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Análisis de las rutas de señalización involucradas en la internalización de *Staphylococcus aureus* en las células epiteliales mamarias bovinas regulada por ácidos grasos

Tesis

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Presenta
M.C. Patricia Nayeli Alva Murillo

Asesor
Dr. Joel Edmundo López Meza

Co-asesora:
Dra. Alejandra Ochoa Zarzosa

Sinodales:
Dr. Carlos Cervantes Vega
Dra. Elva Mayra Toledo Cuevas
Dr. Ciro Estrada Chávez

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... la ciencia es un esfuerzo de colaboración. Los resultados combinados de varias personas que trabajan juntas es a menudo mucho más eficaz de lo que podría ser el de un científico que trabaja solo

John Bardeen (1908-1991)

CONTENIDO

I. RESUMEN

Trabajos previos de nuestro grupo demostraron que moléculas inmunomoduladoras como el butirato (0.5 mM NaB) u octanoato (1 mM NaO) inhiben la internalización de *S. aureus* en las CEMB, o la estimulan (0.25 mM NaO). Sin embargo, no se conocen los mecanismos involucrados en ello, lo que fue el objetivo de este trabajo. Los resultados mostraron que el NaB (0.5 mM, 24 h) activó a las CEMB al favorecer la abundancia en membrana (AM) del TLR2, la fosforilación de p38 y la activación de 8 FT, como AP-1. Además, se indujo la expresión de genes de los péptidos antimicrobianos (PA) TAP, BNBD5 y BNBD10, de la citocina TNF-□ y de la quimiocina IL-8, favoreciendo la respuesta antimicrobiana y pro-inflamatoria. La infección (2 h) inhibió la activación de la ruta TLR2/p38 inducida por el AG. No obstante, se observó una respuesta anti-inflamatoria y una mayor respuesta antimicrobiana, determinada por la expresión del gen de la IL-10 y de los PA LAP, TAP, BNBD5 y BNBD10, respectivamente. El NaO ejerce una acción dual en la internalización de *S. aureus* en las CEMB. El aumento de la internalización regulada por 0.25 mM NaO está relacionado con un incremento de la AM de las integrinas, pero disminuyó la fosforilación de las cinasas, y sólo activó a los FT MEF-1 y Stat-4. Además, indujo la respuesta anti-inflamatoria (aumentó la expresión del gen de la IL-10). Por otro lado, 1 mM NaO (inhibición de la internalización) activó a las CEMB al incrementar la AM de TLR2, la fosforilación de p38, JNK y ERK1/2, y la ligera activación de 14 FT. Asimismo, se indujo la expresión de los genes de los PA LAP y BNBD10, favoreciendo la respuesta antimicrobiana, y se observó una respuesta pro-inflamatoria (aumentó la expresión de los genes de IL-1□, IL-8 y RANTES). La infección revirtió la fosforilación de las cinasas, la activación de los FT y el perfil pro-inflamatoria generada por el AG. Sin embargo, la respuesta antimicrobiana aumentó y se observó un perfil anti-inflamatorio. Estos resultados demuestran que los ácidos grasos de cadena corta (NaB) y media (NaO) regulan diferencialmente la RII de las CEMB. En particular, el NaO ejerce efectos opuestos y dependientes de la

concentración. Lo anterior se relaciona con la modulación de la internalización de *S. aureus* en las CEMB regulada por estos AG.

Palabras clave: butirato, epitelio mamario bovino, octanoato, p38, respuesta antimicrobiana, *Staphylococcus aureus*, TLR2.

II. ABSTRACT

We reported previously that immunomodulatory molecules are able to (i) inhibit the *S. aureus* internalization into bMECs, such as butyrate (0.5 mM NaB) or 1 mM octanoate (NaO), or (ii) increase the bacterial internalization (0.25 mM NaO). Nonetheless, the molecular mechanisms of these processes have not been described, which are the aim of this study. The results showed that NaB treatment (0.5 mM, 24 h) activated the bMECs by increasing the TLR2 membrane abundance (MA), the p38 phosphorylation and the activation of 8 TF, including AP-1. Furthermore, the gene expression of AP (TAP, BNBD5 and BNBD10), the cytokine TNF-□ and the chemokine IL-8 was increased, which favors the antimicrobial and pro-inflammatory responses. The activation of the TLR2/p38 pathway modulated by NaB was inhibited in the NaB-treated and *S. aureus*-challenged bMECs. Nonetheless, an anti-inflammatory profile and a higher antimicrobial response was observed due to an increased in the gene expression of IL-10 and the AP LAP, TAP, BNBD5 and BNBD10. On the other hand, NaO exerts a dual effect on *S. aureus* internalization into bMECs; the 0.25 mM NaO (24 h) treatment increases the bacterial internalization, while this process is reduced in 1 mM NaO-treated cells. The bacterial internalization induction modulated by 0.25 mM NaO is associated with an increase in the integrin MA. The kinases phosphorylation was reduced with this treatment, and the TF MEF-1 and Stat-4 were activated. Additionally, the anti-inflammatory response was induced (up-regulation of gene expression of IL-10). This effect was reverted by infection. Otherwise, 1 mM NaO treatment did not modify the integrin MA; however, this treatment activated the bMECs by increasing the TLR2 MA, the kinases phosphorylation, and the slightly activation of 14 TF. Likewise, the gene expression of AP (LAP and BNBD10), IL-1□, IL-8 and RANTES was up-regulated which favors the antimicrobial and pro-inflammatory responses. When 1 mM NaO-treated cells were *S. aureus*-challenged the kinases phosphorylation -regulated by NaO- was reverted, as well as the TF activation and the pro-inflammatory response. However, we detected an anti-inflammatory profile and the up-regulation of the antimicrobial response. These results show that the short (NaB) and medium

(NaO) chain fatty acids differentially regulate the IIR of bMECs. Particularly, octanoate exerts opposite effects which are concentration dependent. This is related to the modulation of the *S. aureus* internalization into bMECs regulated by these fatty acids.

Keywords: antimicrobial response, bovine mammary epithelium, butyrate, octanoate, p38, *Staphylococcus aureus*, TLR2.

III. INTRODUCCIÓN

III.1 Mastitis bovina

La mastitis bovina se caracteriza por la inflamación de la glándula mamaria, causada generalmente por microorganismos patógenos, aunque también puede ser de origen traumático o tóxico (Kerro Dego et al., 2002). Esta patología es de gran importancia dentro de la industria lechera bovina mundial ya que su prevalencia es del 40-50% en las vacas en un hato lechero, y se estima que las pérdidas económicas (casos subclínicos y clínicos) varían desde €114 o 149 USD hasta €182 o 239 USD por vaca al año (Europe, IFAH, 2013). En México la enfermedad arroja pérdidas económicas de aproximadamente $2,500 anuales por vaca en un hato lechero (Bedolla 2008). Las mermas económicas son provocadas por el costo del tratamiento, la disminución de la producción de la leche, la reducción de la calidad de los productos lácteos, e incluso al sacrifico de los animales, entre otras factores (Hillerton y Berry, 2005). El daño que ocurre en el tejido mamario reduce el número y la actividad de las células epiteliales y contribuye, consecuentemente, a la disminución de la producción lechera. Aunque se utiliza terapia antimicrobiana para tratar la infección, no se protege directamente a la glándula mamaria del daño causado por la enfermedad (Zhao y Lacasse, 2008).

De acuerdo con su duración puede clasificarse en aguda o crónica. En relación a sus manifestaciones clínicas, puede ser subclínica y clínica (Thompson-Crispi et al., 2014). La mastitis clínica se define como una infección de la glándula mamaria que puede ser detectada a simple vista y se caracteriza por la inflamación de uno o varios de los cuartos mamarios; el animal presenta dolor al tacto y en la leche se observan coágulos, descamaciones y en ocasiones sangre (Thompson-Crispi et al., 2014). Por otra parte, la mastitis subclínica es una infección de la ubre donde no se observan cambios externos; no obstante, la infección puede ser detectada por un aumento en el conteo de células somáticas (leucocitos) en la leche (Hillerton y Berry, 2005; Ferreira et al., 2013).

El origen bacteriano es la causa más común de la mastitis y existen cerca de 200 microorganismos que pueden generar esta enfermedad. Los principales microorganismos patógenos relacionados con la mastitis bovina son *Escherichia coli*, *Streptococcus uberis* y *Staphylococcus aureus* (Ferreira et al., 2013). Se ha demostrado una correlación entre el tipo de mastitis y el patógeno prevalente. *E. coli* se asocia con síntomas clínicos e inflamación aguda; mientras que *Strept. uberis* y *S. aureus* producen una respuesta inflamatoria menos severa y se relacionan con la mastitis subclínica (Lahouassa et al., 2007; Günther et al., 2011; Ferreira et al., 2013). Aunque particularmente, *S. aureus* es el patógeno que se aísla con mayor frecuencia de los casos de mastitis subclínica y crónica (Kerro Dego et al., 2002).

III.2 Respuesta inmune innata de la glándula mamaria

La glándula mamaria es susceptible de sufrir lesiones o infecciones; sin embargo, este órgano lleva a cabo funciones inmunológicas conferidas a través de la inmunidad innata y adquirida. En este sentido, se requiere de la interacción y coordinación entre los efectores de ambas respuestas para que exista una defensa efectiva, manteniendo así el balance entre la actividad necesaria para eliminar al estímulo nocivo y la respuesta controlada para evitar el daño al tejido del hospedero.

Las barreras físicas, como el canal del pezón, son parte de los mecanismos de defensa de la glándula mamaria, e incluyen el esfínter y la queratina del canal del pezón (Sordillo y Streicher, 2002; Thompson-Crispi et al., 2014). El lumen intramamario es un ambiente aséptico gracias a la eficiencia de la barrera del canal del pezón (Rainard y Riollet, 2006).

La respuesta inmune innata (RII) de la glándula mamaria está conformada por (i) componentes celulares, como leucocitos (ej. macrófagos, neutrófilos, células dendríticas y asesinas naturales) o células no inmunes (endotelio y epitelio), y (ii) elementos solubles, como el sistema del complemento, enzimas,

citocinas, quimiocinas, eicosanoides, y péptidos antimicrobianos (PA), entre otros (Tosi, 2005; Sordillo et al., 2009; Aitken et al., 2011; Ferreira et al., 2013).

Los macrófagos son el principal tipo celular presente en la leche y poseen la capacidad de fagocitar y eliminar a los patógenos de la mastitis; además, son células presentadoras de antígenos que están implicadas en el inicio de la respuesta inflamatoria (Rainard y Riollet, 2006). Por otro lado, la concentración de los neutrófilos en la leche es muy baja, pero su actividad fagocítica es mayor que la de los macrófagos, y pueden ser reclutados al sitio de la infección (Wellnitz y Bruckmaier, 2012). Las células asesinas naturales juegan un papel crítico para la remoción de patógenos intracelulares y pueden eliminar a las bacterias mediante la liberación de proteínas bactericidas (Rainard y Riollet, 2006). Además de los macrófagos, las células epiteliales mamarias bovinas (CEMB) censan el ambiente intramamario y pueden detectar al patógeno invasor, lo cual favorece el inicio de la inflamación y permite el reclutamiento de los neutrófilos al sitio de la infección (Rainard y Riollet, 2006; Wellnitz y Bruckmaier, 2012).

En cuanto a la aportación de los elementos solubles a la RII de la glándula mamaria, se sabe que el complemento (C5a, estimulador de la fagocitosis de los neutrófilos) está presente en bajas concentraciones en la leche de vacas sanas, y aumenta después de la infección intramamaria (Rainard y Riollet, 2006). La lactoferrina es una proteína quelante de hierro, por lo que posee actividad bacteriostática contra bacterias que tienen altos requerimientos de este elemento. Además, la lactoferrina se asocia con propiedades reguladoras de la inmunidad, como la activación del complemento (Rainard y Riollet, 2006). La lisozima es una proteína bactericida capaz de romper el peptidoglicano de la pared celular de las bacterias Gram positivas (+) o negativas (-); esta enzima puede actuar en sinergia con los anticuerpos, el complemento o la lactoferrina. La xantina oxidasa, enzima presente en los glóbulos de grasa de la leche, cataliza la formación de óxido nítrico a partir del nitrito inorgánico, que en condiciones anaeróbicas genera peroxinitrito, un fuerte agente bactericida (Rainard y Riollet, 2006). Por otro lado, también se encuentran anticuerpos naturales, producidos en ausencia completa

de estimulación antigénica externa, presentes en el suero y leche de vacas; estos son las inmunoglobulinas G2 y M (IgG2 e IgM), que pueden opsonizar (marcar a un patógeno con anticuerpos para su ingestión y eliminación por los fagocitos) a las bacterias causantes de la mastitis bovina (Rainard y Riollet, 2006). Las citocinas pro-inflamatorias como el factor de necrosis tumoral-alfa (TNF-α) y la interleucina (IL)-1β participan en la respuesta inflamatoria temprana, activan al endotelio y son inductores endógenos de la fiebre (Alluwaimi, 2004; Wellnitz y Bruckmaier, 2012). La IL-6 es una citocina clave en la inflamación aguda (Heinrich et al., 2003; Alluwaimi, 2004). Las quimiocinas, como la IL-8 y RANTES, participan en el reclutamiento de leucocitos del torrente sanguíneo al sitio de la infección (Wellnitz y Bruckmaier, 2012). Asimismo, se han encontrado altas concentraciones de PA (β-defensinas) en el tejido mamario expuesto a microorganismos o en tipos celulares involucrados en la defensa del hospedero (Swanson et al., 2004; Rainard y Riollet, 2006).

Una vez que el patógeno penetra las barreras físicas naturales se activa la RII de la glándula mamaria, lo cual se logra cuando el patógeno alcanza niveles críticos de concentración y entra en contacto con las células somáticas de la leche y con la barrera epitelial (Figura 1) (Wellnitz y Bruckmaier, 2012; Ferreira et al., 2013). La respuesta innata inicia cuando los receptores de reconocimiento de patrones (PRR), que se encuentran en la superficie celular o intracelularmente, se unen a moléculas bacterianas llamadas patrones moleculares asociados a patógenos (PAMP). Los PRR se expresan en los leucocitos de la leche y en las células epiteliales mamarias bovinas (Goldammer et al., 2004; Strandberg et al., 2005). Los receptores tipo Toll (TLRs) son los principales PRR caracterizados y su activación inicia la transcripción de varios genes de la respuesta inflamatoria y antimicrobiana (Akira et al., 2006).

El reconocimiento del patógeno permite el inicio de la cascada inflamatoria, que se caracteriza por la liberación de mediadores solubles, la vasodilatación, el aumento del flujo sanguíneo, la extravasación de fluidos (aumento de la permeabilidad de las células endoteliales), la afluencia celular (quimiotaxis) y un

metabolismo celular elevado (Sordillo et al., 2009; Ferreira et al., 2013). Cada una de estas respuestas contribuye a los síntomas clínicos de la inflamación, como son el calor, enrojecimiento, hinchazón y dolor (Sordillo et al., 2009). La respuesta inflamatoria efectiva culmina con la rápida eliminación del patógeno y a menudo el tejido del hospedero no sufre cambios perjudiciales. No obstante, la falta de regulación del proceso inflamatorio se asocia a inflamaciones crónicas (Burvenich et al., 2007).

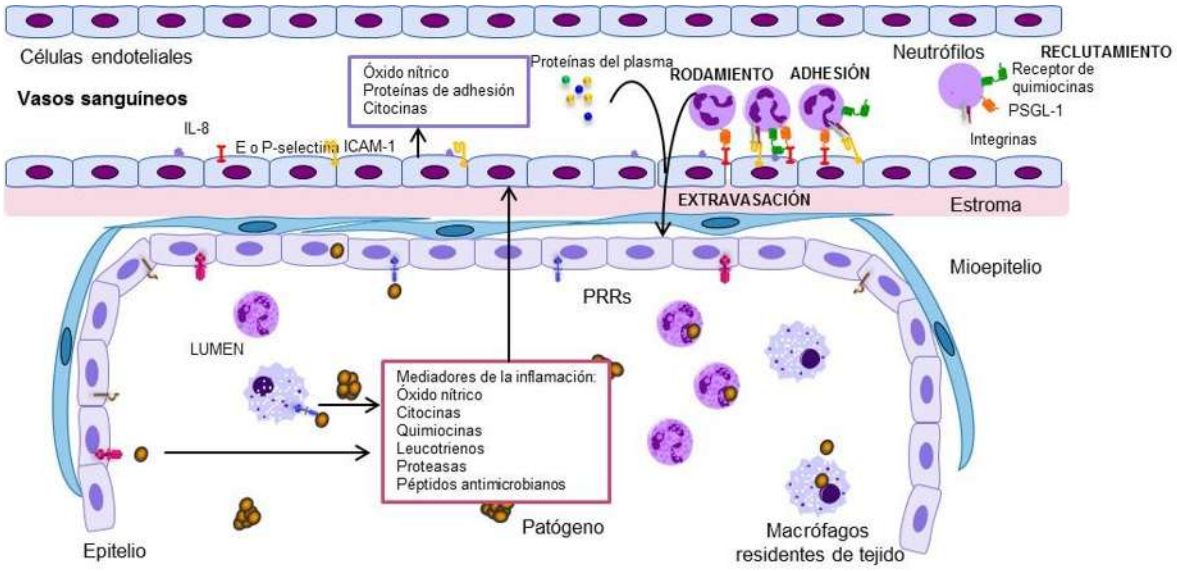


Figura 1. Esquema representativo de la respuesta inflamatoria en la glándula mamaria. Una vez que el patógeno atraviesa las barreras del canal del pezón, se disemina en la cisterna de la glándula mamaria, y es reconocido tanto por los macrófagos residentes de tejido, como por las CEMB. Este reconocimiento es mediado por PRR y permite la activación de estos tipos celulares, lo cual inicia la respuesta inflamatoria y favorece la producción de mediadores de la inflamación, que posteriormente activarán al endotelio mamario bovino. Como consecuencia, éste expresará moléculas de adhesión en su superficie (ej. E o P-selectina, ICAM-1) y producirá más elementos solubles de la RII. Lo anterior permitirá el reclutamiento de leucocitos (neutrófilos), su adhesión, rodamiento y extravasación, para finalmente contribuir a la eliminación del patógeno en la glándula mamaria. ICAM-1: molécula de adhesión intercelular. IL-8; interleucina-8. PRR: receptores de reconocimiento de patrones. PSGL-1: selectina glicoproteína ligando-1.

III.2.1 Participación de las células epiteliales mamarias bovinas en la inmunidad innata

La principal función de las células epiteliales mamarias es la producción de la leche durante la lactancia; sin embargo, se ha demostrado que también son

células efectoras de la inmunidad de la glándula mamaria (Günther et al., 2010). Las CEMB son parte de la primera línea de defensa contra patógenos invasores, debido a su posición y abundancia en la glándula mamaria sana. Los patógenos que entran a la glándula mamaria son censados mediante receptores como los TLRs, así las CEMB son capaces de montar una respuesta diferencial (dependiendo del patógeno), mediante la producción de mediadores de la RII (ej. citocinas, quimiocinas, PA, metabolitos del ácido araquidónico) (Strandberg et al., 2005; Lutzow et al., 2008; Gunther et al., 2010; Schukken et al., 2011; Zbinden et al., 2014).

Las CEMB son capaces de responder diferencialmente al estímulo tanto de patógenos vivos o muertos, como de sus PAMP (Wellnitz y Bruckmaier, 2012). Distintos estudios *in vitro* se han realizado para determinar la participación de estas células en la RII de la glándula mamaria. En este sentido, el LPS (lipopolisacárido, 50 μg/ml, 24 h) estimula la expresión de los genes de citocinas pro-inflamatorias (TNF-α, IL-1α, IL-1β, IL-6), de las quimiocinas (IL-8, CXCL6, CCL2), y del PA BNBD5 (β-defensina de neutrófilos bovinos 5), y TLR2 en las CEMB, y la secreción de la IL-1β, IL-6 e IL-8 (Strandberg et al., 2005; Rainard y Riollet, 2006). El LTA (ácido lipoteicoico, 20 μg/ml, 2 h) también tiene un efecto inductor en la expresión de los genes antes mencionados, aunque en menor proporción que el generado por LPS. Por otro lado, *E. coli* (24 h post-infección, p.i.) favorece una mayor expresión de los genes del TNF-α, IL-1β e IL-8, en comparación con *S. aureus*, aunque a las 3 h p.i. *S. aureus* induce una respuesta más pronunciada en las CEMB (Lahouassa et al., 2007). Además, esta bacteria favorece la secreción de la IL-8 (Rainard y Riollet, 2006). Por otro lado, cuando el epitelio se estimula con concentraciones similares de *E. coli* o *S. aureus,* inactivadas por calor, la respuesta generada por *E. coli* es mayor (expresión de los genes del TNF-α, IL-1β, IL-6, IL-8, RANTES y C3) (Griesbeck-Zilch et al., 2008). La expresión del gen de los PA LAP, TAP y DEFB4 se favorece por el estímulo con *E. coli* muerto por calor (Günther et al., 2009; Liu et al., 2011).

En el caso particular de las infecciones intramamarias generadas por *S. aureus*, la respuesta de las CEMB es moderada o limitada, debido principalmente a que esta bacteria evita la activación de NF-κB, factor transcripcional centinela de la respuesta inflamatoria (Yang et al., 2008; Lara-Zárate et al., 2011). Además, las CEMB también muestran una respuesta inflamatoria diferencial (expresión de genes del TNF-α, IL-1β, IL-8, RANTES, TLR2) dependiendo de la cepa de *S. aureus* utilizada y de la carga bacteriana (Petzl et al., 2008; Zbinden et al., 2014). Adicionalmente, el estímulo bacteriano (*S. aureus*) favorece la expresión de genes de PA (LAP, TAP, BNBD4, BNBD5, BNBD10 y DEFB1) en las CEMB (Ochoa-Zarzosa et al., 2009; Alva-Murillo et al., 2012; Téllez-Pérez et al., 2012; Alva-Murillo et al., 2013a). Todos estos resultados en conjunto sugieren que la respuesta limitada de las CEMB puede favorecer el establecimiento de enfermedades intramamarias por *S. aureus*.

III.3 *Staphylococcus aureus*

S. aureus es una bacteria Gram-positiva, de ~0.8 micrómetros de diámetro y que se agrupa en racimos. Se distingue de las demás especies de *Staphylococcus* porque da un resultado positivo a la prueba de coagulasa, fermenta manitol, presenta hemólisis y un pigmento amarillo-dorado en las colonias en agar sangre (Lowy, 1998). Este un microorganismo patógeno contagioso que se asocia con enfermedades importantes en la medicina humana (ej. osteomielitis, artritis séptica, endocarditis, neumonía y bacteremia) y veterinaria (mastitis bovina) (Brown et al., 2014). La emergencia de cepas resistentes a antibióticos ha dificultado el tratamiento de estas enfermedades, por lo que *S. aureus* se ha convertido en un problema de Salud Pública (Lindsay, 2010).

Actualmente se sabe que algunas cepas de *S. aureus* tienen la capacidad de invadir y persistir dentro de las células fagocíticas profesionales (macrófagos, monocitos) y no profesionales (CFNP) como el epitelio, endotelio, osteoblastos, fibroblastos, denominándose por ello un microorganismo facultativo intracelular

(Fraunholz y Sinha, 2012; Alva-Murillo et al., 2014a). Lo anterior le confiere diversas ventajas: (i) le proporciona acceso a un nicho intracelular protegido, (ii) le permite evadir al sistema inmune del hospedero, facilitando así su reproducción y/o diseminación a otros tejidos, además de que (iii) disminuye la eficacia de la terapia antimicrobiana (Almeida et al., 1996). Las cepas facultativas intracelulares de *S. aureus* se relacionan generalmente con enfermedades crónicas y recurrentes (Garzoni y Kelley, 2009; Fraunholz y Sinha, 2012).

III.4 Mecanismo de internalización de *Staphylococcus aureus* en células fagocíticas no profesionales

El principal mecanismo de internalización de *S. aureus* en las CFNP es un proceso de endocitosis mediada por receptores (mecanismo zipper) (Hauck et al., 2012). En este mecanismo la proteína de unión a fibronectina (FnBP) presente en la superficie bacteriana se une a la fibronectina (Fn) de la matriz extracelular, y este complejo (Fn-FnBP) es reconocido por las integrinas $\alpha 5\beta 1$ (receptor) de la célula hospedera (Sinha et al., 1999; Sinha y Herrmann, 2005; Hauck et al., 2012). Posteriormente, en el sitio de adhesión de la bacteria se induce el agrupamiento de las integrinas y el reclutamiento local de proteínas estructurales, como la rensina y zyxina, y de enzimas de señalización como las tirosina cinasas de la familia Src y las cinasas de adhesión focal (FAK) (Dziewanowska et al., 1999; Selbach y Backert, 2005). La actividad combinada de las FAK y Src favorece la fosforilación de tirosinas en proteínas efectoras, como la cortactina (Selbach y Backert, 2005). Esta proteína se relaciona con la internalización bacteriana debido a su efecto sobre el rearreglo del citoesqueleto por el complejo Arp2/3 (McNiven et al., 2000; Sinha y Herrmann, 2005).

III.4.1 Otros receptores que participan en la internalización de *Staphylococcus aureus*

A pesar de que las integrinas $\alpha 5\beta 1$ son el principal receptor involucrado en la internalización de *S. aureus* en las células eucariotas, existen otros receptores -

de la inmunidad innata- en la célula hospedera que están involucrados en este proceso, como TLR2 y CD36 (Alva-Murillo et al., 2014a).

III.4.2 Receptores tipo Toll o TLRs

Los receptores TLRs forman parte esencial de la respuesta inmune innata y reconocen PAMPs. Los TLRs son receptores transmembranales que se caracterizan por tener un dominio citoplásmico similar al de los receptores de la familia IL-1, por lo que es llamado dominio del receptor Toll/IL-1 o TIR, el cual se requiere para la señalización intracelular, que activará diferentes factores transcripcionales (FT) (Akira et al., 2006). Además, los TLRs poseen un motivo extracelular de repetidos ricos en leucina (LRR), el cual es responsable del reconocimiento de los ligandos (Akira et al., 2006). Al unirse al ligando, los TLRs se multimerizan y activan, actuando en coordinación con otros TLRs o con moléculas accesorias (CD36 o CD14) (Diacovich y Gorvel, 2010). Una vez activados los receptores se inician rutas de señalización que culminan en la inducción de la expresión de genes (ej. citocinas, quimiocinas, péptidos antimicrobianos, enzimas) involucrados en la respuesta inmune del hospedero. Estas rutas conducen a la activación de proteínas cinasas activadas por mitógenos (MAPKs) como p38, JNK o ERK, y de FT como el factor de transcripción nuclear (NF-κB), AP-1 (activator protein-1) o el factor de transcripción regulador de interferones (IRF) (Akira et al., 2006). El receptor TLR2 reconoce al ácido lipoteicoico (LTA) o al peptidoglicano (PGN) de *S. aureus*, y puede interactuar física y funcionalmente con los receptores TLR1 y TLR6. El heterodímero TLR2/TLR6 reconoce lipoproteínas diaciladas provenientes de micoplasma, y participa en la activación de fibroblastos por LTA (Takeuchi et al., 2001). Por otro lado, cuando TLR2 se asocia con TLR1, este heterodímero es capaz de reconocer a los lipopéptidos triacilados (Takeuchi et al., 2002).

En células fagocíticas profesionales (macrófagos) se ha demostrado que TLR2 está involucrado en la internalización bacteriana (Watanabe et al., 2007; Fournier 2012; Yimin et al., 2013; Fang et al., 2014). En estudios *in vivo* se ha

reportado que los ratones deficientes en TLR2 son más susceptibles a las infecciones por *S. aureus* (Hoebe et al., 2005). Sin embargo, poco se sabe de la participación de este receptor en la internalización de *S. aureus* en las CFNP. En este sentido, se ha sugerido que este receptor tiene una participación en la internalización de *S. aureus* en CFNP ya que su bloqueo con un anticuerpo neutralizante, en células cebadas humanas y CEMB, condujo a una disminución en la internalización (Rocha-de-Souza et al., 2008; Medina-Estrada et al., 2015).

III.4.3 CD36

CD36 es una glicoproteína de membrana que pertenece a la familia de receptores "scavenger" clase B. Participa en rutas de señalización donde se asocia (i) con TLR2, (ii) la activación de la MAPK p38 y (iii) la producción de péptidos antimicrobianos en queratinocitos (Li et al., 2013). En macrófagos, CD36 puede interactuar con TLR2 para el reconocimiento de *S. aureus* (Hoebe et al., 2005). Además, actúa como un receptor fagocítico en macrófagos, ya que cuando este tipo celular no expresa el receptor se presenta una reducción en la internalización de *S. aureus* y del LTA, acompañada de una producción deficiente de citocinas (TNF-α e IL-12) (Stuart et al., 2005). La internalización mediada por CD36 requiere del dominio citoplásmico (carboxilo terminal) del receptor para inducir la señalización por TLR2/TLR6 (Stuart et al., 2005). En células HEK293 y HeLa, la sobre-expresión de CD36 aumenta la internalización de *E. coli* o *S. aureus* (Stuart et al., 2005; Baranova et al., 2008). No obstante, el papel de CD36 en la internalización de *S. aureus* en las CEMB es desconocido.

Para una descripción más detallada de los mecanismos de internalización de *S. aureus* en las CFNP, se refiere al lector al capítulo 4 de esta tesis.

III.5 Ácidos grasos

Los ácidos grasos (AG) son compuestos orgánicos constituidos por una cadena hidrocarbonada unida a un grupo carboxilo terminal (-COOH). La cadena hidrocarbonada puede estar saturada (sin dobles enlaces) o insaturada (uno o

más dobles enlaces), ser lineal o ramificada, o puede tener estructuras cíclicas o grupos hidroxilos. De acuerdo a la longitud de la cadena hidrocarbonada estos compuestos se pueden clasificar en (i) ácidos grasos de cadena corta (AGCC), que constan de 2-6 carbonos, (ii) ácidos grasos de cadena media (AGCM), que van de 8-14 carbonos, y (iii) ácidos grasos de cadena larga (mayores a 16 átomos de carbono) (Neville y Picciano, 1997; Bauman y Griinari, 2003).

En la glándula mamaria bovina los AGCC y AGCM se producen por síntesis *de novo*, siendo sus precursores el acetato y el β-hidroxibutirato (Bauman y Griinari, 2003; Mansson, 2008). Ambos se generan en el rumen por la fermentación bacteriana de la fibra dietética. El ácido butírico (butirato, 4C) sólo se encuentra presente en la leche bovina, lo cual la diferencia de las otras leches de mamíferos (Parodi, 1997). Por otro lado, los ácidos grasos de cadena larga son derivados de los triacilglicéridos que forman parte de las lipoproteínas de la sangre -quilomicrones y VLDL (lipoproteínas de muy baja densidad)- después de la hidrólisis en la glándula mamaria por la lipasa de lipoproteínas (LPL) (Mansson 2008). Los AG de 16 carbonos se originan de ambas fuentes (Bauman y Griinari, 2003; Mansson, 2008).

Además de estar presente en la leche bovina, los humanos obtienen los AGCC y AGCM a partir de otras fuentes. En este sentido, los AGCC son los principales productos obtenidos del metabolismo bacteriano de la fibra dietética en el colon, por lo que la mayoría de sus efectos se han descrito en el tracto intestinal (Hamer et al., 2008; Canani et al., 2011). Mientras que los AGCM se obtienen a partir de alimentos como el yogurt, aceite de coco, mantequilla y queso (Nagao y Yanagita, 2010). Los AGCM, unidos a los triacilglicéridos de los alimentos, son hidrolizados y viajan a través de la vena portal para ser absorbidos por el hígado, sirviendo como fuente de energía.

III.6 Propiedades fisiológicas de los AGCC y AGCM

Los AGCC son la fuente principal de energía para los rumiantes, llegando a cubrir cerca del 70% de sus requerimientos energéticos, y en los humanos se ha

estimado que cubren entre un 5-15% (Bergman, 1990). El AGCC más estudiado, tanto en humanos como en animales de granja, es el butirato. Dentro de los efectos que ejerce este AG se encuentran la inhibición de la carcinogénesis, la estimulación del flujo de sangre y la mejora de la barrera de defensa del colon; además, influye en la captación del fluido electrolítico, en la contracción del músculo liso, y disminuye el estrés oxidativo (Hamer et al., 2008; Canani et al., 2011). El butirato es un inhibidor de la proliferación celular y de la angiogénesis, además promueve la diferenciación celular y la apoptosis en diferentes líneas celulares cancerosas, entre ellas las derivadas de cáncer de mama y colon (Mandal y Kumar, 1996; Emenaker et al., 2001; Zeng et al., 2015). Sin embargo, el butirato ejerce un efecto contrario en los colonocitos normales, ya que induce su proliferación, lo cual es conocido como “la paradoja del butirato” (Canani et al., 2011).

En cuanto a los efectos de los AGCM, se sabe que el octanoato (8C) induce la perturbación del ciclo celular en preadipocitos humanos, inhibe la adipogénesis y favorece la lipogénesis, al regular la expresión y activación de FTs como el receptor activado por proliferadores peroxisomales gamma (PPARγ) (Nakajima et al., 2003; Yang et al., 2009). En células epiteliales mamarias bovinas, este AGCM induce la expresión del gen del receptor CD36 y los niveles de la proteína de PPARγ (Yonezawa et al., 2004).

Adicionalmente, se ha descrito que tanto los AGCC como los AGCM pueden ejercer una actividad antimicrobiana *in vitro* contra patógenos del tracto intestinal (*E. coli, Salmonella enterica, Shigella flexneri, Campylobacter jejuni, Clostridium perfringens*) (Skrivanova et al., 2005; Van Immerseel et al., 2006; Van Deun et al., 2008a). En particular, el octanoato y su monoacilglicérido (monocaprilina) actúan también como antimicrobianos contra patógenos de la mastitis bovina, como *S. aureus*, *Streptococcus agalactie*, *Streptococcus dysgalactiae*, *Streptococcus uberis* y *E. coli* (Nair et al., 2005).

III.7 Ácidos grasos de cadena corta y de cadena media como inmunomoduladores

Los AGCC y AGCM no sólo poseen propiedades fisiológicas interesantes, sino que también se han asociado con la regulación de la respuesta inmune en distintos organismos. En este sentido, existen numerosos reportes que evidencian la participación de los AGCC –principalmente el butirato- en el control (inhibición) de los procesos inflamatorios de colon en humanos, e incluso se ha evaluado su uso terapéutico contra estos padecimientos en modelos animales (Tedelind et al., 2007; Hamer et al., 2008; Raqib et al., 2012; Vinolo et al., 2012). En ratones alimentados con una dieta alta en grasas (ambiente inflamatorio) el tratamiento con butirato (tribiturina) protege al animal de la resistencia a la insulina y la dislipidemia, además atenúa la producción del TNF-α e IL-1β tanto en macrófagos como en tejido adiposo, y reduce la infiltración de leucocitos a este último (Vinolo et al., 2012).

La respuesta inmune de las células fagocíticas profesionales o células de la inmunidad puede ser mediada por el butirato. En general, en un contexto de inflamación –inducido por LPS, TNF-α o IL-1β– en células inmunes (monocitos, macrófagos, neutrófilos, células cebadas, células T) de distintas especies (humano, rata, ratón), el butirato ejerce una actividad anti-inflamatoria (Meijer et al., 2010; Vinolo et al., 2011b; Nastasi et al., 2015). Este efecto se logra debido a que el butirato previene la quimiotaxis y la adhesión celular, así como la migración de leucocitos al sitio de la inflamación, lo cual puede tener un efecto protector (Meijer et al., 2010). Adicionalmente, favorece el fenotipo Th2 (anti-inflamatorio) de las células T. A nivel celular, este AGCC ejerce sus efectos al disminuir la expresión en la superficie celular de receptores de quimiocinas (ej. CXCR2), además evita la transcripción de genes de citocinas pro-inflamatorias (TNF-α, IL-1β, IL-6 y IL12p40) y de la quimiocina CCL2, así como la secreción de otras quimiocinas pro-inflamatorias (CCL3, CCL4, CCL5, CXCL9, CXCL10 y CXCL11) (Meijer et al., 2010; Vinolo et al., 2011b; Nastasi et al., 2015).

No obstante, existen reportes, en un contexto sin inflamación, donde el butirato puede actuar como pro-inflamatorio, al aumentar la expresión de moléculas de adhesión (L-selectina) en la superficie de neutrófilos y favorecer la quimiotaxis, o al inducir la expresión del gen de la quimiocina IL-8 en macrófagos de pollo (Meijer et al., 2010; Sunkara et al., 2012).

Los AGCC también ejercen efectos reguladores de la respuesta inmune de las CFNP, como las del endotelio y epitelio. Se ha descrito que el butirato aumenta la expresión de moléculas de adhesión (ICAM-1) en células endoteliales, lo que pudiera favorecer la adhesión celular (Meijer et al., 2010). Diversos estudios han evaluado el efecto inmunomodulador del butirato en células epiteliales en el contexto de enfermedades inflamatorias del tracto gastrointestinal (Hamer et al., 2008; Canani et al., 2011). En el epitelio intestinal el butirato regula la expresión y liberación de quimiocinas (IL-8, MCP-1 y CXCL-1) (Vinolo et al., 2011b). Y en condiciones inflamatorias (generadas por la IL-1β), el butirato disminuye la inducción de los genes de las quimiocinas CXCL5 y CXCL11, y de la IL-1β en el epitelio del colon (Blais et al., 2007). Además, la expresión de la IL-8 es modulada por el butirato, ante el estímulo infeccioso en el epitelio intestinal (Weng et al., 2007). El butirato también ejerce un efecto anti-inflamatorio en un contexto de inflamación en las CFNP.

Aparte de modular la inflamación, los AGCC pueden mejorar la respuesta inmune a través de la regulación de la respuesta antimicrobiana (expresión de PA). En células fagocíticas profesionales (macrófagos y monocitos de pollo) el butirato favorece la expresión de genes de β-defensinas (AvBD3, AvBD5, AvBD9, AvBD14) y de la catelicidina B (Sunkara et al., 2011; Sunkara et al., 2012). Mientras que en las CFNP, como el epitelio nasal, de colon y de pulmón humano, el butirato induce tanto la expresión del gen de la catelicidina (LL-37) como los niveles de la proteína (Schauber et al., 2003; Schauber et al., 2004; Kida et al., 2006; Schauber et al., 2006; Schwab et al., 2007; Chakraborty et al., 2009; Jiang et al., 2013; Liu et al., 2013). También favorece la expresión del gen y de la proteína de la β-defensina 2 (HBD-2) de humanos en células de colon (Schwab et

al., 2008). Además, en células epiteliales de cerdo el butirato y sus análogos favorecen la expresión de β-defensinas (pBD2, pBD3, pEP2C y pG1-5) (Zeng et al., 2013).

Existen pocos estudios en cuanto a las capacidades inmunomoduladoras de los AGCM. En este sentido, el octanoato presenta una dicotomía, ya que puede actuar como (i) pro-inflamatorio, al inducir la expresión del gen de la IL-8, o (ii) anti-inflamatorio, al disminuir la expresión de la quimiocina inducida por IL-1β en epitelio de colon (Andoh et al., 2000; Yoshida et al., 2001). También se ha reportado que el octanoato suprime la secreción de la IL-8 en células epiteliales Caco-2, al inhibir la activación de su promotor (Hoshimoto et al., 2002). Adicionalmente, este AGCM es capaz de inducir la expresión del gen de la defensina AvBD9 en macrófagos y monocitos de pollo (Sunkara et al., 2012).

Se han reportado diferentes mecanismos por los cuales los AGCC y AGCM ejercen su actividad inmunoreguladora. La acción anti-inflamatoria de estas moléculas –butirato- se relaciona principalmente con la inhibición que ejercen sobre la activación del factor NF-κB y la producción de citocinas pro-inflamatorias (ej. en el colon humano) (Inan et al., 2000; Canani et al., 2011). Otro mecanismo anti-inflamatorio conocido de estos AG es mediante la inhibición de la señalización del interferón (IFN)-γ y la regulación positiva de la expresión de PPARγ, que inhibe a su vez al factor NF-κB en células epiteliales del colon humano (Klampfer et al., 2003; Canani et al., 2011). La inducción de la quimiotaxis -de neutrófilos- por parte del butirato es dependiente de la activación de receptores acoplados a proteínas G (GPR43), MAPK (p38 y ERK), PI3Kγ y Rac2 (Vinolo et al., 2011b). Se sabe que el butirato es un inhibidor de las histonas desacetilasas (HDAC), resultando en la hiperacetilación de las histonas y en el relajamiento de la cromatina, lo cual puede favorecer la expresión de distintos genes de la respuesta inmune (Meijer et al., 2010). El mecanismo por el cual el octanoato modula la expresión de la IL-8 no se ha descrito, aunque se sabe que no es mediante la acetilación de histonas (H4), ni por su acción sobre el factor NF-κB (Hoshimoto et al., 2002).

La acción reguladora de la expresión de PA por parte del butirato puede deberse también a distintos mecanismos. En este sentido, las MAPK p38, JNK y ERK, y el FT AP-1 están involucrados en la expresión del gen del PA LL-37 en epitelio de pulmón (Kida et al., 2006). En este modelo, el butirato aumenta la acetilación de la histona H3 y H4 en la región promotora de LL-37. Por otro lado, en la inducción de la expresión de la catelicidina humana en epitelio de colon participan MAPKs, el receptor de la vitamina D (VDR) y TGF-β1, pero no PPARγ (Schwab et al., 2007). En células de colon los FT CREB y AP-1 regulan la inducción de la expresión de la catelicidina por el butirato (Chakraborty et al., 2009). No es de nuestro conocimiento la existencia de reportes que indiquen el mecanismo por el cual el octanoato modula la expresión de genes de PA.

III.8 Efecto de los ácidos grasos en la interacción hospedero-patógeno

El uso indiscriminado de antibióticos en el tratamiento de enfermedades infecciosas ha permitido el surgimiento de microorganismos resistentes a antibióticos. Debido a la amplia gama de efectos que ejercen los AGCC y AGCM, principalmente en el colon, han llamado la atención como tratamiento alternativo en enfermedades infecciosas del tracto intestinal. Es así como se han desarrollado diferentes estudios utilizando a estos compuestos como tratamiento profiláctico o terapéutico, o incluso evaluando su efecto sobre la patogenicidad del microorganismo. La mayoría de los reportes se enfocan en el efecto de los AGCC y AGCM en los procesos de interacción hospedero-patógeno, y en algunos estudios se determinó su participación en la internalización de patógenos en CFNP (ej. epitelio intestinal).

Al evaluarse el efecto terapéutico de los AGCC, se ha reportado que en conejos infectados con *Shigella flexneri* el tratamiento con butirato mejora los síntomas clínicos y físicos respecto a los conejos sin tratamiento, lo que está relacionado con la expresión y actividad del PA CAP-18 (catelicidina) en la superficie del epitelio de colon (Raqib et al., 2006). Adicionalmente, en un estudio en humanos con infección por *Shigella* spp., el enema de butirato disminuyó la

inflamación rectal y la concentración de IL-1β en las heces, pero aumentó la expresión del PA LL-37 en la superficie del epitelio del recto, así como su concentración en las heces (Raqib et al., 2012).

Existen pocos estudios donde evalúen el efecto de los AGCM como terapéuticos, pero los resultados no han sido positivos. En este sentido, se sabe que en pollos colonizados con *Campylobacter jejuni* y alimentados posteriormente con una dieta rica en octanoato, no se ve afectada la cuenta bacteriana en el ciego, lo cual puede deberse a un efecto protector de la mucosa intestinal sobre el patógeno (Hermans et al., 2010).

En general, al usar estos compuestos como tratamiento profiláctico o preventivo se han reportado efectos favorecedores para el hospedero. Al alimentar a pollos con butirato en distintas presentaciones (microperlas impregnadas del ácido, microcápsulas, polvo o disuelto en agua) se observó una disminción de la colonización de *S. enteritidis* en el tracto gastrointestinal (Van Immerseel et al., 2004c; Van Immerseel et al., 2005; Fernandez-Rubio et al., 2009). Sin embargo, se ha reportado que las microperlas cubiertas con butirato no protegen a los pollos de la colonización del ciego por *C. jejuni* (Van Deun et al., 2008a). No obstante, el butirato protege a células de colon humano de la invasión de *C. jejuni* (Van Deun et al., 2008b). En pollos pre-tratados con butirato se reduce el título de *S. enteritidis* recuperado, relacionándose este efecto con una reducción en la colonización bacteriana y un aumento en la expresión de PA (β-defensinas) (Sunkara et al., 2012).

Por otro lado, en conejos alimentados con una dieta rica en octanoato y triacilglicéridos de octanoato e infectados posteriormente con *E. coli* EPEC (enteropatogénica), disminuyó el número de bacterias en el ciego y en las heces, respecto a los conejos sin tratamiento (Skrivanova et al., 2008). Además, en pollos que fueron alimentados con una dieta suplementada con octanoato se recuperaron menos bacterias (*C. jejuni*) en el ciego y no se alteró la flora microbiana (Solis de los Santos et al., 2008; Solis de los Santos et al., 2010). Mientras que al suplementar la comida de pollos con octanoato disminuyó la

colonización de *S. enteritidis*, reduciendo potencialmente la capacidad bacteriana de invadir células epiteliales intestinales, debido a la disminución en la expresión de los genes *hilA* y *hilD* (regulador transcripcional de la isla de patogenicidad de *Salmonella* o SPI1) (Kollanoor-Johny et al., 2012).

Otra forma en la que se ha evaluado el efecto de estos AG en la interacción hospedero-patógeno es directamente sobre el patógeno. En este sentido, al tratar previamente a *S. enteritidis* con octanoato o butirato se redujo su internalización en las células de epitelio del ciego de pollos, mientras que el pretratamiento con otro AGCC (acetato, 2C) aumentó su virulencia y su capacidad de invasión (Van Immerseel et al., 2004a; Van Immerseel et al., 2004b). El efecto de estos AG se relacionó con la supresión de la expresión del gen de virulencia *hilA* (Van Immerseel et al., 2004a; Boyen et al., 2008). Otro gen de virulencia cuya expresión se ve reducida con el octanoato es *fimA* (gen que codifica la subunidad mayor de la fimbria o pilus tipo 1). Como consecuencia de esta inhibición, se observó que la internalización de *Salmonella typhimurium* en células de epitelio intestinal de cerdo disminuyó al pretratarlas con octanoato (2 mM) o butirato (10 mM) (Boyen et al., 2008).

III. 9 Antecedentes

La RII es la primera línea de defensa que se activa ante el ataque de un patógeno. Dentro de este mecanismo de defensa, las células epiteliales juegan un papel muy importante. En particular, las CEMB son centinelas en la glándula mamaria y su activación por el patógeno invasor determina la eficacia de la respuesta del hospedero (Günther et al., 2011). Las CEMB son un blanco para distintos microorganismos, como *S. aureus*, principal patógeno contagioso causante de la mastitis bovina (Kerro Dego et al., 2002). Esta bacteria tiene la capacidad de invadir la célula hospedera, lo cual permite evadir la respuesta inmune del hospedero, disminuye el éxito de la terapia antimicrobiana y se asocia con enfermedades crónicas y recurrentes (Almeida et al., 1996; Fraunholz y Sinha, 2012).

El principal mecanismo de la internalización de *S. aureus* es mediado por las integrinas $\alpha 5\beta 1$ de la célula hospedera y por la proteína de unión a fibronectina (FnBP) de la bacteria (Sinha et al., 1999). En células fagocíticas profesionales se ha reportado la participación de otros receptores, como TLR2 y CD36, en el reconocimiento e internalización de *S. aureus* (Watanabe et al., 2007; Fournier 2012; Yimin et al., 2013; Fang et al., 2014). Sin embargo, se sabe poco acerca de la participación de estos receptores en la internalización bacteriana en las CFNP. En este sentido, el bloqueo de TLR2 -con anticuerpos neutralizantes- en células cebadas y CEMB disminuye el número de *S. aureus* recuperado (Rocha-de-Souza et al., 2008; Medina-Estrada et al., 2015).

En conjunto, la capacidad de *S. aureus* de internalizarse en la célula hospedera y el surgimiento de cepas resistentes a antibióticos, han hecho difícil el tratamiento de estas enfermedades de manera convencional. Por lo que distintos estudios se han enfocado en moléculas inmunomoduladoras que regulen la inmunidad innata del hospedero, para evitar el establecimiento de la enfermedad o para favorecer su resolución (Lippolis et al., 2011; Raqib et al., 2012).

Los AGCC y AGCM no sólo son compuestos interesantes por sus propiedades nutricionales, si no que han llamado la atención por su capacidad de regular la respuesta inmune, particularmente el butirato (Canani et al., 2011). Se ha demostrado el efecto anti-inflamatorio *in vitro* e *in vivo* del butirato en procesos inflamatorios del tracto intestinal (ej. colitis) (Meijer et al., 2010; Canani et al., 2011). Respecto a los AGCM, se sabe que el octanoato actúa como anti-inflamatorio en un ambiente inflamatorio *in vitro* en células de colon (Hoshimoto et al., 2002). Interesantemente, tanto los AGCC como los AGCM son inductores de la expresión de genes de PA, lo cual favorece la RII del hospedero (Sunkara et al., 2012; Jiang et al., 2013). Debido a lo anterior y como alternativa al uso de antibióticos, se han aplicado estos compuestos como tratamiento preventivo o terapéutico en enfermedades infecciosas del tracto intestinal (*E. coli*, *Salmonella* spp, *C. jejuni, Shigella flexneri*) en animales de granja (conejos, cerdos, pollos) e incluso en humanos (Van Immerseel et al., 2005; Raqib et al., 2006; Skrivanova et

al., 2008; Hermans et al., 2010; Raqib et al., 2012; Sunkara et al., 2012). Sin embargo, en pocos de estos estudios se ha evaluado el efecto de estos AG en otros modelos (patógenos intracelulares) y sobre la respuesta inmune del hospedero (Raqib et al., 2006; Raqib et al., 2012; Sunkara et al., 2012).

En este sentido, en nuestro grupo de trabajo se ha demostrado que el butirato (0.5 mM) disminuye la internalización de *S. aureus* (~50%) en las CEMB, además regula positivamente elementos de la inmunidad innata durante la infección, como la expresión de los genes del TAP e iNOS (óxido nítrico sintasa inducible), y la producción del óxido nítrico (Ochoa-Zarzosa et al., 2009). Lo anterior puede relacionarse con el aumento de la acetilación de histonas H3 debido al butirato y al estímulo bacteriano. Adicionalmente, el octanoato (1 mM), también disminuye la internalización de *S. aureus* (~50%) en las CEMB y favorece la expresión de los PA LAP, BNBD4 y BNBD10 durante la infección (Alva-Murillo et al., 2013a). Mientras que este proceso de internalización aumenta (~160%) con 0.25 mM de octanoato, condición en la que prácticamente no se observa respuesta antimicrobiana por parte de las CEMB (Alva-Murillo et al., 2013a).

IV. JUSTIFICACIÓN

Staphylococcus aureus es un patógeno exitoso que genera distintas enfermedades infecciosas relacionadas a su persistencia extracelular, aunque también posee la habilidad de invadir a las células hospederas (células fagocíticas profesionales y no profesionales). Al ser una bacteria facultativa intracelular es capaz de generar enfermedades crónicas y persistentes. La capacidad de internalizarse en las células hospederas le confiere a *S. aureus* dos ventajas importantes: 1) evadir los mecanismos de defensa y 2) disminuir el éxito de la terapia antimicrobiana. Aunado a esto, el surgimiento de cepas resistentes a antibióticos ha hecho difícil el tratamiento de estas enfermedades. Además, esta bacteria evita la adecuada activación de la RII del hospedero (ej. CEMB). Es así como diversos estudios se han enfocado a la utilización de moléculas con efectos reguladores de la inmunidad innata, como los ácidos grasos, como agentes que prevengan el establecimiento de las enfermedades infecciosas.

En este sentido, en nuestro grupo de trabajo se reportó que los AGCC y AGCM modulan tanto la internalización de *S. aureus* como la RII en las CEMB, pero aún no se ha descrito el mecanismo por el cuál estas moléculas regulan dicho proceso. Con base en lo anterior, en el presente trabajo se identificaron las rutas de transducción celular involucradas en la regulación de la internalización de *S. aureus* en las CEMB en presencia de los AG. Para ello, los experimentos planteados se realizaron utilizando un AGGC que disminuye la invasión (0.5 mM butirato) y un AGCM (0.25 y 1 mM octanoato) que posee un efecto dual.

V. HIPÓTESIS

En las células epiteliales mamarias bovinas, el butirato y octanoato de sodio regulan negativamente elementos de las rutas de señalización de los receptores integrinas $\alpha 5\beta 1$, TLR2 y CD36, disminuyendo así la internalización de *Staphylococcus aureus*.

VI. OBJETIVOS

VI.1 Objetivo general

Evaluar la participación de los receptores integrinas $\alpha 5\beta 1$, TLR2 y CD36, y sus rutas de señalización en la internalización de *Staphylococcus aureus* a células epiteliales mamarias bovinas, regulada por butirato y octanoato de sodio.

VI.2 Objetivos específicos

VI.2.1 Establecer la participación de los receptores integrinas $\alpha 5\beta 1$, TLR2 y CD36 en la internalización de *S. aureus* en las células epiteliales mamarias bovinas tratadas con butirato y octanoato de sodio.

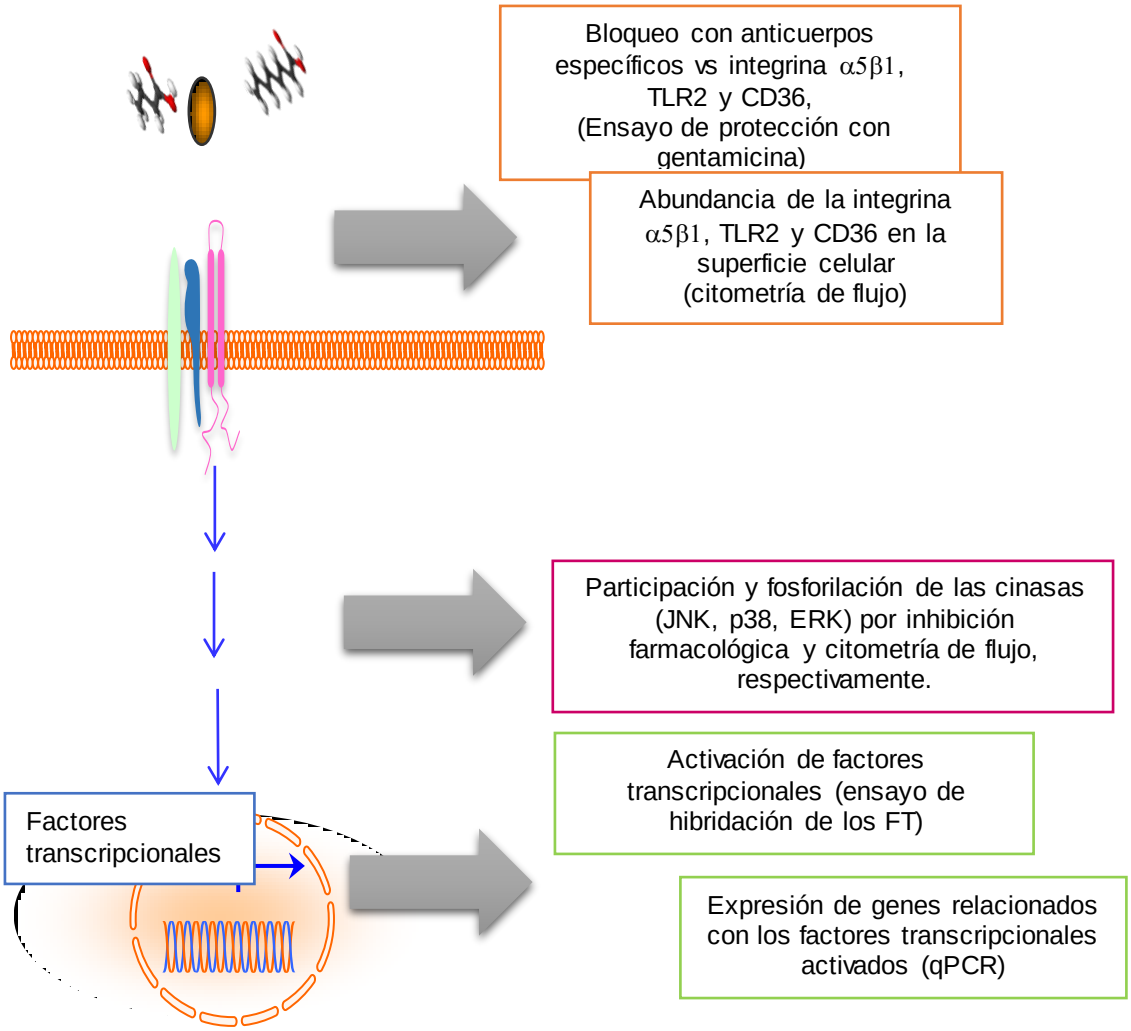
VI.2.2 Determinar el papel de las MAPKs (JNK, p38 o ERK) en la internalización de *S. aureus* a las células epiteliales mamarias tratadas con butirato y octanoato de sodio.

VI.2.3 Establecer el estado de activación de factores transcripcionales de la respuesta inmune innata y su relación con la internalización de *S. aureus*, en las células de epitelio mamario bovino tratadas con butirato y octanoato de sodio.

VI.2.4 Determinar la expresión de los genes de la RII? modulados por factores transcripcionales (ej. citocinas pro y anti-inflamatorias, quimiocinas y péptidos antimicrobianos) y establecer su relación con la internalización de *S. aureus*, en las células de epitelio mamario bovino tratadas con butirato y octanoato de sodio.

VII. ESTRATEGIA EXPERIMENTAL

Análisis de las rutas de señalización involucradas en la internalización de *Staphylococcus aureus*, a las células epiteliales mamarias bovinas, regulada por ácidos grasos



VIII. RESULTADOS

Los resultados generados con la realización del presente trabajo se presentan en 4 capítulos.

1) El capítulo I corresponde al artículo de investigación: “The activation of the TLR2/p38 pathway by sodium butyrate in bovine mammary epithelial cells is involved in the reduction of *Staphylococcus aureus* internalization”. Molecular Immunology. 68 (2): 445-455. ISSN: 0161-5890. doi: 10.1016/j.molimm.2015.09.025. Factor de impacto 2.97 (2014 JCR Science Edition).

2) En el capítulo II se presenta el manuscrito: “Sodium octanoate modulates the innate immunity response of bovine mammary epithelial cells through TLR2/p38/JNK/ERK1/2 pathway: implications in *Staphylococcus aureus* internalization”.

3) El capítulo III corresponde a los resultados de: “Participación de las integrinas $\alpha 5\beta 1$ en la internalización de *Staphylococcus aureus* en las células epiteliales mamarias bovinas regulada por butirato”.

4) El capítulo IV comprende el artículo de revisión: “Nonprofessional phagocytic cell receptors involved in *Staphylococcus aureus* internalization. BioMed Research International, vol. 2014, Article ID 538546, 9 pages, 2014. doi:10.1155/2014/538546. ISSN: 1110-7243. Factor de impacto 1.574 (2014 JCR Science Edition).

CAPÍTULO I

The activation of the TLR2/p38 pathway by sodium butyrate in bovine mammary epithelial cells is involved in the reduction of *Staphylococcus aureus* internalization

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The activation of the TLR2/p38 pathway by sodium butyrate in bovine mammary epithelial cells is involved in the reduction of *Staphylococcus aureus* internalization

Nayeli Alva-Murillo, Ivan Medina-Estrada, Marisol Báez-Magaña, Alejandra Ochoa-Zarzosa, Joel E. López-Meza*

Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 Carr, Morelia-Zinapécuaro, Posta Veterinaria, C.P. 58893 Morelia, Michoacán, Mexico

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ABSTRACT

Staphylococcus aureus is an etiological agent of human and animal diseases, and it is able to internalize into non-professional phagocytic cells (i.e. bovine mammary epithelial cells, bMECs), which is an event that is related to chronic and recurrent infections. bMECs contribute to host innate immune responses (IIR) through TLR pathogen recognition, whereby TLR2 is the most relevant for *S. aureus*. In a previous report, we showed that sodium butyrate (NaB, 0.5 mM), which is a short chain fatty acid (SCFA), reduced *S. aureus* internalization into bMECs by modulating their IIR. However, the molecular mechanism of this process has not been described, which was the aim of this study. The results showed that the TLR2 membrane abundance (MA) and mRNA expression were induced by 0.5 mM NaB ~1.6-fold and ~1.7-fold, respectively. Additionally, 0.5 mM NaB induced p38 phosphorylation, but not JNK1/2 or ERK1/2 phosphorylation in bMECs, which reached the baseline when the bMECs were *S. aureus*-challenged. Additionally, bMECs that were treated with 0.5 mM NaB (24 h) showed activation of 8 transcriptional factors (AP-1, E2F-1, FAST-1, MEF-1, EGR, PPAR, ER and CBF), which were partially reverted when the bMECs were *S. aureus*-challenged. Additionally, 0.5 mM NaB (24 h) up-regulated mRNA expression of the antimicrobial peptides, TAP (~4.8-fold), BNBD5 (~3.2-fold) and BNBD10 (~2.6-fold). Notably, NaB-treated and *S. aureus*-challenged bMECs increased the mRNA expression of all of the antimicrobial peptides that were evaluated, and this was evident for LAP and BNBD5. In the NaB-treated bMECs, we did not detect significant expression changes for IL-1β and IL-6 and only TNF-α, IL-10 and IL-8 were induced. Interestingly, the NaB-treated and *S. aureus*-challenged bMECs maintained the anti-inflammatory response that was induced by this SCFA. In conclusion, our results suggest that 0.5 mM NaB activates bMECs via TLR2/p38, which leads to improved antimicrobial defense before/after pathogen invasion, and NaB may exert anti-inflammatory effects during infection.

1. Introduction

Staphylococcus aureus is the etiological agent in a wide range of human and animal diseases (Lowy, 1998). Chronic and recurrent infections related to this bacterium have been associated with its ability to internalize and survive within professional and nonprofessional phagocytic cells (NPPCs) (Almeida et al., 1996; Garzoni and Kelley, 2009; Fraunholz and Sinha 2012).

S. aureus internalization occurs mainly by a zipper-like process, which is mediated by α5β1 integrin in the membrane of the host cell (Hauck et al., 2012). However, others host cell proteins are involved in this process, such as TLR2 and CD36 (Alva-Murillo et al., 2014a). Reports have described the participation of TLR2 in *S. aureus* internalization into phagocytic cells (Watanabe et al., 2007; Fournier, 2012; Yimin et al., 2013; Fang et al., 2014), but little is known about the participation of TLR2 during *S. aureus* internalization into NPPCs. In this sense, it is known that the blockage of TLR2 with neutralizing antibodies in human mast cells and bovine mammary epithelial cells (bMECs) decreases the number of internalized *S. aureus* (Rocha-de-Souza et al., 2008; Medina-Estrada et al., 2015). Additionally, TLR2 is located in phagosomes and co-

* Corresponding author. Fax: +52 443 295 8029.
E-mail address: elmeza@umich.mx (J.E. López-Meza).

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localizes with different *S. aureus* pathogen-associated molecular patterns (PAMPs) (Müller et al., 2010). Although the role of TLR2 during *S. aureus* internalization is unclear, it has been previously suggested that TLR2 activation is a pre-requisite for this process.

On the other hand, CD36, a membrane glycoprotein that belongs to the class B scavenger receptor family, interacts with TLR2 during *S. aureus* recognition as a coreceptor for diacylglyceride detection through the TLR2/6 complex, which mediates bacterial invasion primarily in phagocytic cells (Hoebe et al., 2005). In HEK 293 and HeLa cells, CD36 overexpression increases *S. aureus* or *Escherichia coli* internalization (Stuart et al., 2005; Baranova et al., 2008). Nevertheless, the role of CD36 in *S. aureus* internalization into bMECs is unknown.

Mammary epithelial cells can display a relevant innate immune response (IIR) in mammary glands (Rainard and Riollet, 2006). In cattle, the bMECs contribution to udder defense is mediated by pattern recognition receptors (PRRs), with TLR2 being the most relevant receptor for *S. aureus* recognition. Additionally, this stimulation leads to the activation of MAPK family members (JNK, ERK and p38) and transcription factors, such as NF-κB, AP-1 or IRF1/2, which in turn stimulates antimicrobial and inflammatory gene expression (Akira et al., 2006; Chiu et al., 2009; Stack et al., 2014). It is well known that p38 activation is triggered by *S. aureus* stimulation (live or killed bacteria or PAMPs) in phagocytic cells (McLeish et al., 1998; Fang et al., 2014; Song et al., 2014; Chekabab et al., 2015). Also, the role of p38 in *S. aureus* internalization has been demonstrated in phagocytic cells (e.g. macrophages and monocytes) by pharmacological inhibition (Kapetanovic et al., 2011). However, in NPPCs (ca. osteoblasts) *S. aureus* did not modify the phosphorylation of p38 (Ellington et al., 2001). In addition, the TLR2/p38 interaction has been related with *S. aureus* stimulation of immune system (antimicrobial peptide expression) but no with bacteria internalization (Menzies and Kenoyer, 2006). An attractive alternative to avoiding bacterial internalization in bMECs is the modulation of their innate immune response to facilitate the elimination of the invading pathogen.

One of the most relevant immunomodulatory molecules is butyrate, which is a short chain fatty acid (SCFA) that is produced in the colonic lumen by the bacterial fermentation of dietary fiber. Additionally, bovine milk is the only mammalian milk that contains this SCFA (2–5% wt) (Jensen, 2002). Butyrate has been associated with several effects at the intestinal and extraintestinal levels, such as cell growth, differentiation, apoptosis induction in transformed epithelial cells, satiety, oxidative stress in the intestine, colonic barrier defense, intestinal epithelial permeability and immune modulation (Hamer et al., 2008; Canani et al., 2011). Moreover, butyrate modulates several signaling pathways in intestinal epithelial cells, including that of the transcription factor AP-1 (Mandal et al., 2001; Hamer et al., 2008; Canani et al., 2011). Additionally, this SCFA possesses anti-inflammatory properties that interfere with NF-κB activation or inhibit interferon-γ signaling, peroxisome proliferator-activated receptor γ (PPARγ) up-regulation, and histone deacetylase (HDAC) (Inatomi et al., 2005; Schwab et al., 2007a; Hamer et al., 2008; Canani et al., 2011). Furthermore, butyrate is a strong inducer of antimicrobial peptides in human and animal cells (Schauber et al., 2003; Zeng et al., 2013; Sunkara et al., 2014).

In previous reports, we demonstrated that short and medium fatty acids reduce *S. aureus* internalization into bMECs (Ochoa-Zarzosa et al., 2009; Alva-Murillo et al., 2012, 2013). In particular, 0.5 mM sodium butyrate (NaB) reduces ~50% *S. aureus* internalization into bMECs and up-regulates tracheal antimicrobial peptide (TAP) gene expression (Ochoa-Zarzosa et al., 2009). However, the molecular mechanisms that are regulated by butyrate regarding bacterial internalization reduction and the modulation of the bMEC innate immune response are unknown. In this study, we showed that 0.5 mM NaB activates bMECs via TLR2/p38, which leads to the improvement of the antimicrobial defense against pathogen invasion and may exert anti-inflammatory effects during infection.

2. Materials and methods

2.1. Staphylococcus aureus *strain*

The *S. aureus* subsp. *aureus* (ATCC 27543) strain used in this study was isolated from a case of bovine clinical mastitis. This strain has a recognized capacity to be internalized into bMECs (Gutiérrez-Barroso et al., 2008). Bacteria were grown overnight in Luria-Bertani broth (LB, Bioxon, México), and the colony forming units (CFU) were adjusted by measuring their optical density at 600 nm (OD 0.2 = 9.2×10^7 CFU/ml).

2.2. Reagents and antibodies

LTA (from *S. aureus*), LPS (from *E. coli* 0111:B4) and sodium butyrate (NaB) were acquired from Sigma–Aldrich (St. Louis, MO, USA). In this study, we used 0.5 mM NaB, which inhibits *S. aureus* internalization into bMECs (Ochoa-Zarzosa et al., 2009). The monoclonal blocking antibodies used were anti-TLR2 (TL2.1, Abcam) and anti-CD36 (FA6-152, Abcam). The MAPK inhibitors, SB20358 (p38), SP600125 (JNK) and U0126 (ERK1/2) were acquired from Cell Signaling Technology^R (Boston, MA). The working solutions were dissolved in dimethyl sulfoxide (DMSO), which was employed as vehicle in the corresponding experiments.

2.3. Primary bovine mammary epithelial cell (bMEC) culture

bMEC isolation was performed on udder alveolar tissue from healthy lactating cows as described previously (Anaya-López et al., 2006). Cells from passages 2–8 were used in all of the experiments. The cells were cultured in petri dishes (Corning-Costar) in growth medium (GM) that was composed of a DMEM medium/nutrient mixture F-12Ham (DMEM/F-12K, Sigma), which was supplemented with 10% fetal calf serum (Equitech Bio), 10 μg/ml insulin (Sigma), 5 μg/ml hydrocortisone (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B (Invitrogen). The bMECs were grown in 5% CO_2 atmosphere at 37 °C. To perform the *S. aureus* challenge, the bMECs were cultured in serum-free growth medium without antibiotics for 24 h, and then, they were infected.

2.4. Invasion assays

Polarized bMEC monolayers were cultivated on 96-well flat-bottom plates that were coated (Corning-Costar) with 6–10 μg/cm^2 rat-tail type I collagen (Sigma). Prior to the invasion assays, the bMECs (~10×10^3 cells/well) were incubated with 0.5 mM NaB in DMEM/F12K (Sigma) without antibiotics and serum for 24 h. Then, the cells were treated separately with different blocking antibodies, including anti-TLR2 (5 μg/ml, 1 h) and anti-CD36 (0.25 μg/ml, 45 min). Mouse IgG (purified from normal mouse serum that was purchased from Pierce) was used as the negative control. The blocking of the invasion assays was performed using gentamicin protection assays as described (Gutiérrez-Barroso et al., 2008). Briefly, the bMECs that were used in the antibody blockage experiments were infected with *S. aureus* (MOI 30:1 bacteria per cell). For this, the bMECs were inoculated with 3.5 μl of bacterial suspensions to 9.2×10^7 CFU/ml and incubated for 2 h in 5% CO_2 at 37 °C. Then, the bMECs were washed three times with PBS (pH 7.4) and incubated in GM without serum and penicillin and streptomycin, and they were supplemented with 50 μg/ml gentamicin for 1 h at 37 °C to eliminate extracellular bacteria. Finally, the bMEC monolayers were detached with trypsin-EDTA (Sigma) and lysed with 250 μl

of sterile distilled water. The bMEC lysates were diluted 100-fold, plated on LB agar in triplicate and incubated overnight at 37 °C. The CFUs were determined with the standard colony counting technique. The data are presented as the ratio CFU/bMEC.

2.5. Flow cytometry analysis

To evaluate the cell-surface expression of the TLR2 and CD36 receptors, $\sim 2 \times 10^5$ bMECs/well were cultured to 80% confluence on 24-well plates (Corning) and then treated with 0.5 mM NaB, *S. aureus*, or both as described for the invasion assays. Then, the bMECs were detached with trypsin/EDTA (Sigma) and the cell pellet was recovered by centrifugation (2500 rpm, 10 min, 4 °C) and washed 2X with cold-PBS (pH 7.4). The cells were fixed with 4% paraformaldehyde for 10 min at 4 °C. The bMECs were blocked with normal goat serum (5% in PBS, Pierce) for 30 min at 4 °C with shaking and then recovered by centrifugation. Further, the bMECs were incubated with primary antibodies, anti-TLR2 (0.666 μg/ml in PBS containing 0.1% BSA) or anti-CD36 (2 μg/ml in PBS containing 0.1% BSA), overnight at 4 °C. Then, the bMECs were incubated with a Rhodamine-conjugated secondary antibody against mouse IgG (1:50, Molecular Probes) for 2 h on ice. The samples were analyzed in a BD Accuri™ C6 flow cytometer with the BD Accuri C6 software. Ten thousand events were collected and analyzed. bMECs that were incubated only with the secondary antibody were used as a negative control. For TLR2 membrane abundance assessments, the positive control was bMECs that were stimulated with LPS (1 μg/ml, Sigma) for 24 h.

2.6. Role of MAP kinases in S. aureus internalization into bMECs

Polarized bMEC monolayers were cultivated on 96-well flat-bottom plates that were coated (Corning-Costar) with 6–10 μg/cm^2 rat-tail type I collagen (Sigma). The bMECs were incubated with 0.5 mM NaB in DMEM/F12K (Sigma) without antibiotics and serum for 24 h. Prior to the invasion assay (30 min, 1 or 2 h), pharmacological inhibitors of p38 (2.5–10 μM, SB203580), JNK (5–20 μM, SP600125) or ERK1/2 (0.62–10 μM, U0126) were added separately to the bMECs. Infection assays were performed with gentamicin protection assays as described above. The cells lysates were plated on LB agar in triplicate and incubated overnight at 37 °C. The CFU numbers were determined with the standard colony counting technique. DMSO (vehicle) was used as a control.

To evaluate the MAP kinase activation levels by flow cytometry, the bMECs were treated with 0.5 mM NaB, *S. aureus* or both, and the samples (30 μg of protein) were prepared according to the manufacturer's protocol for adherent cells (Becton Dickinson, Germany). pp38 (T180/Y182), pJNK1/2 (T183/185), and pERK1/2 (T202/Y204) were quantitatively determined using antibodies from a Flex Set Cytometric Bead Array (Becton Dickinson) according to the manufacturer's protocol. Flow cytometric analyses were performed using the BD Accuri™ C6 and CBA analysis FCAP software (Becton Dickinson). A total of 3000 events were acquired following the supplied protocol. The minimum detection levels for each phospho-protein were 0.38 U/ml for pJNK, and 0.64 U/ml for pp38 and pERK.

2.7. Transcription factor-DNA interactions

Nuclear proteins were obtained from bMECs using the NE-PER Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's instructions (Thermo Scientific, Rockfor, IL). Protein concentrations were determined with the Bradford assay. The nuclear extracts were subjected to the TranSignal Protein/DNA array I (Panomics, Fremont, CA, USA). Briefly, biotin-labeled DNA-binding oligonucleotides (TranSignal™ Probe Mix) were incubated with 15 μg/ml of nuclear extracts to allow for the formation of transcription factor/DNA complexes. These complexes were separated from free probes and hybridized to a protein/DNA array, and then, the complexes were detected using an HRP-based chemiluminescence method according to the manufacturer's protocol.

2.8. Analysis of mRNA expression of antimicrobial and inflammatory response genes

To analyze the effects of 0.5 mM NaB and/or *S. aureus* on the expression of IIR genes in bMECs, monolayers of cells that were cultured in 6 well dishes with 6–10 μg/cm^2 rat-tail type I collagen (Sigma) were incubated with 0.5 mM NaB (24 h) and/or *S. aureus* for 2 h (MOI 30:1). bMEC total RNA (5 μg) was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions, and then, they were used to synthesize cDNA as described (Alva-Murillo et al., 2013). The expression analysis of antimicrobial and inflammatory response genes was performed with qPCR using the comparative Ct method ($\Delta\Delta$Ct) in a StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The reactions were carried out with a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Specific primer pairs were acquired from Invitrogen and Elim Biopharm (Table S1), and their specificity was determined by end point PCR. GAPDH was used as an internal control (Alva-Murillo et al., 2013).

2.9. Data analysis

The data were obtained from three independent experiments, each of which was performed in triplicate, and compared with analysis of variance (ANOVA). The results are reported as the means ± the standard errors (SE), and the significance level was set at $P < 0.05$, except for RT-qPCR analysis where fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. For gene expression and membrane staining assays data were normalized to the untreated cells (control). The results from all of the experiments are shown.

3. Results

3.1. The CD36 receptor is involved in S. aureus internalization into bMECs

The role of the TLR2 receptor in *S. aureus* internalization into bMECs has been reported (Medina-Estrada et al., 2015). However, the role of CD36 in *S. aureus* internalization into bMECs remains unknown. We addressed the role of this receptor in this event using blocking invasion assays, flow cytometry and RT-qPCR. bMECs that were treated with a CD36-specific blocking antibody (0.25–1 μg/ml) and then challenged with *S. aureus* showed a decrease in the recovered CFU level (~50%) (Fig. 1A). This result shows that the scavenger receptor, CD36, was required for *S. aureus* internalization into the bMECs. This effect was specific because the bacterial internalization was not modified in mouse IgG-treated bMECs (data not shown). Moreover, increasing concentrations of antibody did not modify the *S. aureus* internalization level. In agreement with this result, in further experiments, we used the lowest antibody concentration (0.25 μg/ml). Additionally, we evaluated the membrane abundance (MA) and mRNA expression of this receptor. Cells that were challenged with *S. aureus* (2 h) did not show changes in the CD36 MA and mRNA expression (Fig. 1B-D). Only the positive control with LTA (1 μg/ml, 16 h) slightly induced the CD36 mRNA expression (1.8-fold, considered as not significant accordingly to the RQ cut-off established) (Fig. 1D). These results suggest that CD36 is involved in *S. aureus* internalization into bMECs.

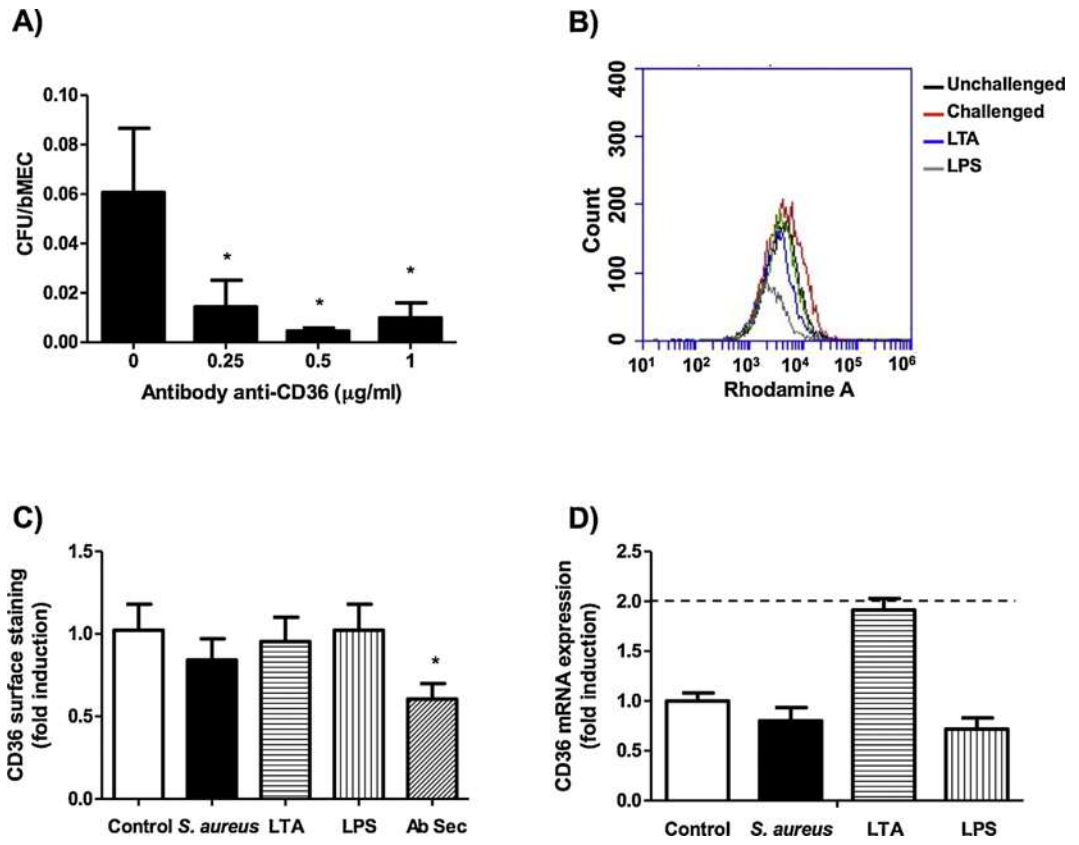

Fig. 1. The CD36 receptor is involved in *S. aureus* internalization into bMECs. (A)bMECs were incubated with different concentrations of a specific antibody against CD36 (45 min), and then, they were challenged with *S. aureus* for 2 h. The number of internalized bacteria is represented by the ratio CFU/bMEC. The symbol "*" indicates significant changes ($P < 0.05$) in relation to bMECs without antibody. (B)A histogram plot shows CD36 staining data in non-challenged bMECs (black line), cells that were challenged with *S. aureus* (red line) or cells that were stimulated with LTA (1 μg/ml, 16 h, blue line) or LPS (1 μg/ml, 24 h, green line). The cells were fixed and stained extracellularly with an anti-CD36 antibody overnight and analyzed with flow cytometry. The cells that were treated only with the secondary antibody (Ab sec) were used as a negative control. (C)The relative fluorescence intensities for the CD36 membrane abundance are shown. The fluorescence intensity was estimated from 10,000 events. The symbol "*" indicates significant changes ($P < 0.05$) in relation to bMECs control. (D)CD36 mRNA expression was analyzed with RT-qPCR. Each value/bar shows the mean of triplicates ±SE of three independent experiments. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. TLR2 and CD36 are not directly involved in the NaB-induced reduction of S. aureus internalization into bMECs

In a previous study, we demonstrated that bMECs that were treated with NaB (0.5 mM, 24 h) showed a reduction in *S. aureus* internalization (~50%) (Ochoa-Zarzosa et al., 2009). However, the role of TLR2 and CD36 receptors in this process is unknown. To evaluate the role of these receptors, bMECs were treated with 0.5 mM NaB (24 h) and the receptors were blocked individually prior to *S. aureus* challenge. As expected, 0.5 mM NaB treatment reduced the bacterial internalization (~50%) into bMECs. Similar results were obtained when the receptors were blocked with specific antibodies against TLR2 (Fig. 2A) or CD36 (Fig. 3A). Moreover, the blockage of each receptor after NaB treatment did not modify the effect, as we recovered similar CFUs from the control cells (Figs. 2 and 3A). These results suggest that both of these receptors are not directly involved in the inhibition of *S. aureus* internalization that is regulated by 0.5 mM NaB.

3.3. TLR2 membrane abundance and mRNA expression are induced by NaB but not CD36

Because receptor blockade in the presence of NaB did not modify the effect of SCFA alone, we evaluated the MA and mRNA expression of both receptors. The TLR2 MA and mRNA expression levels were augmented by 0.5 mM NaB treatment ~1.6-fold and ~1.7-fold, respectively. This behavior was similar to that observed in bMECs that were challenged with *S. aureus* (Fig. 2B and C). Intriguingly, the bacteria did not modify the TLR2 MA and mRNA expression levels that were induced by NaB. On the other hand, neither the CD36 MA nor mRNA expression levels were modified in all of the evaluated conditions (Fig. 3B and C). According to these results, we hypothesized that 0.5 mM NaB could activate bMECs via TLR2.

3.4. NaB activates p38, but not JNK1/2 or ERK1/2 in bMECs

Next, we evaluated whether 0.5 mM NaB induces MAPK activation (p38, JNK or ERK1/2), which is activated by TLR2 and is involved in bacterial phagocytosis, mainly by professional phagocytic cells (McLeish et al., 1998; Ellington et al., 2001; Ninkovic and Roy, 2012). Initially, we investigated whether these kinases participate in *S. aureus* internalization into bMECs using pharmacological inhibitors prior to bacterial invasion. bMECs that were incubated (30 min, 1 or 2 h) with pharmacological inhibitors of p38 (2.5–10 μM, SB203580), JNK (5–20 μM, SP600125) or ERK1/2 (0.62–10 μM, U0126) showed a considerable reduction in *S. aureus* internalization, indicating that these kinases are involved in this process. We observed the highest effect with 5 μM SB203580, 20 μM SP600125 and 2.5 μM U0126 following 30 min of incubation, with a ~50% reduction in bacterial internalization (Fig. S1).

Further, we evaluated p38, JNK and ERK1/2 phosphorylation to relate their activation states with the 0.5 mM NaB-mediated reduc-

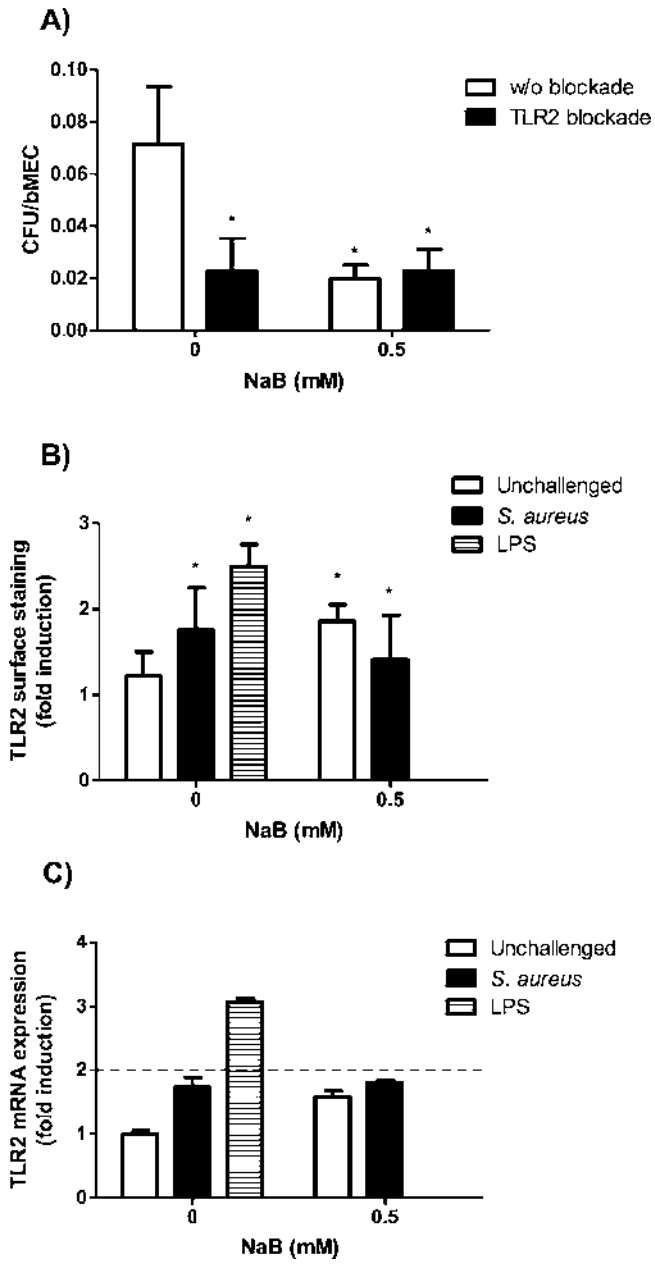

Fig. 2. The role of TLR2 in *S. aureus* internalization into bMECs, as regulated by NaB. (A)bMECs were treated with 0.5 mM NaB for 24 h, then incubated with a specific blocking anti-TLR2 antibody (5 μg/ml) for 1 h, and further challenged with *S. aureus* for 2 h. The number of internalized bacteria is represented by the ratio CFU/bMEC. The symbol "*" indicates significant changes ($P < 0.05$) in relation to bMECs without antibody and NaB. (B)bMECs were treated with NaB and/or challenged with *S. aureus* and the TLR2 abundance was evaluated with flow cytometry. The fluorescence intensity was estimated from 10,000 events. LPS was used as positive control. The symbol "*" indicates significant changes ($P < 0.05$) in relation to bMECs control (unchallenged and without NaB). (C)TLR2 mRNA expression was analyzed with RT-qPCR. Each bar shows the mean of triplicates ±SE of three independent experiments. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs.

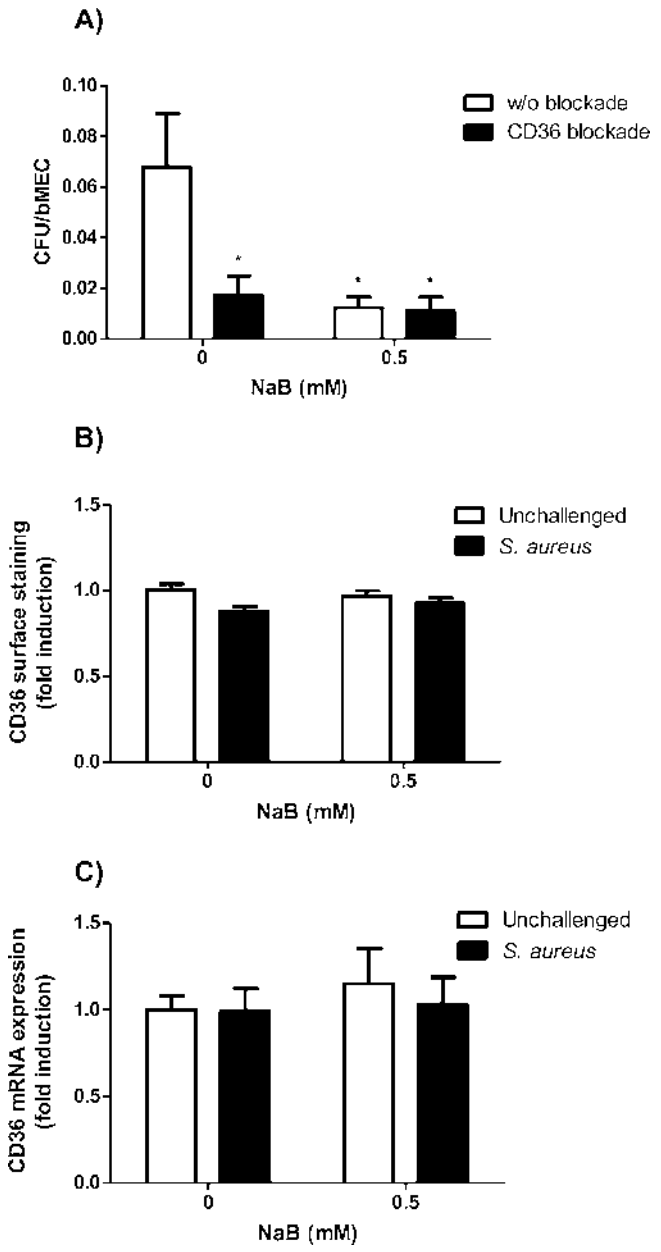

Fig. 3. The role of CD36 in *S. aureus* internalization into bMECs, as regulated by NaB. (A) bMECs were treated with 0.5 mM NaB for 24 h, incubated with an anti-CD36 antibody (0.25 μg/ml) for 45 min, and then challenged with *S. aureus* for 2 h. The number of internalized bacteria is represented by the ratio CFU/bMEC. The symbol "*" indicates significant changes ($P < 0.05$) in relation to bMECs control (without antibody and NaB). (B)The CD36 abundance in the bMECs was evaluated with flow cytometry analyses in the cells treated with NaB and/or challenged with *S. aureus*. The fluorescence intensity was estimated from 10,000 events. (C)The CD36 mRNA expression was analyzed with RT-qPCR. Each bar shows the mean of triplicates ±SE of three independent experiments. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs.

tion of *S. aureus* internalization into bMECs. As expected, the bMECs that were treated with the inhibitors had a reduced activation state of the 3 MAPKs (Fig. 4). When bMECs were *S. aureus*-challenged (2 h), the basal activation of p38 was not modified (Fig. 4A); however, phosphorylated JNK1/2 was augmented ~1.6-fold (Fig. 4B) and ERK1/2 activation was drastically reduced (Fig. 4C). Additionally, this reduction was similar to what was observed in the bMECs that were treated with the inhibitor, U0126. Interestingly, the bMECs that were treated with 0.5 mM NaB demonstrated increased p38 phosphorylation (~2-fold, Fig. 4A), but the JNK1/2 (Fig. 4B) and ERK1/2 activation levels were reduced by ~50% (Fig. 4C). When the NaB-treated cells were *S. aureus*-challenged, the p38 activation level diminished to basal levels; however, the JNK1/2 and ERK1/2 activation levels were essentially not changed. The TLR2 MA and MAPK results indicated that 0.5 mM NaB prior to bacterial invasion favors the defense response of bMEC via an increase in TLR2 MA and p38 MAPK activation.

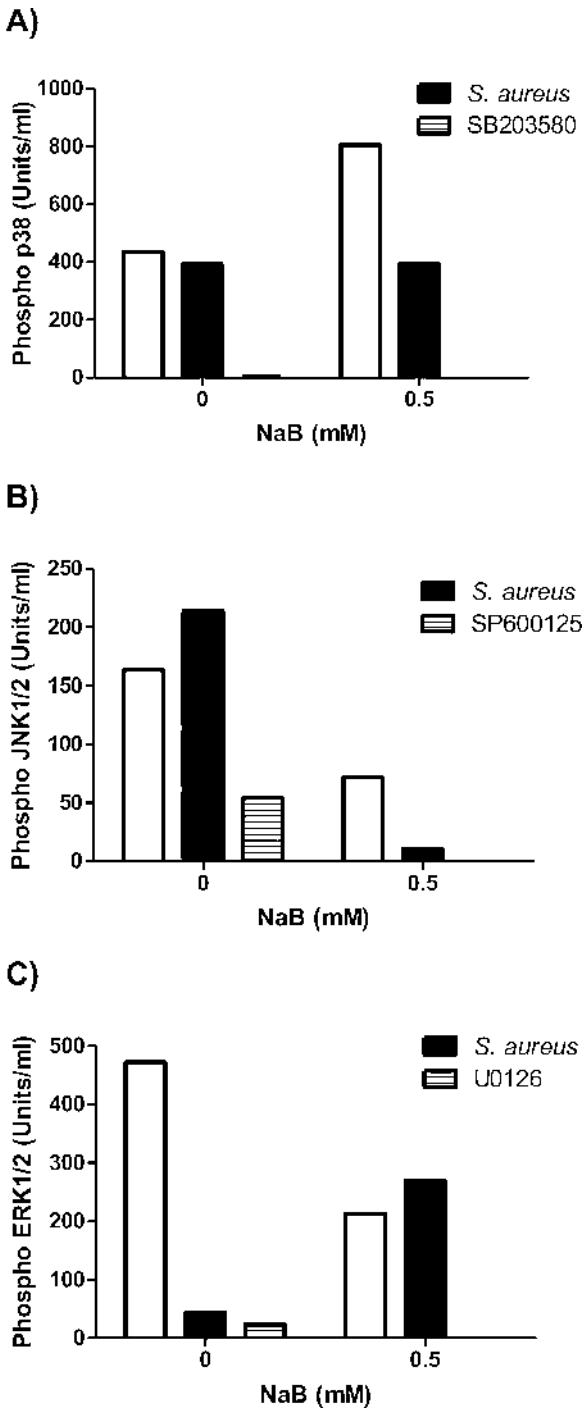

Fig. 4. p38, JNK and ERK1/2 activation, as regulated by NaB in *S. aureus*-challenged bMECs. MAPK phosphorylation was measured in bMECs that were treated with 0.5 mM NaB and/or challenged with *S. aureus* by flow cytometry. The phosphorylated MAPK concentrations (Units/ml) are represented: (A) pp38, (B) pJNK and (C) pERK1/2. Each bar shows the result of one experiment. SB203580: p38 inhibitor. SP600125: JNK1/2 inhibitor. U0126: ERK1/2 inhibitor.

3.5. Activation of transcription factors and IIR element mRNA expression

Stimulation of TLR2 leads to intracellular signaling pathways that result in the activation of transcriptional factors (TFs), such as AP-1, NF-κB, and IRF, among others (Uematsu and Akira 2006). Additionally, NaB is able to modulate the activation of different TFs that are related to host defense (e.g., AP-1, NF-κB, and E2F-1) (Abramova et al., 2006; Kida et al., 2006; Schwab et al., 2007b). In this sense, we evaluated the activation state of 56 TFs that are related to IIR by a Protein/DNA array. In the bMEC control, only the TF EGR showed a clear activated status (Fig. 5A). Notably, the *S. aureus*-challenged bMECs (2 h) had a reduced TF activation level (Fig. 5B), whereas only the TF AP-1 (0.5 ratio) was slightly stimulated in relation to the unchallenged cells. Interestingly, the 0.5 mM NaB treated bMECs (24 h) had 8 activated TFs (AP-1, E2F-1, FAST-1, MEF-1, EGR, PPAR, ER and CBF) (Fig. 5C); however, this effect was partially reverted when the bMECs were *S. aureus*-challenged (Fig. 5D). It is important to note that AP-1 had the highest activation status of the 0.5 mM NaB treated bMECs (1.7 ratio) (Fig. 5E).

Next, we sought to determine the bMEC antimicrobial and inflammatory response element mRNA expression levels via RT-qPCR. *S. aureus* (2 h) induced TAP (~2-fold), LAP (~6-fold), and BNBD5 (~4.5-fold) mRNA expression (Fig. 6A). As we expected, 0.5 mM NaB (24 h) up-regulated TAP (~4.8-fold), BNBD5 (~3.2-fold) and BNBD10 (~2.6-fold) mRNA expression (Fig. 6A). Interestingly, the bMECs that were NaB-treated and *S. aureus*-challenged displayed an increase in the mRNA expression of all of the antimicrobial peptides that were tested, and the LAP and BNBD5 levels were most evident.

Regarding the effect of 0.5 mM NaB on the inflammatory gene mRNA expression levels in bMECs, we focused on (i) pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6; (ii) an anti-inflammatory cytokine (IL-10); and (iii) a chemokine, IL-8. The IL-6 mRNA expression level was not modified under any of the evaluated conditions (Fig. 6B). Contrary to the expected result, 0.5 mM NaB up-regulated the TNF-α (~3-fold) mRNA expression level, and this effect was similar in the *S. aureus*-challenged bMECs (Fig. 6B). Additionally, the treatment with 0.5 mM NaB did not modify *S. aureus* induction. IL-1β mRNA expression was not modified by *S. aureus*; however, its expression was augmented ~2-fold by 0.5 mM NaB (Fig. 6B). On the other hand, the IL-10 and IL-8 mRNA expression levels were not modified in the *S. aureus*-challenged bMECs (Fig. 6C). However, the 0.5 mM NaB treatment induced the IL-10 and IL-8 mRNA expression levels by ~2 and ~4-fold, respectively. Interestingly, in the 0.5 mM NaB treated and *S. aureus*-challenged bMECs, these expression levels remained up-regulated, and even the IL-10 mRNA level was increased. These results suggest that 0.5 mM NaB improves the bMEC antimicrobial defense before/after a pathogen invasion and may exert anti-inflammatory effects during infection.

4. Discussion

IIR modulation may facilitate the development of therapeutic or prophylactic treatment strategies for disease control and prevention in both animals and humans. For this purpose, compounds with immunomodulatory properties are attractive, such as sodium butyrate (NaB). Previously, we showed that 0.5 mM NaB treatment reduced *S. aureus* internalization into bMECs and improved IIR (Ochoa-Zarzosa et al., 2009). This study demonstrates that the TLR2/p38 signaling pathway is involved during the NaB-reduced *S. aureus* internalization into bMECs.

Mammary epithelial cells play an essential role in the surveillance of mammary tissue during infections because they help in immune cell recruitment and bacterial recognition via TLR signaling pathways (Lahouassa et al., 2007; Gilbert et al., 2013). TLR2 is the major receptor for *S. aureus* detection and also mediates immune effector production (Takeda and Akira, 2005). However, the role of TLR2 in *S. aureus* internalization is still not clear. There is evidence indicating that this receptor does not directly participate in bacterial internalization because *S. aureus* engulfment occurs at the same degree in TLR2-deficient macrophages as its wild-type

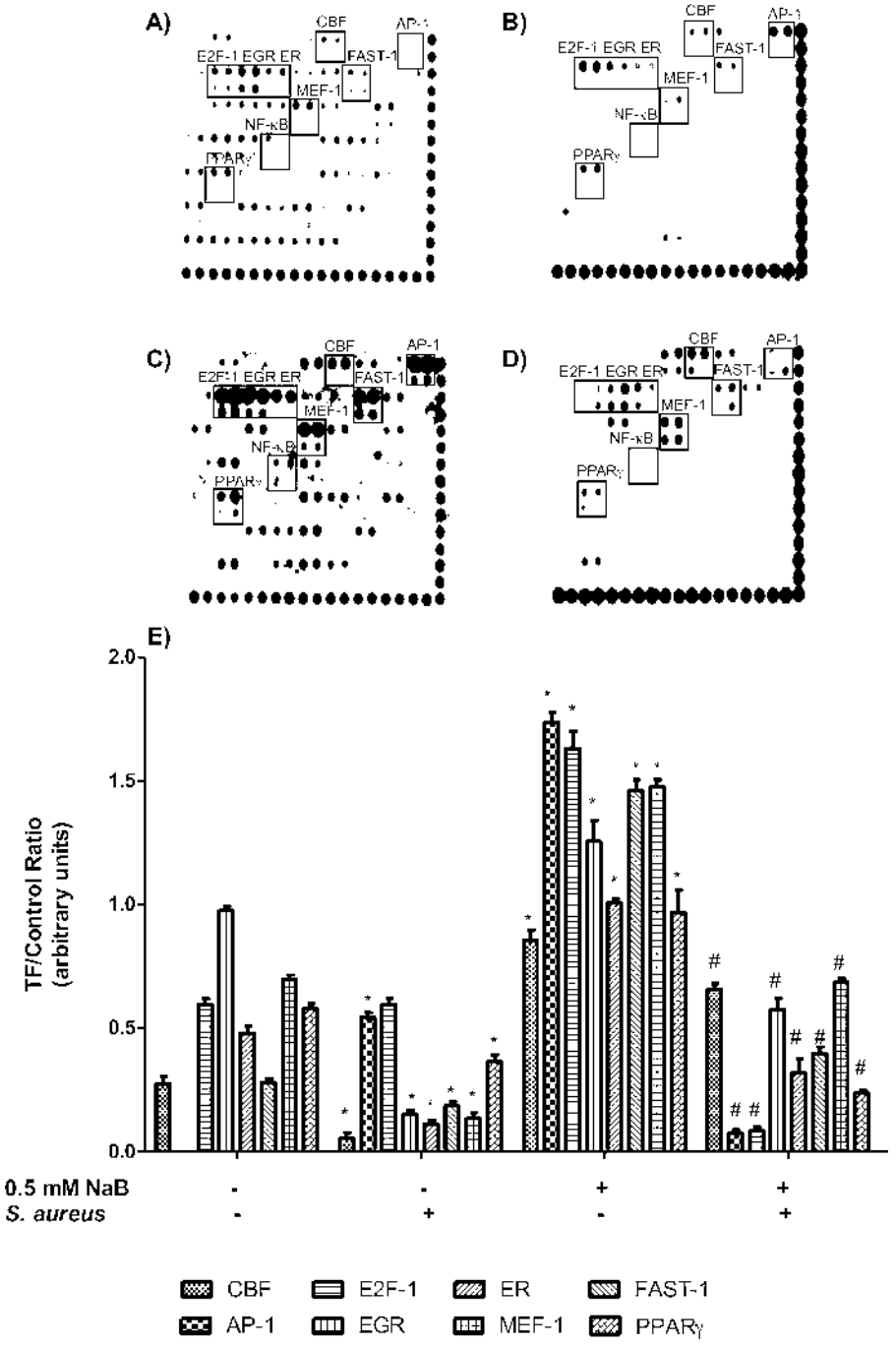

Fig. 5. Transcription factor activation by NaB in *S. aureus*-challenged bMECs. Protein/DNA array blots were utilized to analyze 56 different transcription factor DNA-binding sites from samples that were obtained from (A) bMEC nuclear extracts (control), (B)bMECs that were challenged with *S. aureus*, (C) bMECs that were treated with 0.5 mM NaB for 24 h and (D) cells that were treated with 0.5 mM NaB and challenged with *S. aureus*. The DNA samples were spotted in duplicate in two rows (top: undiluted; bottom: dilution 1/10). Biotinylated DNA was spotted for alignment along the right and bottom sides of the array. (E)The intensity from each spot on the membrane was quantified using ImageJ software. The bars show the mean intensity of duplicates of the ratio TF/control. The symbol "*" indicates significant changes ($P < 0.05$) in relation to the unchallenged bMECs. The symbol "#" indicates significant changes ($P < 0.05$) in relation to the challenged bMECs. CBF: core binding factor. AP-1; activating-protein 1. E2F-1: E2F transcription factor-1. EGR: early growth response protein 1. ER: estrogen receptor. FAST-1: forkhead activin signal transducer-1. MEF-1: myeloid Elf-1 like factor. PPAR: peroxisome proliferator-activated receptor.

counterpart (Yimin et al., 2013). Additionally, it was reported that TLR2 blockage with a functional blocking antibody (clone TL2.1) decreases the number of internalized bacteria into human cord blood-derived cells (Rocha-de-Souza et al., 2008). In agreement, when bMECs were blocked with a functional blocking anti-TLR2 (clone TL2.1) antibody, a reduction in *S. aureus* internalization was observed (Fig. 2A), which indicates that the role that TLR2 plays during internalization is indirect. In support of these data, in bMECs that were previously treated with NaB, TLR2 blockade did not modify the inhibitory effect of this SCFA. Altogether, these results indicate that the TLR2 participation during the NaB-reduced *S. aureus* internalization into bMECs was indirect.

Likewise, there is evidence suggesting that TLR2 may regulate phagocytic receptor expression (i.e., scavenger receptors) instead of acting as a phagocytic receptor per se (Mae et al., 2007). In this sense, we did not detect any modulation regarding mRNA expression or protein membrane abundance of the scavenger receptor CD36 when TLR2 was activated by *S. aureus* and/or 0.5 mM NaB or

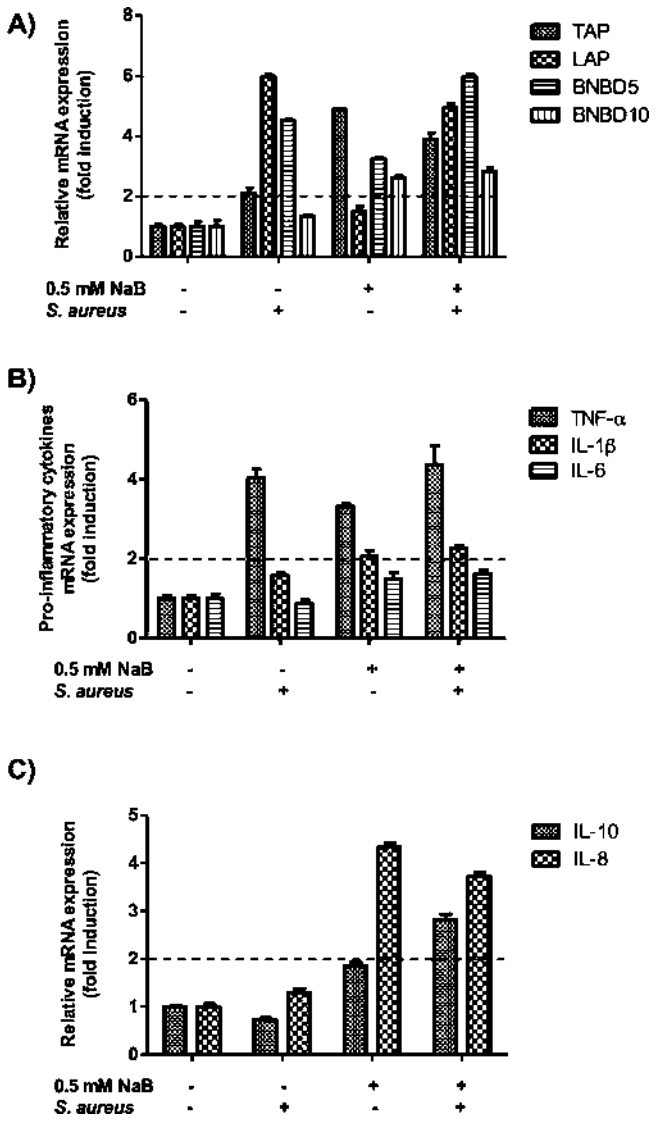

Fig. 6. Innate immune response gene mRNA expression analysis through NaB regulation in *S. aureus*-challenged bMECs. qPCR analysis that shows the effect on antimicrobial peptide (A) and cytokine (B and C) mRNA expression. The bMECs were treated with 0.5 mM NaB and then challenged with *S. aureus* for 2 h. Each bar shows the mean of triplicates ±SE of three independent experiments. GAPDH was used as endogenous gene in all of the conditions. TAP: tracheal antimicrobial peptide. LAP: lingual antimicrobial peptide. BNBD5: bovine neutrophil β-defensin 5. BNBD10: bovine neutrophil β-defensin 10. TNF-α: tumor necrosis factor-alpha. IL-1β: interleukin-1beta. IL-6: interleukin-6. IL-10: interleukin-10. IL-8: interleukin-8. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs.

LPS (Fig. 3B and C). According to these results, we conclude that CD36 is not a relevant participant in the *S. aureus* internalization that is regulated by NaB in bMECs.

There are a few reports regarding the effect of NaB on immune receptors. In this sense, NaB (24 mM) does not modulate TRL2 mRNA expression in Caco-2 cells (Leung et al., 2009). Nevertheless, NaB and a synthetic amide derivate reduced liver inflammation in animals with a high-fat diet and slightly augmented TLR2 mRNA expression in liver tissue (Mattace Raso et al., 2013). Interestingly, in bMECs, TLR2 MA was induced by 0.5 mM NaB (Fig. 2B) and the mRNA expression was slightly increased (Fig. 2C), which supports our hypothesis that this SCFA favors TLR2 pathway activation in bMECs previous to *S. aureus* challenge.

It has been reported that TLR2 activation regulates multiple downstream molecules, including MAPK (Vasselon et al., 2002), resulting in the activation of several transcriptional factors, such as AP-1 and NF-κB (Uematsu and Akira, 2006). Additionally, several reports have shown that p38 MAPK plays an important role in the production of antimicrobial peptides in epithelial cells (i.e., keratinocytes) (Kanda and Watanabe 2008; Lan et al., 2011; Li et al., 2013). Thus, we hypothesized that p38 MAPK could be a downstream molecule upon TLR2 activation in response to 0.5 mM NaB, which in turn leads to the expression of antimicrobial peptides in bMECs and, as consequence, induces a reduction in the *S. aureus* internalization into bMECs. In agreement, we showed that only p38 MAPK phosphorylation was induced in the 0.5 mM NaB treated bMECs (Fig. 4A), whereas the ERK1/2 and JNK1/2 phosphorylation levels were significantly inhibited by this SCFA. Notably, the p38 MAPK phosphorylation was not inhibited beyond the baseline when the bMECs were *S. aureus*-challenged.

Stimulation of the p38 MAPK pathway leads to the activation of different TFs that are related to host defense (e.g., AP-1, NF-κB, E2F-1, and EGR-1), which have also been reported to be modulated by NaB (Abramova et al., 2006; Kida et al., 2006; Schwab et al., 2007b). In agreement, the 0.5 mM NaB treated bMECs had 8 activated TFs that are involved in innate immune responses (e.g., AP-1, E2F-1, FAST-1, MEF-1, EGR, PPAR, ER and CBF), and AP-1 was the highest activated TF (Fig. 5C). The AP-1 activation that was induced by NaB has been previously reported in epithelial cells (Nepelska et al., 2012). However, to our knowledge, this is the first report that correlates AP-1 activation by NaB with *S. aureus* internalization in epithelial cells. Notably, the *S. aureus*-challenge essentially only activated AP-1 in the bMECs. We did not detect NF-κB activation by 0.5 mM NaB or *S. aureus*, which is in agreement with a previous report (Lara-Zárate et al., 2011). Schwab et al. (2006) showed that NaB -via p38- induces PPARγ mRNA expression, protein expression, and activity, which is in agreement with the results of this study. In the same sense, it was reported that SCFAs are able to stimulate PPARγ expression in differentiated adipocytes (Wahli and Michalik, 2012). E2F-1 is a factor that possesses anti-inflammatory activity through the stabilization of IκB, leading to the inhibition of NF-κB-dependent processes (Chen et al., 2002). Additionally, it was reported that this factor (E2F-1) is up-regulated after the inhibition of histone deacetylase activity by trichostatin in the retina (Wallace and Cotter 2009), which may explain its activation by NaB, as demonstrated in this study, because this compound is a recognized inhibitor of histone deacetylase activity.

Further, we analyze the mRNA expression of several antimicrobial peptides and cytokines in bMECs, the expression of which previously has been described (Kim et al., 2011; Schukken et al., 2011; Zbinden et al., 2014). It was reported that *S. aureus* induces antimicrobial peptide expression (Swanson et al., 2004; Yang et al., 2006; Alva-Murillo et al., 2013). In agreement, in this study we showed that the *S. aureus*-challenged bMECs had increased LAP and BNBD5 expression (Fig. 6A). Interestingly, the 0.5 mM NaB treatment induced TAP, BNBD5 and BNBD10 expression, and this up-regulation was maintained even when the bMECs were challenged with *S. aureus*. In agreement, reports from our group and others showed that NaB induces antimicrobial peptide expression (Schauber et al., 2003; Zeng et al., 2013; Sunkara et al., 2014). The induction of the expression of 3 antimicrobial peptides by 0.5 mM NaB in bMECs prior to *S. aureus* challenge may explain the reduction in bacterial internalization. However, additional experiments are required to demonstrate the presence of antimicrobial proteins in the media.

It has been established that *S. aureus* provokes an anti-inflammatory environment in bMECs. Accordingly, we did not detect significant IL-1β, IL-6 and IL-8 mRNA expression level changes, and only TNF-α was induced (Fig. 6B and C). This result is in agreement with previous reports from our group (Alva-Murillo et al., 2014b; Medina-Estrada et al., 2015). On the other

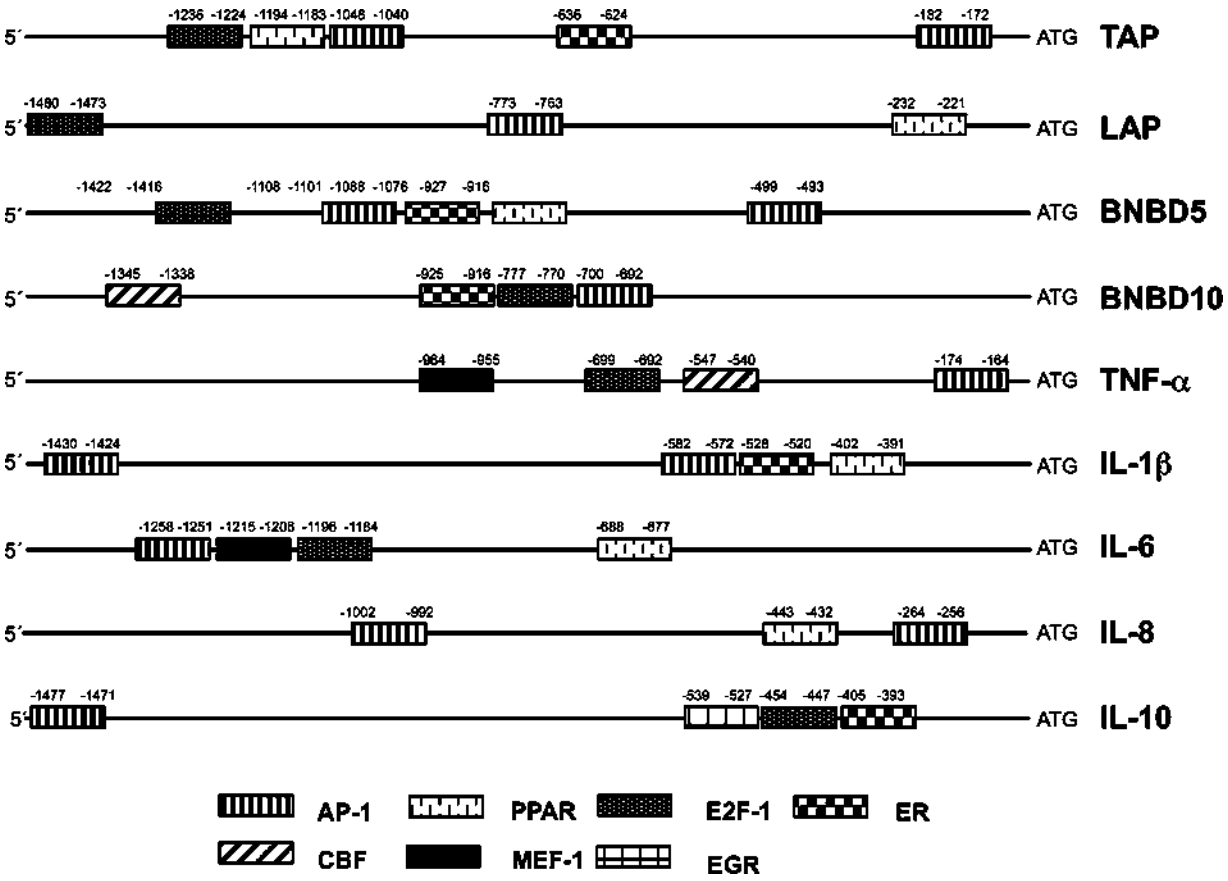

Fig. 7. Transcription binding site analysis in the promoters of innate immune elements. The program PROMO V 3.0.2 was used. The putative binding sites were analyzed in 1.5 kb upstream of the ATG in each gene. TAP: tracheal antimicrobial peptide (Genbank_AC_000184.1). LAP: lingual antimicrobial peptide (Genbank_AC_000184.1). BNBD5: bovine neutrophil β-defensin 5 (Genbank_AC_000184.1). BNBD10: bovine neutrophil β-defensin 10 (Genbank_AC_000184.1). TNF-α: tumor necrosis factor-alpha (Genbank AC_000180.1). IL-1β: interleukin-1beta (Genbank AC_000168.1). IL-6: interleukin-6 (Genbank AC_000161.1). IL-10: interleukin-10 (Genbank AC_000173.1). IL-8: interleukin-8 (Genbank AC_000163.1).

hand, 0.5 mM NaB induced the expression of the anti-inflammatory cytokine, IL-10 and the chemokine, IL-8, which is an important chemotactic factor for attracting neutrophils and T-lymphocytes to infection sites. Interestingly, the NaB-treated and *S. aureus*-challenged bMECs maintained the anti-inflammatory response that was induced by the SCFA.

To correlate the TF activation and the gene expression changes that were regulated by 0.5 mM NaB, we carried out an analysis of the promoter region of antimicrobial peptides and cytokines genes to find putative transcription binding sites for the factors that are activated by this SCFA (Fig. 7). We used the PROMO 3.0.2 program for this analysis (Messeguer et al., 2002). Notably, all of the evaluated genes showed putative transcription binding sites for AP-1, which may explain the induction in the expression of these genes by 0.5 mM NaB in bMECs. However, the gene expression regulation by epigenetic mechanisms should also be considered because of the inhibitory activities of NaB on histone deacetylase. In this sense, Liu et al. (2011) reported that *E. coli* infection loosens chromatin compaction at the lingual antimicrobial peptide (LAP) promoter in the udder. Thus, the control of these genes by epigenetic regulation is very important and needs further research, and it rarely depends on only one TF (Sullivan et al., 2007).

5. Conclusions

The results of this study support the fact that 0.5 mM NaB activates bMECs through the TLR2/p38 pathway. This leads to increased antimicrobial peptide expression, which favors better *S. aureus* elimination.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2015.09.025.

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Table S1. Bovine oligonucleotides used in this study.

Specificity	Primer	Sequence (5´-3´)*	Fragment size (bp)	Annealing temperature (°C)	References
TLR2	Forward Reverse	CGACTGGCCCGATGACTACC TGAGCAGGAGCAACAGGAAGAG	146	58.5	(Alva-Murillo et al., 2014b)
CD36	Forward Reverse	GTGCAGAATCCAGATGAAGTGACA TGCTACAGCCAGGTTGAGAATG	22	54.7	This study
BNBD5	Forward Reverse	GCCAGCATGAGGCTCCATC TTGCCAGGGCACGAGATCG	143	55	(Cormican et al., 2008)
BNBD10	Forward Reverse	GCTCCATCACCTGCTCCTC AGGTGCCAATCTGTCTCATGC	152	54	(Téllez-Pérez et al., 2012)
TAP	Forward Reverse	GCGCTCCTCTTCCTGGTCCTG GCACGTTCTGACTGGGCATTGA	216	57	(Alva-Murillo et al., 2013b)
LAP	Forward Reverse	GCCAGCATGAGGCTCCATC CTCCTGCAGCATTTTACTTGGG	194	54	(Cormican et al., 2008)
TNF-α	Forward Reverse	CCCCTGGAGATAACCTCCCA CAGACGGGAGACAGGAGAGC	101	56	(Mookherjee et al., 2006)
IL-1β	Forward Reverse	GCAGAAGGGAAGGGAAGAATGTAG CAGGCTGGCTTTGAGTGAGTAGAA	198	52	(Alva-Murillo et al., 2014b)
IL-6	Forward Reverse	AACCACTCCAGCCACAAACACT GAATGCCCAGGAACTACCACAA	179	57	(Alva-Murillo et al., 2014b)
IL-8	Forward Reverse	TTCCACACCTTTCCACCCCAA GCACAACCTTCTGCACCCACTT	149	53.5	(Alva-Murillo et al., 2014b)
IL-10	Forward Reverse	GATGCGAGCACCCTGTCTGA GCTGTGCAGTTGGTCCTTCATT	129	59	(Alva-Murillo et al., 2014b)
GAPDH	Forward Reverse	TCAACGGGAAGCTCACTGG CCCCAGCATCGAAGGTAGA	237	56.9	(Yonezawa et al., 2009)

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Figure S1

A)

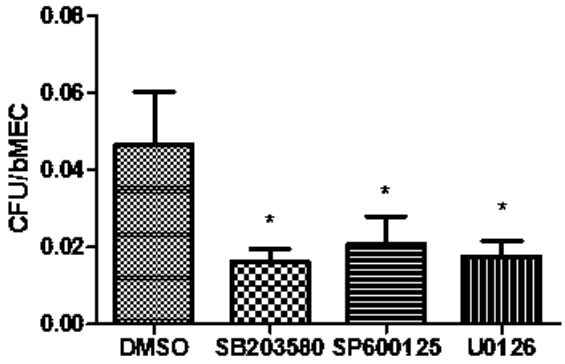

Fig. S1. Participation of MAK kinases (p38, JNK and ERK1/2) in *S. aureus* internalization into bMEC. bMECs were incubated with pharmacological inhibitors of p38 (5 mM, SB203580), JNK (20 mM, SP600125) or ERK1/2 (2.5 mM, U0126). Further, bMECs were challenged with *S. aureus* for 2 h. The number of bacteria internalized was calculated assuming that the control (with DMSO 0.1%) represents 100% internalization. Each bar shows the mean of triplicates ± SE of three independent experiments. "*" $P < 0.05$.

CAPÍTULO II

Sodium octanoate modulates the innate immunity response of bovine mammary epithelial cells through TLR2/p38/JNK/ERK1/2 pathway: implications in *Staphylococcus aureus* internalization

Sodium octanoate modulates the innate immunity response of bovine mammary epithelial cells through TLR2/p38/JNK/ERK1/2 pathway: implications during *Staphylococcus aureus* internalization

Nayeli Alva-Murillo, Alejandra Ochoa-Zarzosa and Joel E. López-Meza*.

Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 Carr, Morelia-Zinapécuaro, Posta Veterinaria, C.P. 58893, Morelia, Michoacán, México.

***Corresponding author. Tel/Fax:** (52)-443-295-8029.

E-mail address: elmeza@umich.mx (J.E. López-Meza)

Correspondance address: Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 Carr, Morelia-Zinapécuaro, Posta Veterinaria, C.P. 58893, Morelia, Michoacán, México. **E-mail address:** elmeza@umich.mx

Abstract

Bovine mammary epithelial cells (bMECs) contribute to mammary gland defense against invading pathogens, such as *Staphylococcus aureus* (intracellular facultative), which is recognized by TLR2. In a previous report, we showed that sodium octanoate (NaO) induces (0.25 mM) or inhibits (1 mM) the *S. aureus* internalization into bMECs, and regulates differentially the innate immune response (IIR). However, the molecular mechanisms have not been described, which was the aim of this study. The results showed that $\alpha 5\beta 1$ integrin membrane abundance (MA) was increased in 0.25 mM NaO-treated cells, but the TLR2 or CD36 MA was not modified. When these receptors were blockage individually, the 0.25 mM NaO-increased *S. aureus* internalization was notably reduced. Interestingly, in this condition the IIR of bMECs was impaired because the MAPK (p38, JNK and ERK1/2) phosphorylation and the TF activation were decreased in relation to basal levels. On the other hand, the 1 mM NaO treatment induced TLR2 MA but neither the integrin nor CD36 MA was modified. The 1 mM NaO-decreased *S. aureus* internalization was reduced when TLR2 was blockage. In addition, the phosphorylation levels of p38, JNK and ERK1/2 were increased, and 14 transcriptional factors related to IIR were slightly activated (Brn-3, CBF, CDP, c-Myb, AP-1, Ets-1/Pea-3, FAST-1, GAS/ISRE, AP-2, NFTA-1, OCT-1, RAR/DR-5, RXR/DR-1 and Stat-3). Moreover, 1 mM NaO treatment up-regulated the gene expression of IL-1β (~2 fold), IL-8 (~2 fold) and RANTES (~2 fold). Notably, when 1 mM NaO-treated bMEC were challenged with *S. aureus* the gene expression of IL-8 and IL-10 was up-regulated (~6 and ~4 fold, respectively); while IL-1β mRNA level was reverted (~1 fold). In conclusion, our results showed that $\alpha 5\beta 1$ integrin, TLR2 and CD36 are involved in 0.25 mM NaO-increased *S. aureus* internalization into bMECs. In addition, 1 mM NaO activates bMECs via TLR2 signaling pathways –p38, JNK1/2 and ERK1/2- which leads to improve the IIR before and after *S. aureus* invasion. Besides, NaO (1 mM) might exert anti-inflammatory effects after bacterial internalization.

Keywords: *Staphylococcus aureus*; octanoate; internalization; TLR2; epithelial cells; inflammatory response.

1. Introduction

Medium chain fatty acids (8-14 carbons, MCFAs) are an important energy source present in several foods as medium chain triglycerides (i.e. coconut and palm kernel oil, butter, milk, yogurt and cheese) (Nagao and Yanagita, 2010). MCFAs possess *in vitro* antimicrobial properties against human gastrointestinal (Marounek et al., 2003; Skrivanova and Marounek, 2007; Aydin et al., 2011) and bovine mastitis pathogens (e.g. *Staphylococcus aureus*) (Nair et al., 2005). In particular, octanoate (8C) has been used in rabbit and poultry farms as preventive treatment for gastrointestinal diseases caused by *Escherichia coli* or *Campylobacter jejuni* (Skrivanova et al., 2008; Skrivanova et al., 2009; Ghareeb et al., 2013). Nonetheless, the host immune response in these studies has not been evaluated. However, a few studies have described the immunomodulation capacity of octanoate in human intestinal epithelial (Andoh et al., 2000; Hoshimoto et al., 2002). Additionally, this MCFA induces antimicrobial peptide (AP) expression in both professional (macrophages and monocytes) and non-professional phagocytic cells (NPPCs) (Sunkara et al., 2012; Jiang et al., 2013).

The innate immune response (IIR) is the first-line defense mechanism against invading pathogens. Epithelial cells (ECs) participate in this defense through pattern recognition of conserved molecules associated with microorganisms by various families of germ-line-encoded pattern-recognition receptors, including the Toll-like receptors (TLRs) (Bulek et al., 2010). The production of innate immune effectors (enzymes, AP, cytokines, chemokines) is due to the stimulation of ECs through diverse receptors, which in turns lead to the activation of MAPK family members (p38, JNK and ERK1/2) and transcription factors (e.g. NF-κB, AP-1, E2F-1, EGR, FAST-1) (Akira et al., 2006; Chiu et al., 2009; Alva-Murillo et al., 2015; Medina-Estrada et al., 2015).

In cattle, bovine mammary epithelial cells (bMECs) are responsible for the production of milk and their response is critical for prompt bacterial clearance and prevention of mastitis (inflammation of the mammary gland) (Brenaut et al., 2014) (Thompson-Crispi et al., 2014). *S. aureus* is a major pathogen in many animal species, and represents one of the leading causes of bovine mastitis, especially the subclinic mastitis, which usually is chronic (Kerro Dego et al., 2002; Scali et al., 2015). This chronicity has been related to the ability of *S. aureus* to internalize into professional and NPPCs such as bMECs (Garzoni and Kelley, 2011; Fraunholz and Sinha, 2012; Scali et al., 2015). In NPPCs, the internalization process depends primarily on the host $\alpha 5\beta 1$ integrin and the bacteria fibronectin-binding proteins (FnBP) (Hauck et al., 2012; Medina-Estrada et al., 2015). However, it has been reported that other host cell receptors are involved in this process (Alva-Murillo et al., 2014a). In this sense, the blockage of TLR2 in bMECs -the most relevant receptor for *S. aureus* recognition- with neutralizing antibodies decreases the number of internalized bacteria (Alva-Murillo et al., 2015; Medina-Estrada et al., 2015). Although, TLR2 is not a phagocytic receptor *per se*, it seems that TLR2 activation is necessary for *S. aureus* internalization into NPPCs (Rocha-de-Souza et al., 2008; Alva-Murillo et al., 2015; Medina-Estrada et al., 2015). Additionally, this receptor has been co-localized intracellularly with *S. aureus* lipoprotein SitC or with both Nod2 and peptidoglycan in keratinocytes (Muller-Anstett et al., 2010; Müller et al., 2010). On the other hand, CD36, an integral membrane scavenger receptor and a fatty acid translocase, has a role in *S. aureus* recognition and internalization mainly in professional phagocytic cells by interacting with TLR2 (Hoebe et al., 2005; Stuart et al., 2005). Particularly, in bMECs, we reported that the blockage of CD36 with a specific antibody reduces the number of *S. aureus* internalized (Alva-Murillo et al., 2015).

It is known that the activation status of MAPK that are involved in TLR2 signaling pathways could be regulated by *S. aureus* infection in NPPCs. Thereby, the phosphorylation of p38 is triggered by *S. aureus* (live or inactivated bacteria or its PAMPs) (Lamprou et al., 2007; Liang y Ji 2007; Adhikary et al., 2008; Chekabab et

al., 2015; Singh and Kumar, 2015). However, in osteoblast and bMECs *S. aureus* did not modify the phosphorylation of p38 (Ellington et al., 2001; Alva-Murillo et al., 2015). On the other hand, the bacterial stimulus (live or inactivated) triggers the JNK activation (Kumar et al., 2004; Lamprou et al., 2007; Adhikary et al., 2008; Alva-Murillo et al., 2015), while ERK1/2 phosphorylation is reduced by live *S. aureus* in epithelial cells or stimulated by UV-killed bacteria (Ratner et al., 2001; Adhikary et al., 2008; Alva-Murillo et al., 2015). Additionally, these MAPKs have an important role during *S. aureus* internalization into bMECs (Alva-Murillo et al., 2015).

The ability of *S. aureus* to internalize into NPPCs allows the bacteria to survive within them, which leads to a low response for conventional therapy and induces chronic and recurrent infections (Kerro Dego et al., 2002; Fraunholz and Sinha, 2012). Thus, it is necessary to develop alternative therapies. In this sense, a few studies are focused on controlling diseases to prevent pathogen internalization by modulating the host IIR. In a previous study, we demonstrated that 0.25 and 1 mM sodium octanoate (NaO) induces or inhibits *S. aureus* internalization into bMECs, respectively (Alva-Murillo et al., 2013a). Additionally, NaO (1 mM) favors the antimicrobial response during inhibition of *S. aureus* internalization. However, the molecular mechanisms involved in NaO modulation of (i) bacterial internalization and (ii) innate immune response are unknown. In this study, we showed that $\alpha 5\beta 1$ integrin, TLR2 and CD36 play an important role in 0.25 mM NaO-increased *S. aureus* internalization, but the IIR of bMECs is impaired. On the other hand, 1 mM NaO activates bMECs via TLR2/p38/JNK/ERK1/2 before pathogen invasion, which leads to a better host defense. In addition, this treatment favors an anti-inflammatory response after *S. aureus* infection.

2. Materials and Methods

2.1 Staphylococcus aureus strain

S. aureus subsp. *aureus* (ATCC 27543) strain was used in this study. This strain was isolated from a case of bovine clinical mastitis and it is able to internalize into bMECs (Gutiérrez-Barroso et al., 2008). Bacteria were grown overnight in Luria-Bertani broth (LB, Bioxon, México). For the different assays the colony forming units (CFU) were adjusted by measuring their optical density at 600 nm (OD 0.2 = 9.2 X 10^7 CFU/ml).

2.2 Reagents and antibodies

LPS (from *E. coli* 0111:B4) and sodium octanoate (NaO) were acquired from Sigma-Aldrich (St. Louis, MO, USA). In this study, we used 0.25 and 1 mM NaO, which induces or inhibits *S. aureus* internalization into bMECs, respectively (Alva-Murillo et al., 2013a). The monoclonal blocking antibodies used were anti-$\alpha5\beta1$ integrin (MAB2514, Millipore), anti-TLR2 (TL2.1, Abcam) and anti-CD36 (FA6-152, Abcam). The MAPK inhibitors SB20358 (p38), SP600125 (JNK) and U0126 (ERK1/2) were acquired from Cell Signaling Technology^R (Boston, MA). The working solutions were dissolved in dimethyl sulfoxide (DMSO), which was employed as vehicle in the MAPK activation assay.

2.3 Primary bovine mammary epithelial cell (bMEC) culture

bMEC isolation was performed on udder alveolar tissue from healthy lactating cows as described (Anaya-López et al., 2006). Cells from passages 2 to 8 were used in all of the experiments. The cells were cultured in petri dishes (Corning-Costar) in growth medium (GM) that was composed of a DMEM medium/nutrient mixture F-12 Ham (DMEM/F-12K, Sigma), which was supplemented with 10% fetal calf serum (Equitech Bio), 10 μg/ml insulin (Sigma), 5 μg/ml hydrocortisone (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B (Invitrogen). The bMECs were grown in 5% CO_2 atmosphere at 37°C.

2.4 Invasion assays

Polarized bMECs monolayers were cultivated on 96-well flat-bottom plates that were coated (Corning-Costar) with 6-10 μg/cm^2 rat-tail type I collagen (Sigma). Prior to the invasion assays, the bMECs (~10x10^3 cells/well) were incubated with 0.25 or 1 mM NaO in DMEM/F12K (Sigma) without antibiotics and serum for 24 h. Then, the cells were treated separately with different blocking antibodies, including anti-$\alpha 5\beta 1$ integrin (10 μg/ml, 30 min), anti-TLR2 (5 μg/ml, 1 h) and anti-CD36 (0.25 μg/ml, 45 min). Mouse or rat IgG (purified from normal mouse or rat serum that was purchased from Pierce) was used as control. Invasion assays were performed using gentamicin protection assays as we described previously (Gutiérrez-Barroso et al., 2008; Alva-Murillo et al., 2015). Briefly, the bMECs that were used in the antibody blockage experiments were infected with *S. aureus* (MOI 30:1 bacteria per cell). For this, the bMECs were inoculated with 3.5 μl of bacterial suspensions from 9.2 X 10^7 CFU/ml and incubated for 2 h in 5% CO_2 at 37°C. Then, the cells were washed three times with PBS (pH 7.4) and incubated in GM without serum and penicillin and streptomycin supplemented with 50 μg/ml gentamicin for 1 h at 37°C to eliminate extracellular bacteria. Finally, the bMEC monolayers were detached with trypsin-EDTA (Sigma) and lysed with 250 μl of sterile distilled water. The cell lysates were plated on LB agar in triplicate and incubated overnight at 37°C. The CFUs were determined with the standard colony counting technique. The data are presented as the ratio CFU/bMECs.

2.5 Flow cytometry analysis

To evaluate the cell-surface expression of the $\alpha 5\beta 1$ integrin, TLR2 and CD36 receptors, ~2x10^5 bMECs/well were cultured to 80% confluence on 24-well plates (Corning) and then treated with 0.25 or 1 mM NaO, *S. aureus* or both, as described for the invasion assays. Then, the bMECs were detached with trypsin/EDTA (Sigma) and the cell pellet was recovered by centrifugation (2,500 rpm, 10 min, 4°C) and washed 2X with cold-PBS (pH 7.4). The cells were fixed with 4% paraformaldehyde for 10 min at 4°C. The bMECs were blocked with normal goat

serum (5% in PBS, Pierce) for 30 min at 4°C with shaking and then recovered by centrifugation. Further, the bMECs were incubated with primary antibodies, anti-$\alpha5\beta1$ integrin (10 μg/ml), anti-TLR2 (0.666 μg/ml in PBS containing 0.1% BSA) or anti-CD36 (2 μg/ml in PBS containing 0.1% BSA), overnight at 4°C. Then, the bMECs were incubated with a FITC-conjugated secondary antibody against rat or mouse IgG (1:50, Molecular Probes) for 2 h on ice. The samples were analyzed in a BD Accuri™ C6 flow cytometer with the BD Accuri C6 Software. 10,000 events were collected and analyzed. bMECs that were incubated only with the secondary antibody were used as a negative control. For TLR2 membrane abundance (MA) assays, the positive control consisted in bMECs stimulated with LPS (1 μg/ml, Sigma) for 24 h.

2.6 MAPK activation during NaO-modulated S. aureus internalization

To evaluate the MAPK activation levels by flow cytometry, the bMECs were treated with 0.25 or 1 mM NaO (24 h), *S. aureus* or both, and the samples (30 μg of protein) were prepared according to the manufacturer's protocol for adherent cells (Becton Dickinson, Germany). pp38 (T180/Y182), pJNK1/2 (T183/185), and pERK1/2 (T202/Y204) were quantitatively determined using antibodies from a Flex Set Cytometric Bead Array (Becton Dickinson) according to the manufacturer´s protocol. Flow cytometric analyses were performed using the BD Accuri™ C6 and CBA analysis FCAP software (Becton Dickinson). A total of 3,000 events were acquired following the supplied protocol. The minimum detection levels for each phospho-protein were 0.38 U/ml for pJNK, and 0.64 U/ml for pp38 and pERK. The negative controls were bMECs treated with pharmacological inhibitors of p38 (5 μM, SB203580), JNK (20 μM, SP600125) or ERK1/2 (2.5 μM, U0126) for 30 min.

2.7 Transcription factor-DNA interactions

Nuclear proteins were obtained from bMECs using the NE-PER Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's instructions (Thermo Scientific, Rockfor, IL). Protein concentrations were determined with the Bradford

assay. The nuclear extracts were subjected to the TranSignal Protein/DNA array I (Panomics, Fremont, CA, USA). Briefly, biotin-labeled DNA-binding oligonucleotides (TranSignal™ Probe Mix) were incubated with 15 μg/ml of nuclear extracts to allow the formation of transcription factor/DNA complexes. These complexes were separated from free probes and hybridized to a protein/DNA array, and then the complexes were detected using an HRP-based chemiluminescence method according to the manufacturer's protocol.

2.8 Analysis of mRNA expression of inflammatory response genes

To analyze the effects of 0.25 and 1 mM NaO and/or *S. aureus* on the expression of IIR genes in bMECs, monolayers of cells that were cultured in 6 well dishes with 6-10 μg/cm^2 rat-tail type I collagen (Sigma) were incubated with 0.25 or 1 mM NaO (24 h) and/or *S. aureus* for 2 h (MOI 30:1). bMEC total RNA (5 μg) was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions, and then, they were used to synthesize cDNA as described (Alva-Murillo et al., 2013a). The expression analysis of inflammatory response genes was performed with qPCR using the comparative Ct method (ΔΔCt) in a StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The reactions were carried out with a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Specific primer pairs were acquired from Invitrogen and Elim Biopharm (Table 1), and their specificity was determined by end point PCR. GAPDH was used as an internal control (Alva-Murillo et al., 2013).

2.9 Analysis of the cytokines genes promoter region

In order to evaluate a possible correlation between the gene expression levels and the putative binding affinity to transcription factors, the PROMO 3.0 program was used for the analysis of gene promoter region (Messeguer et al., 2002). The putative binding sites were analyzed in a 1.5 kb region upstream of the ATG in each gene.

2.10 Data analysis

The data were obtained from three independent experiments, each performed in triplicate, and compared with analysis of variance (ANOVA). The results are reported as the means ± the standard errors (SE), and the significance level was set at $P < 0.05$, except for RT-qPCR analysis where fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. For gene expression and membrane staining assays data were normalized to the untreated cells (control).

3. Results

3.1 $\alpha 5\beta 1$ integrin, TLR2 and CD36 are involved in NaO-induced S. aureus internalization into bMECs, but only TLR2 is involved in NaO-reduced bacterial internalization

Previuosly, we demonstrated that the *S. aureus* internalization was increased in the 0.25 mM NaO-treated bMECs; whereas in the 1 mM NaO-treated cells the number of *S. aureus* recovered was inhibited (Alva-Murillo et al., 2013a). In addition, it is known that $\alpha 5\beta 1$ integrin, TLR2 and CD36 play an important role in the *S. aureus* internalization into bMECs (Alva-Murillo et al., 2015; Medina-Estrada et al., 2015). However, the role of these receptors in the NaO mediated-internalization process is unknown. To determine the roles of these receptors, bMECs were treated with 0.25 or 1 mM NaO (24 h) and then the receptors were blocked independently prior to *S. aureus* challenge. As expected, 0.25 and 1 mM NaO treatments induced (~2-fold) or reduced (~50%) the bacterial internalization, respectively. As we reported previously, the bacterial internalization decreased (~50%) when $\alpha 5\beta 1$ integrin, TLR2 or CD36 were blocked with specific antibodies (Fig. 1). Interestingly, the blockage of these receptors after 0.25 NaO treatment reduced considerably the NaO-induced *S. aureus* internalization (0.015, 0.014 and 0.01 ratio, respectively) (Fig. 1). On the other hand, the *S. aureus* internalization was dramatically decreased (0.003 ratio) in the 1 mM NaO-treated bMECs after

TLR2 blockade, and the CFU recovered were even lesser in 1 mM NaO-treated cells (0.017 ratio) (Fig. 1B). Notably, the blockage of $\alpha5\beta1$ integrin or CD36 after 1 mM NaO treatment did not modify the effect of this MCFA (Fig. 1A, 1C). To notice, these effects were specific because the bacterial internalization was not modified in mouse or rat IgG-treated cells (data not shown). These results suggest that $\alpha5\beta1$ integrin, TLR2 and CD36 are involved in the 0.25 mM NaO-increased *S. aureus* internalization into bMECs. Meanwhile, only TLR2 seems to play an important role in 1 mM NaO-reduced bacterial internalization.

3.2 $\alpha5\beta1$ integrin membrane abundance (MA) is induced by 0.25 mM NaO, but TLR2 MA is increased by 1 mM NaO

Next, we explored a possible correlation between blocking assays and receptors membrane abundance (MA). As we reported previously, *S. aureus* decreases the $\alpha5\beta1$ integrin MA and induces the TLR2 MA in bMECs, but do not modify CD36 (Alva-Murillo et al., 2015; Medina-Estrada et al., 2015). The $\alpha5\beta1$ integrin MA was increased (~3 fold) by 0.25 mM NaO treatment, and this induction did not change after bacterial stimulus (Fig. 2A). Also, the TLR2 and CD36 MA were not modified (Fig. 2B-C), and this data correlated with the gene expressions of both receptors (data not shown). Interestingly, 1 mM NaO (24 h) treatment augmented the TLR2 MA (~2 fold) and this level was maintained after *S. aureus* challenged (Fig. 2B). We observed a similar response in the TLR2 mRNA expression levels; however, it was not statistically significant (data not shown). In this condition, neither the CD36 MA nor the gene expression was modified (Fig. 2C). According to these results, we propose that (i) 0.25 mM NaO treatment may induce the *S. aureus* internalization through $\alpha5\beta1$ integrin; meanwhile (ii) 1 mM NaO could activate bMECs via TLR2 before *S. aureus* challenged.

3.3 MAPK activation by NaO

Because TLR2 activation leads to MAPK phosphorylation (p38, JNK or ERK1/2), and these kinases are involved in bacteria phagocytosis in both phagocytic and NPPCs (Ninkovic and Roy, 2012; Alva-Murillo et al., 2015), we evaluated the phosphorylation status of these MAPK in NaO-treated cells. As expected, the JNK phosphorylation level was increased (1.3 ratio) in *S. aureus*-challenged bMECs but the activation of p38 was reduced (0.48 ratio) and the ERK1/2 activation was not modified (Fig. 3). When bMECs were treated with 0.25 mM NaO (24 h), the basal activation of p38, JNK and ERK1/2 was decreased (0.43, 0.35 and 0.78-fold, respectively); this effect was not modified after *S. aureus* challenged, except for JNK, in this case the inhibition was greater (0.15-fold). The p38 and JNK reduction was similar to that observed in bMECs treated with their pharmacological inhibitors (Fig. 3A-B). Interestingly, 1 mM NaO treatment increased the phosphorylation of the three MAPKs being more evident for p38 activation (~2 fold). However, after *S. aureus* challenged the stimulatory effect of 1 mM NaO was reverted to basal levels, except for JNK1/2 where the activation level it was lower (0.17-fold). Taking together these results we suggest that 0.25 mM NaO inhibits the bMECs IIR because the MAPK phosphorylation was reduced, whereas 1 mM NaO favors the activation of bMECs through p38, JNK and ERK1/2 phosphorylation.

3.4 Activation of transcription factors

In bMECs, the TLR2 activation triggers signaling pathways that induce the activation of transcriptional factors (TFs) related to IIR, such as AP-1, NF-κB, E2F-1, FAST-1, MEF-1, EGR, PPAR, ER and CBF, among others (Alva-Murillo et al., 2015; Bauer et al., 2015; Medina-Estrada et al., 2015). In addition, NaO is able to modulate TF activation related to host defense (PPARγ, CBP/p300) (Yonezawa et al., 2004; Mochizuki et al., 2008). In this sense, we evaluated the activation status of 56 TFs related to IIR by a Protein/DNA array (Fig. 4 and 5). The bMECs showed a basal activation profile where the TF EGR showed the strongest activation (0.98

ratio). Interestingly, the TFs basal activation status was reduced after *S. aureus* challenged, only AP-1 and E2F1 showed an evident signal (0.58 and 0.62 ratio, respectively) (Fig. 4B). Intriguingly, 0.25 mM NaO treatment drastically reduced the FTs basal activation; we only observed signals for MEF-1 and Stat-4 (0.47 and 0.49-ratio) (Fig. 4C and 5B). The basal state of EGR was reduced (0.79 ratio) in 1 mM NaO-treated cells, nevertheless it showed the highest activation. Additionally, 1 mM NaO treatment slightly activated (from a ratio of 0.03 to 0.53) 14 TFs (Brn-3, CBF, CDP, c-Myb, AP-1, Ets-1/Pea-3, FAST-1, GAS/ISRE, AP-2, NFAT-1, OCT-1, RAR/DR-5, RXR/DR-1 and Stat-3) in relation to unchallenged cells; however this effect was abolished after *S. aureus* challenged (Figs. 4F and 5). Markedly, the basal activation of NF-κB and PPARγ was reduced in all of the conditions evaluated. These results suggest that (i) 0.25 mM NaO impairs the IIR of bMECs, and (ii) 1 mM NaO activates bMECs via TLR2 before *S. aureus* challenge.

3.5 Gene expression of inflammatory response elements

The NaO-modulated antimicrobial response was evaluated previously by measuring the gene expression of antimicrobial peptides (TAP, LAP, BNBD4, BNBD5 and BNBD10), as well as the mRNA levels of the cytokine pro-inflammatory TNF-α (Alva-Murillo et al., 2013a). However, a more wide study to determine the effect of NaO on the inflammatory response of bMECs has not been performed. In this work, we evaluated the gene expression regulation of two pro-inflammatory (IL-1β and IL-6) and one anti-inflammatory cytokines (IL-10), as well as 2 chemokines (IL-8 and RANTES) in NaO-treated bMECs during *S. aureus* challenge. *S. aureus* infection did not modify neither IL-1β, IL-6, IL-10, IL-8 or RANTES mRNA levels (Fig. 6). The 0.25 mM NaO treatment increased the IL-10 gene expression (~3 fold), this effect was reduced after bacterial challenged (Fig. 6B). The 1 mM NaO treated-bMECs showed an induction of IL-1β (~2 fold), IL-8 (~2 fold) and RANTES (~2 fold) mRNA levels, which was reverted by *S. aureus* except for IL-8 where the level increased to ~6 fold. In addition, the IL-10 mRNA

level was not modified in cells treated with 1 mM NaO; however after bacterial stimulus the mRNA level was up-regulated (~4 fold) (Fig. 6B). Taking together these results, we could suggest that 0.25 mM NaO acts as anti-inflammatory due to the up-regulation of the IL-10 gene expression. Meanwhile, 1 mM NaO may exerts pro-inflammatory effects before infection, but it can act as anti-inflammatory during infection.

3.6 Analysis of the promoter regions of cytokines genes

In this study we carried out an analysis of the promoter region of the cytokine genes evaluated to find putative transcription binding sites for the factors that were activated by NaO (Fig. 7). Interestingly, all of the evaluated genes showed putative transcription binding sites for AP-1 and c-Myb, which might explain the induction in the gene expression by 1 mM NaO in bMECs through TLR2 pathway. Although EGR showed the strongest activation in this condition, only the IL-10 gene showed a putative binding site for this TF.

4. Discussion

Most of the diseases provoked by *S. aureus* involve extracellular bacteria or biofilm formation; however, some strains are able to internalize into phagocytic cells and NPPCs, which allows to bacteria avoid the host immune response that favors chronic and recurrent infections, such as bovine mastitis. In this sense, bMECs are one of the target cells for *S. aureus* internalization. The modulation of the IIR is essential for the improvement of prophylactic or therapeutic treatments in order to avoid diseases in animals or humans. In this way, several studies have been focused in compounds with immunomodulatory properties, like fatty acids. We reported previously, that butyrate (0.5 mM) -a short chain fatty acid- reduces *S. aureus* internalization into bMECs and improved the IIR via TLR2/p38 pathway (Alva-Murillo et al., 2015). In addition, we showed that NaO treatment modulates differentially the internalization of *S. aureus* into bMECs (Alva-Murillo et al., 2013a). This study demonstrates that (i) in 0.25 mM NaO-treated bMECs the IIR mediated

by TLR2 and its signaling pathway (MAPKs and TF) is impaired, and (ii) that 1 mM NaO pre-treatment activates bMECs via TLR2/p38/JNK/ERK1/2 pathway, which might be involved in the 1 mM NaO-reduced *S. aureus* internalization into bMECs.

It is known that the main mechanism described for *S. aureus* internalization into NPPC (i.e. bMECs) is mediated by the $\alpha5\beta1$ integrin (Alva-Murillo et al., 2014a). In agreement, the $\alpha5\beta1$ integrin blockage in bMECs reduced the CFU recovered (Fig. 1A). We observed that in 0.25 mM NaO-treated cells the integrin MA was increased (~3 fold) and the receptor blockage reduced drastically the *S. aureus* internalization (Fig. 1 and 2). Whereas, 1 mM NaO did not modify the integrin MA and the receptor blockage did not alter the 1 mM NaO-reduced bacterial internalization. In agreement, the cell surface $\beta1$ integrin expression was not affected by this MCFA (1 mM, 24 h) in human bladder cancer cells (Yamasaki et al., 2014). These results suggest that the integrin availability may favor the 0.25 mM NaO-induced *S. aureus* internalization. However, this receptor is not directly involved in the 1 mM NaO-reduced bacterial internalization.

The epithelial cells are part of the first-line defense mechanisms of mammals. This response is mediated through receptors, as TLR2 -the major receptor for *S. aureus* recognition- and it is known that this receptor is involved in *S. aureus* internalization into NPPCs (Rocha-de-Souza et al., 2008; Medina-Estrada et al., 2015). Also, the TLR2 blockage with a functional antibody (clone TL2.1) reduced the number of CFU recovered into human cord blood-derived cells or bMECs (Rocha-de-Souza et al., 2008; Medina-Estrada et al., 2015). In this study we observed the same result (Fig. 1A): in 0.25 or 1 mM NaO-treated bMECs and then TLR2 blockage, we recovered less CFU internalized than in cells without blocking (Fig. 1B). In addition, we detected an increase in the TLR2 MA in 1 mM NaO-treated cells but not in 0.25 mM-treated bMECs (Fig. 2B). To our knowledge, this is the first report indicating that octanoate modulates the TLR2 MA. It has been reported that fatty acids are able to modulate TLR expression. In this sense, rats fed with a high-fat diet (HFD) showed levels increased of gene and protein expression of TLR2 in the skeletal muscle (Zhu et al., 2015). On the other hand,

short chain fatty acids (butyrate) increased TLR2 gene expression in liver tissue during an inflammatory event (Mattace Raso et al., 2013). Also, we reported previously that butyrate induced the TLR2 MA and gene expression levels in bMECs (Alva-Murillo et al., 2015). These results support our hypothesis that 1 mM NaO activates bMECs via TLR2 before *S. aureus* challenged.

There is evidence suggesting that TLR2 is not a phagocytic receptor *per se*, however for TLR2 bacterial recognition, it can cooperate with others phagocytic receptors (i.e., scavenger receptors) (Mae et al., 2007). In a previous work, we reported that CD36 is involved in *S. aureus* internalization into bMECs, because when receptors were blocked with a specific blocking antibody the CFU internalized were reduced (Alva-Murillo et al., 2015). However, we did not find a role of CD36 in butyrate-reduced *S. aureus* internalization. In this sense, we detected a participation of CD36 in 0.25 mM NaO-induced bacterial internalization, but not in 1 mM NaO (Fig. 1C). Nonetheless, the CD36 MA was not modified in any condition evaluated (Fig. 2C). It is known that NaO induces CD36 gene expression in bMECs; however, this induction was with 10 mM NaO for 7 days (Yonezawa et al., 2004). This led us to suggest that the modulation of CD36 depends on the concentration and exposure time of the stimulus. Altogether, these results suggest that TLR2 and CD36 are involved in 0.25 mM NaO-induced *S. aureus* internalization, but only TLR2 is implied in 1 mM NaO-reduced bacterial internalization.

TLR2 stimulation leads to the activation of signaling pathways where MAPKs (p38, JNK1/2 or ERK1/2) and transcription factors (AP-1, NF-κB, IRF) are activated (Akira and Takeda, 2004). It is known that p38, JNK1/2 and ERK1/2 are involved in the internalization of *S. aureus* into bMECs, and the bacterial stimulus induces the JNK phosphorylation (Alva-Murillo et al., 2015). Interestingly, we observed that the phosphorylation levels of the MAPKs were down-regulated in 0.25 mM NaO-treated cells (Fig. 3). In the same condition, the profile of activated TF was almost abolished (Figs. 4 and 5). Contrary, the 1 mM NaO treatment up-regulated the MAPKs phosphorylation levels. In accordance, the activation of ERK1/2, p38 and

JNK were induced in neurons treated with octanoate (3 mM, 24 h) (Kamata et al., 2007). The cells treated with 1 mM NaO showed a slightly activation of 14 TF (Brn-3, CBF, CDP, c-Myb, AP-1, Ets-1/Pea-3, FAST-1, GAS/ISRE, AP-2, NFTA-1, OCT-1, RAR/DR-5, RXR/DR-1 and Stat-3) that are involved in IIR (Fig. 4E-F and 5). This effect was reverted after *S. aureus* challenge. In a previous report, we showed that butyrate induces the activation of 8 TFs, and this is strongest than the effect provoked by NaO (Alva-Murillo et al., 2015). To our knowledge, this is the first report that correlates these TFs activation by NaO with *S. aureus* internalization into NPPCs. It is important to notice that there are only a few reports related to NaO and TF activation. In this sense, NaO enhances the IL-1β-induced activation of NF-κB in intestinal epithelial cells (Andoh et al., 2000). Nonetheless, in Caco-2 cells the NaO does not modify the activation of NF-κB (Hoshimoto et al., 2002). We did not observe an activation of this TF by NaO in bMECs. On the other hand, PPARγ -a TF with anti-inflammatory properties- protein level is increased in 10 mM NaO-treated bMECs (Yonezawa et al., 2004). Nevertheless, 1 mM NaO treatment reduced the basal activation of PPARγ (Fig. 4E, 5B). In accordance, NaO (1-3 mM) in 3T3-L1 cells (preadipocytes) reduces the protein level of this TF (Han et al., 2002). In addition, this MCFA did not alter AP-1 activity in Caco-2 cells (Hoshimoto et al., 2002). However, the 1 mM NaO treated bMECs showed a slightly induction in the activation status of AP-1 (Fig. 4E, 5B). These discrepancies could be due to (i) the different concentration used, (ii) the incubation periods, and (iii) the cell type used.

A previous report from our group showed an immunomodulatory ability of NaO (Alva-Murillo et al., 2013a). In this sense, the antimicrobial response in bMECs treated with 1 mM NaO (24 h) is more effective (regarding to LAP and BNBD10 gene expression) than 0.25 mM NaO-treated cells. Notably, *S. aureus* challenged increased the antimicrobial response in 1 mM NaO-treated bMECs by showing and up-regulation of BNBD4, LAP and BNBD10 mRNA levels. Additionally, this MCFA (1 mM) reduces TNF-α gene expression. This evidence suggested that NaO not only modulates the antimicrobial defense but the inflammatory response. We

analyzed the gene expression of cytokines and chemokines in bMECs, which could be modulated by *S. aureus* challenge (Kim et al., 2011; Schukken et al., 2011; Alva-Murillo et al., 2014b). It is known that *S. aureus* provokes an anti-inflammatory environment in bMECs (Lahouassa et al., 2007; Günther et al., 2011; Schukken et al., 2011). We did not detect a significant change in gene expression of any gene evaluated (Fig. 6). These results are in agreement with previous reports from our group (Alva-Murillo et al., 2015). The 0.25 mM NaO treatment only induced IL-10 mRNA levels but it was downregulated after *S. aureus* challenge (Fig. 6B). In 1 mM NaO-treated bMECs the IL-1β, IL-8 and RANTES mRNA levels were up-regulated; however, after challenged with *S. aureus* the levels of IL-1β and RANTES were reduced, while the anti-inflammatory cytokine IL-10 gene expression was increased. Evidence suggests that NaO is able to modulate the inflammatory response in an intestinal level by showing a dichotomy. In intestinal epithelial cells treated with octanoato (1-10 mM, 12 h) the IL-1β-induced IL-8 gene expression and secretion are augmented (Andoh et al., 2000). On the other hand, in Caco-2 cells treated with NaO (1.3 mM, 24 h) the IL-1β-induced IL-8 secretion is suppressed (Hoshimoto et al., 2002). This demonstrates that NaO could exert either pro-inflammatory or anti-inflammatory activities.

We analyze the promoter region of the cytokine genes evaluated in this study, as well as the AP genes evaluated previously, in order to find putative transcription factor binding sites, and therefore to correlate the TF activation and the gene expression changes (Fig. 7). Interestingly, all of the evaluated genes showed putative transcription binding sites for AP-1 and c-Myb. Which might explain the AP and cytokines mRNA up-regulation by 1 mM NaO, although, other mechanisms could exist in this model. It is known that fatty acids (e.g. butyrate) regulate gene expression by epigenetic mechanisms (Tedelind et al., 2007). However, little is known about the histone deacetylase inhibitor property of NaO, and additional experiments are required to demonstrate this.

To notice, we observed a contrary and dose-dependent response in relation to (i) the $\alpha 5\beta 1$ integrin and TLR2 MA, (ii) the MAPK (p38, JNK and ERK1/2)

phosphorylation, (iii) the TF activation, (iv) the antimicrobial and (v) inflammatory gene expression evaluated. However, the way in which NaO exerts this differential response on the modulation of innate immune elements is unknown.

Conclusion

Altogether these results showed that NaO induces a dose-dependent differential response on the innate immunity of bMECs. In this way, 0.25 mM abolishes the IIR by decreasing the MAPK phosphorylation, TF activation, the antimicrobial and inflammatory response, which favors the *S. aureus* internalization into bMECs. On the other hand, bMECs are activated by 1 mM NaO –via TLR2/p38/JNK/ERK1/2- and this leads to improve the IIR and to diminish *S. aureus* internalization. Additionally, NaO (1 mM) might act as anti-inflammatory during infection.

Conflict of interest

The authors declare no conflict of interest.

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Figure captions

Fig. 1 The role of α5β1 integrin, TLR2 and CD36 in *S. aureus* internalization into bMECs modulated by NaO. bMECs were treated with 0.25 or 1 mM NaO for 24 h, then incubated with a specific blocking **(A)** anti-α5β1 integrin (10 µg/ml), **(B)** anti-TLR2 (5 µg/ml) or **(C)** anti-CD36 (0.25 µg/ml) antibody for 30 min, 1 h or 45 min, respectively, and further challenged with *S. aureus* for 2 h. The number of

internalized bacteria is represented by the ratio CFU/bMEC. Each bar shows the mean of triplicates ± SE of three independent experiments.

Fig. 2 The $\alpha 5\beta 1$ integrin, TLR2 and CD36 membrane abundance regulated by NaO. bMECs were treated with 0.25 or 1 mM NaO (24 h) and/or challenged with *S. aureus* and the **(A)** $\alpha 5\beta 1$ integrin, **(B)** TLR2 and **(C)** CD36 receptor abundance was evaluated by flow cytometry. The fluorescence intensity was estimated from 10,000 events. For TLR2 MA, LPS was used as a positive control. Each bar shows the mean of triplicates ± SE of three independent experiments.

Fig. 3. p38, JNK and ERK1/2 activation regulated by NaO in *S. aureus*-challenged bMECs. MAPK phosphorylation was measured in bMECs that were treated with 0.25 or 1 mM NaO and/or challenged with *S. aureus* by flow cytometry. The phosphorylated MAPK concentrations (U/ml) are represented: **(A)** pp38, **(B)** pJNK and **(C)** pERK1/2. Each bar shows the result of one experiment. SB203580: p38 inhibitor. SP600125: JNK1/2 inhibitor. U0126: ERK1/2 inhibitor.

Fig. 4. Transcription factor activation by NaO in *S. aureus*-challenged bMECs. Protein/DNA array blots were utilized to analyze 56 different transcription factor DNA-binding sites from samples that were obtained from **(A)** bMEC nuclear extracts (control), **(B)** bMECs that were challenged with *S. aureus*, **(C and E)** bMECs that were treated with 0.25 or 1 mM NaO for 24 h and **(D and F)** cells that were treated with 0.25 or 1 mM NaO and challenged with *S. aureus*. The DNA samples were spotted in duplicate in two rows (top: undiluted; bottom: dilution 1/10). Biotinylated DNA was spotted for alignment along the right and bottom sides of the array. AP-1; activating-protein 1. AP-2: activating enhancer binding protein 2. Brn-3: brain-specific homoebox/POU domain protein. CBF: core binding factor. CDP: CCAAT displacement protein. c-Myb: c-myeloblastosis transcription factor. DR1: death receptor 1. DR5: death receptor 5. EGR: early growth response protein 1. Ets-1: Ets-1 transcriptional factor. FAST-1: forkhead activin signal transducer-1.

GAS: interferon-gamma activated sequence. ISRE: interferon-stimulated response element. MEF-1: myeloid Elf-1 like factor. NF-κB: nuclear factor-κB. NFTA-1: nuclear factor or activated T cells. PPAR: peroxisome proliferator-activated receptor. OCT-1: octamer transcription factor. PPAR: RAR: retinoic acid receptor. RXR: retinoid X receptor. Stat: signal transducer and activator of transcription.

Fig. 5. Transcription factor activation analysis. The intensity from each spot from the membranes was quantified using ImageJ software. The bars show the mean intensity of duplicates of the ratio TF/control. The symbol "*" indicates significant changes ($P < 0.05$) in relation to the unchallenged bMECs. The symbol "#" indicates significant changes ($P < 0.05$) in relation to the challenged bMECs.

Fig. 6. **Inflammatory response gene expression analysis through NaO regulation in *S. aureus*-challenged bMECs.** qPCR analysis that shows the effect on pro-inflammatory **(A)** and anti-inflammatory cytokines **(B),** and chemokines **(C)** gene expression. The bMECs were treated with 0.25 or 1 mM NaO and then challenged with *S. aureus* for 2 h. Each bar shows the mean of triplicates ± SE of three independent experiments. GAPDH was used as endogenous gene in all of the conditions. IL-1β: interleukin-1beta. IL-6: interleukin-6. IL-10: interleukin-10. IL-8: interleukin-8. RANTES: Regulated on Activation, Normal T Expressed and Secreted. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs.

Fig. 7. Transcription binding site analysis in the promoters of innate immune elements. The program PROMO V 3.0.2 was used. The putative binding sites were analyzed in 1.5 kb upstream of the ATG in each gene. TAP: tracheal antimicrobial peptide (Genbank_AC_000184.1). LAP: lingual antimicrobial peptide (Genbank_AC_000184.1). BNBD4: bovine neutrophil β-defensin 4 (Genbank_AC_000184.1). BNBD5: bovine neutrophil β-defensin 5 (Genbank_AC_000184.1). BNBD10: bovine neutrophil β-defensin 10

(Genbank_AC_000184.1). TNF-α: tumor necrosis factor-alpha (Genbank AC_000180.1). IL-1β: interleukin-1beta (Genbank AC_000168.1). IL-6: interleukin-6 (Genbank AC_000161.1). IL-10: interleukin-10 (Genbank AC_000173.1). IL-8: interleukin-8 (Genbank AC_000163.1).

Figure 1

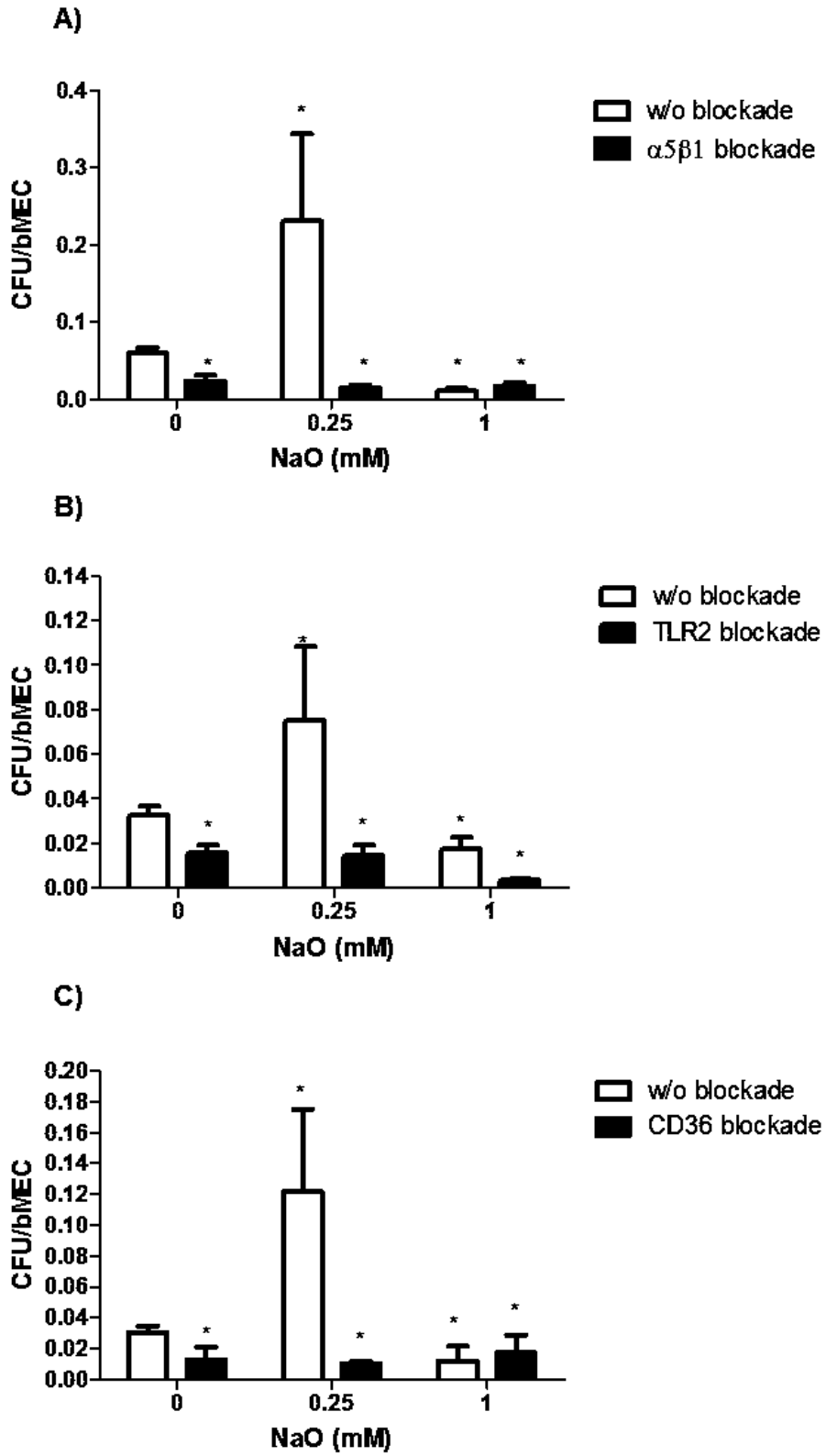

A)
0.4
0.3
0.2
0.1
0.0
CFU/bMEC
0
0.25
1
NaO (mM)
w/o blockade
α5β1 blockade
B)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
CFU/bMEC
0
0.25
1
NaO (mM)
w/o blockade
TLR2 blockade
C)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
CFU/bMEC
0
0.25
1
NaO (mM)
w/o blockade
CD36 blockade

Figure 2

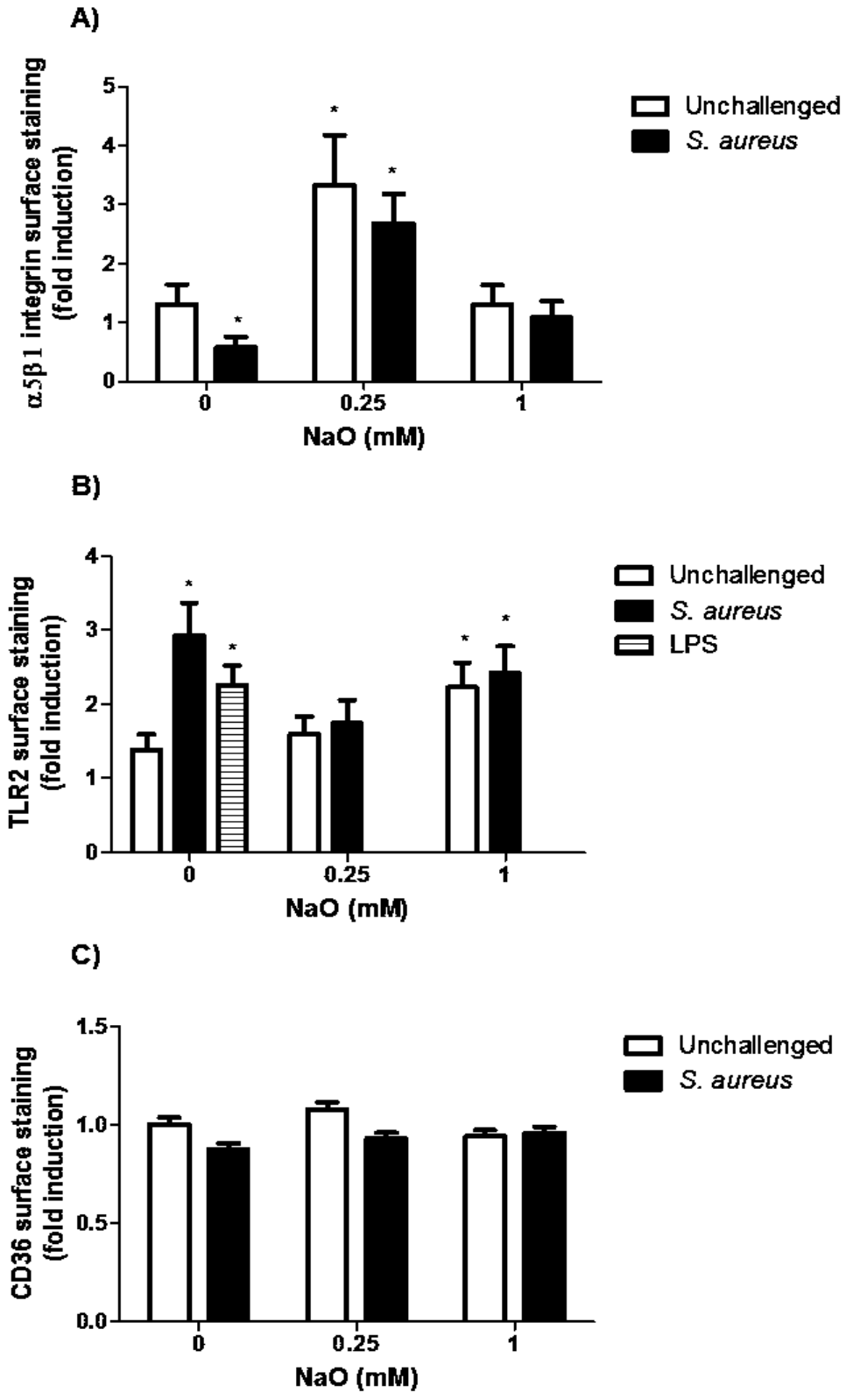

A)
α5β1 integrin surface staining (fold induction)
5
4
3
2
1
0
0
0.25
1
NaO (mM)
Unchallenged
S. aureus
B)
TLR2 surface staining (fold induction)
4
3
2
1
0
0
0.25
1
NaO (mM)
Unchallenged
S. aureus
LPS
C)
CD36 surface staining (fold induction)
1.5
1.0
0.5
0.0
0
0.25
1
NaO (mM)
Unchallenged
S. aureus

Figure 3

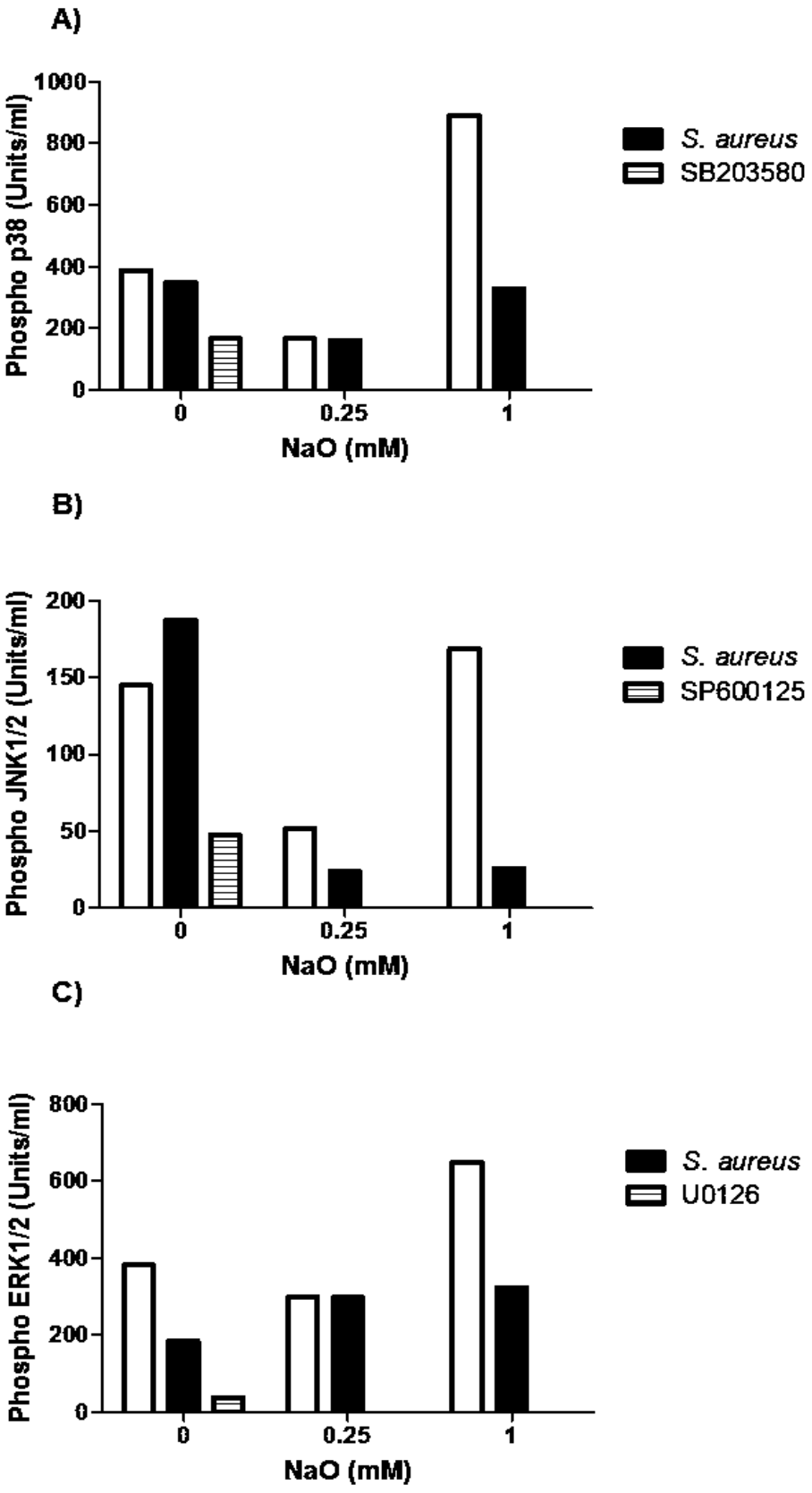
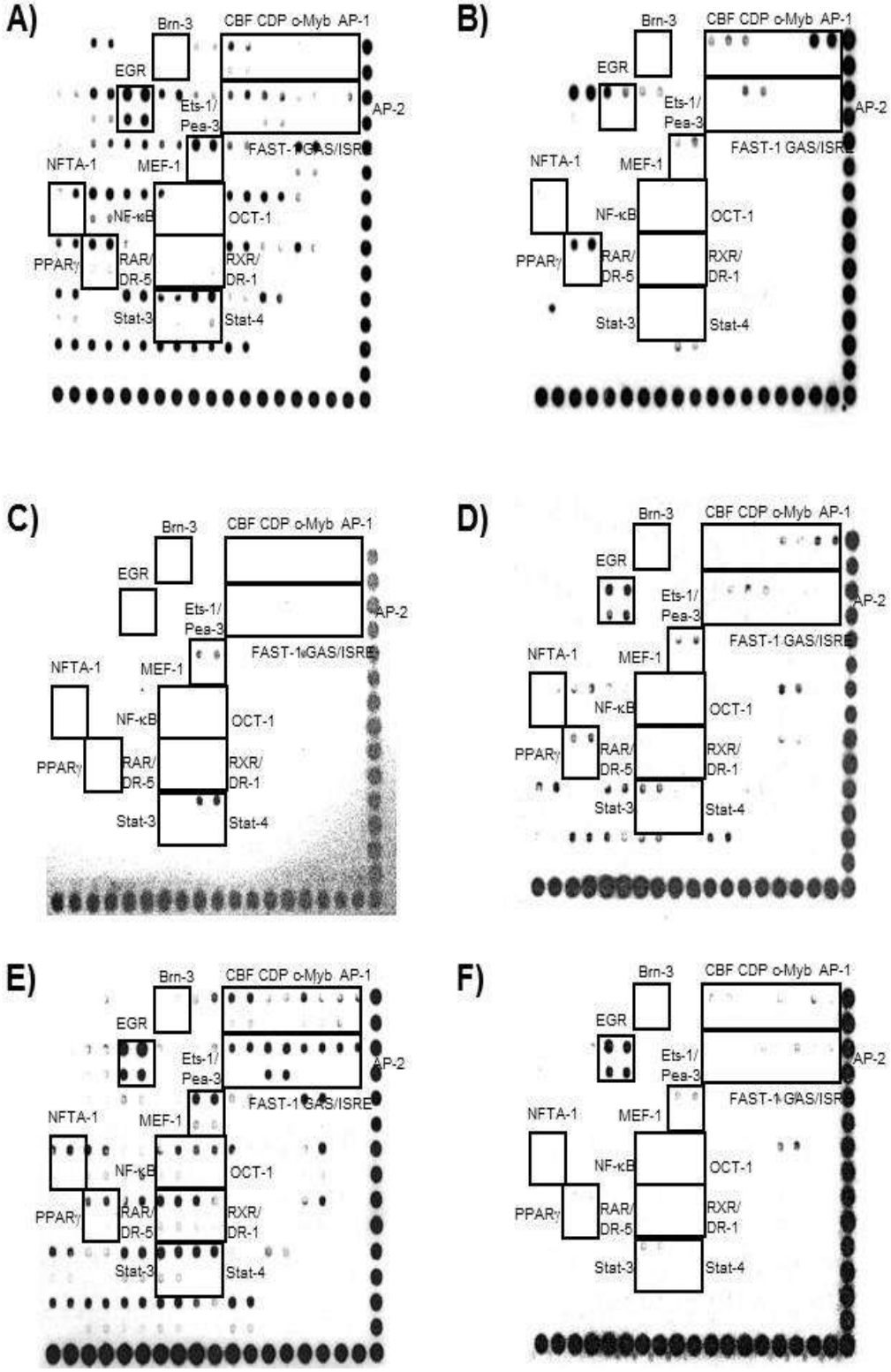

A)
Phospho p38 (Units/ml)
1000
800
600
400
200
0
0
0.25
1
NaO (mM)
S. aureus
SB203580
B)
Phospho JNK1/2 (Units/ml)
200
150
100
50
0
0
0.25
1
NaO (mM)
S. aureus
SP600125
C)
Phospho ERK1/2 (Units/ml)
800
600
400
200
0
0
0.25
1
NaO (mM)
S. aureus
U0126

Figure 4


A)
Brn-3
CBF CDP c-Myb AP-1
EGR
Ets-1/
Pea-3
AP-2
FAST-1 GAS/ISRE
NFTA-1
MEF-1
NF-κB
OCT-1
PPARγ
RAR/
DR-5
RXR/
DR-1
Stat-3
Stat-4
B)
Brn-3
CBF CDP c-Myb AP-1
EGR
Ets-1/
Pea-3
AP-2
FAST-1 GAS/ISRE
NFTA-1
MEF-1
NF-κB
OCT-1
PPARγ
RAR/
DR-5
RXR/
DR-1
Stat-3
Stat-4
C)
Brn-3
CBF CDP c-Myb AP-1
EGR
Ets-1/
Pea-3
AP-2
FAST-1 GAS/ISRE
NFTA-1
MEF-1
NF-κB
OCT-1
PPARγ
RAR/
DR-5
RXR/
DR-1
Stat-3
Stat-4
D)
Brn-3
CBF CDP c-Myb AP-1
EGR
Ets-1/
Pea-3
AP-2
FAST-1 GAS/ISRE
NFTA-1
MEF-1
NF-κB
OCT-1
PPARγ
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DR-5
RXR/
DR-1
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Brn-3
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Pea-3
AP-2
FAST-1 GAS/ISRE
NFTA-1
MEF-1
NF-κB
OCT-1
PPARγ
RAR/
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RXR/
DR-1
Stat-3
Stat-4
F)
Brn-3
CBF CDP c-Myb AP-1
EGR
Ets-1/
Pea-3
AP-2
FAST-1 GAS/ISRE
NFTA-1
MEF-1
NF-κB
OCT-1
PPARγ
RAR/
DR-5
RXR/
DR-1
Stat-3
Stat-4

Figure 5

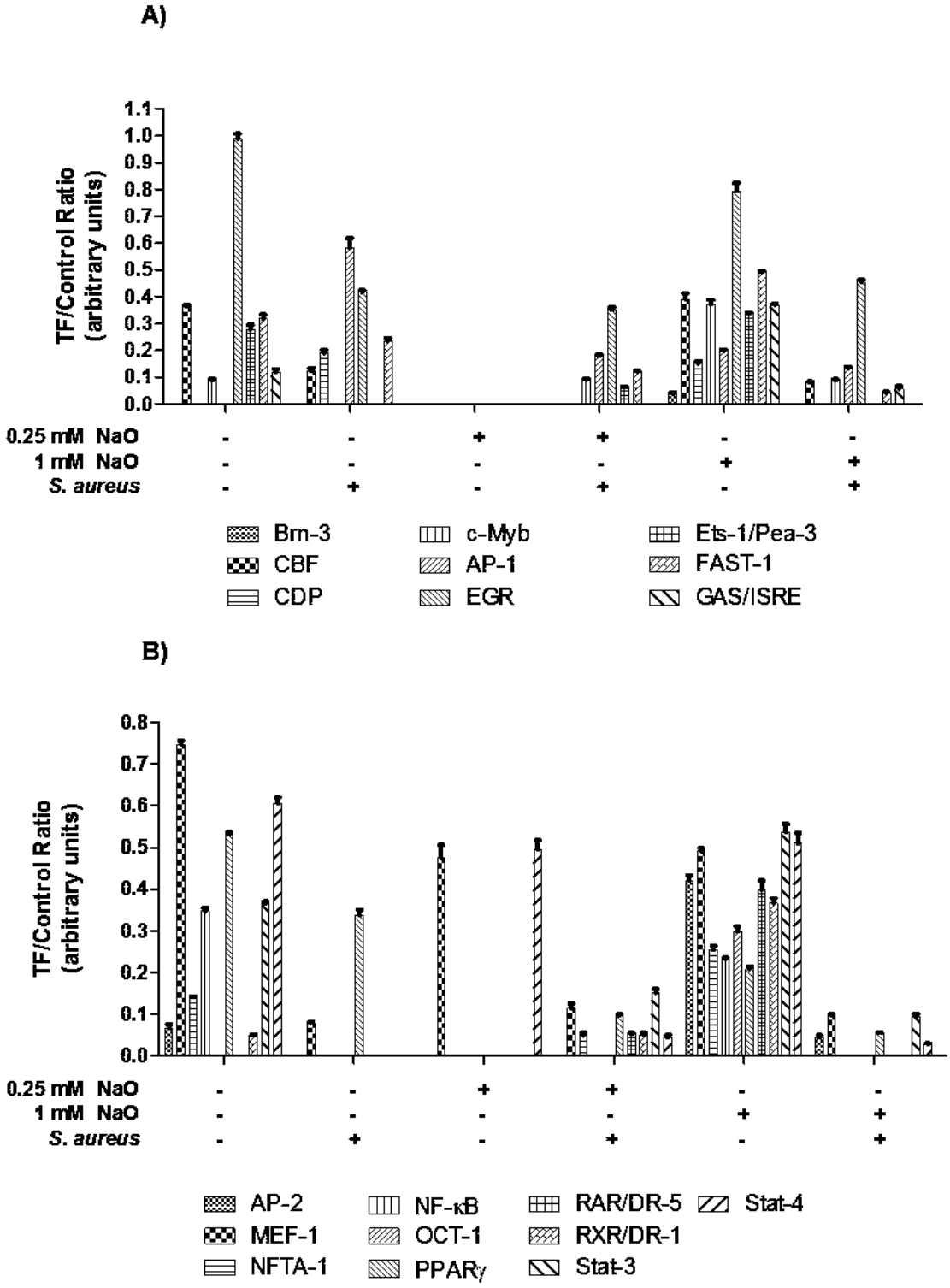
A)
TF/Control Ratio (arbitrary units)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.25 mM NaO - - + + - -
1 mM NaO - - - - + +
S. aureus - + - + - +
Brn-3
CBF
CDP
c-Myb
AP-1
EGR
Ets-1/Pea-3
FAST-1
GAS/ISRE
B)
TF/Control Ratio (arbitrary units)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.25 mM NaO - - + + - -
1 mM NaO - - - - + +
S. aureus - + - + - +
AP-2
MEF-1
NFTA-1
NF-κB
OCT-1
PPARγ
RAR/DR-5
RXR/DR-1
Stat-3
Stat-4

Figure 6

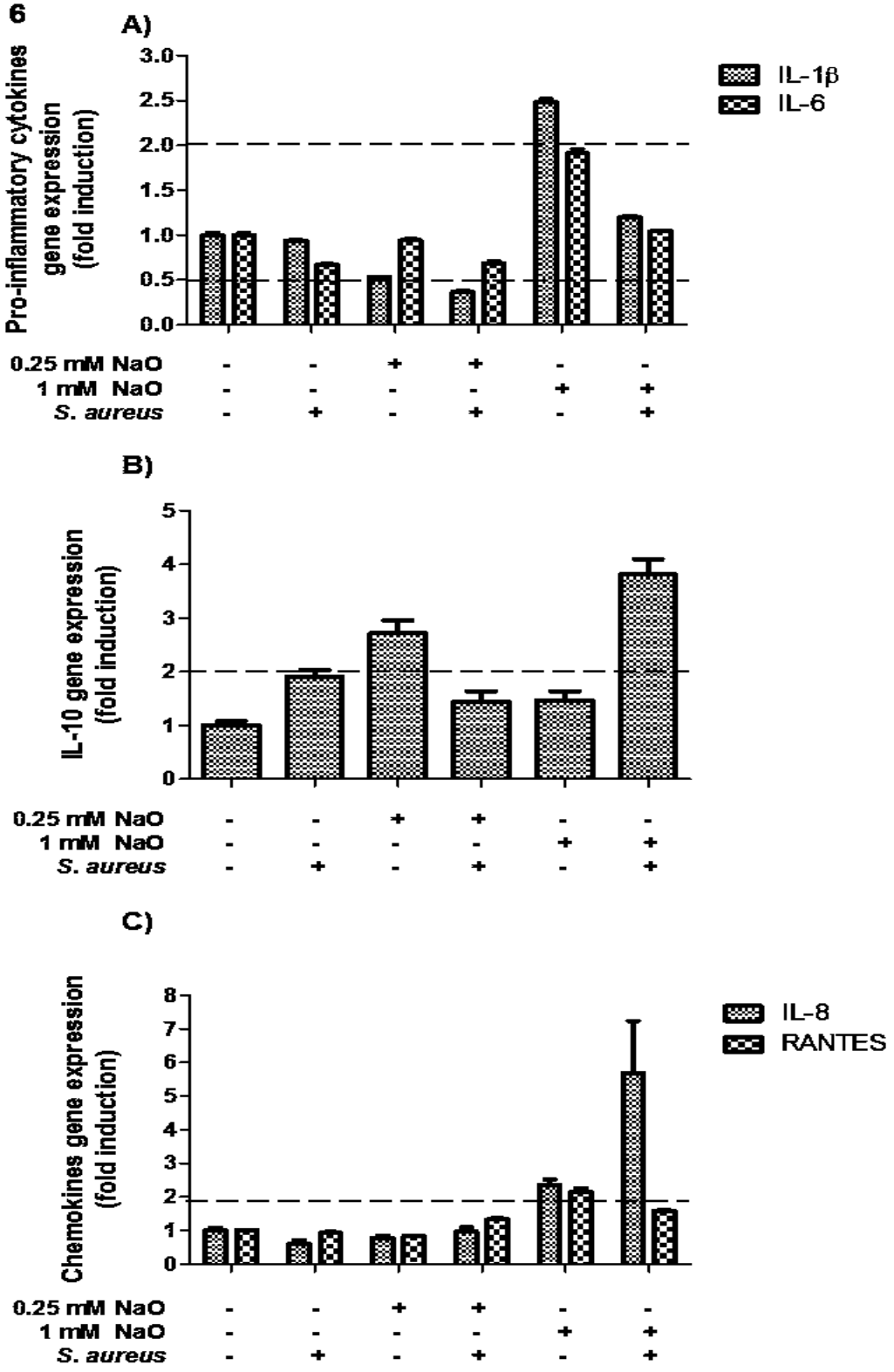
A)
Pro-inflammatory cytokines gene expression (fold induction)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IL-1β
IL-6
0.25 mM NaO - - + + - -
1 mM NaO - - - - + +
S. aureus - + - + - +
B)
IL-10 gene expression (fold induction)
5
4
3
2
1
0
0.25 mM NaO - - + + - -
1 mM NaO - - - - + +
S. aureus - + - + - +
C)
Chemokines gene expression (fold induction)
8
7
6
5
4
3
2
1
0
IL-8
RANTES
0.25 mM NaO - - + + - -
1 mM NaO - - - - + +
S. aureus - + - + - +

Figure 7

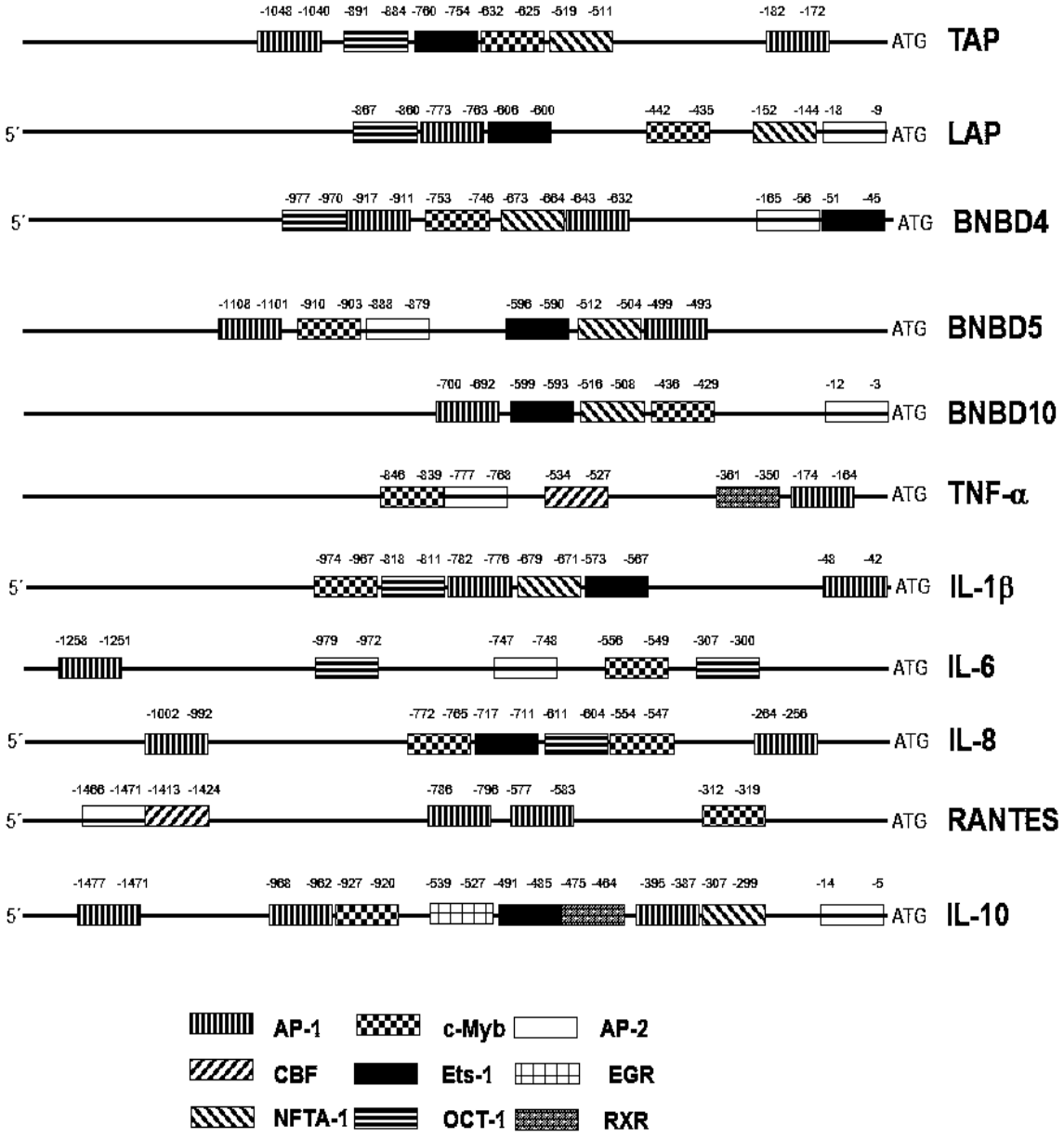
-1048 -1040 -891 -884 -760 -754 -632 -625 -519 -511
-182 -172
ATG
TAP
-867 -860 -773 -763 -606 -600
-442 -435
-152 -144 -18 -9
5′
ATG
LAP
-977 -970 -917 -911 -753 -746 -673 -664 -643 -632
-165 -56 -51 -45
5′
ATG
BNBD4
-1108 -1101 -910 -903 -888 -879
-596 -590 -512 -504 -499 -493
ATG
BNBD5
-700 -692 -599 -593 -516 -508 -436 -429
-12 -3
ATG
BNBD10
-846 -839 -777 -768
-534 -527
-361 -350 -174 -164
ATG
TNF-α
-974 -967 -818 -811 -782 -776 -679 -671 -573 -567
-48 -42
5′
ATG
IL-1β
-1258 -1251
-979 -972
-747 -748
-556 -549 -307 -300
ATG
IL-6
-1002 -992
-772 -765 -717 -711 -611 -604 -554 -547
-264 -256
5′
ATG
IL-8
-1466 -1471 -1413 -1424
-786 -796 -577 -583
-312 -319
5′
ATG
RANTES
-1477 -1471
-968 -962 -927 -920
-539 -527 -491 -485 -475 -464
-395 -387 -307 -299
-14 -5
5′
ATG
IL-10
AP-1
c-Myb
AP-2
CBF
Ets-1
EGR
NFTA-1
OCT-1
RXR

Table 1. Bovine oligonucleotides used in this study.

Specificity	Primer	Sequence (5´-3´)*	Fragment size (bp)	Annealing temperature (°C)	References
TNF-α	Forward	CCCCTGGAGATAACCTCCCA	101	56	(Mookherjee et al., 2006)
	Reverse	CAGACGGGAGACAGGAGAGC			
IL-1β	Forward	GCAGAAGGGAAGGGAAGAATGTAG	198	52	(Alva-Murillo et al., 2014b)
	Reverse	CAGGCTGGCTTTGAGTGAGTAGAA			
IL-6	Forward	AACCACTCCAGCCACAAACACT	179	57	(Alva-Murillo et al., 2014b)
	Reverse	GAATGCCCAGGAACTACCACAA			
IL-8	Forward	TTCCACACCTTTCCACCCCAA	149	53.5	(Alva-Murillo et al., 2014b)
	Reverse	GCACAACCTTCTGCACCCACTT			
IL-10	Forward	GATGCGAGCACCCTGTCTGA	129	59	(Alva-Murillo et al., 2014b)
	Reverse	GCTGTGCAGTTGGTCCTTCATT			
RANTES	Forward	CACCCACGTCCAGGAGTATT	117	54	(Nelson et al., 2010)
	Reverse	CTCGCACCCACTTCTTCTCT			
GAPDH	Forward	TCAACGGGAAGCTCACTGG	237	56.9	(Yonezawa et al., 2009)
	Reverse	CCCCAGCATCGAAGGTAGA			

CAPÍTULO III.

Participación de las integrinas $\alpha 5\beta 1$ en la internalización de *Staphylococcus aureus* en las células epiteliales mamarias bovinas regulada por butirato.

Participación de las integrinas α5β1 en la disminución de la internalización de *Staphylococcus aureus* en las células epiteliales mamarias bovinas regulada por butirato

Nayeli Alva-Murillo, Alejandra Ochoa-Zarzosa and Joel E. López-Meza*.

Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo. Morelia, Michoacán, México.

*Autor responsable:

Joel E. López Meza

Centro Multidisciplinario de Estudios en Biotecnología

Facultad de Medicina Veterinaria y Zootecnia

Universidad Michoacana de San Nicolás de Hidalgo

Km 9.5 Carr, Morelia-Zinapécuaro, Posta Veterinaria

C.P. 58893

Morelia, Michoacán, México.

Tel/Fax: (52)-443-295-8029.

E-mail: elmeza@umich.mx

Resumen

El principal mecanismo empleado para la internalización de *Staphylococcus aureus* en células fagocíticas no profesionales es mediado por las integrinas $\alpha5\beta1$ que se encuentran en la superficie de la célula hospedera. Existen moléculas inmunomoduladoras, como el butirato de sodio (NaB) que disminuye la internalización de esta bacteria en las células de epitelio mamario bovino (CEMB). Sin embargo, se desconoce el papel de las integrinas $\alpha5\beta1$ en este proceso, lo cual fue el objetivo de este trabajo. El bloqueo funcional de las integrinas $\alpha5\beta1$ no modificó la disminución de la internalización generada por NaB (0.5 mM). Por otro lado, el NaB incrementó la abundancia membranal del receptor, efecto que se mantuvo aún después del estímulo bacteriano. No se observó modificación en la expresión de los genes de las subunidades del receptor de integrinas en ninguna de las condiciones evaluadas. Estos resultados sugieren que las integrinas no tienen una participación directa sobre la inhibición de *S. aureus* modulada por 0.5 mM NaB.

Abstract

The main mechanism described for *Staphylococcus aureus* internalization into eukaryotic cells is mediated through the host receptor integrin $\alpha5\beta1$. It is known that some immunomodulatory compounds, such as sodium butyrate (0.5 mM NaB), are able to inhibit the bacterial internalization into bovine mammary epithelial cells (bMECs). However, the role of the receptor integrin $\alpha5\beta1$ in this event is unknown, which was the aim of this study. When this receptor was blocked with a specific antibody, we did not observe changes in the 0.5 mM NaB- reduced internalization. On the other hand, NaB treatment induced the membrane abundance of this receptor; which was maintained after the challenge with *S. aureus*. Meanwhile, the $\alpha5$ or $\beta1$ subunits gene expression was not modified in all of the conditions evaluated. These results suggest that this receptor does not have a direct role in the *S. aureus* internalization inhibition mediated by 0.5 mM NaB.

1. Introducción

En el ganado lechero bovino *Staphylococcus aureus* es el principal patógeno contagioso asociado con la mastitis bovina crónica, patología que se caracteriza por la inflamación de la glándula mamaria (Kerro Dego et al., 2002). Esta enfermedad es de gran importancia a nivel mundial, ya que genera grandes pérdidas económicas (Hogeveen et al., 2011). Este microorganismo tiene la capacidad de internalizarse en la célula hospedera (ej. células fagocíticas no profesionales, CFNP), esto le confiere las ventajas de evadir al sistema inmune y reducir la efectividad de la terapia antimicrobiana (Almeida et al., 1996). El mecanismo de internalización de *S. aureus* mejor descrito es el "zipper"(Medina-Estrada et al., 2015), en el cual la proteína de unión a fibronectina (FnBP) presente en la superficie bacteriana se une a la fibronectina (Fn) de la matriz extracelular formando un complejo (Fn-FnBP) que es reconocido por las integrinas $\alpha5\beta1$ de la célula hospedera -como las células epiteliales mamarias bovinas (CEMB)- lo que induce el rearreglo del citoesqueleto mediante la polimerización de actina y la consecuente invaginación del microorganismo (Sinha et al., 1999; Medina-Estrada et al., 2015)

Las integrinas son receptores heterodiméricos transmembranales, formados por la asociación no covalente de una subunidad α y otra β, no poseen actividad intrínseca de tirosina cinasa y entre sus ligandos están la laminina, Fn, fibrinógeno, vitronectina, colágena y las moléculas de adhesión intracelular de células adyacentes (Kumar, 1998). Participan en diferentes procesos fisiológicos como la migración, proliferación, diferenciación, adhesión celular, apoptosis, fagocitosis, polaridad y en la expresión de genes (Hynes, 2002; Martin et al., 2002). También participan en la respuesta inmune, cicatrización de heridas, tráfico de leucocitos y cáncer (Hynes, 2002). Las integrinas sufren un ciclo endocítico en el cual se internalizan de la membrana plasmática hacia compartimentos endosomales y después regresan a la superficie celular (Bretscher, 1992). Cuando se unen al ligando, las integrinas promueven la acumulación de los receptores en la membrana celular y la formación de estructuras o focos de adhesión. En estos puntos de adhesión se reclutan proteínas estructurales y de señalización, al interactuar con el

dominio citoplásmico de la subunidad β. Algunas de las proteínas cinasas que se concentran en los focos de adhesión son las cinasas FAK, c-Src, la PKC y la cinasa asociada a integrinas. Las integrinas participan en la activación de las MAPKs, como la cinasa regulada por señales extracelulares, la cinasa activada por p21 (PAK), la cinasa NH_2 terminal c-Jun (JNK) y FAK/Src (Giancotti, 2000). Como respuesta a la activación de las integrinas, se inician rutas de señalización que se asocian con la modificación del citoesqueleto de actina, así como con la expresión de genes de la respuesta inmune temprana, como las citocinas IL-1β, IL-8 y TNF-α (Hynes, 2002). La proteína FAK juega un papel muy importante en la internalización de *S. aureus* en las CFNP. En este sentido, en los fibroblastos deficientes de FAK se ha demostrado una disminución considerable de la internalización bacteriana, en relación a los fibroblastos WT (Agerer et al., 2005).

En nuestro grupo de trabajo hemos reportado que los ácidos grasos de cadena corta, como el butirato, disminuyen la internalización bacteriana en las CEMB (Ochoa-Zarzosa et al., 2009; Alva-Murillo et al., 2012). Sin embargo, no se ha estudiado la participación de las integrinas α5β1 en la internalización de *S. aureus* en las CEMB modulada por estos ácidos grasos, lo cual fue el objetivo de este trabajo.

2. Materiales y Métodos

2.1 Reactivos y cepas

El butirato de sodio (NaB) se adquirió de la casa comercial Sigma. Se utilizó el anticuerpo monoclonal anti-integrina α5β1 de rata (MAB2514, Chemicon-Millipore).

Para el presente estudio se utilizó una cepa de *S. aureus* subsp. *aureus* (ATCC 27543) aislada de un caso de mastitis bovina clínica, con la capacidad de invadir endotelio y epitelio mamario bovino (Gutiérrez-Barroso et al., 2008; Ochoa-Zarzosa et al., 2009). Para obtener el inóculo, la bacteria se creció en caldo Luria

Bertani (LB, 5 g de NaCl, 10 g de peptona y 5 g de extracto de levadura, por cada litro de agua, BIOXON), incubándose a 37°C durante 16-18 h. Para los experimentos se ajustó el cultivo bacteriano a una $D.O._{600}$ de 0.2, equivalente a $9.2x10^7$ UFC/ml.

2.2 Cultivo primario de células de epitelio mamario bovino

El cultivo primario de CEMB se obtuvo del tejido alveolar de la ubre de vacas lactantes, se aisló y cultivó como se ha descrito previamente por nuestro grupo de trabajo (Anaya-López et al., 2006). Se utilizaron CEMB del pasaje 2 al 8 y se cultivaron en cajas de Petri (Costar-Corning) con medio mínimo esencial de Dulbecco y F12 de Ham (DMEM/F12K, Sigma) suplementado con 10% de suero de ternera (Equitech-Bio), 1% de penicilina-estreptomicina (400 U/ml, GIBCO), 10 µg/ml insulina (Sigma), 10 µg/ml hidrocortisona (Sigma), 250 ng/ml de anfotericina B (Invitrogen), ajustado a un pH de 7.4. Las células se incubaron en una atmósfera de 5% de CO_2 a 37°C.

2.3 Bloqueo del receptor de integrinas $\alpha 5\beta 1$ durante la internalización de S. aureus en las CEMB

Se adicionó colágena tipo I de cola de rata a cajas de cultivo de 96 pozos (Costar-Corning), donde se cultivaron las monocapas confluentes de las CEMB (~$40x10^3$ células/pozo). Se agregó medio DMEM/F12K sin suero y sin antibióticos 24 h antes de adicionar el tratamiento de NaB. Se utilizó la concentración de 0.5 mM de butirato, en la cual se inhibe la internalización de *S. aureus*, por 24 h. Cumplido este tiempo se incubaron las CEMB con 10 µg/ml del anticuerpo anti-integrina (MAB2514) durante 30 min. Se llevó a cabo el ensayo de protección con gentamicina, infectando a las CEMB con la cepa de *S. aureus*, empleando una multiplicidad de infección (MDI) de 30 bacterias por 1 célula. El proceso de infección se llevó a cabo durante 2 h a 37°C. Después de este tiempo se lavaron 3 veces las CEMB con PBS, y se agregó gentamicina (50 µg/ml) para eliminar a las bacterias no internalizadas, incubándose 1 h a 37°C. Nuevamente se lavaron las células 3

veces con PBS, y se despegaron del plato con 0.05% tripsina-EDTA (Gibco), se recuperaron por centrifugación (600 x g/10 min), y posteriormente se lisaron con 250 µl de agua destilada estéril. Se distribuyeron 200 µl del lisado en agar LB (caldo LB con 10 g/l de agar bacteriológico, BIOXON) y se incubaron a 37°C por 18 h. Finalmente, se realizó el conteo total de las UFC.

2.4 Abundancia del receptor de integrinas $\alpha 5\beta 1$ en la membrana plasmática de las CEMB

Se adicionó colágena tipo I de cola de rata a cajas de cultivo de 24 pozos (Costar-Corning), donde se cultivaron las monocapas confluentes de las CEMB (~$2x10^5$ células/pozo). Se agregó medio DMEM/F12K sin suero y sin antibióticos 24 h antes de poner el tratamiento con NaB (24 h). Se llevó a cabo el ensayo de protección con gentamicina como se describió anteriormente. Se lavaron las células 3 veces con PBS frío y se despegaron con 0.05% tripsina-EDTA (Gibco). Se recuperaron las células por centrifugación (10 min/600 x g) a 4°C, y se lavó la pastilla 2 veces con PBS frío para luego recuperar las células por centrifugación (600 x g /2 min) a 4°C, sin resuspender. Posteriormente se adicionó suero de cabra al 5% en PBS (para bloquear las uniones inespecíficas) y se resuspendió, se incubó 30 min en hielo. Se recuperó la pastilla centrifugando y se incubó con el anticuerpo anti-integrina MAB2514 (10 µg/ml) por 2 h a 4° C. Esto se hizo en agitación y evitando el contacto con la luz. A los controles que solamente llevaron anticuerpo secundario no se les adicionó el anticuerpo primario, solamente BSA 0.1% en PBS. Cumplido el tiempo, se recuperaron las células por centrifugación y se lavaron dos veces con PBS frío. Se volvieron a centrifugar y se incubaron con el anticuerpo secundario acoplado a isotiocianato de fluoresceína o FITC (1:50 en PBS). Luego, las células se resuspendieron y se incubaron por 1 h en agitación, en hielo y cubierto de la luz. Se recuperó la pastilla por centrifugación y se lavó 2 veces con PBS, y se fijaron las CEMB con paraformaldehído al 4% en PBS (25 µl por tubo) durante 10 min en hielo. Se lavaron 3 veces con PBS y se recuperó la pastilla. Se resuspendió en 100 µl de PBS frío. Finalmente, se llevó a cabo la medición de la abundancia de la proteína en un citómetro de flujo Accuri BD C6, midiendo 10,000 eventos por condición.

2.5 Análisis de la expresión relativa de los genes de la subunidad $\alpha 5$ y $\beta 1$ del receptor de integrinas en las CEMB

El ARN total (5 µg) de las CEMB se extrajo de las condiciones evaluadas usando Trizol (Invitrogen), de acuerdo a las instrucciones del proveedor y después se usó para sintetizar el ADNc. La muestra obtenida se trató con DNAsa I (Invitrogen) para remover cualquier contaminación con ADN. La síntesis de ADNc se realizó por la reacción de transcripción reversa (RT) en un volumen final de 20 µl conteniendo: 25 µg/ml de oligo $d(T)_{15\text{-}18}$ (Invitrogen), 500 nM de dNTP´s Mix (Invitrogen). La reacción se incubó a 65°C por 5 min y después se guardó 5 min en hielo. Se centrifugó y se añadieron 4 µl de 5X First-Strand Buffer (Invitrogen), 10 mM de ditiotreitol (Invitrogen) y 2 U/µl de inhibidor de RNAsa (RNAsa Out, Invitrogen), luego se incubó a 37°C durante 2 min. Finalmente, se adicionaron 10 U/µl de la enzima transcriptasa reversa M-MLV (Invitrogen) y se incubó a 37°C por 50 min. La reacción se inactivó a 70°C por 15 min. La integridad del ADNc sintetizado se confirmó por medio de PCR con oligonucleótidos específicos para el gen constitutivo de la enzima gliceraldehído-3-fosfato deshidrogenasa (GAPDH).

La cuantificación relativa de la expresión de genes se realizó con el método Ct comparativo (ΔΔCt) en el sistema StepOne Plus Real-Time PCR Systems (Applied Biosystems) de acuerdo a las instrucciones del proveedor. La reacción se llevó a cabo con SYBR Green PCR Master Mix (Applied Biosystems), con 0.9 pmol/µl de cada oligonucleótido, 250 ng de ADNc y agua grado biología molecular. Se utilizaron oligonucleótidos específicos para amplificar los genes que codifican para la subunidad $\alpha 5$ y $\beta 1$ de las integrinas (Medina-Estrada et al., 2015). Como control interno (gen endógeno) se utilizó el gen de la GAPDH (Yonezawa et al., 2009).

2.6 Análisis de datos

Los datos se obtuvieron de tres experimentos independientes por triplicado, y se compararon con análisis de varianza (ANOVA). Los resultados se reportan

como el promedio ± el error estándar (EE) con nivel de significancia de *P<0.05*. Para el análisis de qPCR los niveles de expresión relativa de genes mayores a 2 o menores a 0.5 se consideraron estadísticamente significativos.

3. Resultados y discusión

3.1 Las integrinas $\alpha 5\beta 1$ no están directamente involucradas en la disminución de la internalización de S. aureus en las CEMB mediada por 0.5 mM de NaB

Debido a que las integrinas $\alpha 5\beta 1$ son uno de los principales receptores que participan en la internalización de *S. aureus* en células epiteliales, se evaluó si el bloqueo funcional de este receptor modificaba el efecto del butirato en la internalización de esta bacteria en las CEMB. En congruencia con lo reportado previamente por nuestro grupo de trabajo (Medina-Estrada et al., 2015), al bloquear a las integrinas $\alpha 5\beta 1$ con el anticuerpo específico se recuperaron menos UFC (~50%) respecto al control (células sin bloqueo) (Fig. 1A). Este efecto fue específico ya que al tratar a las CEMB con 10 μg/ml de IgG de rata, no hubo modificación en el número de UFC recuperadas en relación a las células sin bloqueo ni tratamiento (datos no mostrados). Por otro lado, en las células tratadas con NaB (0.5 mM, 24 h) el bloqueo de las integrinas no modificó el efecto generado por este ácido graso, ya que las UFC recuperadas fueron similares en ambas condiciones (Fig. 1A). Estos resultados sugieren que las integrinas $\alpha 5\beta 1$ no están participando directamente en la disminución de la internalización bacteriana regulada por 0.5 mM de NaB. En un reporte previo de nuestro grupo de trabajo, se demostró que en el aumento de la internalización bacteriana modulada por prolactina (~200%), las integrinas $\alpha 5\beta 1$ juegan un papel importante, ya que al bloquearlas con un anticuerpo específico la internalización de *S. aureus* disminuye notablemente (Medina-Estrada et al., 2015).

3.2 El butirato induce la abundancia membranal de las integrinas $\alpha 5\beta 1$

Con el fin de establecer alguna relación entre la presencia en membrana de las integrinas $\alpha 5\beta 1$ y la internalización de *S. aureus* en las CEMB, se evaluó su abundancia membranal mediante citometría de flujo. El estímulo bacteriano disminuyó (~50%) la abundancia membranal de este receptor (Fig.1B). Lo anterior coincide con un reporte previo de nuestro grupo de trabajo (Medina-Estrada et al., 2015), y sugiere que la bacteria favorece la disminución membranal a través de las vesículas de endocitosis. Además, el NaB (0.5 mM) indujo la abundancia del receptor en la membrana (~2 veces), efecto que no se modificó después de la infección (Fig. 1B). No se encontró una correlación directa entre la abundancia membranal del receptor y la internalización bacteriana. En este sentido, se sabe que la activación de FAK es necesaria para la internalización bacteriana y que el NaB suprime la actividad de FAK en macrófagos (Maa et al., 2010). Por lo anterior, se sugiere que el NaB pudiera estar interfiriendo la ruta de señalización de las integrinas $\alpha 5\beta 1$ afectando la activación de FAK, y disminuyendo la internalización bacteriana ya que se afectaría el rearreglo del citoesqueleto necesario para la endocitosis. Sin embargo, se requiere de análisis adicionales para confirmar dicha hipótesis

A pesar de que no se observó claramente una modificación en la expresión relativa de las subunidades de la integrina en ninguna de las condiciones evaluadas (Fig. 2A-B), el estímulo bacteriano generó un ligero aumento y disminución en la expresión del gen de la subunidad $\alpha 5$ y $\beta 1$, respectivamente, efecto reportado previamente por nuestro grupo de trabajo (Medina-Estrada et al., 2015).

4. Conclusión

Los resultados arrojados hasta el momento indican que las integrinas $\alpha 5\beta 1$ no están involucradas directamente en la disminución de la internalización de *S. aureus* a las CEMB, generada por 0.5 mM de butirato de sodio.

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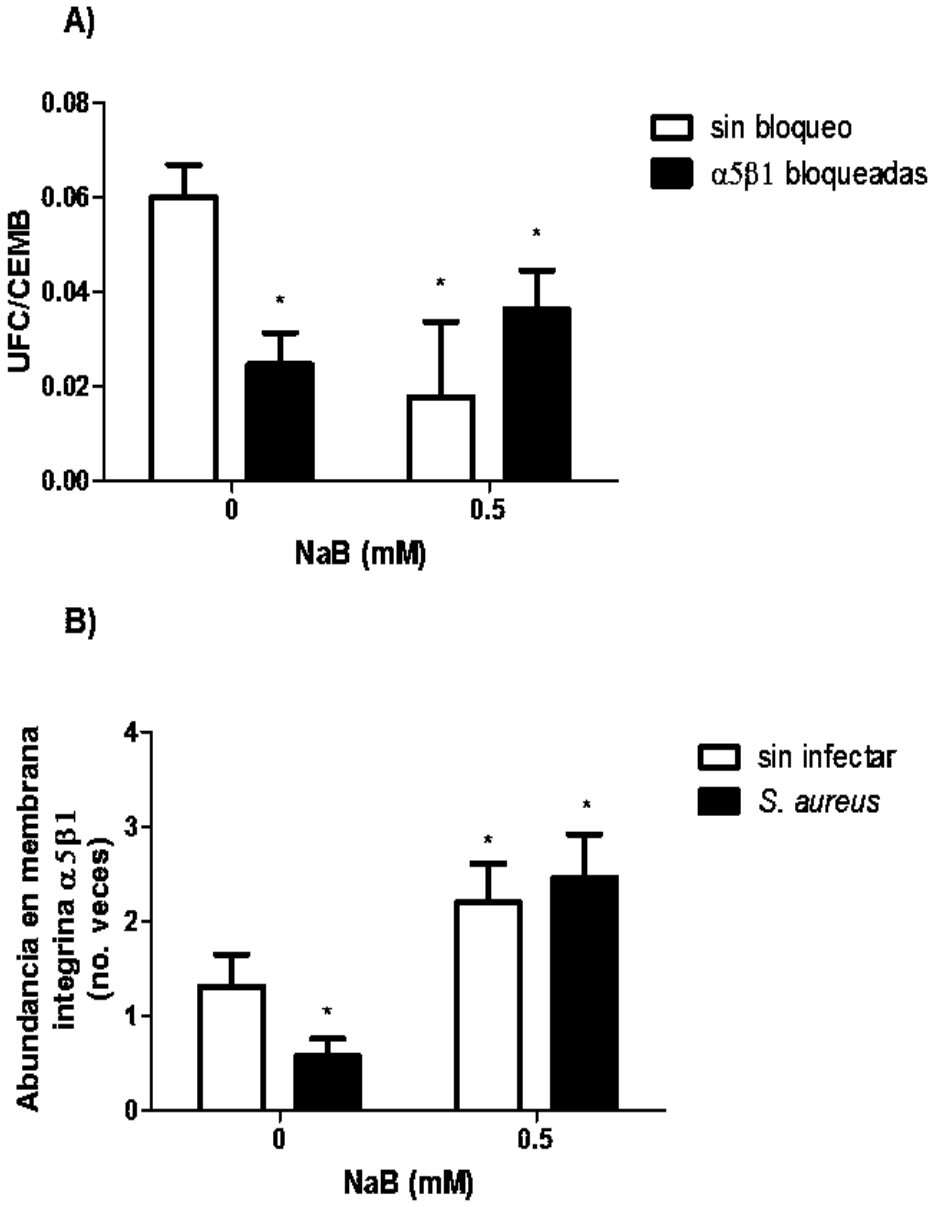
Leyendas de figura

Fig.1 Participación de la integrina $\alpha5\beta1$ en la internalización de *S. aureus* en las CEMB modulada por butirato. (A) Las células se trataron con 0.5 mM NaB por 24 h, después se incubaron con el anticuerpo específico anti-integrina $\alpha5\beta1$ (10 μg/ml, 30 min). Posteriormente, se infectaron por 2 h. El número de bacterias internalizadas se representa como la proporción UFC/células. (B) Las CEMB se trataron con NaB, y/o se infectaron con *S. aureus*, y se evaluó la abundancia del receptor en la membrana celular por citometría de flujo. La intensidad de fluorescencia se estimó de 10,000 eventos. Cada barra muestra el resultado de tres experimentos independientes por triplicado ± SE.

Fig.2 Expresión relativa de las subunidades $\alpha5$ y $\beta1$ de la integrina en las CEMB mediante qPCR. Las células se trataron con 0.5 mM NaB por 24 h, posteriormente se infectaron por 2 h (MDI 30:1). Se muestra la expresión relativa del gen de la subunidad (A) $\alpha5$ y (B) $\beta1$ de la integrina, utilizando como control de referencia a GAPDH. Cada barra muestra el promedio ± EE de tres experimentos independientes por triplicado.

Figure 1


A)
0.08
0.06
0.04
0.02
0.00
UFC/CEMB
sin bloqueo
α5β1 bloqueadas
*
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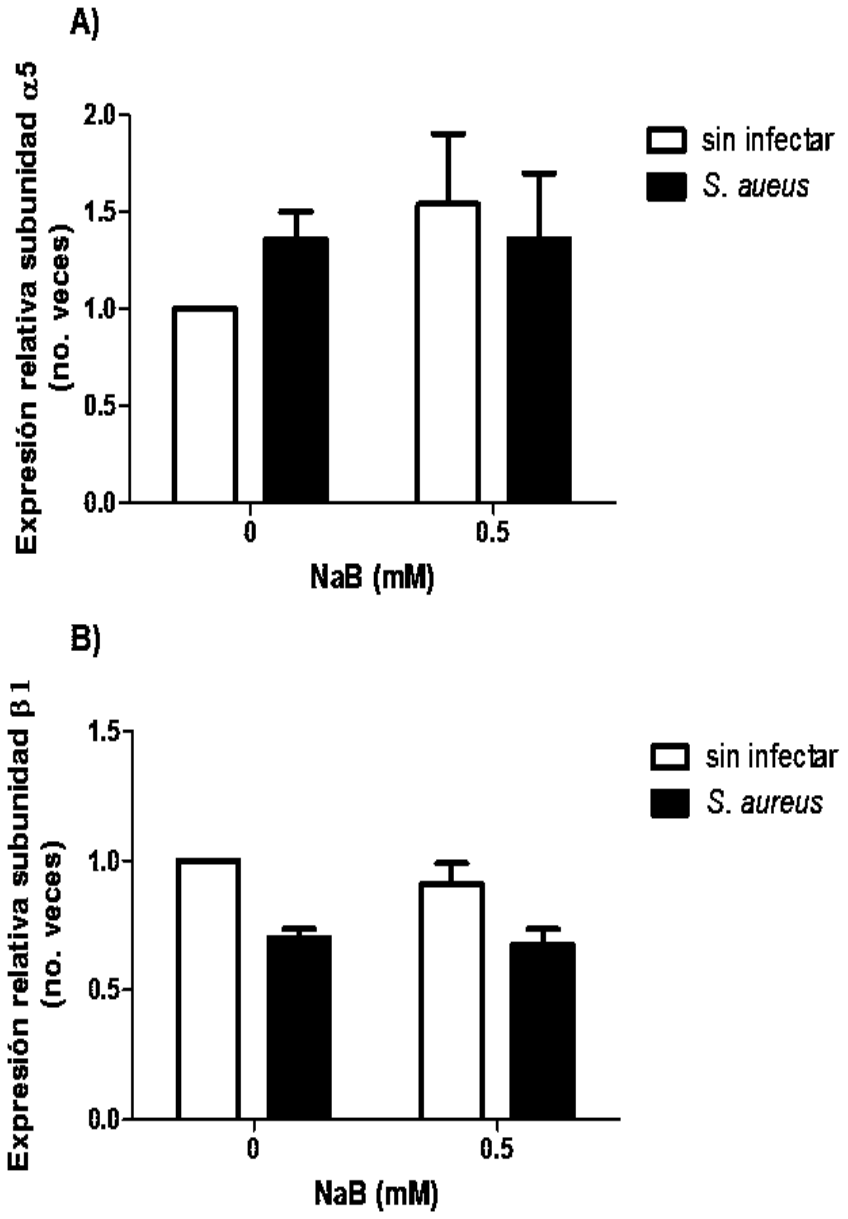
0
0.5
NaB (mM)
B)
4
3
2
1
0
Abundancia en membrana integrina α5β1 (no. veces)
sin infectar
S. aureus
*
*
*
0
0.5
NaB (mM)

Figure 2


A)
Expresión relativa subunidad α5 (no. veces)
2.0
1.5
1.0
0.5
0.0
0
0.5
NaB (mM)
sin infectar
S. aueus
B)
Expresión relativa subunidad β1 (no. veces)
1.5
1.0
0.5
0.0
0
0.5
NaB (mM)
sin infectar
S. aureus

CAPÍTULO IV.

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Review Article

Nonprofessional Phagocytic Cell Receptors Involved in *Staphylococcus aureus* Internalization

Nayeli Alva-Murillo, Joel Edmundo López-Meza, and Alejandra Ochoa-Zarzosa

Centro Multidisciplinario de Estudios en Biotecnología-FMVZ, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 Carretera Morelia-Zinapécuaro, CP 58893 La Palma. MICH, Mexico

Correspondence should be addressed to Alejandra Ochoa-Zarzosa; ochoaz@umich.mx

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Staphylococcus aureus is a successful human and animal pathogen. The majority of infections caused by this pathogen are life threatening, primarily because *S. aureus* has developed multiple evasion strategies, possesses intracellular persistence for long periods, and targets the skin and soft tissues. Therefore, it is very important to understand the mechanisms employed by *S. aureus* to colonize and proliferate in these cells. The aim of this review is to describe the recent discoveries concerning the host receptors of nonprofessional phagocytes involved in *S. aureus* internalization. Most of the knowledge related to the interaction of *S. aureus* with its host cells has been described in professional phagocytic cells such as macrophages. Here, we showed that in nonprofessional phagocytes the $\alpha 5\beta 1$ integrin host receptor, chaperons, and the scavenger receptor CD36 are the main receptors employed during *S. aureus* internalization. The characterization and identification of new bacterial effectors and the host cell receptors involved will undoubtedly lead to new discoveries with beneficial purposes.

1. Introduction

Staphylococcus is a Gram-positive commensal and opportunistic human pathogen that causes serious community-acquired and nosocomial infections, including abscess formation, wound infection, endocarditis, osteomyelitis, pneumonia, and sepsis/septic shock [1, 2]. Additionally, strains of *S. aureus* cause diseases in cattle (mastitis), poultry, pigs, and horses [3, 4]. Treatment of these infections has become difficult because of the emergence of antibiotic-resistant strains [5].

Evidence exists that several strains of *S. aureus* have the ability to invade and persist within nonprofessional phagocytic cells (NPPCs), such as epithelial [6–8], endothelial [9, 10], osteoblast [11, 12], fibroblast [13, 14], and kidney cells [15, 16]. This ability enables the bacteria to evade the host innate immune system and to survive inside a wide variety of mammalian cells. Bacteria initially adhere to the cell membrane and extracellular matrix substrates through surface proteins (adhesins) [17, 18] and are then internalized by different NPPCs.

Several reviews have discussed the intracellular persistence of this bacterium [19], the role of small colony variants (SCVs) [20], and the fate of the infected phagosome in professional phagocytes as well as in different NPPCs [21]. In this review, we will focus on the host NPPC receptors that are involved in the molecular interaction with *S. aureus* to accomplish bacterial internalization. Finally, we will discuss the medical implications derived from this knowledge and show a summary of the host receptors related to *S. aureus* internalization in NPPCs in Figure 1.

2. Bacterial Adhesion and Internalization

Bacterial internalization is a strategy that allows bacteria to evade the host immune response and to survive in the host cells. Several bacteria require initial adhesion to the host cell before the internalization process. Therefore, the adhesion and invasion into eukaryotic cells are major steps in bacterial pathogenesis [18].

Bacteria are capable of adhering to extracellular matrix components (i.e., collagen, vitronectin, fibrinogen, and especially fibronectin (Fn)) through protein-protein interactions mediated by "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) or "secreted

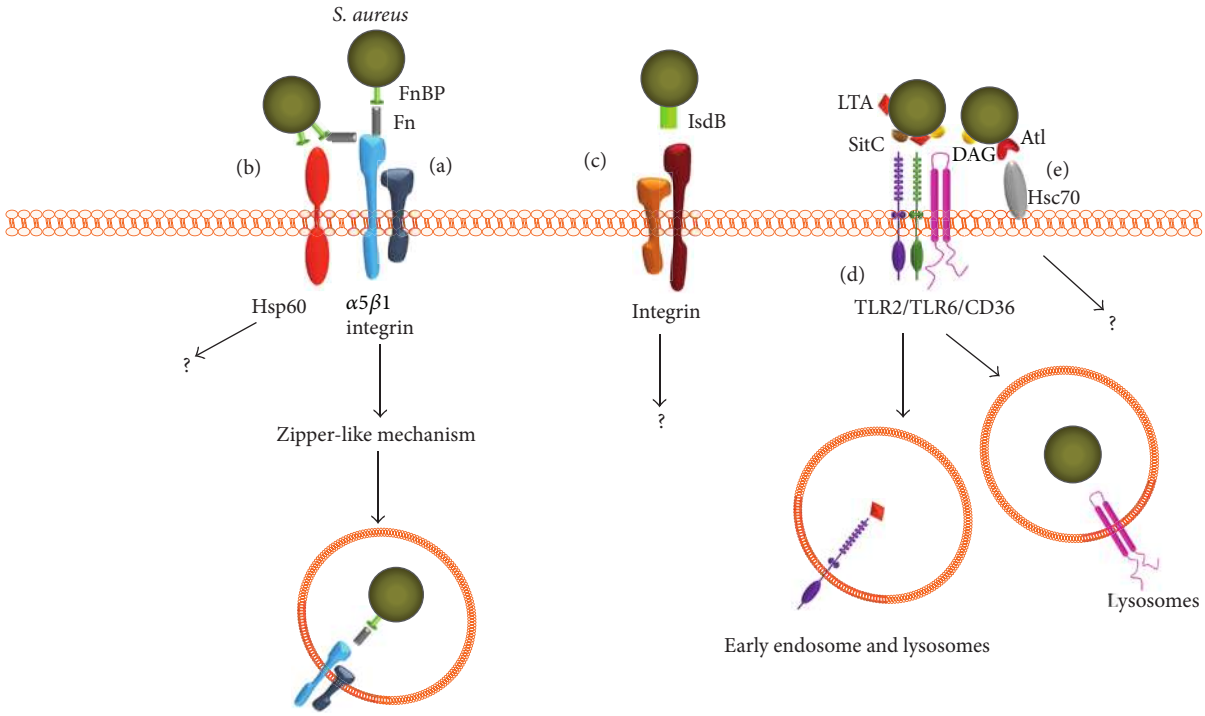

Figure 1: Different receptors and mechanisms involved in *S. aureus* internalization into nonprofessional phagocytic cells. (a) The first mechanism described for *S. aureus* internalization involved the $\alpha5\beta1$ integrin host receptor and is mediated by bacterial FnBPs via Fn as a linking molecule; bacterial endocytosis is accomplished through a zipper-like mechanism [9, 16, 22, 23]. (b) FnBPs interact directly with host Hsp60 or with integrin and Hsp60 as a coreceptor through a Fn bridge [24], but the mechanism of endocytosis remains unknown. (c) The *S. aureus* iron-regulated surface determinant-B (IsdB) contributed to invasion, and IsdB most likely interacts with integrins that bind ligands with the RGD motif [25]; however, the endocytic pathway has not been determined. (d) TLR2 is involved in *S. aureus* internalization. CD36 acts as a coreceptor and is capable of recognizing diacylglycerides, whereas TLR2/TLR6 dimers recognize different PAMPs, such as LTA and SitC [26–28]. In monocytes TLR2 colocalizes with LTA in early endosomes and lysosomes [29]. In HeLa cells, internalized *S. aureus* colocalizes with CD36 [30]. (e) The host chaperone Hsc70 binds directly to autolysin (Atl) and mediates *S. aureus* internalization [31], but the endocytic routes remain uncharacterized.

expanded repertoire adhesive molecules." Additionally, bacterial adhesins recognize host cell surface elements such as integrins, cadherins, and selectins [18]. Pathogen adhesion occurs in two ways: (1) adhesins directly engage the host cell surface receptor, that is, *Listeria* spp. [37], *Yersinia* spp. [38, 39], and *Neisseria gonorrhoeae* [39, 40], and (2) bacterial connections form indirectly with the host receptor via the recruitment of extracellular matrix proteins (e.g., *S. aureus*) [16, 41].

The bacterial engagement of eukaryotic receptors such as integrins often triggers a receptor-mediated internalization process that facilitates access to a protected intracellular niche, promoting bacterial replication [6, 42].

3. The Interaction between Nonprofessional Phagocyte Cell Receptors and *Staphylococcus aureus* Virulence Factors Promotes Internalization

S. aureus possesses a wide arsenal of virulence factors (adhesins, invasins, enzymes, toxins, and surface components) that contribute to the pathogenesis of infection (reviewed in Zecconi and Scali, 2013) [43]. These components promote the bacterial evasion of the host immune system as well as the colonization, dissemination, tissue damage, and transmission [1, 43]. *S. aureus* expresses adhesins such as fibronectin-binding proteins (FnBPs), fibrinogen-binding proteins, elastin-binding proteins, collagen-binding proteins, clumping factor, extracellular adhesion protein (Eap), and protein A [43–45]. *S. aureus* also possesses other cell-associated components such as capsular polysaccharide, peptidoglycan (PGN), and lipoteichoic acid (LTA) and secretes components such as enzymes (coagulase, lipase, hyaluronidase, and protease) and toxins (enterotoxins, toxic shock syndrome, hemolysins, and leukocidin), which are very important for the establishment of infection [1, 43, 46]. In the next sections, we will describe the *S. aureus* components and their cognate receptors in NPPCs that lead to bacterial internalization.

4. $\alpha5\beta1$ Integrin and Fibronectin Receptors

Integrins are cation-dependent glycoprotein transmembrane receptors containing noncovalently associated α- and β-subunits [47]. In vertebrates, at least 18 α- and 8 β-subunits have been described [48]. Integrins have an extracellular

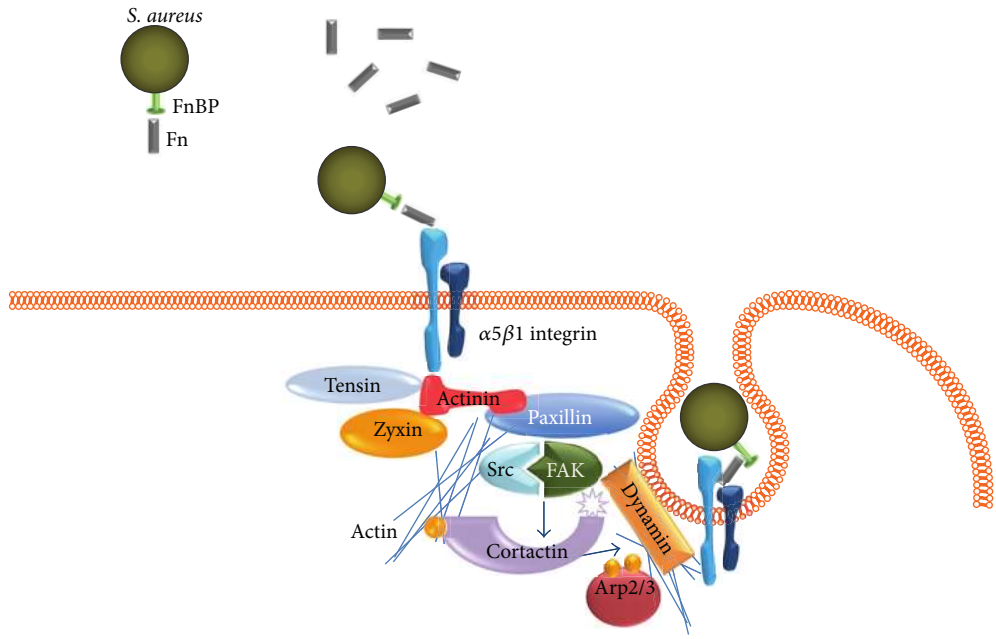

Figure 2: Summary of $\alpha5\beta1$ integrin-mediated internalization of *S. aureus* into NPPCs. The RGD motif in fibronectin (Fn) is the crucial attachment site for fibronectin receptors, such as integrins. The activation and clustering of $\alpha5\beta1$ integrin trigger particular signaling pathways and the accumulation of a focal adhesion-like protein complex in the vicinity of attached bacteria, as characterized by the recruitment of actinin, paxillin, zyxin, tensin, focal adhesion kinase (FAK), and Src kinase [32–34]. A crucial step in these signaling events is the reorganization of the actin cytoskeleton. Cortactin, an actin-binding protein, has been identified as one of the effectors of activated FAK and Src kinases, which associates with Arp2/3 complex to promote actin polymerization and binds to dynamin-2, a regulator of endocytosis [33, 35, 36].

binding domain that recognizes RGD or LVD sequences in ligands such as Fn, fibrinogen, vitronectin, and laminin [47, 48]. These receptors mediate a wide range of physiological and pathological processes, including cellular adhesion, migration, differentiation, apoptosis, phagocytosis, wound healing, and cancer. In addition, many integrins participate in pathogen recognition and host defense response in NPPCs; that is, $\beta1$ integrin mediates adhesion and endocytosis of *Yersinia* [39] and *S. aureus* [16, 41]. This event is mediated by a zipper-like process and depends on remodeling the actin cytoskeleton and membrane dynamics [49, 50]. The detailed mechanism for zipper-like-mediated internalization of *S. aureus* in NPPCs is shown in Figure 2.

Fn is a key dimeric glycoprotein in the extracellular matrix. The ability to bind to Fn is a characteristic of bacterial adhesion, which is a well-known mechanism described for many pathogens, including *S. aureus*. This bacterium expresses two closely related FnBPs encoded by the genes *fnbA* and *fnbB* [51], which are both contained in the majority of isolates with invasive properties [52].

Since the 1980s, it has been well recognized that *S. aureus* adhesion and internalization via a zipper-like process in NPPCs are mediated by integrins, Fn, and FnBPs. The role of FnBPs during *S. aureus* invasion has been established in endothelial cells [9, 10], osteoblasts [53], keratinocytes [54, 55], fibroblast [56], and epithelial cells [16, 22]. The events of internalization that occur via a zipper-like process were elucidated by experiments that included the following: (1) FnBP-deletion mutants of invasive strains; (2) noninvasive strains that express FnBPs; (3) the Fn-binding soluble domain isolated from FnBP; and (4) the blockage of receptors using anti-$\alpha5\beta1$ or anti-Fn antibodies. The results of these approaches showed that FnBPA has a relevant role in invasion because its deletion in the *S. aureus* Cowan strain diminished the level of invasiveness (~80%) into a human embryonic kidney cell line (HEK 293) [16]. Similarly, an isogenic mutant (DU5883) of *S. aureus* (8325-4) that does not express FnBPs showed reduced internalization into transformed bovine mammary epithelial cells (MAC-T cells) [22], osteoblasts [53], and keratinocytes [57]. The role of FnBPs in host invasion was confirmed using complementation assays in which noninvasive strains transformed with plasmid overexpressing FnBPs were able to invade NPPCs [16]. The presence of FnBPs on the surface of *S. aureus* confers the advantage for tissue colonization *in vivo*, as observed in mammary glands, and confers the induction of severe infection [58, 59]. In addition, Dziewanowska et al. (1999) showed that FnBP-mediated bacterial uptake by NPPCs requires actin polymerization and is dependent on tyrosine kinases [22].

In contrast, the role of Fn was initially elucidated in HEK 293 cells. The preincubation of these cells with a soluble recombinant protein fragment composed of the Fn-binding domain of FnBP completely abolished the invasion by *S. aureus* Cowan and P1 strains, presumably by competing with the *S. aureus* FnBP to interact with the host cell receptor [16]. The use of polyclonal anti-Fn antibodies corroborated the role of Fn during *S. aureus* internalization in other cell types, for example, endothelial cells [9, 16, 24]. These data

demonstrated that Fn mediates the interaction of *S. aureus* FnBPs with NPPCs.

The role of integrins during *S. aureus* internalization into NPPCs has been demonstrated by blockage experiments with antibodies. The blockage of integrin $\alpha5\beta1$ by specific antibodies in HEK 293 [16], in HUVEC [60] cells, or in keratinocytes [57] demonstrated that these receptors have a relevant role during *S. aureus* internalization because their blockage leads to a significant reduction of internalized bacteria. Additionally, a monoclonal antibody specific for $\beta1$ integrins dramatically reduced *S. aureus* invasion into human Hep-2 cells [24]. In addition, a mutant mouse fibroblast line (GD25) lacking $\beta1$ integrin showed significantly reduced bacterial invasion [23]. Recent work by Ridley et al. (2012) showed that both the availability and functional state of integrin $\alpha5\beta1$ are crucial for *S. aureus* invasion in different epithelial cells [61]. The use of GRGDS, a competitive inhibitor of $\beta1$ integrin ligands, has demonstrated the role of integrin during the internalization of *S. aureus* into alveolar epithelial cells (A549) by reducing the number of CFU recovered. In this work, the siRNA-mediated knockdown of $\beta1$ integrin expression in A459 cells significantly reduced *S. aureus* internalization (~50%) [8]. In addition, indirect evidence from our group established that the blockage of this integrin with latex beads covered with Fn inhibits *S. aureus* internalization into primary bovine mammary epithelial cells [62].

Overall, these results strongly suggest that *S. aureus* FnBPs and $\alpha5\beta1$ integrin are necessary for efficient *S. aureus* internalization into NPPCs; however, other mechanisms are employed by this bacterium favoring its internalization that we will describe below.

5. Heat Shock Proteins

Heat shock proteins (Hsps) are a group of evolutionarily highly conserved molecules that are expressed by prokaryotic and eukaryotic cells. These proteins perform important intracellular functions regarding protein folding and transport [63].

The role of Hsps during *S. aureus* internalization into NPPCs was first reported by Dziewanowska et al. (2000) [24]. Using a ligand blotting assay, Dziewanowska and colleagues identified that Hsp60 interacts with FnBP and showed that the pretreatment of epithelial cells with a monoclonal antibody specific for eukaryotic Hsp60 significantly reduces *S. aureus* internalization. Another Hsp related to *S. aureus* internalization in NPPCs is Hsc70. This protein is associated with viral infections by acting as a receptor for human T-cell lymphotropic virus type 1 (HTLV-1) [64] or rotaviruses [65, 66]. Hsc70 interacts with *S. aureus* hydrolases such as autolysin (Atl) during the bacterial internalization process. Atl participates in biofilm formation and mediates binding to the extracellular matrix and plasma proteins [31, 67, 68]. Hirschhausen et al. (2010) analyzed the *atl*-deficient *S. aureus* mutant SA113*atl* strain for its capability to be internalized into endothelial cells, and they showed the impaired ability of this strain to be endocytosed by these host cells [31]. Additionally, they reported that Atl binds directly to endothelial Hsc70 without a bridging molecule such as Fn. In addition, antibody blockade of Hsc70 decreases *S. aureus* internalization in these cells, and this protein has also been involved during *Brucella abortus* invasion into trophoblast giant cells [69], which suggests that this receptor is used as a generalized pathway during bacterial internalization.

6. Toll-Like Receptors

TLRs offer an efficient and immediate response to bacterial, fungal, and viral infections by recognizing PAMPs. The TLR family consists of 13 mammalian members, and each member mediates an intrinsic signaling pathway and induces specific biological responses against microorganisms [70]. The cytoplasmic domain (Toll/IL-1 receptor domain) of TLRs is required for the signaling response leading to the activation of transcription factors such as NF-κB [70]. The leucine-rich repeat (LRR) extracellular motif is responsible for the recognition of PAMPs [71]. TLRs are activated by ligand-induced multimerization and act by cooperating with several proteins such as other TLRs or coreceptors.

For *S. aureus* infections, TLR2 is the most relevant receptor involved in this process. TLR2 recognizes different PAMPs such as lipopeptides from Gram-positive and Gram-negative bacteria, lipoarabinomannan, LTA, PGN, atypical lipopolysaccharide, a phenol-soluble modulin from *S. epidermidis*, and others [72]. Additionally, TLR2 interacts with TLR1 and TLR6 in the process of ligand recognition, and the TLR2/TLR6 heterodimer recognizes the PGN in the macrophage phagosome [73] and a diacylated mycoplasma lipoprotein [74], while the TLR2/TLR1 heterodimer recognizes triacylated lipopeptides [75]. Reports have described the participation of TLR2 during *S. aureus* internalization in NPPCs; however, the results are not conclusive because TLR2 participation in phagocytosis may be indirect. For example, Rocha-de-Souza et al. (2008) indicated that TLR2 is involved in *S. aureus* internalization into human cord blood-derived mast cells using neutralizing antibodies [26]. The blockage of TLR2 in these cells decreases the number of bacteria internalized. In our work, we observed a similar result in primary bovine mammary epithelial cells (data unpublished); however, it remains to be clarified whether TLR2-mediated internalization is the consequence of the signaling activity of this receptor or whether the recognition of bacterial PAMPs by TLR2 is a key step for endocytosis. Although TLRs are not phagocytic receptors *per se*, they are also internalized in the process and participate in the link between phagocytosis and inflammatory responses by triggering the production of cytokines [76]. In addition, TLR2 is located in phagosomes and colocalizes with different *S. aureus* PAMPs. In NPPCs, the predominant triacylated lipoprotein of *S. aureus*, SitC, is located intracellularly with TLR2 in murine keratinocytes and stimulates proinflammatory cytokine expression [77]; however, SitC is internalized in a TLR2-independent manner. The results described above suggest that although no clear role of TLR2 has been observed during *S. aureus* internalization, this process appears to be a prerequisite for full TLR2

activation in both professional phagocytic cells as well as in NPPCs [76].

7. Coreceptors for TLR2 Mediate *Staphylococcus aureus* Recognition

CD36 is a membrane glycoprotein that belongs to the class B scavenger receptor family that interacts with other membrane receptors such as TLRs. This receptor plays a role during tumor growth, inflammation, wound healing, and angiogenesis and is able to recognize PAMPs or pathogen-infected cells by acting as a phagocytic receptor [78, 79]. During the host recognition of *S. aureus* mediated by TLR2, CD36 may act as a facilitator or coreceptor for diacylglyceride recognition through the TLR2/6 complex mediating bacterial invasion primarily in phagocytic cells [27]. In the NPPC line HEK 293, the overexpression of CD36 confers binding and uptake of *S. aureus*, suggesting a role for CD36 during the endocytosis of Gram-positive bacteria [28]. In addition, Lee-lahavanichkul et al. (2012) have demonstrated that intracellular *S. aureus* colocalizes with CD36 in HeLa cells [30]. CD14, a glycosylphosphatidylinositol-anchored membrane protein, is another coreceptor that participates in bacterial recognition by TLRs and enhances PGN and LTA signal transmission through TLR2 [80]. CD14/TLR2 is an essential receptor complex involved in Panton-Valentine leukocidin recognition [81]. CD14 and CD36 play a prominent role in LTA binding and enhancing LTA-induced signaling in human monocytes [29]. The aforementioned involvement suggests that CD14 may have a similar role as CD36 in *S. aureus* internalization; however, this effect remains to be fully explored.

8. Other *Staphylococcus aureus* Virulence Factors that Participate in the Internalization Process Interact with Uncharacterized Host Cell Receptors

As we have described above, several host receptors are used by *S. aureus* to invade NPPCs (Figure 1). Nonetheless, reports have indicated that different uncharacterized host receptors may be involved in *S. aureus* internalization in NPPCs. In the next section, we will describe several bacteria virulence factors involved in internalization whose host receptors remain to be characterized.

9. The Extracellular Adherence Protein

The extracellular adherence protein (Eap) in *S. aureus* binds to matrix extracellular components, inhibits leukocyte adhesion to endothelial cells, acts like an anti-inflammatory factor [82], and causes *S. aureus* agglutination [83]. This protein stimulates the adherence of *S. aureus* to epithelial cells [83] and fibroblasts [84]. Eap also participates during the bacterial internalization process because its absence reduces the adherence and internalization of *S. aureus* into fibroblast and epithelial cells [14], while the addition of exogenous Eap increases *S. aureus* internalization [85, 86]. This invasion process may be influenced by the 32 kDa neutral phosphatase that is located on the bacterial surface that binds to Eap [87]; however, no reports have yet described the identification of a host receptor for Eap.

10. Glyceraldehyde-3-Phosphate Dehydrogenase-C

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme, and several GAPDH homologs present in bacteria are able to bind to Fn, lysozyme, plasminogen, and the cytoskeletal proteins myosin and actin. Therefore, this enzyme plays a role during *S. aureus* colonization and internalization [88, 89]. *S. aureus* has two GAPDH homologs termed *gapA* (also known as *gapC* in a bovine mastitis isolate) and *gapB* [90], and both proteins are important in the pathogenesis of *S. aureus* in a *Galleria mellonella* model of infection [91].

GapC plays an important role during *S. aureus* internalization into MAC-T cells [92]. The number of CFUs recovered from an isogenic *gapC* mutant H330 strain that were adhered and internalized into MAC-T cells was lower than the number corresponding to the WT strain. Nevertheless, the absence of *gapC* does not completely abolish the attachment and internalization of the bacteria, which is most likely due to the presence of other bacterial adhesins [92]. No reports have yet described the identification of a host receptor that recognizes *gapC*.

11. Iron-Regulated Surface Determinant-B

S. aureus acquires iron from host hemoglobin due to the bacterial expression of iron-regulated surface determinants (Isd) [93]. Zapotoczna et al. (2013) reported that iron-regulated surface determinant-B (IsdB) promotes the invasion of *S. aureus* into 293T and HeLa cells [25]. Additionally, they proposed that soluble *S. aureus* IsdB binds to and stabilizes the active conformation of integrins, enabling them to interact with RGD-containing ligands, which leads to bacterial internalization in an integrin-dependent pathway. In addition, IsdB adheres to platelets through the integrin receptor GPIIb/IIIa (aIIIbb3) [94]; however, this receptor has not been implicated in bacterial internalization.

12. Conclusions

Phagocytosis is an essential component of innate and adaptive immune responses. In NPPCs, phagocytosis plays major roles in tissue maintenance, regeneration, and remodeling. However, pathogenic bacteria also employ many of the receptors involved in phagocytosis during the interplay between the host cell defense response and tissue colonization. Thus, phagocytosis, endocytosis, and intracellular trafficking can be exploited for therapeutic objectives such as intracellular drug delivery (for a wide and detailed description of these beneficial strategies, see Duncan and Richardson, 2012) [95]. In addition, the manipulation of the host cell membrane affects numerous

events, including actin remodeling and phagocytosis. The characterization and identification of new bacterial effectors and the host cell receptors involved will undoubtedly lead to new discoveries with beneficial purposes. Many of the pathways operating during the intracellular trafficking of bacteria (e.g., autophagosome formation) may have roles in multiple pathologies such as cancer, metabolic diseases, or neurological disorders (reviewed in Rubinsztein et al. 2012) [96]. Furthermore, a very important role of integrins during apoptosis clearance has been established, which may be related to autoimmune disorders, atherosclerosis, cancer, or human age-related macular degeneration (reviewed in Sayedyahossein and Dagnino, 2013) [97]. All of these medical implications highlight the relevance of the study of phagocytic receptors in the infection of NPPCs by *S. aureus* (Figure 1) because diseases related to intracellular strains (e.g., *S. aureus*) are chronic and recurrent, and many of them are life threatening.

Conflict of Interests

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

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IX. DISCUSIÓN GENERAL

Los AGCC y AGCM son compuestos que modulan la respuesta inmune de distintos organismos. En un ambiente inflamatorio -a nivel intestinal- estos AG actúan como anti-inflamatorios (Hoshimoto et al., 2002; Meijer et al., 2010; Vinolo et al., 2011b). Además, regulan la expresión de genes de PA (Jiang et al., 2013). Debido a estas características, se les ha utilizado en distintos estudios para evaluar su efecto profiláctico o terapéutico en enfermedades infecciosas del tracto intestinal tanto en animales de granja (conejos, pollos, puercos) como en humanos (Van Immerseel et al., 2006; Skrivanova et al., 2008; Raqib et al., 2012). No obstante, poco se sabe de su efecto sobre otros modelos de interacción hospedero-patógeno. En este sentido, reportes previos de nuestro grupo de trabajo demostraron que el NaB (0.5 mM, 24 h) disminuye la internalización de *S. aureus* en las CEMB, y que el NaO puede disminuir la internalización bacteriana (1 mM) o favorecerla (0.25 mM) (Ochoa-Zarzosa et al., 2009; Alva-Murillo et al., 2012). Sin embargo, el mecanismo molecular de estos procesos infecciosos es desconocido.

Los resultados arrojados en este estudio demostraron que en las CEMB tratadas con NaO (0.5 mM, 24 h) o con NaO(1 mM, 24 h), se activaron algunos elementos en común de las rutas de señalización. En ambos casos, los AG –independientemente- favorecieron la presencia en la membrana plasmática del receptor TLR2, lo cual indica que las CEMB son activadas previo al estímulo bacteriano. Además, la fosforilación de p38 se indujo tanto en las CEMB tratadas con NaO como con NaO. Es importante señalar que se observó una respuesta diferencial en cuanto a los FT activados y su intensidad, así como en la respuesta antimicrobiana, la cual fue dependiente del compuesto utilizado. El NaB y el NaO estimularon la activación de FT relacionados con la RII; sin embargo, es más notoria la activación producida por el NaB (0.85-1.73 U.A.). AP-1 mostró la mayor activación en las CEMB tratadas con butirato, mientras que Stat-3 fue el FT más activado por el tratamiento con 1 mM de NaO. En cuanto a la respuesta antimicrobiana, el NaO indujo la expresión de los genes de los PA TAP, BNBD5 y BNBD10; mientras que el NaO solo aumentó la expresión del gen de BNBD10. En las CEMB tratadas con

ambos AG –de manera independiente- se observó una respuesta pro-inflamatoria en cuanto a la expresión del gen de la citocina IL-1β y de la quimiocina IL-8, en una proporción similar. En estas condiciones las principales diferencias radican en que (i) el NaB favoreció la presencia en membrana de las integrinas $\alpha 5\beta 1$, mientras que el NaO no la modificó; (ii) los ensayos de bloqueo funcional del TLR2 sugieren que la participación de este receptor es directa en la reducción de la internalización mediada por 1 mM de NaO, ya que las UFC recuperadas disminuyeron drásticamente; (iii) y el NaB (0.5 mM) redujo la fosforilación de las MAPKs ERK1/2 y JNK, mientras que el NaO la aumentó.

La respuesta inmune de las CEMB inducida por el NaB o por el NaO se revirtió después del estímulo bacteriano en cuanto a la fosforilación de las MAPKs, la activación de los FT y la expresión de genes de la respuesta inflamatoria. Sin embargo, la respuesta antimicrobiana generada por estos AG aumentó después del estímulo con *S. aureus*, en relación a LAP y BNBD5 en el caso del NaB; así como BNBD4, LAP y BNBD10 con el NaO.

Estos resultados corroboraron que es importante el contexto en el que se evalúe el efecto de los AGCC y AGCM. Al igual que a nivel intestinal, el NaB y el NaO actúan como pro-inflamatorios en epitelio mamario bovino (Andoh et al., 2000; Meijer et al., 2010; Sunkara et al., 2012). Mientras que en un contexto inflamatorio en las CEMB (estímulo con *S. aureus*) ambos AG ejercen un efecto anti-inflamatorio, como en el intestino (Hoshimoto et al., 2002; Blais et al., 2007; Weng et al., 2007; Vinolo et al., 2011).

Por otro lado, TLR2 y CD36 participan en la inducción de la internalización bacteriana modulada por 0.25 mM de NaO, ya que al bloquear a los receptores –independientemente- con anticuerpos específicos, se recuperaron menos UFC. Adicionalmente, las integrinas $\alpha 5\beta 1$ juegan un papel importante en la internalización de *S. aureus* inducida por 0.25 mM de NaO, ya que este AG favoreció la presencia en membrana de las integrinas, además se recuperaron menos UFC al bloquear este receptor. En las CEMB tratadas con 0.25 mM de NaO (24 h) la RII fue

prácticamente abatida, ya que (i) el estado de fosforilación de las MAPKs p38, JNK y ERK1/2 fue menor al basal, (ii) la activación de los FT disminuyó drásticamente, (iii) no se modificó la expresión de los genes de los PA evaluados, y (iv) se observó un efecto anti-inflamatorio (aumento la expresión del gen de la citocina anti-inflamatoria IL-10). Interesantemente, después del estímulo bacteriano la respuesta inmune no se modificó, excepto que disminuyó la expresión del gen de la IL-10.

Es notorio que el efecto del NaO sobre la RII de las CEMB no sólo depende del contexto inflamatorio en el que se evalúe, sino también de la concentración del AGCM usada. En las CEMB tratadas con 0.25 mM de NaO (antes de la infección) se detectó un efecto anti-inflamatorio, mientras que con 1 mM actuó como pro-inflamatorio.

Estos resultados indican que el NaO (0.5 mM) y NaO (1 mM) activan a las CEMB mediante la ruta de señalización de TLR2/p38 o TLR2/p38/ERK/JNK, respectivamente, favoreciendo la respuesta antimicrobiana antes y después de la internalización de *S. aureus*. Además, ambos AG pueden actuar como anti-inflamatorios durante el proceso infeccioso. Por otro lado, el NaO (0.25 mM) evita la activación de las CEMB y disminuye drásticamente la RII, lo cual puede conducir al aumento en la internalización de *S. aureus*.

X. CONCLUSIÓN GENERAL

El NaB (0.5 mM, 24 h) y el NaO (1 mM, 24 h) activan a las CEMB vía TLR2/p38 y TLR2/p38/JNK/ERK1/2, respectivamente, favoreciendo la respuesta antimicrobiana antes -y después- de un proceso infeccioso, lo cual se relaciona con la disminución de la internalización de *S. aureus* en las CEMB. Además, ambos ejercen un efecto anti-inflamatorio durante la infección. Por otro lado, 0.25 mM de NaO induce la AM de las integrinas $\alpha 5\beta 1$ pero inhibe la vía de las MAPKs y abate la RII de las CEMB lo que favorece la internalización.

XI. BIBLIOGRAFÍA COMPLEMENTARIA

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