/E-cadherin complex. It was demonstrated that AHL directly interacts with β-catenin, resulting in the hyperphosphorylation of its tyrosine residues and translocation to the cytoplasm where it is phosphorylated by the *destruction complex* and degraded via the proteasome pathway [28]. Infection of mice urinary tract with *P. aeruginosa* strain PAO1, which is a producer of AHL and other quorum sensing molecules, results in high neutrophil recruitment and cytokine production compared with mutant strains unable to secrete this type of molecules [29].

P. aeruginosa also regulates the c-jun N-terminal kinases (JNKs) activity in corneal epithelial cells [26]. Interestingly, JNKs modulate adherent junction integrity by phosphorylating β-catenin at Ser33/37 and Thr41, the same residues that are phosphorylated by GSK3. However, it is not yet clear whether this JNK-dependent β-catenin phosphorylation induces its degradation [30]. Finally, it was also shown that corneal epithelial cells infected with *P. aeruginosa* express IL-6, IL-8, and TNF-α through a JNK-dependent mechanism [26]. These results suggest that *P. aeruginosa* targets β-catenin

degradation to induce proinflammatory cytokine expression by secreting AHL and regulating the activity of JNKs.

6. *Helicobacter pylori*: Role of LRP6

The human pathogen *Helicobacter pylori* resides in the stomach of about half of the world population. The infection with this bacterium is associated with development of gastritis, peptic ulcer, and, in some cases, cancer. Although experimental data have shown that *H. pylori* induces activation of Wnt/β-catenin signaling through β-catenin stabilization in epithelial cells, a protein effector responsible for β-catenin stabilization has not yet been identified [21]. The cytotoxins VacA and CagA, expressed by virulent *H. pylori* strains, were not strictly required for β-catenin-induced stabilization [22]. Infections of human gastric epithelial cells NCI-N87 with *H. pylori* K1 induced phosphorylation of the LRP6 coreceptor at Ser1490 [31]. This phosphorylation was regulated by DVL2/DVL3 and was required for β-catenin nuclear translocation and transcriptional activity because knockdown of either of these two proteins reduced β-catenin-induced Axin2 expression. Moreover, evidence that LRP6 phosphorylation was dependent on a bacterial effector was based on experiments with *H. pylori* type four secretion system mutants or heat killed bacteria [31]. Nevertheless, no report so far has shown that stabilization of β-catenin by *H. pylori* influences the expression of inflammatory cytokines.

7. Concluding Remarks

The inflammatory response is a tightly regulated process because chronic or uncontrolled inflammation may cause tissue injury. Several signaling transduction pathways have been well characterized to induce inflammation; however, much less is known about the suppression and resolution mechanisms that control it. The evidence accumulated so far has pointed out that activation of the Wnt/β-catenin pathway reduces several molecular inflammatory processes that are triggered by bacterial pathogens. The fact that proinflammatory stimuli such as TNFα, IFNγ, and NO are able to increase the expression of Wnt/β-catenin signaling molecules indicate that these pathways should be interconnected. Also, it is likely that proinflammatory stimulation by bacterial infections is a requisite to activate Wnt signaling, indicating that β-catenin activity is required for late stages of the inflammatory response.

It is predictable that different specific combinations of Fzd receptors and Wnt ligands may promote or inhibit inflammation induced by bacterial pathogens. This is because bacterial pathogens modulate distinct key proteins that regulates Wnt β-catenin pathway and manipulates cell functions to increase its survival and spread invasion through different mechanisms. Future studies with different pathogenic bacteria and cell types will open new scenarios in which the knowledge of interconnection points in space and time of several signaling pathways may be used to design biotechnological approaches to resolve uncontrolled inflammation and enhance bacterial clearance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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15. Discusión general

Staphylococcus aureus es un patógeno reemergente exitoso que es causante de una amplia gama de infecciones en el hombre y los animales. Los factores de virulencia que es capaz de expresar esta bacteria le permiten invadir y colonizar cualquier tejido (Tong *et al*., 2015). La fisiopatología de las infecciones provocadas por la bacteria está representada por el modelo “trojan horse”, caracterizado por una respuesta inmune de inflamación intensa en la primera etapa y su inhabilitación en etapas posteriores. Esta estrategia le permite a la bacteria evadir la respuesta inmune innata y establecer infecciones crónicas o recurrentes que son potencialmente fatales para el hospedero (Liu, 2009).

Se ha propuesto que *S. aureus* manipula la respuesta inmune del hospedero por su habilidad de expresar factores de virulencia cuya actividad se ha asociado a la expresión de citocinas pro- o anti-inflamatorias (Thammavongsa *et al*., 2015). Por ejemplo, las moléculas de pared celular PGN y LTA inducen la expresión de IL-1β, TNFα o IL-8 (Kumar & Kumar, 2015). Estas moléculas de pared celular se expresan constitutivamente por la bacteria y son algunas de las primeras en ser reconocidas por el sistema inmune, lo que explica la intensa respuesta inflamatoria en la primera etapa de la infección (Bekeredjian-Ding *et al.*, 2015).

La expresión de otros factores de virulencia en *S. aureus* depende de su densidad poblacional o la formación de biofilms; por ejemplo, las leucocidinas, proteína A o modulinas (Kanangat *et al.*, 2007). Entre estas proteínas, las modulinas se han reportado que son esenciales para inducir la represión de las citocinas IL-8, IL-1 (Deplanche *et al*., 2016). Simultáneamente, las modulinas también se han reportado como responsables en la expresión de citocinas anti-inflamatorias como IL-10 lo que conlleva la evasión de la respuesta inmune y favorece el establecimiento de infecciones por la bacteria (Schreiner *et al.*, 2013).

Dentro de los mecanismos de inactivación del sistema inmune es importante mencionar la inducción de apoptosis de neutrófilos, que constituye el principal tipo celular de la respuesta inmune innata y se ha reportado que juega un papel fundamental en la prevención de infecciones provocadas por *S. aureus*. En este contexto, es sabido que la estimulación de neutrófilos con la citocina IL-8 constituye un mecanismo por el cual estas células son atraídas a los focos de infección provocados por bacterias y además favorecen su sobrevivencia en presencia de TNF-α (Zurek *et al*., 2015). En contraste, la estimulación de neutrófilos con citocinas anti-inflamatorias como IL-10 en presencia de TNF-α induce su apoptosis e inactivan la fagocitosis de

875 microorganismos (Roilides *et al.*, 2000, Ward *et al.*, 2005). Este efecto ha sido
876 observado dentro de lesiones provocadas por *S. aureus* que se caracterizan por la
877 presencia de núcleos formados por neutrófilos apoptóticos que rodean las colonias
878 bacterianas (Kobayashi *et al*., 2015). Se ha propuesto que estas estrategias son
879 mecanismos inducidos por la bacteria más que una respuesta celular debido a que la
880 generación de distintas mutantes bacterianas reducen su capacidad de generar
881 lesiones celulares y son eliminadas más eficientemente (Thomer *et al*., 2016). Sin
882 embargo, aunque se han reconocido distintas moléculas bacterianas que son
883 esenciales en la manipulación de la respuesta inmune, los blancos moleculares en las
884 células hospederas son menos conocidos.

885 En este contexto, nuestro equipo de trabajo reportó previamente que la vía de
886 transducción de señales PI3K/Akt es importante en la internalización de la bacteria, lo
887 que resultó en la fosforllación de las isoformas de la enzima GSK3α y GSK3β (Ver
888 Anexo I) y (Anexo II). Este trabajo es el primero que propone y analiza la participación
889 de GSK3α en la regulación de la expresión de citocinas inducida por una bacteria.
890 Como se observa en la Figura 3 del Capítulo I, la isoforma GSK3α es más sensible a
891 fosforilación por estimulación con *S. aureus* que la isoforma GSK3β. Estos
892 incrementos de fosforilación indujeron la reducción de su actividad cinasa como se
893 evidencia en la disminución de la fosforilación del sustrato específico fosfo-glucógeno
894 sintasa Ser641. Efectos similares han sido reportados en otros contextos de infección
895 con otras especies de bacterias con respecto a la isoforma GSK3β.

896 La infección de endotelio con *S. aureus* indujo la fosforilación de la subunidad de NF-
897 κB p65 en ser536 y su translocación al núcleo durante los primeros 40 minutos. En
898 etapas posteriores no se observaron cambios en NF-kB, lo que indica que este factor
899 transcripcional solo se mantuvo activo hasta los 40 minutos de contacto con la
900 bacteria. La inhibición de Akt con 1 μM de AktIV produjo un incremento en la activación
901 de NF-κB por fosforilación que podría explicar por qué la actividad de GSK3 es
902 importante para estimular la actividad de NF-κB. Sin embargo, la inhibición de GSK3
903 con su inhibidor específico SB216763 no alteró los niveles de fosforilación de NF-κB ni
904 su translocación al núcleo, lo que sugiere que el mecanismo por el que GSK3 favorece
905 la actividad de NF-κB no involucra una regulación directa. Estos resultados
906 concuerdan con lo observado en el desarrollo embrionario de ratones en donde la
907 actividad de GSK3β favorece la actividad de NF-κB (Götschel *et al.*, 2008, Hoeflich *et*
908 *al*., 2000). Sin embargo, no se ha determinado aún que GSK3α o GSK3β participen
909 directamente en los mecanismos involucrados en la activación de NF-κB (Cortés-
910 Vieyra *et al*., 2012).

Si bien la actividad transcripcional de NF-κB es importante para la expresión de IL-8, los factores de transcripción c-Jun, AP1 y STAT-3 también se han implicado (Roebuck, 1999, Piperi *et al.*, 2011). La inhibición de NF-κB con su inhibidor específico partenólido redujo la expresión de IL-8 inducida por *S. aureus* (Figura 7-Capítulo I). Diversos estudios han reportado resultados similares; por ejemplo, la inhibición de NF-κB en neutrófilos reprime la expresión de IL-8 inducida por la cepa de *S. aureus* USA300 (Zurek *et al*., 2015) y además se ha relacionado con la inducción de apoptosis de células en infecciones con *Escherichia coli, Yersinia pestis y Salmonella typhimurium* (Neish & Naumann, 2011). De manera interesante, nuestro equipo de trabajo previamente ha reportó que la actividad de NF-κB es importante para favorecer la fagocitosis y eliminación *S. aureus* intracelular (Oviedo-Boyso *et al.*, 2008), por lo que su inactividad podría estar relacionada con la evasión de la fagocitosis y la muerte celular, lo que resultaría en la sobrevivencia de la bacteria.

Por otra parte el factor transcripcional CREB es esencial para estimular la expresión de IL-10 en distintos contextos celulares (Avni *et al.*, 2010). En este trabajo se observó que la fosforilación de CREB en Ser133, que es importante para estimular su actividad transcripcional, fue promovida por la infección con *S. aureus* principalmente durante el tiempo en el que las isoformas de GSK3 presentaron una actividad cinasa baja (después de 40 minutos). De acuerdo con una revisión exhaustiva de la literatura, este es el primer reporte que ubica la activación del factor transcripcional CREB como parte de la respuesta celular ante la infección *con S. aureus*. Además, observamos que la incubación de las células con el inhibidor específico de GSK3 aceleró la fosforilación de CREB en Ser133 y, en contraste, la incubación de células con el inhibidor de Akt AktIV (1 μM) abatió la estimulación del incremento de fosforilación de CREB Ser133. Aunque ya se ha relacionado la actividad de Akt con la estimulación de CREB (Du & Montminy, 1998), este es el primer reporte en el contexto de la estimulación inducida por bacterias. Además, mediante los experimentos de silenciamiento de las isoformas de GSK3 observamos que el silenciamiento de la isoforma GSK3α indujo un mayor incremento en la abundancia de fosfo-CREB Ser133 inducido por la bacteria que el silenciamiento de la isoforma GSK3β. Experimentos en células de corteza cerebral de ratón señalan que GSK3α es la isoforma predominante de la regulación de CREB y su actividad transcripcional (Liang & Chuang, 2006). No obstante, debido a que GSK3 no fosforila a CREB, a menos que este factor se encuentre previamente fosforilado en Ser133 (Sweatt, 2001), el mecanismo por el cual GSK3 reprime el incremento de fosfo-CREB Ser133 no está claro. Es probable entonces que exista una cinasa de CREB aún no identificada y cuya actividad es reprimida por la actividad de GSK3.

Esta regulación sobre CREB, inducida por *S. aureus*, regula la expresión de IL-10 debido a que la expresión de mutantes de CREB incapaces de interaccionar con el co-activador CBP no expresan IL-10 a los niveles que las células control, lo que confirma la función de CREB en la expresión de IL-10. La expresión de IL-10 es un evento posterior a la expresión de citocinas pro-inflamatorias, es decir, que son citocinas que se expresan durante etapas posteriores al contacto inicial con bacterias (Witchell *et al.*, 2010, Tuchscherr *et al*., 2011). La actividad constitutiva de las isoformas de GSK3 participaría en este "*reloj biológico*" favoreciendo así la expresión de citocinas pro-inflamatorias en la etapa inicial, a través de la estimulación de NF-κB y represión de CREB, mientras que en etapas posteriores cuando GSK3 reduce su actividad cinasa, lo opuesto ocurre y se favorece la expresión de citocinas anti-inflamatorias. Este modelo parece sustentarse por el hecho de que la inhibición de las isoformas de GSK3 con SB216763 acelera la interacción física de CBP con CREB y reprime la interacción de NF-κB/CBP. Tal efecto se ha reportado previamente en monocitos estimulados con LPS o por bacterias como *Helicobacter pylori o Francisella tularensis* (Nakayama *et al*., 2009, Zhang *et al*., 2009) pero no para *S. aureus*.

Estos experimentos revelan que el silenciamiento de GSK3α induce preferencialmente la interacción de CREB/CBP y simultáneamente reduce la asociación de NF-κB/CBP. Esto significa que el modelo de competencia por CBP entre NF-κB y CREB puede ser promovido por la isoforma GSK3α, de una manera similar como lo hace la isoforma GSK3β en otros contextos (Martin *et al*., 2005). Al respecto, estos resultados sugieren que los programas de regulación de la inflamación como respuesta celular en el contexto de las isoformas de GSK3 puede ser específica dependiendo del estímulo y sugieren también que GSK3α es la isoforma de GSK3 mediante la que *S. aureus* promueve el cambio en la síntesis de citocinas con actividad pro-inflamatoria en la etapa inicial a anti-inflamatoria en etapas posteriores. Además, experimentos iniciales de silenciamiento en células HeLa revelaron que el silenciamiento de GSK3α induce apoptosis, lo que indica que la actividad de GSK3α puede ser importante para favorecer la supervivencia celular y señala que la apoptosis de neutrófilos inducida por *S. aureus* puede ser consecuencia de la inactivación de GSK3α, puesto que en los neutrófilos GSK3α es la principal isoforma expresada (Giambelluca *et al.*, 2013).

La cuantificación de la expresión de las citocinas IL-8 e IL-10 reveló que GSK3α es la isoforma responsable de su regulación inducida por *S. aureus*, debido a que el silenciamiento de esta isoforma promueve la expresión de IL-10 y la represión de IL-8 en mayor magnitud que el silenciamiento de la isoforma GSK3β. Este conocimiento permitirá facilitar el diseño de estrategias orientadas al control de las infecciones

causadas por este patógeno, alternas al uso de terapia antimicrobiana (Ver anexo III) e inclusive como se ha reportado previamente, la capacidad de *S. aureus* para inhibir la inflamación puede ser benéfica en ciertos contextos de enfermedades autoinmunes como la esclerosis múltiple donde se ha observado que la capacidad de la bacteria para regular negativamente la inflamación favorece la sobrevivencia neuronal en ratas con esclerosis múltiple inducida (Kumar *et al.*, 2015). El estudio de los mecanismos por los cuales *S. aureus* controla negativamente la inflamación podría ser utilizado como una terapia alterna al uso de medicamentos anti-inflamatorios esteroideos y no esteroideos.

Finalmente, como se muestra en el Capítulo II, la vía de transducción de señales Wnt/β-catenina parece ser activada por la estimulación con *S. aureus.* Aparentemente, S. aureus provoca un incremento en la estabilización del co-activador β-catenina como resultado de la inhibición de la actividad de las isoformas de GSK3, lo que podría explicar la alteración de la arquitectura celular inducida por las infecciones crónicas de *S. aureus* (García-Betancur *et al*., 2017). Sin embargo, se requiere obtener mayor evidencia experimental que corrobore los incrementos de la expresión de genes de la vía Wnt a la estabilización inducida de β-catenina por *S. aureus.* Otra posibilidad derivada de la estabilización de β-catenina sería la inhibición de la vía de NF-κB, puesto que en periodos prolongados de la infección de las CEB con la bacteria se ha observado la inactivación de la vía (Silva-García *et al*., 2018). Estas observaciones sientan las bases para estudios futuros que profundicen en la caracterización molecular de la patogénesis de *S. aureus*.

16. Anexos

16.1. Anexo I

Los factores de transcripción NF-κB y CREB requieren la asociación con co-activadores como la acetil-transferasa CBP para inducir la expresión de sus genes dependientes como las citocinas pro- y anti-inflamatorias, respectivamente. En modelos de infección con ligandos bacterianos, virales y fúngicos está reportado que el balance de este proceso depende de la influencia que la enzima GSK3 tiene sobre estos factores transcripcionales. Sin embargo, para el patógeno *Staphylococcus aureus* este proceso no había sido probado hasta ahora a pesar de que el desbalance en la producción de citocinas y la coordinación de la respuesta inflamatoria son características esenciales de las infecciones producidas por esta bacteria. Los experimentos preliminares aunados a la bibliografía escrita hasta el momento nos permitieron plasmar esta idea en al artículo de revisión que está actualmente publicado en la Revista de Educación Bioquímica. En esta revisión participé como co-autor brindando ideas para el escrito, escritura de secciones y elaboración de diagramas.

LA ENZIMA GLUCÓGENO SINTASA CINASA 3 REGULA LA ACTIVIDAD DE LOS FACTORES DE TRANSCRIPCIÓN NF-κB Y CREB DURANTE LA RESPUESTA INFLAMATORIA CAUSADA POR *Staphylococcus aureus**

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RESUMEN

La enzima glucógeno sintasa cinasa 3 (GSK3) es una proteína cinasa de Ser/Thr, que regula la expresión de citocinas durante la respuesta inflamatoria. GSK3 modula la actividad del factor nuclear κB (NF-κB) y de la proteína de unión al elemento de respuesta de AMPc (CREB). En esta revisión se presenta uno de los mecanismos reguladores de la actividad de GSK3 y su efecto en las infecciones por *Staphylococcus aureus*.

PALABRAS CLAVE: GSK3, NFκB, CREB, inflamación, *Staphylococcus aureus*.

ABSTRACT

The enzyme glycogen synthase kinase 3 (GSK3) is a Ser/Thr kinase that regulates the expression of cytokines during the inflammatory response by modulating the activity of nuclear factor κB and cAMP response element-binding protein (CREB). This review presents one of the mechanisms that regulate GSK3 activity and its effect on *Staphylococcus aureus* infections.

KEY WORDS: GSK3, NF-κB, CREB, inflammation, *Staphylococcus aureus*.

INTRODUCCIÓN

La inmunidad innata es la primera línea de defensa contra agentes infecciosos, y consiste de una red interactiva de sistemas moleculares y celulares responsable de reconocer y eliminar microorganismos patógenos. Este tipo de inmunidad incluye una amplia variedad de cambios en la célula, cuyo objetivo es producir moléculas que coadyuven a eliminar al patógeno (1). La habilidad del sistema inmune innato de reconocer y responder a componentes microbianos se debe a la presencia de receptores tipo Toll (TLRs) (2, 3). Los TLR están presentes tanto en fagocitos profesionales (monocitos, macrófagos y células dendríticas) como en no profesionales como las células epiteliales y endoteliales. Estos receptores pueden discriminar entre distintos patrones moleculares asociados a patógenos (PAMPs) que conducen a la activación de una variedad de vías de transducción de señales, las cuales regulan la naturaleza, magnitud y duración de la respuesta inflamatoria (4). La reacción inflamatoria es muy importante en la respuesta inicial a la infección y en ella participan neutrófilos, monocitos, macrófagos, moléculas del complemento, citocinas, quimiocinas, así como también proteínas y péptidos con actividad antimicrobiana, que contribuyen coordinadamente a eliminar al patógeno del tejido infectado. Aunque la producción de citocinas pro-inflamatorias es importante para regular la defensa inicial del hospedero contra los patógenos invasores, la incapacidad de modular la intensidad o duración de la respuesta inflamatoria puede ser perjudicial y dar lugar a enfermedades inflamatorias crónicas (5).

En la última década, el interés se ha enfocado en explicar el mecanismo por el que las células participantes en la respuesta inflamatoria inicial

*Recibido: 6 de septiembre de 2017 Aceptado: 21 de octubre de 2017

expresan citocinas pro-inflamatorias (p.ej. interleucina 8, IL-8) y más tarde apagan esta expresión y activan la síntesis de citocinas anti-inflamatorias (p.ej. IL-10), disminuyendo con ello la inflamación. En este sentido, se ha observado que uno de los principales mecanismos moduladores de la expresión de citocinas con actividad pro- y anti-inflamatoria es el que está mediado por la vía de señalización constituida por las enzimas fosfatidilinositol 3-cinasa (PI3K), Akt (denominada también proteína cinasa B, PKB) y glucógeno sintasa cinasa 3 (GSK3), activada por TLR (6, 9). Se ha propuesto que la activación de GSK3 es fundamental para inducir la producción de citocinas que promueven la inflamación (IL-8) o la disminuyen (IL-10), por medio de un mecanismo que involucra la participación de los factores de transcripción factor nuclear-kappa B (NF-κB) y la proteína de unión al elemento de respuesta de AMPc (CREB), respectivamente (7).

En este trabajo se explicarán brevemente algunos de los principales conceptos de la respuesta inflamatoria, para después discutir la función de GSK3 como una enzima mediadora importante de la vía de transducción PI3K/Akt. Con esta información abordaremos con mayor detalle algunos aspectos mecanísticos de GSK3, lo que dará lugar a entender mejor la regulación que ejerce esta enzima sobre NF-κB y CREB en la respuesta inflamatoria. Por último, se describirán algunos resultados de nuestro laboratorio, donde evaluamos la regulación de la expresión de citocinas por GSK3 y una de las perspectivas de investigación que tenemos a mediano plazo.

LA RESPUESTA INFLAMATORIA

La inflamación es el primer proceso fisiológico de respuesta a un daño o infección de un tejido y es fundamental tanto para la inmunidad innata como la adaptativa. Cuando un tejido es infectado por bacterias patógenas se activa un programa de respuesta que incluye la expresión de citocinas, quimiocinas, mediadores lipídicos y aminas bioactivas por diversos tipos de células como monocitos, células dendríticas, células asesinas naturales ("*natural killer cells*") y mastocitos. Entre las múltiples funciones de estos factores, una muy importante es el incremento de la permeabilidad vascular que conduce a la extravasación de leucocitos polimorfonucleares al sitio de infección. La mayor cantidad de información molecular que se tiene hasta ahora sobre el proceso inflamatorio proviene de estudios sobre componentes de las vías de transducción activados por el receptor al factor de necrosis tumoral (TNFR), el receptor a IL-1 y los TLR (8). Las citocinas pro-inflamatorias predominantemente producidas son el factor de necrosis tumoral (TNF), IL-1, IL-8, IL-12, IL-15 IL-18, IL-23 e IL-27 (9). Estos péptidos activan procesos citotóxicos, celulares, humorales y alérgicos, que en conjunto tienen la finalidad de eliminar al agente causal del daño al tejido.

Es importante mencionar que la respuesta inflamatoria está sujeta a una regulación estricta, de tal forma que, una vez cumplido el objetivo de eliminar la causa de la inflamación, las mismas células involucradas cambian su programa genético para expresar ahora mediadores anti-inflamatorios, cuyo objetivo es restaurar la homeostasis. Cuando este programa anti-inflamatorio falla puede haber daño al tejido, lo que en ocasiones deriva en inflamación crónica y enfermedad (10). Se han identificado citocinas que tienen principalmente una función anti-inflamatoria, por ejemplo el antagonista del receptor a IL-1 (IL-1Ra), el factor de crecimiento transformante beta (TGF-β), IL-10 e IL-35 (11).

Tal cambio de actividad celular pro-inflamatoria a anti-inflamatoria resulta relativamente fácil de entender si echamos andar nuestra lógica al formular preguntas como ¿qué sucedería si la respuesta inflamatoria continuara indefinidamente? La respuesta creo que es obvia, nuestros órganos se alterarían a tal grado que se perderían las funciones que mantienen al organismo en homeostasis. Para explicar el tránsito de mecanismos pro-inflamatorios a anti-inflamatorios, lo primero que se pensó fue que a medida que transcurría la inflamación, el tejido iba recuperando la homeostasis porque los mediadores pro-inflamatorios se iban degradando. Es decir, no se consideraba que las mismas células iniciadoras de la inflamación pudieran ser capaces de inhibir la expresión de moléculas pro-inflamatorias y activar la expresión y secreción de moléculas anti-inflamatorias. Esta forma de conceptualizar la respuesta inflamatoria ha cambiado y ahora se acepta que hay moléculas que son críticas para controlar el cambio de actividad pro- a anti-inflamatoria. Una de estas moléculas es la enzima GSK3 descubierta en 1980 por su efecto regulador de la síntesis de glucógeno (12). Sin embargo, la evidencia acumulada indica sin lugar a dudas que GSK3 tiene múltiples funciones, como su participación en el control de la inflamación (13). Durante la respuesta inflamatoria la activación de Akt regula la actividad de GSK3, por lo que a continuación estudiaremos un poco acerca de la vía de transducción que activa a Akt.

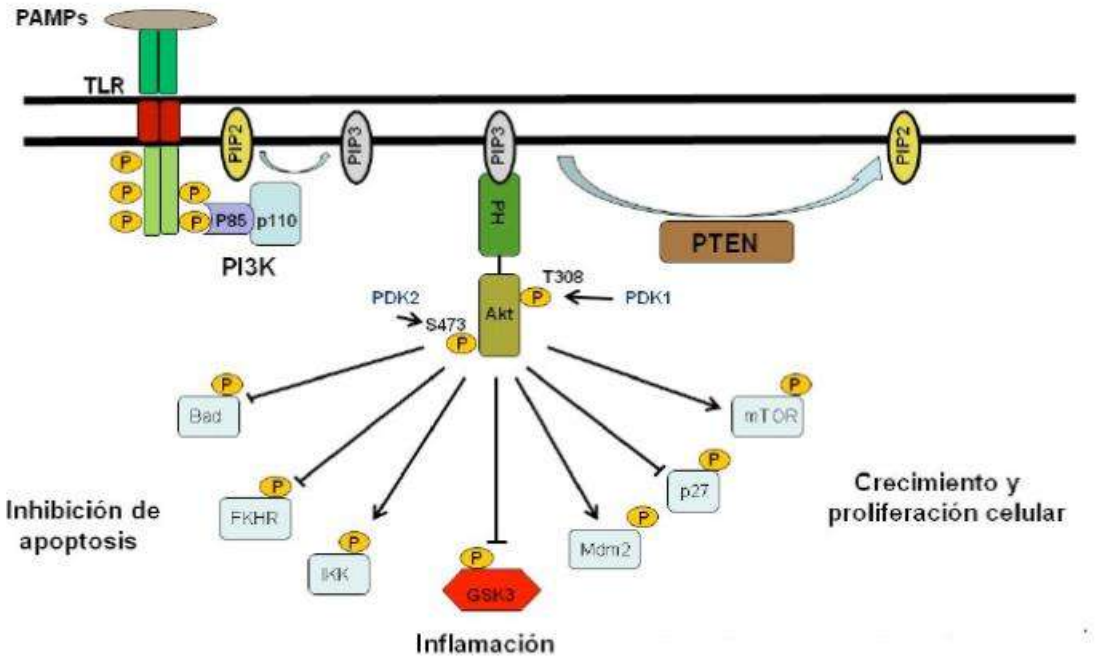

Figura 1. Modificado de referencia 14

CARACTERÍSTICAS Y MECANISMO DE ACTIVACIÓN DE LA VÍA DE TRANSDUCCIÓN PI3K/Akt/GSK3

La enzima PI3K es un heterodímero constituido por una subunidad catalítica de 110 kDa (p110) y una subunidad reguladora de 85 kDa (p85). De acuerdo con su estructura y mecanismo de activación las PI3K se clasifican en varias familias. Las enzimas de la clase IA (principales responsables de la producción de 3'-fosfoinosítidos) son activadas por receptores que tienen actividad de tirosina cinasa (RPTKs) y las que pertenecen a la clase IB por receptores acoplados a proteínas G (GPCR). Dentro de la clase IA se han caracterizado tres isoformas de p110, denominadas α, β y γ, y siete isoformas de p85 que se sintetizan por procesamiento alternativo de tres genes, *p85α*, *p85β* y *p85γ* (14). La segunda enzima de la vía, denominada Akt/PKB, fue caracterizada como un homólogo del oncogén viral *v-akt*, que se considera responsable de causar leucemia en ratones (15). Debido a que *v-akt* y su contraparte en humanos presentan similitud con PKA y PKC, a esta nueva proteína cinasa de Ser/Thr se le llamó inicialmente PKB. Existen tres isoformas de Akt, Akt1, Akt2 y Akt3, con un 80% de homología entre ellas, aunque son codificadas por genes diferentes. Las tres Akt tienen en su región N-terminal un dominio de unión a plekstrina (PH) de aproximadamente 100 aminoácidos, que le permite anclarse a la membrana plasmática mediante su interacción con 3'-fosfoinosítidos. Para activarse al 100%, Akt debe ser fosforilada en Thr 308 (localizada en su dominio catalítico) y en Ser 473 (localizada en su región C-terminal). La interacción de factores de crecimiento con RPTKs y de PAMPs (p.ej. péptidoglicano, PGN; ácido lipoteicoico, LTA y lipopolisacárido, LPS) con TLRs da lugar a la activación de Akt (16, 17).

La activación de la vía PI3K-Akt se inicia cuando un estímulo (p.ej. factores de crecimiento o PAMPs) promueve la fosforilación de residuos de Tyr en el dominio citosólico de los receptores (Fig. 1). Estas fosfo-Tyr son reconocidas por el dominio SH2 de p85, lo que trae como consecuencia la activación alostérica de p110 y la producción de fosfatidilinositol-3,4,5-trisfosfato (PIP_3). La presencia de PIP_3 en la capa interna de la membrana plasmática funciona como un mediador esencial para el funcionamiento de la vía PI3K-Akt, en la que participan PI3Ks de la clase IA. En esta vía, las dos enzimas que contienen dominios PH y, por lo tanto, pueden reconocer a PIP_3 son la cinasa de Ser/Thr dependiente de 3'-fosfoinositido (PDK1), constitutivamente activa, y Akt. La interacción del dominio PH de Akt con PIP3 cambia la conformación de Akt y expone sus sitios de fosforilación Thr308 y Ser473, los cuales son fosforilados por PDK1 y PDK2 (denominada también complejo diana de rapamicina de mamíferos, mTORC2), respec-

tivamente. Una vez activada, Akt fosforila una diversa variedad de sustratos, entre los cuales se encuentra GSK3, modulando así una variedad de procesos celulares como apoptosis, proliferación y sobrevivencia celular, e inflamación.

GSK3 MODULA LA EXPRESIÓN DE CITOCINAS CON ACTIVIDAD PRO- Y ANTI-INFLAMATORIA

GSK3 es una enzima constitutivamente activa muy importante en múltiples procesos biológicos, debido a que es el punto de convergencia de varias vías de señalización y porque regula la actividad de más de 50 sustratos (18). Su secuencia de aminoácidos está altamente conservada en eucariontes y se han caracterizado dos isoformas α y β, que aparentemente no son redundantes porque la interrupción de la isoforma β en ratones resulta en letalidad embrionaria; esto significa que GSK3α es incapaz de compensar la pérdida de GSK3β (19). Debido a la amplia influencia que GSK3 tiene en diversas funciones celulares, la actividad de GSK3 está regulada por diferentes mecanismos. Por ejemplo, su actividad puede ser inhibida por la fosforilación de residuos de Ser en la región N-terminal (Ser21 en GSK3α y Ser9 en GSK3β). Además, la actividad de GSK3 también está controlada por su asociación con proteínas de anclaje y su localización intracelular (6). Se ha demostrado que la activación o inhibición de GSK3 tiene una función importante en la regulación de la respuesta inmune al ejercer un control, mediado por TLRs, de la producción de citocinas con actividad anti- y pro-inflamatoria (7).

Las vías de señalización activadas por TLR2, TLR4, TLR5 y TLR9 son fundamentales para regular la producción de las citocinas pro-inflamatorias (p.ej. IL-1β, TNF-α, IL-8) y anti-inflamatoria (p.ej. IL-4, IL-10) (20, 7, 21). El mecanismo por el que estos TLR regulan la expresión de citocinas que intervienen en la inflamación se estudió en monocitos estimulados con LPS (7). Los datos obtenidos indicaron que la inhibición de GSK3β, pero no de GSK3α, redujo los niveles de las citocinas pro-inflamatorias y aumentó la expresión de IL-10, en respuesta a los agonistas de TLR, LTA (TLR2), lípido A sintético (TLR4), flagelina (TLR5) y CpG (TLR9). La inhibición de GSK3 ejerció un efecto regulador diferencial en la asociación de NF-κB (promueve la expresión de citocinas pro-inflamatorias) y CREB (promueve la expresión de citocinas anti-inflamatorias) con el co-activador nuclear, proteína de unión a CREB (CBP), es decir, GSK3 inactiva causó un aumento en la actividad de unión de CREB al DNA y, al mismo tiempo, inhibió apreciablemente la actividad nuclear de NF-κB (7). De esta manera, la interacción de NF-κB y CREB con CBP, regulada por la actividad de GSK3, ocupa un lugar preeminente con respecto a la naturaleza y magnitud de la producción de citocinas pro- y anti-inflamatorias, lo que resulta en la modulación adecuada y precisa de la inflamación.

GSK3α Y GSK3β REGULAN DIFERENCIALMENTE LA EXPRESIÓN DE CITOCINAS EN CÉLULAS ENDOTELIALES INFECTADAS CON *Staphylococcus aureus*

Staphylococcus aureus es una bacteria Gram$^+$ causante de importantes enfermedades infecciosas en seres humanos y animales. Esta bacteria expresa una amplia variedad de factores de virulencia y PAMPs que inducen inflamación y son responsables de dañar al tejido (22). Las células del hospedero reconocen los PAMPs de *S. aureus* por medio del receptor TLR2. La interacción de estos PAMPs con TLR2 activa la vía de señalización PI3K/Akt/GSK3, lo cual da lugar a cierto número de respuestas celulares tales como proliferación, diferenciación, sobrevivencia, apoptosis e inflamación (23).

La participación de GSK3α y GSK3β en infecciones por *Staphylococcus aureus* se observó por primera vez cuando la internalización de esta bacteria en células endoteliales produjo un incremento en la fosforilación e inhibición de la actividad de ambas isoformas de GSK3 (24). Un aspecto interesante de ese estudio fue que GSK3α alcanzó un nivel de fosforilación y de inhibición mayor que GSK3β. Esta evidencia experimental indicó que no solo GSK3β está involucrada en procesos de infección e inflamación causados por bacterias patógenas, como se ha descrito ampliamente en la literatura (13), sino que también GSK3α podría tener una función preponderante. Más adelante se pudo demostrar que ambas isoformas de GSK3 regulan la expresión de IL-12p40 en células endoteliales estimuladas con PGN de *S. aureus* (25). Esta citocina es muy importante porque controla la respuesta inmune innata durante la infección bacteriana y determina el tipo y duración de la respuesta inmune adaptativa. IL-12p40 es altamente inducible por LPS, LTA y PGN, por su interacción con TLRs y activación de la vía NF-κB (26). Aunque la función de IL-12p40 promueve un desarrollo apropiado de la respuesta inflamatoria inicial, su sobreexpresión es perjudicial para el tejido. Por ello, las células contrarrestan los efectos de esta citocina produciendo IL-10, que disminuye la actividad de NF-κB y aumenta la actividad de CREB. Los datos de Cortés-Vieyra et al. (25) indicaron que la vía PI3K/Akt/GSK3 se activó por efecto de PGN, lo que se correlacionó con cambios en la actividad de GSK3α y GSK3β. Los autores pudieron establecer que la inhibición de la actividad de GSK3α indujo un incremento en la expresión de IL-12p40, mientras

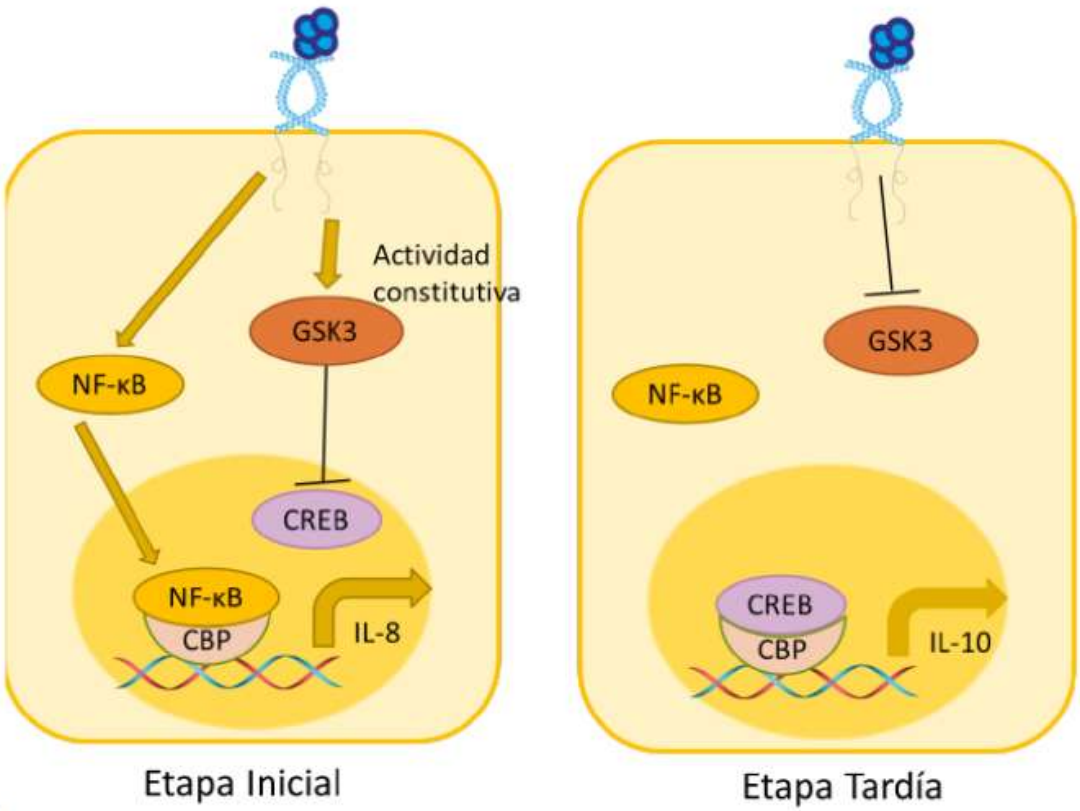

Figura 2.

que la inhibición de la actividad de GSK3β resultó en una expresión disminuida de esta citocina.

Los resultados más recientes obtenidos en nuestro grupo (Silva-García O y Baizabal-Aguirre VM, manuscrito enviado) han mostrado que la actividad de GSK3α y, en menor grado, GSK3β en células endoteliales infectadas por *S. aureus* durante 60 min ejerció un efecto inhibidor de la activación de CREB, limitando con ello su actividad transcripcional y promoviendo la actividad de NF-κB, el cual a su vez indujo la expresión de la citocina pro-inflamatoria IL-8. Cuando se ensayaron tiempos de infección de 120 min observamos que GSK3α/β alcanzó su fosforilación e inhibición máxima por Akt, lo cual inhibió indirectamente la actividad transcripcional de NF-κB. Esto causó un aumento en la actividad transcripcional de CREB, lo que estimuló la expresión de la citocina anti-inflamatoria y tolerogénica IL-10, y disminuyó simultáneamente la expresión de IL-8 (Fig. 2). Con base en estos resultados creemos que este mecanismo de "*switch*" molecular de la activación de un factor transcripcional pro-inflamatorio (NF-κB) en la fase inicial de la infección, a la activación de un factor transcripcional anti-inflamatorio (CREB) en la fase tardía de la infección por *S. aureus* explicaría en parte por qué las infecciones causadas por esta bacteria transitan de una fase de inflamación aguda a una fase de inflamación crónica.

Estos datos plantean la posibilidad de que GSK3α y GSK3β ejerzan una regulación diferencial de la expresión de genes pro- y anti-inflamatorios, por medio de la modulación de la actividad transcripcional de NF-κB, CREB y probablemente otros factores, dependiendo del tipo de célula infectada y de la bacteria en turno. En este contexto, uno de los objetivos a mediano plazo en nuestro laboratorio es realizar un análisis transcriptómico y proteómico global de los genes regulados por cada una de las isoformas de GSK3 cuando *S. aureus* infecta células endoteliales y epiteliales de humano. Esta evaluación *ómica* se combinará también con la identificación de factores de virulencia expresados por la bacteria y que son los responsables de activar NF-κB en fase temprana y CREB en fase tardía de la infección. Toda esta información que se genere sentará las bases para diseñar una estrategia biotecnológica cuya finalidad será eliminar *S. aureus* y promover la recuperación de la homeostasis celular.

AGRADECIMIENTOS

VMBA agradece los apoyos recibidos por el Consejo Nacional de Ciencia y Tecnología (CONACYT, Ciencia Básica 152518), y Coordinación de la Investigación

Científica de la Universidad Michoacana de San Nicolás de Hidalgo (UMSNH). RRM, recibió beca de CONACYT para estancia posdoctoral. AHM recibió beca de CONACYT para estudios de doctorado y MCMP recibe beca de CONACYT para estudios de maestría.

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16.2. Anexo II

Los experimentos de estimulación de células de endotelio bovino (CEB) con la cepa de *Staphylococcus aureus* ATCC 27543 indican que la fosforilación de las isoformas de GSK3 se incrementa por efecto de la infección, con una preferencia hacia la isoforma GSK3α. Esto es importante porque la isoforma GSK3α no se ha asociado a la regulación de la expresión de citocinas en contextos de infección bacteriana. Sin embargo, la estimulación con la bacteria completa (Oviedo-Boyso *et al.* (2011) resultó en una amplia variedad de posibilidades por las que la bacteria puede inducir la fosforilación de las isoformas de GSK3. Para probar si ciertas estructuras específicas de la pared celular de *S. aureus* son responsables de la fosforilación de GSK3, se decidió probar la respuesta celular ante la molécula péptidoglicano (PGN), que es el componente principal de la pared celular de *S. aureus*. Se observó que la estimulación de las CEB con PGN purificado indujo la fosforilación de las isoformas de GSK3 con preferencia sobre la isoforma GSK3α, de manera similar a lo observado con la bacteria completa. Además, los experimentos de silenciamiento con ARN de interferencia sobre las isoformas de GSK3α/β indican que el silenciamiento de la isoforma GSK3β reduce la expresión de la citocina IL-12p40, mientras que el silenciamiento de la isoforma GSK3α induce el incremento de la misma citocina en las CEB estimuladas con PGN. Este es el primer trabajo que valora la participación de GSK3α en la regulación de la expresión de citocinas inducida por modelos bacterianos. Estos resultados están actualmente publicados en la revista Plos One. Durante la elaboración de este trabajo aporté ideas para los experimentos llevados a cabo, el silenciamiento de las isoformas de GSK3 y la medición de IL-12p40 por PCR semicuantitativo y ELISA, lo que me permitió ocupar un lugar como primer autor del trabajo, junto a mi compañera Ricarda Cortes Vieyra.

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RESEARCH ARTICLE

The Glycogen Synthase Kinase 3α and β Isoforms Differentially Regulates Interleukin-12p40 Expression in Endothelial Cells Stimulated with Peptidoglycan from *Staphylococcus aureus*

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Citation: Cortés-Vieyra R, Silva-García O, Oviedo-Boyso J, Huante-Mendoza A, Bravo-Patiño A, Valdez-Alarcón JJ, et al. (2015) The Glycogen Synthase Kinase 3α and β Isoforms Differentially Regulates Interleukin-12p40 Expression in Endothelial Cells Stimulated with Peptidoglycan from *Staphylococcus aureus*. PLoS ONE 10(7): e0132867. doi:10.1371/journal.pone.0132867

Editor: Yi-Hsien Hsieh, Institute of Biochemistry and Biotechnology, TAIWAN

Received: February 2, 2015

Accepted: June 19, 2015

Published: July 22, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by CONSEJO NACIONAL DE CIENCIA Y TECNOLOGÍA (CONACyT)-MÉXICO (grant number 152518 VMB), Coordinación de Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo, grant-2014-2015 to VMBA.

Abstract

Glycogen synthase kinase 3 (GSK3) is a constitutively active regulatory enzyme that is important in cancer, diabetes, and cardiovascular, neurodegenerative, and psychiatric diseases. While GSK3α is usually important in neurodegenerative and psychiatric diseases GSK3β is fundamental in the inflammatory response caused by bacterial components. Peptidoglycan (PGN), one of the most abundant cell-wall structures of Gram-positive bacteria, is an important inducer of inflammation. To evaluate whether inhibition of GSK3α and GSK3β activity in bovine endothelial cells (BEC) regulates the expression of the pro-inflammatory cytokine IL-12p40, we treated BEC with SDS-purified PGN from *Staphylococcus aureus*. We found that PGN triggered a TLR2/PI3K/Akt-dependent phosphorylation of GSK3α at Ser21, GSK3β at Ser9, and NF-κB p65 subunit (p65) at Ser536, and the phosphorylation of GSK3α was consistently higher than that of GSK3β. The expression of IL-12p40 was inhibited in BEC stimulated with PGN and pre-treated with a specific neutralizing anti-TLR2 antibody that targets the extracellular domain of TLR2 or by the addition of Akt-i IV (an Akt inhibitor). Inhibition of GSK3α and GSK3β with LiCl or SB216763 induced an increase in IL-12p40 mRNA and protein. The effect of each isoform on IL-12p40 expression was evaluated by siRNA-gene expression silencing of GSK3α and GSK3β. GSK3α gene silencing resulted in a marked increase in IL-12p40 mRNA and protein while GSK3β gene silencing had the opposite effect on IL-12p40 expression. These results indicate that the TLR2/PI3K/Akt-dependent inhibition of GSK3α activity also plays an important role in the inflammatory response caused by stimulation of BEC with PGN from *S. aureus*.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Staphylococcus aureus causes important infectious diseases in animals and humans because it expresses a wide arrange of virulence factors and cell-wall associated structures that are responsible for damaging tissues [1]. One of the main cell wall structures of *S. aureus* is the peptidoglycan (PGN) that activates the innate immune system of the host and promotes inflammation [2] via the PI3K/Akt signaling pathway [3–5]. While it has been proposed that the intracellular receptors NOD1/2, but not TLR2, are the main proteins involved in PGN signaling [2], some studies have shown that TLR2 is a major receptor that senses PGN. For example, treatment of RAW264.7 macrophages with PGN induced the TLR2-dependent recruitment of p85α, Rac1 and Ras that mediates IKKα/β-NF-κB activation, and COX2 expression through the Rac1/PI3K/Akt and Ras/Raf1/Erk1-2 signaling pathways [3, 6]. Also, in BV-2 microglia, PGN activates the TLR2/MyD88/PI3K/Akt pathway, which leads to IκBα degradation, phosphorylation of NF-κB p65 subunit (p65) at Ser536, and expression of pro-inflammatory cytokines, iNOS, and COX2 [4]. Moreover, PGN binds to TLR2 in fibroblasts and activates the FAK/PI3K/Akt signaling and the transcription factor AP-1, leading to an increase in IL-6 expression [5].

The phosphoinositide 3-kinase/Akt (PI3K/Akt) signaling pathway mediates a variety of cellular responses such as survival, proliferation, differentiation, apoptosis, and as mentioned above, inflammation [7, 8]. Activation of PI3K/Akt pathway leads to the PI3K-dependent synthesis of phosphatydilinositol-3,4,5-triphosphate (PIP_3) and phosphorylation of Akt at Thr308 and Ser473 by the constitutively active PDK1 [9, 10] and mTORC2 [11, 12]. Akt in turn regulates the activity of a wide range of substrates, among which glycogen synthase kinase 3 (GSK3) is important in the modulation of the inflammatory response [8, 13]. GSK3 refers to two mammalian paralogs that are commonly called GSK3α and GSK3β isoforms [14], which are constitutively active and can be inactivated by phosphorylation at Ser21 (GSK3α) or Ser9 (GSK3β) by Akt [15]. Since its discovery, GSK3β has been shown to be involved in the regulation of many cellular functions including growth, differentiation, embryonic development, cell cycle progression, apoptosis [16, 17] and in the inflammatory response caused by bacterial infection through the regulation of NF-κB activity [13, 15]. In regard to GSK3α most of the glucose/glycogen homeostasis appears to depend mainly on this isoform, with a minor contribution of GSK3β in skeletal muscle [18–20]. Also, GSK3α plays a potential role as a regulatory enzyme of the central nervous system [21]. This isoform, but not GSK3β, has recently been identified in the maintenance and/or proliferation of Th17 cells stimulated with the pro-inflammatory cytokine IL-1 [22]. However, participation of GSK3α in the modulation of inflammation, triggered by microbial products has not been well documented. Studies in murine models indicate that both isoforms of GSK3 are not physiologically redundant [16, 18, 23]. Recently, it was found that LiCl inhibition of GSK3α in lipopolysaccharide (LPS)-activated neutrophils and in the murine dorsal air-pouch model lead to a large increase in TNF-α secretion by affecting the translational mechanism of the TNF-α protein without altering its mRNA levels [24]. Furthermore, in a previous report we demonstrated that internalization of *S. aureus* by endothelial cells is associated with the PI3K/Akt activity [25]. Although the correlation between *S. aureus* internalization and GSK3α or GSK3β activity was not analyzed in that report, we indeed observed a higher phosphorylation of GSK3α at Ser21 than GSK3β at Ser9 [25]. Thus, it is likely that GSK3α has also regulatory functions in the inflammatory response induced by *S. aureus*.

Interleukin (IL)-12 is an important pro-inflammatory cytokine because its expression during bacterial infection regulates the innate response and determines the type and duration of the adaptive immune response [26, 27]. Structurally, this cytokine is a heterodimer composed of two subunits designated p35 and p40 linked by disulfide bonds [28, 29]. The *IL-12p40* gene is highly inducible by microbial products such as LPS, lipoteichoic acid (LTA) and PGN via

Toll-like receptor signaling and NF-κB activation [27]. Antigen-presenting cells and phagocytic cells are the primary producers of IL-12 [27], although human endothelial cells also produce it [30]. Despite IL-12 is essential for host defense, its overexpression can cause persistent inflammation giving rise to autoimmune disorders. To counterbalance the action of IL-12, immune cells produce IL-10 that decreases NF-κB and AP-1 activity, and at the same time increases CREB activity [31, 32].

Stimulation of human monocytes and peripheral blood mononuclear cells (PBMCs) with agonists of TLR2 (LTA from *Streptococcus pneumoniae*), TLR4 (LPS or synthetic lipid A), TLR5 (flagellin from *Salmonella* Typhimurium), or TLR9 (human CpG), reduced the expression of IL-12p40 through the inhibition of GSK3β with no participation of the GSK3α isoform [32]. In contrast, data presented in this work indicate that stimulation of bovine endothelial cells (BEC) with PGN from *S. aureus* modulates the expression of IL-12p40 through the inhibition of GSK3α and GSK3β. Interestingly, inhibition of GSK3α with pharmacological drugs or its gene expression silencing with interference RNA in BEC stimulated with PGN produced a marked increase in the expression of IL-12p40. In similar experiments, directed to GSK3β, we observed a reduced expression of IL-12p40. In both cases the mechanism involved the activation of the TLR2/PI3K/Akt signaling pathway. Altogether, the biochemical evidence presented indicates that both isoforms of GSK3 differentially modulate the expression of IL-12p40, a pro-inflammatory cytokine.

Materials and Methods

Media and Chemicals

F-12 Ham (HF-12) of Dulbecco's modified Eagle's medium, bovine serum albumin (BSA), trypsin-EDTA, Igepal CA-930, PGN from *S. aureus* 77140, Wortmannin (Wort), Akt-i IV, LY294002 (LY), SB216763 (SB), NaCl, LiCl, puromycin, and Bradford reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Fetal calf serum (FCS) was acquired from Equitech-Bio, Inc. (Kerrville, TX, USA). A cocktail of sodium penicillin G, streptomycin sulfate, and amphotericin B was purchased from Gibco-BRL (Gaithesburg, MD, USA). Akt Inhibitor II, D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy) propyl hydrogen phosphate (SH-5) was acquired from Calbiochem (Darmstadt, Germany). Halt Phosphatase inhibitor cocktail was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Protease inhibitor cocktail was acquired from GE Healthcare Bio-sciences (Little Chalfont, UK). Trizol reagent and EXPRESS One-Step SYBR GreenER Universal Kit were purchased from Invitrogen (Carlsbad, CA, USA). Bovine Interleukin 12 (IL-12/p40) TSZ ELISA kit was purchased from Biotang (Massachusetts, USA). All other reagents were acquired from Sigma-Aldrich.

Antibodies

Rabbit polyclonal antibodies against the extracellular domain of TLR2 (N-17; sc-8689), the isotype unspecific IgG, and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against phospho-glycogen synthase (Ser641) and rabbit monoclonal antibodies against phospho-Akt (Ser473), phospho-GSK3α (Ser21), phospho-GSK3β (Ser9), phospho-p65 (Ser536), Akt, GSK3β, and NF-κB p65 subunit were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell Line and Culture Conditions

The endothelial cell line used as a model cell in this study was obtained from bovine umbilical veins and immortalized by transfection with an expression vector containing the E6-E7

oncogenes of human papillomavirus 16 (BVE-E6E7) [33]. This immortalized bovine endothelial cell line, called BEC in this study, was grown and maintained in HF-12 supplemented with 10% FCS and a cocktail of sodium penicillin G, streptomycin sulfate, and amphotericin B, unless otherwise noted.

Purification of PGN

We eliminated lipopeptides from commercial PGN preparations from *S. aureus* (Sigma-Aldrich) as previously described by Dziarski and Gupta (2005) [34]. Briefly, 2 mg of PGN per mL were treated with 8% sodium dodecyl sulfate (SDS) at 90°C for 30 min, followed by 10 washes with H_2O to remove SDS. The concentration of purified PGN was calculated spectrophotometrically at 450 nm by adjusting the value of the dispersion to the same value obtained from commercial PGN that was prepared at 1 μg/mL.

BEC Transfection

BEC (2×10^5) were grown in six-well culture plates with HF-12K without serum and antibiotics for 24 h. Then, each well was transfected with the X-tremeGENE HP DNA Transfection Reagent kit (Roche). We used either 3 μg of siRNA-GSK3α plasmids (Sigma Aldrich, clone IDs, pLKO.1-GSK3α1: NM_019884.1-948s1c1; pLKO.1-GSK3α2: NM_019884.1-567s1c1, and pLKO.1-GSK3α3: NM_019884.2-1207s1c1) or siRNA-GSK3β plasmids (pLKO.1-GSK3β1 and pLKO.1-GSK3β2) that were gifts from Alex Toker (Addgene plasmid # 32496 and # 32497). Control cells were transfected with 3 μg of the pLKO.1 plasmid (pLKO.1) that was a kind gift from Bob Weinberg (Addgene plasmid # 8453). For GSK3α the targeting sequences were: 5'-TACATCTGTTCTCGCTACTA-3' (nucleotides 1535–1554, pLKO.1-GSK3α1); 5'-CCAGGACAAGAGGTTCAAGAA-3' (nucleotides 1153–1173, pLKO.1-GSK3α2); 5'-CCTGGACAAAGGTGTTCAAAT-3' (nucleotides 1788–1808, pLKO.1-GSK3α3); 5'-GAAGTCAGCTATACAGACACT-3' (nucleotides 587–607, pLKO.1-GSK3β1) and 5'-GAAAGCTAGATCACTGTAACA-3' (nucleotides 735–755, pLKO.1-GSK3β2). After 24 h, the culture medium was changed to HF-12K with serum plus 0.8 μg/mL of puromycin and cells were incubated for 15 days to select for stable transfections. BEC were recovered and sub-cultured in six-well culture plates in HF-12K without serum, incubated for 24 h and stimulated with PGN, as described below.

Protein Extraction and Western Blot Assays

To test for the relative abundance of phosphorylated and non-phosphorylated proteins, BEC were grown in six-well tissue culture plates (Ultra Cruz) to approximately 90% confluence before serum starvation for at least 4 h. Total protein (cytosolic plus nuclear) from control and treated cells was obtained by washing the cells 2X with cold PBS and lysing them with 100 μl of a cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-930, 10 mM Na-pyrophosphate, 50 mM NaF and 1 mM Na-orthovanadate supplemented with 1X protease inhibitor cocktail and 1X phosphatase inhibitor cocktail, which were added immediately before lysing the endothelial cells. The lysates were centrifuged at 16,000 xg for 20 min at 4°C and the supernatant was transferred to ice-cold Eppendorf tubes. Protein concentration was measured by the Bradford method [35] using BSA as standard. Then, 30–40 μg of protein was separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and electroblotted in a wet chamber onto 0.45 μm nitrocellulose membrane (Bio-Rad) at 250–300 mA for 1 h. Membranes were probed with primary polyclonal antibodies to phosphorylated forms of Akt, GSK3α, GSK3β, or p65. Then, membranes were stripped, reprobed with secondary monoclonal antibodies to the non-phosphorylated form of Akt or polyclonal antibodies to GSK3β or

p65 as controls of protein loading, and detected with the Immobilon Western Chemiluminescent HRP substrate kit from Millipore (Billerica, MA, USA).

RNA Extraction and qRT-PCR

To analyze the relative expression of IL-12p40 mRNA, BEC were grown in six-well culture plates to approximately 90% confluence before serum starvation for at least 4 h. Then 10 μg/mL of PGN was added to the cultured cells, centrifuged at 130 xg for 5 min and incubated for 2, 4 or 8 h at 37°C in 5% CO_2, or pretreated with 10 μM SH-5, 10 μM SB216763, 10 mM NaCl, 10 mM LiCl or 5 μg/mL anti-TLR2 for 1 h, or with Akt-i IV for 0.5 h followed by washing with HF-12 without serum and stimulation with 10 μg/mL PGN, centrifuged at 130 xg for 5 min and incubated for 4 h at 37°C in 5% CO_2. At the end of the incubation period, BEC were washed 2X with cold PBS and total RNA was extracted using 1 mL Trizol of reagent following the isolation procedure described by the supplier. One-step reverse transcription and real-time quantitative PCR (qRT-PCR) was performed using the EXPRESS One-Step SYBR GreenER Universal Kit and the real-time StepOnePlus thermocycler from Applied Biosystems. Each reaction was performed with 100 ng/μL of RNA under the standard 20 μL reaction provided by Invitrogen. The one-step cycling program conditions was: 58°C (for *IL12p40*) and 50°C (for *β-actin*) for 5 min (cDNA synthesis); 95°C for 2 min; 40 cycles at 95°C for 15 s and 55°C (for *IL12p40*), 60°C (for *β-actin*) for 1 min. The oligonucleotide primers used were based on the sequences published by Konnai et al. 2003 [36]. Amplification of the expected single products (186 pb for *IL12p40* and 227 pb for *β-actin*) was confirmed by visualization on 1% agarose gels stained with ethidium bromide. Relative transcript levels of IL-12p40 mRNA were calculated with the delta-delta C_t method, using β-actin as the reference gene.

Measurement of IL-12p40 protein levels

Bovine IL-12p40 protein in culture supernatants was measured by sandwich ELISA, according to manufacturer instructions (Biotang).

Statistical analysis

The relative abundance of phosphorylated proteins was quantitated by densitometric analysis with the Image Processing and Analysis in Java Program ImageJ (http://rsbweb.nih.gov/ij). To calculate the densitometric values, the intensity of the phosphorylated band was divided by the intensity of the non-phosphorylated ones. These intensity values were referred to a value of 1.0 that was arbitrarily assigned to the untreated control. The statistical significance of triplicate blots, IL-12p40 mRNA levels and IL-12p40 protein levels were evaluated with the Tukey multiple-comparison test and One-Way analysis of variance (ANOVA) by using the SIGMASTAT program version 3.0 (SPSS Inc., Chicago, IL, USA). P values <0.05 or <0.01 or <0.001 were considered statistically significant.

Results

Phosphorylation of Akt, GSK3α and GSK3β in BEC Stimulated with PGN Depends on TLR2

In macrophages, microglia and fibroblasts PGN induces the expression of pro-inflammatory molecules by activation of the PI3K/Akt signaling pathway in a TLR2-dependent manner [3–6], and Akt-dependent inhibition of GSK3β by phosphorylation at Ser9 [15]. Therefore, we first decided to explore the involvement of TLR2 in the phosphorylation of Akt, GSK3α and GSK3β in BEC stimulated with PGN. We observed that in BEC stimulated with 10 μg/mL of

PGN for 15 min (S1A and S1B Fig), the relative abundance of phosphorylated Akt (~2-fold), GSK3α (~6-fold) and GSK3β(~3-fold) were inhibited in BEC pre-treated with a neutralizing antibody against the extracellular domain of TLR2 (Fig 1A, 1B and 1C). Phosphorylation of Akt was not affected in BEC pre-treated with an unspecific isotype IgG (Fig 1D). It has been reported that PGN induces the expression of TLR2 in microglia and fibroblasts [4, 5]. To rule out this effect, we evaluated the relative abundance of TLR2 in BEC stimulated with PGN at different times and no change was observed in the levels of TLR2 (S1C Fig). A complete loss of Akt phosphorylation was observed when BEC were pre-treated with LY-294002 (LY, an inhibitor of PI3K) and then stimulated with PGN (S1D Fig). Analysis of GSK3α/β phosphorylation confirmed that stimulation of BEC with 10 μg/mL of PGN for 15 to 60 min induced a strong increase in GSK3α phosphorylation at Ser21 compared with the minor increase in GSK3β phosphorylation at Ser9 (S2A and S2B Fig). These results indicate that 1) PGN induces TLR2-dependent phosphorylation of Akt, GSK3α and GSK3β in endothelial cells and 2) GSK3α was consistently the isoform with the highest level of phosphorylation.

PGN Activates the PI3K/Akt-Dependent Phosphorylation of GSK3α and GSK3β in BEC

Next, we tested if phosphorylation of GSK3α and GSK3β in endothelial cells stimulated with PGN required activation of PI3K and Akt. Pre-treatment of BEC with LY or Wortmannin (Wort, an inhibitor of PI3K) and SH-5 (an inhibitor of Akt) and stimulated with PGN induced a significant decrease in GSK3α and GSK3β phosphorylation (Fig 2A and 2B). No significant changes in phosphorylation levels were observed when cells were treated with the inhibitors for PI3K (LY), Akt (Akt-i IV) and GSK3 (SB-216763, SB) alone (S3A–S3C Fig). These data indicate that phospho-inhibition of GSK3α and GSK3β in BEC depends on the PI3K and Akt activity.

Phosphorylation of GSK3α and GSK3β by Akt in BEC Stimulated with PGN Inhibited the Ability of both Isoforms to Phosphorylate Glycogen Synthase

To test if phosphorylation of GSK3α at Ser21 and GSK3β at Ser9 resulted in the inhibition of their enzymatic activity, we evaluated the phosphorylation of glycogen synthase (GS) at Ser641, one of the downstream targets of both isoforms. Incubation of BEC with PGN caused the inhibition of 40% to 60% GS phosphorylation, compared with the untreated control (Fig 3). Pre-treatment of BEC with LiCl or SB (two inhibitors of GSK3), SB plus PGN or LiCl plus PGN caused an even stronger reduction in GS phosphorylation. These effects were not due to an osmolarity effect because treatment of BEC with 10 mM NaCl did not change the phosphorylation level of GS in cells not stimulated or stimulated with PGN (Fig 3). These data indicate that phosphorylation of GSK3 in BEC stimulated with PGN induced a significant reduction of GSK3 activity.

PGN Regulates the Expression of IL-12p40 through a Mechanism that Involves TLR2/Akt Activation and GSK3 Inhibition

One of the most important cytokines produced during the initial stages of the inflammatory response is the IL-12p40. The expression of this cytokine in macrophages stimulated with different PAMPs is down-regulated due to the inhibitory effect on GSK3β activity [32]. Therefore, we determined if IL-12p40 expression is regulated in a similar manner in endothelial cells stimulated with PGN from *S. aureus*. When transcript levels of IL-12p40 were analyzed in BEC stimulated with PGN we observed an increase in IL-12p40 mRNA at 4 h, which decreased to

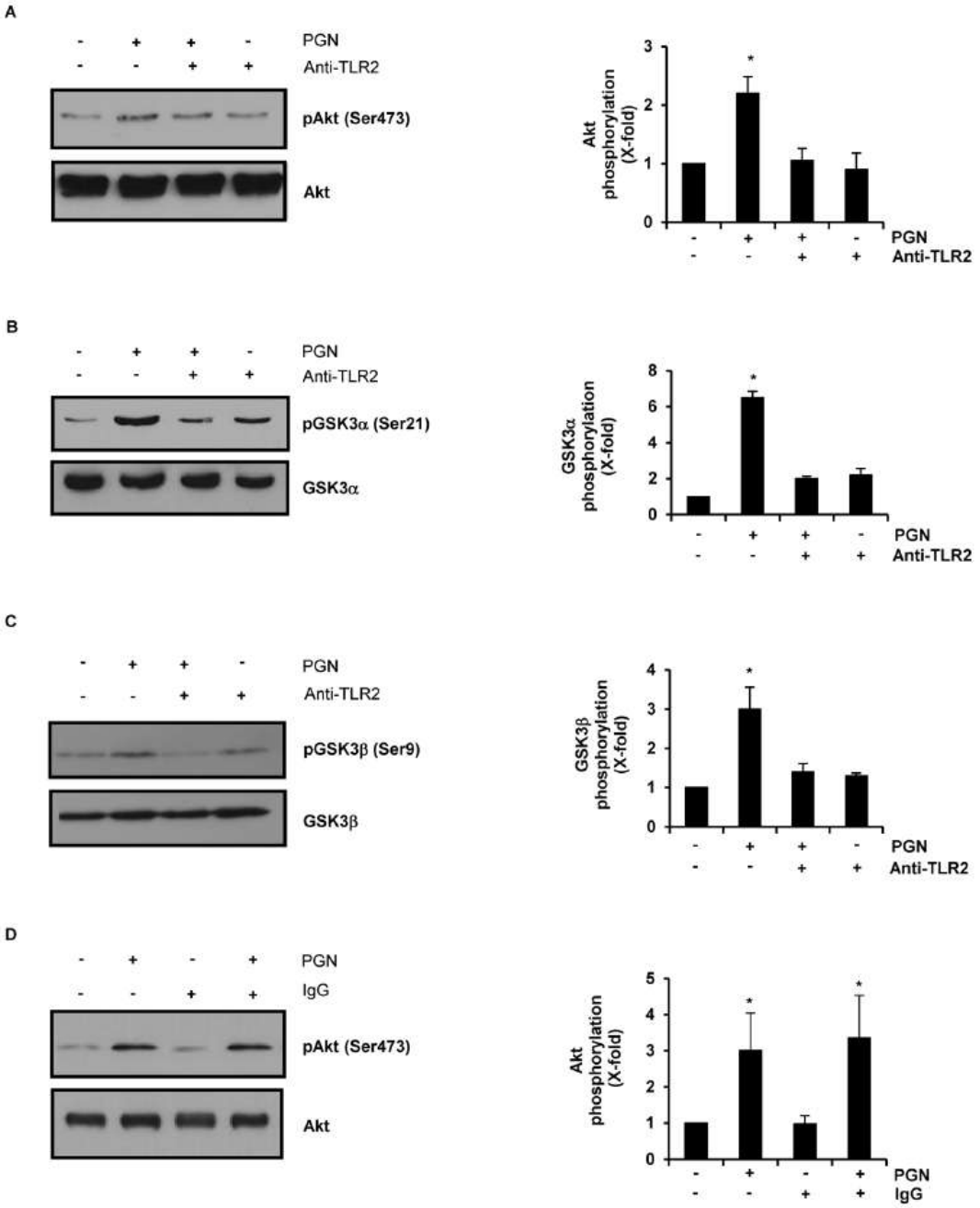

Fig 1. Peptidoglycan (PGN) induces phosphorylation of Akt, GSK3α and GSK3β in bovine endothelial cells (BEC) through TLR2 activation. A, B, C) BEC were either stimulated with 10 μg/mL of PGN for 30 min, pre-incubated with 5 μg/mL of neutralizing antibody against the extracellular domain of TLR2 (anti-TLR2) for 60 min and stimulated with 10 μg/mL of PGN for 30 min or pre-incubated with 5 μg/mL of anti-TLR2 for 60 min. D) BEC were either stimulated with 10 μg/mL of PGN for 30 min, pre-incubated with 5 μg/mL of an isotype anti-IgG for 60 min or pre-incubated with 5 μg/mL of anti-IgG for 60 min and stimulated with 10 μg/mL of PGN for 30 min. As control in all cases A-D, BEC were left untreated (-). After treatments, protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of Akt1 (pAkt Ser473), GSK3α (pGSK3α Ser21) or GSK3β (pGSK3β Ser9). Blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated forms of Akt (A, and D), GSK3α (B) or GSK3β (C) to verify equal protein loading. Blots are representative of three independent experiments. Graphs on the right panel indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$, compared with the untreated control.

doi:10.1371/journal.pone.0132867.g001

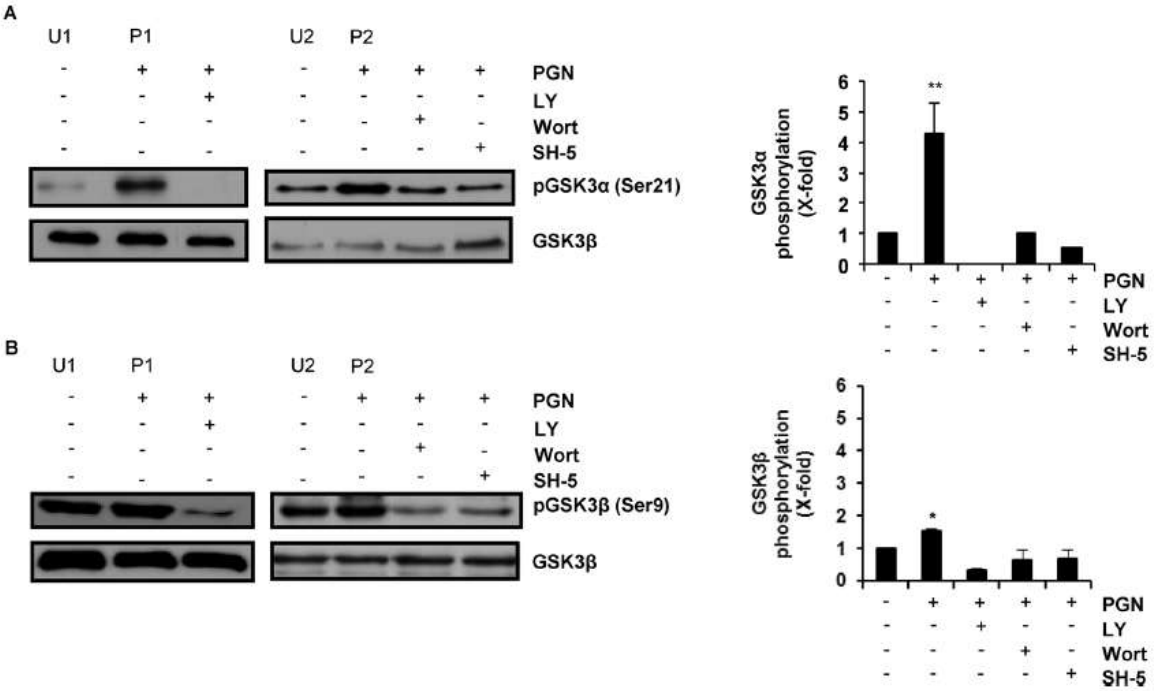

Fig 2. PGN induces PI3K/Akt-dependent phosphorylation of GSK3α and GSK3β in BEC. A-B) BEC were untreated and unstimulated (U1 and U2), stimulated with 10 μg/mL of PGN for 30 min (P1 and P2) or treated with either 10 μM of LY294002 (LY), 100 nM of Wortmannin (Wort) or 10 μM of SH-5 for 30 min, and then stimulated with 10 μg/mL of PGN for 30 min. Protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of GSK3α (pGSK3α Ser21) or GSK3β (pGSK3β Ser9. To verify for equal amount of proteins, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of GSK3β. Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis. In each graph, the densitometric control values plotted were the average of U1 + U2 while the values plotted for the PGN-stimulated cells were the average of P1 + P2. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$; **$p < 0.01$, compared with the unstimulated control.

doi:10.1371/journal.pone.0132867.g002

the control level at 8 h (Fig 4A). This increase was dependent on TLR2 activation because treatment of BEC with a neutralizing anti-TLR2 reduced the IL-12p40 to the level of control mRNA (Fig 4B and 4C). Inhibition of Akt activity strongly reduced the IL-12p40 protein levels (Fig 4D). In contrast, inhibition of GSK3 activity with LiCl or SB caused a strong increase in IL-12p40 protein amounts (Fig 4E and 4F). These results indicate that IL-12p40 expression in BEC stimulated with PGN was associated with activation of TLR2/Akt and inhibition of GSK3.

GSK3 can both positively and negatively affect different transcription factors such as NF-κB and CREB that are responsible for the regulation of pro- and anti- inflammatory cytokine production, respectively [32]. Thus, we tested the relative abundance of NF-κB p65 subunit phosphorylation at Ser536 (one of the phosphorylation sites at the transactivation domain) when both isoforms of GSK3 were inhibited with LiCl and stimulated with PGN. Data obtained show a ~5-fold increase in p65 phosphorylation in BEC stimulated with PGN alone and an even stronger increase (~9 fold) in BEC pre-treated with LiCl (S4A Fig). Pretreatment of BEC with NaCl was included to rule out any osmolarity effect (S4A Fig). Next, we performed GSK3α/β gene silencing. Surprisingly, siRNA gene silencing of either GSK3α or GSK3β in BEC stimulated with PGN produced a strong increase in p65 phosphorylation (S4B Fig). We reproducibly observed less phospho-p65 relative abundance when expression of GSK3β was silenced by siRNA. Perhaps, this may imply that phosphorylation at Ser536 contributes differentially not only to the expression of IL-12p40 but also to other NF-κB-regulated genes.

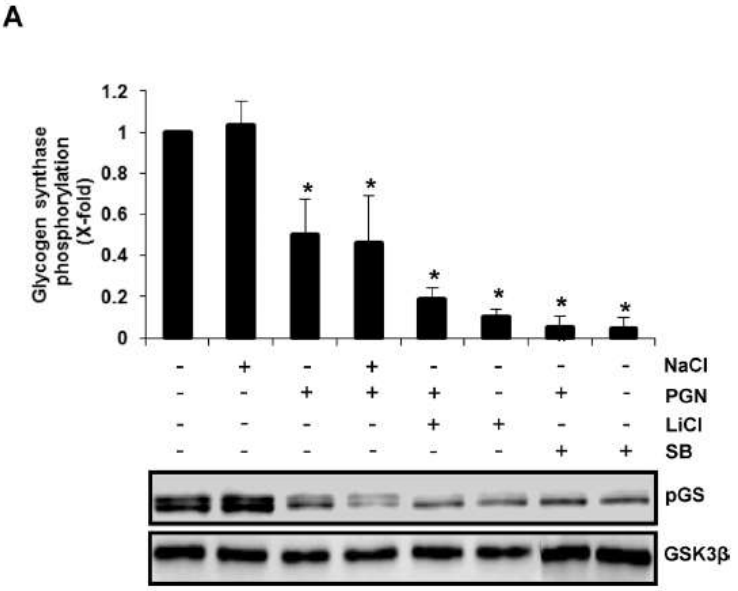

Fig 3. Inhibition of glycogen synthase phosphorylation at Ser641 in BEC treated with PGN, LiCl or SB, and stabilization of β-catenin levels by GSK3α or GSK3β gene silencing. A) BEC were either stimulated with 10 μg/mL of PGN for 30 min, treated with 10 mM NaCl for 60 min, 10 mM LiCl or 10 μM SB and then stimulated with 10 μg/mL of PGN for 30 min. As controls BEC were left untreated (-) or incubated with NaCl, LiCl or SB alone for 60 min. B) BEC were transfected with siRNA targeting GSK3α (siRNA GSK3α), siRNA targeting GSK3β (siRNA GSK3β), control siRNA (siRNA control) or left untransfected (-) for 16 days. After transfection, BEC were incubated for 24 h. Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated form of glycogen synthase (GS Ser641) or the nonphosphorylated form of β-catenin. To verify that equal amount of protein was loaded in each lane, blots were stripped and reprobed with antibodies that recognize the nonphosphorylated forms of GSK3β (A) or β-actin (B). Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p<0.05$, compared with the untreated (A) or untransfected (B) control.

doi:10.1371/journal.pone.0132867.g003

Inhibition of GSK3α Activity is Responsible for IL-12p40 Increase

A number of reports have confirmed that GSK3β is the isoform involved in the regulation of pro- and anti-inflammatory cytokines expression. Such experimental evidence, along with the fact that in our case we consistently observed that GSK3α was the isoform more phosphorylated when BEC were stimulated with PGN, prompted us to explore which GSK3 isoform was responsible for the IL-12p40 increase. First, we obtained the evidence of gene silencing for each isoform (Fig 5A). Next, as another control of GSK3α/β gene silencing, we measured the total (cytoplasmic and nuclear) relative abundance of β–catenin because it is known that in resting cells the constitutive activity of GSK3 negatively regulates the Wnt/β-catenin signaling. That is GSK3-dependent phosphorylation of β-catenin promotes its ubiquitylation and subsequent degradation by the proteasome 26S [20]. Thus, when the activity of GSK3 isoforms is inhibited by chemical compounds or the genes of the isoforms are silenced by siRNA treatment, the phosphorylation of β-catenin is blocked and this in turn induces an intracellular accumulation of β-catenin. Transfection of BEC with siRNA targeting GSK3α (siRNA GSK3α) or with siRNA targeting GSK3β (siRNA GSK3β) affected the activity of GSK3 because we detected an increase in total β-catenin levels compared with un-transfected or transfected BEC with control siRNA (siRNA control) (Fig 5B, S1 Dataset). These data demonstrate that the activity GSK3α

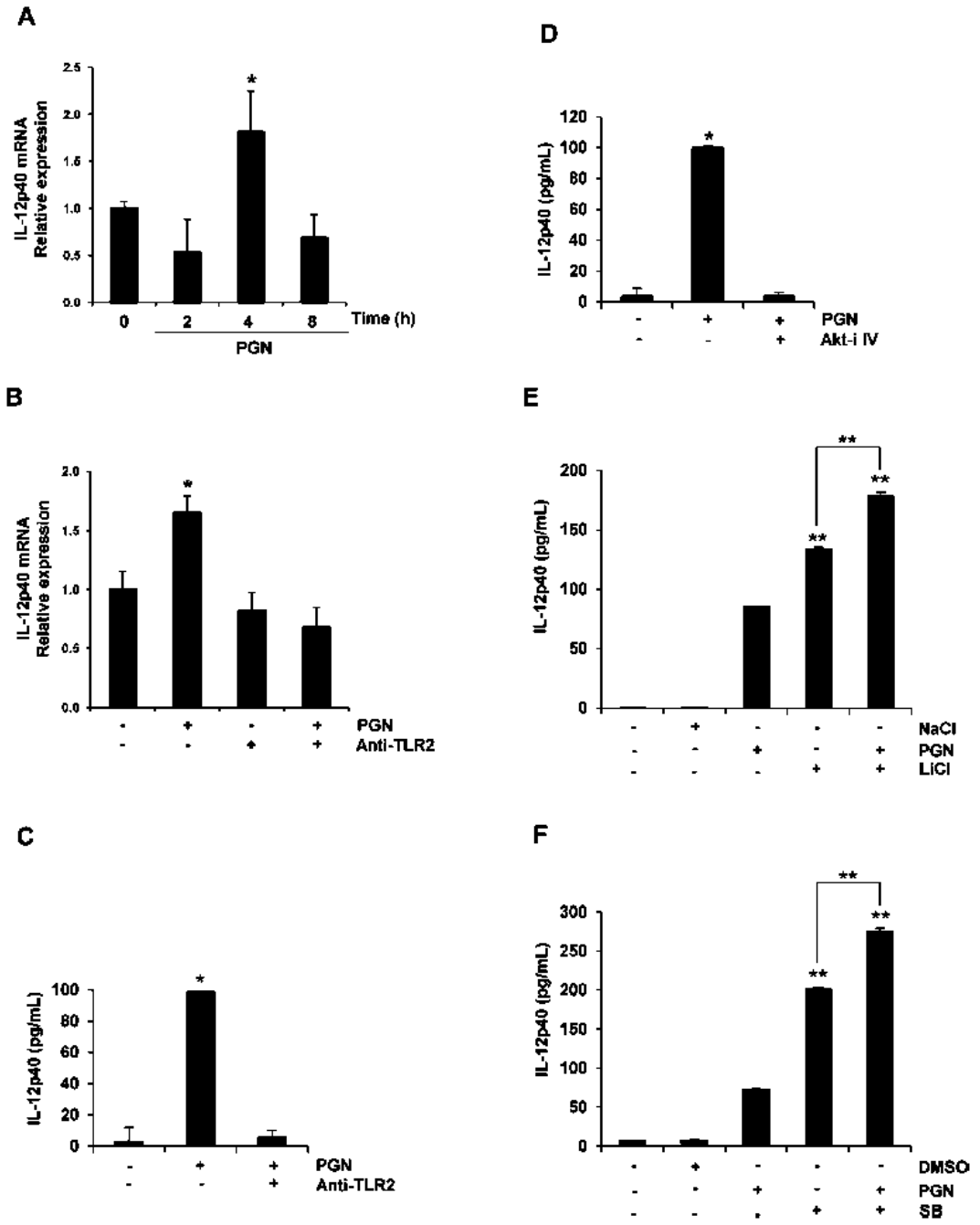

Fig 4. PGN Induces IL-12p40 expression through TLR2/Akt activation and GSK3 Inhibition in BEC. A) BEC were left untreated and unstimulated (0) or stimulated with 10 μg/mL of PGN-prep for 2, 4 or 8 h. B) BEC were stimulated with 10 μg/mL of PGN for 4 h, treated with 5 μg/mL of neutralizing anti-TLR2 for 60 min, or treated with 5 μg/mL of anti-TLR2 for 60 min and then stimulated with 10 μg/mL of PGN for 4 h. C) BEC were stimulated with 10 μg/mL of PGN for 9 h or treated with 5 μg/mL of anti-TLR2 for 60 min and then stimulated with 10 μg/mL of PGN for 9 h. D) BEC were stimulated with 10 μg/mL of PGN for 9 h or pretreated with 1 μM of Akt inhibitor IV (Akt-i IV) for 30 min and then stimulated with 10 μg/mL for 9 h. E) BEC were treated with 10 mM NaCl for 60 min, stimulated with 10 μg/mL of PGN for 9 h, treated with 10 mM of LiCl for 60 min or treated with 10 mM of LiCl for 60 min and then stimulated with 10 μg/mL of PGN for 9 h. F) BEC were treated with 10 μM of DMSO, stimulated with 10 μg/mL of PGN for 9 h, treated with 10 μM of SB 216763 (SB) for 30 min or treated with 10 μM of SB 216763 (SB) for 30 min and then stimulated with 10 μg/mL of PGN for 9 h. As controls, in B-F BEC were left untreated and stimulated (-). Total RNA was extracted and relative transcript level of IL-12p40 was quantitated by qRT-PCR using the delta-delta Ct method, and amplification of β-actin

as a reference gene (A-B). Cell-free supernatants were analyzed by ELISA for production of IL-12p40 (C-F). Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$; **$p < 0.01$. All data were compared with the untreated and unstimulated controls.

doi:10.1371/journal.pone.0132867.g004

and GSK3β regulates the stability of β–catenin in BEC, as it has been confirmed in other types of cells [20].

Analysis of IL-12p40 expression at the level of mRNA and protein showed a significant increase in BEC transfected with siRNA GSK3α and stimulated with PGN, compared with BEC stimulated with PGN alone or BEC transfected with siRNA control and stimulated with PGN (Fig 5C and 5D, S2 Dataset). On the other hand, and according to Martin et al. (2005) [32], a significant down-regulation of IL-12p40 was obtained in BEC transfected with siRNA GSK3β and stimulated with PGN (Fig 6A and 6B, S3 Dataset). To rule out any off-target effect of the siRNA sequence used, we tested other siRNA sequences for each GSK3 isoform. Quantitation of the IL-12p40 protein levels after silencing GSK3α or GSK3β with different siRNA sequences gave comparable values to those previously obtained (S5 Fig, and S4 Dataset). Altogether these results indicate that both GSK3α and GSK3β differentially modulate the expression of IL-12p40 in endothelial cells stimulated with PGN from *S. aureus*.

Discussion

The novel findings of this work can be summarized as follows (Fig 7): 1) GSK3 functions as a modulator of the inflammatory response in endothelial cells stimulated with PGN. Previous reports have documented that PGN activates TLR2/PI3K/Akt and induces the expression of pro-inflammatory molecules [3–5]; however, they did not explore how this activation and production of pro-inflammatory cytokines was correlated with changes in GSK3 activity; 2) although it has been accepted that GSK3β is the only isoform that mediates inflammation in cells stimulated with bacterial virulence factors and other stimuli [13] we have found that both GSK3α and GSK3β regulate IL-12p40 expression in endothelial cells stimulated with PGN; 3) the effect of the isoforms on IL-12p40 was different because inhibition of GSK3α activity resulted in the increased expression of IL-12p40 while inhibition of GSK3β activity lead to a decreased expression of the same cytokine and 4) the inhibition of GSK3α and GSK3β was linked to the activation of the TLR2/PI3K/Akt signaling pathway.

Endothelial cell activation during an inflammatory process may be divided into rapid and slow responses that are independent and dependent on new gene expression, respectively [37]. TLRs of endothelial cells play a fundamental role in the regulation of the inflammatory response upon exposure to any of the currently known TLR ligands [38]. PGN, one of the major cell-wall structures of *S. aureus*, is an important inducer of the inflammatory response [39]. Although, it is known that activation of the TLR2/PI3K/Akt signaling pathway by PGN induces the activation of NF-κB and the expression of pro-inflammatory molecules such as cytokines, COX2 and iNOS (3, 4), no report has shown that GSK3α or GSK3β inhibition by this signaling pathway regulates pro-inflammatory cytokine expression in response to PGN from *S. aureus*. Our findings, using BEC as a model cell, are the first to show that phosphoinhibition of both GSK3α and GSK3β, but predominantly GSK3α, links the TLR2/PI3K/Akt signaling pathway activation with phosphorylation of the NF-κB p65 subunit at Ser536. Furthermore, the inhibition of both isoforms is associated with a differential regulation of IL-12p40 expression (Figs 4–6), a multifunctional cytokine with important tasks in the innate and adaptive immune responses [26, 27].

A number of reports have documented that TLR2 plays a crucial role in the host response against *S. aureus* because knockout mice deficient in TLR2 are highly susceptible to

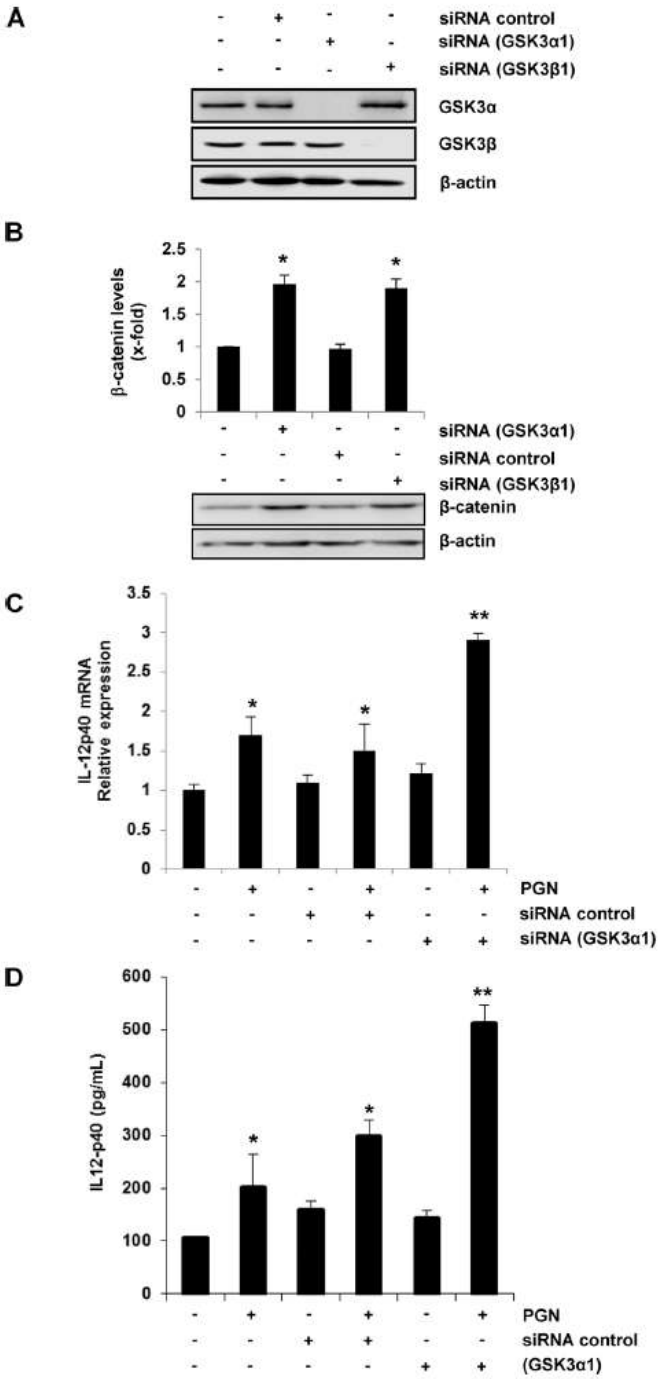

Fig 5. GSK3α inhibition up-regulates IL-12p40 expression in BEC stimulated with PGN. A) BEC were left untransfected (-), transfected with control siRNA (siRNA control), transfected with siRNA targeting GSK3α (siRNA GSK3α1) or transfected with siRNA targeting GSK3β (siRNA GSK3β1). B) BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 4 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL PGN for 4 h, transfected with siRNA targeting GSK3α (siRNA GSK3α1) or transfected with siRNA targeting GSK3α (siRNA GSK3α1) and then stimulated with 10 μg/mL of PGN for 4 h. C) BEC were left untransfected and unstimulated (-),

stimulated with 10 μg/mL of PGN for 9 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 9 h, transfected with siRNA targeting GSK3α (siRNA GSK3α1) or transfected with siRNA targeting GSK3α1 and then stimulated with 10 μg/mL of PGN for 9 h. Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated forms of GSK3α and GSK3β (A). To verify that equal amount of proteins was loaded in each lane, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of β-actin (A). Blots are representative of three independent experiments. Total RNA was extracted and relative transcript level of IL-12p40 was quantitated by qRT-PCR, using the delta-delta Ct method and amplification of β-actin as a reference gene (B). Cell-free supernatants were analyzed by ELISA for production of IL-12p40 (C). Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$; **$p < 0.01$. All data were compared with the untreated and untransfected controls.

doi:10.1371/journal.pone.0132867.g005

staphylococcal infections [40]. However, the specificity of TLR2 for PGN is still an issue of debate. According to Travassos et al. (2004) [41] highly purified PGN did not activate TLR signaling. In contrast, several other authors have proposed that PGN activates TLR2 [3–6, 42] and studies with PGN from *S. aureus* lacking lipidated prelipoproteins have co-localized them with Nod2, TLR2 and TLR4 in keratinocytes from murine oral epithelium and HEK293/hTLR2 cells, demonstrating that staphylococcal PGN, and not the associated lipoproteins, is able to trigger a TLR2 specific immune response [43]. Our data support the notion that PGN activates signaling that are associated with TLR2 activation in BEC because blocking of TLR2 with a TLR2 specific neutralizing antibody inhibited the phosphorylation of Akt, phospho-inhibition of GSK3α and GSK3β (Fig 1) and expression of IL-12p40 (Fig 4). Moreover, the PGN used in this study to stimulate BEC was purified with hot SDS, which eliminates the lipopeptides [39]. In agreement with our work, Zhang et al. (2012) [44] observed that TLR2 was activated in dendritic cells stimulated with heat-killed *Brucella abortus*, and this was an essential step for IL-12p40 induction through the activation of subpathways that regulate TLR9 signaling. In a different report, Satta et al. (2008) [45] detected an induction of TLR2 expression in human endothelial cells that served to amplify the inflammatory response to lipopeptides. This was not the case in our study because levels of TLR2 protein in BEC were not modified by PGN treatment, which means that an increase in TLR2 abundance is not a requirement for IL-12p40 expression in BEC stimulated with PGN from *S. aureus*.

Although it is well established that Akt phosphorylates and inactivates GSK3α and GSK3β, as we have observed in this study, Gulen et al. (2012) [22] showed that GSK3α, but not GSK3β, can reversely phosphorylates and suppresses Akt activation in resting Th17 cells. These authors also demonstrated that activation of Th17 treated with IL-1 leads to an increase of IKKi activity and GSK3α phosphorylation at Ser21, promoting Akt-mTOR activation [22]. Previous evidence from our lab indicated that GSK3α and GSK3β phosphorylation, as a consequence of BEC infected by *S. aureus*, may be involved in the internalization process and perhaps the inflammatory response caused by this bacterium [25].

In the last few years experimental evidence on the different functions of GSK3α and GSK3β is accumulating [14]. Our data demonstrate that inhibition of both GSK3α and GSK3β activity exerts an opposed function on IL-12p40 expression and this is in part different from data obtained by Martin et al. (2005) [32]. These authors found that inhibition of GSK3β, but no GSK3α, activity by treatment of macrophages with LPS or synthetic lipid-A as specific ligands of TLR4 or LTA from *S. pneumoniae* as a specific ligand of TLR2, reduced the expression of IL-12p40 [32]. In contrast, our data clearly indicate that phospho-inhibition of GSK3α by treatment of BEC with PGN from *S. aureus* increased IL-12p40 expression. This suggests that although GSK3β is the isoform generally associated with the inflammatory response to bacterial infections, as reported by several authors [13, 32], GSK3α may also play an important role in this process through the regulation of pro-inflammatory cytokine expression. Evidence that

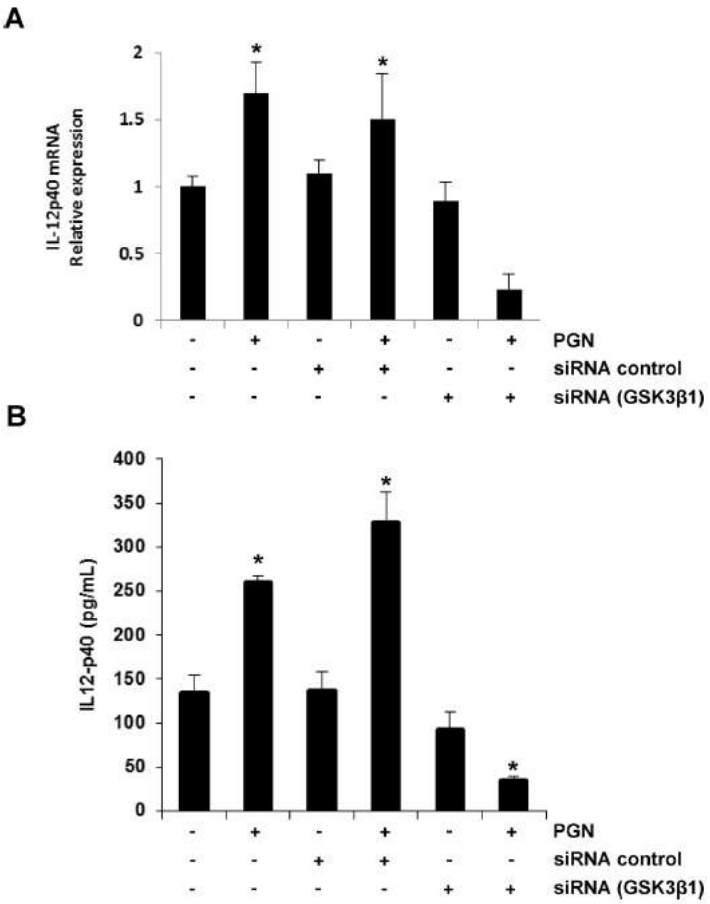

Fig 6. GSK3β inhibition down-regulates IL-12p40 expression in BEC stimulated with PGN. A) BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 4 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 4 h, transfected with siRNA targeting GSK3β (siRNA GSK3β1) or transfected with siRNA targeting GSK3β (siRNA GSK3β1) and then stimulated with 10 μg/mL of PGN for 4 h. B) BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 9 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 9 h, transfected with siRNA targeting GSK3β (siRNA GSK3β1) or transfected with siRNA targeting GSK3β (siRNA GSK3β1) and then stimulated with 10 μg/mL of PGN for 9 h. Total RNA was extracted and relative transcript level of IL-12p40 was quantitated by qRT-PCR, using the delta-delta Ct method, and amplification of β-actin as a reference gene (A). Cell-free supernatants were analyzed by ELISA for production of IL-12p40 (B). Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$. All data were compared with the untreated and untransfected controls.

doi:10.1371/journal.pone.0132867.g006

GSK3α is important in an inflammatory process was recently reported by Giambelluca et al. (2014) [24]. They found that in human neutrophils, in which the main isoform is GSK3α, the addition of LiCl resulted in a significant postranscritptional up-regulation of TNF-α secretion [24]. Interestingly, our results point out that inhibition of GSK3α activity resulted in a marked transcriptional up-regulation of IL-12p40. One explanation for these opposite results may be that active GSK3β phosphorylates NF-κB at its transactivation domain allowing the expression of IL-12p40. In a different scenario and when the activity of GSK3β is inhibited by phosphorylation at Ser9 or gene silencing, this isoform is unable to phosphorylate and activates NF-κ,

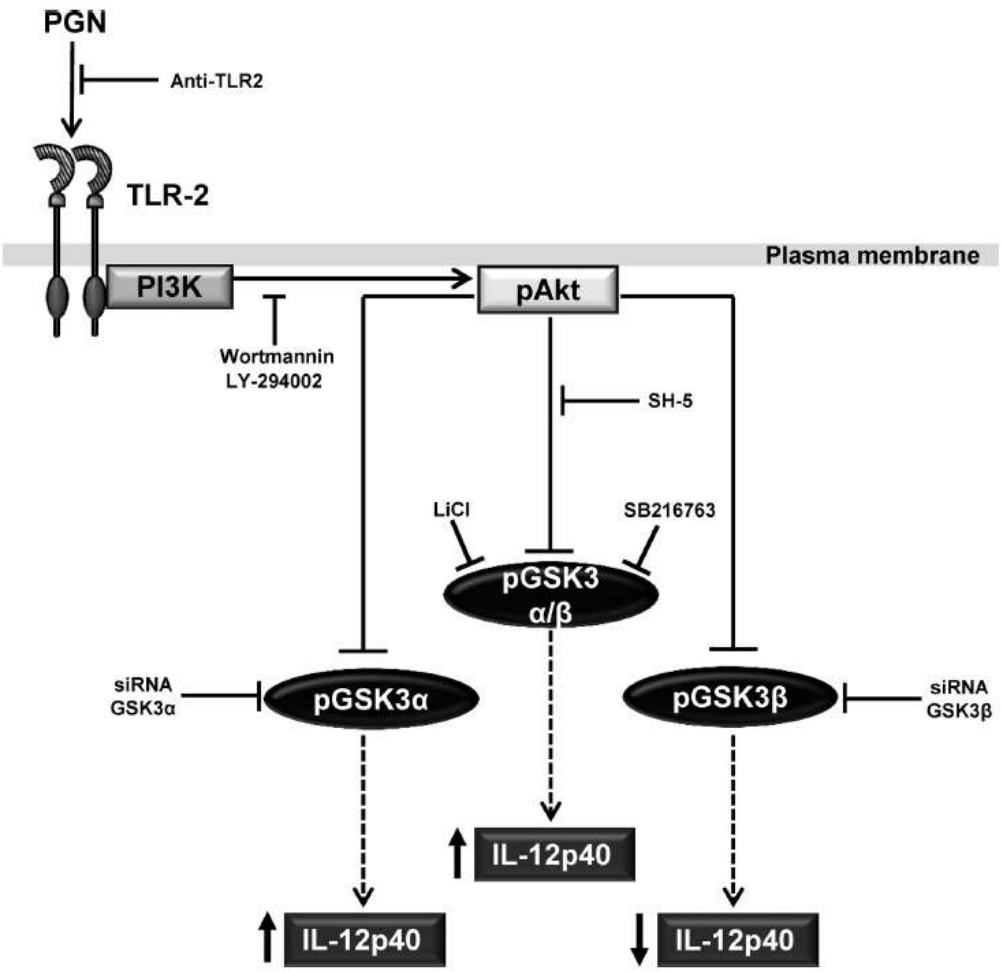

Fig 7. A working model of the signaling pathway involved on the IL-12p40 expression induced by PGN in BEC. PGN induces the activation of the TLR2/PI3K/Akt pathway, which in turn induces GSK3α and GSK3β phosphorylation/inhibition and this in turn up- or down-regulates IL-12p40 expression. Anti-TLR2, Wortmannin (PI3K inhibitor), LY-294002 (PI3K inhibitor) or SH-5 (Akt inhibitor) blocks the phosphorylation/inhibition of GSK3α and GSK3β. LiCl (GSK3 inhibitor), SB-216763 (GSK3 inhibitor) or siRNA GSK3α increases the expression of IL-12p40 while siRNA GSK3β decreases the expression of IL-12p40. Not represented in the diagram is the decrease in IL-12p40 expression when TLR2 is blocked with anti-TLR2 and when Akt is inhibited with Akt-i IV.

doi:10.1371/journal.pone.0132867.g007

causing the decrease in IL-12p40 levels. It is also likely that the type of cell used, the stimulus applied, and even the type of cytokine evaluated may explain the mechanistic differences between our results and those reported by Martin et al. (2005) [32] and Giambelluca et. al. (2014) [24]

IL-12 is a cytokine required for innate immune defense and adaptive immunity to pathogens because stimulation of peripheral blood lymphocytes and NK cells with IL-12 produced as a result of infection, induces IFN-γ secretion and increases the cytotoxicity activity as well as proliferation of these cells [26, 27]. Interestingly, it has been proposed the existence of an IL-12-regulated circuit between endothelium and lymphocytes through IFN-γ, resulting in a reciprocal modulation of cellular responses [46, 47]. Moreover, it is likely that IL-12p40 produced by endothelium recruits macrophages to the site of infection because this cytokine has been shown to have chemotactic properties [48]. Although IL-12p40 expression by CD154

stimulation was already detected in endothelium [30], we are the first to demonstrate expression of this cytokine in endothelial cells stimulated with a bacterial structure. Other authors have detected expression of IL-12-related molecules, but not expression of IL-12p40 in human intestinal microvascular endothelial cells stimulated with pro-inflammatory compounds (TNF-α, IFN-γ, IL-1β) and microbial structures [LPS, LTA, PGN, CpG-DNA, flagellin, and poly(I:C)] [49]. In this context, our data suggest that production of IL-12p40 by endothelial cells stimulated with PGN might reflect innate and adaptive immune roles of the endothelium in response to Gram positive microbial antigens. More importantly, the fine modulation in the IL-12p40 expression is of paramount importance because this cytokine is critical for host defense; however, excessive increase in its production can cause severe inflammatory disorders [31]. Therefore, it is likely that a switch in the up- and down-regulation of IL-12p40 expression should co-exist, which might depend on the differences in spatio-temporal participation of the two isoforms of GSK3 and the diverse mechanisms controlling their activity such as phophorylation, subcellular distribution and formation of molecular complexes [14, 15]. More experiments will be undoubtedly needed to clarify the mechanistic details of the differential actions of GSK3α and GSK3β on the phosphorylation of NF-κB and the expression of IL-12p40 and other cytokines during the inflammatory response caused by pathogenic bacteria.

Supporting Information

S1 Fig. PGN does not induce changes in the expression of TLR2 but activates PI3K-dependent phosphorylation of Akt in BEC. A) BEC were left untreated and unstimulated (0) or stimulated with 10 μg/mL of PGN for 15, 30, 60, 120 or 240 min. B) BEC were left unstimulated (U) or stimulated with 1, 10, 20 or 30 μg/mL of PGN for 30 min. C) BEC were left unstimulated (U) or stimulated with 10 μg/mL of PGN for 15, 30, 60 or 120 min. D) BEC were left untreated and unstimulated (U), untreated (-) or treated with 10 μM of LY294002 (LY) for 30 min and then stimulated with 10 μg/mL of PGN for 30 min. Protein extracts were analyzed by western blot and probed with a polyclonal antibody against TLR2 (A) or the phosphorylated form of Akt1 (pAkt Ser473) (B-D). To verify that equal amount of proteins was loaded in each lane, blots were stripped and reprobed with antibodies that recognize β-actin (A) or the nonphosphorylated form of Akt (B-D). Blots are representative of three independent experiments. Graphs on the right indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$, compared with the unstimulated control.
(PDF)

S2 Fig. Temporal course of GSK3α and GSK3β phosphorylation induced by PGN in BEC. A and B) BEC were left unstimulated (0) or stimulated with 10 μg/mL of PGN for 15, 30, 60 or 120 min. Protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of GSK3α (pGSK3α Ser21) or GSK3β (pGSK3β Ser9). To verify equal protein loading, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of GSK3β. Blots are representative of three independent experiments. Graphs on the right indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the unstimulated control.
(PDF)

S3 Fig. Phosphorylation of Akt at Ser473, GSK3α at Ser21 and GSK3β at Ser9 in BEC treated with inhibitors. BEC were left untreated, treated with 10 μM of LY294002 (LY) for 30 min, treated with 1 μM of Akt inhibitor IV (Akt-i IV) for 30 min or treated with 10μM of SB216763 (SB) for 30 min. Untreated cells were incubated with 10 μM of DMSO. Then, total

protein from untreated and treated cell was obtained. A) Phosphorylation of Akt at Ser473; B) Phosphorylation of GSK3α at Ser21; C) Phosphorylation of GSK3β at Ser9. Detection of β-actin and GAPDH were used as control of protein loading. Data presented are representative of two independent experiments.
(PDF)

S4 Fig. GSK3 inhibition induces phosphorylation of NF-κB. A) BEC were left untreated and unstimulated (-), treated for 60 min with 10 mM of NaCl, stimulated with 10 μg/mL of PGN for 30 min, treated for 60 min with 10 mM of LiCl and then stimulated with 10 μg/mL of PGN for 30 min or treated for 60 min with 10 mM of LiCl. B) BEC were transfected with control siRNA (siRNA control), transfected with siRNA control and then stimulated with 10 μg/mL of PGN for 30 min, transfected with siRNA targeting GSK3α (siRNA GSK3α), transfected with siRNA GSK3α and then stimulated with 10 μg/mL of PGN for 30 min, transfected with siRNA targeting GSK3β (siRNA GSK3β) or transfected with siRNA GSK3β and then stimulated with 10 μg/mL of PGN for 30 min. Protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of p65 (NF-κB p65 Ser536). To check for equal amount of proteins, blots were stripped and reprobed with antibodies that recognize the nonphosphorylated forms of p65 (A) or β-actin (B). Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the unstimulated control.
(PDF)

S5 Fig. GSK3α and GSK3β gene silencing differentially regulates IL-12p40 expression in BEC stimulated with PGN. BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 9 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 9 h, transfected with siRNA targeting GSK3α (siRNA GSK3α2 or siRNA GSK3α3) or transfected with siRNA targeting GSK3β (siRNA GSK3β1 or siRNA GSK3β2) and then stimulated with 10 μg/mL of PGN for 9 h. A) Cell-free supernatants were analyzed by ELISA for production of IL-12p40 and B) Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated forms of GSK3α and GSK3β. To verify that equal amount of protein was loaded in each lane, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of β-actin. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$.
(TIF)

S1 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

S2 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

S3 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

S4 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

Acknowledgments

To Dr. Rosa Elvira Núñez-Anita for comments to the manuscript.

Author Contributions

Conceived and designed the experiments: RCV OSG VMBA. Performed the experiments: RCV OSG JOB AHM. Analyzed the data: RCV OSG JOB. Contributed reagents/materials/analysis tools: ABP JJVA JOB BBF VMBA. Wrote the paper: RCV VMBA.

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16.3. Anexo III

Recientemente, se han sintetizado moléculas sintéticas como los péptidos inmunoreguladores (Innate Defense Regulator, IDR) que pueden inducir el control de la expresión de citocinas pro-inflamatorias, al mismo tiempo que inducen la expresión de ciertas quimiocionas como MCP-1 y MCP-3. Esto se traduce en una mayor eficiencia por parte del sistema inmune para eliminar microorganismos invasores en modelos de infección bacteriana. Además, estos péptidos han demostrado eficacia para eliminar *Staphylococcus aureus,* aun cuando se infecten ratones con cepas multiresistentes. Sin embargo, los mecanismos por los cuales los péptidos IDR como IDR-1002 controlan este proceso están poco explorados. Para probar si los péptidos IDR regulan la expresión de citocinas de una manera similar a la infección producida por *S. aureus* se analizó el efecto del péptido IDR-1002 en la activación de la vía de NF-κB y CREB. Estos experimentos demostraron que la estimulación de células tipo macrófago de ratón con el péptido IDR-1002 produjo la estabilización del inhibidor de NF-κB, la proteína IκBα y simultáneamente se produjo la activación del factor transcripcional CREB por la vía dependiente de p38-ERK-1/2-MSK1 lo que se tradujo en la disminución de la expresión del factor de necrosis tumoral α (TNFα) y la enzima ciclo-oxigenasa 2 (COX-2). Mi participación en este trabajo consistió en la co-inmunoprecipitación de los complejos CBP/CREB, CBP/NF-κB, NF-κB/IκBα, así como la detección de la fosforilación de las cinasa de IκBα e IKKα. Además, aporté ideas para experimentos y escritura parcial del artículo, lo que me permitió ser primer autor, junto a mi compañero Alejandro Huante Mendoza.

frontiers in Immunology

ORIGINAL RESEARCH
published: 25 November 2016
doi: 10.3389/fimmu.2016.00533

Peptide IDR-1002 Inhibits NF-κB Nuclear Translocation by Inhibition of IκBα Degradation and Activates p38/ERK1/2–MSK1-Dependent CREB Phosphorylation in Macrophages Stimulated with Lipopolysaccharide

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OPEN ACCESS

Edited by:
Takayuki Yoshimoto, Tokyo Medical University, Japan

Reviewed by:
Kushagra Bansal, Harvard Medical School, USA
Taishin Akiyama, University of Tokyo, Japan

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *27 July 2016*
Accepted: *11 November 2016*
Published: *25 November 2016*

Citation:
Huante-Mendoza A, Silva-García O, Oviedo-Boyso J, Hancock REW and Baizabal-Aguirre VM (2016) Peptide IDR-1002 Inhibits NF-κB Nuclear Translocation by Inhibition of IκBα Degradation and Activates p38/ERK1/2–MSK1-Dependent CREB Phosphorylation in Macrophages Stimulated with Lipopolysaccharide. Front. Immunol. 7:533. doi: 10.3389/fimmu.2016.00533

The inflammatory response is a critical molecular defense mechanism of the innate immune system that mediates the elimination of disease-causing bacteria. Repair of the damaged tissue, and the reestablishment of homeostasis, must be accomplished after elimination of the pathogen. The innate defense regulators (IDRs) are short cationic peptides that mimic natural host defense peptides and are effective in eliminating pathogens by enhancing the activity of the immune system while controlling the inflammatory response. Although the role of different IDRs as modulators of inflammation has been reported, there have been only limited studies of the signaling molecules regulated by this type of peptide. The present study investigated the effect of IDR-1002 on nuclear factor κB (NF-κB) and cAMP-response element-binding protein (CREB) transcription factors that are responsible for triggering and controlling inflammation, respectively, in macrophages. We found that TNF-α and COX-2 expression, IκBα phosphorylation, and NF-κB nuclear translocation were strongly inhibited in macrophages pre-incubated with IDR-1002 and then stimulated with lipopolysaccharide (LPS). IDR-1002 also increased CREB phosphorylation at Ser133 *via* activation of the p38/ERK1/2–MSK1 signaling pathways without detectable expression of the cytokines IL-4, IL-10, and IL-13 involved is suppressing inflammation or alternative activation. Transcriptional activation of NF-κB and CREB is known to require interaction with the transcriptional coactivator CREB-binding protein (CBP). To test for CBP–NF-κB and CBP–CREB complex formation, we performed co-immunoprecipitation assays. These assays showed that IDR-1002 inhibited the interaction between CBP and NF-κB in macrophages stimulated with LPS, which might explain the inhibition of TNF-α and COX-2 expression. Furthermore, the complex between CBP and CREB in macrophages stimulated with IDR-1002 was also inhibited, which might explain why IDR-1002 did not lead to expression of IL-4, IL-10,

and IL-13, even though it induced an increase in phospho-CREB relative abundance. In conclusion, our results indicated that IDR-1002 has a dual effect. On one hand, it inhibited NF-κB nuclear translocation through a mechanism that involved inhibition of IκBα phosphorylation, and on the other, it activated a protein kinase signaling cascade that phosphorylated CREB to selectively influence cytokine gene expression. Based on these results, we think IDR-1002 could be a potential good biopharmaceutical candidate to control inflammation.

Keywords: IDR, peptides, inflammation, macrophages, IκBα, NF-κB, CREB, TNF-α

INTRODUCTION

The inflammatory response is one of the main defense mechanisms of the innate immune system that is activated by host infection with microbial pathogens or tissue damage. This response is fundamental to identify and neutralize the causative agent and restore tissue homeostasis (1). However, in some cases, inflammation can be detrimental, especially when the control or resolution mechanisms fail, leading to chronic inflammatory conditions such as arteriosclerosis, insulin resistance, rheumatoid arthritis, and Alzheimer's disease (2).

The balance between the inflammatory response and its resolution is maintained in part by the activity of two transcription factors with opposite roles, the nuclear factor κB (NF-κB) and the cAMP-response element-binding protein (CREB). Activation of NF-κB induces expression of pro-inflammatory genes (e.g., TNF-α, IL-6, IL-8, and COX-2), while activation of CREB leads to gene expression of cytokines that have an anti-inflammatory role (e.g., IL-4, IL-10, and IL-13) (3, 4). NF-κB has five subunits but the heterodimer of NF-κB, constituted of p65 and p50 subunits, is one of the most studied active forms of this family of transcription factors (5, 6). When cells are not stimulated, NF-κB is anchored in the cytosol to IκBα protein. After stimulation, IκBα becomes phosphorylated, ubiquitinated, and degraded by the 26S proteosome. Degradation of IκBα releases NF-κB that is translocated to the nucleus where it initiates a complex transcriptional response (7). Once in the nucleus NF-κB can interact with a transcriptional coactivator CREB-binding protein (CBP) and initiate a distinct transcriptional pattern. Activation of NF-κB can also involve phosphorylation of several residues in the transactivation domain of p65 subunit, among which Ser536 is well characterized (8–10).

cAMP-response element-binding protein is a cellular transcription factor that belongs to the basic leucine-zipper domain family of proteins (11). This family is formed by dozens of proteins, including CREB, the cAMP-response element modulator (CREM), and the activating transcription factor-1 (ATF-1). In cells activated by growth factors, hormones, lipopolysaccharide (LPS), and other stimuli (12, 13), CREB is phosphorylated at Ser133 by protein kinase A, protein kinase B (Akt), MSK, or other enzymes (14, 15). Phosphorylation at this residue promotes CREB binding to transcriptional coactivator CBP, which can lead to displacement of NF-κB from the same interaction domain on CBP (16, 17). Formation of CREB–CBP complex promotes the expression of cytokines, typically involved in anti-inflammatory events or alternative activation of monocytes, thereby suppressing expression of pro-inflammatory cytokines activated by the NF-κB–CBP complex (18). Although this mechanism has been widely proposed (19–22), other groups have reported no expression of genes when CREB is phosphorylated at Ser133 downstream of mitogen-activated protein kinase (MAPK) pathways (15, 23, 24). It may imply that control of inflammation by CREB phosphorylation does not necessarily involve the expression of anti-inflammatory genes induced by this transcription factor.

Innate defense regulator (IDR) peptides were designed from the natural theme of cationic host defense peptides (25) as an alternative to traditional anti-inflammatory and antimicrobial drugs. The immunomodulatory effects of IDRs have attracted the attention because of the worldwide problem of multiple antibiotic resistance in bacteria (26–28). In addition to downregulation of the pro-inflammatory response to bacterial ligands, IDRs stimulate protective immunity (enhancing cellular recruitment and promoting differentiation of immune cells) leading to lowering of the bacterial burden without any direct interaction with bacteria (26–28). In addition to their anti-infective and anti-inflammatory effects due to modulation of the immune response, these peptides may also be considered good potential therapeutic candidates to combat chronic inflammation associated with autoimmune diseases (29, 30). One of these peptides, IDR-1002, efficiently enhanced the immune response to eliminate *Staphylococcus aureus* and *Escherichia coli* from infected tissues, even when these bacterial pathogens were multi-resistant to antibiotics (26, 27). Moreover, in synovial fibroblasts, IDR-1002 effectively controlled inflammation by selectively modulating the expression of interleukin (IL)-1Ra and IL-10 and reducing NF-κB p50 nuclear translocation induced by IL-1β (29).

Although several studies have previously reported the potential beneficial effects of IDR-1002, the molecular transduction mechanisms involved in the IDR-1002 activity have not been well described to date (26, 27, 31, 32). Therefore, we focused our attention on the NF-κB and CREB transcription factors, because they are important in triggering and resolving the inflammatory response, respectively. We found that, in RAW264.7 macrophages, stimulated with LPS IDR-1002 was able to inhibit NF-κB nuclear translocation by inhibiting IκBα degradation, leading to a reduction of cyclooxygenase-2 (COX-2), and TNF-α expression. Co-immunoprecipitation analysis of CBP–NF-κB molecular complex showed that IDR-1002 inhibited the

interaction between CBP and NF-κB induced by LPS. We also observed that IDR-1002 induced high levels of CREB phosphorylation at Ser133 through activation of p38/ERK1/2–MSK1/2 signaling pathways. Interestingly, we could not detect expression of the anti-inflammatory cytokines IL-4, IL-10, and IL-13, which are known to be regulated by CREB. By applying a similar co-immunoprecipitation analysis, the molecular complex between CBP and CREB was not detected in macrophages stimulated with IDR-1002, which may explain the lack of anti-inflammatory gene expression.

MATERIALS AND METHODS

Media and Chemicals

Innate defense regulator peptides (IDR) 1002 (VQRWLIVWRIRK-NH_2), HH2 (VQLRIRVAVIRA-NH_2), and 1 (KSRIVPAIPVSLL-NH_2) were synthesized using solid phase F-moc chemistry by CPC Scientific, Inc. (Sunnyvale, CA, USA) and were >95% pure. RPMI 1640 cell culture medium, Bradford reagent, bovine serum albumin (BSA), Wortmannin (inhibitor of PI3K), Akt IV (inhibitor of Akt), Rapamycin (inhibitor of p70S6K), SB216763 (inhibitor of GSK3), PD98059 (inhibitor of ERK1/2), and Ro-318220 (inhibitor of MSK1) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). SB203580 (inhibitor of p38) and SP600125 (inhibitor of JNK) were purchased from Calbiochem (San Diego, CA, USA). Lymphoprep™ was purchased from Axis-Shield PoC AS (Oslo, Norway). Upon arrival, all inhibitors were solubilized in DMSO and stored in aliquots at −20°C. Newborn calf serum was acquired from Hyclone Laboratories, Inc. (South Logan, UT, USA). Penicillin G and streptomycin were purchased from Gibco-BRL (Gaithesburg, MD, USA). EDTA-free protease inhibitor cocktail (50×) was purchased from Roche Applied Science (Manheim, Germany). Antibodies against β-actin (Sc-47778), COX-2 (Sc-1747), GAPDH (Sc-25778), laminin A/C (6215), IκBα (Sc-203), and phospho-IκBα (Sc-52943) for the detection of phosphorylated Ser32 and Ser36, CREB-1 (Sc-186), NF-κB p65 (Sc-8008), CBP (Sc-7300), anti-rabbit IgG-HRP (Sc-2004), rabbit anti-goat IgG-HRP (Sc-2768), and goat anti-mouse IgG-HRP (Sc-2031) were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against the phosphorylated forms of CREB at Ser133 (9196), p38 at Thr180/Tyr182 (4511), NF-κB p65 at Ser536 (3033), GSK3α at Ser21 (9316), and GSK3β at Ser9 (9336) were purchased from Cell Signaling Technology (Boston, MA, USA). Antibodies to detect phosphorylated MSK1 at Thr581 (AF2518) were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies to detect the phosphorylated forms of IKKα and β at Thr23 were obtained from Santa Cruz Biotechnology (Cat. Sc-21660, Santa Cruz, CA, USA). Non-fat dry milk was acquired from Bio-Rad (California, CA, USA) and Luminol reagent from Millipore (Billerica, MA, USA).

Cell Line and Culture Conditions

The mouse leukemic monocyte macrophage cell line RAW 264.7 used was obtained from the American Type Culture Collection (ATCC). This immortalized cell line was grown and maintained in RPMI medium supplemented with 10% FCS, unless otherwise noted.

Isolation of Human Peripheral Blood Mononuclear Cells

Venous blood (20 ml) from healthy human donors was collected in tubes containing EDTA in accordance with the approval and ethical guidelines of the Universidad Michoacana de San Nicolás de Hidalgo. Blood was diluted in the same volume of RPMI medium. Then, 20 ml of diluted blood was carefully added to a tube containing 10 ml of Lymphoprep™ and centrifuged at 800 × *g* for 20 min at room temperature (23°C). The phase containing peripheral blood mononuclear cells (PBMC) was separated and washed 3× with enough PBS to complete 50 ml for each wash and centrifuged at 250 × *g* for 10 min. After washing, PBMC were resuspended in RPMI medium and seeded in six-well plate at 2×10^6 cells/well. Cells were left resting for at least 3 h before the experiments.

Gene Knockdown of MSK1 and MSK2

MSK1 and MSK2 gene expression were silenced with siRNA acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The siRNA for MSK1 (sc-35978), MSK2 (sc-75837), and control siRNA (sc-37007) were added to macrophages at the final concentration of 40 nM for 48 h, according to the manufacturer's instructions. To test for MSK1/MSK2 participation in CREB phosphorylation at Ser133, macrophages were first gene silenced for MSK1 and/or MSK2 and then stimulated with 20 μg/ml of IDR-1002 for 15 min.

Protein Extraction and Western Blot Analysis

The relative abundance of phosphorylated and non-phosphorylated proteins was evaluated in protein extracts from macrophages grown in six-well culture plates to ~90% confluence before serum starvation for at least 4 h. For each assay, total protein (cytosolic plus nuclear from control and treated cells) was obtained by washing cells with cold PBS (2×) and lysing them with 80 μl of a cold lysis buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Igepal CA-930, 10 mM Na-pyrophosphate, and 50 mM NaF, supplemented with 1 mM Na-orthovanadate and 1× protease inhibitor cocktail that was added immediately before use. The lysates were centrifuged at 13,000 × *g* for 20 min at 4°C, and the supernatant was transferred to ice-cold Eppendorf tubes. Nuclear and cytoplasmic fractions were prepared from cells using the NE-PER Reagent kit from Pierce (Rockford, IL, USA), and stored in aliquots at −80°C. Protein concentration was measured by the Bradford method (33) using BSA as standard. Then, 40 μg of protein was separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels and electroblotted in a wet chamber to 0.45 μm nitrocellulose membrane (Bio-Rad) at 250–300 mA for 1 h. Membranes were then probed with the indicated antibody and the abundance of the phosphorylated and unphosphorylated forms of each protein was detected using the Immobilon Western Chemiluminescent HRP substrate kit from

Millipore (Billerica, MA, USA). Membranes were exposed to an X-ray film (Kodak) with two intensifying screens (DuPont) at room temperature (23°C).

Co-Immunoprecipitation Assays

Macrophages Raw 264.7 were seeded in 10-cm cell plate culture until confluence, left in serum-free media for 4 h, and then stimulated as indicated. For immunoprecipitation technique, we use the kit Pierce™ Protein A/G Magnetic Beads (Thermo Fischer Scientific). In brief, ~1 × 10^7 cells were lysed in washing/lysis buffer supplemented with 1× protease inhibitor cocktail, and cells debris were collected by centrifugation at 18,000 × *g* for 10 min at 4°C. The supernatant was collected and quantified for protein concentration. Then, 4 µg of each immunoprecipitation antibody (CBP or IκBα) was added to 500 µg of protein extract and the volume adjusted to 500 µl. The mixture was incubated with continuous agitation all night at 4°C. Next day, 25 µg of magnetic beads were added and incubated 1 h in agitation at room temperature (23°C); beads were washed 2× with washing buffer and 1× with water. The target antigen was eluted with lane marker buffer 1× plus 50 mM DTT in the final volume of 100 µl. For immunoblots, 20 µl of target antigen in elution buffer was used and detected as described in the western blot analysis section.

Cytokine Quantitation

Mouse cytokines, IL-4, IL-6, IL-10, IL-13, TNF-α, and PGE2 levels, were measured by ELISA, according to the manufacturer's instructions (R&D, Minneapolis, MN, USA).

Cytokine Array

Macrophages were incubated for 1 h with 20 µg/ml of IDR-1, IDR-HH2, or IDR-1002 and then stimulated for 2–24 h with 10 ng/ml of LPS. Supernatants were collected and cytokines were detected using a mouse cytokine antibody pair-based array spotted on a membrane (Abcam, ab133993) according to the manufacturer's instructions (Cambridge, UK). Briefly, analysis of cytokines present in the macrophages supernatant is carried out by chemiluminescent western blot assay, using biotinylated detector antibodies and streptavidin HRP. Targets of this ELISA-like array are granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-2, 3, 4, 5, 6, 9, 10, 12 p40/p70, 12p70, 13, 17, interferon gamma (IFN-γ), monocyte chemoattractant protein 1 and 5 (MCP-1 and MCP-5), regulated on activation normal T cell expressed and secreted (RANTES), stem cell factor (SCF), soluble tumor necrosis receptor factor 1 (sTNFR1), tumor necrosis factor alpha (TNF-α), thrombopoietin, and vascular endothelial growth factor (VEGF).

Statistical Analysis

Statistical significance was evaluated using Student's paired *t*-test using the SIGMASTAT program version 3.0 (SPSS Inc., Chicago, IL, USA). Band densitometric analysis was performed using the Image Processing and Analysis module in Java Program ImageJ (http://rsbweb.nih.gov/ij).

RESULTS

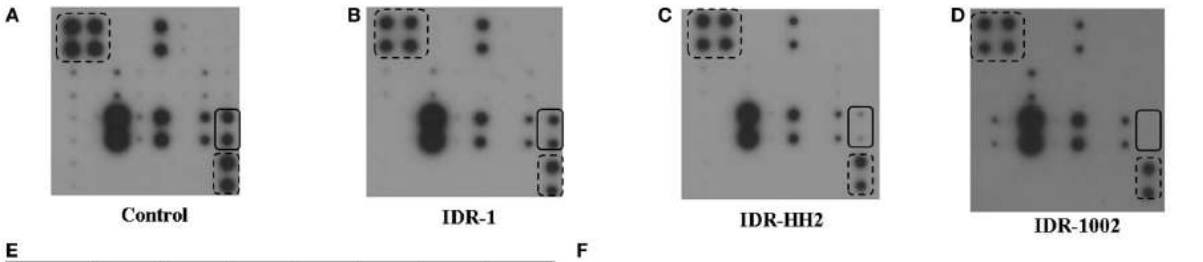
IDR-1002 Reduced the LPS-Induced Expression of TNF-α and COX-2

To explore the effect of IDR-1, IDR-HH2, and IDR-1002 on cytokine expression, we used an antibody array of 22 cytokines. After 12 h of incubation with each of the IDR peptides, we evaluated the level of cytokine expression in the supernatants. Compared to control (**Figure 1A**), IDR-1 had a modest effect on TNF-α expression (**Figure 1B**). However, when macrophages were treated with IDR-HH2 or IDR-1002, the basal levels of TNF-α were significantly reduced. We observed that IDR-1002 was a somewhat stronger inhibitor than IDR-HH2 (**Figures 1C,D**). A map of the cytokine array is shown in **Figure 1E**. To determine the kinetics of the effect of IDR-1002 on TNF-α production, macrophages were pre-incubated with IDR-1002 for 1 h and then stimulated, for periods of 2–24 h, with LPS from *E. coli*, an agonist that induced a strong release of TNF-α. We observed that production of TNF-α activated by LPS was strongly reduced in the presence of IDR-1002 (**Figure 1F**). These results indicate that IDR-1002 had an inhibitory effect on the basal and LPS-induced expression of TNF-α.

Another important gene expressed in the inflammatory response and induced by LPS is COX-2. This enzyme catalyzes the conversion of arachidonic acid into prostaglandins and thromboxane, playing an important role in the inflammatory response (34). To determine the effect of IDR-1002 on COX-2 production, macrophages were pre-incubated with IDR-1002 for 1 h and then stimulated with LPS for a period of 2–24 h. Under this condition, we observed that COX-2 expression was strongly and significantly reduced in the presence of IDR-1002 (**Figure 2A**). Incubation of macrophages with IDR-1002 alone for 2, 4, and 8 h did not have any effect on COX-2 (**Figure 2B**). These results indicate that IDR-1002 had an inhibitory effect on the LPS-induced expression of COX-2.

IDR-1002 Reduced the Phosphorylation of NF-κB p65 Subunit at Ser536 and Its Nuclear Translocation

Activation of NF-κB stimulates its nuclear translocation leading to the expression of many pro-inflammatory genes, among which TNF-α and COX-2 play important roles in the inflammatory response. To explore if IDR-1002 inhibited TNF-α and COX-2 expression by inhibiting NF-κB activity, we evaluated phosphorylation of the p65 subunit at Ser536 (a residue located in the transactivation domain), and its nuclear relative abundance. An ~20–40% reduction of p65 phosphorylation at Ser536 was observed when macrophages were incubated with IDR-1002 for 5, 30, or 60 min (**Figure 3A**). Next, we investigated the relative abundance of p65 in the cytoplasm and nucleus of macrophages incubated with LPS and IDR-1002. From data shown in **Figure 3B**, it was clear that LPS stimulated p65 nuclear translocation, which was inhibited by IDR-1002. The co-immunoprecipitation of NF-κB and coactivator CBP was detected exclusively in macrophages stimulated with LPS, but not in those stimulated with IDR-1002 followed by LPS or IDR-1002 alone (**Figure 3C**). These



E

Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
Trombopoietin	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	Pos
Trombopoietin	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	Pos

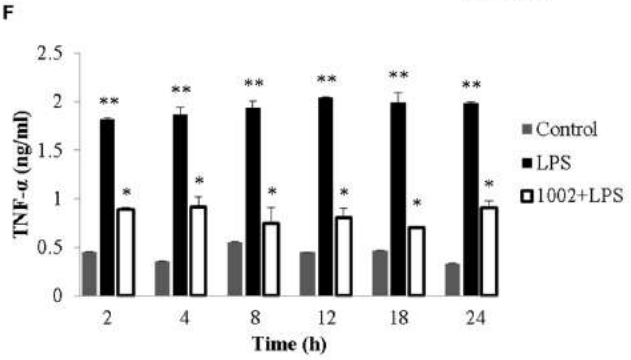


FIGURE 1 | IDR peptides reduced the expression of TNF-α in RAW 264.7 macrophages. Macrophages were left unstimulated (control) **(A)** or stimulated with 20 μg/ml of IDR-1 **(B)**, IDR-HH2 **(C)**, or IDR-1002 **(D)** for 12 h. Conditioned media were then collected and centrifuged at 18,800 × *g* for 10 min. The supernatants were transferred to ice-cold tubes and stored at −80°C. Cytokine expression was evaluated with an antibody base-paired array kit (Abcam ab133993), in which antibodies against 22 different cytokines were spotted on a membrane. **(E)** Cytokines array map. In each array, the position of TNF-α is indicated with continuous-line rectangles while positive controls are indicated with dashed-line rectangles. **(F)** Cells were left unstimulated (control), stimulated with 10 ng/ml LPS, or pretreated with 20 μg/ml IDR-1002 for 1 h followed by incubation with 10 ng/ml LPS for 2–24 h. Supernatants were collected and treated as described above. TNF-α quantitation was performed by ELISA (R&D), according to the manufacturer's instructions. Data represent means ± SEM ($n = 3$). $^{**}P < 0.05$ for LPS assays compared with the unstimulated control value. $^{*}P < 0.05$ for 1002 + LPS assays compared with LPS treatment.

results indicate that IDR-1002 reduced the relative abundance of p65 phosphorylated at Ser536 and its LPS-induced nuclear translocation and interaction with CBP.

IDR-1002 Inhibited the Phosphorylation and Degradation of IkBα Induced by LPS

In unstimulated cells, NF-κB is anchored to IκBα in the cytoplasm. Release of NF-κB requires phosphorylation of IκBα, which marks it to become ubiquitinated and then degraded in the proteosome. This mechanism implies that if phosphorylation of IκBα is inhibited, then NF-κB would not be released and its nuclear translocation would not occur. Our data indicate that IDR-1002 strongly inhibited the LPS-induced phosphorylation of IκBα (**Figure 4A**) and its degradation (**Figure 4B**). This effect of IDR-1002 on IκBα seemed to be independent of the stimulus because inhibition of IκBα phosphorylation and degradation by IDR-1002 was equally effective in macrophages stimulated with TNF-α or IL-1β (**Figures 4A,B**). Interaction of NF-κB p65 and IκBα was stabilized as a consequence of IDR-1002 inhibition of IκBα phosphorylation and degradation. **Figure 4C** shows that there was an increase in co-immunoprecipitation NF-κB p65 bound to IκBα in macrophages stimulated with IDR-1002. These data may explain the inhibitory effect exerted by IDR-1002 on TNF-α and COX-2 expression.

Activation of CREB Phosphorylation at Ser133 by IDR-1002 Was Dependent on ERK1/2, p38, and MSK1 Activity

A major mechanism for controlling the inflammatory response is the suppression of pro-inflammatory cytokine expression, which can be promoted when NF-κB forms a molecular complex with CBP (16, 17). This process takes place during the inflammatory response when CREB is also activated by phosphorylation at Ser133. Phosphorylation of CREB enables it to compete with NF-κB for the same interaction domain on CBP (18). The consequence of NF-κB separation from CBP is a reduction in inflammatory cytokine expression and simultaneously an increment, in many but not all cases, of anti-inflammatory cytokine expression due to CREB activity. To explore if IDR-1002 activated CREB phosphorylation at Ser133, macrophages were incubated with IDR-1002 for 5– 60 min. Under these conditions, a significant increase in CREB phosphorylation was observed for up to 45 min of incubation with IDR-1002 (**Figure 5A**). Because CREB phosphorylation can be mediated by Akt (PKB), ERK1/2,

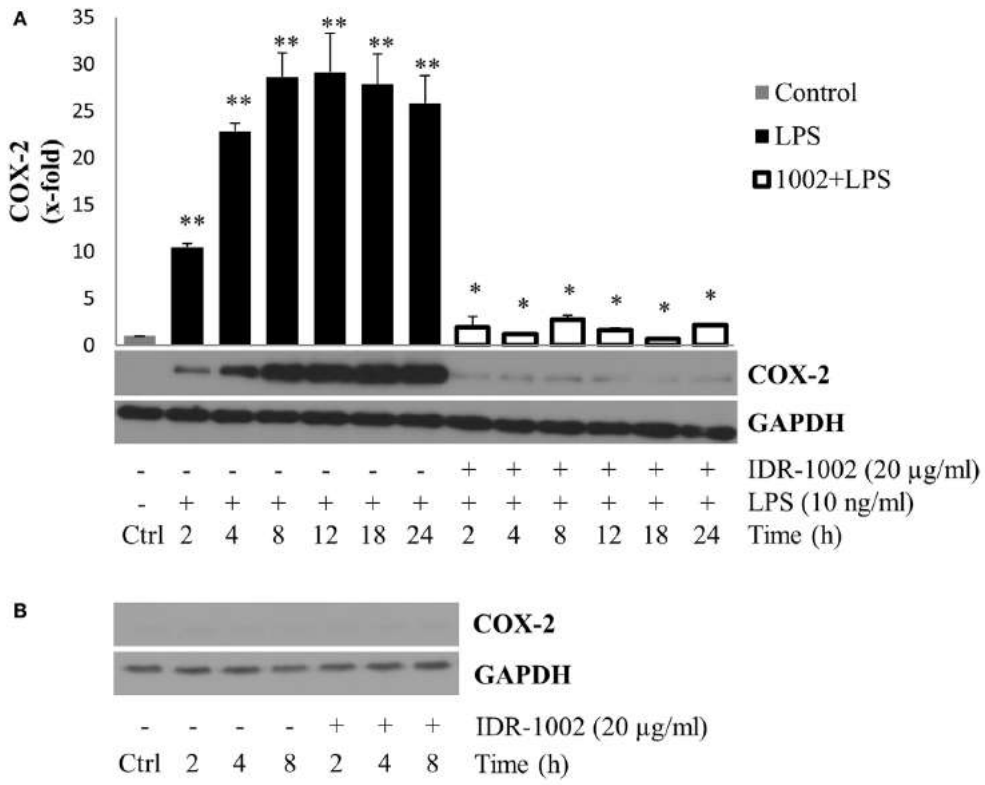


FIGURE 2 | IDR-1002 inhibited the LPS-induced COX-2 expression in RAW 264.7 macrophages. **(A)** Macrophages Raw 264.7 were left unstimulated (Ctrl), stimulated with 10 ng/ml of LPS, or pretreated with 20 μg/ml of IDR-1002 for 1 h followed by incubation with 10 ng/ml of LPS for 2–24 h. At the end of each incubation period, COX-2 was analyzed by Western blotting. Protein from Ctrl cells were collected at 4 h. A representative immunoblot of three independent experiments is shown. GAPDH detection was used as control to ensure equal protein loading. **(B)** At each indicated incubation time, COX-2 expression was measured in untreated control and IDR-1002-treated cells. Values were normalized with GAPDH as protein loading control. This graph is representative of two independent experiments. Data represent means ± SEM ($n = 3$). $^{**}P < 0.05$ for LPS compared with the unstimulated control value. $^{*}P < 0.05$ for 1002 + LPS compared with LPS treatment.

p38, and MSK1/2, we performed experiments utilizing specific inhibitors for each one of these enzymes. Macrophages stimulated with IDR-1002 showed an increased relative abundance of CREB phosphorylated at Ser133 and this was reduced by specific inhibitors of p38 and ERK1/2 (**Figures 5B,C**). Correspondingly, IDR-1002 induced a strong phosphorylation of ERK1/2 and p38 (Figures S1A,B in Supplementary Material), as described previously (27). However, specific inhibitors of PI3K, GSK3, and Akt did not have any effect on CREB phosphorylation (Figures S2A,B in Supplementary Material).

Other authors have reported that ERK1/2 and p38 do not directly phosphorylate CREB but instead they first phosphorylate MSK1/2, which in turn phosphorylates CREB at Ser133 (35). To test if MSK1/2 linked the activation of ERK1/2 and p38 with CREB phosphorylation, we silenced the MSK1 and MSK2 genes using siRNA. When these genes were independently or simultaneously silenced, a significant reduction in CREB phosphorylation was observed (**Figure 6A**), which indicated that both MSK1 and MSK2 could participate. However, only MSK1 (**Figure 6B**), but not MSK2 (data not shown), phosphorylation was detected in macrophages stimulated with IDR-1002. Evidence of MSK1 gene knockout by siRNA is presented in **Figure 6A**. Furthermore, incubation of macrophages with either ERK1/2- or p38-specific inhibitors almost completely prevented phosphorylation of MSK1 (**Figure 6B**).

However, although CREB was strongly phosphorylated, we observed no increase in the anti-inflammatory cytokines IL-4, IL-10, and IL-13 as measured by ELISA (data not shown). The lack of expression of these cytokines associated with M2 macrophage response might be due to the absence of interaction between phosphorylated CREB and CBP. The co-immunoprecipitation of CREB with CBP from macrophages stimulated with IDR-1002 showed that no complex was formed between CBP and CREB (**Figure 6C**). Together, these genetic and biochemical data indicated that IDR-1002-induced phosphorylation of CREB at Ser133 depended on the ERK1/2/p38–MSK1 signaling pathway, and that, at least in our system, phosphorylation of CREB by MSK1 did not promote interaction between CREB and CBP.

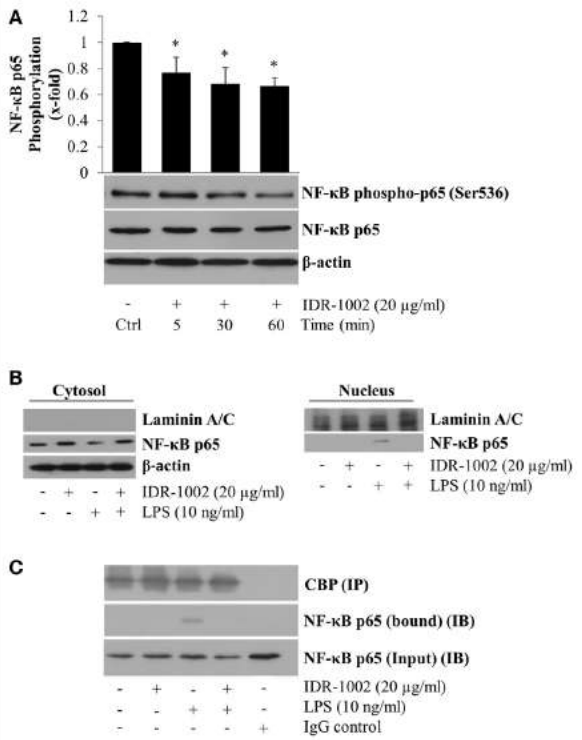


FIGURE 3 | IDR-1002 reduced NF-κB p65 phosphorylation at Ser536 and inhibited its LPS-activated nuclear translocation in RAW264.7 macrophages. **(A)** Macrophages were left unstimulated (Ctrl) or stimulated with 20 μg/ml of IDR-1002 for 5–60 min. Phosphorylation of NF-κB p65 subunit was detected by Western blotting. Detection of β-actin and unphosphorylated p65 in each sample was performed to ensure equal protein loading. **(B)** Macrophages were pretreated with 20 μg/ml of IDR-1002 for 1 h and then with 10 ng/ml of LPS for 2 h. Non-phosphorylated NF-κB p65 subunit was detected by Western blotting in nuclear and cytosolic protein enriched fractions. Laminin A/C and β-actin were detected as controls for nuclear and cytosolic fractions, to observe equal protein loading and no cross-contamination between nuclear and cytosolic fractions, respectively. **(C)** Macrophages were treated as described in **(B)**. Eluates from CBP immunoprecipitates (IP) were subjected to Western blot analysis with NF-κB p65 antibody to detect the p65 bound to CBP and its amount present in the input (IB). Control with isotype IgG was also included. Detection of CBP was performed to show equal protein loading. *$P < 0.05$ for IDR-1002 compared with the unstimulated control value.

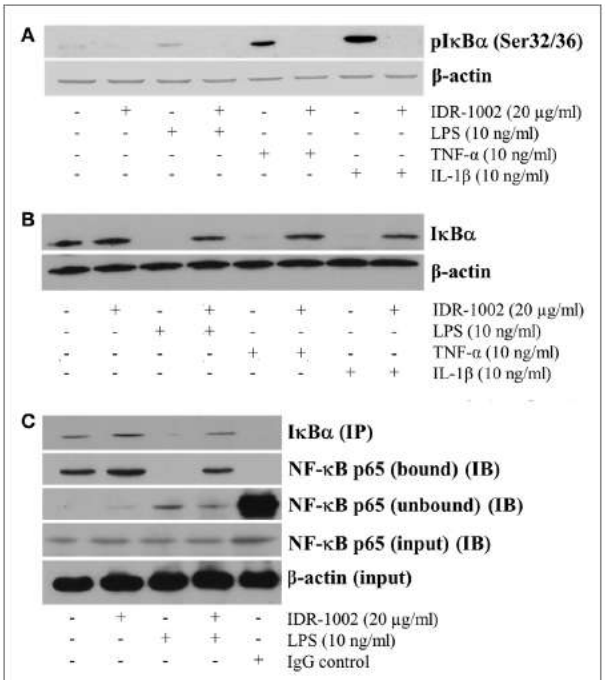


FIGURE 4 | IDR-1002 inhibited IκBα phosphorylation and degradation in RAW 264.7 macrophages. **(A)** Macrophages were left unstimulated or pretreated with 20 μg/ml of IDR-1002 for 1 h. Then, macrophages were stimulated for 10 min with either 10 ng/ml of LPS, 10 ng/ml of TNF-α, or 10 ng/ml of IL-1β. Phosphorylated IκBα was detected by Western blot analysis and β-actin was detected to ensure equal protein loading. **(B)** Macrophages were treated as in **(A)** except that incubation with LPS, TNF-α, or IL-1β was for 30 min. Total IκBα was detected by Western blot analysis in total cell lysates extracts. β-actin detection was performed to ensure total protein loading. **(C)** Macrophages were left unstimulated or stimulated with 20 μg/ml of IDR-1002 for 1 h followed by stimulation with 10 ng/ml of LPS for 10 min. IκBα was immunoprecipitated (IP) and eluates were subjected to Western blot to detect NF-κB p65 in the immunoprecipitates of IκBα (bound) or supernatants (unbound) samples (IB). β-actin detection was performed in the input cell extracts to ensure equal protein.

DISCUSSION

Data presented here demonstrated that in RAW264.7 macrophages/monocytes stimulated with LPS, IDR-1002 was able to inhibit IκBα phosphorylation and degradation, completely blocking NF-κB p65 nuclear translocation. This would explain the inhibition of the interaction between NF-κB and CBP, and partially inhibition of NF-κB p65 Ser536-phosphorylation, which can enhance transactivation. These effects of IDR-1002 on NF-κB p65, together with others previously reported (36, 37), were sufficient to reduce the expression of TNF-α and COX-2. In addition, IDR-1002 induced a significant increase in CREB phosphorylation at Ser133 through the activation of p38/ERK1/2 and MSK1/2. Although the relative abundance of phosphorylated CREB was higher in macrophages stimulated with IDR-1002 than in untreated controls, no interaction between CREB and CBP occurred, and thereby preventing the enhanced expression of cytokines IL-4, IL-10, and IL-13.

Inhibition of TNF-α expression in RAW264.7 macrophages has been demonstrated by *in vitro* administration of different compounds in response to LPS from *E. coli* (38–41). For example, the anti-inflammatory effect of extracellular phospho-ceramide analog-1 (PCERA-1) was found to depend on suppression of TNF-α production, an increase in cAMP levels, and increased expression of IL-10 (38). The low molecular weight chemical compounds madecassic and asiatic acid, and the isoflavone genistein potently inhibited the expression of TNF-α, COX-2, NO, prostaglandin E_2, IL-1β, and IL-6 by blocking both IκBα

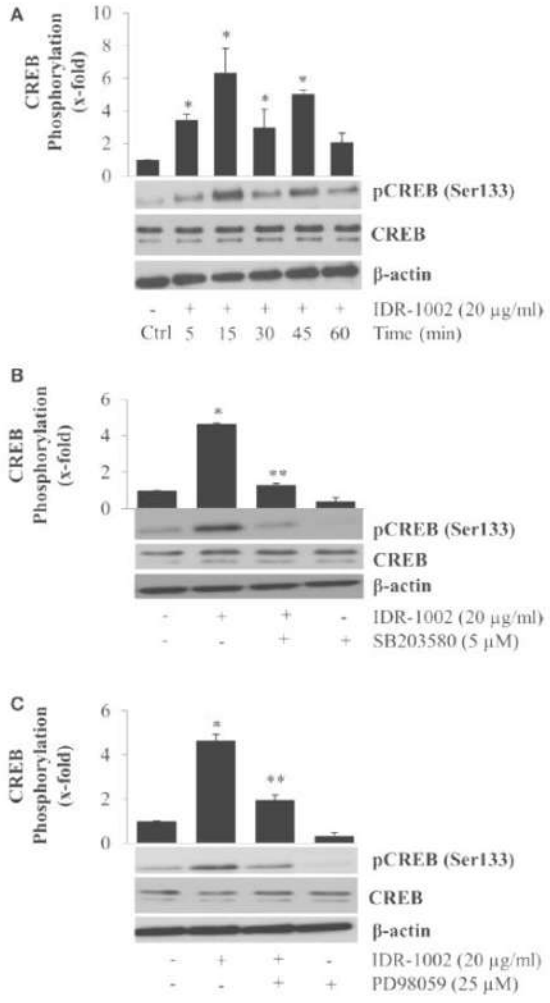


FIGURE 5 | Inhibition of p38 and ERK1/2 activity reduces CREB phosphorylation at Ser133 in Raw 264.7 macrophages activated by IDR-1002. **(A)** Macrophages were left unstimulated (Ctrl) or stimulated with 20 μg/ml of IDR-1002 for 5–60 min. After stimulation, CREB phosphorylation was detected by Western blotting. **(B)** Cells were left untreated (–) or treated with 5 μM of SB203580 (SB, a p38 inhibitor) for 30 min and then stimulated with 20 μg/ml of IDR-1002 for 15 min. **(C)** Cells were left untreated (–) or treated with 25 μM of PD98059 (PD, an ERK1/2 inhibitor) for 30 min and then stimulated with 20 μg/ml of IDR-1002 for 15 min. Additional controls in which cells were treated with 5 μM of SB or 25 μM of PD alone were also included. Phosphorylation of CREB was analyzed by Western blotting. Detection of β-actin and unphosphorylated CREB were performed to ensure equal protein loading. Blots are representative of three independent experiments. The data represent means ± SEM ($n = 3$). *$P < 0.05$ for IDR-1002 compared with the unstimulated control value. **$P < 0.05$ for inhibitor + IDR-1002 assays compared with the IDR-1002 assay.

degradation and NF-κB nuclear translocation (39–41). The effects in macrophages induced by IDR-1002 on TNF-α/COX-2 expression, and NF-κB inhibition, resemble those triggered by the compounds mentioned above, suggesting that parallel mechanisms mediate the effect of IDR-1002 to control inflammation. Specifically, we found in RAW 264.7 macrophages that IDR-1002 was able to inhibit LPS-induced phosphorylation of IKKα and IKKβ, which may explain the inhibitory effect of this peptide on IκBα phosphorylation (Figure S3 in Supplementary Material). Recently, Liu et al. (42) discovered that a long non-coding RNA inhibits IκB phosphorylation by forming a ternary complex with NF-κB and IκBα, which caused a strong inhibition of metastasis in breast cancer. Based on these results, it is possible that IDR-1002, as an inhibitor of IKK and IκBα phosphorylation, may be a good candidate for metastasizing cancers. We are currently performing experiments to test IDR-1002 in various cancer cell lines.

COX-2 is important in the control of inflammation, pain, fever, and other physiological processes. Inhibition of COX-2 activity by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin has been the clinicians' choice to alleviate medical conditions. However, NSAIDs induce tolerance over time (43) and administration for even short periods can cause gastrointestinal and renal secondary effects in ~25% of individuals and almost 5% of them suffer serious health problems (44). Because of these detrimental effects induced by NSAIDs, a search for new anti-inflammatory drugs without side effects is of primary importance in the pharmaceutical industry. The strong inhibition by IDR-1002 of LPS-induced COX-2 expression observed in our study suggests that IDR peptides may be a viable alternative. However, more experiments *in vivo* and chemical modification/formulation of IDR-1002 to increase its potency, specificity, and half-life time must be considered before its administration to human beings.

Apart from the inhibitory effect of IDR-1002 on LPS-induced NF-κB and COX-2, this cationic peptide induced the phosphorylation/activation of p38, ERK1/2, and MSK1, which led to the phosphorylation of CREB at its Ser133 activation site. The contribution of the signaling pathway PI3K/Akt/GSK3 as an effector of IDR-1002-induced CREB phosphorylation was ruled out (Figure S2 in Supplementary Material). According to one current model, once CREB becomes phosphorylated at Ser133, it is able to interact with CBP and induce expression of anti-inflammatory cytokine genes, among which IL-4, IL-10, and IL-13 are the most important for reducing inflammation (23). However, it has been documented that in some cases phosphorylation of CREB at Ser133 by MSK1/2 is not absolutely required to promote recruitment of CBP and, consequently, expression of some CREB target genes does not take place (15). Similarly, activation of T-cell receptor (TCR) promotes a strong phosphorylation of CREB at Ser133 without formation of CREB–CBP complex, unless low amounts of cAMP were present as a costimulus (24). In our case, IDR-1002-activated MSK1-dependent phosphorylation of CREB did not promote the interaction of CREB with CBP, which might explain lack of effects on gene expression of IL-4, IL-10, and IL-13. This conclusion was drawn after several attempts to co-immunoprecipitate CBP with CREB in protein extracts recovered from macrophages stimulated with IDR-1002. More experiments to find out the

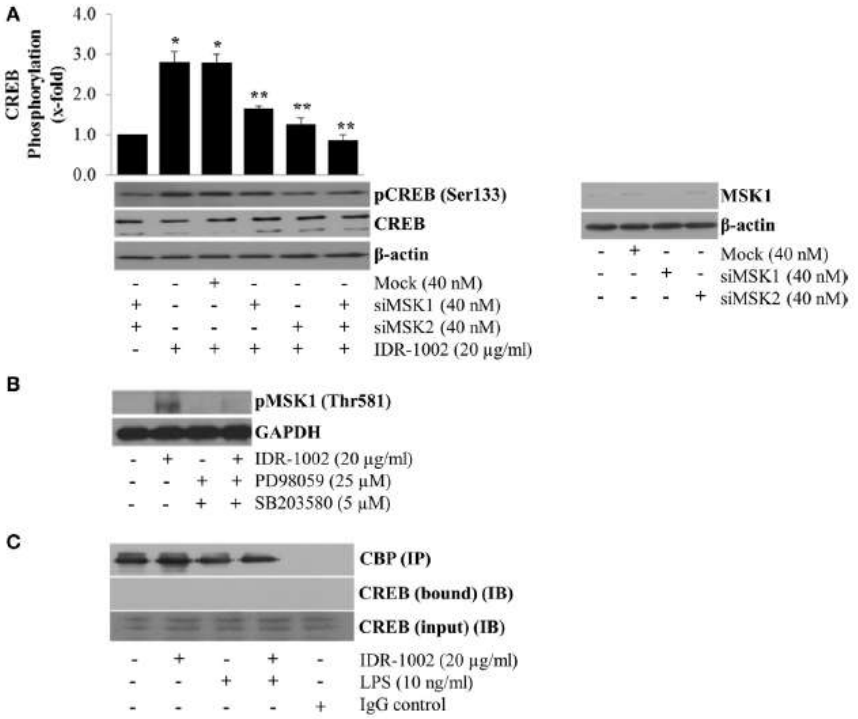


FIGURE 6 | MSK1-mediated CREB phosphorylation at Ser133 in RAW 264.7 macrophages stimulated with IDR-1002. (A) Macrophages were transfected with non-specific siRNA control (Mock), MSK1 (siMSK1), MSK2 (siMSK2) or MSK1, and MSK2 simultaneously for 48 h. Cells were then stimulated with 20 μg/ml of IDR1002 for 15 min. CREB phosphorylation was analyzed by Western blotting. Detection of β-actin and unphosphorylated CREB in each sample was performed to ensure equal protein loading. The right panel shows detection of MSK1 gene expression when macrophages were treated with MSK1-specific siRNA. In **(B)**, macrophages were left unstimulated (–) or pretreated with 25 μM of PD98059 (PD, an ERK1/2 inhibitor) and 5 μM of SB203580 (SB, a p38 inhibitor) for 30 min, and then stimulated with IDR-1002 for 15 min. Additional controls with 25 μM of PD and 5 μM of SB alone were also included. Under these conditions, phosphorylation of MSK1 was detected by Western blotting. **(C)** Macrophages were left unstimulated or stimulated with 20 μg/ml of IDR-1002 followed by stimulation with 10 ng/ml of LPS. Immunoprecipitation was performed as previously described and eluates from CBP immunoprecipitates (IP) were subjected to Western blot analysis with CREB antibodies. Then, we detected CREB immunoprecipitated (bound) with CBP and in the input (IB). Control with isotype IgG was also included. Detection of CBP was performed to show equal protein loading. The blots are representative of three independent experiments. Data represent means ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the unstimulated control value. $^{**}P < 0.05$ for siRNA treatment compared with the IDR-1002 value.

molecular details of this mechanism are currently underway in our laboratory.

The experimental evidence obtained in RAW 264.7 macrophages indicating that IDR-1002 inhibits LPS-induced COX-2 expression, activates CREB phosphorylation at Ser133, and inhibits LPS-induced IκBα degradation was confirmed in PBMC (Figures S4A–C in Supplementary Material). This novel spatiotemporal combination of both effects (inhibition of NF-κB signaling and activation of CREB phosphorylation) makes IDR-1002 an attractive anti-inflammatory pharmaceutical drug.

AUTHOR CONTRIBUTIONS

AH-M performed most of the experiments and helped with the writing of the manuscript. OS-G performed many experiments. JO-B helped to perform some experiments. RH financially supported the research (provided the peptide IDR-1002) and critically reviewed the manuscript. VMB-A financially supported most of the supplies and wrote the manuscript.

FUNDING

This work was supported by Consejo Nacional de Ciencia y Tecnología (CONACyT)-México, grant-152518 to VMB-A and Coordinación de la Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo, grant-2015-2016 to VMB-A, and by the Canadian Institutes for Health Research to RH. AH-M and OS-G received a doctoral scholarship from CONACyT, while RH was supported by a Canada Research Chair and a UBC KIlliam Professorship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00533/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

17. Referencias complementarias.

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