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Respuesta de queratinocitos frente a la interacción con diferentes serotipos de *Aggregatibacter actinomycetemcomitans*: aspectos inflamatorios y osteodestructivos.

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RESUMEN

La microbiota de la cavidad oral está compuesta por variados biofilms con diferentes características determinadas en función de las propiedades (pH, disponibilidad de nutrientes, cantidad de oxígeno, etc.) del nicho ecológico donde se desarrollan. En este sentido, existe una fuerte relación entre las características cualitativas de las biopelículas orales y los estados clínicos de salud y enfermedad, donde el equilibrio u homeostasis de estos *biofilms* puede ser modificado, alcanzando un potencial patogénico asociado al desarrollo de enfermedades orales de alta prevalencia, tal como caries, gingivitis, periodontitis, mucositis o periimplantitis.

En el caso de la periodontitis, definida como una inflamación de los tejidos de soporte del diente, con destrucción del ligamento periodontal y hueso alveolar, diferentes patógenos han sido asociados con la progresión y severidad de esta patología, de los cuales *Porphyromonas gingivalis* y *Aggregatibacter actinomycetemcomitans* son considerados patógenos claves, los cuales teniendo condiciones favorables de crecimiento, tienen un rol protagónico en la generación de un microambiente disbiótico que favorece el inicio y progresión de la patología ya mencionada

A. actinomycetemcomitans, un cocobacilo Gram negativo, no móvil, anaerobio facultativo y capnofílico presenta 7 serotipos (a-g) en función de las variantes estructurales de su lipopolisacardio (LPS). De estos, los serotipos a, b y c son los de mayor prevalencia a nivel oral, el serotipo b ha sido aislado desde pacientes con periodontitis, el serotipo a de individuos con o sin signos de periodontitis y el c con mayor frecuencia en individuos sanos. Esta distribución, resulta indicativa de una virulencia heterogénea y un mayor potencial inmunogénico por parte del serotipo b, donde abundante evidencia sustenta la idea de que este serotipo resulta más virulento que los otros ya mencionados, determinando respuestas diferenciales de mayor magnitud como producción de citoquinas, receptores de quimioquinas, estimulación de células precursoras de osteoclastos, entre otras, comparado con los serotipos a y c.

Esta respuesta diferencial frente a los serotipos de *A actinomycetemcomitans* descrita anteriormente, también ha sido estudiada en células del estroma periodontal como células epiteliales, específicamente queratinocitos; sin embargo, la totalidad de los aspectos de dicha respuesta, y cómo ésta influye en los fenómenos inflamatorios y osteodestructivos, no está del todo definida. Por esta razón, la presente investigación tiene como objetivo evaluar la respuesta de queratinocitos frente a los diferentes serotipos de *A. actinomycetemcomitans* y determinar la importancia de esta respuesta en el desarrollo de los aspectos inflamatorios y osteodestructivos, hipotetizando que al igual que en otros tipos celulares el serotipo b de *A. actinomycetemcomitans* es también capáz de inducir respuestas diferenciales en células epiteliales.

Para este objetivo, se utilizó como línea celular de estudio la línea de queratinocitos OKF6/TERT2, estimulados con los serotipos a, b y c de *A. actinomycetemcomitans*, evaluando por qPCR la de citoquinas pro-inflamatorias, moléculas inductoras de destrucción tisular dependientes y expresión de receptores tipo *toll*. Sumado a lo anterior, se estimularon también células macrofágicas de la línea THP-1, con el fin de contrastar si esta capacidad de respuesta diferencial era exclusiva de células inmunes especializadas, o bien era trasnversal a cualquier tipo celular, como células epiteliales o de la inmunidad innata.

De estas experiencias y considerando lo que plantea la evidencia disponible para otros tipos celulares, se demostró que la respuesta tanto de queratinocitos como de células macrofágicas en torno a la producción de citoquinas, expresión de receptores TLR, moléculas de destrucción tisular y moléculas de polarización de macrófagos, frente a *A. actinomycetemcomitans* no presenta - al menos en términos de expresión génica - una respuesta serotipo dependiente, sino que es una respuesta genérica, cuyo rol sería actuar como primera señal de alarma para el reclutamiento de células capaces de discriminar estas diferencias estructurales y montar una respuesta inmune más específica en función del patógeno frente al cual se está respondiendo, siendo esta respuesta solo una primera “ola” de citoquinas capaz de activar la respuesta inflamatoria sin una especifidad determinada.

ABSTRACT

The microbiota of the oral cavity is composed of various biofilms with different characteristics determined based on the properties (pH, availability of nutrients, amount of oxygen, etc.) of the ecological niche where they develop. In this sense, there is a strong relationship between the qualitative characteristics of oral biofilms and the clinical states of health and disease, where the balance or homeostasis of these biofilms can be modified, reaching a pathogenic potential associated with the development of highly prevalent oral diseases. , such as cavities, gingivitis, periodontitis, mucositis, or peri-implantitis.

In the case of periodontitis, defined as an inflammation of the supporting tissues of the tooth, with destruction of the periodontal ligament and alveolar bone, different pathogens have been associated with the progression and severity of this pathology, of which *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are considered key pathogens, which, having favorable growth conditions, play a leading role in the generation of a dysbiotic microenvironment that favors the onset and progression of the aforementioned pathology

A. actinomycetemcomitans, a nonmotile, facultative anaerobic, capnophilic, Gram-negative coccobacillus has 7 serotypes (a-g) based on the structural variants of its lipopolysaccharide (LPS). Of these, serotypes a, b and c are the most prevalent at the oral level, serotype b has been isolated from patients with periodontitis, serotype a from individuals with or without signs of periodontitis, and serotype c more frequently from healthy individuals. This distribution is indicative of a heterogeneous virulence and a greater immunogenic potential by serotype b, where abundant evidence supports the idea that this serotype is more virulent than the others already mentioned, determining differential responses of greater magnitude such as cytokine production, chemokine receptors, osteoclast precursor cell stimulation, among others, compared to serotypes a and c.

This differential response against the serotypes of *A actinomycetemcomitans* described above has also been studied in periodontal stromal cells as epithelial cells,

specifically keratinocytes, however, the totality of the aspects of this response, and how it influences inflammatory and osteodestructive phenomena, is not completely defined. For this reason, the present research aims to evaluate the response of keratinocytes against the different serotypes of *A. actinomycetemcomitans* and to determine the importance of this response in the development of inflammatory and osteodestructive aspects, hypothesizing that, as in other cell types *A. actinomycetemcomitans* serotype b is also capable of inducing differential responses in epithelial cells.

For this objective, the OKF6 / TERT2 keratinocyte line, stimulated with serotypes a, b and c of *A. actinomycetemcomitans*, were used as a study cell line, evaluating pro-inflammatory cytokines, dependent tissue destruction inducing molecules and expression of toll-like receptors. In addition to the above, macrophage cells of the THP-1 line were also stimulated, in order to contrast whether this differential response capacity is exclusive to specialized immune cells, or is transversal to any cell type, such as epithelial cells or cells. innate immunity.

From these experiences and considering the evidence available for other cell types, it was demonstrated that the response of both keratinocytes and macrophage cells around the production of cytokines, expression of TLR receptors, tissue destruction molecules and polarization molecules of Macrophages, against *A. actinomycetemcomitans*, do not present - at least in terms of gene expression - a serotype-dependent response, but rather a generic response, whose role would be to act as the first alarm signal for the recruitment of cells capable of discriminating these structural differences. and mount a more specific immune response depending on the pathogen to which it is responding, this response being only a first "wave" of cytokines capable of activating the inflammatory response without a determined specificity.

CAPITULO 1

Introducción

La periodontitis - cuya ultima definición la describe como una enfermedad crónica, no comunicable, inflamatoria y destructiva de los tejidos de soporte del diente asociada a la presencia de un biofilm disbiótico - corresponde a una de las patologías orales de mayor prevalencia a nivel nacional, y a la más prevalente a nivel mundial de las patología que producen daño en tejido óseo (Palmer y cols., 2014; Newman y cols., 2015; Tonetti & Claffey 2005; Gamonal y cols., 1998). Esta inflamación que ocurre a nivel oral, no solo afecta al hospedero de manera local sino que además producto de la cronicidad de esta condición, induce una inflamación sistemica crónica de bajo grado, que vincula y posiciona a esta enfermedad como un potente factor de riesgo para otras patologías sistémicas como artritis reumatoide, diabetes, accidentes cardiovasculares, parto prematuro, Alzheimer, entre otras (Kalakonda y cols., 2016; Tonetti & Claffey 2005).

Diferentes lineas de investigación han buscado entender los mecanismos mediante los cuales opera esta enfermedad, siendo el componente microbiológico su factor etiológico principal. En este sentido, diversas teorías han intentado describir cómo el biofilm participa en el inicio y el desarrollo de la enfermedad, siendo la teoría del sinergismo microbiano y el modelo de disbiosis la más aceptada y documentada a la fecha (Hajishengallis G, 2012).

Este modelo, plantea que los mismos microorganismo presentes en un estado de salud, en determinadas condiciones o perturbaciones de su ambiente, pueden alterar su abundancia y activar mecanismos de virulencia capaces de trasnformar un *biofilm* compatible con salud en un biofilm disbiótico inductor de la enfermedad (Hajishengallis G, 2012). Es en este contexto en que diversas bacterias cobran protagonismo como piezas claves en el desarrollo de esta disbiosis, de las cuales *Aggregatibacter actinomycetemcomitans,* resulta interesante de ser estudiada dada su aparente virulencia heterogénea, la cual estaría determinada por variantes estructurales de su lipopolisacarido (LPS) que determinarían respuestas inflamatorias de diversas magnitudes, que clínicamente se evidenciarían como

niveles variables de severidad en la enfermedad (Aberg y cols., 2012; Chen y cols., 2010; Jentsch y cols., 2012; Roman-Torres y cols., 2010; Sakellari y cols., 2011).

A la fecha, variadas aristas han sido estudiadas sobre los mecanismos de patogenicidad que esta bacteria esgrime cuando se le presentan condiciones de crecimiento favorable, sin embargo, existe escasa evidencia sobre la interacción de este microorganismos con células epiteliales y macrofágicas, que son las primeras células que tiene el hospedero como línea de defensa frente a patógenos, y cuyo entendimiento sería clave para comprender a cabalidad las primeras etapas de infección y para el desarrollo de terapias, herramientas diagnósticas y criterios pronósticos que permitan mediante el uso de herramientas microbiológicas y moleculares, la determinación del riesgo del hospedero, la prevención de la patología y la reducción del daño irreversible que esta condición causa y que deja a los individuos con secuelas funcionales y estéticas y que además produce un alto costo en el gasto en salud, dada la concomitancia de patologías con las que la periodontitis es asociada.

CAPÍTULO 2

Antecedentes Generales

2.1. Contexto microbiológico oral

La comunidad bacteriana oral representa el grupo mejor caracterizado de bacterias asociadas al hospedero humano. Los distintos hábitats presentes en la boca (mucosa bucal, paladar duro, encía queratinizada, tonsilas palatinas, saliva, sitios sub- y supra-gingivales, orofaringe y dorso de la lengua), permiten la formación y desarrollo de *biofilms*, cuyas poblaciones bacterianas están determinadas por las características biológicas de cada nicho, como pH, disponibilidad de nutrientes y presencia de oxígeno, entre otras (Takahashi y cols., 2015; Faran Ali & Tanwir, 2012).

Existe una fuerte correlación entre la composición cualitativa de la microbiota oral y los estados clínicos de salud y enfermedad. En este sentido, la microbiota oral aporta de diferentes formas a la homeostasis tanto local como general, estimulando el sistema inmune mucoso y potenciando los mecanismos de tolerancia oral. Por ejemplo, en ratones la presencia de bacterias orales, comparados con ratones libres de gérmenes, se relaciona con un mayor desarrollo del sistema inmune intestinal y tejido linfático, y una mayor cantidad de linfocitos (Macpherson & Harris, 2004). Sin embargo, cuando la homeostasis de dicho biofilm es alterada, la composición de estas comunidades bacterianas se modifica, alcanzando en muchos casos un potencial patogénico asociado al desarrollo de enfermedades como caries, gingivitis, periodontitis, mucositis y peri-implantitis (Palmer y cols., 2014; Socransky y cols., 1998; Feres y cols., 2004; Newman y cols., 2015).

De las aproximadamente setecientas especies de bacterias cultivables descritas a nivel oral, unas cuatrocientas componen el biofilm sub-gingival, ubicándose en el espacio limitado entre el diente y la encía, constituyéndose como el principal factor etiológico de las enfermedades periodontales asociadas a placa bacteriana (Avila y cols., 2009; Mash y cols., 2009).

Dentro de este grupo de patologías, encontramos a la periodontitis, definida como una inflamación de los tejidos de soporte del diente, con destrucción progresiva del ligamento periodontal, hueso alveolar y la formación de un saco periodontal con o sin recesión gingival; que se diferencia de las enfermedades gingivales, por la presencia de una pérdida de inserción clínicamente detectable (Armitage, 1995; Socransky y cols., 2003; Sbordone y cols., 2003).

Con relación a esta patología, se puede presentar de forma crónica o agresiva. En el caso de la periodontitis crónica, se caracteriza por una mayor prevalencia en adultos y un nivel de destrucción consistente con factores locales como depósitos de placa y cálculos sub-gingivales; mientras que la periodontitis agresiva se manifiesta por una pérdida de inserción y destrucción ósea rápidas, con escasos depósitos de placa en relación a la gravedad de la enfermedad, ocurre principalmente en pacientes jóvenes, clínicamente sanos a nivel sistémico, pudiendo además presentarse en varios miembros de una misma familia (Henderson y cols., 2003).

En Chile, la periodontitis crónica alcanza una prevalencia del 90% en adultos y a nivel mundial es la patología ósea de mayor prevalencia (Tonetti & Claffey 2005; Gamonal y cols., 1998), indicándola además como factor de riesgo en diversas patologías y condiciones sistémicas, tal como enfermedad cardiovascular, diabetes mellitus tipo 2, parto prematuro, bajo peso al nacer, y últimamente con enfermedad de Alzheimer, psoriasis, disfunción eréctil, artritis reumatoide, entre otras (Kalakonda y cols., 2016; Tonetti & Claffey 2005).

En relación a la patogenia de estas enfermedades, las bacterias del *biofilm* sub-gingival, por medio de sus factores de virulencia, inducen en variados tipos de células la producción de citoquinas y quimioquinas pro-inflamatorias, que aumentan la permeabilidad capilar y permiten la quimiotaxis de células polimorfonucleares a través del epitelio de unión hacia el surco gingival, generando un ambiente inflamatorio propicio para la destrucción de los tejidos (Socransky y cols., 1998; Mombelli 2003; Haffajee 2005). Si este proceso es mantenido en el tiempo, la infección, que comienza como una inflamación solo de los tejidos gingivales (gingivitis), se extiende profundamente provocando la pérdida del tejido conjuntivo de sostén y el hueso alveolar, dando lugar a una migración apical del epitelio de

unión y formación del saco periodontal (Hernández y cols., 2011). En el interior de este saco, aún cuando la superficie epitelial está en constante reemplazo, diversas especies bacterianas son capaces de invadir las células del epitelio gingival, evadiendo la acción del sistema inmune y dando curso a la progresión de la enfermedad (Socransky & Haffajee, 2002; Van Winkelhoff y cols., 2002; Hajishengallis G, 2012).

Diferentes patógenos han sido asociados con la progresión y severidad de estas patologías, muchos de los cuales se detectan incluso en estado de salud periodontal, y que frente a condiciones favorables y por medio de interacciones con otros microorganismos, tienen la capacidad de expresar su potencial patogénico, aumentando la inmunogenicidad del biofilm. Estas bacterias se han denominado *"keystone pathogens"*, de los cuales *Porphyromonas gingivalis* y *Aggregatibacter actinomycetemcomitans* son considerados los mayores periodontopatógenos (Slots & Ting 1999, Van Winkelhoff y cols., 2002, Socransky & Haffajee 2005). Es así como en Chile la frecuencia de detección de *A. actinomycetemcomitans* alcanza un 16.6% para los casos de periodontitis localizada y un 33.3% para los casos de periodontitis generalizada, mientras que *P. gingivalis* alcanza una frecuencia de detección del 76.2% en pacientes con periodontitis localizada y de un 100% en los pacientes con periodontitis generalizada (Gajardo y cols., 2005).

Si bien estas bacterias pueden causar de forma directa la destrucción de los tejidos de soporte del diente, es la propia respuesta inflamatoria del hospedero la que induce la activación de mecanismos osteodestructivos y suministra nutrientes al medio, como péptidos derivados de la destrucción de los mismos tejidos, que permiten a estas bacterias aumentar su número, incrementar su virulencia, potenciar la infección y perpetuar el ciclo patogénico de la enfermedad (Gemmell y cols., 2007; Socransky & Haffajee, 2002; Van Winkelhoff y cols., 2002; Hajishengallis G, 2012). En este sentido, los casos en que gingivitis progresa a periodontitis, es probable que la misma inflamación provocada por la acumulación de placa bacteriana actúe como presión selectiva para el desarrollo de una microbiota disbiótica e inflamofílica, de la cual surgen miembros que pueden evadir la respuesta inmune del hospedero *(Keystone pathogens)* y contribuir al establecimiento de la enfermedad (Hajishengallis 2014).

2.2. Aggregatibacter actinomycetemcomitans

Aggregatibacter actinomycetemcomitans es un cocobacilo Gram negativo, no móvil, anaerobio facultativo y capnofílico (Henderson y cols., 2003), descrito como el principal patógeno en el inicio y progresión de la periodontitis agresiva (Schacher y cols., 2007; Socransky & Haffajee, 2005).

Con relación a sus factores de virulencia, podemos agruparlos en aquellos que son moduladores del sistema inmune como leucotoxina, superantígenos, toxina de distención citoletal, proteínas Omp y proteínas moduladoras e inhibidoras de la función y migración de células polimorfonuclares; además de un segundo grupo, inductores de la destrucción tisular donde se encuentran proteínas de estrés celular y el lipopolisacárido (LPS) (Raja y cols., 2014).

Con respecto al LPS, existen 6 variantes estructurales en el componente O-polisacárido de dicha estructura, en base a la cual esta bacteria ha sido clasificada en 6 serotipos (a-f). Los serotipos a, b y c, corresponden a los más prevalente en poblaciones Caucásicas, Asiáticas, Africanas y Latinoamericanas (Aberg y cols., 2012; Chen y cols., 2010; Jentsch y cols., 2012; Roman-Torres y cols., 2010; Sakellari y cols., 2011), y la composición estructural de sus LPS esta dada en el caso del serotipo b, por unidades repetidas de trisacáridos compuestos de α-D-fucosa, α -L-rhamnosa y β-D-N-acetil-galactosamina, mientras que en los serotipos a y c estas unidades repetitivas son de 6-deoxy- α-D-talosa y 6-deoxy- α -L-talosa, respectivamente (Perry y cols., 1996)

En cuanto a la relación de estos serotipos con las diferentes formas de periodontitis, el serotipo b ha sido aislado con mayor frecuencia desde lesiones periodontales en pacientes con periodontitis crónica o agresiva, mientras que el serotipo a, se asocia a individuos con o sin signos de periodontitis (Socranski y cols., 1998) y el c con mayor frecuencia en individuos sanos (Dogan y cols., 1999; Lakio y cols., 2002;). Esta distribución de los serotipos en las diferentes formas de periodontitis resulta indicativa de una virulencia heterogénea y un mayor potencial inmunogénico por parte del serotipo b (Hacker & Carniel, 2001; Chen y

cols., 2010; Cortelli y cols., 2012; Jentsch y cols., 2012; Roman-Torres y cols., 2010; Sakellari y cols., 2011). Al respecto, se ha reportado que cepas del serotipo b de A. actinomycetemcomitans producen microvesículas citotóxicas de membrana que no están presentes en los otros serotipos. Además, este serotipo muestra una expresión temprana de la fase soluble del LPS, comparado con los serotipos a y c; interfiere su propia co-agregación con *Fusobacterium nucleatum* contrario a lo descrito para serotipo a que no tiene efecto inhibitorio en su unión, muestra también una mayor resistencia a la fagocitosis y muerte por linfocitos polimorfonucleares que los otros serotipos e induce mayor expresión de ICAM-1 e interleuquina (IL)-8 en células epiteliales gingivales y una mayor expresión de IL-1 β en macrófagos comparado con los serotipos a y c (Kikuchi y cols., 2012; Nowotny y cols., 1982; Rosen y cols., 2003; Shimada y cols., 2008; Takahashi y cols., 1991; Yamaguchi y cols., 1995).

2.3. Respuesta inmune frente a *A. actinomycetemcomitans*

A nivel periodontal, la respuesta frente a *A. actinomycetemcomitans* comienza con su reconocimiento a través de moléculas receptoras, que reconocen patrones estructurales comunes a varios tipos de microorganismos, conocidos como patrones moleculares asociados a patógenos (PAMPs). Estos receptores se encuentran ampliamente distribuidos en las células de nuestro organismo, existiendo diversos tipos, tal como los receptores Toll-like (TLR), receptores tipo NOD (dominio de oligomerización de nucleótidos), proteínas CD14, receptor de la proteína 3 del complemento, receptores de lectinas y receptores del tipo scavenger o basurero (Akira y cols., 2001; Arancibia y cols., 2007).

De los diez receptores de tipo toll descritos en humanos, TLR-2 y TLR-4 son los mas estudiados. TLR-2 está implicado en el reconocimiento de componentes bacterianos como péptidoglican o lipoproteínas, y TLR-4 en el reconocimiento específicamente del antígeno O del LPS de bacterias Gram (-) (Kikkert y cols., 2007; Mori y cols., 2003). La expresión de estos receptores en los tejidos periodontales guarda relación con la interacción entre las células de los tejidos periodontales y los microrganismos del biofilm sub-gingival, donde la señalización de los receptores toll-like tiene un rol fundamental en la respuesta inmune innata y el mantenimiento de la salud periodontal, mediando la expresión de citoquinas pro-

inflamatorias y modulando el tipo de respuesta inmune que se desplegará. No obstante, la sobre producción de citoquinas pro-inflamatorias, producto de la estimulación crónica de estos receptores, culmina en la mayoría de los casos en la destrucción de los tejidos periodontales (Beklen y cols., 2008; Watanabe y cols., 2011). Se ha descrito que los receptores TLR2 y TLR4 se expresan en mayor cantidad en los tejidos gingivales humanos de pacientes con periodontitis comparados con individuos sanos, estando el grado de expresión relacionado con la severidad de la periodontitis (Rojo-Botello y cols., 2012).

En base a un modelo experimental de periodontitis con ratones *knockout* para TLR2 y TLR4, se han estudiado los mecanismos que modulan el resultado de una infección periodontal por *A. actinomycetemcomitans*. Se observó que ratones TLR2 -/- desarrollaron periodontitis más severa y mayor perdida ósea luego de ser infectados. Además, en un modelo de infección intraperitoneal de *A. actinomycetemcomitans* usado para caracterizar el rol del TLR2 en el reclutamiento de macrófagos y neutrófilos, la respuesta inflamatoria inicial en ratones TLR2 -/- fue menor comparado con el grupo control, evidenciándose que el infiltrado de neutrófilos y macrófagos en la cavidad peritoneal, disminuyó significativamente, y los niveles de IL-1β, factor de necrosis tumoral (TNF)-α, CXCL2 y CCL5 fueron significativamente menores en los ratones TLR2 -/-. En el caso de ratones TLR4 -/- infectados oralmente con *A. actinomycetemcomitans*, estos desarrollaron menor grado de severidad de periodontitis, menor pérdida ósea y una disminución en la migración de células inflamatorias y producción de IL-1 β y TNF-α en el tejido periodontal, comparado con el grupo control (Gelani y cols., 2009).

Como resultado del reconocimiento de *A. actinomycetemcomitans*, diversas citoquinas son producidas por las células de los tejidos periodontales; las primeras células en responder son las células del estroma periodontal, tales como fibroblastos ya sea gingivales o periodontales, donde se induce la producción de IL-6, y queratinocitos del epitelio gingival que producen TNF- α, IL-1β, IL-6, IL-8 e IL-15, muchas de las cuales tienen acción quimioatrayente sobre células inmunes como neutrófilos y macrófagos (Herbert y cols., 2016).

Frente a este ambiente inflamatorio dado por el reconocimiento de *A. actinomycetemcomitans*, las primeras células en migrar hacia el sitio de infección periodontal son los neutrófilos polimorfonucleares, los cuales se desplazan siguiendo gradientes de concentración quimioatrayentes, tal como IL-8 secretado por las células que ya reconocieron el patógeno (Denison y cols., 1997). Estos neutrófilos, son células de vida corta, que mueren en gran cantidad en el sitio de inflamación y que contienen gránulos citoplasmáticos que degradan elementos estructurales del tejido, donde en el caso de periodontitis la acumulación y muerte masiva de estos neutrófilos polimorfonucleares está entre las principales causas de destrucción tisular (Denison y cols., 1997).

En estudios de estimulación de neutrófilos con extractos de patógenos periodontales como *P. gingivalis* o *A. actinomycetemcomitans* se ha observado que estas células se vuelven más reactivas y aumentan la producción de metaloproteinasa de matriz (MMP)-9 e IL-8 respecto a los no estimulados, lo que es indicativo de que esta hiperreactividad sería consecuencia de la respuesta inflamatoria del tejido periodontal (Restaíno y cols., 2007). Además, estos neutrófilos modulan su fenotipo en función del grado de inflamación existente a nivel periodontal, hipotetizándose actualmente la existencia de tres subtipos, definidos a partir de la expresión de marcadores CD asociados a activación/degranulación, inmuno-regulación, adhesión y regulación del complemento; donde encontramos neutrófilos naive o vírgenes, que están presentes de forma normal en la circulación sanguínea; neutrófilos para-inflamatorios, reclutados de forma constitutiva hacia los tejidos periodontales en estado de salud, principalmente en respuesta a las bacterias de la microbiota normal o daños tisulares menores sin signos clínicos de inflamación; y un tercer fenotipo denominado pro-inflamatorio que deriva de los para-inflamatorios y que se encuentra asociado a una periodontitis establecida (Fine y cols., 2016).

Este ambiente inflamatorio, no sólo van a determinar las células que migran al sitio de infección, sino que también el tipo de respuesta adaptativa que se va llevar a desarrollar (Cutler y cols., 2004). Grupos específicos de citoquinas polarizantes están involucrados en la diferenciación de subpoblaciones de células T helper, IL-12 e INF-y para células Th1; IL-4, IL-5 e IL-2 para células Th2; TGF-β, IL-6, IL-21, IL-17 e IL-23 para Th17 y TGF-β, IL-10 e IL-2 para inducir diferenciación de células T reguladoras. Estas citoquinas, estimulan la

expresión de factores de transcripción específicos involucrados en la diferenciación de células T, como lo son T-bet para el caso de las Th1, GATA3 para Th2, RORC2 para Th17 y Foxp3 para células T reguladoras (Bluestone y cols., 2009; Kalinski y cols., 2005; Murphy y cols., 2002).

Una vez diferenciadas, estas células expresan un patrón de citoquinas y quimioquinas característico, que determina una migración selectiva de las células T *helper* hacia el foco de repuesta. Las citoquinas producidas por células Th1 han sido asociadas con destrucción ósea, como consecuencia de un fenómeno inflamatorio de origen infeccioso; mientras que las citoquinas Th2 minimizan la pérdida ósea (Colic y cols., 2009; Graves y cols., 2011), en el caso de las Th17, se ha descrito actividad inflamatoria involucradas en una serie de infecciones y procesos osteolíticos y autoinmunes (Dong, 2008; Jeffcoat & Reddy, 1991; Takayanagi y cols., 2000); y en células Treg un efecto supresor en la osteolisis inflamatoria modulado por citoquinas como TGF-□, IL-10 y otras moléculas como CTLA-4 (Bettelli y cols., 2006; Jeffcoat & Reddy 1991).

Esta polarización y activación de linfocitos T CD4 vírgenes es mediada por la actividad de células dendríticas (DCs), las cuales son un link entre los fenómenos de inmunidad innata y adaptativa, donde los componentes microbianos como el LPS activan a estas células y determina en ellas la expresión de un patrón de citoquinas, que induce la subsecuente polarización de linfocitos T para una respuesta antígeno específica (Gemmell y cols., 2007; Garlet, 2010; Houri-Haddad y cols., 2007; Graves cols., 2011).

En base a lo anteriormente expuesto, y considerando la variabilidad en la capacidad inmuno-estimulatoria de los diferentes serotipos de *A. actinomycetemcomitans*, abundante evidencia sustenta la idea que esta respuesta tanto a nivel de células dendríticas como de linfocitos T es variable en función del serotipo con que dichas células son estimuladas. En este sentido, cuando células dendríticas son expuestas a diversos serotipos de A. actinomycetemcomitans (a, b y c) o sus LPS purificados, la respuesta en cuanto a la expresión y secreción de citoquinas como IFN-y, TNF- α, IL-1 β, IL-6, IL-12 e IL-23 es mayor frente al serotipo b que a los serotipos a y c, donde estas citoquinas pertenecientes a los grupos polarizantes para Th1 y Th17 serían moduladoras durante la presentación de antígenos, de

una respuesta inmune pro-inflamatoria que facilita la destrucción del tejido conectivo y la reabsorción del hueso alveolar (Díaz-Zúñiga y cols., 2014).

Sumado a lo anterior, se ha demostrado que esta respuesta diferencial serotipo dependiente por parte de las células dendríticas es TLR2/TLR4 dependiente. Cuando estas células son estimuladas con A. actinomycetemcomitans previo bloqueo de TLR2 y/o TLR4, existe una inhibición en la expresión de mRNA para IL-12, IL-23 y CCR6, observándose además, una correlación positiva entre los niveles de mRNA de TLR2 e IL-23 y CCR6, y los niveles de mRNA para TLR4 e IL-1β, IL-12 y CCR5. Esta respuesta tendría relación con la capacidad de TLR4 de reconocer el lípido A del LPS, mientras que TLR2 responde reconociendo la fracción O-polisacárido de dicha estructura, donde el serotipo b determina una sobreexpresión de dicho receptor (Díaz-Zúñiga y cols., 2015).

Así mismo, en el caso de linfocitos T estimulados con células dendríticas previamente inducidas ante el serotipo b de A. actinomycetemcomitans, se observó una mayor expresión de quimioquinas (CCLs) CCL2, CCL3, CCL5, CCL20, CCL21, Y CCL28 y de los receptores de quimioquinas (CCRs) CCR1, CCR2, CCR5, CCR6 CCR7 Y CCR9 (todas asociadas a patrones de respuesta Th1 y Th17) comparado con experimentos similares con los serotipos a y c, además de una correlación positiva entre los niveles de CCLs y CCRs y los niveles de expresión de los factores Tbet y RORC2 frente a la estimulación con el serotipo b, respecto a los otros serotipos, siendo esto, tal como se mencionó anteriormente, indicativo de una modulación hacia la polarización Th1 y Th17 por parte del serotipo b (Álvarez y cols., 2015).

Considerando lo anteriormente mencionado, al estudiar la función de linfocitos T a partir de la expresión de factores de transcripción y producción de citoquinas, en respuesta a la estimulación con DCs inducidas con los diferentes serotipos de esta bacteria, se observa que el serotipo b y su LPS purificado inducen altos niveles de factores de transcripción y citoquinas asociadas a un perfil de tipo Th1 y Th17, en comparación con los otros serotipos (Díaz-Zúñiga y cols., 2015). Además, se ha demostrado que la expresión y secreción de RANKL asociada a la diferenciación y activación de células precursoras de osteoclastos TRAP+, junto con la expresión de factores de trascripción asociados a Th17 y la inducción in vitro de reabsorción ósea por parte de linfocitos T, es mayor cuando estos son estimulados

con DCs autólogas expuestas al serotipo b de *A. actinomycetemcomitans* comparado con los serpotipos a y c, reafirmando la hipótesis de que el serotipo b modula la respuesta inmune hacia un perfil Th1/Th17 responsable de propiciar el ambiente inflamatorio para la destrucción de los tejidos periodontales (Melgar-Rodríguez y cols., 2016).

Numerosas citoquinas han sido descritas como estimuladoras de la reabsorción ósea, tales como TNF-α, IL-1 β, IL-1α, IL-6, IL-11, IL-15 e IL-17, mientras otras tales como IL-4, IL-5, IL-10, IL-13, IL-18 y TGF- β1 inhiben la reabsorción ósea (Takayanagi, 2005; Walsh y cols., 2006). En este contexto, durante la respuesta inflamatoria característica de la periodontitis, las citoquinas pro-inflamatorias asociadas a los fenotipos Th1 y Th17 estimulan la expresión del factor osteo-destructivo ligando del receptor activador del factor nuclear □B (RANKL) en la membrana de los osteoblastos. RANKL, al unirse a su receptor específico RANK presente en la membrana de células precursoras de osteoclastos, induce su diferenciación y activación como osteoclastos maduros (Gaffen y cols., 2008; Graves y cols., 2008). Por otro lado, RANKL también es producida por otros tipos celulares como células polimorfonucleares, linfocitos Th17, células epiteliales y linfocitos B (Kaliski y cols., 2005), existiendo abundante evidencia que el aumento en los niveles de RANKL en los tejidos periodontales estimula la diferenciación de células precursoras de osteoclastos, su maduración y sobrevivencia (Crotti y cols., 2003; Gaffen y cols., 2008; Hofbauer y cols., 2001).

Este daño en los tejidos producto del ambiente inflamatorio y la persistencia de la infección bacteriana, no sólo está determinado por un aumento en los niveles de RANKL, sino que también por la expresión y actividad de proteinasas específicamente de la familia de las MMPS, que contribuyen también a la destrucción progresiva de los tejidos periodontales (Sorsa y cols., 2006). Estas MMPs representan un grupo de endopeptidasas dependientes de zinc, que degradan componentes de la matriz extracelular y que además tienen actividad regulatoria, actúan como co-factores enzimáticos y como citoquinas o quimioquinas (Sbardella y cols., 2012; Overall y cols., 2002).

Alrededor de 24 tipos de MMPs han sido identificadas en humanos, las cuales son clasificadas de acuerdo con sus propiedades estructurales y sustratos específicos en

colagenasas, gelatinasas, estromalisinas, matrilisinas y metaloproteinasas de matriz tipo membrana (Folgueras y cols., 2004). Dado que el colágeno tipo 1 es el principal componente de la matriz extracelular de los tejidos periodontales, el grupo MMP-1, MMP-8, MMP-13 y MMP-14, pertenecientes a las colagenasas, juegan un rol fundamental en la pérdida de soporte periodontal (Sorsa y cols., 2010). La MMP-8 o colagenasa-2, es la principal colagenasa en los tejidos gingivales y fluido crevicular gingival, siendo producida tanto por células residentes como por células inflamatorias, representado el 80% de las colagenasas del fluido crevicular gingival, seguido de la MMP-13 que alcanza un 18% aproximadamente (Golub y cols., 2008).

Se han observado diferentes niveles de MMP-8 en fluido crevicular gingival y saliva en gingivitis, periodontitis y en estado de salud periodontal, dependiendo del nivel de inflamación existente; además, factores como el tabaquismo y la diabetes mellitus tipo 2 aumenta los niveles de MMP-8 en pacientes con periodontitis; mientras que por el contrario, se ha evidenciado que el tratamiento convencional no quirúrgico en periodontitis reduce los niveles de MMP-8 y MMP-9 en concordancia con la remisión de los signos clínicos, no obteniéndose la misma respuesta en aquellos sitios asociados a altas concentraciones sostenidas de MMP-8 (Mantyla y cols., 2006; Marcaccini y cols., 2010; Hernánedez y cols., 2010; Sorsa y cols., 2006).

2.4. Interacción de queratinocitos con *Aggregatibacter actinomycetemcomitans*

La respuesta diferencial frente a los distintos serotipos de A. *actinomycetemcomitans* descrita anteriormente, también ha sido estudiada en células del estroma periodontal como queratinocitos y fibroblastos, sin embargo, la totalidad de los aspectos de dicha respuesta y cómo esta influye en los fenómenos inflamatorios y osteodestructivos no está del todo dilucidada. Actualmente, se reconoce que las células epiteliales no son entidades pasivas a nivel periodontal, sino que por el contrario, son metabólicamente activas y reaccionan ante los estímulos externos sintetizando citoquinas y moléculas de adhesión que dirigen la migración y activación de leucocitos, además de producir factores de crecimiento y variadas enzimas. Con relación a esto, los resultados de experiencias en células epiteliales gingivales

estimuladas con *A. actinomycetemcomitans* muestran una mayor expresión de IL-8 e ICAM-1 frente al serotipo b, que ante los otros serotipos (Shimada y cols., 2008).

Esta interacción de células epiteliales con *A. actinomycetemcomitans* es un proceso activo que demanda la participación de receptores celulares del hospedero. El receptor de transferrina en el queratinocito es un posible candidato específico de invasión de *A. actinomycetemcomitans,* donde bloqueos in vitro de dicho receptor muestran una inhibición de la invasión por parte de este microorganismo (Meyer y cols., 1997). Por otro lado, las integrinas de la superficie de las células epiteliales, podrían ser una ruta de entrada de *A. Actinomycetemcomitans.* Esta hipótesis se basa en la capacidad de unión a integrinas de una proteína constitutiva de *A. actinomycetemcomitans*, que en estudios con cepas de *Escherichia coli* no invasivas transformadas con esta proteínas, dichas bacterias cambian su fenotipo a uno de mayor invasividad (Meyer y cols., 1997). Además, algunas cepas de *A. actinomycetemcomitans* tienen motivos en su superficie que contienen fosforilcolina, una molécula que media la invasión de *Streptococcus pneumoniae* a través de las células endoteliales, de modo que *A actinomycetemcomitans* podría invadir también dichas células usando este mecanismo (Cundell y cols., 1996; Schenkein y cols., 2000).

Una característica de *A. actinomycetemcomitans* dentro del fenómeno de invasión de la célula epitelial, es la capacidad de atravesar el epitelio moviéndose desde una célula a otra por medio de la formación de protrusiones citoesqueletales (Henderson y cols., 2003). En este sentido, la evidencia disponibles muestra en modelos in vitro que la migración de *A. actimonycetemcomitans* a través del epitelio está relacionada con un aumento en la producción de citoquinas pro-inflamatorias, tal como TNF- α, IL-1β, IL-6, IL-8, IL-15, y un aumento en la apoptosis a través de la vía de las caspasas 3/7 por parte de estas células (Dickinson y cols., 2011; Kang y cols., 2012; Suga y cols., 2013).

Al mismo tiempo, se ha reportado que las células epiteliales responden al contacto con A. actinomycetemcomitans alterando la transcripción de genes específicos, especialmente aquellos involucrados en la remodelación de tejidos y la reabsorción ósea (Umeda y cols., 2012). Esté fenómeno también ocurre en la propia bacteria, donde el contacto con las células epiteliales determina la sobre-expresión de genes de virulencia como flp,

relacionado con la expresión de fimbrias, cdtB que codifica para toxina de distención citoletal (CDT), aae y apaH, involucrados en adherencia e invasión de células epiteliales, orf859 asociados con la sobrevivencia intracelular de A. actinomycetemcomitans y otros como omp29, omp100 ltxA y cagE que son sobre-expresados en el serotipo b en presencia de células epiteliales (Umeda y cols., 2013). Sumado a lo anterior, se ha descrito además, la capacidad de las células epiteliales gingivales de producir RANKL en condiciones in vitro y de potenciar la diferenciación y actividad de osteoclastos, confirmado su potencial actividad en la osteodestrucción periodontal (Usui y cols., 2015). Además, muchas de las citoquinas pro-inflamatorias que esta célula es capaz de producir, tales como IL-1, IL-6, IL-8 y TNF-α, tienen una potente acción estimuladora de la diferenciación de osteoclastos, siendo reguladas positivamente por la presencia de A actinomycetemcomitans (Garlet y cols., 2005; Lima y cols., 2010; Dunmyer y cols., 2012). Algunas de estas citoquinas, tal como IL-8, son ligandos de CXCR1 y CXCR2 expresado en neutrófilos, mediando la migración de estas células que se constituyen como las primeras del sistema inmune innato en llegar al sitio de infección y responder a la infección (Kang y cols., 2012).

Sobre la base de las ideas expuestas y considerando la heterogeneidad en la capacidad inmunogénica de *A. actinomycetemcomitans* y la capacidad de respuesta de las células epiteliales frente a este microorganismo, la presente investigación tiene como objetivo evaluar la respuesta de los queratinocitos frente a los diferentes serotipos de A. *actinomycetemcomitans* y determinar la importancia de esta respuesta en el desarrollo de los aspectos inflamatorios y osteodestructivos de las enfermedades periodontales asociadas a placa bacteriana.

CAPÍTULO 3

Hipótesis, Objetivo General y Objetivos Específicos

3.1. Hipótesis

Con relación a la variabilidad fenotípica de *A. actinomycetemcomitans* a partir de las características estructurales de su LPS, se han descrito 7 serotipos (a-g), siendo los serotipos a, b y c los de mayor prevalencia en la cavidad oral en humanos. De estos, la presencia del serotipo a se a vinculado a cuadros de periodontitis crónica, mientras que el b se ha caracterizado como el más virulento y con mayor capacidad inmunogénica, siendo frecuentemente aislado de lesiones periodontales de sujetos con periodontitis Estadios III y IV, a diferencia del serotipo c que es aislado con mayor frecuencia de sitios periodontales sanos en sujetos sin enfermedades periodontales.

Diferentes tipos celulares estimulados con *A. actinomycetemcomitans* muestran una mayor respuesta frente al serotipo b respecto a los otros dos serotipos antes mencionados, tanto por su capacidad inmuno-estimulatoria a nivel de membrana vía TLR, como probablemente a la expresión diferencial de genes asociados a virulencia cuando se encuentra interactuando con células epiteliales, específicamente queratinocitos.

Dentro de este marco, la hipótesis que sustenta este proyecto plantea que ***"La respuesta de queratinocitos en torno a la producción de citoquinas, expresión de receptores de tipo toll, producción de MMP-9 y RANKL es mayor frente al serotipo b de A. actinomycetemcomitans comparado con los serotipos a y c, contribuyendo en mayor medida al desarrollo de los procesos inflamatorios y osteodestructivos característicos de la periodontitis desde las primeras etapas de interacción patógeno-hospedero".***

3.2. Objetivo General

Evaluar la respuesta de células epiteliales frente a los serotipos más prevalentes de *Aggregatibacter actinomycetemcomitans*, en un modelo in vitro de infección celular,

determinando si la respuesta inflamatoria diferencial "serotipo-dependiente" que ha sido descrita en otros tipos celulares es replicable a nivel epitelial.

3.3. Objetivos específicos:

3.3.1. Evaluar la expresión de citoquinas pro-inflamatorias, receptores tipo *toll* y moléculas inductoras de destrucción tisular en queratinocitos orales infectados con los serotipos a, b, y c de *Aggregatibacter actinomycetemcomitans.*

3.3.2. Evaluar la expresión de citoquinas pro-inflamatorias, receptores tipo *toll* y moléculas inductoras de destrucción tisular en macrófagos de la línea THP-1 infectados con los serotipos a, b, y c de *Aggregatibacter actinomycetemcomitans.*

3.3.3. Analizar mediante la expresión de marcadores de superficie y expresión de citoquinas, la capacidad de polarizar a fenotipos M1 y M2 de células macrofágicas de la línea THP-1 luego de ser infectadas con los serotipos a, b, y c de *Aggregatibacter actinomycetemcomitans.*

3.3.4. Contrastar la expresión de citoquinas pro-inflamatorias, receptores tipo *toll* y moléculas inductoras de destrucción tisular en células del epitelio de unión aisladas mediante microdisección láser de mucosa gingival en un modelo murino de periodontitis inducido a partir de los serotipos a, b, y c de *Aggregatibacter actinomycetemcomitans.*

CAPÍTULO 4:

Paper 1

Betancur D, Muñoz Grez C, Oñate A. **Comparative Analysis of Cytokine Expression in Oral Keratinocytes and THP-1 Macrophages in Response to the Most Prevalent Serotypes of *Aggregatibacter actinomycetemcomitans***. Microorganisms. 2021 Mar 17;9(3):622. doi: 10.3390/microorganisms9030622. PMID: 33802988; PMCID: PMC8002688.

4.1. Abstract

Background: Periodontitis is a chronic inflammatory disease associated with a dysbiotic biofilm. Many pathogens have been related with its progression and severity, one of which is *Aggregatibacter actinomycetemcomitans*, a Gram-negative bacterium with seven serotypes (a–g) according with the structure of its LPS, with serotype b defined as the most virulent compared with the other serotypes. The aim of this study was to evaluate the response of oral keratinocytes and macrophages to *A. actinomycetemcomitans*. Methods: Oral keratinocytes (OKF6/TERT2) and macrophages (THP-1) were infected with *A. actinomycetemcomitans* serotypes a, b and c. The expression of IL-1β, IL-6, IL- 8, IL-18, TNF-α, MMP-9, RANKL, TLR-2, TLR-4, TLR-6, thymic stromal lymphopoietin (TSLP), and ICAM-1 was evaluated by qPCR at 2 and 24 h after infection. Results: An increase in the expression of these molecules was induced by all serotypes at both times of infection, with macrophages showing higher levels of expression at 24 h compared to epithelial cells in which the highest levels were observed in the first hours after infection. Conclusions: Keratinocytes and macrophages contribute to the inflammation in periodontitis from the early stages of infection, producing the first waves of cytokines, acting as the first signal for professional immune cell recruitment and modulation of more specific immune responses.

4.2. Keywords

Cytokines; epithelial cells; keratinocytes; macrophages; *Aggregatibacter actinomycetemcomitans*; periodontitis

4.3. Introduction

According with the new classification of periodontal and peri-implant diseases and conditions, periodontitis is defined as a chronic multifactorial inflammatory disease associated with a dysbiotic biofilm, characterized by the inflammation and destruction of the tooth-supporting tissues with clinical attachment loss, alveolar bone loss, presence of periodontal pocketing and gingival bleeding [1]. This disease not only has an effect at the local level in the oral cavity, but also has an effect in systemic health, inducing low-grade chronic systemic inflammation, and being a risk factor for other conditions such as cardiovascular diseases, type 2 diabetes mellitus, premature delivery, and low birth weight, as well as being associated more recently with pathologies like Alzheimer's disease, psoriasis, erectile dysfunction, and rheumatoid arthritis [2–9].

This pathology has an infectious etiology, associated with the presence of a pathogenic biofilm in teeth, at the subgingival level, where of the approximately 700 cultivable oral bacterial species described, 400 make up this subgingival biofilm, located in the limited space between the tooth and the gum and being the main etiological factor of periodontitis and other periodontal diseases associated with biofilms [10,11]. These bacteria, in health, are in a constant balance in terms of quantity and diversity, as well as with the host's defenses, establishing a symbiotic state, free of inflammation that favors the growth of non-pathogenic commensal bacteria and stimulates a basal immune response [12]. However, various factors (poor oral hygiene, smoking, diabetes, individual predisposition, among others) can modify the environmental characteristics of the site and disturb the balance of this biofilm, providing the conditions for microorganisms that are normally found in low numbers, named opportunistic pathogens or pathobionts to increase in number and virulence (mainly through density-dependent cell signaling) giving place to a dysbiotic and pathogenic biofilm that favors the development and progression of this inflammatory disease [13–16].

Many pathogens have been associated with the progression and severity of this pathology, defined as *"keystone pathogens"* due to their central role in the development of

the dysbiotic biofilm, and the subversion of the host's immune system, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* [17–19].

A. actinomycetemcomitans, a member of the periodontal dysbiotic biofilm, is a Gram-negative, non-motile, facultative anaerobic, and capnophilic coccobacillus [20,21]. Its virulence factors can be grouped into two groups, those that are modulators of the immune system such as leukotoxin (Ltx), superantigens, cytolethal distending toxin (CDT); and a second group associated with tissue destruction which includes cell stress proteins and lipopolysaccharide (LPS) [21–23].

According to the structure of its LPS, seven different serotypes (a–g) have been described based on the composition of the O-polysaccharide chain of this structure, being serotypes a, b and c the most prevalent in the oral cavity in humans [24,25]. Serotype b, has been found to be the most virulent and prevalent in periodontitis patients [17], and in different cell types has shown the ability to induce a greater response in terms of production of cytokines, chemokines, cytokine receptors, and tissue destruction molecules such as metalloproteinases or receptor activator nuclear kappa ligand (RANKL), compared to serotypes a and c [26,27]. This differential response has been widely studied in cells such as dendritic cells and lymphocytes, which show a greater induction by serotype b of the production of cytokines associated with the differentiation of subpopulations of T cells Th1 and Th17, both linked to the destruction of bone tissue and chronic inflammation; however, there is little evidence about this differential character of the response induced by these serotypes in other cell types such as epithelial cells and macrophages [28–30].

During the pathogenesis of periodontitis, *A. actinomycetemcomitans* colonizes the gingival sulcus, infiltrating epithelial cells until reaching the underlying connective tissue, triggering an inflammatory immune response that induces the activation of mechanisms of destruction of both connective tissue and bone resorption [31–33]. To achieve this, *A. actinomycetemcomitans* must first break through the host's front line of defense in the periodontium, made up of epithelial cells and macrophages. The latter, through a chemotactic gradient produced by the epithelial cells, cross the epithelium and are released into the

gingival sulcus where they participate in the control of pathogens that try to colonize this site [34,35].

Extensive evidence has shown the ability of macrophages and epithelial cells to recognize periodontal pathogens, including *A. actinomycetemcomitans*, and respond to these through the production of cytokines, chemokines, antibacterial peptides, alarmins, reduction of integrins and cell–cell adhesion molecules, and others [36–39]. However, the possible differential response of these cell types against the aforementioned bacterial serotypes is not entirely clear yet, there is little knowledge about whether this variable response is a characteristic of only of professional immune cells [26,27], or if it also occurs at the first lines of host defense by modulating inflammation and tissue destruction, in a greater or lesser degree depending on the serotype, from the earliest stages of infection.

This study aimed to analyze whether the response of both oral epithelial cells and macrophages against *A. actinomycetemcomitans* presents a serotype-dependent differential character as described for other cell types [26–28]. For this purpose, an in vitro infection model has been developed using the most prevalent *A. actinomycetemcomitans* serotypes and oral keratinocytes and macrophages cells to simulate the initial stage of periodontitis pathogenesis.

4.4. Materials and Methods

4.4.1. Bacteria Stains

The *A. actinomycetemcomitans* strains ATCC® 43717 (serotype a), ATCC® 43718 (serotype b), and ATCC® 43719 (serotype c) were cultured in BHI broth (70138 GranuCult. Merck®, Darmstadt, Germany) supplemented with 10% horse serum (H1270 Sigma-Aldrich®, Gillingham, UK) at 37 °C in capnophilic conditions (8% O2 and 12% CO2) for 24 h according to the growth curves previously made by our group using standard conditions. The bacteria were used at the exponential growth phase in order to obtain a reliable number of bacteria with full antigenic potential.

4.4.2. Oral Keratinocytes Culture

The immortalized human oral keratinocyte derived from floor mouth OKF6/TERT2 cell line (obtained from Dr. Rolando Vernal, Laboratory of Periodontal Biology, Universidad de Chile, Chile.) was incubated in keratinocyte serum-free medium (KSFM) (37010- 022 Gibco®, Carlsbad, CA, USA) supplemented with bovine pituitary (13028-014 Gibco®, Carlsbad, CA, USA), epidermal growth factor (10450-013 Gibco®, Carlsbad, CA, USA), calcium chloride solution 0.3M (102382 Merck®, Darmstadt, Germany) and penicillin/streptomycin (15140-122 Gibco®, Carlsbad, CA, USA) at final concentration of 100 U/mL of penicillin and 100 µg/mL of streptomycin. Keratinocytes were incubated at 37 °C in 5% CO2 and humidified atmosphere.

4.4.3. THP-1 Derived Macrophages Culture

The human monocytic leukemia THP-1 cell line (ATCC® TIB-202) was cultured in RPMI-1640 medium (R8758 Gibco®, Carlsbad, CA, USA) containing 10% fetal bovine (F2442 Sigma-Aldrich®) serum and supplemented with glucose (G8270 Sigma-Aldrich®, Gillingham, UK) (14 mM final concentration), pyruvic acid (107360 Sigma-Aldrich®, Gillingham, UK) (1mM final concentration), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (H3375 Sigma-Aldrich®, Gillingham, UK) (10 mM final concentration. pH 7.35), 2-mercaptoethanol (M6250 Sigma-Aldrich®, Gillingham, UK) (0.5 mM final concentration) and penicillin/streptomycin (15140-122 Gibco®, Carlsbad, CA, USA) (100 U/mL final concentration of penicillin and 100 µg/mL of streptomycin) under a humidified atmosphere at 37 °C in 5% CO2.

To induce differentiation into macrophages, THP-1 cells were incubated at the same conditions in the aforementioned medium, using phorbol 12-myristate 13-acetate (PMA) (16561-29-8 Sigma-Aldrich®, Gillingham, UK) (100 nM final concentration) for 2 days, as previously described [40,41].

4.4.4. Infection Assay

OKF6/TERT2 monolayers were obtained by seeding 1×10^6 cells per well of a 6 wells plate and incubating for 24 h at 37 °C in 5% CO2. In the case of THP-1 cells, these were prepared

using 1 × 106 cells per well of a 6 wells plate and were activated for 48 h as mentioned above. Bacteria were grown, washed once with phosphate buffered saline solution (PBS), suspended in PBS and added to OKF6/TERT2 and THP-1 cultures at a multiplicity of infection (MOI) of 100 approximately. The plates were centrifuged at 300× *g* (RCF) for 10 min to ensure the contact between the cell layer and the bacteria; after centrifugation, plates were incubated for 90 min at 37 °C in 5% CO2 to allow for internalization of bacteria. Cells were then washed and incubated with fresh medium (KSFM and RPMI as appropriate) supplemented with gentamicin (G1914 Sigma-Aldrich®, Gillingham, UK) and metronidazole (M3761 Sigma-Aldrich®, Gillingham, UK), (300 µg/mL and 200 µg/mL respectively) for the post infection times defined (2 or 24 h).

4.4.5. RNA Extraction and RT-PCR

Total RNA was isolated from the cells at each condition using TRIzol® Reagent (T9424 Invitrogen® Sigma-Aldrich®, Gillingham, UK) reagent according with the manufacturer's instructions. The reverse transcription reaction was performed using 2000 ng of the extracted RNA and using the First-Strand cDNA Synthesis SuperMix kit (18080400 Invitrogen®. Thermofisher®, Waltham, MA, USA) following the manufacturer's protocol for re- verse transcription, using DNAse digestion.

4.4.6. qPCR

The mRNA expression of cytokines and the other molecules of interest was determined by quantitative real-time polymerase chain reaction (qPCR). For this, 30 ng of cDNA were amplified using the appropriated primers (Table 1) previously designed in the platform Ensembl Genome and Primer-BLAST (NCBH-NIH), with the Takyon® No Rox SYBR® MasterMix dTTP Blue (UF-NSMT-B0701 Eurogentec®, Seraing, Belgium) reagent in an AriaMx Real-time PCR System (Agilent®, Santa Clara, CA, USA) as follows: 95 °C for 3 min, followed by 40 cycles of 90 °C for 5 s and 60 °C for 30 s, ending with a melt curve of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s, for detection of non-specific amplification products that could lead to false positive signals. 18S rRNA expression levels were used as a normalizing endogenous control.

4.4.7. Statistical Analysis

The qPCR data were analyzed using the GraphPad Prism 8.0 software (GraphPad Software.Inc, San Diego, CA, USA) and the relative quantification was obtained by normalizing each gene mRNA expression to 18S rRNA expression using the $2^{-\Delta\Delta Ct}$ method [42]. The normality distribution of data was determined using Kolmogorov-Smirnov test and the differences among groups was evaluated using Tukey`s test and two ways ANOVA analysis. Asterisks were used to indicate a value of p considered statistically significant ($p < 0.05$). Data were expressed as fold-change means and standard deviation for 3 independent experiments performed at different times and qPCR reactions for each gene and sample were performed in duplicates.

Table 1. Sequences of primers used.

Target	Forward	Reverse
IL-1β	ctgtcctgcgtgttgaaaga	ttgggtaatttttgggatctaca
IL-6	gccagctatgaactccttct	gaaggcagcaggcaacac
IL-8	agatctgaagtgtgatgactc	gaagcttgtgtgctctgctgtctc
IL-18	gatagccagcctagaggtatgg	ccttgatgttatcaggaggattca
TNF-α	cagcctcttctccttcctgat	gccagagggctgattagaga
MMP-9	gccactactgtgcctttgagtc	ccctcagagaatcgccagtact
TLR-2	ctctcggtgtcggaatgtc	aggatcagcaggaacagagc
TLR-4	ccctcccctgtacccttct	tccctgccttgaataccttc
TLR-6	actgaccttcctggatgtggca	tgacctcatcttctggcagctc
TSLP	gccatgaaaactaaggctgc	cgccacaatccttgtaattg
ICAM	agcggctgacgtgtgcagtaat	tctgagacctctggcttcgtca
RANKL	tgattcatgtaggagaattaaacagg	gatgtgctgtgatccaacga
18S	ctcaacacgggaaacctcac	cgctccaccaactaagaacg

Primers were designed using the platform Ensembl Genome and Primer-BLAST (NCBH-NIH).

4.5. Results

4.5.1. Expression of Pro-Inflammatory Cytokines and Chemokines by Oral Epithelial Cells

We investigated the influence of *A. actinomycetemcomitans* serotypes on expression of pro-inflammatory cytokines and chemokines by oral epithelial cells. For the case of interleukin

1 beta (IL-1β) a statistically significant overexpression was observed for the three serotypes evaluated compared to the non-infected control at 2 h ($p = 0.0004$, $p < 0.0001$, and $p < 0.0001$ for the serotypes a, b, and c respectively) as well as at 24 h, when the three serotypes are compared with the control condition ($p < 0.0001$ for all serotypes). Regarding the differences among serotypes at 2 h, the only statistically significant differences were observed between serotypes a and c, with a higher expression induced by serotype c ($p = 0.0006$). After 24 h of infection there was a statistically significant greater expression for serotype a compared with serotype b ($p < 0.0001$) and c ($p < 0.0001$), without differences between the latter two ($p = 0.7213$) (Figure 1A).

When the expression of interleukin 6 (IL-6) was measured, a statistically significant higher expression was observed for the three serotypes studied at both times of infection compared with the control condition ($p < 0.0001$); at 2 h, a statistically significant higher expression for serotype c was observed compared to the other two serotypes ($p < 0.0001$), and for serotype b over the serotype a ($p < 0.0001$). At 24 h a lower expression of this molecule was observed in all conditions compared to 2 h, although maintaining statistically significant higher relative expression for the three serotypes compared to the uninfected control, and showing statistically significant increased expression for serotypes a and c compared to serotype b ($p = 0.0029$ and $p = 0.0055$ respectively) (Figure 1B).

Interleukin 8 (IL-8 or CXCL8), showed a higher expression by epithelial cells in response to the serotypes a ($p < 0.0001$), b ($p = 0.0007$) and c ($p < 0.0001$) compared with non- infected condition at 2 h of infection. Likewise, this pattern of expression was similar at 24 h of infection for serotype a ($p < 0.0001$), b ($p = 0.0023$) and c ($p < 0.0001$) in comparison with the control condition. In terms of the differences among serotypes at 2 h, the highest expression of this molecule was induced by serotype c over serotypes a ($p = 0.0014$) and b ($p = 0.0002$), and at 24 h a statistically significant higher expression for serotype c over serotypes a and b ($p < 0.0001$) was detected, but also of serotype a over serotype b ($p = 0.0021$) (Figure 1C).

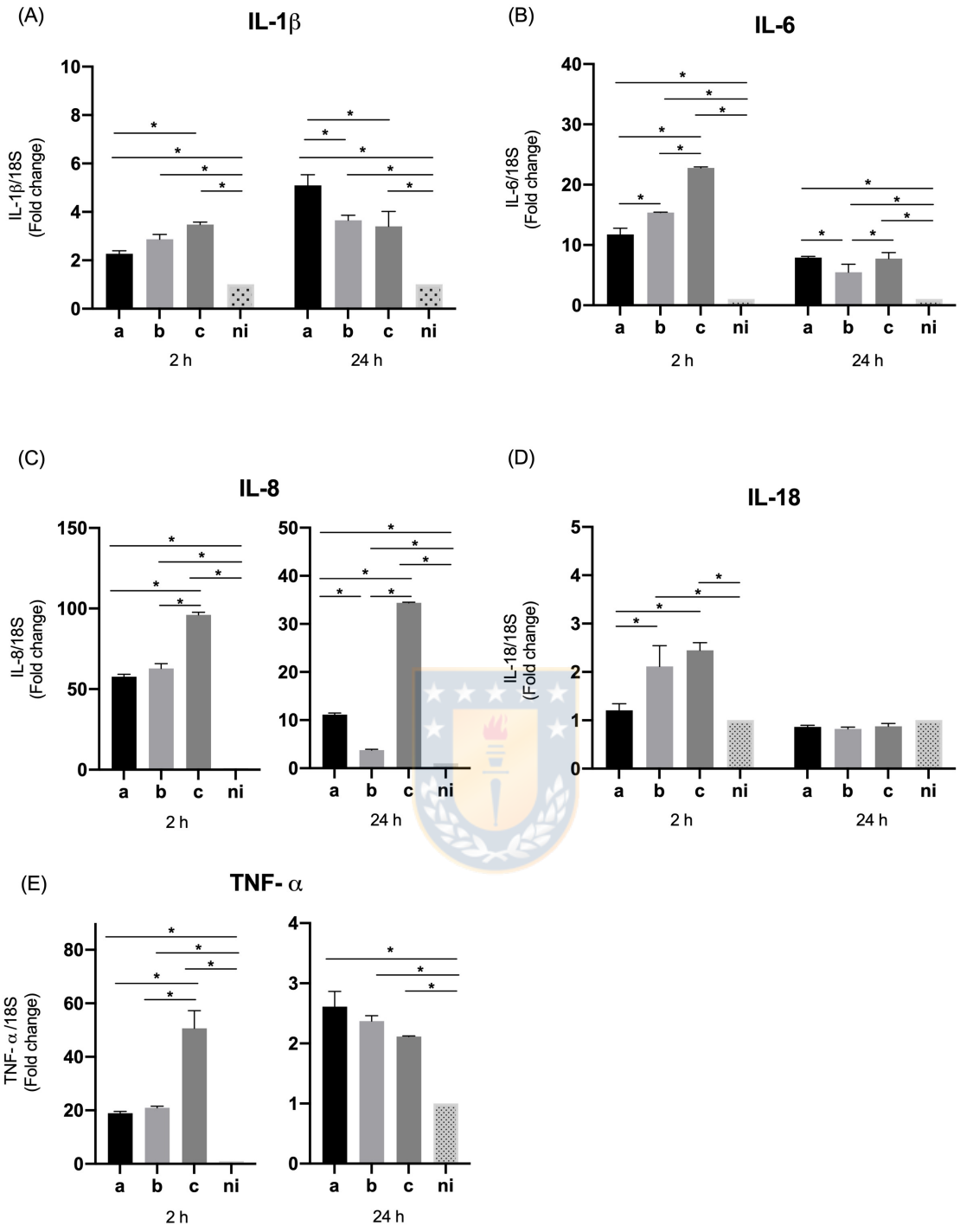

Figure 1. Cytokines and chemokines mRNA expression by *Aggregatibacter actinomycetemcomitans*- induced oral epithelial cells (OKF6/TERT2). Expression in oral epithelial cells infected at a MOI = 102 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b), and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) oral epithelial cells was

considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. (**A**) IL-1β, (**B**) IL-6, (**C**) IL-8, (**D**) IL-18, and (**E**) TNF-α. Asterisks were used to indicate a value of *p* considered statistically significant (* $p < 0.05$).

In the case of interleukin 18 (IL-18), statistically significant differences were only observed at 2 h of infection, with an overexpression of this molecule induced by serotypes b ($p < 0.0001$) and c ($p < 0.0001$) with respect to the control condition, and among serotype b ($p < 0.0001$) and c ($p < 0.0001$) over the serotype a. No statistically significant differences were observed in the expression induced by the serotype a in comparison with the non-infected condition, and no statistically significant differences were observed among conditions at 24 h (Figure 1D).

On the other hand, when the levels of tumor necrosis factor alpha (TNF-α) were measured, a statistically significant increase in expression was observed by the cells studied at 2 h of stimulation in response to the serotype a ($p < 0.0001$), b ($p < 0.0001$) and c ($p = 0.0148$) in comparison with the non-infected condition; in the same way, this over expression was observed also at 24 h with statistically significant differences for the serotype a ($p = 0.0199$), b ($p = 0.0030$), and c ($p < 0.0001$) compared with the non-infected condition. Regarding the differences among serotypes, statistically significant higher levels of TNF- α were expressed only after 2 h of infection by serotype c compared to serotypes a ($p = 0.0354$) and b ($p = 0.0440$) (Figure 1E).

4.5.2. Expression of Molecules Associated with Tissue Destruction by Oral Epithelial Cells

When the levels of metalloproteinase 9 (MMP-9) were measured, a statistically significant increased expression was observed in response to the serotype a ($p = 0.0158$), b ($p = 0.0187$), and c ($p = 0.0052$) with respect to non-infected condition at 2 h of stimulation, and in response to serotype a ($p = 0.0002$), b ($p < 0.0001$) and c ($p = 0.0033$) compared with the control condition after 24 h of infection. Regarding the differences among serotypes, serotype c was able to induce statistically significant higher expression levels of this molecule than serotype a ($p = 0.0438$), and the same way serotype b over serotype a ($p = 0.0430$), at 2

h of infection in both cases. On the other hand, when the differences among serotypes are analyzed after 24 h of stimulation statistically significant higher relative expression of serotype c over the serotypes a ($p = 0.0046$) and b ($p = 0.0083$) becomes evident, as well as of the serotype b over the serotype a ($p < 0.0001$) (Figure 2A).

Regarding expression levels of RANKL, the serotypes a ($p < 0.0001$), b ($p < 0.0001$) and c ($p < 0.0001$) were capable of inducing a statistically significant overexpression of this molecule compared to the non-infected condition at both times of infection. At two hours we observed higher levels of RANKL expression for serotype b over serotypes a ($p < 0.0001$) and c ($p < 0.0001$) and the serotype c over the serotype a ($p = 0.0287$). On the other hand, at 24 h of stimulation statistically significant differences in the expression were observed regarding the b serotype in comparison with the serotype a ($p < 0.0001$) and c ($p < 0.0001$) (Figure 2B).

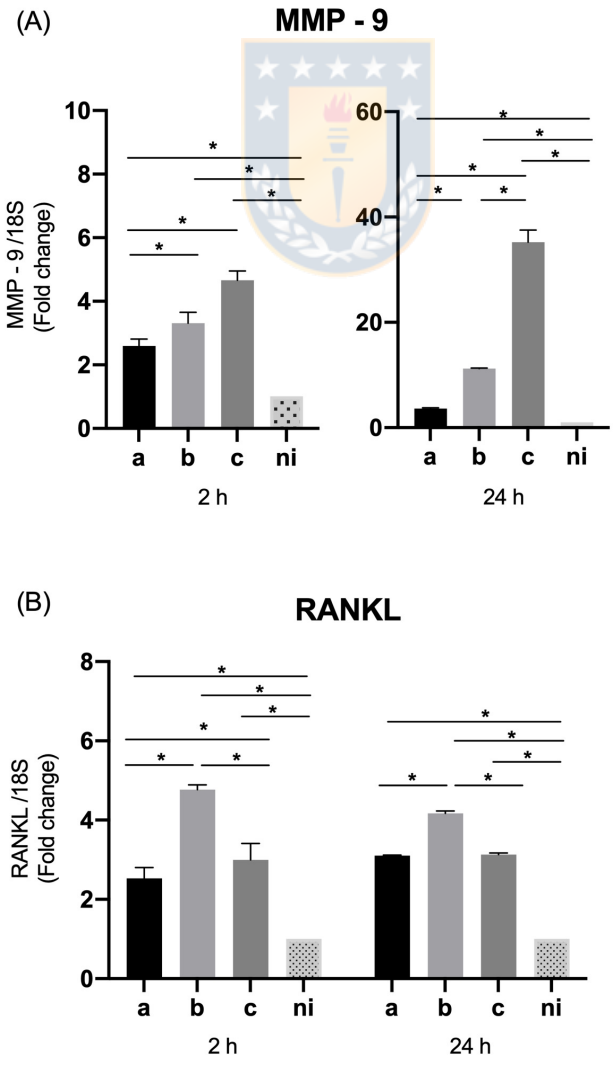

Figure 2. Molecules associated with tissue destruction mRNA expression by *A. actinomycetemcomitans*-induced oral epithelial cells (OKF6/TERT2). Expression in oral epithelial cells infected at a MOI = 10^2 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b), and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) oral epithelial cells was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. (**A**) MMP- 9. (**B**) RANKL. Asterisks were used to indicate a value of *p* considered statistically significant (* $p < 0.05$).

4.5.3. Expression of TLR Receptors in Oral Epithelial Cells

In the case of TLR-2, at 2 h post infection, a statistically significant increase in its expression was observed in response to serotypes a ($p = 0.0003$) and b ($p < 0.0001$) compared to the uninfected condition, without statistically significant difference between the serotype c and control condition ($p = 0.0821$). When comparing serotypes, the expression of this molecule was statistically significant higher for serotype b than for c ($p = 0.0002$), with no statistically significant differences among serotype a and the remaining serotypes. On the other hand, at 24 h of infection, a statistically significant overexpression was only observed among the infected conditions and the control condition ($p = 0.0018$, $p = 0.0011$, and $p = 0.0073$ for the serotypes a, b, and c respectively), without statistically significant differences among serotypes (Figure 3A).

In terms of the expression of TLR-4, at 2 h of infection, a statistically significant overexpression was observed induced by the serotype a ($p = 0.0048$), b ($p = 0.0055$) and c ($p = 0.0018$) compared to the control condition; and likewise at 24 h of stimulation a higher levels of transcription were detected in response to the three serotypes over the non-infected condition ($p < 0.0001$); no evidence of statistically significant differences among serotypes was observed for 2 h and 24 h after infection (Figure 3B).

When levels of TLR-6, are measured, a statistically significant higher expression can be observed after 2 h of infection when the cells are stimulated with serotypes b ($p < 0.0001$) and c ($p < 0.0001$) compared to the uninfected condition, with no differences between serotype a and the control condition ($p = 0.9880$). While respecting the differences in expression among serotypes, a statistically significant higher expression was observed for serotypes b ($p < 0.0001$) and c ($p < 0.0001$) over serotype a. When TLR-6 expression is observed after 24 h of infection, a statistically significant overexpression of this molecule is evidenced for all stimulated conditions, for serotypes a ($p < 0.0001$), b ($p = 0.0009$), and c ($p < 0.0001$) compared to the non-infected control. While regarding the differences among infected conditions, the lowest expression is given by serotype b, being statistically significant higher for serotypes a ($p = 0.0013$) and c ($p = 0.0013$) over serotype b, without differences between them (Figure 3C).

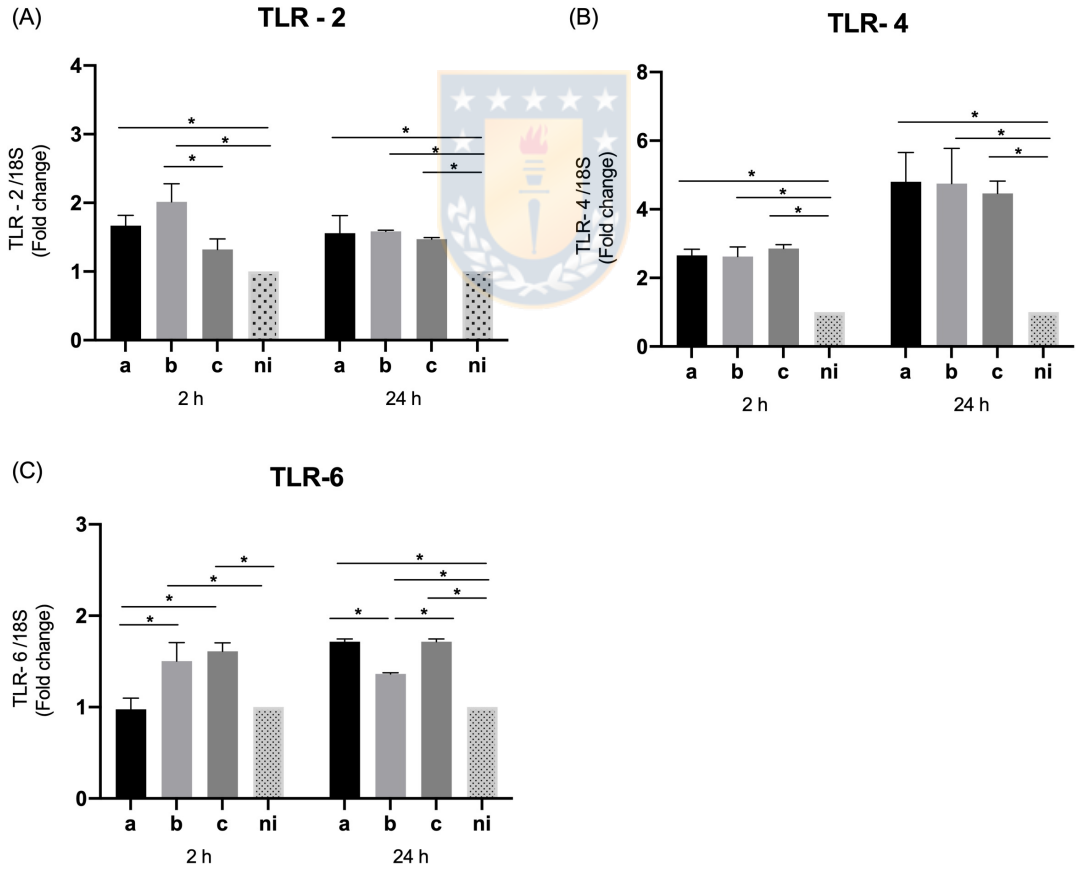

Figure 3. Toll like receptors (TLR) mRNA expression by *A. actinomycetemcomitans*-induced oral epithelial cells (OKF6/TERT2). Expression in oral epithelial cells infected at a MOI = 10^2 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b),

and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) oral epithelial cells was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. (**A**) TLR-2, (**B**) TLR-4, and (**C**) TLR-6. Asterisks were used to indicate a value of p considered statistically significant (* $p < 0.05$).

4.5.4. Expression of Thymic Stromal Lymphopoietin (TSLP) and ICAM-1 by Oral Epithelial Cells

In the case of TSLP levels of expression, the serotypes a ($p < 0.0001$), b ($p < 0.0001$), and c ($p < 0.0001$), induced an overexpression of this molecule compared to the non-infected condition at 2 h of infection; similarly, at 24 h of infection, serotype a ($p < 0.0001$), b ($p = 0.0327$), and c ($p < 0.0001$) were able to induce statistically significant higher relative expression than the uninfected condition. Regarding the differences among serotypes, the expression was statistically significant higher for the serotype a ($p < 0.0001$) and c ($p = 0.0037$) over serotype b at 2 h; and for the serotype a ($p = 0.0076$) and c ($p < 0.0001$) over the serotype b, and for the serotype c over the serotype a ($p = 0.0064$) at 24 h after infection (Figure 4A).

The expression of ICAM-1 in the studied conditions was statistically significant higher for the serotype a ($p < 0.0001$), b ($p < 0.0001$), and c ($p = 0.0006$) respect to the control condition at 2 h and for the serotype a ($p = 0.0152$), b ($p = 0.0201$), and c ($p = 0.0039$) compared with control condition at 24 h after infection. In relation to the differences among serotypes, only at 2 h of infection variations were observed with statistically significant higher levels of expression induced by the serotype a ($p = 0.0035$) and c ($p = 0.0035$) over serotype b (Figure 4B).

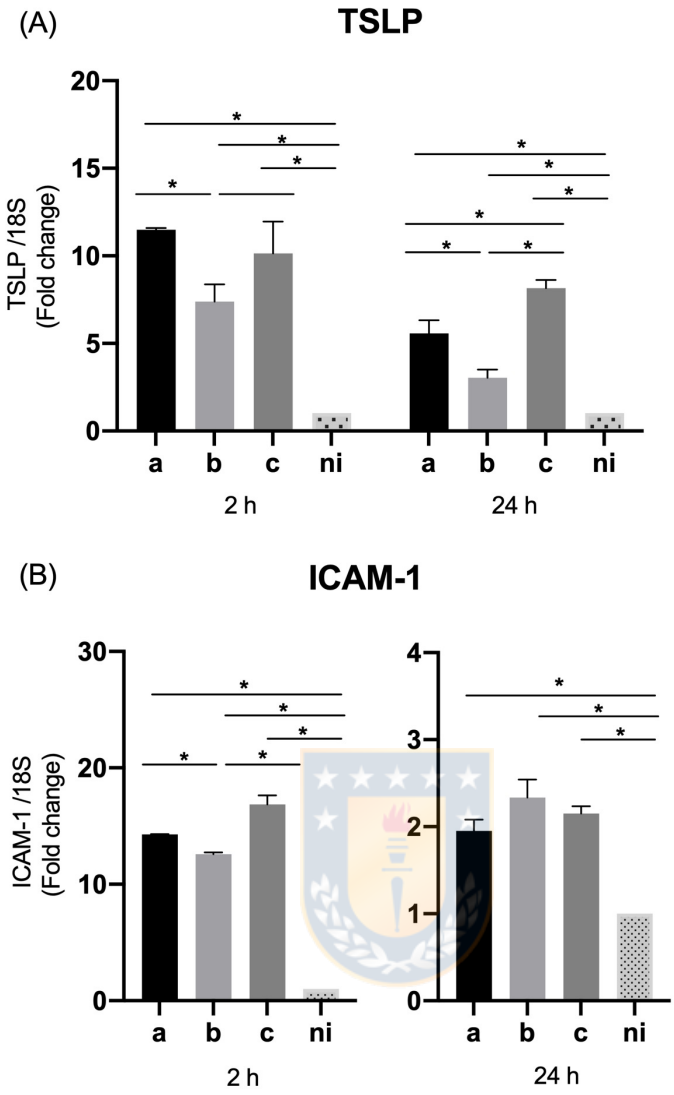

Figure 4. Thymic stromal lymphopoietin (TSLP) and ICAM-1 mRNA expression by *A. actinomycetemcomitans*-induced oral epithelial cells (OKF6/TERT2). Expression in oral epithelial cells infected at a MOI = 10^2 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b), and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) oral epithelial cells was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. (**A**) TSLP. (**B**) ICAM-1. Asterisks were used to indicate a value of *p* considered statistically significant (* $p < 0.05$).

4.5.5. Expression of Pro-Inflammatory Cytokines and Chemokines by THP-1 Macrophage Cells

The qPCR data reveal that in the case of IL-1β, the serotypes a ($p < 0.0001$), b ($p = 0.0041$) and c ($p = 0.0108$) induced a statistically significant overexpression with respect to the non-infected condition at two hours; similarly, at 24 h of infection by the serotype a ($p = 0.0003$), b ($p < 0.0001$) and c ($p = 0.005$) respect to the non-infected control. When the differences among serotypes are analyzed at 2 h, a statistically significant greater expression is induced by serotype b over serotypes a ($p = 0.0228$) and c ($p = 0.0310$), and in the case of 24 h post infection, the highest expression is given by serotype b on serotypes a ($p = 0.0006$) and c ($p = 0.03$), followed by serotype c on serotype a ($p = 0.0226$) (Figure 5A).

In the case of IL-6, at 2 h post infection all the serotypes studied induce a statistically significant greater expression of this molecule compared to the uninfected condition, serotype a ($p < 0.0001$), b ($p = 0.0010$) and c ($p = 0.0002$), being also statistically significant higher for serotypes a ($p = 0.0064$) and b ($p < 0.0275$) than for serotype c. Similarly, at 24 h, all infected conditions showed statistically significant higher expression levels of IL-6 than the uninfected condition, serotype a ($p = 0.0066$), b ($p < 0.0001$), and c ($p < 0.0001$), showing statistically significant differences only between serotype a and serotype c ($p = 0.0315$) (Figure 5B).

The IL-8 expression data by macrophage cells samples showed a statistically significant increased expression of this cytokine in response to the three serotypes analyzed compared to the unstimulated control condition, serotype a ($p = 0.0025$), b ($p = 0.0231$), and c ($p = 0.0062$) at 2 h of infection and serotype a ($p = 0.0014$), b ($p = 0.0102$) and c ($p = 0.0070$) at 24 h of infection. On the other hand, the differences among bacteria revealed that at 2 h the highest expression was induced by serotype a over serotype b ($p = 0.0006$) and c ($p = 0.0099$), without statistically significant differences between the latter two. While at 24 the overexpression induced by serotype a alone was statistically significant higher compared to serotype c ($p = 0.0292$), without statistically significant differences with respect to serotype b ($p = 0.1630$), or between serotypes b and c ($p = 0.9675$) (Figure 5C).

In terms of the expression of IL-18 by THP-1 macrophage cells, the three serotypes analyzed were able to induce a statistically significant expression of this cytokine with respect to the control condition serotype a ($p = 0.0056$), b ($p = 0.0051$) and c ($p = 0.0190$) at 2 h of infection and serotype a ($p = 0.0074$), b ($p = 0.0017$) and c ($p = 0.0002$) at 24 h of infection. There were no statistically significant differences among serotypes, except for a significantly greater expression of serotype a over serotype b ($p = 0.0066$) at 2 h of infection (Figure 5D).

We also evaluated the expression of TNF- α by macrophage cells infected with the 3 previously mentioned serotypes of *A. actinomycetemcomitans*, observing for both time points studied and all serotypes, the ability to induce a statistically significant overexpression of this cytokine compared to the uninfected condition ($p < 0.0001$). The analysis of the differences among serotypes showed at 2 h significantly higher levels of expression in response to serotypes b ($p = 0.0005$) and c ($p < 0.0098$) over serotype a, without marked differences between serotype b and c; while on the contrary, at 24 h, the most considerable overexpression was given by serotype a over serotypes b ($p < 0.0001$) and c ($p < 0.0001$), without statistically significant differences between the latter two (Figure 5E).

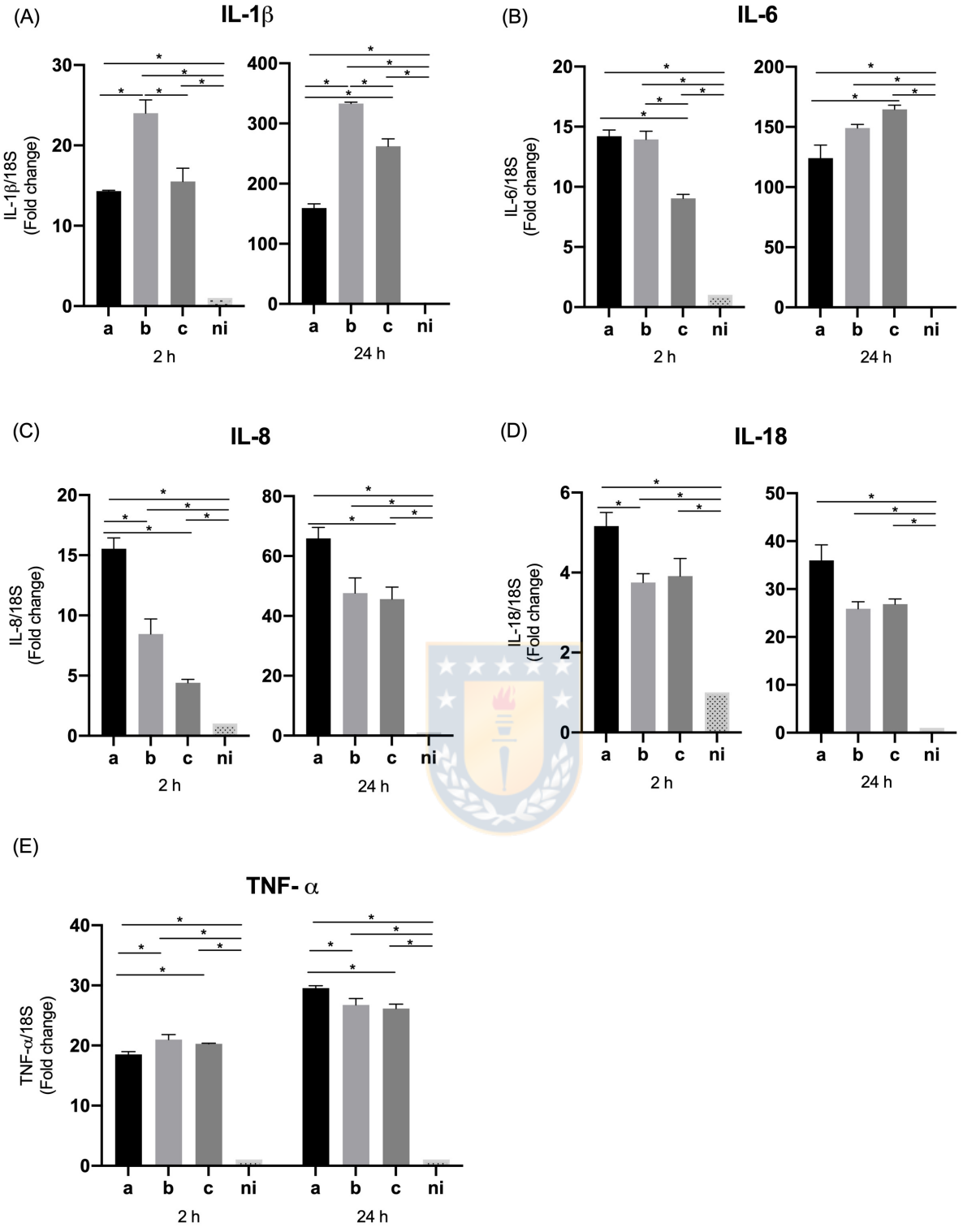

Figure 5. Cytokines and chemokines mRNA expression by *A. actinomycetemcomitans*-induced THP-1 human macrophages (ATCC® TIB-202TM). Expression in macrophages infected at a MOI = 10^2 with strains ATCC® 43717TM (serotype a), ATCC® 43718TM (serotype b), and ATCC® 43719TM (serotype c), 2 and 24 h after infection. For relative

expression, mRNA expression in non-infected (ni) macrophages was considered as 1, as a reference for fold-change in expression using 18S rRNA ex- pression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. (**A**) IL-1β, (**B**) IL-6, (**C**) IL-8, (**D**) IL-18, and (**E**) TNF-α. Asterisks were used to indicate a value of *p* considered statistically significant (* $p < 0.05$).

4.5.6. Expression of MMP-9 and RANKL by THP-1 Macrophage Cells

As evaluated for epithelial cells, the expression MMP-9 was measured in THP-1 cells in response to the aforementioned bacterial serotypes.

MMP-9 showed a significantly increased expression ($p < 0.0001$) in response to stimulation with the three serotypes used with respect to the control condition at both times analyzed. Showing statistically significant differences among serotypes only at 2 h, with a higher expression of serotype b over serotypes a ($p < 0.0001$) and c ($p < 0.0001$), with no marked difference between the latter, while at 24 h no differences in expression were observed among serotypes (Figure 6A).

For its part, RANKL was significantly overexpressed in response to the 3 bacteria studied compared to the non-infected condition, serotype a ($p = 0.0015$), b ($p = 0.0028$), and c ($p = 0.0154$) at 2 h of infection and serotype a ($p < 0.0001$), b ($p = 0.0002$), and c ($p < 0.0001$) at 24 h of infection. The differences among serotypes were observed only 24 h after infection with a significantly greater overexpression of serotype c over serotype b ($p = 0.0073$), and without statistically significant differences with serotype a ($p = 0.8094$) or differences between serotypes a and b ($p = 0.1856$) (Figure 6B).

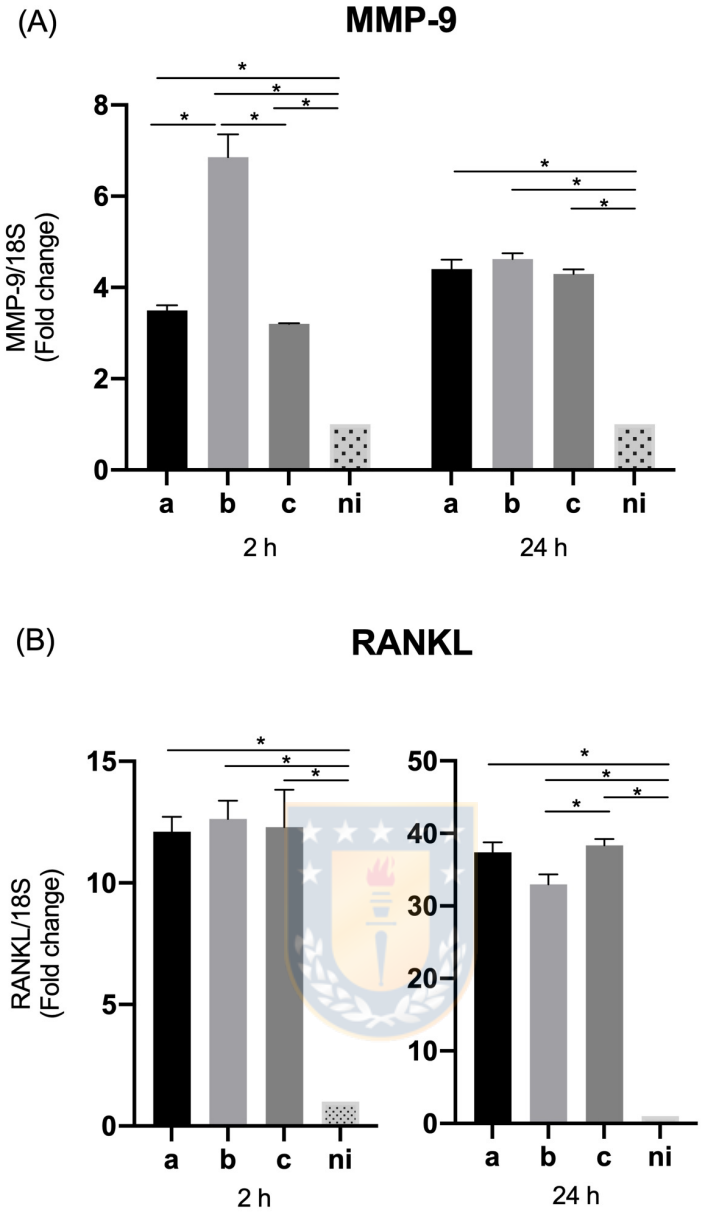

Figure 6. Molecules associated with tissue destruction mRNA expression by *A. actinomycetemcomitans*-induced THP-1 human macrophages (ATCC® TIB-202TM). Expression in macrophage cells infected at a MOI = 10^2 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b), and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) macrophages was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. (**A**) MMP-9. (**B**) RANKL. Asterisks were used to indicate a value of *p* considered statistically significant (* $p < 0.05$).

4.5.7. Expression of TLR Receptors in THP-1 Macrophage Cells

We also evaluate the expression of TLR-2, TLR-4, and TLR-6 by THP-1 cells in response to infection with the aforementioned bacterial serotypes. The qPCR data reveal that the expression of these three receptors was significantly higher ($p < 0.0001$) in response to the three serotypes studied both at 2 and 24 h post infection (Figure 7), with no differences among serotypes for any of the aforementioned receptors at both times studied, with exception of a significantly higher overexpression of TLR-6 at 24 h induced by serotype b over serotypes a ($p = 0.0027$) and c ($p = 0.0002$) without marked differences between the latter two ($p = 0.6301$) (Figure 7C).

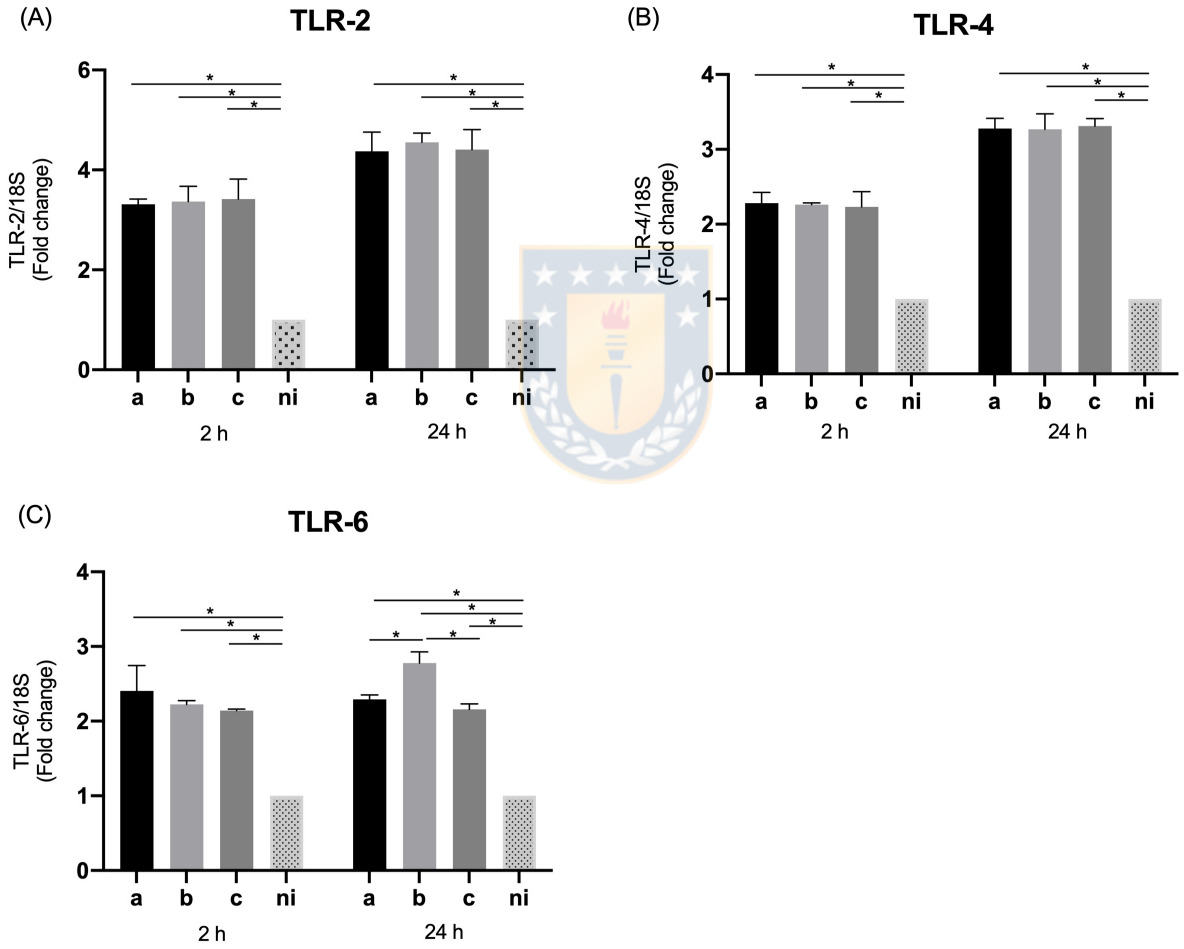

Figure 7. Toll like receptors (TLR) mRNA expression by *A. actinomycetemcomitans*-induced THP-1 human macrophages (ATCC® TIB-202TM). Expression in macrophages infected at a MOI = 10^2 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b), and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative

expression, mRNA expression in non-infected (ni) macrophages was considered as 1, as a reference for fold-change in expression using 18S rRNA ex- pression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. (**A**) TLR-2, (**B**) TLR-4, and (**C**) TLR-6. Asterisks were used to indicate a value of p considered statistically significant (* $p < 0.05$).

4.5.8. Expression of TSLP and ICAM-1 in THP-1 Macrophage Cells

Similar to what was done in epithelial cells, the expression levels of TSLP and ICAM-1 were measured in THP-1 cells infected with the three bacterial serotypes under study.

TSLP showed significantly higher levels of expression ($p < 0.0001$) by THP-1 cells for the three serotypes used, compared to the uninfected condition at both 2 and 24 h. While the differences among serotypes were only statistically significant at 2 h, showing a greater expression in response to serotype a over serotypes b ($p < 0.0001$) and c ($p < 0.0001$), and serotype b over serotype c ($p < 0.0001$). No differences were observed among serotypes at 24 h of infection (Figure 8A).

Regarding the expression levels of ICAM-1 in response to infection with *A. actinomycetemcomitans*, all the serotypes studied were capable of inducing a statistically significant increase in the expression of this molecule compared to the non-stimulated condition, serotype a ($p = 0.0035$), b ($p < 0.00201$), and c ($p = 0.0011$) at 2 h of infection, and serotype a ($p < 0.0001$), b ($p < 0.0001$), and c ($p < 0.0001$) at 24 h of infection (Figure 8B).

No marked differences were observed among serotypes, except at 24 h between serotypes a and b ($p = 0.0291$), being greater for serotype a versus serotype b (Figure 8B).

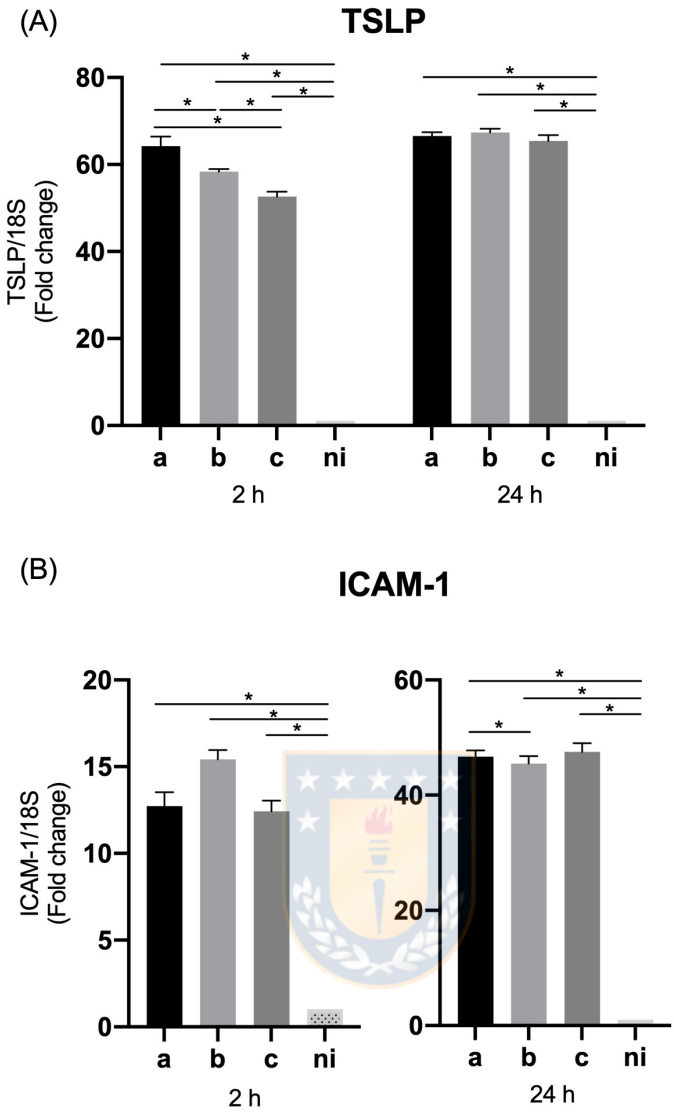

Figure 8. TSLP and ICAM-1 mRNA expression by *A. actinomycetemcomitans*-induced THP-1 human macrophages (ATCC® TIB-202TM). Expression in macrophages infected at a MOI = 10^2 with strains ATCC® 43717^{TM} (serotype a), ATCC® 43718^{TM} (serotype b), and ATCC® 43719^{TM} (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) macrophages was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard de- viation of three independent experiments performed in duplicate. (**A**) TSLP, (**B**) ICAM-1. Asterisks were used to indicate a value of *p* considered statistically significant (* $p < 0.05$).

The fold changes of mRNA relative expression for all molecules mentioned above are summarized in the Table 2.

Table 2. Fold changes of mRNA relative expression in oral epithelial cells and macrophages stimulated with *A. actinomycetemcomitans.*

	Oral Epithelial Cells (OKF6/TERT2) Mean (S.D.)								Macrophage Cells (THP-1) Mean (S.D.)							
	2 h				24 h				2 h				24 h			
	a	**b**	**c**	**n.i.**	**a**	**b**	**c**	**n.i.**	**a**	**b.**	**c.**	**n.i.**	**a**	**b**	**c**	**n.i.**
IL-1β	2.26 (±0.1)	2.87 (±0.1)	3.48 (±0.09)	1	5.09 (±0.4)	3.65 (±0.2)	3.40 (±0.6)	1	14.32 (±0.09)	24 (±1.6)	15.52 (±1.6)	1	159.52 (±7.0)	333.16 (±2.3)	262.03 (±12.3)	1
IL-6	11.75 (±1.0)	15.39 (±0.05)	22.78 (±0.1)	1	7.91 (±0.1)	5.48 (±1.3)	7.73 (±1.0)	1	14.19 (±0.5)	13.94 (±0.6)	9.03 (±0.3)	1	124.13 (±10.7)	149 (±3.0)	164.62 (±3.4)	1
IL-8	57.69 (±1.4)	62.75 (±3.0)	96.01 (±1.6)	1	11.14 (±0.3)	3.77 (±0.1)	34.41 (±0.1)	1	15.53 (±0.9)	8.46 (±1.2)	4.40 (±0.2)	1	65.93 (±3.6)	47.60 (±5.1)	45.63 (±4.0)	1
IL-18	1.20 (±0.1)	2.11 (±0.4)	2.44 (±0.1)	1	0.86 (±0.03)	0.82 (±0.03)	0.87 (±0.05)	1	5.16 (±0.3)	3.75 (±0.2)	3.91 (±0.4)	1	35.98 (±3.2)	25.91 (±1.4)	26.85 (±1.0)	1
TNF-α	18.90 (±0.6)	20.97 (±0.5)	50.64 (±6.6)	1	2.61 (±0.2)	2.37 (±0.09)	2.11 (±0.009)	1	18.51 (±0.4)	20.99 (±0.8)	20.29 (±0.07)	1	29.54 (±0.3)	26.74 (±1.0)	26.15 (±0.7)	1
MMP-9	2.59 (±0.2)	3.31 (±0.3)	4.66 (±0.2)	1	3.64 (±0.1)	11.23 (±0.07)	35.21 (±2.3)	1	3.49 (±0.1)	6.86 (±0.4)	3.20 (±0.01)	1	4.40 (±0.2)	4.62 (±0.1)	4.3 (±0.09)	1
RANKL	2.53 (±0.2)	4.77 (±0.1)	2.99 (±0.4)	1	3.10 (±0.01)	4.17 (±0.05)	3.12 (±0.04)	1	12.10 (±0.6)	12.63 (±0.7)	12.29 (±1.5)	1	37.40 (±1.3)	32.99 (±1.3)	38.34 (±0.9)	1
TLR-2	1.66 (±0.1)	2.01 (±0.2)	1.32 (±0.1)	1	1.55 (±0.2)	1.58 (±0.01)	1.47 (±0.02)	1	3.31 (±0.1)	3.36 (±0.3)	3.41 (±0.4)	1	4.37 (±0.3)	4.55 (±0.1)	4.40 (±0.3)	1
TLR-4	2.65 (±0.1)	2.62 (±0.2)	2.85 (±0.1)	1	4.80 (±0.8)	4.75 (±1.0)	4.46 (±0.3)	1	2.28 (±0.1)	2.26 (±0.02)	2.23 (±0.2)	1	3.27 (±0.1)	3.27 (±0.2)	3.31 (±0.1)	1
TLR-6	0.97 (±0.1)	1.50 (±0.2)	1.61 (±0.09)	1	1.71 (±0.03)	1.36 (±0.01)	1.71 (±0.03)	1	2.40 (±0.3)	2.22 (±0.04)	2.14 (±0.02)	1	2.29 (±0.06)	2.77 (±0.1)	2.15 (±0.07)	1
TSLP	11.49 (±0.1)	7.38 (±0.9)	10.14 (±1.8)	1	5.57 (±0.7)	3.04 (±0.4)	8.15 (±0.4)	1	64.22 (±2.2)	58.37 (±0.5)	52.62 (±1.1)	1	66.54 (±0.8)	67.38 (±0.8)	65.44 (±1.3)	1
ICAM-1	14.29 (±0.01)	12.59 (±0.1)	16.87 (±0.7)	1	1.95 (±0.1)	2.33 (±0.2)	2.15 (±0.08)	1	12.72 (±0.8)	15.42 (±0.5)	12.42 (±0.6)	1	46.69 (±1.1)	45.48 (±1.2)	47.50 (±1.5)	1
18S	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Data are represented as fold-change for 3 independent experiments, mean and standard deviation (S.D.) for each condition are showing. For relative expression, mRNA expression in non-infected (n.i.) macrophages and oral epithelial cells were considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. (**a:** Sereotype a, **b:** Serotype b, **c:** Serotype c, **n.i.:** Non-infected condition).

4.6. Discussion

Epithelial cells and macrophages are a natural physical barrier and the first defense mechanisms at the level of periodontal sulcus or pocket, preventing the penetration of microorganisms into the deep connective tissue. They also act as a surveillance system to mount an inflammatory response to microbial colonization, expression of cytokines, chemokines, tissue destruction molecules, and modification of the expression of receptors associated with the recognition of microorganisms [43].

Regarding the expression of cytokines, our data are consistent with the available evidence that supports the expression of inflammatory mediators by macrophages and epithelial cells in response to periodontal pathogens, showing an increase in these molecules at the level of the gingival crevicular fluid in sites that exhibit destruction of periodontal tissues [44–47].

The role of cytokines in the progression of periodontitis is extremely important. These molecules are modulators of both homeostasis and inflammation, being the first response signal against pathogens by stimulating the barrier site and communicating it with professional immune cells such as T and B lymphocytes, dendritic cells, and NK cells [48]. The cytokines participating in the immune response against periodontal pathogens can be categorized into three stages. A first wave of cytokines expressed by epithelial cells, gingival fibroblasts and innate immune cells, produced directly due to the contact of the microorganisms with the host cells. A second group of cytokines associated with the differentiation of specific subpopulations of lymphocytes, secreted by polymorphonuclear cells, antigen-presenting cells, phagocytes and local lymphocytes in response to the stimulus of the microbiome. Finally, a third wave of cytokines, expressed by the already differentiated lymphocyte subpopulations, which participates either as feedback mechanisms or direct effector of inflammation and activation of tissue destruction pathways in case of disease [49].

IL-1β, a cytokine member of the IL-1 family involved in both innate and adaptive immunity and associated with inflammation, is overexpressed by both epithelial and macrophage cells in response to the three bacterial serotypes studied, as shown by Dickinson

et al., where gingival epithelial cells were stimulated with *A. actinomycetemcomitans* VT1169 at 2 and 24 h, showing a greater secretion of IL-1β, TNF-α, IL-8, and IL-6 mainly at 24 h compared to the control condition [50]. There is little evidence that analyzes the expression of IL-1β in macrophages comparing among *A. actinomycetemcomitans* serotypes, however in a study in which macrophage-like cells were pretreated for 24 h with *A. actinomycetemcomitans* LPS, high concentrations of IL-1β were observed in the cell supernatant compared to the control, this study also highlights that the LPS of *A. actinomycetemcomitans* induces a tolerance response in macrophages that alters the secretion of IL- β and TNF- α as well as of the tissue-degrading enzyme MMP-9, modulating the host inflammatory response [51].

In the case of IL-18, a chemokine whose main activity is polynuclear neutrophil attractant with an important role in periodontitis development and described as critical in driving the pathologic breakdown of barrier integrity in different cells models, our results are consistent with those obtained in other studies such as Ando-Suguimoto et al., where the transcription of IL -1β and IL-18 was upregulated in macrophages and oral epithelial cells after 1 h interaction with *A. actinomycetemcomitans*, and similar to our results, this positive regulation persisted only in macrophages, in our case for 24 h post infection [52].

There is little evidence of the role of IL-18, a pro-inflammatory cytokine also known as an interferon-gamma inducing factor in the response to periodontal pathogens, how- ever the evidence available in other epithelial cell models suggests this molecule plays a key function in the control of the barrier function of epithelia, being critical in driving the pathologic breakdown of barrier integrity allowing the passage of microorganisms to sub-epithelial tissues [53,54], and probably its dissemination regulating the expression of matrix metalloproteinase 9 (MMP-9) [55].

Both IL-1β and IL-18 expression have been described as a consequence of the activation of the inflammasome, a macromolecular complex that, through various signals, participates in the processing of inflammatory pro-interleukins to their active forms. These signals range from the recognition of molecular patterns associated with microorganisms

(MAMPs) and damage (DAMPs), as well as the entry of various molecules, and in the case of the entry of some virulence factors into cells [56,57].

The expression of NLRP3, which is involved in inflammasomes, plays a key role in periodontitis, being higher in periodontitis gingival tissues than in healthy tissues, especially at the epithelium layer [58]. And at the salivary level, higher levels of NLRP3 were found in cases of periodontitis compared to healthy subjects [59].

Some components of the inflammasome, such as apoptosis-associated speck-like protein containing a CARD (ASC) and absent in melanoma 2 (AIM2) proteins and NLRP3, are upregulated by *A. actinomycetemcomitans* infection in THP-1 cells and human mononuclear leukocytes [60,61]. However, in the case of epithelial cells, different signaling and activation pathways of the inflammasome are induced in the response against *A. actinomycetemcomitans*, where it has been shown that this bacterium enhances the expression of NLRP3, TLR4, TLR2, and NOD2 in macrophages but not in human gingival epithelial cells [52].

In our case, the differences in magnitude in the expression of IL-1β and IL-18 between macrophages and oral epithelial cells, could reinforce the idea that the response to a pathogen not only depends on the stimulus (type of bacteria, serotype, strain, etc.) but also on cell type, as in the case of osteoblastic cells which, when stimulated with *A. actinomycetemcomitans*, apoptosis is promoted at least partially through the NLRP3 inflammasome activation [62].

The future study of the expression of inflammasome components in response to the different serotypes of *A. actinomycetemcomitans* and the role of its virulence factors, could be a way to elucidate the mechanisms of the differential response against this bacterium that is observed only in some cell types such as professional immune cells, but not in innate immune cells [26–30].

MMP-9, the main gelatinase in oral fluids involved in the degradation of the extracellular matrix [63], is expressed by various cell types and activated or released by pro-inflammatory cytokines such as IL-1 β, TNF- α and some proteases derived from pathogens of the periodontal biofilm [64]. Our results for the cells types studied, suggests that it begins

to be expressed by epithelial cells from the initial stages of infection of periodontal tissues, similar to how it occurs in other epithelia and macrophages [65–67]. In the case of macrophages, the LPS of *A. Actinomycetemcomitans* induces the expression of MMP-9 as described previously [68]; likewise, in our hands, using the complete bacteria, we found a higher expression of this molecule but without a differential expression pattern by a specific serotype, except at 2 h on the part of serotype b compared with serotypes a and c, but reaching similar levels for all serotypes at 24 h post-infection.

The expression of RANKL, a molecule associated with bone destruction for its ability to activate osteoclasts, by oral epithelial cells and macrophages has been extensively studied, with special attention to the effect of other cytokines such as TNF- α on the modulation of its expression, particularly at the epithelial level [69–71].

There is little evidence regarding the effect of the stimulation of oral epithelial cells with periodontal microorganisms and its effect on the expression of this mediator of bone destruction. As such, the expression levels by epithelial cells and macrophages stimulated with the three *A. actinomycetemcomitans* serotypes suggest that these cell types support bone destruction, from the earliest stages of infection, or at least configure an inflammatory environment conducive to other cells, such as osteoclasts or pre-osteoclasts to activate their bone destruction mechanisms.

A. actinomycetemcomitans has lipopolysaccharide, an endotoxin recognized by TLR2 *(*a molecule that recognizes acylated bacterial lipoproteins and signals as a heterodimer with either TLR1 or TLR6) and TLR4 (a transmembrane protein that participates in the recognition of LPS in Gram-negative bacteria), being TLR4 the main receptor for LPS [72]. The overexpression of these receptors together with their role in the expression of pro-inflammatory cytokines by various cell types, in response to periodontal pathogens has been demonstrated in vitro and in vivo; for example, macrophages TLR2 -/- and TLR4 -/- stimulated with *A. actinomycetemcomitans* exhibited an attenuated production of the pro-inflammatory cytokines TNF-α and IL-6 [72].

On the other hand, it has been observed that the expression of TLR2 and TLR4 in gingival epithelial cells is higher in individuals with diabetes and is positively regulated

according to the severity of periodontal disease [73], and that TLR2 and TLR4 would have a role in the control of infection by *A. actinomycetemcomitans* [74,75]. This evidence is consistent with our results that show an increase in the expression of toll-like receptors on both macrophages and epithelial cells in response to the three bacterial serotypes at both time points analyzed. However, in the case of TLR6, even though its expression has been demonstrated both in oral epithelial cells and in macrophages, there are no studies to date that analyze in depth its role in the cellular response against bacteria with structural variables such as those of *A. actinomycetemcomitans*, both independently or as forming TLR2/TLR6 dimers. In our case, and for the previously described experimental conditions, a discrete increase in TLR6 expression could be observed regardless of the serotype used, mainly in macrophage cells. However, these data could be different under other experimental conditions or reference parameters.

The expression and function of TLR in the oral cavity are key to the maintenance of homeostasis of oral tissues, given the constant presence of commensal microorganisms. The expression of these receptors is not only mediated by the state of the tissues (inflamed versus non-inflamed) but they are also regulated in terms of their location. TLR2 and TL4 are highly expressed in the epithelial basal layers but less so in the cells of the superficial layers where the exposure to the environment and microorganisms is greater [76], an aspect against which the analysis of this study is limited, as we worked with a cell monolayer models.

More studies on the role of Toll-like receptors in the recognition of the structural variables of *A. actinomycetemcomitans* are required, mainly for the case of TLR4. This receptor dimerized with myeloid differentiation factor 2 (MD-2) forms the heterodimer responsible for recognizing a common pattern in the structurally diverse LPS molecules [77]. Lipid A corresponds to the conserved molecular pattern of LPS and is the main inducer of the immune response against LPS, with TLR4 together with MD-2 being responsible for the physiological recognition of LPS [78]. In this sense, additional evidence is required to support how this receptor capable of recognizing the conserved fraction of LPS (lipid A), could be modulating a serotype-dependent differential immune response (as it occurs in some cell types) [26–30], based on structural variables typical of the O antigen, a region not described as recognized by this receptor. Probably other receptors such as NOD2 or NLRP3, and other

virulence factors could be contributing to the inflammatory response induced by these bacteria [79].

Thymic stromal lymphopoietin (TSLP) an analogous of IL-7 cytokine, is a critical fac- tor linking responses at interfaces between the body and the environment, expressed by various cell types such as epithelial cells and epidermal keratinocytes, mast cells, airway smooth muscle cells, fibroblasts, dendritic cells, trophoblasts, and cancer or cancer-associated cells [80]. Environmental factors such as Toll-like receptor ligands, a NOD2 ligand, viruses, microbes, allergen sources and cigarette smoke trigger TSLP production, and pro- inflammatory cytokines induce or enhance TSLP production [80,81]. To date, there is no evidence of the expression of this molecule by macrophages and oral epithelial cells in response to periodontal pathogens such as *A. actinomycetemcomitans.* However, our data show that *A. actinomycetemcomitans* has the ability to induce TSLP expression in epithelial cells and to a much greater extent in macrophages compared to unstimulated cells, opening the possibility of studying this molecule as one of the key mediators in the response of the first cells that come into contact with the pathogen in the initial stages of periodontitis.

In the case of ICAM-1, also known as CD54, an increase in its expression has been described in macrophages stimulated with *A. actinomycetemcomitans* LPS [82], as well as gingival epithelial cells induced with 5 different strains of *A. actinomycetemcomitans* [83,84]. Our data are in agreement with the available evidence showing an increase in ICAM-1 expression for both types of cells studied in response to the three bacterial serotypes, without a sustained tendency to a differential response of one serotype over an- other. Based on these data, the role of periodontal pathogens in the increase of intercellular adhesion molecules can be affirmed, which could serve as a mechanism to retain inflammatory cells at the site of infection, limiting the spread of pathogens and concentrating inflammatory cells from the first contact by the host with the microorganisms [85,86].

The molecules expressed by the cell types used in this study correspond to molecules belonging to the first and second wave of mediators expressed in the initial stages of periodontitis. In the case of epithelial cells, for most of the molecules studied, the highest levels of expression were observed at 2 h post stimulation, probably due to the ability of these

cells to be rapid responders and to give the first warning signal when encountering some pathogens, contrary to what happened in macrophages, where a large part of the genes evaluated showed their highest expression levels at 24 h post-infection. In this way, the synchrony as a function of time of the different cell types present in periodontal tissues is confirmed, from keratinocytes and macrophages involved in the first discharges of inflammatory mediators to the subsequent expression of molecules by specialized cell sub-populations capable of inducing specific cytokines profiles. [27,28]

In agreement with this, when bacterial serotypes of the present study have been used in other cell models (such as professional immune cells), the expression patterns of cytokines, chemokines and TLR receptors is different from those found by us, showing a differential response pattern against one serotype over others, which would be a property of specialized cells in the recognition of structural variables in the most advanced stages of infection [26–30], contrary to the first phases of infection where the cells involved would develop a generic inflammatory response, without discriminating the structural variables of the pathogen, but rather acting as a first alarm signal for the assembly of a more specific response by cells with the ability to recognize virulence variability.

In addition, it should be noted that the heterogeneity in the virulence of *A. actinomycetemcomitans* is not only present among serotypes, also differences in expression induced by strains of the same serotype were observed in in vitro cell models, as strains ATCC® 29524, ATCC® 29522 and ATCC® 43718, all belonging to serotype b, which show substantial differences between them in the expression, for example, of IL-8 and ICAM-1, in epithelial cells at 1, 3, 6, 12, and 24 h of infection, demonstrating the wide variability of virulence of this pathogen [83], and supporting the idea that the mere presence of serotype b would not necessarily implicate greater severity of periodontitis and would not be enough to establish a new pathological entity, as aggressive periodontitis was until a not long ago [87].

Finally, the study of cytokines and chemokines from all their angles is important considering the nexus among periodontal inflammation, polymicrobial biofilm and disease. Inflammation has been described with a central role in the modulation of the periodontal biofilm and in the continuous passage from a state of health, to gingivitis and later

periodontitis, known as the IMPEDE model (inflammation-mediated-polymicrobial-emergence and dysbiotic-exacerbation), where the initial inflammation contributes to biofilm dysbiosis, modulating the expression of virulence factors by periodontal microorganisms (many of them known as inflammophilic bacteria) and modifying the gene expression of the host cells [88]. As such, although many bacteria are not fundamentally responsible for tissue damage, they contribute to the inflammatory environment, which is conducive to the development of other pathobionts present in the periodontal niche [89].

More studies are needed to understand the mechanisms by which *A. actinomycetemcomitans* modulates the response of different cell types, and how this host-pathogen interaction modifies the gene expression of both the microorganism and the infected cells [90].

4.5. Conclusions

A. actinomycetemcomitans serotypes a, b, and c have the ability to induce an increase in the expression of cytokines, chemokines, tissue destruction molecules (MMP9 and RANKL), TSLP and ICAM-1 in oral epithelial cells and macrophages, showing an increase in the earlier expression for epithelial cells compared to macrophages, and in both cases without a serotype-specific pattern associated with the increase of the production of the molecules mentioned above.

4.6. Author Contributions

Conceptualization, D.B. and A.O.; methodology, D.B and A.O.; writing original draft preparation, D.B. and C.M.G.; writing review and editing, A.O. D.B.; supervision, A.O.; project administration, A.O.; funding acquisition, A.O. All authors have read and agreed to the published version of the manuscript.

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4.9. Conflicts of Interest

The authors declare no conflict of interest.

4.10. References

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CAPÍTULO 5:
Paper 2

Betancur D, Muñoz Grez C, Soto R, Oñate A. **Expression of macrophage polarization markers in response to the different serotypes of *Aggregatibacter actinomycetemcomitans*.** Biomolecules. 2021 (in press)

5.1. Abstract

Aggregatibacter actinomycetemcomitans, a Gram-negative bacterium with seven serotypes (a–g) according to the structure of its LPS, has been defined as one of the most important pathogens in the development of a dysbiotic periodontal biofilm and the onset of periodontitis (an inflammatory chronic diseases of the tissues around the teeth), where the serotype b is characterized as the most virulent compared with the other serotypes. The aim of this study was to evaluate the expression of macrophages polarization markers M0, M1 and M2 against *A. actinomycetemcomitans*. Methods: THP-1 cells were infected with A. actinomycetemcomitans serotypes a, b and c. The expression of CD11b, CD4, CD14, CD68 for M0; IL-6, HLA/DRA, CXCL10 for M21 and IL-10, CD163, fibronectin 1 FN1 and CCL17 was evaluated by qPCR at 2 and 24 h after infection. Results: An increase in the expression of these molecules was induced by all serotypes at both times of infection, showing higher levels of expression to the M1 panel at 2 and 24 hours compared to other markers. Conclusions: *A. actinomycetemcomitans* has a role in the macrophage polarization to M1 phenotype in a non-serotype dependent manner.

5.2. Keywords

Macrophages, periodontitis, *Aggregatibacter actinomycetemcomitans*, intracellular bacteria, macrophages polarization, innate immunity.

5.3. Introduction

The oral microbiome has been defined as a community of microorganisms of at least 1000 microbial species including bacteria, viruses, fungi, archaea and protozoa that live in

the oral cavity distributed according to the characteristics of each ecological niche available in the mouth [1, 2]. This microbiome has been proposed as an etiological agent of various pathologies of the oral cavity such as cavities, gingivitis, periodontitis and diseases associated with the placement of implants and intraoral medical devices [3, 4]. However, the evidence available on bacterial populations at the oral level shows that the mere presence of bacteria is not enough to produce these pathologies [5], and that a disruption of the balance of interactions between microbial communities and the host is necessary [6].

The term *"oralome"* has been coined to define all the interactions that take place between the oral microbiome and the host, in processes such as the maturation and development of an appropriate oral immune response, and making evident the importance of the interaction between host and pathogens in the initiation and development of highly prevalent diseases such as tooth decay or periodontitis [7-9].

Currently periodontitis is defined as a non-communicable chronic, multifactorial, and inflammatory disease associated with a dysbiotic biofilm, with inflammation and destruction of the tooth-supporting and protection tissues with clinical attachment loss, alveolar bone destruction, formation of periodontal pocketing and gingival bleeding [10].

Periodontitis has an infectious etiology, associated with the presence of a pathogenic bacterial communities in the tissues surrounding the tooth, where of the approximately 700 cultivable oral bacterial species described, 400 make up this subgingival biofilm, located in the limited space between the tooth and the gum [11,12].

Many pathogens defined as *"keystone pathogens"* due to their central role in the development of the dysbiotic biofilm have been associated with the progression and severity of this pathology, the level of tissue destruction and the subversion of the host's immune system, such as Porphyromonas gingivalis and *Aggregatibacter actinomycetemcomitans* [13-15].

A. actinomycetemcomitans, is a Gram negative, non-motile, facultative anaerobic, and capnophilic coccobacillus present in both health and periodontal pathologies with a

heterogeneous virulent behavior depending on the conditions available in the environment in which it is found [16-17]. The structural differences of its lipopolysaccharide (LPS) have led to the description, based on the composition of the O-polysaccharide chain of this structure seven different serotypes (a–g) being serotypes a, b and c the most prevalent in the oral cavity in humans [18, 19].

In many studies, serotype b has been described to the most virulent and prevalent in periodontitis patients, and in different cell models it has shown to induce a greater response in terms of production of chemokines, cytokines, cytokine receptors, and tissue destruction molecules such as metalloproteinases (MMP) or receptor activator nuclear kappa ligand (RANKL), compared to the other serotypes present in the oral cavity [20-24]. Despite this, to this day there is little evidence about this differential character of the response induced by these serotypes in other cell types such as macrophages and the effect that this variable virulence could have on phenomena such as the polarization to M1 or M2 phenotype during both the progression and resolution stages of periodontitis.

In relation to this, extensive evidence has shown the ability of macrophages and other immune and structural cells to recognize periodontal pathogens, including *A. actinomycetemcomita*ns, and produce cytokines, chemokines, antibacterial proteins, alarmins molecules, and other molecules in response to this bacterium [25–28].

Nevertheless, the possible differential response of these cell types against the bacterial serotypes a, b and c is not entirely clear yet, with little knowledge about if this variable response is a characteristic of only professional immune cells such as dendritic cells, lymphocytes among others [20-24], or if the structural differences of this microorganism could have an effect at the level of innate host defense from the earliest stages of infection, modulating in a serotype-dependent manner phenomena such as macrophage polarization, currently defined as key in the development and resolution of infectious and inflammatory processes.

This study aimed to analyze the expression of macrophages polarization markers (M0, M1 and M2) against *A. actinomycetemcomitans*, and to measure if this genic expression has a serotype-dependent differential character as described for other cell models [20-22]. For this, an in vitro infection model previously used by our team [29] was employed, using the most prevalent *A. actinomycetemcomitans* serotypes and macrophages cells to simulate the initial stage of periodontitis.

5.4. Methods

5.4.1. Bacteria Stains

The *A. actinomycetemcomitans* serotype a (ATCC® 43717), serotype b (ATCC® 43718), and serotype c (ATCC® 43719) were cultured at 37 °C in capnophilic conditions (8% O_2 and 12% CO_2) in BHI broth (70138 GranuCult. Merck®, Darmstadt, Germany) supplemented with 10% horse serum (H1270 Sigma-Aldrich®, Gillingham, UK) for 24 hours based on the growth curves previously obtained by our group under standard conditions. For each experiment, bacteria were used at the exponential growth phase in order to obtain a reliable number of microorganisms with full immunogenic potential.

5.4.2. THP-1 Derived Macrophages Culture

The cell line ATCC®TIB-202 of human monocytic leukemia (THP-1) was cultured in RPMI-1640 medium (R8758 Gibco®, Carlsbad, CA, USA) with 10% fetal bovine serum (F2442 Sigma-Aldrich®) and supplemented with glucose (G8270 Sigma-Aldrich®, Gillingham, UK) (14 mM final concentration), pyruvic acid (107360 Sigma-Aldrich®, Gillingham, UK) (1mM final concentration), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (H3375 Sigma-Aldrich®, Gillingham, UK) (10 mM final concentration. pH 7.35), 2-mercaptoethanol (M6250 Sigma-Aldrich®, Gillingham, UK) (0.5 mM final concentration) and penicillin/streptomycin (15140-122 Gibco®, Carlsbad, CA, USA) (100 U/mL final concentration of penicillin and 100 µg/mL of streptomycin) under a humidified atmosphere at 37 °C in 5% CO_2.

To produce differentiation into macrophagic cells, THP-1 cells were grown at the same aforementioned conditions, using phorbol 12-myristate 13-acetate (PMA) (16561-29-8 Sigma-Aldrich®, Gillingham, UK) (100 nM final concentration) for 2 days, according with the protocol previously described (30, 31).

5.4.3. Infection Assay

THP-1 cells were prepared using 1×10^6 cells per well of a 6 wells plate and activated for 48 hours as mentioned above. Bacteria were grown, washed with phosphate buffered saline solution (PBS), suspended in PBS and added to THP-1 cultured using a multiplicity of infection (MOI) of 100, approximately. The plates were centrifuged for 10 minutes at 300×g (RCF) to achieve the contact between the cell layer at the bottom of the plate and the bacteria. Plates were incubated for 90 minutes at 37 °C in 5% CO_2 allowing for the internalization of bacteria. Finally, cells were washed and incubated with fresh medium supplemented with gentamicin (G1914 Sigma-Aldrich®, Gillingham, UK) and metronidazole (M3761 Sigma-Aldrich®, Gillingham, UK), (300 µg/mL and 200 µg/mL respectively) for the post infection times defined (2 and 24 hours).

5.4.4. RNA Extraction and RT-PCR

Total RNA was isolated from the cells using TRIzol® reagent (T9424 Invitrogen® Sigma-Aldrich®, Gillingham, UK) according to the manufacturer's instructions; 2000 ng of the extracted RNA were used in the reverse transcription reaction using the First-Strand cDNA Synthesis SuperMix kit (18080400 Invitrogen®. Thermofisher®, Waltham, MA, USA) following the manufacturer's protocol for reverse transcription with DNAse digestion.

5.4.5. qPCR

Three panels of markers were used to define the macrophagic phenotypes. For M_0 macrophages we used the expression of CD11b, CD4, CD14 and CD68. M_1 were evaluated by the expression of interleukine 6 (IL-6), HLA class II histocompatibility antigen DR alpha chain (HLA/DRA) and C-X-C Motif Chemokine Ligand 10 (CXCL10) and finally M_2

phenotype using the markers CD206, interleukine 10 (IL-10), CD163, fibronectin 1 (FN1) and C-C motif chemokine ligand 17 (CCL17).

The mRNA expression of the markers was measured using quantitative real-time polymerase chain reaction (qPCR). For this purpose, 30 ng of cDNA were amplified with the primers previously designed in the platform Ensembl Genome and Primer-BLAST (NCBH-NIH), using the following sequences: CD11b: forward primer 5′-cagcctttgaccttatgtcatgg -3′ and reverse primer 5′-cctgtgctgtagtcgcact -3′; CD4: forward primer 5′-cctcctgcttttcattgggctag -3′ and reverse primer 5′- tgaggacactggcaggtcttct -3′; CD14: forward primer 5′- ctggaacaggtgcctaaaggac-3′ and reverse primer 5′- gtccagtgtcaggttatccacc-3′; CD68: forward primer 5′- cgagcatcattctttcaccagct-3′ and reverse primer 5′-atgagaggcagcaagatggacc-3′; IL-6: forward primer 5′- agacagccactcacctcttcag-3′ and reverse primer 5′- ttctgccagtgcctctttgctg-3′; HLA-DRA: forward primer 5′-aaaaggagggagttacactcagg-3′ and reverse primer 5′- gctgtgagggacacatcagag-3′; CXCL10: forward primer 5′- ggtgagaagagatgtctgaatcc-3′ and reverse primer 5′-gtccatccttggaagcactgca-3′; CD206: forward primer 5′- gcaaagtggattacgtgtcttg-3′ and reverse primer 5′- ctgttatgtcgctggcaaatg-3′; IL-10: forward primer 5′- tctccgagatgccttcagcaga-3′ and reverse primer 5′- tcagacaaggcttggcaaccca-3′; CD163: forward primer 5′-ccagtcccaaacactgtcct-3′ and reverse primer 5′- atgccagtgagcttcccgttcagc-3′; FN1: forward primer 5′- acaacaccgaggtgactgagac-3′ and reverse primer 5′- ggacacaacgatgcttcctgag-3′; CCL17: forward primer 5′- ccagggatgccatcgtttttgtaactgtgc-3′ and reverse primer 5′-cctcactgtggctcttcttcgtccctggaa-3′; 18S: forward primer 5′- ctcaacacgggaaacctcac-3′ and reverse primer 5′- cgctccaccaactaagaacg-3′;

Takyon® No Rox SYBR® MasterMix dTTP Blue (UF-NSMT-B0701 Eurogentec®, Seraing, Belgium) reagent was used in an AriaMx Real-time PCR System (Agilent®, Santa Clara, CA, USA) using this thermal profile: 95 °C for 3 min, followed by 40 cycles of 90 °C for 5 seconds and 60 °C for 30 s, ending with a melt curve of 95 °C for 15 seconds, 60 °C for 1 min, and 95 °C for 15 seconds, for detection of non-specific amplification products that could lead to false positive signals. 18S rRNA expression levels were used as a normalizing endogenous control

5.4.6. Statistical Analysis

The relative quantification was obtained normalizing each gene mRNA expression to 18S rRNA expression using the $2^{-\Delta\Delta Ct}$ method (32). The qPCR data were analyzed using the software GraphPad Prism 8.0 (GraphPad Software.Inc, San Diego, CA, USA). The normality distribution of data was evaluated using Kolmogorov-Smirnov test and the differences among groups using Tukey`s test and two ways ANOVA analysis. Asterisks indicate a p-value considered statistically significant ($p<0.05$). The data were expressed as fold-change means and standard deviation for 3 independent experiments performed at different times as biological replicates and qPCR reactions for each gene and sample were performed in duplicates as technical replicates.

5.5. Results

5.5.1. Expression of M_0 markers in response to Aggregatibacter actinomycetemcomitans serotypes.

We evaluated the effect of *A. actinomycetemcomitans* serotypes on expression of M_0 markers by THP-1 cells. For the case of CD11b a statistically significant overexpression was observed for the three serotypes evaluated compared to the non-infected control at 2 hours ($p=0.0001$, $p=0.0004$, and $p<0.0001$ for the serotypes a, b, and c respectively) as well as at 24 hours, when the three serotypes are compared with the non-infected condition ($p<0.0001$ for all serotypes). Regarding the differences among serotypes at 2 hours, statistically significant differences were observed between serotypes b and c, with a higher expression induced by serotype b ($p=0.0194$). On other hand, after 24 hours of infection there was a statistically significant greater expression for serotype c compared with serotype a ($p=0.0021$) and b ($p=0.0033$), without differences between the last two mentioned ($p=0.9962$) (Figure 1A).

When the expression of CD4 was measured, a statistically significant higher expression was observed for the three serotypes studied at both times of infection compared with the control condition ($p < 0.0001$); however, no statistically significant differences were observed among the serotypes at both times of infection (Figure 1B).

In the case of CD14, statistically significant differences were observed for the three serotypes studied at both times of bacteria stimulation compared with the control condition ($p<0.0001$). In terms of the differences among serotypes just at 24 hours of infection an overexpression of this molecule was induced by serotypes c with respect to the serotype a ($p=0.0232$) and b ($p=0.0002$) (Figure 1C).

CD68 was measured and a statistically significant higher expression was observed for the three bacteria studied at 2 and 24 hours of infection compared with the non-infected condition ($p < 0.0001$); however, statistically significant differences were observed among the serotypes only at 2 hour of infection by the serotype a over the serotype b ($p=0.0087$) (Figure 1D).

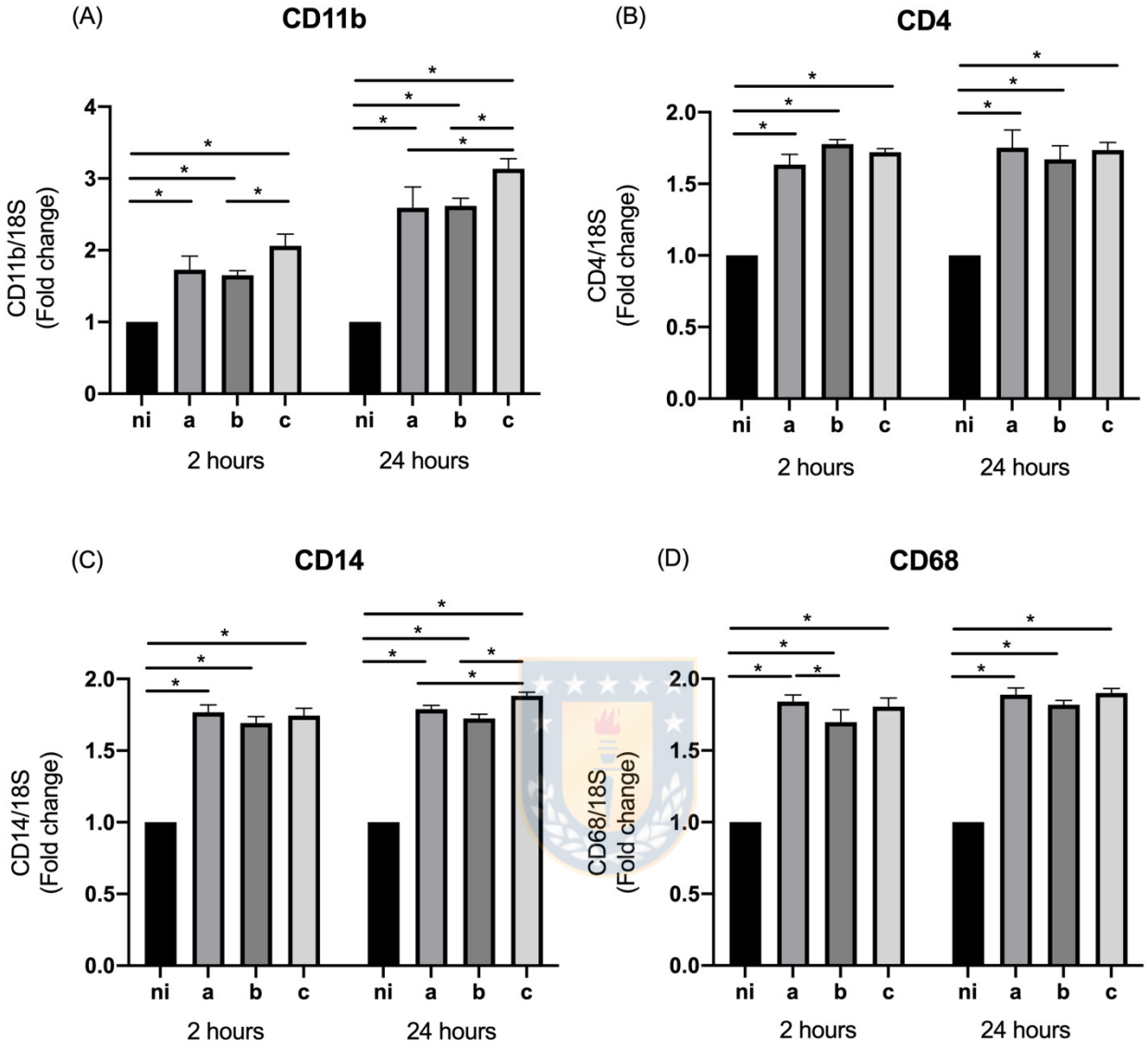

Figure 1. M_0 markers expression by *Aggregatibacter actinomycetemcomitans*-induced THP-1 human macrophages (ATCC® TIB-202TM). Expression in macrophages infected at a MOI = 10^2 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b), and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) macrophages was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. Asterisks were used to indicate a value of p considered statistically significant (* $p < 0.05$).

5.5.2. Expression of M_1 markers in response to Aggregatibacter actinomycetemcomitans serotypes.

When the M_1 markers panel is measured, IL-6 showed statistically significant differences for the three serotypes studied at both times of bacteria stimulation compared with the control condition ($p=0.0006$, $p=0.0060$, and $p=0.0138$ for the serotypes a, b, and c respectively at 2 hours of infection and $p=0.0005$, $p=0.0008$, and $p< 0.0001$ for the serotypes a, b, and c respectively at 24 hours). The differences among the serotypes for this target just were observed at 2 hours of stimulation by the serotype a ($p=0.0050$) and b ($p=0.0215$) over the serotype c, without statistically significant differences between them, and at 24 hours for the case of serotype c over the serotype a ($p=0.0275$) (Figure 2A)

Regarding expression levels of HLA/DRA, the serotypes a, b and c were capable of inducing a statistically significant overexpression of this molecule compared to the non-infected condition at both times of infection ($p=0.0004$, $p=0.0110$, and $p=0.0045$ for the serotypes a, b, and c respectively at 2 hours of infection and $p=0.0042$, $p< 0.0001$, and $p< 0.0001$ for the serotypes a, b, and c respectively at 24 hours). At two hours we observed higher levels of HLA/DRA expression for serotype a over serotype b ($p=0.0303$) and for serotype c over serotype b ($p<0.0001$). On the other hand, at 24 h of stimulation statistically significant differences in the expression were observed regarding the b serotype in comparison with the serotype c ($p= 0.0127$) (Figure 2B).

In terms of the expression of CXCL10, data by macrophage cells samples for all serotypes studied showed statistically significant differences in comparison with the non-infected control condition at 2 and 24 hours of infection ($p=0.0036$, $p=0.0004$, and $p=0.0050$ for the serotypes a, b, and c respectively at 2 hours of infection and $p < 0.0001$ for the three serotypes at 24 hours). The statistically significant differences among the serotypes were observed just at 24 hours of infection with a higher expression induced by the serotype a over the serotype b ($p=0.0002$) (Figure 2C).

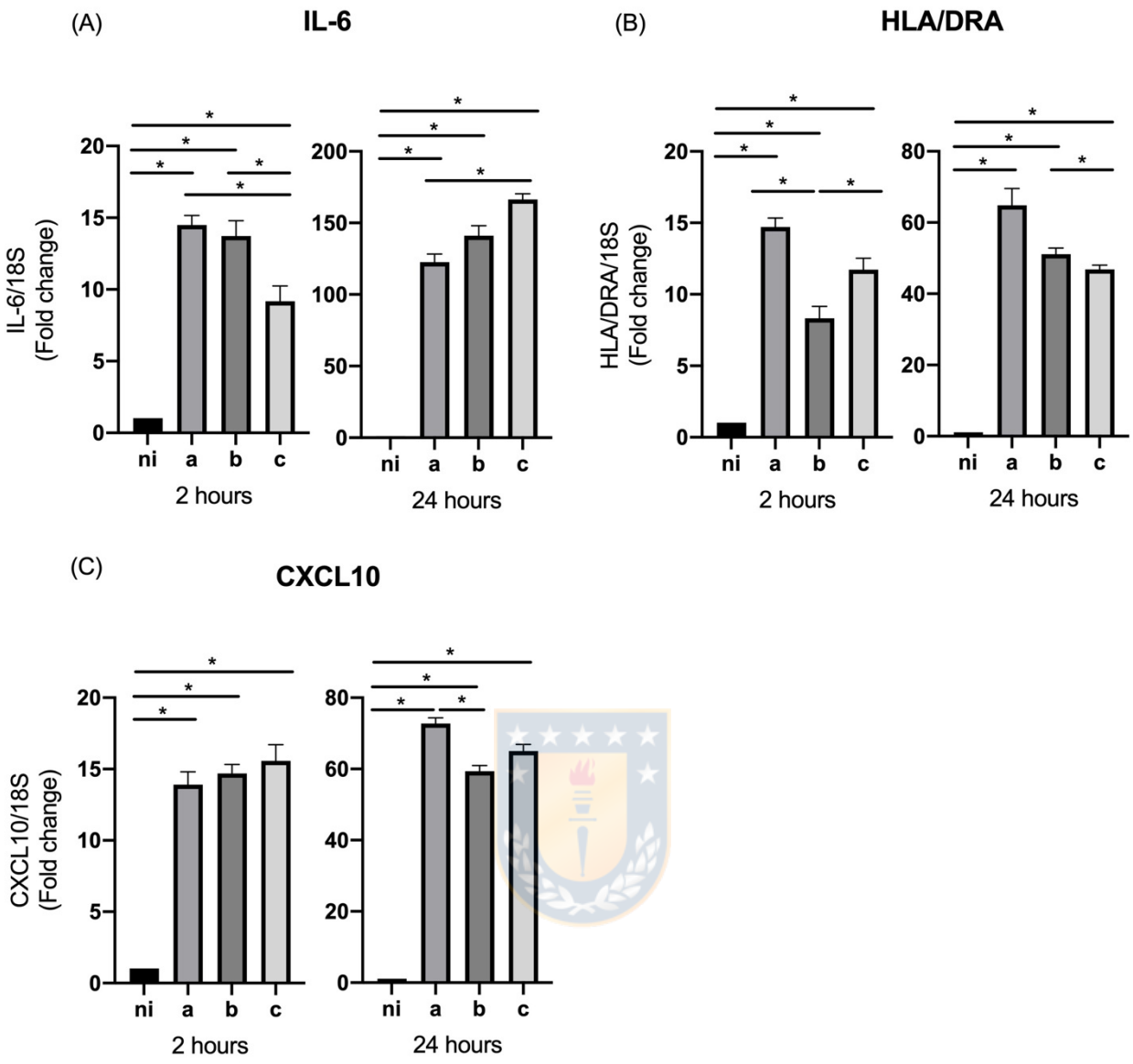

Figure 2. M_1 markers expression by *Aggregatibacter actinomycetemcomitans*-induced THP-1 human macrophages (ATCC® TIB-202TM). Expression in macrophages infected at a MOI = 10^2 with strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b), and ATCC® 43719™ (serotype c), 2 and 24 h after infection. For relative expression, mRNA expression in non-infected (ni) macrophages was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. Asterisks were used to indicate a value of p considered statistically significant (* $p < 0.05$).

5.5.3. Expression of M_2 markers in response to Aggregatibacter actinomycetemcomitans serotypes.

The expression of CD206 in the studied conditions was statistically significantly higher for the three serotypes ($p<0.0001$) with respect to the control conditions at 2 and 24 hours of infection. The statistically significant differences among the serotypes were observed at 2 hours of infection between the serotypes a and b ($p=0.0109$), b and c ($p<0.0001$) and between the serotypes a and c ($p=0.0397$), with a higher expression induced by the serotype b over the other serotypes. In contrast, at 24 hours of infection differences statistically significant among the serotypes showed a higher expression induced by the serotype c over the serotypes b ($p=0.0001$) and c ($p=0.0059$) (Figure 3A).

The qPCR data reveal that in the case of IL-10, the expression induced by all serotypes studied was statistically significantly higher compared to the control conditions at both times of infection ($p<0.0001$ for all serotypes). When the differences among serotypes are analyzed, our data shows an expression statistically significant higher for the serotype b over the serotype a at 2 hours of infection ($p=0.0227$) and for the serotype c over the serotype b ($p=0.0108$) at 24 hours of stimulation (Figure 3B).

In terms of the expression of CD163, the three serotypes of *A. actinomycetemcomitans* studied were capable of inducing an expression statistically significantly higher than control condition at the two time points of infection ($p<0.0001$ for all serotypes), and the differences among serotypes for this marker were statistically significantly only at 2 hours of infection for the serotype c over the serotype a ($p=0.0004$) without differences statistically significantly between serotypes at 24 hours (Figure 3C).

In the same way, when the levels of expression of FN1 are measured, the three bacteria analyzed in this study induce an expression statistically significant higher in comparison to the non-infected condition ($p<0.0001$ for all serotypes) at both times of infection, and the differences among serotypes only were observed at 2 hours of infection for the serotype c on the serotype a ($p=0.0103$) (Figure 3D), just as it happens with CD163.

Finally, we also evaluated the expression of CCL17, by THP-1 cells infected with the three previously mentioned serotypes of *A. actinomycetemcomitans*, observing for both time points studied and all serotypes, the ability to induce a statistically significant overexpression of this marker compared to the uninfected condition ($p < 0.0001$). The analysis of the differences among serotypes did not show a difference statistically significant for none of the evaluated times (Figure 3E).

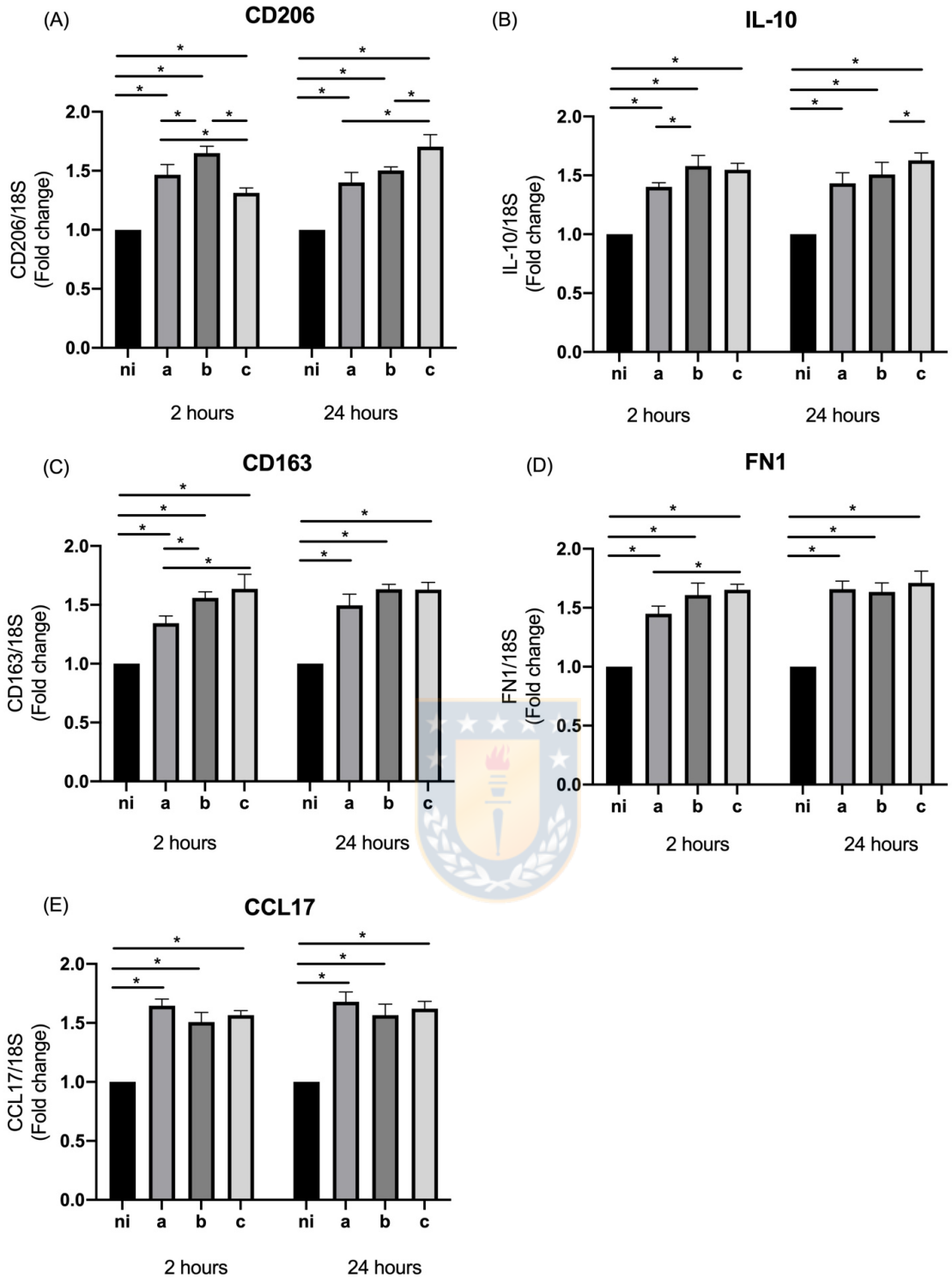

Figure 3. M_2 markers expression by *Aggregatibacter actinomycetemcomitans*-induced THP-1 human macrophages (ATCC® TIB-202TM). Expression in macrophages infected at a MOI = 10^2 with strains ATCC® 43717^{TM} (serotype a), ATCC® 43718^{TM} (serotype b), and ATCC® 43719^{TM} (serotype c), 2 and 24 h after infection. For relative expression, mRNA

expression in non-infected (ni) macrophages was considered as 1, as a reference for fold-change in expression using 18S rRNA expression levels as a normalizing endogenous control. Data are represented as fold-change means and standard deviation of three independent experiments performed in duplicate. Asterisks were used to indicate a value of p considered statistically significant (* $p < 0.05$).

5.6. Discussion

In periodontitis and in other diseases, the innate immune system produces a protective inflammatory response against damage signals like pathogens presence or tissue destruction. eliminating microorganisms and removing cellular debris to recover cell integrity and maintaining homeostasis of the tissues (33).

Macrophage polarization into different subtypes seems to shape macrophage responses and clusters them according to the stimuli, in terms of the diversity of microenvironment, the amounts of cytokines present in the tissues, and the duration and size of exposure (34). This polarization has been classified into two major macrophage polarization clusters, classically activated macrophages or M1 and alternatively activated macrophages or M2, each related to both progression and resolution of inflammation (35).

Classically activated macrophages or M1 constitute the first line of defense with epithelial cells at level of the periodontal pocket against intracellular pathogens such as A. actinomycetemcomitans and promote the Th1 polarization of CD4 cells in in the later stages of the periodontal immune response (36). This subset of macrophages exhibited a high level of phagocytic activity, and markers that best characterized them were IL-6 and CXCL10, however the level of expression of these markers is dependent on the nature of the stimulus such as interferon gamma, LPS or both (37). However, in our hands, the heterogenic virulence of A. actinomycetemcomitans LPS based on the structural differences, under the experimental conditions used, did not show a marked tendency to induce macrophage polarization to an M1 phenotype of one serotype over another, when the markers IL-6, CXCL10 and HLA/DRA were measured .

This macrophage phenotype, produce proinflammatory cytokines such as IL-1β, IL-6, IL-12, IL-18 and IL-23, TNF-α, and type I IFN; and several chemokines such as CXCL1, CXCL3, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CXCL13, and CXCL16; CCL2, CCL3, CCL4, CCL5, CCL8, CCL15, CCL11, CCL19, and CCL20, most of which have been defined as part of the "first wave" of cytokines during the development of periodontal inflammation, acting as the first alarm signal for the assembly of a more complex and specific immune response and for the recruitment of cells to the site of infection (38).

M2 macrophages are mainly identified based on the expression of CD163, IL-10, CCL17 and other markers (39). They produce high amounts of IL-8, monocyte chemo-attractant protein-1 (MCP)-1, IP-10, macrophages inflammatory protein (MIP)-1β, and CCL5 or Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) in order to recruit neutrophils, monocytes, and T lymphocytes in an anti-inflammatory or regulatory response, participating mainly in the remodeling of tissues and the resolution of the inflammatory condition (40, 41).

The role of the balance between M1 and M2 macrophages in the onset and development of periodontitis is not fully studied yet. When the M1/M2 status of macrophage polarization in healthy, gingivitis, and periodontitis patient samples is measured using biopsies, the evidence shows that gingivitis and periodontitis differ from each other by the levels of macrophage infiltrate, but not by changes in macrophage polarization (42) in contrast with some studies that have shown a higher amount of M1 macrophages (higher M1 / M2 ratio) in biopsies of patients with periodontitis compared with healthy or gingivitis individuals (43).

On other hand the presence of M2 macrophages in some study models has been indicative of the role of this cell cluster in the repair process, for instance the shift in the polarization towards M2 macrophages in the early stage of tissue repair contributed to the enhanced periodontal regeneration after stem cell transplantation in a rat model (44).

When the magnitude of expression of the panels of markers selected for this study is observed, our data show that the three bacterial serotypes analyzed have the ability to induce greater expression of markers associated with an M1 phenotype, compared to the markers of the M2 phenotype, without marked differences for one serotype over another, adding evidence that the heterogeneity of the virulence of A. actinomycetemcomitans, would not be explained at least in its entirety by the structural variables of the LPS, and that many other virulence factors would be responsible for inducing greater responses against one serotype over another probably in specialized immune cells capable of discriminating these differences, in contrast to innate immune cells responsible for assembling generic responses. These results should be analyzed considering the limitations of this test in terms of the use of a single microorganism in an environment of a single cell type, which differs from the real context of periodontitis where the participation of various cell types is conjugated, a biofilm bacterial complex and the presence of cytokines, chemokines, resolvins, metalloproteinases and other molecules intertwined in a complex network of signals that allow the activation of the mechanisms of the disease and its resolution (38).

The role of macrophage polarization in periodontal disease is a gap yet to be closed. How is it that these subpopulations are regulated? What is the role of the subpopulations of M2 macrophages M2a, M2b and M2c? where M2b would play a key role in the response to LPS, a molecule common to most periodontal pathogens (45, 46).

Macrophage polarization-modulating drugs might be the future of the immune regulation for the prevention, treatment, and reduction in patient susceptibility for periodontal diseases (47). The complete study of the role of macrophages in periodontal disease could be a new area in the study of diagnostics and therapeutic tools, development of biomarkers and upgrade of the clinical protocols.

5.7. Conclusion

Our study found that the most prevalent serotypes of *A. actinomycetemcomitans* in the mouth induce the expression of M1 macrophages markers in a manner non serotype dependent.

5.8 Conflict of Interest

The authors declare no conflict of interest.

5.9. Author Contributions

All authors contributed equally to this work

5.9. Funding

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CAPÍTULO 6

Discusión General

La importancia de células epiteliales y macrófagos en el desarrollo de la periodontitis radica es su capacidad de actuar como una barrera física natural y ser los primeros mecanismos de defensa a nivel del surco o bolsa periodontal, impidiendo la penetración de microorganismos en el tejido conectivo profundo, siendo los primeros responsables del reconocimiento de patógenos y de pesquisar las perturbaciones que el *biofilm* pudiese sufrir cuando varían las condiciones ambientales en que este se desarrolla (Palmer y cols., 2014; Socransky y cols., 1998; Feres y cols., 2004; Newman y cols., 2015). .

En cuanto a la expresión de citoquinas, nuestros datos son consistentes con la evidencia disponible que sustenta la expresión de mediadores inflamatorios por macrófagos y células epiteliales en respuesta a patógenos periodontales.

Las citoquinas que participan en la respuesta inmune contra patógenos periodontales se pueden clasificar en tres etapas. Una primera ola de citoquinas expresadas por células epiteliales, fibroblastos gingivales y células inmunes innatas, producidas directamente por el contacto de los microorganismos con las células huésped, y que al menos en base a los datos obtenidos en esta investigación no tiene, un patrón específico o microorganismo dependiente (serotipo dependiente en este caso), sino mas bien, es genérica y carece de una capacidad de discriminar por ejemplo variables estructurales que pudiesen hacer variar en su magnitud o composición (Pan y cols., 2019).

Existe además una segunda ola de citoquinas, asociadas con la diferenciación de subpoblaciones específicas de linfocitos, secretadas por células polimorfonucleares, células presentadoras de antígenos, fagocitos y linfocitos locales en respuesta al estímulo del microbioma, la cual eventualmente a la luz de la evidencia disponible podría ser sensible a variantes estructurales y tener una composición o magnitud microorganismo-dependiente (Pan y cols., 2019)..

Finalmente, una tercera ola de citoquinas, expresada por las subpoblaciones de linfocitos ya diferenciadas, que participa como mecanismos de retroalimentación o como efector directo de la inflamación y activación de las vías de destrucción tisular en caso de enfermedad (Pan y cols., 2019)..

Con relación a las moléculas asociadas al reconocimiento de estos patógenos, se requieren más estudios sobre el papel de los receptores tipo *toll* en la identificación de las variables estructurales de *A. actinomycetemcomitans*, principalmente para el caso de TLR4. Este receptor dimerizado con el factor de diferenciación mieloide 2 (MD-2) forma el heterodímero responsable de reconocer un patrón común en las moléculas de LPS estructuralmente diversas. El lípido A corresponde al patrón molecular conservado de LPS y es el principal inductor de la respuesta inmune contra LPS, siendo TLR4 junto con MD-2 los responsables del reconocimiento fisiológico de LPS. En este sentido, se requiere evidencia adicional para definir de que manera este receptor es capaz de reconocer la fracción conservada de LPS (lípido A), y cómo podría estar modulando una respuesta inmune diferencial dependiente del serotipo (como ocurre en algunos tipos de células), basado en variables estructurales típicas del antígeno O, una región no descrita como reconocida por este receptor. Probablemente otros receptores como NOD2 o NLRP3, y otros factores de virulencia podrían estar contribuyendo a la respuesta inflamatoria diferencial inducida por estas bacterias (Gelani y cols., 2009; Rojo-Botello y cols., 2012).

El estudio de las citoquinas y quimioquinas desde todos sus ángulos es importante considerando el nexo entre la inflamación periodontal, la biopelícula polimicrobiana y la enfermedad. La inflamación se ha descrito con un papel central en la modulación del biofilm periodontal y en el paso continuo de un estado de salud a periodontitis, conocido como modelo IMPEDE (inflamación mediada por aparición polimicrobiana y exacerbación disbiótica), donde la inflamación inicial contribuye a la disbiosis de la biopelícula, modulando la expresión de factores de virulencia por los microorganismos periodontales (muchos de ellos conocidos como bacterias inflamofílicas) y modificando la expresión génica de las células huésped. Como tal, aunque muchas bacterias no son fundamentalmente

responsables del daño tisular, contribuyen al entorno inflamatorio, que favorece el desarrollo de otros patobiontes presentes en el nicho periodontal (Loos y cols., 2020).

Sobre la base de las ideas anteriormente expuestas es que, a la luz de los datos obtenidos, se rechaza la hipótesis planteada en esta investigación, demostrándose que este tipo celular (queratinocitos y macrófagos) no muestra una respuesta diferencial frente al serotipo b, comparado con los otros serotipos estudiados. En esta línea, se necesitan más estudios para comprender los mecanismos por los cuales *A. actinomycetemcomitans* modula la respuesta de diferentes tipos de células, y cómo esta interacción huésped-patógeno modifica la expresión génica tanto del microorganismo como de las células infectadas.

Sumado a lo anterior, el desarrollo de modelos de estudio replicables que emulen la complejidad del microbioma oral como cultivos multibacterianos, co-cultivos con especies fúngicas, transfecciones virales, entre otras, además del uso de herramientas como secuenciación masiva, microarrays y edición genética para el silenciamiento de genes serían cruciales en la comprensión mas a cabalidad de las interacciones hospedero-patógeno que ocurren en la cavidad oral.

CAPÍTULO 7:

Conclusiones

Las células epiteliales orales de la línea OKF6/TERT2 presentan la capacidad de responder frente a la infección con los serotipos a, b y c de *A. actinomycetemcomitans*, sin embargo, a diferencia de otros tipos celulares dicha respuesta carece de un carácter serotipo dependiente.

Por su parte, cuando células macrofágicas son estimuladas con los mismos serotipos, estas también muestran una respuesta no diferencial pero de una magnitud mayor a tiempos de infección mas prolongados, siendo esto posiblemente indicativo de la sincronía temporal con la que se articula la respuesta inmune, donde las células epiteliales actuarían como respondedores rápidos, mientras que las células macrofágicas tendrían un papel amplificador de esta primera señal de daño y/o contacto con el patógeno.

A su vez, estas células macrofágicas en respuesta a la estimulación con los serotipos mas prevalentes de *A. actinomycetemcomitans,* polarizan a un fenotipo pro-inflamatorio M_1, sin importar el serotipo con el cuál están siendo infectadas, por lo que posiblemente este microorganismo modularía mecanismos capaces de perpetuar la inflamación desde las primeras etapas de infección, al inducir la diferenciación de fenotipos celulares específicos

Pese a lo anteriormente mencionado, más estudios son necesarios para dilucidar los mecanismos mediante los cuales *A. actinomycetemcomitans* interactúa con estos tipos celulares y cuál es el real efecto que tiene esta interacción en el desarrollo final de la enfermedad, el mantenimiento de la disbiosis del microcosmos periodontal, y las modificaciones del oraloma tanto en salud como en enfermedad.

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ANEXO 1

Otras Publicaciones

Chaparro A, Atria P, Realini O, Monteiro LJ, **Betancur D**, Acuña-Gallardo S, Ramírez V, Bendek MJ, Pascual A, Nart J, Beltrán V, Sanz A. Diagnostic potential of peri-implant crevicular fluid microRNA-21-3p and microRNA-150-5p and extracellular vesicles in peri-implant diseases. J Periodontol. 2021 Jun;92(6):11-21. doi: 10.1002/JPER.20-0372. Epub 2020 Nov 13. PMID: 33185898.

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