Centro de Investigación en Alimentación y Desarrollo, A.C.

CARACTERIZACIÓN INMUNOQUÍMICA Y MOLECULAR DE ANTIGENOS INMUNODOMINANTES DE *Giardia lamblia* EN UN MODELO MURINO DE GIARDIASIS

Por:

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A Dios…

Por que sin Él nada sería posible.

Pero con Él, nada es imposible.

(Filipenses 4:13)

Adquiere sabiduría, adquiere inteligencia;
No te olvides ni te apartes de las razones de mi boca;
No la dejes, y ella te guardará;
Ámala, y te conservará.
Sabiduría ante todo; adquiere sabiduría;
Y sobre todas tus posesiones adquiere inteligencia.
Engrandécela, y ella te engrandecerá;
Ella te honrará, cuando tú la hayas abrazado.
Adorno de gracia dará a tu cabeza;
Corona de hermosura te entregará.
(Proverbios 4:5-9)

"Lo conocido es finito, lo desconocido infinito; desde el punto de vista intelectual estamos en una pequeña isla en medio de un océano ilimitable de inexplicabilidad. Nuestra tarea en cada generación es recuperar algo más de tierra".

Thomas Henry Huxley

CONTENIDO

LISTA DE FIGURAS

LISTA DE TABLAS

RESUMEN

Giardia lamblia es un parásito protozoario intestinal que infecta a humanos y algunos mamíferos. Se conocen ocho genotipos del parásito clasificados del A al H, pero solo A y B infectan humanos. La eliminación de *Giardia* del intestino requiere la activación de mecanismos inmunológicos en el hospedero mediados por células B y T. No obstante, el conocimiento acerca de las proteínas del parásito que inducen una respuesta inmunológica eficaz es limitado. Estudios previos de nuestro grupo de investigación reportaron el antígeno 5G8 de *Giardia* GS/M83-H7 como altamente inmunogénico, y sugirieron que podría ser una proteína de choque térmico (HSP70) llamada BIP. El objetivo del presente trabajo fue caracterizar inmunoquímica y molecularmente los antígenos de *G. lamblia* 5G8 y BIP en un modelo de infección murino. Para ello, se sobreexpresó y purificó BIP recombinante con la que se generaron anticuerpos policlonales. Mediante Citometría de Flujo y Western blotting se determinó que el perfil de expresión de 5G8 y BIP en la superficie de trofozoítos GS/M83-H7 y GS/M83-H7-5G8(+) fue distinto (5G8 en 2-95% y BIP en < 1% de la población), lo que sugiere que son antígenos diferentes. El análisis de la respuesta inmune humoral por ELISA y Western blotting determinó que BIP es reconocido por sueros de ratones reinfectados. La respuesta inmune celular se evaluó mediante ensayos de proliferación por MTT, empleando esplenocitos de ratones infectados. Sin embargo, en las condiciones evaluadas no se detectó estimulación por parte de BIP. Se determinó mediante algoritmos de predicción de epítopes de células B y T, que BIP posee en su secuencia regiones con alta probabilidad de ser reconocidas por el sistema inmune del hospedero. Incluso, el epitope 590-609 podría ser reconocido por ambas células. Por otro lado, el análisis molecular de 5G8 determinó que pertenece a la familia de proteínas variables de superficie (VSP) de *Giardia*. El gen *vsp-5g8* se amplificó por PCR y se obtuvo la secuencia nucleotídica (1824 pb) que codifica para VSP-5G8 (607 aminoácidos, 61 kDa), presente en *Giardia* genotipo B. Los análisis de homología y predicción de epítopes de VSP-5G8 indicaron que posee epítopes de alta probabilidad que además están conservados en otras VSP de diferentes genotipos de *Giardia*. En conclusión, los antígenos BIP y VSP-5G8 son proteínas inmunogénicas de *Giardia*, su caracterización

contribuirá a entender mejor los mecanismos de interacción hospedero-*Giardia* y a establecer las bases moleculares para el desarrollo de una vacuna racional contra esta infección.

Palabras clave: *Giardia lamblia*, proteínas inmunogénicas, respuesta inmune

ABSTRACT

Giardia lamblia is a protozoan parasite that infects the small intestine of humans and some mammals. Eight genotypes of the parasite (A-H) have been described, but only A and B can infect humans. Elimination of *Giardia* from intestine require the activation of host immunological mechanisms mediated by B and T cells. Nevertheless, the knowledge about parasite proteins able to induce an immunological response is limited. Previous studies of our research group reported that 5G8 antigen of *Giardia* GS/M83-H7 is highly immunogenic and suggested that 5G8 could be a heat shock protein (HSP70) named BIP. The aim of this work was to characterize immunochemical and molecularly the antigens 5G8 and BIP of *G. lamblia* in a murine infection model. Thus, BIP protein was overexpressed, purified and used to generate polyclonal antibodies. FACS and Western blotting assays determined that the expression profile of 5G8 and BIP in surface of trophozoites GS/M83-H7 and GS/M83-H7-5G8 (+) was different (5G8 express 2-95% and BIP < 1% of trophozoites population), this suggest they are different antigens of *Giardia*. The analysis of humoral response against BIP by ELISA and Western blotting determined that BIP is recognized by serum from reinfected mice. Cellular immune response was evaluated by MTT proliferation assays using splenocytes from infected mice. However, we did not detected a positive stimulation by BIP at evaluated conditions. It was determined that BIP possess sequence regions with high probability to be recognized by the host immune system. Thus, epitope "590-609" could be recognized by B and T cells. On the other hand, the molecular analysis of 5G8 placed this antigen into the *Giardia* variant surface proteins family. The *vsp-5g8* gene was amplified by PCR and its nucleotide sequence of 1824 pb obtained. The encoded VSP-5G8, is composed of 607 aminoacids (61 kDa) and it is present in the genotype B of *Giardia*. However, the homology and epitope prediction analysis indicated that VSP-5G8 has high probability epitopes that are also conserved among other VSP of different *Giardia* genotypes. In conclusion, the antigens BIP and VSP-5G8 are immunogenic proteins of *Giardia*, their characterization will contribute to the better knowledge of the interaction host-*Giardia* mechanisms and to establish the molecular basis for the rational development of an effective vaccine against this infection.

Keywords: *Giardia lamblia*, immunogenic proteins, immune response

SINOPSIS

Las enfermedades gastrointestinales causadas por infecciones parasitarias constituyen uno de los principales problemas de salud pública de México y otros países en desarrollo. Generalmente la vía de transmisión es fecal-oral, por lo que su incidencia se ve relacionada a las deficiencias en las condiciones sanitarias de la población. Sin embargo, países industrializados se muestran afectados principalmente en viajeros, niños y ancianos. La giardiasis, una de las enfermedades gastrointestinales más comunes en todo el mundo (Feng et al. 2011) es ocasionada por la infección con *Giardia lamblia*. La prevalencia de esta enfermedad en países industrializados varía entre un 2-5%, mientras que es en países en desarrollo la prevalencia es significativamente más alta, afectando a un 20-60% de la población y es considerada una enfermedad re-emergente (Moya-Camarena et al. 2002; Savioli et al. 2006; Cedillo-Rivera et al. 2009; Vazquez et al. 2009; Hernandez et al. 2011). En Sonora, el Sistema Nacional de Vigilancia Epidemiológica indica que hasta noviembre del 2016, se han reportado 496 casos de giardiasis (Secretaría-de-Salud 2016). Sin embargo, los datos reportados podrían estar subestimados ya que estudios epidemiológicos en zonas susceptibles del estado indican que la tasa real es mucho mayor a la reportada, especialmente en escolares, con cifras de 16 a 37 casos por cada 100 habitantes (Moya-Camarena et al. 2002; Valenzuela-Félix 2006; Miranda-Ozuna 2009; Quihui-Cota et al. 2012).

G. lamblia (*G. intestinalis* o *G. duodenalis*) es un parásito protozoario que infecta y coloniza el intestino delgado superior de humanos y algunos mamíferos (Adam 2001). *Giardia* presenta dos etapas de desarrollo, las cuales son estructural y bioquímicamente diferentes. El quiste es la forma resistente del microorganismo, es inmóvil pero tiene una pared celular y doble membrana interna, lo que le hace ser resistente a las condiciones del ambiente por largos periodos de tiempo. El trofozoíto tiene cuatro pares de flagelos que le proporcionan movilidad y un disco ventral que le permite adherirse al epitelio

intestinal y causar las manifestaciones clínicas y patológicas de la enfermedad (Adam 2001; Carranza et al. 2010) (ver Figura 1, página 16). La infección inicia con la ingesta de alimentos o bebidas contaminadas, exposición a aguas recreacionales contaminadas, contacto persona-persona y estudios recientes han reportado el potencial carácter zoonótico de esta enfermedad (Ekdahl et al. 2005; Roxstrom-Lindquist et al. 2006; Sprong et al. 2009; Feng et al. 2011; Lujan et al. 2011; Fantinatti et al. 2016). La dosis infecciosa es baja ya que cada quiste al exponerse al bajo pH del estómago desenquista en dos trofozoítos, los cuales posteriormente se dividen por fisión binaria y rápidamente colonizan el lumen intestinal. Cuando los trofozoítos migran hacia el tracto gastrointestinal bajo, la concentración de colesterol, bilis y el pH inducen el proceso de enquistamiento. Los quistes son liberados del hospedero al ambiente a través de las heces y el ciclo de transmisión se completa con la infección de un nuevo hospedero (ver Figura 2, página 17) (Lujan et al. 1998; Adam 2001; Ankarklev et al. 2010; Lopez-Romero et al. 2015).

Una característica notoria de la giardiasis es el amplio espectro de manifestaciones clínicas, las cuales varían desde infecciones asintomáticas, hasta enfermedad sintomática aguda o crónica con dolor abdominal, diarrea severa, lesiones intestinales y síndrome de mala absorción de nutrientes, el cual puede durar varios meses y en casos severos se ha asociado a desnutrición (Farthing 1992; Adam 2001). Esto es de especial importancia en zonas endémicas, en las cuales las reinfecciones y la enfermedad crónica son muy comunes. La organización mundial de la salud (OMS) menciona a *Giardia* como uno de los agentes patógenos más comúnmente asociado a enfermedades diarreicas y desnutrición aguda severa, las cuales constituyen las principales causas de morbilidad y mortalidad en niños de países en desarrollo (Manary et al. 2012). Los tratamientos actualmente sugeridos por la OMS incluyen el uso de antiparasitarios, rehidratación oral y dietas suplementadas con micronutrientes como el zinc (Manary et al. 2012). No obstante, los antiparasitarios usados comúnmente no son del todo eficaces, se asocian a una serie de efectos secundarios y los esquemas de tratamiento no son los adecuados, incluso ya se han reportado casos de cepas resistentes (SEGOB 1996; Gardner et al. 2001; Cedillo-Rivera et al. 2009; Leitsch 2015). Además,

no generan una memoria ante reinfecciones, lo que hace susceptibles de recaídas frecuentes a poblaciones en zonas endémicas.

Giardia pertenece a la orden de los Diplomonados, un grupo de protozoarios binucleados y flagelados que son encontrados en ambientes anaeróbicos o microaerofílicos. *Giardia* spp representa a uno de los grupos evolutivos más ancestrales de las eucariotas (Ankarklev et al. 2010). El género *Giardia* consiste de 6 especies, entre ellos, solo *G. lamblia,* puede infectar humanos y algunos otros mamíferos (Adam 2001). Estudios genómicos han confirmado ocho genotipos o ensambles (A-H). Solo los genotipos A y B pueden infectar humanos, y el genotipo B es el más frecuente alrededor del mundo (Adam 2001; Franzen et al. 2009; Jerlstrom-Hultqvist et al. 2010; Ankarklev et al. 2015). Otros ensambles de *Giardia* son C y D que infectan a perros, E que infecta a animales ungulados como cabras y cerdos, F infecta a gatos, G a roedores y H a animales marinos (ver Tabla 1, página 15) (Adam 2001; Lasek-Nesselquist et al. 2010). Sin embargo, estudios recientes han reportado casos de zoonosis por *Giardia* principalmente para el genotipo E (Sprong et al. 2009; Feng et al. 2011; Fantinatti et al. 2016). Diversos factores pueden influenciar los síntomas, el curso y la patofisiología de la enfermedad. Algunos de estos factores son la edad, el estado nutricio y la immunocompetencia del hospedero en el momento de la infección, mientras otros son referentes al parásito, tales como la carga parasitaria, la virulencia de la cepa, el genotipo y las co-infecciones con otros microorganismos (Faubert 2000; Thompson 2001; Solaymani-Mohammadi et al. 2010; Lujan et al. 2011; Benere et al. 2012; Jerlstrom-Hultqvist et al. 2012; Koh et al. 2013; Bartelt et al. 2015).

Las infecciones por *G. lamblia* en individuos inmunocompetentes, generalmente son procesos autolimitados, lo que evidencia la existencia de mecanismos de defensa en el hospedero eficaces contra el parásito (Eckmann 2003). El sistema inmune de la mucosa intestinal es especialmente complejo pues debe ser capaz de distinguir los antígenos de agentes patógenos entre antígenos propios, de la microbiota y de los alimentos. La mayoría de los estudios *in vivo* sugieren que *G. lamblia* no invade la mucosa intestinal y se caracteriza por ser un proceso no inflamatorio. Sin embargo, es capaz de estimular no solo una respuesta inmune de mucosas sino también una respuesta a nivel sistémico (Eckmann 2003; Lopez-Romero et al. 2015).

En el Capítulo 1 del presente trabajo de tesis, se presenta un artículo de revisión en el cual se describen a detalle los procesos inmunológicos del hospedero que intervienen en la eliminación de *Giardia* (ver Figura 3 página 18) (Lopez-Romero et al. 2015). La inmunidad innata representa los primeros mecanismos de defensa del hospedero. Ante una giardiasis los mecanismos de la inmunidad innata más importantes involucran la liberación de especies reactivas de oxígeno como el óxido nítrico, la activación de células como los mastocitos y las dendríticas, así como la producción de moco por las células de Goblet y el peristaltismo. La liberación de citocinas proinflamatorias como IL-6 e IL-17 durante la infección por *Giardia* es necesaria para la generación de una respuesta inmune eficaz y a su vez, la liberación de citocinas anti-inflamatorias podría estar relacionada a la progresión de la enfermedad crónica. No obstante, los mecanismos cruciales para la eliminación del parásito dependen de procesos mediados por células B (humoral), así como por células T (celular). Generalmente, en un hospedero inmunocompetente, la carga parasitaria disminuye de 3-4 semanas post infección, lo cual correlaciona con el establecimiento de la respuesta inmune humoral. Principalmente se ha demostrado que las inmunoglobulinas tipo IgA juegan un papel importante (Singer et al. 2000; Langford et al. 2002; Velazquez et al. 2005). Se ha demostrado que las células T CD4+ son esenciales para combatir al parásito, ya que en ausencia de ellas, los animales de experimentación desarrollan giardiasis crónica (Singer et al. 2000). Se ha propuesto que los linfocitos T pueden actuar estimulando la maduración y activación de linfocitos B para la generación de inmunoglobulinas mas específicas y eficientes o que pueden actuar mediante una respuesta proinflamatoria tipo Th17 (Dann et al. 2015; Saghaug et al. 2015; Singer 2015). Sin embargo, los mecanismos por los cuales actúan contra la giardiasis sin causar inflamación significativa son poco conocidos.

El sistema inmune puede reconocer proteínas de *Giardia* y activar una serie de mecanismos con el fin de eliminar al parásito del intestino. En las últimas décadas, se han descrito proteínas que pueden ser reconocidas por el sistema inmune del hospedero (ver Tabla 2, página 20). Sin embargo, a pesar de la importancia que tienen los mecanismos mediados por linfocitos T en la respuesta anti-*Giardia*, la mayoría de los estudios se enfocan en proteínas reconocidas por la respuesta inmune humoral (Palm et

al. 2003; Velazquez et al. 2005; Quintero et al. 2013; Tako et al. 2013). En nuestro grupo de investigación se ha evaluado la respuesta inmune ante antígenos de *G. lamblia* en un modelo de infección murino. Entre estos antígenos destaca el reconocimiento hacia una banda proteica de ≈ 70 kDa. La proteína 5G8 de ≈ 70 kDa además de ser fuertemente reconocida por anticuerpos de ratones infectados, es capaz de inducir en el hospedero la producción de anticuerpos que pueden aglutinar (neutralizar) trofozoítos de *Giardia in vitro* (ver Figura 4, página 21) (Velazquez et al. 2005; Quintero et al. 2013).

Estudios preliminares de secuenciación por espectrometría de masas sugirieron que 5G8 podría tratarse de la proteína BIP, una proteína de choque térmico de 70 kDa (HSP70, por sus siglas en inglés). Además, un estudio reciente reportó que la proteína BIP de *G. lamblia* es potencialmente reconocida por anticuerpos IgG de ratones BALB/C infectados con la cepa GS/M83-H7 y es capaz de estimular la activación de células dendríticas y la subsecuente presentación de antígeno (Lee et al. 2014). Sin embargo, el diseño experimental de este estudio no evaluó como tal la activación de una respuesta celular en un modelo de infección, sino la capacidad de las células dendríticas de llevar a cabo la presentación de BIP hacia células T vírgenes. Con base en lo anterior, se planteó como hipótesis que los antígenos inmunodominantes 5G8 y BIP de *G. lamblia* poseen características que les permiten estimular una respuesta adaptativa humoral y celular en un modelo de giardiasis murino.

Las HSP son expresadas en las células con el objetivo de ayudar en la sobrevivencia bajo condiciones de estrés intrínseco o extrínseco (Beckmann et al. 1992). Datos recientes reportan que proteínas pertenecientes a la familia de las HSP70 de varios organismos presentan características inmunogénicas y algunas de ellas ha sido propuestas como candidatos para vacunas o adyuvantes de vacunas contra diversas enfermedades (Segal et al. 2006; Tamura et al. 2011; Dhakal et al. 2013; Assadian et al. 2014; Batra et al. 2014). El conocimiento acerca de las proteínas HSP70 de *Giardia* es aun limitado, éstas han sido empleadas principalmente como marcadores de organelos, etapas del ciclo celular (enquistación) e incluso para diagnóstico. La proteína BIP o GRP-78 es una proteína chaperona residente de retículo endoplásmico, pero que también ha mostrado asociarse a vesículas de enquistación (ESV) (Lujan et al. 1996; Soltys et al. 1996; Stefanic et al. 2006). Aun cuando existe evidencia del potencial inmunogénico de

BIP, aun no se sabe si éste antígeno puede inducir en el hospedero infectado el desarrollo de mecanismos inmunológicos efectivos contra *Giardia* y promover una protección hacia futuras infecciones.

En el Capítulo 2 del presente documento, se presenta un artículo de investigación original el cual tuvo como objetivo la caracterización inmunoquímica de la proteína BIP de *G. lamblia*. El primer objetivo de este estudio fue determinar si 5G8 y BIP eran el mismo antígeno. Para ello, se sobreexpresó la proteína BIP en *E. coli*, se purificó por cromatografía de afinidad a metales inmovilizados (IMAC) y se utilizó para generar anticuerpos policlonales. Mediante ensayos de citometría de flujo y western blotting se determinó el perfil de expresión de 5G8 y BIP en trofozoítos GS/M83-H7 y GS/M83-H7-5G8 (+). El análisis de western blotting determinó que BIP se expresa en ambas cepas de *Giardia* a un nivel similar. Sin embargo, 5G8 solo puede ser detectada con esta técnica en la cepa 5G8 (+). Estos resultados fueron confirmados por citometría de flujo, en la cual se observó también que el perfil de expresión de los antígenos en trofozoítos es distinto, mientras que 5G8 se encuentra en la superficie de un 2 hasta un 95% de los trofozoítos analizados, BIP se encuentra en menos del 1% de la población. El perfil de expresión distinto de ambos antígenos indica que se trata de proteínas diferentes de *Giardia*.

El análisis de la respuesta inmune humoral ante BIP por ELISA y western blotting determinó que BIP es reconocido por sueros de ratones reinfectados a partir de la tercera semana post-reinfección. La respuesta inmune celular se evaluó mediante ensayos de proliferación por MTT, empleando esplenocitos de ratones infectados. Sin embargo, en las condiciones evaluadas no se detectó una estimulación por parte de BIP. Mediante algoritmos de predicción de epítopes para células B y T se determinó que la secuencia aminoacídica de BIP posee regiones con alta probabilidad de ser reconocidas por el sistema inmune del hospedero. Incluso, el epitope "590-609" podría ser reconocido por ambas células. Estos datos indican que aunque BIP no es 5G8, posee características con potencial inmunogénico. Estudios futuros podrían determinar si BIP es capaz de estimular algún tipo de respuesta celular y con ello, elucidar un posible mecanismo de interacción con el hospedero.

G. lamblia, al igual que muchos otros parásitos, realiza procesos de variación antigénica, la cual involucra las proteínas variables de superficie (VSP) (Prucca et al. 2009). Las VSP de *Giardia* son proteínas ricas en motivos cisteína, poseen una región amino terminal altamente variable y una región conservada en el carboxilo terminal, la cual incluye una cola citoplasmática de cinco aminoácidos (CRGKA) (Nash 1992). El repertorio de VSPs difiere entre aislados-genotipos de *Giardia*, el aislado GS (genotipo B) tiene más de 270 genes predichos, mientras que los aislados WB (genotipo A), DH (genotipo A2) y P15 (genotipo E) tienen 186, 121 y 123 respectivamente (Adam et al. 2013). No obstante, solo una VSP se expresa en la superficie del trofozoíto en determinado momento (Nash 1997).

Las VSP cubren densamente la superficie de trofozoítos, incluyendo los flagelos, lo que representa una interfase entre el hospedero y el parásito (Pimenta et al. 1991; Nash 2002). Lo anterior explica la fuerte respuesta inmune humoral en el hospedero particularmente dirigida hacia estas proteínas. De hecho, los anticuerpos anti-VSP son citotóxicos a altas concentraciones, mientras que a bajas concentraciones inhiben el crecimiento del parásito. Entonces, en un hospedero infectado, la respuesta inmune humoral podría tener un papel selectivo, ya que su establecimiento coincide con la eliminación de la VSP original en la superficie del trofozoíto (Prucca et al. 2009). Aunque la variación antigénica en *Giardia* se ha propuesto como mecanismo de evasión de la respuesta inmune, se ha observado que el cambio de estas proteínas ocurre incluso *in vitro* por factores aún desconocidos. La variación ocurre a diferentes tasas según el genotipo; por ejemplo, en *Giardia* WB (A) ocurre un cambio cada 12-13 generaciones, mientras que en GS (B) el cambio ocurre en promedio cada 6.5 generaciones (Nash 2002; Luján et al. 2011). La expresión de determinada VSP se regula a nivel post-transcripcional por un mecanismo similar al RNA de interferencia (RNAi), mediante el cual se eliminan todos los transcritos excepto los de una VSP (Prucca et al. 2011).

Debido a que el antígeno altamente inmunogénico 5G8 resultó no ser BIP, se buscó aislarlo y caracterizarlo molecularmente. En el Capítulo 3 se presentan un segundo artículo de investigación original en el cual se describen los experimentos y análisis realizados para la identificación de la proteína 5G8. Esta proteína se purificó mediante cromatografía de afinidad empleando un anticuerpo monoclonal específico.

Posteriormente, ésta fue reducida, alquilada y digerida enzimáticamente, los péptidos generados se separaron por HPLC y fueron analizados mediante espectrometría de masas (ESI-MS/MS). Se identificaron péptidos presentes en la secuencia y se compararon con la base de datos del genoma de *Giardia* (GiardiaDB). A partir de este análisis, se identificaron dos proteínas diferentes de masas moleculares relativas de 28 y 38 kDa, ambas etiquetadas como parte de la familia de las VSP. Sin embargo, la evidencia bioquímica preliminar sugiere que se trata de una sola proteína de alrededor de 70 kDa. Mediante un análisis bioinformático se encontró que ambas compartían en común regiones aminoacídicas (carboxilo terminal y amino terminal). Al realizar el análisis en el genoma de GS_B se encontraron los genes reportados para las secuencias de 28 y 38 kDa consecutivos y traslapados en una región. Se determinó una secuencia teórica para 5G8 constituida por 607 aminoácidos a partir del traslape de los fragmentos de 28 y 38 kDa. El análisis posterior de los dominios en la secuencia teórica determinó que 5G8 es una VSP y sugiere que está constituida por una cadena polipeptídica codificada por un solo gen. Sin embargo, fue necesario comprobar esta hipótesis secuenciando el gen de 5G8.

En el Capítulo 4 del presente trabajo de tesis, se presenta un tercer articulo de investigación original en el cual el objetivo principal fue determinar la secuencia de VSP-5G8, y elucidar el papel que puede tener esta proteína en la inmunogenicidad de *G. lamblia*. Para ello, se diseñaron oligonucleótidos con base en la secuencia teórica de VSP-5G8, se amplificó el gen *vsp-5g8* y se determino su secuencia. Los resultados coincidieron con los obtenidos previamente en el análisis por espectrometría de masas e indicaron que *vsp-5g8* se encuentra en *Giardia* genotipo B. Mediante análisis de comparación de secuencias aminoacídicas se determinó que 5G8 posee regiones conservadas entre otras VSP de diferentes genotipos y aislados de *Giardia*. Es de destacar, que algunas de estas regiones incluyen regiones importantes para el reconocimiento inmunológico, tales como residuos de anclaje para epítopes de células T. El análisis comparativo de *vsp-5g8* y el genoma reportado para GS_B sugiere la presencia un polimorfismo en la posición 801 del gen, este podría ser determinante de que se exprese una variante completa (61 kDa) o truncada (28 kDa, depleción de nucleótido 801) de VSP-5G8. Estudios adicionales sugieren que la expresión de *vsp-5g8*

podría ser un determinante de la inmunogenicidad de la cepa incluso si se expresa en una pequeña proporción de trofozoítos. Sin embargo, son necesarios más experimentos para elucidar los mecanismos involucrados en la regulación de esta y otras VSPs así como el rol de VSP-5G8 durante el curso de la giardiasis.

Se sabe que *Giardia* al ser un organismo tetraploide posee cuatro copias de cada alelo para determinada *vsp*. De hecho, pueden presentarse alelos múltiples, de los cuales, solo uno se expresa. Esta expresión alelo-específica sugiere mecanismos de regulación epigenética que median la presencia de una *vsp* en el fenotipo de *Giardia* en un momento definido (Prucca et al. 2009; Prucca et al. 2011). Estudios recientes han demostrado que *Giardia* presenta diversidad genética a lo largo del genoma y no solo en cuanto a alelos de *vsps* (Caccio et al. 2008; Sprong et al. 2009; Wielinga et al. 2011). Sin embargo, esta característica difiere entre genotipos, ya que el genotipo B (GS) presenta hasta 50% mas heterocigosidad alélica de secuencia (ASH) que el A (WB), lo cual complica los estudios de caracterización y genotipificación de este parásito (Franzen et al. 2009; Ankarklev et al. 2012; Ankarklev et al. 2015; Wielinga et al. 2015).

En resumen, en el presente trabajo se determinó que BIP y 5G8 son diferentes antígenos de *Giardia*. BIP fue reconocida por la respuesta inmune humoral sistémica en un modelo de infección murino. Sin embargo, en las condiciones evaluadas, no se detectó una respuesta inmune celular inducida por BIP. Por otro lado, se determinó que 5G8 es una proteína perteneciente a la familia de las VSP, constituida por una sola cadena polipeptídica codificada en un gen de 1824 pb presente en el genotipo B de *Giardia.* VSP-5G8 posee regiones conservadas entre VSPs de distintos genotipos de *Giardia* pero la expresión de la variante completa de VSP-5G8 podría determinar la inmunogenicidad de la cepa de *Giardia*. Con base en lo anterior se determinó que efectivamente, los antígenos 5G8 y BIP de *G. lamblia* poseen características que les permiten estimular una respuesta adaptativa humoral y celular en un modelo de giardiasis murino.

Estudios más a fondo podrían determinar si estos antígenos pueden inducir en el hospedero una respuesta inmune eficaz capaz de proteger contra futuras reinfecciones del mismo u otro genotipo de *Giardia*. La identificación y caracterización de proteínas de *Giardia* que puedan activar efectivamente respuestas mediadas por células B y T

permitirá mejorar el entendimiento de los mecanismos de interacción hospedero-parásito, así como también contribuirá proporcionando información útil para el desarrollo de nuevas estrategias terapéuticas y profilácticas que estimulen al sistema inmunológico como la generación de vacunas.

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Tabla 1. Genotipos de *Giardia lamblia* y sus hospederos

Ensamble/genotipo	**Hospedero**
A (Grupo 1)	Humano, castor, gato, lémur, oveja, becerro, perro, chinchilla, alpaca, caballo, cerdo, vaca
A (Grupo 2)	Humano, castor
B	Humano, puerco de guinea, perro, mono
C	Perro
D	Perro
E	Vaca, oveja, alpaca, cabra, puerco
F	Gato
G	Roedor
H	Marinos

Tabla modificada del original (Adam 2001; Lasek-Nesselquist et al. 2010)

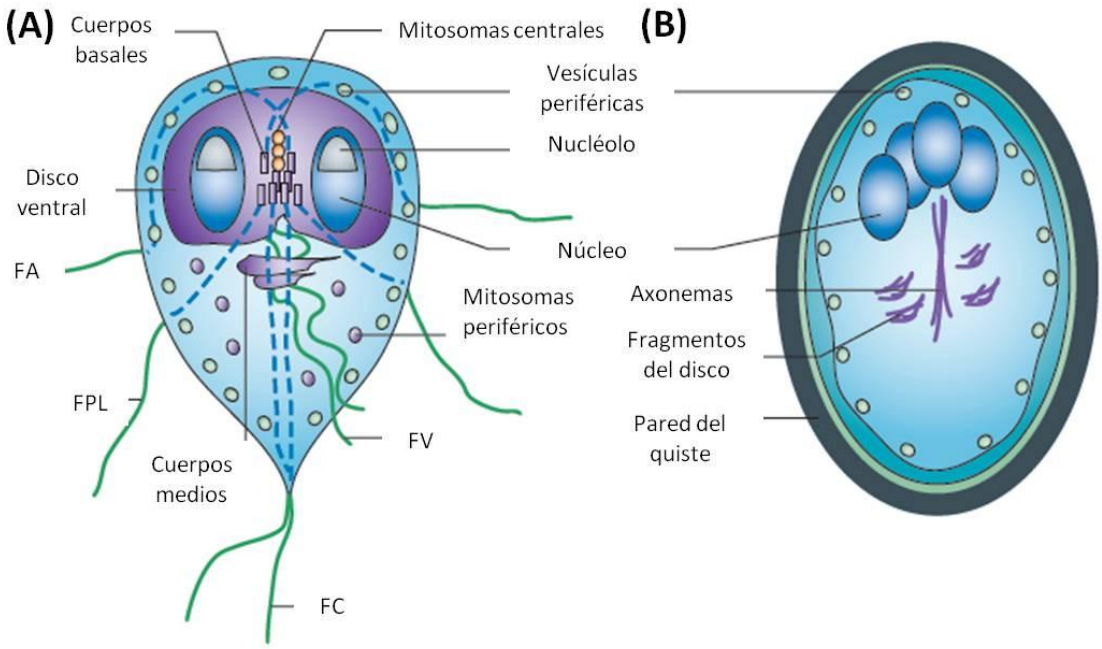

Figura 1. Características morfológicas principales de *Giardia lamblia* (modificado de Ankarklev, Jerlstrom-Hultqvist et al. 2010). (A) El trofozoíto tiene mide de 12-15 μm en longitud y 5-9 μm de grosor. El trofozoíto se presenta de cara dorsal. Posee dos núcleos diploides, cuatro pares de flagelos: anteriores (FA), ventrales (FV), posterior/ laterales (FPL), y caudales (FC). Los flagelos nacen a partir de los cuerpos basales. La función de los cuerpos medios aún se desconoce. El disco ventral es amplio, rígido y compuesto de microtúbulos y proteínas que facilitan la adhesión. *Giardia* presenta además, mitosomas, y vesículas similares a lisosomas cercanas a la membrana. B) El quiste posee cuatro núcleos tetraploides, una pared y una doble membrana interna que lo protegen de las condiciones ambientales adversas. No son móviles y tienen una forma ovalada que mide de 8-12 μm de largo por 7-10 μm de ancho. La pared del quiste es 0.3- 0.5 μm. de grosor y constituida de filamentos que van de los 7- 20 nm de diámetro. El principal componente de la pared es la N-acetilglucosamina y tres proteínas principales CWP1, CWP2 y CWP3 (Ankarklev et al. 2010).

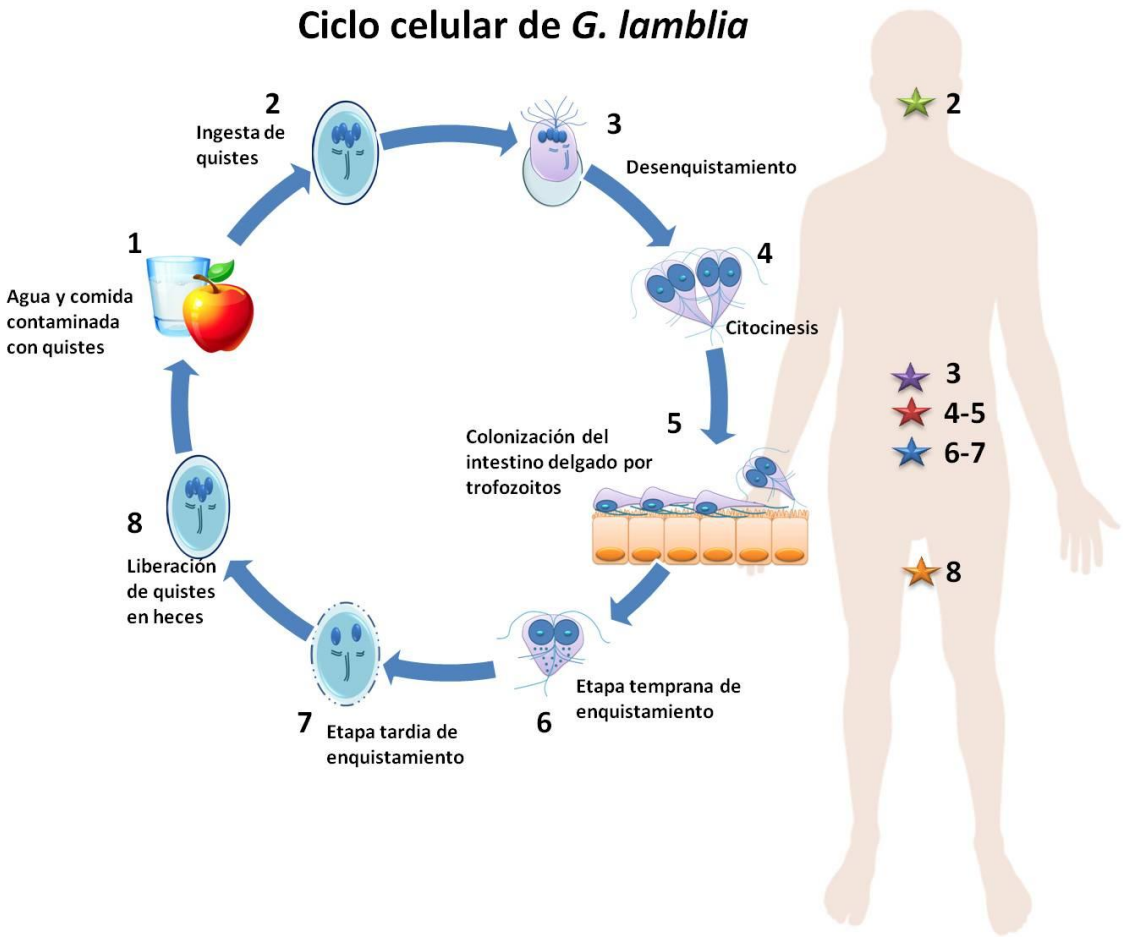

Figura 2. Ciclo de vida de *Giardia lamblia*. La ruta de transmisión es fecal-oral. La infección por *Giardia* comienza con la ingesta de quistes a través de alimentos contaminados (1 y 2). La exposición al ambiente ácido del estómago, induce el proceso de desenquistamiento de *Giardia* (3). Cada quiste produce dos trofozoítos (4). Los trofozoítos se replican y colonizan el intestino adhiriéndose al epitelio (5). A medida que los trofozoítos viajan hacia el tracto gastrointestinal inferior, el bajo nivel de colesterol, alta concentración de bilis y el pH ligeramente alcalino inducen una etapa de enquistamiento temprana en la cual los trofozoítos se redondean y se transportan proteínas específicas para la formación del quiste a través de vesículas hacia la superficie (6). El disco adhesivo se desensambla y la célula replica su DNA para generar una célula que contenga dos núcleos tetraploides (4N cada uno). Durante la etapa de enquistamiento tardío, los núcleos se dividen, lo que genera células de cuatro núcleos diploides (2N cada uno), el DNA se replica de nuevo para generar una célula 16N (7). Los quistes son liberados en las heces, permitiendo el ciclo completo con la infección de un nuevo hospedero (8) (Lopez-Romero et al. 2015).

Mecanismos de defensa del hospedero contra *G. lamblia*

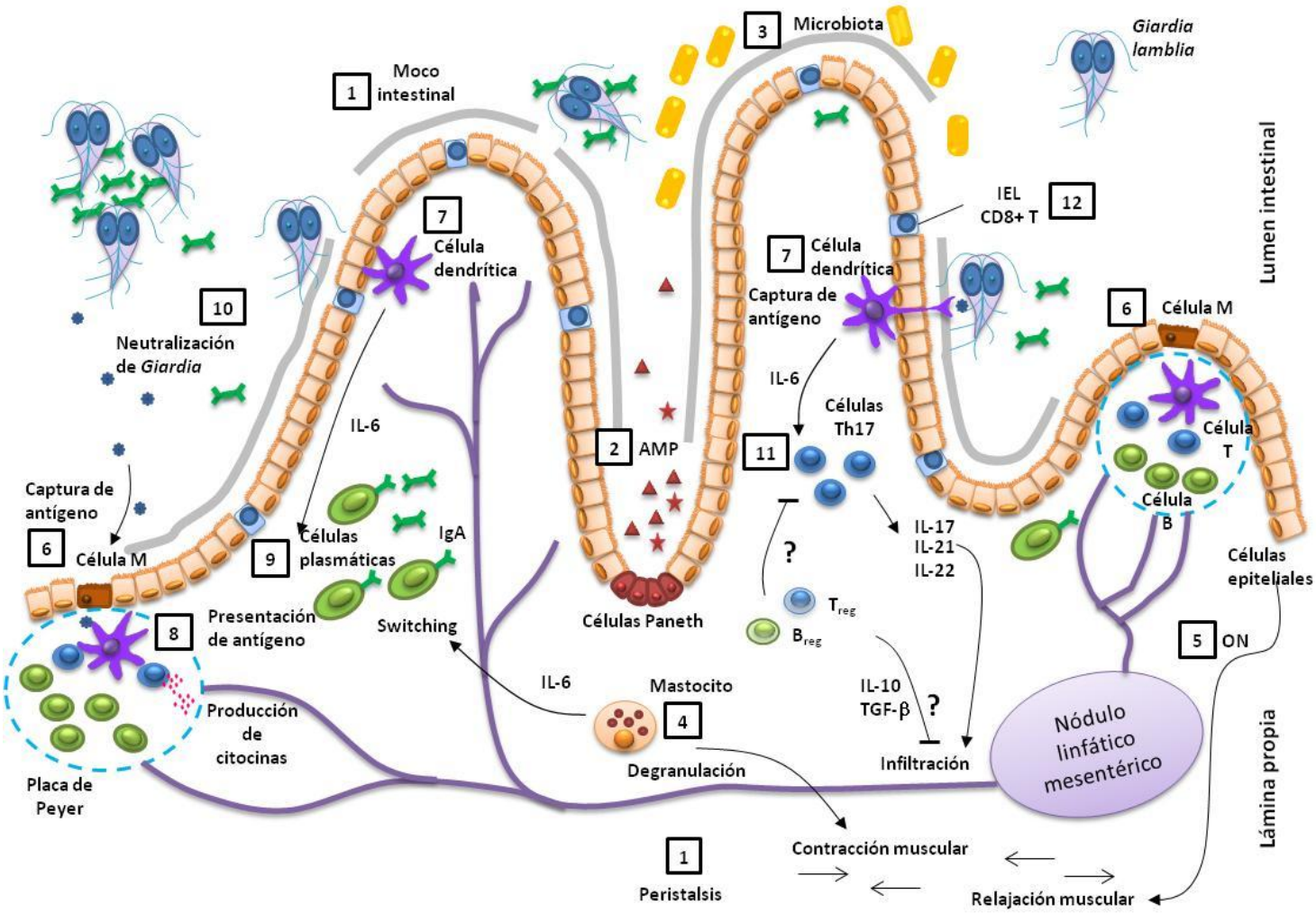

Figura 3. Mecanismos de defensa del hospedero contra *Giardia lamblia*. La respuesta inmune innata y adaptativa actúan en sincronía ara el control de la infección por *Giardia*. Los mecanismos de inmunidad innata son la primera línea de defensa. La capa de moco en la superficie del intestino y los movimientos peristalticos constituyen barreras mecánicas para la adhesión del parásito (1). Los péptidos antimicrobianos liberados por las células de Panneth (AMP) y otras células, pueden matar a trofozoítos (2). La microbiota tiene un efecto anti-*Giardia* por competencia, toxicidad directa o por modulación de la respuesta inmune. Y además, contribuye a preservar la integridad del intestino (3). Los mastocitos liberan citocinas pro-inflamatorias como la IL-6, la degranulación de su contenido promueve la peristalsis (4). Las células epiteliales y algunos leucocitos liberan óxido nítrico (ON), el cual tiene efecto citostático, inhibe los procesos de desenquistamiento y enquistamiento y contribuye a la peristalsis (5). Las células M son importantes para la captura de antígeno hacia placas de Peyer (6). Las células dendríticas juegan un papel como conectores de la inmunidad innata y adaptativa. Se localizan en lámina propia y placas de Peyer donde pueden tener contacto con el antígeno (7). Las dendríticas fagocitan y procesan antígenos de *Giardia* para presentarlos a células T a través de sus MHC-II . Las células T activadas liberan un panel de citocinas que modulan la respuesta anti-*Giardia* (8). La Il-6 liberada por mastocitos, dendríticas o células T, modula la maduración de células B e induce un cambio de isotipo ("switching") hacia IgA (9). Estos anticuerpos pueden actuar como neutralizantes (10). Las células T CD4+ Th17 liberan citocinas IL-17, Il-21, Il-22, los cuales tienen un papel pro-inflamatorio anti-*Giardia* (11). Los linfocitos intraepiteliales (IEL) contribuyen en el daño patológico durante la giardiasis. Existen algunas interrogantes importantes para el entendimiento de la relación hospedero-parásito: ¿Cuál es el papel de las células B y T reguladoras en la modulación de la respuesta inflamatoria durante la giardiasis? Debido a que los trofozoítos no invaden la mucosa, ¿Cómo puede el parásito inducir una respuesta mucosa y sistémica? ¿Cómo la infección por *Giardia* rompe la tolerancia de mucosas? ¿Qué antígenos de *Giardia* inducen una respuesta inmune protectora? ¿*Giardia* podría estar modulando la presentación de antígeno por las células dendríticas? (Lopez-Romero et al. 2015).

Tabla 2. Principales proteínas inmunogénicas de *Giardia lamblia*.

Nombre	Masa molecular (kDa)	Localización	Inmunidad Protectora
Proteínas estructurales			
α-1-giardina	32	Disco Ventral	Yes
α-2-giardina	33	Disco Ventral	NE
α-7.3-giardina	33	Disco Ventral	NE
α-7.1-giardina	34	Disco Ventral	NE
α-11-giardina	35	Disco Ventral	NE
SALP-1	27	Disco Ventral	NE
β-giardina	27	Citoesqueleto	NE
α-2-tubulina	36	Citoesqueleto	NE
β-tubulina	55	Citoesqueleto	NE
GHSP-115	115	Intracelular	NE
Proteínas del metabolismo			
ADI	25	Intracelular	NE
OCT	33.5	Intracelular	NE
FBA	37	Intracelular	NE
UPL-1	38	Intracelular	NE
Enolasa	50	Intracelular	NO
Proteínas Variables de Superficie			
VSPH7	70	Membrana	NE
VSP9B10, VSP1267, VSPA6, VSPS1, VSPS2, VSPS7, VSPS12 y VSPS6**	39-76	Membrana/ Intracelular	SI
TSA 417	25	Membrana	NE
Proteínas de Choque Térmico			
BIP	71	RE/ESV	NE
Proteínas del Quiste			
CWP-1	26	ESV	NE
CWP-2	39	Pared del quiste	SI
Otras			
GTA-1	20		NE
GTA-2	27	Intracelular	NE

**Expresión simultánea por disrupción de variación antigénica. NE, no evaluado; SALP, proteína similar a las de ensamble de fibras estriadas; GHSP, proteína de tallo y cabeza; ADI arginina deaminasa; OCT, ornitin carbamoil transferasa; FBA, aldosa fructosa 1,6-bifosfato; UPL-1, uridin fosforilasa; VSP, proteína variable de superficie; CWP, proteína de pared de quiste; GTA, antígeno de trofozoíto de *Giardia*; ESV, vesículas específicas de enquistamiento; RE, retículo endoplásmico (Lopez-Romero et al. 2015).

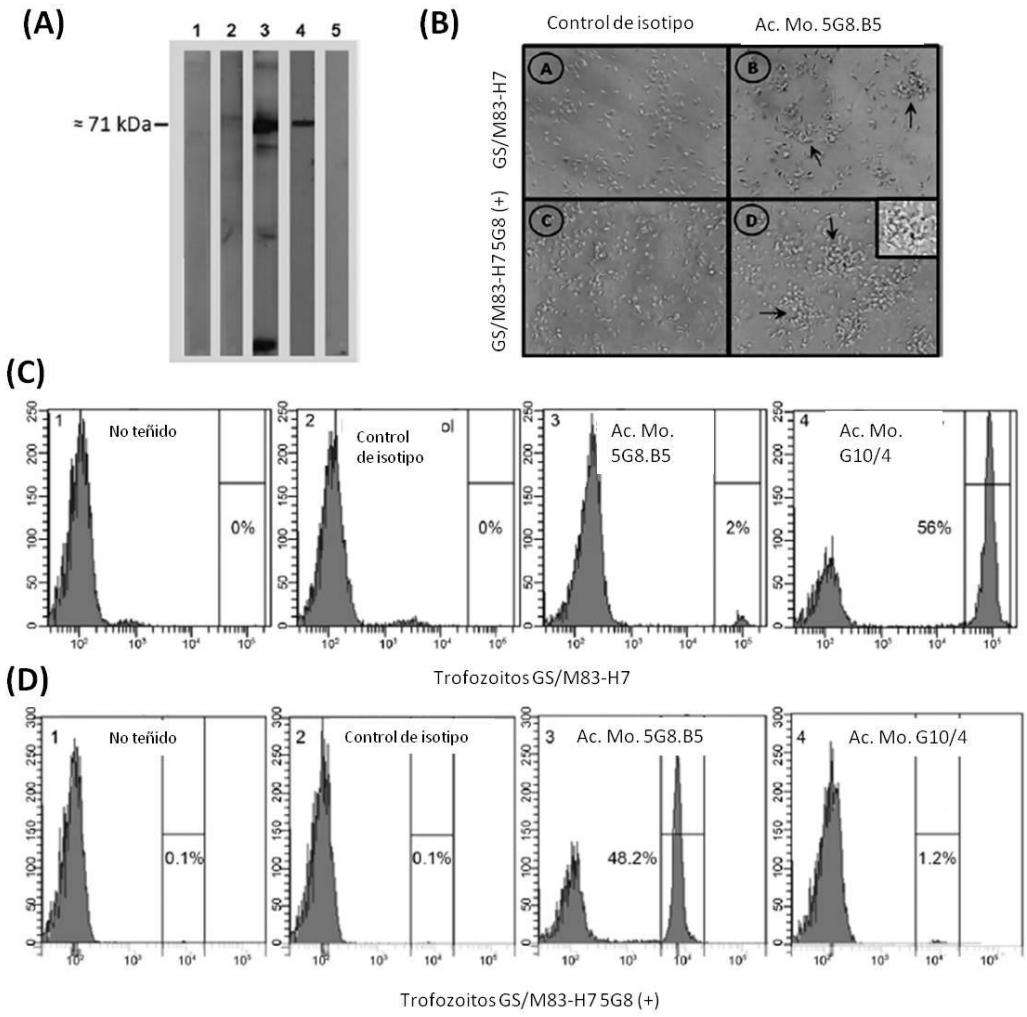

Figura 4. Antecedentes en el estudio de VSP-5G8. A) La proteína 5G8 de *Giardia lamblia* es capaz de estimular en el hospedero infectado la generación de anticuerpos específicos. 1: suero preinmune control, 2: suero de ratones infectados (5ta semana post-infección), 3: suero de ratones re-infectados (2da semana post-reinfección), 4: anticuerpo monoclonal 5G8.B5, 5: control de isotipo. B) Los anticuerpos generados son capaces de inducir aglutinación de trofozoítos in vitro. C) 5G8 es expresada en la superficie de un 2-6% de trofozoítos GS/M83-H7. Para ayudar en el estudio del antígeno 5G8 se seleccionó por inmunoafinidad, usando un anticuerpo monoclonal, la población de trofozoítos que expresan en superficie 5G8, esta población se denominó GS/M83-H7 5G8 (+) y un 60-95% de ellos expresan en superficie 5G8 (Velazquez et al. 2005; Quintero et al. 2013).

CAPITULO 1

Artículo:

Host defences against *Giardia lamblia*

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Keywords: *Giardia lamblia*, host-parasite, immune response, immunogenic proteins

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Review Article

Host defences against *Giardia lamblia*

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SUMMARY

Giardia spp. is a protozoan parasite that inhabits the upper small intestine of mammals and other species and is the aetiological agent of giardiasis. It has been demonstrated that nitric oxide, mast cells and dendritic cells are the first line of defence against Giardia. IL-6 and IL-17 play an important role during infection. Several cytokines possess overlapping functions in regulating innate and adaptive immune responses. IgA and CD4$^+$ T cells are fundamental to the process of Giardia clearance. It has been suggested that CD4$^+$ T cells play a double role during the anti-Giardia immune response. First, they activate and stimulate the differentiation of B cells to generate Giardia-specific antibodies. Second, they act through a B-cell-independent mechanism that is probably mediated by Th17 cells. Several Giardia proteins that stimulate humoral and cellular immune responses have been described. Variant surface proteins, α-1 giardin, and cyst wall protein 2 can induce host protective responses to future Giardia challenges. The characterization and evaluation of the protective potential of the immunogenic proteins that are associated with Giardia will offer new insights into host–parasite interactions and may aid in the development of an effective vaccine against the parasite.

Keywords giardia lamblia, immune response, immunogenic proteins

INTRODUCTION

Giardia spp. is a binucleated flagellated protozoan that lives and reproduces in the small intestine of a diverse array of vertebrate species (1, 2). This parasite is the aetiological agent of giardiasis, one of the most common gastrointestinal diseases worldwide. Giardiasis is most prevalent in developing countries; in these, it exhibits a highly variable prevalence that ranges from 1 to 50%, whereas in developed countries its prevalence ranges from 0·4 to 7% (3–6). The clinical manifestations of giardiasis vary from no symptoms to acute or chronic diarrhoea with abdominal pain, flatulence, weight loss, intestinal lesions and malabsorption syndrome that can last for several months (2).

Giardia has a relatively simple life cycle (Figure 1) that consists of two different stages of structural and biochemical development: the trophozoite (vegetative form), which colonizes the host intestine, and the cyst (infective form), which is resistant to environmental conditions (1, 7, 8). Infection begins with the ingestion of food or water that is contaminated with cysts (2, 9–11). The acidic environment of the stomach induces an excystation process in which one cyst generates two trophozoites that can then colonize the proximal small intestine without invading the epithelia. Trophozoites are resistant to removal via bulk flow in the intestinal lumen because they can attach to intestinal epithelium. When trophozoites migrate into the lower gastrointestinal tract, they encounter changes in the environment, including pH and levels of bile and cholesterol that can trigger encystation. The cysts are then released from the host through the faeces, and the transmission cycle is completed upon cyst infection of a new host (1, 8, 12–14).

The genus *Giardia* consists of six species; of these, only *G. lamblia* (also named *G. intestinalis* or *G. duodenalis*) can infect humans, in addition to several other mammals. *G. muris* is found in rodents, *G. agilis* in amphibians, *G. psittaci* in parakeets, *G. ardae* in herons and *G. microti* in muskrats (1). Genomic studies of *Giardia* have confirmed that eight genotypes of *G. lamblia* exist, named A–H (5, 15). It is known that the A and B genotypes infect humans, although the B genotype is the most frequent worldwide (3, 5). The *Giardia* genotypes that do

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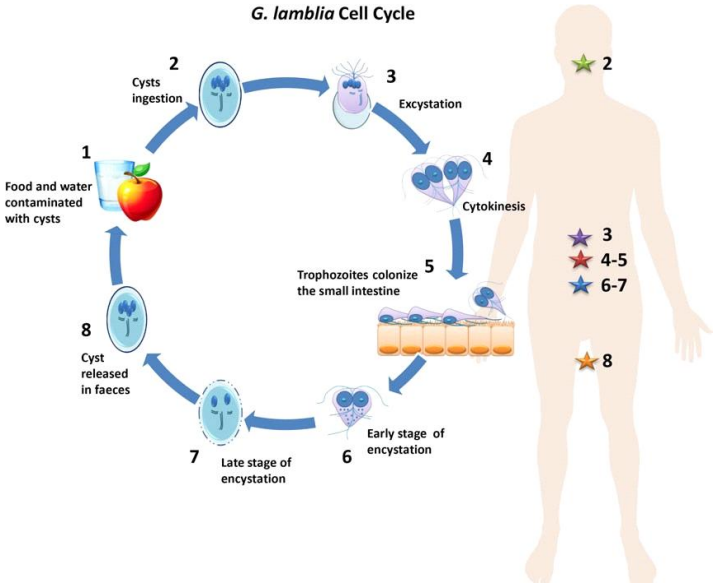

Figure 1 *Giardia lamblia* life cycle. The transmission route of giardiasis is faecal–oral. *Giardia* infection begins with ingestion of cysts from contaminated foods or water (1 and 2). Exposure to the acidic environment of the stomach induces *Giardia* excystation process (3). Each cyst produces two trophozoites (4). Trophozoites colonize and replicate in the small intestine and can attach to intestinal epithelium (5). As *trophozoites* travel down through the intestine, the environment low in cholesterol, high in bile and slightly alkaline can induce an early encystation stage, in which trophozoites round up and specific encysting proteins are transported by vesicles to the cell surface to form the cyst wall (6). The adhesive disc disassembles and the cell undergoes DNA replication to give a cell containing two nuclei (4N each one). During the late encystation stage, nuclei divide, giving four nuclei (2N each one), and DNA is duplicated again to generate a mature cyst with four nuclei and a total ploidy of 16N (Ankarklev *et al.*) (7). Cysts are released in faeces, allowing completion of the transmission cycle by infecting a new host (8).

not infect humans include genotypes C and D, which infect dogs, genotype E, which infects hoofed animals, genotype F, which infects cats, genotype G, which infects rodents, and genotype H, which infects seals (5, 15). However, only genotypes A, B and E can be efficiently grown *in vitro* (2).

Giardiasis infection becomes a self-limited illness in over 85% of affected individuals, indicating the existence of effective host defence mechanisms against the parasite. Interestingly, cases of chronic giardiasis have been documented even in immunocompetent individuals (16). In this review, we describe the most relevant immune mechanisms that have been observed in the interactions between *Giardia* and hosts during giardiasis.

HOST DEFENCE MECHANISMS AGAINST *GIARDIA LAMBLIA*

The intestinal mucosal immune system is extremely complex, as it can recognize and discriminate among food antigens and commensal bacteria and pathogens and still adequately respond to pathogens via innate and adaptive mechanisms. Because *G. lamblia* does not invade the epithelial layer, it causes little to no mucosal inflammation. Although current knowledge regarding the mechanisms of immunity to *Giardia* is limited, several studies have produced significant advances in the understanding of innate and adaptive host responses against the parasite (Figure 2) (9, 16–18).

Mechanisms of innate immunity

The innate immune system is a rapid, nonspecific first line of defence against colonization by pathogens. *Giardia* primarily colonizes the duodenum and jejunum, both of which are hostile environments that limit the survival of microbes because of their high concentrations of digestive enzymes and bile. Intestinal epithelial cells are renewed every 3–5 days. Therefore, *Giardia* must constantly adhere and detach to the epithelia to avoid being entrapped by mucus and eliminated by peristaltic movement. In chronic giardiasis, patients lose intestinal barrier function, which

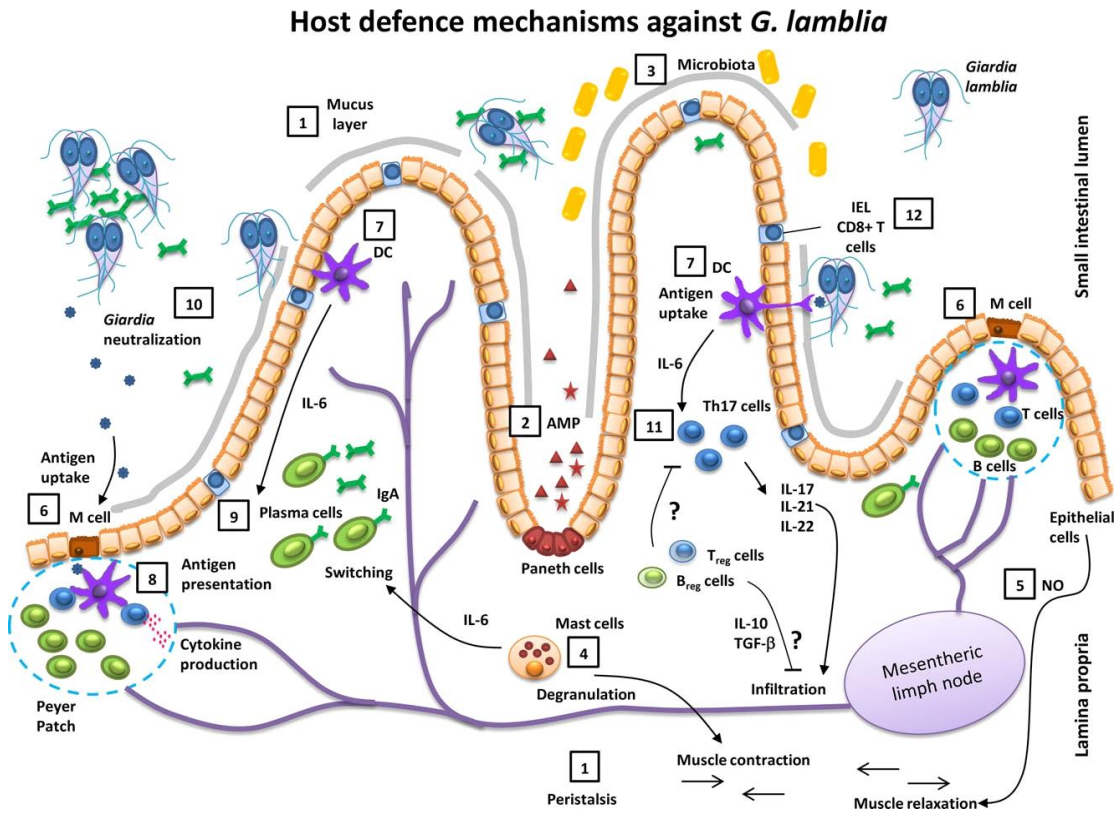

Figure 2 Host defence mechanisms against *Giardia lamblia*. Innate and adaptive immune systems act in synchrony to control *Giardia* infection. Innate immune mechanisms are the first line of defence against *Giardia* colonization. The mucus layer on the intestine surface and peristaltic movements constitute mechanical barriers to *Giardia* attachment (1). Antimicrobial peptides (AMP) released by paneth and other cells can kill trophozoites (2). Gut microbiota have an anti-*Giardia* effect by competition, direct toxicity or modulating the immune response. Additionally, microbiota contributes to preserve gut integrity (3). Mast cells release pro-inflammatory cytokines such as IL-6; mast cell degranulation promotes peristalsis (4). Gut epithelial and immune cells produce nitric oxide (NO), which has a cytostatic effect on *Giardia* trophozoites, inhibits excystation/encystation processes and contributes to peristalsis (5). M cells take up antigens from the lumen of the gut by endocytosis into peyer patches (PP) to induce immune responses (6). Dendritic cells (DC) play a role as a 'connector' between innate and adaptive immunity. Dendritic cells are localized in lamina propria and PP where can recognize antigens. Dendritic cells also can expand their dendrites into the intestinal lumen to take up antigens (7). Dendritic cells ingest and process *Giardia* antigens for further presentation to naive T cells by MHC class II molecules (MHC-II). Activated T cells release a panel of cytokines, which modulate the anti-*Giardia* response (8). IL-6 released by mast cell, DC or T cells is an important modulator of B-cell maturation, and it induces antibody class switching to produce IgA (9). Plasma cells migrate to lamina propria to release IgA, which can inhibit *Giardia* attachment to intestinal epithelial cells (10). Th17 $CD4^+$ T cells are activated during early adaptive immunity against *Giardia*, and release cytokines such as IL-17, IL-21, IL-22, which play a pro-inflammatory role (11). Intra-epithelial lymphocytes (IEL) are mainly $CD8^+$ T cells and play a role in pathological damage of intestine during giardiasis (12). Important gaps in our knowledge on host–*Giardia* relationship: What roles are playing the Breg and Treg cells in the regulation of the inflammatory process during *Giardia* infection? As *Giardia* trophozoites do not invade mucosa, how can the parasite antigens efficiently induce both mucosal and systemic immune responses? How does *Giardia* infection break mucosal tolerance? Which *Giardia* antigens can induce a protective immune response? Does *Giardia* modulate antigen handling by antigen presenting cells (DC, macrophages)?

results in reduced secretion of mucus and enzymes and can contribute to the clinical manifestation of the infection (19, 20). The mucus that is secreted by intestinal goblet cells protects epithelial cells from digestive enzymes and impedes the pathogen access to the epithelia, including *Giardia* (9, 16–18).

Antimicrobial peptides (AMP) are small peptides (<40 aa) that have a broad spectrum of microbicidal

activities. They are produced by a variety of host cells, including epithelial paneth cells (21). Over 500 AMP have been discovered, and they are classified according to their structure as maganins, cathelicidins and defensins. It has been shown *in vitro* that the paneth cell-derived defensins (cryptidins) 2 and 3, the neutrophil defensin NP-2 and the cathelicidin indolicidin can reduce trophozoite viability (22). However, studies conducted *in vivo* demonstrated that metalloproteinase-7-deficient mice (which cannot produce active defensins) were able to control and eradicate *G. muris* (16), but not *G. lamblia* (23), suggesting that *Giardia* susceptibility to AMP is species dependent.

Gut epithelial and immune cells can synthesize nitric oxide (NO), which has immunomodulatory and cytotoxic activities. NO is produced enzymatically from L-arginine through the action of NO synthase (NOS), which can exist in three different isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). The major isoform that is expressed by intestinal epithelial cells, iNOS, is inducible by cytokines and microbial products (16, 17). It has been observed that patients infected with *G. intestinalis* exhibit increased NO levels (24). Studies conducted *in vitro* have demonstrated that NO inhibits the excystation process and also induces a cytostatic effect on *G. lamblia* trophozoites (25). Interestingly, genotype B and E trophozoites are more susceptible to NO than genotype A (26), suggesting that NO acts in a genotype-dependent manner. Although iNOS deficiency did not significantly affect the clearance of *Giardia*, nNOS deficiency delayed parasite clearance (27). This might be because nNOS also plays a role in gastrointestinal transit and motility, which together form an important barrier against *Giardia* colonization (28). Despite the potential effects of NO on *Giardia* trophozoites, the parasite has developed strategies to evade this host defence mechanism. Trophozoites downregulate the expression of iNOS in intestinal epithelial cells (29). Similarly, paediatric patients infected with *Giardia* exhibit reduced expression of iNOS (30). *Giardia* can inhibit the production of epithelial NO by taking up and consuming arginine as a source of energy (25, 31). As a product of this reaction, ornithine is released, which can competitively inhibit arginine uptake by the intestinal epithelium (30). Arginine depletion is known to induce apoptosis in human cell lines (31, 32). Interestingly, human giardiasis exhibits an increased rate of apoptosis of intestinal epithelial cells, which is one of the pathogenic mechanisms of the disease (33, 34).

The presence of microbiota in the gastrointestinal tract is an innate defence mechanism against pathogens (35, 36). It has been proved that certain commensal bacterial species enable mice to be resistant against *Giardia* colonization (37). Intestinal microbiota can interfere with the pathophysiology of *Giardia* infection and limit the growth of the parasite through diverse mechanisms, including resource competition, the release of inhibitory factors (38, 39), direct toxicity and the induction of cross-reactive immune responses (37, 40–42). Mice that are fed with probiotics (*Lactobacillus rhamnosus, Lactobacillus casei*) exhibit significant increases in antioxidant and superoxide dismutase production, elevated levels of intestinal disaccharidases, and a restorative gut morphology that reduces the severity and duration of giardiasis (43, 44), suggesting that commensal microbiota not only have an anti-*Giardia* effect but also protect and preserve gut integrity during infection. Therefore, differences in microbiotic composition between individuals and species could explain variability in pathology and susceptibility to infection (18).

Some cells of the innate host system can act directly or indirectly against *G. lamblia*. Previous studies have shown that macrophages derived from lymphoid organs and milk can engulf *Giardia* trophozoites both *in vitro* and during infection (45–50). Additionally, a slight increase in the macrophage population of the lamina propria in infected mice has been observed, although they have been described as possible regulatory cells (51). Neutrophils may participate as effectors during giardiasis. The incubation of human polymorphonuclear neutrophil (PMN) with *Giardia* trophozoites and hyperimmune serum triggers an oxidative response in PMN (52). There is no evidence that PMN infiltration is associated with giardiasis. However, *in vivo* neutrophil infiltration has been observed in mice as an indirect consequence of microbiotic invasion into the mucosa (53). Eosinophilia may be result of parasite exposure (54). It has been proved that IL-5 plays a central role in eosinophil development, activation and survival (55).

Mast cells are currently recognized as effector cells of the immune response against several parasites. Mast cell-deficient mice (c-kit$^{w/wv}$) that were infected with *G. lamblia* failed to produce parasite-specific IgA and could not eliminate the infection, whereas wild-type mice cleared the infection normally (56). Interestingly, in tissues from the small intestine that have been infected with *G. duodenalis*, the most strongly induced transcripts are mast cell proteases (23). *Mast cells* and NO may act together to induce peristalsis (27, 57). *Mast cells* can also contribute to B-cell survival, activation and differentiation into plasma cells, driving the development of IgA-oriented humoral immune responses (58). These results suggested that mast cells play a significant role in the protective immune response against *Giardia*.

In intestinal epithelia, antigens are preferentially uptaken through specialized areas in the follicle-associated epithelium. Microfold cells (M cells) are specialized for delivering foreign material from the intestinal lumen to the

organized lymphoid tissues within the mucosa by transepithelial transport (59). These cells are being considered as a target for mucosal vaccine strategies (60). However, little is known about their involvement during giardiasis.

Dendritic cells (DC) also play a role in the clearance of giardiasis. *G. lamblia* is a weak activator of murine and human DC (61, 62). However, *Giardia* lysates, excretory–secretory products and other specific proteins can activate the maturation of DC by causing increases in the expression of pro-inflammatory cytokines, such as IL-6, TNF-a, IL-12, and of surface molecules, such as CD80, CD86, MHC class II molecules (63, 64). Accordingly, the presence of *Giardia* may activate the maturation and migration of DC to the infection site and the subsequent release of immunomodulatory cytokines.

As *Giardia* does not invade epithelia, it is notable that the parasite can induce local and systemic antibody and T-cell responses (65–67). The understanding of how *Giar- dia* is able to induce efficient systemic immune responses has not yet been resolved. Nevertheless, M cells and DC might be implicated in the transport of *Giardia*-specific antigens to local and systemic immune organs. M cells could enable the movement of *Giardia*-specific antigens into peyer patches, where an army of B and T lympho- cytes would be waiting. DC express tight junction proteins and therefore might establish connections with intestinal endothelial cells by extending their dendrites into the intestinal lumen, enabling *Giardia*-specific antigens to be detected (68). Once activated, DC induce the downregula- tion of tight junctions so that they can migrate to other sites and induce adaptive immune mechanisms (69).

Cytokines

Cytokines are small soluble proteins that modulate the differentiation, activation and proliferation of cells. They are produced by a variety of cell types, and several of them possess overlapping functions for regulating innate and adaptive immune responses (70). Some cytokines, such as IL-6, IL-12 and IL-17, play a double role in activating both innate and adaptive mechanisms (71–73). Previous studies on animal models of experimental giardiasis have reported elevated levels of cytokines, including TNF-a, IFN-c, IL-6, IL-12, IL-5, IL-4, IL-2, IL-13, IL-17, IL-22 and IL-23 (62, 64, 74–83). The cytokine that is mainly elevated during giardiasis is IL-6, a pro-inflammatory cytokine that induces innate cellular responses and also mediates B-cell switching to IgA and T-cell development into Th17 cells (73, 84–86). IL-6-deficient mice fail to eliminate *Giardia* and also exhibit altered intestinal cytokine responses despite having normal levels of IgA (80, 81). Although there are many sources of this cytokine, DC-derived IL-6 is fundamental to the clearance of *Giardia*. This is probably because the early production of IL-6 by DC is important for T-cell differentiation (79, 87). The presence of IL-6 during initial contact between DC and T cells could be a key determinant of the type of response that ensues. Interestingly, studies in mice and in cattle that had been infected with *Giardia* have shown that the parasite induces upregulation of IL-17 in the host (76, 82, 88). Moreover, IL-17 receptor A (IL-17AR)-knockout mice infected with *Giardia* were unable to eliminate the parasite (88). IL-6 and IL-17 participate in the development of Th17 $CD4^+$ T cells, which mediate early adaptive immune mechanisms (64, 82, 88–90). These results suggest that Th17 lymphocytes play an important role in the elimination of *Giardia*.

TNF-a is another pro-inflammatory cytokine that is released during *Giardia* infection (64, 76, 82). TNF-a-deficient mice exhibit an obvious delay in the clearance of *Giardia*, suggesting that TNF-a plays an important role in giardiasis (75). Nevertheless, the mechanisms by which TNF-a affects *Giardia* are still unknown. TNF-a is not believed to be a component of the immune mechanisms that have been previously described for *Giardia*, such as IgA production, mast cell responses or IL-6 expression (75). IFN-c and IL-4 are the main cytokines that are produced during Th1 and Th2 responses, respectively, and they appear to play a role in giardiasis because they are elevated in infection models (76, 80, 91). However, IFN-c- and IL-4-deficient mice can eliminate the parasite normally, suggesting that neither Th1 nor Th2 responses are absolutely necessary for the clearance of *Giardia* (92). Sera samples collected from infected humans have also revealed a high induction of Th1, Th2 and Th17 cytokines, including IL-2, IL-5, IL-6, IL-8, IL-12, IL-13, IL-23, IFN-c and TNF-a (93–95). Additionally, high serum levels of IL-4, IL-5, IFN-c and MCP-1 have been correlated with an increased duration of *G. lamblia* infection, whereas high levels of IL-8 have been associated with shorter infections (96). Therefore, *Giardia* infections in animals and humans produce a wide array of cytokines that integrate innate and adaptive mechanisms to eliminate the parasite.

Giardia does not induce significant cell infiltration during infections in humans, mice or cattle (78, 83). The recruitment of DC and granulocytes and the activation of lymphocytes might even be diminished in response to the downregulation of pro-inflammatory cytokines (34, 62, 78, 83, 97, 98). Moreover, the release of IL-10 and TGF-b impedes the recruitment of inflammatory cells (62, 64, 74, 76, 91). It is noteworthy that these cytokines are mainly produced by $CD4^+$ T lymphocytes (76). Collectively, the evidence detailed above suggests that active regulatory responses in the intestinal tract are either induced by the

host to reduce inflammation and immune-mediated pathology or induced by *Giardia* to promote its persistence in the intestine (75).

Adaptive immunity mechanisms

If innate immune mechanisms cannot eliminate the pathogen, it continues growing and expressing and releasing antigens, which triggers adaptive immune responses (70). Specific humoral and cellular immune responses play a role in *Giardia* clearance. Several reports have indicated that the clearance of *Giardia* in immunocompetent murine models occurs at approximately 3–5 weeks post-infection, which correlates with the establishment of humoral immunity (16, 67, 92, 99). Repeated infections in humans and animal models have shown that after an initial exposure to *Giardia*, individuals develop immunity to a second infection or at least present with a lesser degree of pathological damage in epithelia (18). Host control of *Giardia* requires mucosal humoral and cellular immune responses, such as a balanced response of antigen-specific $CD4^+$ T cells, the release of cytokines, including IL-6, TNF-a, IFN-c and IL-4, and the production of specific IgA or IgG antibodies against parasite antigens (9, 75, 79, 91, 100).

It has been established that antibodies, particularly of the IgA isotype, contribute to the maintenance of protective immunity against giardiasis (99). The protective and clearance capacity of IgA during giardiasis has been investigated. Anti-*Giardia* secretory IgA can be detected in human saliva and breast milk (101, 102). Mice deficient in antibody production, mainly of type IgA, fail to eliminate *G. muris* and *G. lamblia* (99). However, B-cell-deficient mice that are infected with *G. lamblia* and *G. muris* can eliminate both *Giardia* species (92). Additionally, mice deficient in the poly-Ig receptor (pIgR) fail to transport secretory IgA into the intestinal lumen. pIgR-deficient mice develop chronic infections when infected with *G. muris* but not in the case of *G. lamblia*, suggesting a higher sensibility of *G. lamblia* to Ab-independent mechanisms compared to *G. muris* or, conversely, that *G. muris* is more sensitive to Ab-dependent cytotoxicity (103).

Giardia infection in children induces the production of secretory antibodies in saliva and stool. Salivary (IgA) and serum (IgG) responses against *G. duodenalis* infection were significantly higher in infected patients than in controls. This observation could lead to the development of a diagnostic tool that measures specific salivary IgA and specific serum IgG antibodies to monitor the exposure of various populations to *G. duodenalis* (102, 104). Therefore, B cells and especially IgA antibodies are important but not strictly required for the control of *Giardia* infections.

T-cell-mediated immune responses play an essential role in the clearance of *Giardia* infections. In humans and animals, a decrease in $CD4^+$ T-cell populations contributes to the development of chronic giardiasis (2, 92). Moreover, performing adoptive transfer of $CD4^+$ T cells into T-cell-deficient mice restores their resistance to infection in *G. muris* and *G. lamblia* models (2, 92). Although there is evidence of the important role that T cells play during the course of *Giardia* infection, the mechanisms of action are poorly understood. Hanevik *et al.* demonstrated a host immunological memory to *Giardia* in humans. They found *Giardia*-reactive $CD4^+$ T cells in the peripheral blood of individuals who were infected 5 years prior during an outbreak of assemblage B. It is noteworthy that memory cells were activated in response to A and B genotype antigens, suggesting that cellular immune responses are not assemblage specific (105). It is possible that $CD4^+$ T cells can cooperatively induce B-cell activation, maturation and differentiation into plasma cells to produce more specific and efficient immunoglobulins. B-cell-deficient mice can eliminate the vast majority of parasites when infected with *G. muris* or *G. lamblia*, whereas T-cell-deficient mice are not able to control these infections (92). These results suggest a B-cell-independent mechanism of $CD4^+$ T-cell control during giardiasis. Recent publications have revealed a possible mechanism by which T cells act independently of B cells. Calves infected with *G. duodenalis* exhibited a significantly increased proliferation of $CD4^+$ ab T cells. T-cell characterization revealed increased expression of IL-17, and only a few cells were found to express FoxP3 (82). Moreover, spleen and mesenteric lymph node cells from C57BL/6 mice that were infected with *G. muris* exhibited a prominent induction of IL-17A beginning at 1 week p.i. When IL-17A action was impaired by the deletion of the IL-17RA receptor, the infected animals could not clear the parasite (88). In summary, these findings suggest that T-cell-mediated protective immune responses to giardiasis could be partially modulated by Th17 responses.

Interestingly, T cells are also associated with pathological damage that causes alterations of the apical membranes of enterocytes during giardiasis. Athymic nu/nu mice showed no histological changes after *Giardia* infection (106). Further studies revealed that alterations of enterocyte membranes are parasite independent and can be achieved by injecting primed $CD8^+$ T cells, but not $CD4^+$ T cells, taken from normal mice that were infected with *G. muris* (107). Intra-epithelial lymphocytes (IEL) are T cells that reside between intestinal epithelial cells, and they are comprised mainly of $CD8^+$ T cells. Despite the protective roles of IEL in other parasitic infections such as helminthiasis, increased numbers of IEL have been found in patients with chronic giardiasis; these induce brush border

injury and malabsorption syndrome, including the impairment of glucose/sodium uptake (33). T cells also contribute to gut damage by causing the impairment of disaccharidase, which occurs through an immune-based mechanism that requires $CD8^+$ T cells (76). In this context, whereas $CD4^+$ T cells are important mediators of *Giardia* elimination, $CD8^+$ T cells play a role in the immunopathology of the disease, indicating that T cells play dual roles during giardiasis. Collectively, these results suggest that either immunotherapy or vaccines that can promote $CD4^+$ T-cell responses without generating $CD8^+$ T-cell responses could lead to host protection without contributing to gut damage.

Intestinal Treg and Breg cells regulate immune responses to food, microbiota and autoantigens through secreting anti-inflammatory cytokines (69, 108–110). Studies on cat- tle that were infected with *Giardia* indicated a proliferation of T cells and $CD4^+FoxP3^+$ Treg cells, which demonstrates that regulatory mechanisms exist in the host during giardiasis (82). Further studies will be necessary to elucidate and highlight which regulatory mechanisms are involved during the disease. Tr1, Treg (IL-10 dependent), Th3 (TGF-b dependent) (69, 109) and Breg (IL-10 and TGF-b dependent) cells have all been implied in reducing the inflammation that occurs in response to infectious diseases (108). Because IL-10 and TGF-b are strongly upregulated in hosts suffering from *Giardia* infection, it would be interesting to determine whether Treg and Breg cells participate in the regulation of inflammatory responses during giardiasis.

IMMUNOGENIC PROTEINS OF *GIARDIA*

Little is known about which *Giardia* antigens stimulate effective immune responses. It is important to continue to increase our knowledge regarding the immunogenic proteins of *Giardia*, which can induce protective host humoral and cellular responses. Despite the importance of both T cells and B cells in anti-*Giardia* immunity, the majority of previous studies have been focused on the proteins that are recognized by the humoral response (Table 1).

The major proteins that have been identified as target antigens differ in their function and localization in the parasite and include the following immunogenic antigens: variant surface proteins (VSP), giardins, tubulins (cytoskeletal proteins), heat-shock proteins (HSPs), cyst wall proteins (CWPs) and proteins related to the metabolism of the parasite, such as enolase-a, fructose-1,6-biphosphate aldose (FAB), arginine deaminase (ADI) and ornithine carbamoyl transferase (OCT), among others. The first proteomic analysis that was performed for *Giardia* described 16 immunoreactive proteins that were identified by the serum (IgG) of infected individuals (111). Further studies uncovered similar results when secretory IgA antibodies from infected patients were analysed (112). Velazquez *et al.*, using a model of *G. lamblia* infection in C3H-heJ mice, described a group of protein bands that were recognized by secretory and systemic antibodies. During primary infection, the recognized bands corresponded to proteins of 63, 71 and 86 kDa, whereas during secondary infection, additional proteins were detected (48, 55, 106 and 159 kDa). These changes to the pattern of antibody recognition could be due to antigenic variation in the parasite (67). Previous studies have reported a highly immunogenic *Giardia* protein of 71 kDa. This protein can stimulate host defences to produce antibodies, which can then induce *in vitro* agglutination of *Giardia* trophozoites (113). However, additional experiments are needed to evaluate the protective role of this protein and to characterize it on a molecular level.

Variant surface proteins are cysteine-rich proteins with molecular masses that range between 20 and 200 kDa. The most characterized VSP is VSPH7, a 56-kDa protein that is highly immunogenic (2). Mechanisms of antigenic variation in *Giardia* are controlled by interfering RNA (iRNA), and the disruption of this pathway generates trophozoites that simultaneously express numerous VSP. Furthermore, gerbils subjected to primary infection with *Giardia* expressing many VSP were protected from subsequent infections (114), suggesting that antigenic variation is essential for immune evasion; this information might be useful for vaccine development strategies.

Giardins are small, structural, constitutive proteins (29–38 kDa) that can be classified into four groups: a-, b-, c- and d-giardins. They are associated with microtu- bules in the ventral discs and plasma membranes of trophozoites (100). Palm *et al.* detected a 32-kDa immu- noreactive protein in serum samples that were taken from patients infected with *Giardia*; it was identified as being a-1-giardin (111). Characterization of this protein revealed that it contains an epitope between amino acids 160 and 200 that is highly immunogenic (115). Further studies have demonstrated that a-1-giardin not only stimulates the production of anti-*Giardia* antibodies (IgA and IgG2a) but also establishes protection against posterior challenges (116).

Several metabolic proteins *that* have been identified in *Giardia* extracts act to activate humoral responses in infected humans and mice. The *Giardia* enzymes ADI and OCT use arginine to generate ATP and are considered to be *Giardia* virulence factors (103, 111, 112). Arginine depletion modulates the immunophenotypes and cytokine secretions of DC during giardiasis (117). The FAB protein

Table 1 Main immunogenic proteins of *Giardia lamblia*

Name	Molecular weight (kDa)	Localization	Protective immunity	References
Structural proteins				
a-1-Giardin	32	Ventral disc	Yes	(103, 111, 112, 116)
a-2-Giardin	33	Ventral disc	NE	(103, 111, 112)
a-7.3-Giardin	33	Ventral disc	NE	(111, 112)
a-7.1-Giardin	43*	Ventral disc	NE	(103, 111, 112)
a-11-Giardin	35	Ventral disc	NE	(103, 111)
SALP-1	27	Ventral disc	NE	(111, 112, 135)
b-Giardin	27	Cytoskeleton	NE	(103, 111, 112)
a-2-Tubulin	50*	Cytoskeleton	NE	(103, 111, 136)
b-Tubulin	55	Cytoskeleton	NE	(111, 112)
GHSP-115	115	Intracellular	NE	(137)
Metabolism proteins				
ADI	64*	Intracellular	NE	(103, 111, 112, 138, 139)
OCT	33·5	Intracellular	NE	(103, 111, 112, 116)
FBA	37	Intracellular	NE	(103, 111, 112)
UPL-1	38	Intracellular	NE	(103, 111)
Enolase	50	Intracellular	NO	(103, 111, 112, 116)
Variant surface proteins				
VSPH7	57*	Membrane	NE	(111, 140, 141)
VSP9B10, VSP1267, VSPA6, VSPS1, VSPS2, VSPS7, VSPS12 and VSPS6[a]	39–76	Membrane/Intracellular	Yes	(114)
TSA 417	25	Membrane	NE	(111, 112, 140, 141)
Heat-shock proteins				
BIP	71	ER/ESV	NE	(63, 127, 129, 130, 142, 143)
Cyst proteins				
CWP1	26	ESV	NE	(144, 145)
CWP2	39	Cyst wall	Yes	(74, 77, 133, 144, 146)
Others				
GTA-1	20		NE	(111)
GTA-2	27	Intracellular	NE	(111)

[a]Simultaneous expression by antigenic variation disruption.
NE, no evaluated; SALP, striated fibre-assembling-like protein; GHSP, *Giardia* head stalk protein 115; ADI, arginine deaminase; OCT, ornithine carbamoyl transferase; FBA, fructose-1,6-biphosphate aldose; UPL-1, uridine phosphorylase; VSP, variant surface protein; TSA, trophozoite surface antigen; BIP, binding immunoglobulin protein; CWP, cyst wall protein; GTA, *Giardia* trophozoite antigen; ESV, encystation-specific vesicles; ER, endoplasmic reticulum.
*Data obtained from Giardia database (www.giardiadb.org)

catalyses the cleavage of fructose-1,6-biphosphate. This enzyme was shown to be recognized by hyperimmune serum samples taken from humans and mouse with giardiasis (103, 111, 112), and also it has been identified in humans who were infected with *Onchocerca volvulus* (118) and *Schistosoma mansoni* and in murine models (119, 120). Enolase is secreted by *Giardia* in the presence of epithelial cells (103, 111, 112, 121); this hydrolase catalyses a reversible elimination of water from 2-phosphoglycerate to form phosphoenolpyruvate. Enolase has been shown to be localized in the cytosol and on the cell surface of several organisms (122, 123).

Heat-shock proteins are chaperone proteins that are expressed in live cells. HSPs contribute to cell surveillance under conditions of intrinsic or extrinsic stress. The most well-studied HSP is HSP70 (70 kDa), which has been reported in other organisms as being capable of *stimulating* the innate immune system. In fact, HSP70 have been proposed as candidate adjuvants for vaccines against several diseases and also as vaccine candidates against other infections different from *Giardia* (124–126). Current knowledge regarding *Giardia* HSP70 is limited; they have been used as molecular markers of organelles (endoplasmic reticulum (ER), encystation-specific vesicles (ESV)), life cycle stages (encystation) and giardiasis diagnosis (127–131). GRP-78, or binding immunoglobulin protein (BIP), is an HSP70 chaperone protein that was identified in *Giardia* as a resident protein of the ER, and it has also been shown to be associated with ESV (127, 129, 130). However, in human cells, specifically in tumour cells, BIP

can also be found at the cell surface (132). Previous studies in BALB/c mice indicated that BIP could be recognized by infected and immunized mice serum (IgG) (63).

Cyst wall proteins are expressed during the process of encystation and during the lifetime of a cyst, and they can be classified into two different groups. Group I proteins are expressed during the early stages of encystation and are localized to ESV, whereas group II proteins are localized exclusively into cyst wall surface. This group of proteins represent a candidate antigen that may potentially be used in the development of a vaccine that blocks *Giardia* transmission. The CWP2 protein is found in the cell wall of cysts. Larocque *et al.* (74) observed that when mice were immunized with CWP2, the production of anti-*Giardia* IgA and IgG2a was induced, and an inhibitory effect on cyst liberation was revealed when the mice were challenged with trophozoites (74). Further studies have shown that the administration of CWP2 DNA vaccines by bactofection delivery leads to the elimination of at least 60% of cysts (77, 133).

Studies investigating the specific *Giardia* antigens that can be recognized by T cells to effectively activate an immune response have been limited. Cellular immune response plays an important role in the control of *Giardia* infection. Astiazaran *et al.* generated the first described *Giardia*-specific T-cell hybridomas to evaluate T-cell activation by *G. lamblia* proteins. Interestingly, proteins of 90–110, 65–77 and 40–64 kDa stimulated several hybridomas (134). These results suggested that T cells actively participate in *Giardia* antigen recognition. However, additional studies are needed to characterize these immunoreactive proteins. Interestingly, BIP is able to induce DC maturation and the release of pro-inflammatory cytokines (TNF-a, IL-12 and IL-6) *in vitro*. Stimulated DC induce the activation of naive $CD4^+$ T cells (63). Although there is evidence that BIP has immunogenic potential, it is unfortunately still unknown whether this antigen may induce the development of effective immune mechanisms against *Giardia* in an infected host. The identification of immunoreactive proteins that can effectively stimulate T-cell and B-cell responses will be important to the development of new anti-*Giardia* drugs and vaccines.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The clearance of *Giardia* from a host requires both innate and adaptive immunity effector mechanisms. Innate immunity mechanisms are the first line of defence against colonization by pathogens (mucus, peristalsis, cytotoxic factors, NO, microbiota, *mast cells* and DC). Cytokines act as a bridge between innate and adaptive immune responses. IL-6, which is derived from DC, is a major cytokine in the defence against giardiasis; it modulates B-cell maturation and switching to produce IgA, and it also mediates T-cell differentiation. It was previously thought that IgA was the main mechanism that drove *Giardia* elimination from the intestine. However, it is cur- rently known that additional Ab-independent mechanisms exist. It has been proved that $CD4^+$ T cells are fundamental in the control of *Giardia*. Recently, Th17 $CD4^+$ T cells have been described to exert control of *Giardia* infections. Although the production of pro-inflammatory cytokines is increased, little to no inflammation is observed during giardiasis. Numerous questions remain unanswered. For instance, what roles are playing the Breg and Treg cells in the regulation of the inflammatory process during *Giardia* infection? How can the parasite antigens efficiently induce both local and systemic immune responses when it does not invade mucosa? How does *Giardia* infection break mucosal tolerance? Which *Giardia* antigens can induce a protective immune response? Does *Giardia* modulate antigen handling by antigen presenting cells (DC, macrophages)? It would also be worthwhile to study how *Giardia* infection affects the following: (i) immunity against other micro-organisms, (ii) oral tolerance to food, microbiota and vaccine antigens and (iii) inflammatory diseases. Future studies are still needed to fully understand the host–*Giardia* relationship and the immunological host defences that control and eliminate *Giardia* infection.

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CAPITULO II

Artículo:

Characterization of BIP protein of *G. lamblia* as a potential immunogen in a mouse infection model

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ABSTRACT

Giardia lamblia is a protozoan parasite that causes one of the most common gastrointestinal diseases worldwide. To eliminate the parasite from the host intestine, it is necessary the activation of B-cell and T-cell dependent mechanisms. The knowledge about *Giardia* antigens that can stimulate the host immune response is limited. In addition to that, the few reports about immunogenic proteins of the parasite are mainly focused on humoral response. Recent evidence suggests that a 70 kDa protein of *G. lamblia* may activate both, humoral and cellular systemic immune responses. Previously, we have reported the 5G8 protein (70 kDa) as a highly immunogenic antigen from *G. lamblia*, Based on this and other results we hypohesized that 5G8 could be the HSP70 BIP. In the present study, we characterized immunochemically the HSP70 BIP of *Giardia*. Flow cytometric assays and western blotting were used to determine the expression profile of 5G8 and BIP in *Giardia* trophozoites. The differences in their expression profiles indicated that BIP and 5G8 are different proteins. ELISA and Western blotting assays of infected mice serum revealed that BIP can also be recognized by antibodies produced during the infection process of C3H-HeN mice. MTT assays did not revealed the activation of cellular immune response induced by BIP *in vitro*. In addition, we identified the potential B-cell and T- cell epitopes of the BIP protein. BIP is a conserved protein among *Giardia* strains and other pathogens. The complete immunological characterization of this antigen will contribute to a better understanding of the host-parasite mechanisms of *Giardia* as well as other common pathogens.

Keywords: *Giardia lamblia*; immune response; immunogenic protein; BIP.

1. INTRODUCTION

Giardia lamblia is a binucleated flagellated protozoan that lives and reproduces in the small intestine of some mammals including humans (Adam 2001; Luján et al. 2011). This parasite is the etiological agent of giardiasis, one of the most common gastrointestinal diseases worldwide (Adam 2001). Giardiasis infection becomes a self-limited illness in over 85 % of affected individuals, indicating the existence of effective host defence mechanisms against the parasite. Interestingly, cases of chronic giardiasis have been documented even in immunocompetent individuals (Eckmann 2003).

It has been reported the importance of both T cells and B cells in anti-*Giardia* immunity (Lopez-Romero et al. 2015). However, little is known about which *Giardia* antigens stimulate effective immune responses. In addition to that, most of the studies about *Giardia* immunogenic proteins, have been mainly focused on characterizing the host humoral response. We have previously described a group of protein bands (SDS-PAGE) that were recognized by secretory and systemic antibodies, using a model of *G. lamblia* infection in C3H-HeJ mice (Velazquez et al. 2005). During primary infection, the recognized bands corresponded to proteins of 63, 71 and 86 kDa, whereas during secondary infection, additional proteins were detected (48, 55, 106 and 159 kDa). Additionally, we generated the first described *Giardia*-specific T cell hybridomas to evaluate T cell activation by *G. lamblia* proteins. Interestingly, proteins of 90-110 kDa, 65-77 kDa and 40-64 kDa stimulated several hybridomas (Astiazaran-Garcia et al. 2009). We realized that *Giardia* proteins of ≈ 70 kDa can strongly stimulate humoral and also probably cellular immune responses. Further studies have indicated that one 70 kDa proteins named 5G8 can stimulate host defences to produce antibodies, which can then induce *in vitro* agglutination of *Giardia* trophozoites (Quintero et al. 2013). Mass spectrometry analysis (ESI-MS/MS) of this protein identified, among others, the Binding Immunoglobulin Protein (BIP), a heat shock protein of 70 KDa (HSP 70). In addition, it has been reported that *G. lamblia* BIP protein is potentially recognized by IgG antibodies from an infection model in BALB/C mice and also can induce activation of DCs and antigen presentation to naive T cell (Lee et al. 2014).

HSPs are chaperone proteins that are expressed in live cells, they contribute to cell surveillance under conditions of intrinsic or extrinsic stress (Beckmann et al. 1992).

The HSP70 have been reported in other organisms as being capable to stimulate the innate immune system (Bolhassani et al. 2008). In fact, HSP70 have been proposed as candidate adjuvants for vaccines against several diseases, and also as vaccine candidates against other infections different from *Giardia* (Segal et al. 2006; Tamura et al. 2011; Dhakal et al. 2013; Assadian et al. 2014; Batra et al. 2014). Current knowledge regarding *Giardia* HSP70 is limited, members of this family have been used as molecular markers of organelles, life cycle stages (encystation) and giardiasis diagnosis (Lujan et al. 1996; Soltys et al. 1996; Stefanic et al. 2006; Kim et al. 2009; Lee et al. 2009). GRP-78 or BIP is an HSP70 chaperone protein that was identified in *Giardia* as a resident protein of the endoplasmic reticulum, and it has also been shown to be associated with encystation specific vesicles (ESV) (Lujan et al. 1996; Soltys et al. 1996; Stefanic et al. 2006). However, in human cells, specifically in tumour cells, BIP can also be found at the cell surface (Zhang et al. 2010). Although there is evidence that BIP has immunogenic potential, it is unfortunately still unknown whether this antigen may induce the development of effective immune mechanisms against *Giardia* in an infected host that could lead a protection to future *Giardia* challenges. The identification of immunoreactive proteins that can effectively stimulate B- and T- cell responses will be important for the development of new anti-*Giardia* drugs and vaccines. The aim of the present study is to determine whether 5G8 and BIP are the same antigens of *Giardia* and characterize the humoral and cellular host immune response against the protein rBIP of *G. lamblia*.

2. MATERIALS AND METHODS

2.1. Mice and *Giardia* strains

C3H/HeN female mice of six to eight weeks old were used for these assays. Mice were reproduced and maintained at Universidad de Sonora bioterium with light/dark cycles of 12h at 25 °C and sterile water and food *ad libitum*. Mice were maintained according to official Mexican standard (NOM-062-ZOO-1999) and according to Departamento de Investigación y Posgrado en Alimentos bioterium guidelines. Mice were euthanized according to NOM-033, 1995 (NOM-033 1995; NOM-062 1999).

2.2. *G. lamblia* trophozoites culture

G. lamblia trophozoites (clone GS/M-83-H7 (ATCC 50581)) were obtained from the American Type Culture Collection. *G. lamblia* GS/M83-H7-5G8 (+) (50-95% 5G8 (+)), were previously obtained by immunobsorption assay (Quintero et al. 2013). Axenic *G. lamblia* cultures were maintained in the TYI-S-33 medium, which was supplemented with newborn calf serum (NBCS) and antibiotics at 37 °C (Ceftriaxone 100μg/mL).

2.3. *G. lamblia* protein lysates

G. lamblia soluble extract proteins were obtained using a modified method from the described by Gottstein et al. in 1990. *G. lamblia* trophozoites cultures were harvested during log-phase growth and were incubated on ice during 15 min to detach cells. The harvested trophozoites were washed three times with sterile phosphate buffered saline (PBS). Then, the trophozoites were lysed by three cycles of frozen (-80 °C) and thawed [room temperature (RT)] in the presence of a protease inhibitor cocktail [23 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, 0.3 mM pepstatin A, 0.3 mM E-64, 2 mM bestatin and 100 mM sodium ethylenediamine tetraacetic acid (Sigma, St. Louis, MO, USA)]. The *G. lamblia* lysate was sonicated three times during 60 sec and 10% amplitude (Brandon Sonifier S-250D, Shelton, CT, USA). Cell debris were removed by centrifugation (10,000 *g* for 30 min). Protein concentration values were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

2.4. Expression plasmid design

The peptide sequence of the Binding Immunolobulin Protein (BIP) (EES99538.1) of *G. lamblia* GS/M-83-H7 (ATCC 50581) was taken from *Giardia* database (http://giardiadb.org/giardiadb/). This sequence was modified following Soltys et. al. 1996 severances (deletion of 47 aminoacids at N-end) to ensure the recombinant expression, since N-terminal includes the signal peptide which is not present in mature protein and this sequence could affect the expression efficiency in a recombinant system (Soltys et al. 1996). We add a 6xHIS sequence (at N-end) and a thrombin sensible sequence in order to facilitate future purifications. The sequence modified was cloned by DNA 2.0 (Newark, CA, USA) in the expression plasmid pJexpress404.

2.5. Recombinant protein expression and purification

We transformed the competent bacteria *E. coli* Rosetta gami with 10 ng of pJexpress404-BIP vector and cultured in LB (Luria Bertani) agar plates. After a 16 h culture, positive colonies were selected by their ampicillin resistance (100 μg/mL). To obtain soluble rBIP protein, we optimized the over-expression conditions. Over-expression was carried by culturing 1 L of transformed *E. coli* Rosetta gami bacteria in Terrific Broth (TB) medium. Once the culture reached $D.O._{600nm}$ = 0.6-0.8 it was induced with 0.5 mM IPTG during 4h at 37 °C. Cells were pelleted by centrifugation at 3600 g, 30 min, 4 °C and washed two times with ice-cold PBS. After this step cells were always on ice. Then, the pellet was resuspended in an equivalent volume of column binding buffer (1 μL of pellet - 4 μL of buffer). Once re-suspended, bacteria were lysed enzimatically with lisozyme 2 mg/mL and mechanically by sonication [6 cycles of 10 seconds pulses each and 20% amplitude (Brandon Sonifier S-250D, Shelton, CT, USA)]. Soluble proteins were separated by centrifugation at 10 000 X g, 30 min, 4 °C. Supernatant was collected and clarified by filtering with 0.45 μm membrane. rBIP protein was purified by IMAC chromatography, [pre-packed GE HisTrap column (1ml)], the clarified sample was passed through the column with a peristaltic low pressure bomb (Bio-Rad, Hercules, CA, USA) at 0.2 mL/min. Protein was eluted using 6 buffers at different concentrations of imidazole (5, 20, 50, 100, 200, 500 mM). rBIP purified protein was obtained at 500 mM with a purity of at least ~80%.

2.6. Mouse infection

Five millions trophozoites were resuspended in 200 μL of sterile PBS and administered by using a sterile animal feeding needle for peroral inoculation. Primary infection occurred at day 0, while the second challenge took place at day 42. The infected mice were bled from the tail vein weekly during 6 weeks after primary infection, and second challenge. Serum was recovered and stored at -80 °C.

2.7. Generation of Polyclonal antibodies specific to *Giardia*-lysate antigens and to *G. lamblia* rBIP.

To generate anti-*Giardia* polyclonal antibodies 6-14 week old C3H-HeN mice were inoculated by intraperitoneal route with *G. lamblia* lysate (200 μg) emulsified with an equal volume of Freund´s complete adjuvant (CFA) (Sigma, St. Louis MO, USA). All mice were boosted weekly during eight weeks with the same dose in Freund´s incomplete adjuvant (IFA) (Sigma, St. Louis MO, USA).

Anti-BIP polyclonal antibodies, were prepared using the recombinant protein rBIP. To prepare the sample, 200μg of the purified protein rBIP (≈80% purity) was separated by electrophoresis SDS-PAGE (12%) under modified reducing conditions. Gel was stained with coomassie blue and then distained (50% distilled water, 40% methanol, 10% acetic acid). The band corresponding to 70 kDa was cut, triturated with a blade and then passed by a syringe with 1mL of sterile PBS several times. Each peritoneal immunization comprised rBIP (≈10 μg) emulsified with an equal volume of CFA. Mice were boosted with the same dose of rBIP emulsified in IFA each 10 days during a period of two months.

All the immunized mice were bled from the tail vein every week and the serum was recovered and stored at -80 °C. Antibodies titter was determined by an indirect ELISA assay.

2.8. Flow cytometric analysis of *G. lamblia* trophozoites.

Cell surface phenotype of *Giardia* trophozoites was determined by FACS. Trophozoites were washed twice with ice-cold PBS. Then, the cells were incubated with the polyclonal Ab anti-BIP (1:500) or pre-immune mice serum (1:500) in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) [D5F] with 0.05% NaN_3 for 1 h at 4 °C. After three washes with cold PBS, the trophozoites were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:200 with D5F-0.05% NaN_3) (Sigma, St. Louis, MO, USA) for 1 h at 4 °C. Then, the trophozoites were washed three times with cold PBS and fixed for 15 min at RT with 1% paraformaldehyde (PFA) (Sigma, St. Louis MO, USA) in D5F-0.05% NaN_3. At least 10,000 trophozoites were acquired using

fluorescence-activated cell sorting (FACS) (Canto II FACS, Becton Dickinson, CA, USA).

2.9. Western blotting assays

G. lamblia protein lysate (30 µg) and purified rBIP (0.5 µg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) under modified reducing conditions (Velazquez et al. 2005). The proteins were electrotransferred to a nitrocellulose membrane for 30 min using a semi-dry blotting system (Bio-Rad, Hercules, CA, USA) at 15 volt. The nitrocellulose membranes were blocked with PBS containing 5% fat-free dry milk for 1 h at RT. The blocked membranes were then incubated during 1 h at RT with mouse serum (from pre-infected infected and re-infected mice (diluted 1:10) or immunized mice (with *G. lamblia* protein extract (1:100) or with polyclonal anti-BIP serum (1:5000)). After five washes with PBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:7500 with PBS-0.1% BSA) (Sigma, St. Louis MO, USA) for 1 h at RT. The membranes were washed and developed using a SuperSignal West Pico Chemoluminescent Substrate kit (Thermo Scientific, Rockford, IL, USA).

2.10. *In vitro* splenocyte stimulation

In vitro lymphocyte proliferation induced by rBIP was measured by the MTT assay (Mosmann 1983). We used five groups of C3H/HeN mice (n=5 mice/group) from six to seven weeks old. Group 1 was a noninfected control. Groups 2-5 were infected with five millions of *G. lamblia* trophozoites. One group of mice was sacrificed every week post-infection to harvest their spleens. To isolate splenocytes, spleens were disrupted in a Petri dish with the back of a syringe and then filtered through a 70 µm strainer. Cells were pellet by spinning at 4 °C, 500 g for 5 min. The RBC were lysed by resuspending in 25 mL of ACK buffer during 5 minutes, after that, 25 mL of cold DMEM were added and suspension was spinned again at 4 °C, 500 g for 5 min. Pellet was resuspended in 25 mL of PBS to filter it again through a 70 µm strainer. Cells were pellet by spinning at 4 °C, 500 g for 5 min and resuspended in 5 mL of DMEM supplemented with 10% SFB to count. Cells were plated in "u" bottom 96 well plate at a density of 500,000 cells per well. Triplicates for each condition were carried out in the same plate. Cells were

stimulated with three doses (2, 10, 50 nM) of rBIP, lysozyme (HEL) as an irrelevant protein control or phytohemagglutinin (PHA) as a positive control. Cells were incubated with the respective treatment 72, 96 and 120 h, except PHA treatments which was measured only at 72h. Results were normalized with controls of medium and cells without treatment. After incubation time, MTT reagent was added and incubated during four hours more to generate formazan crystals, which were resuspended by using acid isopropanol and results were measured at by spectrophotometry (570-630 nm). Positive induction was considered above an arbitrary proliferation index of 3.0 (IP= O.D. stimulated cells/ O.D. nonstimulated cells).

2.11. B-cell and T-cell epitope prediction

G. lamblia rBIP protein sequence was analysed to predict B and T cell probable epitopes. For identification of B-cell epitopes the algorithms Bcpred, FBCPred, AAP, ABCPred, BcePred and Bepipred were used. These algorithms consider features like antigenicity, flexibility, hydrophobicity, accessibility and lineal continuous disposition of residues in a possible epitope and the score value indicates the probability of an epitope to be recognized by antibodies. T-cell epitopes were predicted with SYFPEITHI algorithm which define the ligation strength of the epitopes to the MHC-II type I-Ak; defined for the mice strain used in our study (C3H-HeN). The score value indicates the probability that a determined epitope has to be processed and presented.

2.12. Homology analysis by bioinformatics

Alignment of BIP protein and nucleotide sequence using BLAST, Clustalw and MEGA tools was developed to determine conserved regions with other HSP70 reported with immunogenic potential.

3. RESULTS

3.1. *Giardia lamblia* proteins BIP and 5G8 are different molecules.

In a previous study, we have demonstrated the immunogenic potential of a *Giardia lamblia* protein of 70 kDa (5G8) (Velazquez et al. 2005; Quintero et al. 2013). In the present work, we performed experiments to elucidate whether the immunodominant antigen 5G8 is the same molecule that HSP70 BIP. Recombinant BIP of *Giardia* was purified by IMAC and used to produce polyclonal antibodies in mice. We developed a western blotting with *G. lamblia* rBIP protein, *G. lamblia* GS/M83-H7 and *G. lamblia*

GS/M83-H7-5G8 (+) protein extracts (Figure 1a). In this experiment, the monoclonal antibody 5G8.B5 detected a protein band of 70 kDa in GS/M83-H7-5G8 (+) but it was not detected in GS/M83-H7. In addition, 5G8.B5 moAb did not reacted with the rBIP antigen. On the contrary, rBIP and the 70 kDa band correspondent to native BIP in *Giardia* extracts were strongly recognized by anti-BIP serum at a similar level between strains. Isotype control antibody and preimmune polyclonal antibodies did not reacted with *Giardia* antigens at the evaluated conditions. In order to confirm those results, we evaluated the expression of 5G8 and BIP antigens on the surface of *Giardia* trophozoites surface by FACS analysis with anti-BIP polyclonal antibodies and moAb 5G8.B5 (Figure 1b). The percentage of trophozoites GS/M83-H7-5G8 (+) expressing 5G8 in surface was higher than in GS/M83-H7 strain (61% and 2% respectively). However, BIP protein was not recognized on the surface of the evaluated *Giardia* trophozoites. Neither isotype control antibody nor preimmune serum antibodies showed any reactivity to these trophozoites. These assays revealed a differential expression profile in *Giardia* strains surface.

3.2. *G. lamblia* BIP protein is recognized by the systemic humoral immune response of infected C3H-HeN mice.

Since 5G8 and BIP are different antigens, it was of our interest to characterize immunologically the protein BIP of *G. lamblia*. To evaluate whether humoral immune response can recognize this protein, serum from infected and re-infected mice were obtained during 6 week post-infection (PI) and 6 week post-reinfection (PRI). IgG antibody titles to *Giardia* GS/M83-H7 and GS/M83-H7-5G8(+) soluble antigens as well as for rBIP were measured by ELISA assay (Figure 2a). The results showed high antibody IgG titles for rBIP, and also for *Giardia* soluble antigens in PRI serum samples. However, antibodies in PRI of GS/M83-H7-5G8(+) PRI mice serum is more reactive than GS/M83-H7 PRI serum. Serum from pre-infected (PRE) mice did not show any reactivity. Hiperimmune GS/M83-H7 mice serum (HI) was used as a positive control. In order to determine the protein bands recognized by PRI mice serum, we developed western blotting assays (Figure 2b) using *G. lamblia* rBIP protein, as well as protein extracts of *G. lamblia* GS/M83-H7 and *G. lamblia* GS/M83-H7 5G8 (+). The 70 kDa protein BIP was strongly recognized by the GS/M83-H7-5G8 (+) PRI serum but

slightly recognized by GS/M83-H7 PRI antibodies. Pre-infection serum did not recognized any protein bands.

3.3. *G. lamblia* BIP protein does not induce proliferation of splenocytes from infected mice.

Cellular immune response plays an essential role in elimination of *Giardia* from the host intestine. However, there are just few reports about the role of *Giardia* antigens that can induce an activation of cellular immune mechanisms. With the aim of evaluate cell proliferation under stimulus of *Giardia* rBIP, splenocytes from non-infected and infected C3H-HeN mice were used (Figure 3). *G. lamblia* rBIP at 72h post-treatment showed a dose dependent effect on splenocytes proliferation. At the higher treatment dose (50 nM), especially at 21 DPI slightly differences on rBIP protein treatment with respect to HEL treatment were detected, with PI of 1.6 and 12.9 respectively. However, at the evaluated conditions we did not observed significant positive stimulation with HEL or BIP (PI > 3.0). In contrast, PHA positive control with PI up to 9.1 showed the ability of tested cells to proliferate.

3.4. Identification of B- and T- cell epitopes of *G. lamblia* BIP.

In order to elucidate the potential epitopes of BIP to be recognized by B- or T- cells a series of prediction algorithms was used. For identification of B cell epitopes were used Bcpred, FBCPred, AAP, ABCPred, BcePred and Bepipred algorithms. In the selected algorithms characteristics like antigenicity, flexibility, accessibility and lineal continuous disposition were considered. From the comparative analysis ten B-cell epitopes were identified with the best scores average for the evaluated resources (Table 1). The most of the epitopes are located near of the carboxi- end of the protein. In addition, we used the SYFPEITHI algorithm to predict epitopes that strongly interacts with MHC-II haplotype (I-Ak). A total of 413 possible epitopes which varies on score values from 2 to 32 were identified (Table 2). In contrast to B-cell epitopes, the aminoacid sequences with more probability to bind to MHC class II are near of amino-end of the protein. Despite this, it can be observed that three of the most probable epitopes could interact with both B-cell (# 2, 7 and 9 epitopes, table 1) and T-cells (# 10, 4 and 7 epitopes, table 2).

4. DISCUSSION

Specific humoral and cellular immune responses play a role in *Giardia* clearance. Several reports have indicated that the clearance of *Giardia* in immunocompetent murine models occurs at approximately 3-5 weeks post-infection, which correlates with the establishment of humoral immunity (Lopez-Romero et al. 2015). Previously we have reported a 70 kDa (5G8) antigen of *Giardia* GS/M83-H7 that can be strongly recognized by serum from infected and reinfected mice, and also can induce in the host the production of antibodies which then can agglutinate *Giardia* trophozoites *in vitro* (Velazquez et al. 2005; Astiazaran-Garcia et al. 2009; Quintero et al. 2013). Since we know the immunodominance of 5G8, it was of our interest to elucidate more about this antigen. We had evidence to think that 5G8 could be the HSP70 BIP (data not shown). In this study, we compared expression profile of 5G8 and BIP in two clones of *Giardia*. We observed a different expression profile of both antigens by FACS and western blotting. Because trophozoites GS/M83-H7-5G8 (+) is an enriched clone from GS/M83-H7 trophozoites, it was expected that GS/M83-H7-5G8 (+) had higher expression percentage of 5G8 than GS/M83-H7 (Figure 1b). Interestingly, BIP expression was null in surface of both clones, but the protein was recognized in western blotting assays of *Giardia* lysates (Figure 1a). The clear difference in the expression profile of 5G8 and BIP antigens indicates that 5G8 and BIP are not the same antigen of *Giardia*. In addition, it was observed that at least during trophozoite stage, BIP is expressed in the inner part of the cell.

Once we knew 5G8 and BIP are different molecules it was of our interest to evaluate the potential of *Giardia lamblia* BIP protein to be recognized and to stimulate systemic humoral and cellular immune mechanisms in the host. The recombinant BIP protein as well as native in *Giardia* extracts were recognized by antibodies present in serum of immunized and reinfected mice (PRI). Nevertheless, we found some differences in the recognition intensity (figure 2a) when tested with serum from primo-infection (PI). Our results contrast with a recent study which indicates *G. lamblia* rBIP protein can be strongly recognized by infected BALB/C mice serum (Lee et al. 2014). These differences can be explained on the basis of the model of infection, BALB/C and C3H-HeN differ genetically, which can significantly influence the immune response to

this protein. In addition to that, since our rBIP protein sequence has been modified in order to produce it in a recombinant *E. Coli* system, we are depleted the first 47 aminoacids in the N-end of the protein. To our model C3H-HeN (I-A^k) this fragment does not represent a potential epitope, but if we analyze prediction for BALB/c MHC-II haplotype (I-A^d) for example, the most probable recognized epitope start at residue 36 (data not shown). This implications suggest we have to be careful to compare results between two different models of study.

Antigens (Ag) recognised by T-cells need to be previously processed by antigen presenting cells (APC) such as DCs and then presented through an MHC. To evaluate the role of BIP in the induction of cellular immune response we performed a proliferation assay and measure the proliferation index by MTT. The results obtained indicated that at least at the conditions evaluated, BIP did not show a positive effect in proliferation rate of slenocytes of infected or uninfected mice. However, since a cellular immune response activation is needed to induce specific antibodies production and we have in previous experiments proven that mice produce antibodies against rBIP and native BIP in Giardia clones, we do not discard the possibility that the method was not sufficient sensible to detect the changes *in vitro*. There are some reports of BIP homologues of other organisms that have been proven to induce activation and maturation of DC, release of pro-inflammatory cytokines and stimulation of some T lymphocytes (Table 3) (Zhang et al. 2010; Tamura et al. 2011; Dhakal et al. 2013; Guzhova et al. 2013; Batra et al. 2014; Chen et al. 2014; Chu et al. 2014; Fang et al. 2014; Rasouli et al. 2014; Wang et al. 2015). Additionally, the *in silico* epitope prediction of B-cell and T cell reveal several regions in rBIP with high probability scores of being recognized by T and B cells (Tables 1 and 2). The B- and T- cell epitopes with higher score were found to be conserved in the sequence of HSP70s from other organisms (Figure 4). Their presence have demonstrated to be immunogenic in the pathogenesis of several infectious and chronic diseases such as cancer (Tamura et al. 2011; Dhakal et al. 2013; Chu et al. 2014; Fang et al. 2014; Rasouli et al. 2014). Suggesting that BIP could be acting in a similar mechanism to activate the immune response. Nevertheless, more test are necessary to elucidate the role of BIP cellular response.

In summary, in this study we evaluated the potential of *Giardia lamblia* BIP protein to be recognized by and effectively activate humoral and cellular systemic immune mechanisms in the host. However, additional studies are needed to evaluate the local immune mechanisms and to determine whether it has a protective role during *Giardia* future challenges. It is also important to continue developing our knowledge on the immunogenic proteins of *Giardia,* which can induce protective host humoral and cellular responses. The characterization and evaluation of the protective potential of the immunogenic proteins that are associated with *Giardia* will offer new insights into host-parasite interactions and may aid in the development of an effective vaccine against the parasite.

5. DISCLOSURES

The authors declare that there is not conflict of interest.

6. FUNDING

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HIGHLIGHTS

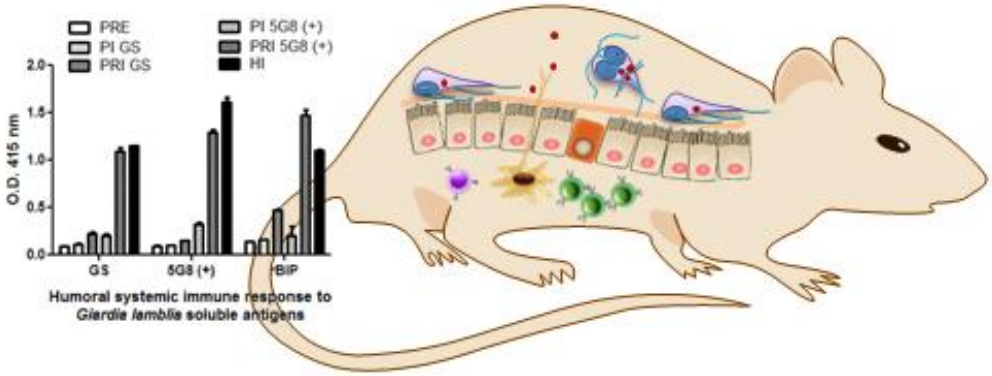
* The previously reported 5G8 protein and BIP are different antigens of *G. lamblia*.

* BIP is able to induce humoral systemic immune response in C3H-HeN mice.

* Epitopes from BIP were predicted to B and T cells.

* BIP Residues were found conserved in other HSP70 reported as immunogenic.

GRAPHICAL ABSTRACT



FIGURES

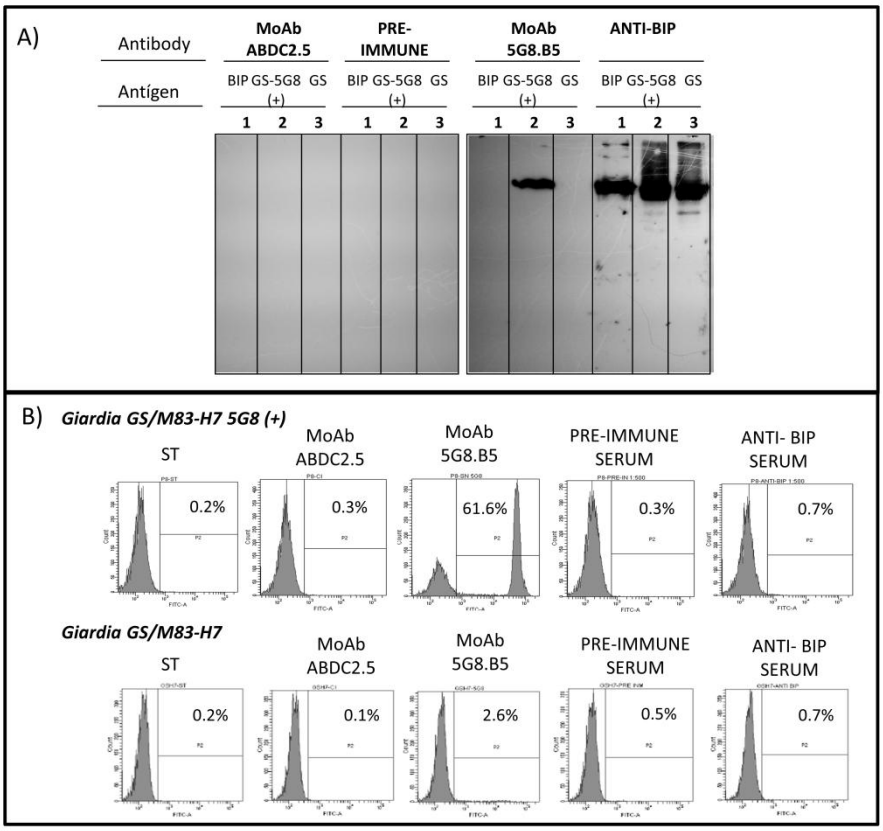


Figure 1. *Gl* antigens BIP and 5G8 are different molecules of the parasite. A) Western blot analysis with the specific antibodies, moAb 5G8.B5 only detected 5G8 protein in *Giardia* GS-5G8 (+), but anti-BIP serum [1:500] detected recombinant and native BIP in both clones at a similar level. Controls of preimmune serum and isotype control moAb were negative. B) FACS analysis of trophozoites *G. lamblia* GS/M83-H7 5G8 (+) from left to right is a dot plot of non stained trophozoites, histogram of non stained cells, cells stained with isotype control moAb [1µg/mL], anti-5G8 moAb [1µg/mL], pre-immune serum [1:500] and cells stained with anti-BIP serum [1:500]. B) Analysis of trophozoites *G. lamblia* GS/M83-H7. Each histogram shows the percentage of trophozoites that was positive to each antibody.

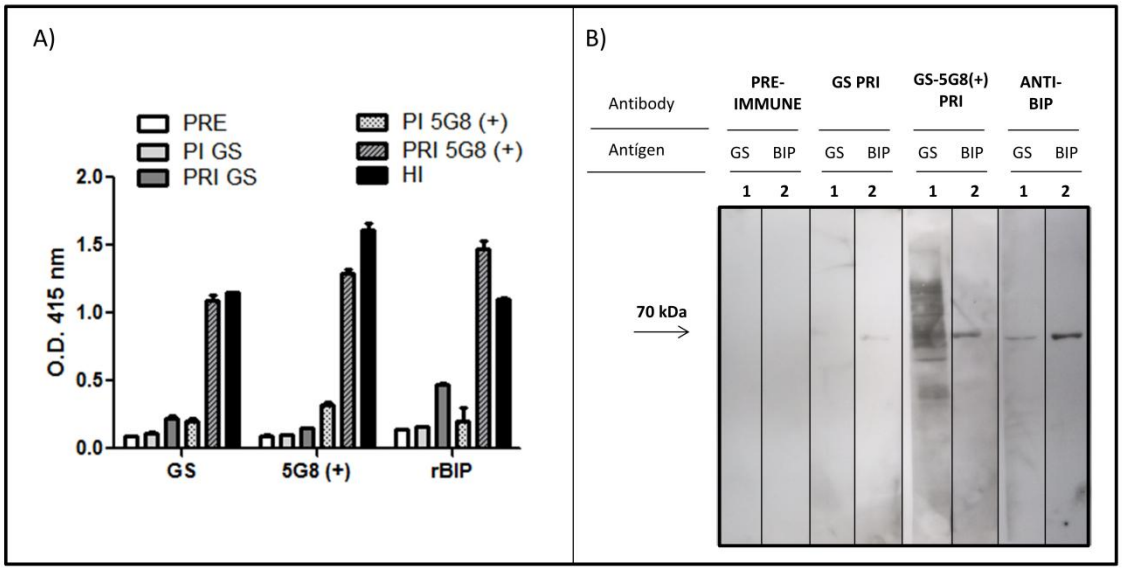


Figure 2. BIP antigen is recognized by humoral systemic immune response. A) Determination of IgG antibodies title to *Gl* soluble antigens and rBIP by ELISA. Serum from C3H-HeN mice post-infection (PI) and post-reinfection (PRI) with *Giardia* GS or 5G8 (+) strains were collected from 1-6w PI and 1-6w PRI. Serum from 3rd week PI and PRI were tested with *Gl* soluble antigens or rBIP. B) Western blotting assay of *Gl* soluble antigens with serum of infected and immunized mice. Recombinant purified protein BIP (0.5 μg) or protein extract of *G. lamblia* GS/M83-H7 (30 μg) were incubated with anti-BIP serum [1:500], *Giardia* GS PRI serum [6th w 1:10], *Giardia GS*-5G8 (+) PRI serum [6th w 1:10]. Controls of preimmune serum were negative in both assays. Immunedetection of BIP is recognized by serum from reinfected mice and response increases with *Giardia* GS-5G8(+) infection.

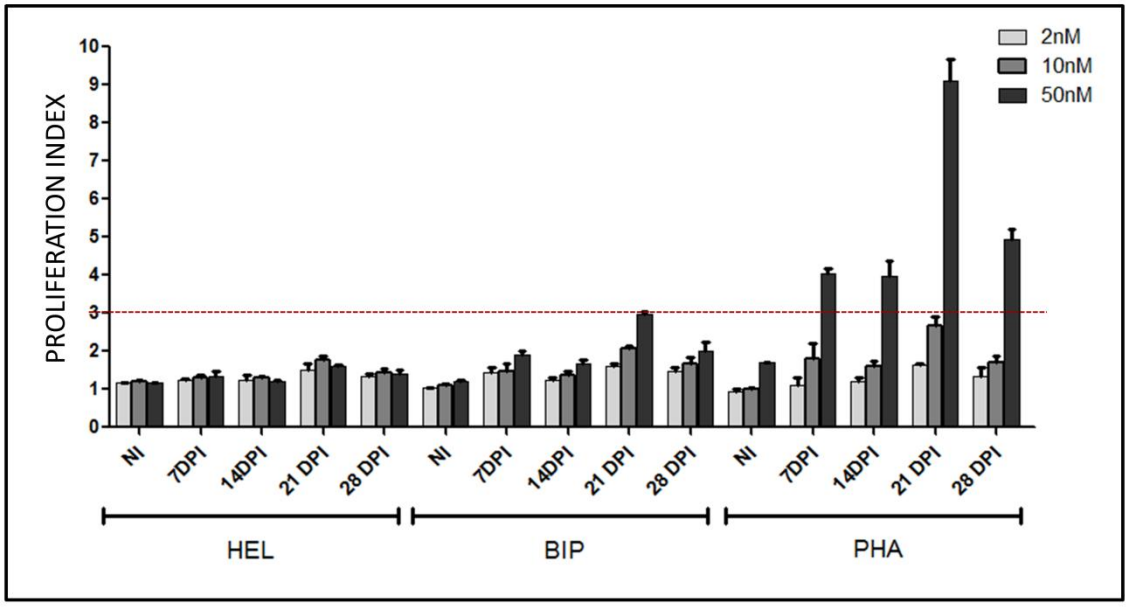


Figure 3. BIP antigen does not induce splenocytes proliferation. Cell proliferation *in vitro* was evaluated by MTT assay. Splenocytes of C3H-HeN mice (n=5 each group) infected with *Giardia* GS/M83-H7 at 0, 7, 14, 21 and 28 days post infection (DPI) were incubated with rBIP, HEL or PHA at the final concentrations of 2nM, 10nM and 50nM. The proliferation index was evaluated at 72, 96 and 120hrs (only 72h effect is shown). The protein rBIP did not showed positive effect in splenocyte proliferation in infected groups. The results were compared with noninfected control (NI) and irrelevant protein HEL. PHA treatment was used as a control of viability at the same concentrations but it was evaluated only at 72h post treatment.

Table 1. B-cell epitope prediction for *G. lamblia* rBIP protein

#	POSITION	EPITOPE	SCORE AVERAGE						
			Bcpred	FBCpred	AAP	ABCPred	Bcepred	Bepipred	Antigenicity
1	**470-485**	NDIPPAPRGTPQIEVT	1.00	1.00	1.00	0.71	1.13	1.52	1.02
2	**590-609**	DWLRDNTDASKEEIEEEKSK	1.00	1.00	1.00	0.87	1.53	1.02	0.94
3	**64-81**	LIGRKFDDPEVQKDMKLL	0.97	0.99	1.00	0.71	1.53	1.08	1.00
4	**495-516**	TVSAVEKSSGKEESITIKNDRG	0.97	0.95	1.00	0.77	1.54	0.95	0.99
5	**553-570**	IVSITTTQTTADKEGNIV	1.00	1.00	1.00	0.91	1.20	1.05	0.98
6	**622-643**	FGRSASAGGSGPEYDYAEKDEL	1.00	1.00	1.00	0.85	0.91	1.39	0.97
7	**250-271**	NIDLSITNTGDKAKDMAVKKAIS	0.92	1.00	1.00	0.84	1.11	0.95	0.98
8	**181-198**	LDKKTQETSGKAKNILVF	0.33	0.88	1.00	0.83	1.36	1.29	1.00
9	**526-548**	LVREAEEFAEEDKINRERAEARN	1.00	1.00	1.00	0.80	1.10	0.77	0.95
10	**398-418**	DVLLIDVTPLTLGIETQGGIM	0.96	1.00	1.00	0.76	0.88	0.44	1.05

Bcpred, FBCPred, AAP, ABCPred, BcePred , Bepipred algorithms identified a series of B-cell epitopes of *G. lamblia* rBIP protein. From the comparative analysis ten epitopes with the highest score average are shown.

Table 2. T-cell epitope prediction for *G. lamblia* rBIP protein

#	POSITION	EPITOPE	SCORE
1	**210 - 224**	LSVDSGVFEVLATAG	32
2	**370 - 384**	LNKDINADEAVAWGA	26
3	**203 - 217**	GTHDVSILSVDSGVF	24
4	**261 - 275**	KAKDMAVKKAISRLR	24
5	**340 - 354**	TDIDEVVLVGGSTRI	24
6	**374 - 388**	INADEAVAWGAAVQA	24
7	**534 - 548**	AEEDKINRERAEARN	24
8	**163 - 177**	LDVVRIINEPTSSSI	22
9	**415 - 429**	GGIMTPLIERNSYIP	22
10	**597 - 611**	DASKEEIEEEKSKFE	22

SYFPEITHI algorithm was used to predict epitopes that strongly interacts with MHC-II haplotype (I-Ak).

Table 3. BIP protein is an HSP70 well conserved protein between pathogens

Protein	Access Number	Origin	Immune effect		Homology with *Gl*-BIP	Reference
HSP70	ACR82496.1	*Leishmania infantum*	Immune-modulator	Activates DCs, ↑ IL-12p70, IFN-g, ↓ IL-4 = Th1	55%Id, 70%+	Rasouli 2014. Immunotherapy
HSP70	AAK85149.2	*Trichinella spiralis*	Vaccine	Activates DCs, ↑IL-1,IL-12p70, TNF-a, IL-6, CD11c, MHC II, CD40, CD80, CD86. ↑ IgG, IgG1 and IgG2a ↑Th1/2 cytokines (IFN-, IL-2, IL-4, IL-6), ↓38% larvae production	56% Id, 75%+	Fang 2014. Vaccine
HSP70	AAC72002.1	*Toxoplasma gondii*	DNA Vaccine	Activates DCs, induces Lc T CD8+ y CD4+	57%Id, 75%+	Chu 2014. Parasitol Int
HSP70	ACE79189.1	*Mycobacterium tuberculosis*	Adjuvant for vaccines Cancer Infection: *Yersinia pestis*	Activation and maturation de DCs. ↑ IL-2, IFN-g, TNA-a. Lc T CD4+ y CD8+	44% Id, 63%+	Dhakal 2013. Indian Journal of Medical Microbiology Batra 2014. PLOS Neglected Tropical Diseases
Hsp70/DDA	AAF65842.1	*Mycobacterium avium*	Vaccine	CD21(+) B cell and CD4(+) T, IgG1, IFN-g (temporal)	46%Id, 62%+	Vrieling 2013. Vaccine
HSP70	AAA02807.1	*Homo sapiens, Trasnformed cells*		Activates DCs, Lc T CD8+	58%, 73%+	Wang 2015. Chin Med J Zhang 2015. J Immunol Guzhova 2013. Int J Hyperthermia
BIP	CAA61201.1	*Homo sapiens, Transformed cells*	Vaccine	Activates DCs, Lc T CD8+	56%Id, 74%+	Tamura 2011. J immunol

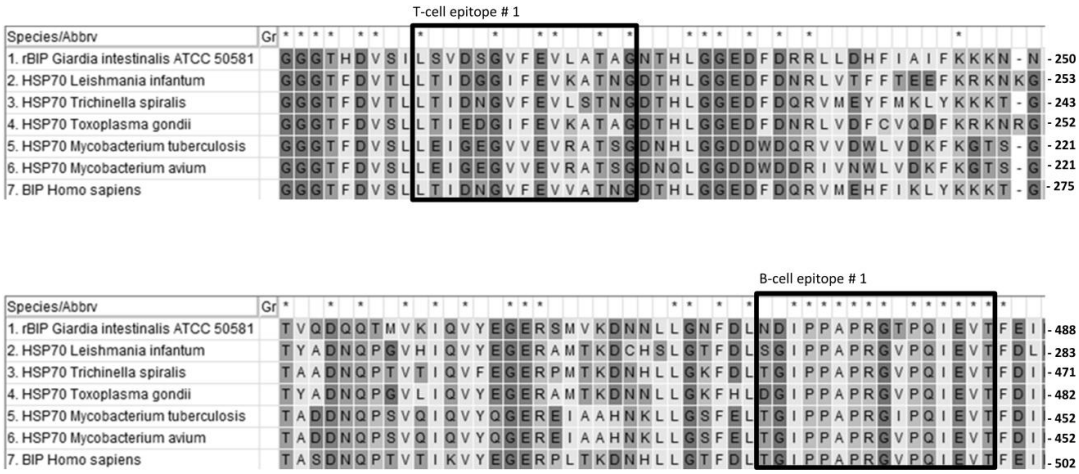


Figure 4. ***Giardia lamblia*** **BIP protein has conserved regions with other HSP70 from different organisms.** The B- and T- cell epitopes with higher score (B cell epitope # and T cell epitope # 1) were found to be conserved in the sequence of HSP70s from other organisms. Their presence have demonstrated to be immunogenic in the pathogenesis of several infectious and chronic diseases such as cancer.

CAPITULO III

Artículo:

Isolation and partial characterization of an immunogenic antigen of *Giardia lamblia*

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ABSTRACT

Humoral and cellular immune responses play an important role during *Giardia* lamblia infection. Several *Giardia* proteins have been identified as immunogenic antigens based on their elicited humoral immune response. Poorly is known about *Giardia* antigens that stimulate a cellular immune response. The main purpose of this study was to isolate and partial characterize an immunogenic antigen (5G8) of *G. lamblia*. The 5G8 protein was isolated from *G. lamblia* trophozoite lysates by affinity chromatography using moAb 5G8-coupled CNBr-Sepharose. The isolated protein was analysed by electrospray tandem mass spectrometry (ESI-MS/MS), and by diverse bioinformatics tools (GiardiaDB, BLASTn, BLASTp and ExPASy). Additionally, several biochemical and immunological characteristics of the isolated protein were analysed. By ESI-MS/MS the amino acidic 5G8 sequence was deduced. The 5G8 antigen belongs to the VSP family proteins of *G. lamblia*. This protein is composed by one polypeptide chain (± 71 kDa). Using the algorithm SYFPHEITI, we identified candidate CD4+ T-cell epitopes from the 5G8 antigen, which can elicit cell-mediated immune responses. In this study, we have identified a *G. lamblia* protein that induces a strong immune response in infected mice. The biochemical and immunological characterization of the immunogenic 5G8 antigen may contribute to the rational design of a *Giardia* vaccine.

Key words: *Giardia lamblia*, immunogenic protein, humoral response, cellular response.

1. Introduction

Giardia lamblia is a non-invasive flagellate protozoan that inhabits the intestinal tract of humans, among other species [1,2], and it is the causal agent of giardiasis, one of the most common diarrheal infections in humans. This parasite presents two stages of life, the vegetative form (trophozoite) and the infective form (cyst). Giardiasis initiates by the consumption of food or water contaminated with cysts. From each hatching cysts, two trophozoites emerge and colonize the small intestine by attaching to the intestinal epithelial cells inducing the pathology of the infection. Infected hosts may present a wide spectrum of symptoms, which can vary from asymptomatic carrier state to severe diarrhoea, abdominal pain, flatulence, nutrient malabsorption syndrome, and even to develop chronic disorders in the gastrointestinal track [3]. Those clinical manifestations of giardiasis could be due to the competence of the host immune system [4,5]. Giardiasis is a self-limiting infection in immunocompetent individuals, which demonstrate the presence of defence mechanisms in the host [3]. Little is known about the immunological mechanisms that eradicate the parasite; however, several studies have demonstrated the importance of B-cells [2,6-12] and T-cells to control *Giardia* infection [1,5,13-16]. *Giardia* trophozoites remain in the intestinal mucosal surface, and the parasite immunogenic proteins may interact with the host immune cells inducing a humoral immune response, enhancing the production of immunoglobulins in mucosa (IgA) and in serum (IgG), as well as activated the cellular immune response, both immune responses may operate to control *Giardia* infection [1,5,17,18]. There have been identified several immunogenic *Giardia* proteins which are recognized by the immune system of humans and infected animals. Those protein antigens have diverse biological functions and localization in the parasite [5], among them are: variant surface proteins (VSP), cyst wall proteins (CWP), heat shock proteins (HSP), *Giardia* trophozoite antigens (GTA-1 and GTA-2), giardin and tubulin (cytoskeletal proteins), enolase, fructose-1, 6-biphosphate aldose (FBA), ornithine carbamoyl transferase (OCT), arginine deaminase (ADI) and uridine phosphorylase (UPL) (metabolic proteins), among others [1,5,6]. However, our knowledge about the immunological characterization of those antigens, and the mechanisms involved in controlling *Giardia* infection are limited.

Previously, we have identified the 5G8 *G. lamblia* protein (± 71 kDa) as an immunogenic antigen of the parasite [19]. This protein was recognized by sera from infected and re-infected mice [19]. Additionally, the 5G8.B5 moAb recognized the 5G8 protein on the surface and inner part of *G. lamblia* trophozoites, and it induced a strong in vitro agglutination *of G. lamblia* trophozoites [19]. Interestingly, we confirmed that the 5G8 protein and the VSP H7 are different proteins (The *G. lamblia* clone GS/M-83-H7 expresses the immunogenic VSP H7). We evaluated the expression of those antigens on the cell surface of *G. lamblia* trophozoites [GS/M-83-H7 and 5G8 (+)] by FACS analysis, using moAbs specific to theVSP H7 (G10/4 moAb) and 5G8 (5G8.B5 moAb) proteins. The antigen recognition by both moAbs was clearly different, indicating that the 5G8 protein and the VSP H7 protein are not the same molecule [19]. Interestingly, in previous studies we observed that *Giardia* proteins at the molecular weight range from 65 to 77 kDa activated specific *Giardia* T-cell hybridomas [18]. These results suggest that the 5G8 protein can elicit both humoral and cellular immune responses. In the present study, we isolated and partially characterized the immunogenic 5G8 protein of *G. lamblia*.

2. Materials and methods

2.1. *G. lamblia* culture

Trophozoites from *G. lamblia* clone GS/M-83-H7 (ATCC 50581) were obtained from the American Type Culture Collection. *G. lamblia* trophozoites (90-50 % 5G8 (+)) were previously obtained by immunoabsorption assay [8,19]. The axenic trophozoite cultures were maintained in TYI-S-33 medium supplemented with 10 % of newborn calf serum (NBCS) with antibiotics (ceftriaxone, 100 µg/mL) at 37 ºC [20].

2.2. *G. lamblia* soluble protein extract

The protein extracts from *G. lamblia* were generated by the method described by Gottstein [21]. Cultured 5G8 (+) trophozoites were incubated on ice for 10 min and harvested [8]. Detached trophozoites were washed (three times) with sterile phosphate buffered saline (PBS) solution. Subsequently, cell culture was frozen (liquid nitrogen) and thawed three times with a protease inhibitor cocktail (23 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, 0.3 mM pepstatin A, 0.3 mM E-64, 2 mM bestatin and 100

mM sodium ethylenediamine tetraacetic acid (Sigma, St. Louis MO, USA)). The lysate was sonicated for 2 min (30 cycles) (Brandon Sonifier 250, Shelton, CT, USA). By centrifugation, cell debris was removed (10,000 g for 30 min) and soluble protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

2.3. Isolation of 5G8 *G. lamblia* protein

To isolate the 5G8 protein, we performed an immunoaffinity chromatography by using the 5G8.B5 moAb (IgG2b), which was previously purified from culture supernatant by passage through an affinity chromatography column with protein A sepharose beads (Protein A- Sepharose® 4B, Fast Flow (Sigma, St. Louis MO, USA)). The supernatant flow rate was adjusted to 60 drops per min. The moAb was eluted by using 0.1 M glycine (pH 2.5) and immediately neutralized with 1 M trizma (pH 8). Afterwards, the 5G8.B5 moAb was coupled to a cyanogen bromide-activated Sepharose beads (Sigma, St. Louis MO, USA). Twenty mg of soluble protein extract (20 mL PBS) generated by lysis of *G. lamblia* 5G8 (+) trophozoites, were passed three times through the column (12 drops per min). The 5G8 protein was eluted with 0.1 M glycine (pH 2.5) and neutralized with 1 M trizma base (pH 8) solution. The eluted 5G8 protein was analysed by SDS-PAGE 12 % under modified reducing conditions of sample buffer (0.2 % SDS, 0.2 % 2-mercaptoethanol). The above modifications, were carried out due to that previous experiments indicated that normal SDS and β-mercaptoethanol concentrations abolished the 5G8.B5 moAb recognition [17,18]. The 5G8 protein was electrotransferred to a nitrocellulose membrane and blocked for 1 h at room temperature (RT) with 5 % fat-free dry milk with 1 % bovine serum albumin (BSA). Then, the membranes were incubated (1 h, RT) with 5G8.B5 moAb (IgG2b, 1 µg/mL with PBS-0.1 % BSA) or the isotype-matched control antibody aBDC.2 [22] (IgG2b, 1 µg/mL with PBS-0.1 % BSA)[19]. After five washes with PBS, nitrocellulose membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:7500 with PBS-0.1 % BSA) (Sigma, St. Louis MO, USA) for 1 h at RT. Finally, membranes were washed and developed by using a SuperSignal West Pico Chemoluminescent Substrate kit (Thermo Scientific, Rockford, IL, USA) [19]. The 5G8 protein recognized by the 5G8.B5 moAb was dissected from the polyacrylamide gel and prepared to mass spectrometry analysis.

2.4. SDS-PAGE

In order to analyse the electrophoretic pattern of *G. lamblia* soluble extract proteins or the 5G8 isolated protein, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12 %) was performed. The SDS-PAGE was conducted under modified reducing conditions by using a sample buffer (0.2 % SDS, 0.2 % 2-mercaptoethanol) or under denaturing conditions (4 % SDS, 2 % 2-mercaptoethanol, and protein sample was boiled at 96° C for 3 min.). Subsequently, the gels were stained with Coomassie Brilliant Blue or silver staining (Bio-Rad) when it needed. Ç

2.5. Flow Cytometry

To analyze the 5G8 protein expression on the surface of *G. lamblia*, trophozoite cultures during log-phase growth were incubated on ice for 15 min and harvested. Trophozoites were incubated with the 5G8.B5 moAb (IgG2b) or the aBDC.2 (IgG2b), the moAbs were tested at 1 μg/mL in D5F-0.05 % NaN3 for 1 h at 4ºC. After 2 washes with cold PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti- mouse IgG (diluted 1:200 with D5F-0.05 % NaN3) (SIGMA, St. Louis, MO, USA) for 1 h at 4°C in the dark. Finally, the trophozoites were washed two times and resuspended in 200 μL of D5F-0.05 % NaN3 and fixed with 200 μL of 1 % paraformaldehyde (PFA) (SIGMA, St. Louis, MO, USA). The acquisition analysis was performed in a FACS Canto II flow cytometer (Canto II FACS, Becton Dickinson, CA, USA).

2.6. Mass Spectrometry Analysis

In order to identify the *G. lamblia* 5G8 protein, high-performance liquid chromatography (HPLC) and electrospray tandem mass spectrometry (ESI-MS/MS) analysis were performed. The 5G8 protein recognized by the 5G8.B5 moAb was cut from the polyacrylamide gel and destained with acetonitrile (ACN) (50 %) and ammonium bicarbonate (NH4HCO3) (50 mM, 50 %). The 5G8 protein was reduced with DTT (100 mM) (Sigma, St. Louis MO, USA), and alkylated with iodoacetamide (55 mM) (Sigma, St. Louis MO, USA). Finally, the 5G8 protein was enzymatically digested by trypsin (12.5 ng/mL) (Sigma, St. Louis MO, USA). The peptides were analysed by HPLC (Surveyor, Thermo, San Jose, CA, USA) tandem mass spectrometry (ESI-MS/MS) (LCQ Deca (Finnigan, San Jose, CA, USA)). Columns (6 cm × 100 μm) were prepared by packing 100 Å, 5 μm Zorbax C18 resin at 500 psi pressure into

columns with integrated electrospray tips made from fused silica, pulled to a 5 µm tip using a laser puller (Sutter Instrument Co., Novato, CA, USA). An electrospray voltage of 1.8 kV was applied using a gold electrode via a liquid junction upstream of the column. Samples were introduced onto the analytical column using a Surveyor autosampler (Surveyor, Thermo, San Jose, CA, USA). The HPLC column eluent was eluted directly into the electrospray ionization source of a Thermo LCQ Deca ion trap mass spectrometer (Thermo, San Jose, CA, USA). The peptides from the 5G8 protein were eluted in a gradient using buffer A (0.1 % formic acid) and buffer B (acetonitrile and 0.1 % formic acid), at a flow rate of 500 nL/min. Following an initial wash with buffer A for 10 min, peptides were eluted with a linear gradient from 0 to 50 % buffer B over a 60 min interval, followed by 50–98 % B over 5 min and a 5 min wash at 98 % B. Automated peak recognition, dynamic exclusion, and daughter ion scanning of the top three most intense ions were performed using the Xcalibur software: (i) full mass survey scan 400–1500 amu, (ii) MS/MS of the most abundant ion from survey scan, (iii) MS/MS of the second most abundant ion from survey scan, and (iv) MS/MS of the third most abundant ion from survey scan. Other instrument parameters included the following: collision energy, 35 %; activation Q, 0.25; activation time, 30 ms; isolation width, 2.0 amu; dynamic exclusion enabled with repeat count, 2; duration, 0.5 min; exclusion duration, 5 min; and exclusion mass width low, 1.5 amu, and high, 1.5 amu. MS/MS data were analysed using SEQUEST run under Bioworks 3.1 (Thermo, San Jose, CA, USA). Spectra were searched against *G. lamblia* protein sequences [23] and a database of common laboratory contaminants was added. Dynamic peptide modifications by oxidation of methionine and carbamidomethylation of cysteine were considered. The peptide and fragment mass tolerances were 2.5 and 0.5 Da, respectively. Only peptides with a Sequest XCorr score of 1.5 (1+), 2.5 (2+), and 3.5 (3+), and a Delta Corr score of 0.8 were considered and 2 or more peptides were required to accept a protein identification.

2.7. Bioinformatics analysis

In order to determine the genetic features of the 5G8 protein, peptide sequences identified by ESI-MS/MS were analysed by using the databases: the *Giardia* protein and genomic data base (GiardiaDB) [23], BLASTn and BLASTp tools. The estimated

molecular mass of the 5G8 protein was obtained by ExPASy [24]. To identify the VSPs family motifs in the 5G8 protein sequence, a detailed comparative bibliographic analysis was conducted [25,26]. Additionally, we used a peptide prediction algorithm, SYFPEITHI [27], in order to predict the possible 5G8 amino acid sequences that have the major probability of MHC class II binding, by using a 5G8 constructed theoretical protein (61 kDa) and as a control protein the protein Henn Egg withe Lysozyme (HEL).

2.8. Statistical analysis

The data were analysed by the Kruskal-Wallis method by using the Statistical Package for the Social Sciences 2002. Statistical significance was accepted when $p < 0.05$.

3. Results

3.1. Isolation of the 5G8 *G. lamblia* protein

In order to isolate the 5G8 protein, a heterogenic extract from axenic cultured *G. lamblia* 5G8 (+) trophozoites was generated. Sixty to eighty percent of cell population expressed the 5G8 protein on the cell surface (Fig. 1A). Immunoaffinity chromatography was conducted, and the isolated 5G8 protein was analysed by polyacrylamide gel electrophoresis (Fig. 1B, line 2). Only one band (≈ 71 kDa) was observed in the SDS-PAGE, and it was recognized by the 5G8.B5 moAb in a Western blot assay (Fig. 1B, line 4). The 5G8 protein band was cut from the gel and processed for further analysis by mass spectrometry.

3.2. Identification of the 5G8 *G. lamblia* protein

To identify the isolated 5G8 protein, electrospray tandem mass spectrometry analysis was carried out. The isolated protein was reduced, alkylated and enzymatically digested. The generated peptides were separated by HPLC and analysed by ESI-MS/MS. The amino acid sequences of the peptides were identified by ESI-MS/MS and compared to the protein sequences reported in the *G. lamblia* sequence Database [23]. From the tandem mass spectrometry (MS/MS) analysis of the 5G8 peptides, were identified two different proteins of relative molecular mass ± 28 (gi|559179812) and ± 38 kDa (gi|559177553). Fourteen peptides from the ± 28 kDa protein were identified with 76 % protein sequence coverage. From the ± 38 kDa protein, were identified 13 peptides with

46 % protein sequence coverage (Table 1). We found that both amino acid sequences were associated to the *G. lamblia* VSP family.

3.3. *Giardia* 5G8 Protein is composed by one polypeptide chain

In order to know whether the 5G8 protein was constituted by one or two polypeptide chains, we performed an electrophoresis (SDS-PAGE) under denaturing conditions (4 % SDS, 2 % 2-mercaptoethanol) and boiling the protein sample at 96° C for 3 min. Under those electrophoretic conditions, we only observed one protein band (± 71 kDa), confirming that the 5G8 protein is composed by one polypeptide chain (Fig. 2).

3.4. *Giardia* 5G8 protein is expressed by one gene

To better understand the characteristics of the 5G8 protein, we performed bioinformatics analysis, by using the GiardiaDB, the tools BLASTn and BLASTp, and the bioinformatics resource ExPASy. The amino acid sequence analysis exhibited a homology of 39 amino acids between the C-terminus of ± 28 kDa, and the N-terminus of the ± 38 kDa product (Fig. S1). In order to identify the genomic localization of each product (± 28 and 38 kDa), we contrasted the nucleotide sequences against the genome sequence of the GS/M-83-H7 strain (http://giardiadb.org/giardiadb/) [23]. We found that the nucleotide sequence of the 28 kDa product was localized in a shotgun sequenced DNA fragment (contig AHHH01000109) with 100 % protein sequence coverage and identity, next to this fragment was found a region which coincides with the 38 kDa product sequence with 100 % coverage and 96 % of identity. Both sequences share the overlapping nucleotide region that was seen at the preliminary analysis. The genomic analysis showed that the nucleotide sequences or products are contiguous and even overlapping. Following to this analysis, we found a mutation (deletion of guanine at position 801) that if corrected it generates an ORF (open reading frame) that translates to a protein of 607 amino acids with theoretical molecular mass of 61 kDa, which coincides with the sum of the molecular mass of both products (± 28 and 38 kDa) considering the overlapping region. Those data indicate that the 5G8 protein is expressed by one gene.

3.5. The 5G8 protein is a VSP

We identified several conserved domains of *G. lamblia* proteins in the 61 kDa theoretical protein by achieving a detail comparative bibliographic analysis in regard to VSPs [25,26]. We observed several VSP features motifs in the 5G8 protein sequence, all

of those domains have been reported to be characteristic in members of the *G. lamblia* VSP family [26]. The N-terminus region of the 5G8 protein possesses a signal peptide (1-18 aa), twenty four CXXC motifs (15-18, 28-31, 43-46, 61-64, 69-72, 76-79, 78-81, 108-111, 112-115, 132-135, 140-143, 141-144, 152-155, 154-157, 189-192, 200-203, 210-213, 219-222, 243-246, 245-248, 256-259, 254-257, 287-290, 301-304 aa), two GGCY motifs (88-91, 201-204 aa), two CXXCXXXCXXC motifs (90-100, 270-280 aa), a transmembrane domain in its C-terminus (351-373 aa), and a cytoplasmic CRGKA domain (375-379 aa) (Fig. 3).

In order to identify homologous proteins in the *Giardia* genome, we performed a BLAST alignment (using the 5G8 amino acid sequence). We found several proteins (VSPs) with high homology (QR46_3107 (97 %), GSB_153033 (70 %), GSB_154953 (63 %)) and coverage (QR46_3107 (100 %), GSB_153033 (87 %) and GSB_154953 (100 %)) to the 5G8 sequence. Those identified VSPs are expressed in different *G. lamblia* genotypes (assemblages A, A2, B and E) (Fig. S2) [28-32].

3.6. T-cell epitope prediction

By using the algorithm SYFPHEITI, we identified candidate CD4+ T cell epitopes, which can elicit cell-mediated immune responses [27]. We evaluate the most probably MHC class II binding sequences by using the 5G8 constructed theoretical protein (61 kDa), and as a control protein, HEL [33,34]. We found several amino acid sequences in the 5G8 protein with chemical affinity to bind the MHC class II molecules (haplotype I-Ak) (Table 2). The predicted amino acid sequence with the highest affinity for the MHC class II molecules was TKQDTQNGTCKSCAA (position 67-81, score 26), followed by QSPGSLICQTASNTD (position 95-109, score 22), YYTDDTSSEPNGKTC (position 186-200, score 20), LCADGFYGSSCSKCH (position 308-322, score 18) and KACDLTIDGTKYCSA (position 370-384, score 18) (Table 2). Comparing those data with the obtained from the control protein HEL, we found peptides with high probability to bind to the MHC class II molecules, such as GSTDTGILQINSRWW (position 49-63, score 32), followed by the sequences GSRNLCNIPCSALLS (position 71-85, score 20), LSSDITASVNCAKKI (position 84-98, score 18), KRHGLDNYRGYSLGN (position 13-27, score 14).

4. Discussion

In this study, we isolated and partial characterized the immunogenic 5G8 protein of *G. lamblia*. The mass spectrometry data clearly demonstrate that 5G8 protein is a member of the VSP family of *G. lamblia*, and it is composed by one polypeptide chain (± 71 kDa). We found several structural features on the amino acid sequence of the 5G8 protein, which defined the VSP nature of this antigen; in the extracellular region we found several cysteine-rich motifs (CXXCXXXCXXC, CXXC and GGCY) and a cytoplasmic CRGKA conserved domain in the C-terminus region [35]. VSPs are immunogenic proteins that cover the surface of several species [5,36]. One VSP is expressed at any given time [37-39]. *G. lamblia* undergo surface antigenic variation in vitro and in vivo [6,25,26], and may be stimulated as an immune evasion strategy, thus endorsing chronic and repeated infections [40] or by non-immune mechanisms [41]. About 303 vsp genes have been identified encoding a VSP protein. It has been estimated that the frequency of change (in vitro) for an individual VSP in *G. lamblia* is approximately once every six-13 generations (48-72 h). We evaluated the expression (in vitro) of the 5G8 protein on *G. lamblia* trophozoites during 45 days. We found a relative constant expression of the 5G8 antigen on the parasite surface for at least 6 weeks. Eighty-nine percent (89.0 % ± 2.2) of the cell population expressed the 5G8 protein on the cell surface at day 0. We observed that the level of expression of the 5G8 protein was maintained by the majority of the cells (at days 21 to 45) (Fig. S3). It is important to notice that in certain trophozoite culture conditions, we observed a decreased in the 5G8 protein expression on the cell surface of the parasite. These observations suggest that the 5G8 expression could be modifiable regarding to culture conditions or external stimuli. It is important to notice that VSP switching may vary in the different *Giardia* strains and isolates [6,40].

Surface antigenic variation was observed as a spontaneous process, but recent sights in *Giardia* gene regulation demonstrate that switching of a VSP to another is not a process of spontaneous development, contrastingly, is a reversible process, regulated by RNA molecules (sRNA, dsRNA, siRNA, miRNA) involved in post-transcriptional control gene expression (silencing) in *Giardia* [38]. This regulation of VSPs expression, post-transcriptional modifications, may not be the same by all VSPs. Culture conditions or

experimental infections (from early stages) could modify the VSP expression [36]. Currently, we are performing several experiments in order to understand how expression of the 5G8 protein on the surface of trophozoites could be regulated by several culture conditions (i.e. serum starvation conditions, oxidative stress, temperature and pH modifications, L-cysteine and arginine concentration, bile deprivation, induction of trophozoite endoplasmic reticulum stress, among others). Previously, we confirmed that the 5G8 protein and the VSP H7 are different proteins (The *G. lamblia* clone GS/M-83-H7 expresses the immunogenic VSP H7). We evaluated the expression of those antigens on the cell surface of *G. lamblia* trophozoites [GS/M- 83-H7 and 5G8 (+)] by FACS analysis, using moAbs specific to the VSP H7 (G10/4 moAb) and 5G8 (5G8.B5 moAb) proteins [19]. The antigen recognition by both moAbs was clearly different, indicating that the 5G8 protein and the VSP H7 protein are not the same molecule [19]. Future experiments will be needed to in order to know more about the VSP-like behavior of the 5G8 protein.

An important characteristic of *Giardia* regarding evolution is that it represents a model organism of an early-branching eukaryote [37,39]. Early findings exhibited that different *Giardia* isolates presents evolutionarily conserve genes, among them are the vsp family gens [1,37,38]. Based on the BLAST alignment analysis we found a highlight homology on the 5G8 sequence among other VSP *Giardia* proteins, and found on different *G. lamblia* genotypes (assemblages A, A2, B, and E). This highlight homology represents an attractive characteristic to the potential future development of an effective *Giardia* vaccine. Additionally, we found that several of the T-cell and B-cell epitopes (data not show) predicted in the 5G8 protein are conserved in the most of those VSPs (Fig. S2). Based on those observations, we consider that the 5G8 protein is an attractive candidate to evaluate its potential protective role during *Giardia* infection.

Previously, we found that the 5G8 *G. lamblia* antigen is an immunogenic protein recognized by sera from infected and re-infected mice (C3H/HeJ), and is expressed on the surface and inside of *Giardia* trophozoites [19]. Additionally, the 5G8 moAb induced in vitro agglutination of trophozoites, and potentially may prevent duodenum trophozoite colonization [19]. Based on the ESI-MS/MS analysis, we identified two different proteins of relative molecular mass ± 28 (gi|559179812) and 38 kDa

(gi|559177553) belonging to the VSP family of *Giardia*. Bioinformatics analysis revealed an ORF that translates to a protein of 607 amino acids with theoretical molecular mass of 61 kDa which contains all the identified peptides by mass spectrometry, and match with the molecular mass of the identified protein (*Giardia* 5G8). These data suggest the existence of only one gen that encoded one protein of a theoretical mass of 61 kDa. Additionally, when we analysed the isolated 5G8 protein by SDS-PAGE under denaturing conditions, we only observed one protein band, indicating that the 5G8 protein is composed by one polypeptide chain. An important immunological aspect of this protein is that induces a strong humoral immune response and promotes in vitro agglutination of *G. lamblia* trophozoites [19]. We know little about the potential role of the 5G8 protein in eliciting a cellular immune response. Previously, we generated a panel of *Giardia*-specific T-cell hybridomas and we observed that a *Giardia* protein with relative molecular mass of ± 71 kDa induced the activation of T-cell hybridomas [18]. By using the algorithm SYFPEITHI, we found amino acid sequences present in the 5G8 constructed theoretical protein that have high probability of binding to the MHC class II molecule. When we compare the highest scoring sequences from 5G8 protein (positions 67-81, 95-109, 186-200, 308-322 and 370-384) and HEL protein (positions 49-63, 71-85, 84-98 and 13-27), we found sequences with similar scores that could potentially stimulate a cellular immune response through via CD4+ T-cell participation [33-34] (Table 3). Interestingly, the HEL sequence 23-39 (SLGNWVCAAKFESNF) presents a low probability to bind the MHC class II molecule (score 6). Nonetheless, induces an efficient T-cell immune response [33,34] (Table 3). This data suggest that the 5G8 *Giardia* protein have molecular determinants that potentially could stimulate both cellular and humoral immune responses. Further studies will be needed to fully understand the molecular and cellular basis of the immune response induced by the 5G8 protein during giardiasis. The identification and characterization of immunogenic antigens of *G. lamblia* such as the 5G8 protein will contribute to the better understanding of the molecular and immunological mechanisms involved in the host-parasite interaction.

5. Acknowledgements

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Table 1. Identified peptides in ± 28 kDa and ± 38 kDa proteins.

Peptide	Sequence	m/z[a]	Charge
	±28 kDa		
1	(R)AACTPGTETTNcK(D)	677.29	2
2	(K)DGAcNVQIGGETYcSQcYTTSEAPVDGVcTASTDSK(C)	1,296.87	3
3	(K)ScAANYFLFK(G)	610.79	2
4	(K)GGcYQIGQSPGSLIcQTASNTDGIcQTcK(D)	1,054.46	3
5	(K)DGYFTVSDATATQDScVAcGDENcATcTVGAEQQK(C)	1,272.85	3
6	(K)KNTGSETGTcVTADEcTAAK(D)	1,050.96	2
	(K)KNTGSETGTcVTADEcTAAK(D)	700.97	3
7	(K)KNTGSETGTcVTADEcTAAKDYYTDDTSSEPNGK(T)	1,225.18	3
	(K)KNTGSETGTcVTADEcTAAKDYYTDDTSSEPNGK(T)	919.14	4
8	(K)NTGSETGTcVTADEcTAAK(D)	658.28	3
	(K)NTGSETGTcVTADEcTAAK(D)	986.21	2
9	(K)NTGSETGTcVTADEcTAAKDYYTDDTSSEPNGK(T)	1,182.49	3
10	(K)DYYTDDTSSEPNGK(T)	796.32	2
11	(K)AcSAKVENcAScSSEGAcQK(C)	735.29	3
12	(K)VENcAScSSEGAcQK(C)	843.82	2
	(K)VENcAScSSEGAcQK(C)	562.88	3
13	(K)cASGFVLEGSNcVK(S)	764.35	2
14	(K)SDcSTENcKTcTNPK(A)	601.24	3
	±38 kDa		
1	(K)SDcSTENcKTcTNPK(A)	601.2429	3
2	(K)NcKGATASDcTAcPAGR(A)	599.5864	3
3	(K)GATASDcTAcPAGR(A)	697.7932	2
4	(R)ALIYGDDPTK(G)	546.7814	2
	(R)ALIYGDDPTK(G)	1,092.56	1
5	(R)ALIYGDDPTKGTcGEGcTTGTGK(G)	787.0222	3
	(R)ALIYGDDPTKGTcGEGcTTGTGK(G)	1,180.03	2
	(K)AcDLTIDGTK(Y)	547.2628	2
6	(K)YcSAcDTATEYPQNGVcATPTAR(A)	865.0294	3
	(K)YcSAcDTATEYPQNGVcATPTAR(A)	1,297.04	2
7	(R)ASScQSQNVASGAcNTcENGFFK(M)	842.0145	3
	(R)ASScQSQNVASGAcNTcENGFFK(M)	1,262.52	2
8	(K)mNGGcYSTSQLPGSTVcVTAPTGGTcTK(S)	970.0893	3
9	(K)MNGGcYSTSQLPGSTVcVTAPTGGTcTK(S)	964.7591	3
10	(K)cIAcDKSDGSIAGVK(D)	790.8727	2
	(K)cIAcDKSDGSIAGVK(D)	527.5823	3
11	(K)SDGSIAGVK(D)	417.222	2
12	(K)SDGSIAGVKDcLScAAPSGSTGPVLcYLVR(D)	1,033.16	3
13	(K)DcLScAAPSGSTGPVLcYLVR(D)	1,142.03	2

[a]m/z: Mass to charge ratio

Table 2. Predicted sequences from the proteins 5G8 and HEL that have high probability of binding to the MHC class II molecules[a].

Peptide	Position	Epitope sequence														Score
								5G8								
1	67-81	T	K	Q	**D**	T	Q	**N**	G	**T**	C	K	**S**	C	A	A	26
2	95-109	Q	S	P	**G**	S	L	**I**	C	**Q**	T	A	**S**	N	T	D	22
3	186-200	Y	Y	T	**D**	D	T	**S**	S	**E**	P	N	**G**	K	T	C	20
4	308-322	L	C	A	**D**	G	F	**Y**	G	**S**	S	C	**S**	K	C	H	18
5	370-384	K	A	C	**D**	L	T	**I**	D	**G**	T	K	**Y**	C	S	A	18
								HEL								
1	49-63	G	S	T	**D**	T	G	**I**	L	**Q**	I	N	**S**	R	W	W	32
2	71-85	G	S	R	**N**	L	C	**N**	I	**P**	C	S	**A**	L	L	S	20
3	84-98	L	S	S	**D**	I	T	**A**	S	**V**	N	C	**A**	K	K	I	18
4	13-27	K	R	H	**G**	L	D	**N**	Y	**R**	G	Y	**S**	L	G	N	14
5	23-39	S	L	G	**N**	W	V	**C**	A	**A**	K	F	**E**	S	N	F	6

[a]MHC class II molecule haplotype I-A^k.

.

FIGURE CAPTION

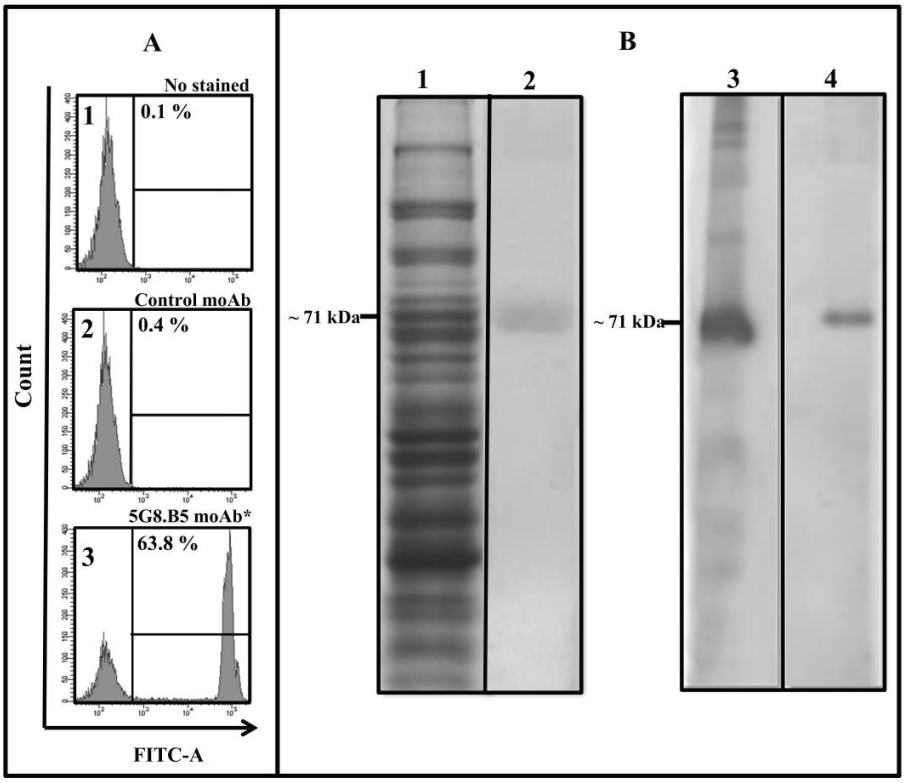


Fig. 1. Isolation of the immunogenic 5G8 protein from the *G. lamblia* soluble protein extract. Fluorescence-activated cell sorting analysis was performed by using the 5G8.B5 moAb and the control aBCD.2 moAb on trophozoites cell surface A: cell surface staining of *G. lamblia* trophozoites [1: non-stained control cells; 2: control moAb (aBCD.2); 3: 5G8.B5 moAb]. After, trophozoites were lysed and the 5G8 protein was isolated by immunoaffinity chromatography. The protein extract was separated by SDS-PAGE (12 %) and the 5G8 protein was recognized by the 5G8.B5 moAb in a Western blot analysis. B: SDS-PAGE electrophoretic pattern [1: *G. lamblia* protein extract; 2: isolated 5G8 protein], 5G8 protein recognition using the 5G8.B5 moAb [3: *G. lamblia* protein extract; 4: isolated 5G8 protein]. Statistical significance was accepted when $p < 0.05$ from control antibody (marked with asterisks).

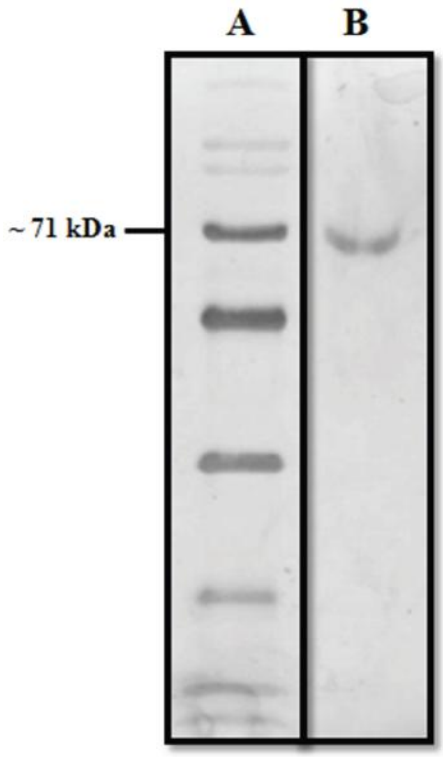


Fig. 2. The 5G8 *G. lamblia* protein is composed by one polypeptide chain. Isolated 5G8 protein (2 μg) was separated by SDS-PAGE (12 %) under denaturing conditions (4 % SDS, 2 % 2-mercaptoethanol, and boiled protein sample at 96° C for 3 min.). A: molecular weight marker, B: isolated 5G8 protein. The lane indicates the ± 71 kDa protein. The gel was stained by silver nitrate standard protocol.

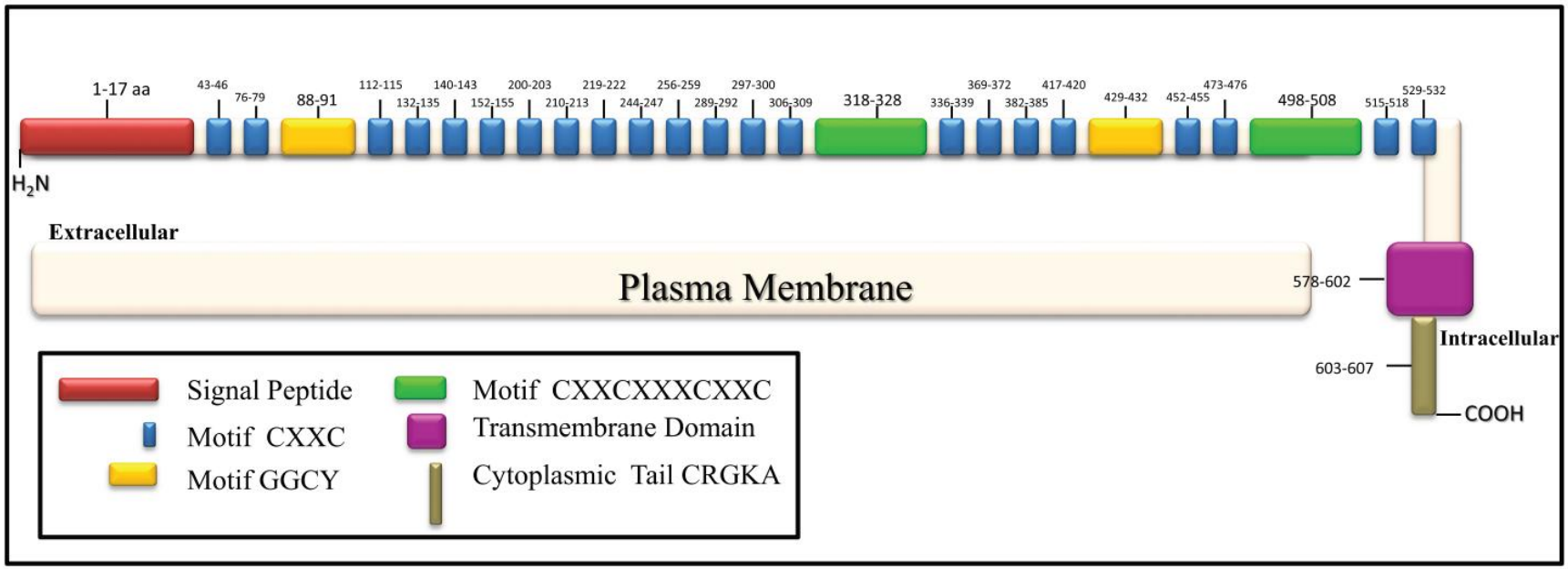
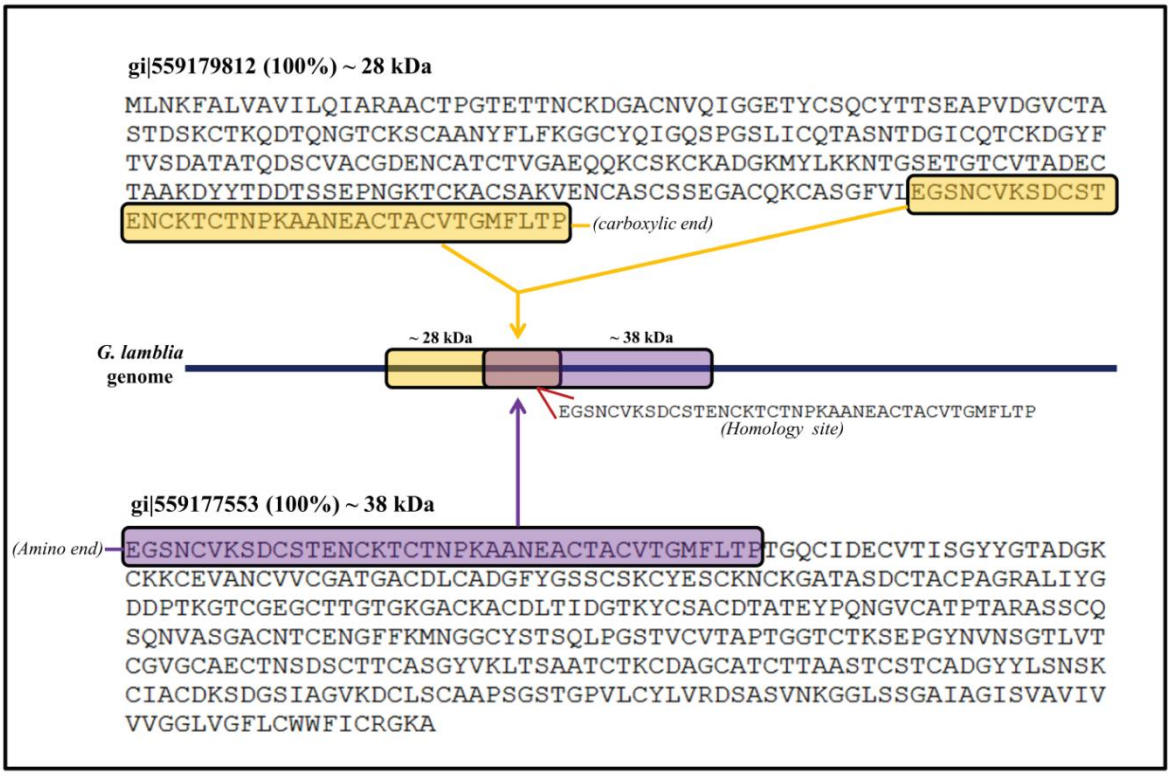


Fig. 3. Structural features of the *Giardia* 5G8 protein. Polypeptide chain (607 amino acids), signal peptide (red), CXXC motif (blue), CXXCXXXCXXC motif (green), GGCY motif (yellow), transmembranal domain (purple) and a cytoplasmic tail CRGKA (brown).



Supplementary Data 1. Homology between ± 28 kDa C-terminus region and the N-terminus of the ± 38 kDa product.

Supplementary Data 2. 5G8 sequence alignment with other VSPs of *G. lamblia*.

#	Name[a]	Access number[b]	Isolate	Genotype	Protein size (aa)	Cover (%)[c]	Identity (%)[c]	Positives (%)[c]	Gaps (%)[c]	Reference
1	QR46_3107	KWX12887.1	BAH15c1	B	607	100	97	98	0	Wielinga C. 2015
2	GSB_153033	ESU41143.1	GS_B	B	732	87	70	79	2	Adam R.D. 2013
3	GSB_154953	ESU40281.1	GS_B	B	679	100	63	72	3	Adam R.D. 2013
4	TSA4	gi|136441|	WB	A	713	97	51	61	7	Gillin,F.D. 1990
5	GLP15_2640	EFO61461.1	P15	E	745	100	48	59	11	Jerlstrom-Hultqvist,J. 2010
6	DHA2_150066	ESU39141.1	DH	A2	747	81	48	58	12	Adam R.D. 2013
7	VSP-54	EDO80151.1	WB	A	682	99	46	57	12	Morrison,H.G. 2007
8	DHA2_150893	ESU38743.1	DH	A2	702	100	46	56	13	Adam R.D. 2013
9	VSP-116	EDO78106.1	WB	A	699	99	44	53	16	Morrison,H.G. 2007
10	GLP15_625	EFO62362.1	P15	E	682	99	42	53	14	Jerlstrom-Hultqvist,J. 2010

a. All of the protein analyzed are members of the VSP family.

b. Accession number from GeneBank is shown.

c. Percentage values were obtained using BLAST.

Supplementary Data 3. In vitro expression of the 5G8 protein on the surface of *G. lamblia* trophozoites.

Days in culture	Trophozoite 5G8 + (%)*
0	89.0 ± 2.2
10	81.3 ± 9.7
15	82.0 ± 11.4
18	81.2 ± 8.3
21	75.0 ± 12.5
35	69.6 ± 14.5
45	67.5 ± 6.5

* 5G8 *G. lamblia* cells were thawed (Day 0), and the changes in the 5G8 protein expression were evaluated during *G. lamblia* cell culture (0-45 days). Data shown are the mean ± SD from triplicate determinations and are representative of three similar experiments.

HIGHLIGHTS

- The isolated *G. lamblia* immunogenic protein (5G8) was identified as a member of the VSPs family proteins.
- The localization of the 5G8 protein in *G. lamblia* resulted in the surface and inner part of trophozoites.
- The 5G8 epitopes induce a B-cell response and potentially a T-cell immune response.

CAPITULO IV

Artículo:

Molecular characterization of the immunodominant VSP-5G8 of *G. Lamblia*

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ABSTRACT

Giardia lamblia is a protozoan parasite that causes intestinal infection. Immunological mechanisms mediated by humoral and cellular responses are needed to eliminate the parasite. *Giardia* undergoes antigenic variation to evade the immune system. One of the VSP family proteins is expressed at the time and switch every 6-13 generations depending of the genotype. Since VSP cover the entire surface infections by *G. lamblia* are characterized of a strong humoral response to VSP members. The VSP-5G8 of 60 kDa is an immunodominant antigen that can induce activation of humoral and cellular immune mechanisms. In this work we amplified the VSP-5G8 transcript from two strains of *Giardia* (genotype B) and determined its nucleotide sequence. Using BLASTp algorithm we determined that VSP-5G8 has conserved regions with other VSP antigens from other *Giardia* genotypes. In addition to that, conserved regions include important immunological residues for predicted T cell epitopes. Subsequenttly flow cytometric analysis revealed that VSP-5G8 is a strong immunogenic protein in mice infection model and even if expressed in a small percentage of trophozoites at a time, VSP-5G8 can determine the immunogenicity of a strain. However, additional experiments are needed to determine the complete role of VSP-5G8 during giardiasis. This knowledge will contribute to understand how this and other similar immunodominant antigens mediate host-parasite interactions.

Keywords: *Giardia lamblia*, immune response, immunogenic proteins, VSP.

2. INTRODUCTION

Giardiasis is one of the most common diarrheal diseases in the world (Feng et al. 2011). It is caused by infection with *G. lamblia* (syn. *G. intestinalis* or *G. duodenalis),* a protozoan that can infects the upper part of small intestine (Adam 2001; Luján et al. 2011). The genus *Giardia* consists of six species which infect different host, but only *G. lamblia* can infect humans and other mammals (Adam 2001). Eight *G. lamblia* genotypes (A-H) also named assemblages have been reported (Lasek-Nesselquist et al. 2010; Luján et al. 2011). Humans can be infected by A and B genotypes, meanwhile C and D infect dogs, E infects hoofed animals, F infects rodents and genotype H can infects marine animals (Lasek-Nesselquist et al. 2010; Luján et al. 2011). However, only A, B and E genotypes can be efficiently cultivatded (Luján et al. 2011).

Giardia elimination from the host intestine requires the activation of humoral and cellular immunological mechanisms (Lopez-Romero et al. 2015). Although some *G. lamblia* immunogenic proteins have been proposed, available studies are focused in humoral immune response recognition (Lopez-Romero et al. 2015). Among them, variant surface protein family (VSPs) has shown to induce a strong immune response in its host (Nash 1997; Lopez-Romero et al. 2015). *Giardia lamblia* VSPs are cystein rich proteins that densely coat the surface of trophozoites (Nash 1992; Nash et al. 1992; Nash 2002; Adam et al. 2010; Luján et al. 2011). The VSP repertoire differs between *Giardia* isolates, GS_B has more than 270 predicted VSP genes (Morrison et al. 2007; Adam et al. 2013), but only one is expressed on the trophozoite surface at the time (Nash 1997; Luján et al. 2011). Antigenic variation of *Giardia* has been proposed as a mechanism to immune system evasion (Luján et al. 2011). However, switching occurs even *in vitro* at different rates depending on the genotype (Prucca et al. 2009; Luján et al. 2011). A switch event occur every 12-13 generations of WB isolate (A), meanwhile GS (B) change its VSP every 6.5 generations (Luján et al. 2011).

Previously, we defined the immunological characteristics of VSP-5G8 (Velazquez et al. 2005; Astiazaran-Garcia et al. 2009; Quintero et al. 2013). Additional MS/MS analysis indicated high homology with two products of smaller molecular mass than expected (28 and 38 kDa). However, biochemical and bioinformatics analysis suggest that 5G8 is constituted by only one polypeptide chain (Quintero-Vargas

Unnpublished). In this work we focused on the nucleotide sequencing of *vsp-5g8* gene from *Giardia* GS/M83-H7 and its polypeptide product as well as immunological implications of VSP-5G8 expression in immunogenicity of *Giardia* strains.

3. MATERIALS AND METHODS

3.1. Mice and *Giardia* strains

Syngenic C3H/HeJ mice of eight to fourteen weeks old were used for these assays. Mice were obtained from "The Jackson Laboratory of Maine" (USA). Mice were reproduced and maintained at University of Sonora bioterium with light/dark cycles of 12h at 25 °C and sterile water and food *ad libitum*.

3.2. *G. lamblia* trophozoites culture

G. lamblia trophozoites of the clone GS/M-83-H7 (ATCC 50581) were obtained from the American Type Culture Collection. *G. lamblia* GS/M83-H7-5G8 (+) (50-95% 5G8 (+)), were previously obtained by immunoabsorption assay. Axenic *G. lamblia* cultures were maintained in the TYI-S-33 modified medium, which was supplemented with newborn calf serum (NBCS) and 100μg/mL ceftriaxone at 37 °C.

3.3. Nucleic acids extraction from *Giardia* strains

Giardia cultures at 80% of confluence ($10x10^6$ trophozoites) were used for nucleic acid extrction. DNA samples were obtained using QIAmp® DNA Mini Kit (Qiagen) and RNA samples using RNeasy® Mini Kit (Qiagen) following manufacturer instructions. Purity, quality and quantity of DNA or RNA samples were evaluated by Nanodrop 2000 spectrophotometer. RNA samples were stored at -80 °C until use. To evaluate 5G8 amplification from transcripts, RNA samples were used to synthesize cDNA using QuantiTect® Reverse Transcription Kit (Qiagen).

3.4. Amplification and sequencing of *vsp-5g8* and fragments

Amplification of *vsp-5g8* gene was done using TaqDNA polymerase (Quiagen, cat # 201205) in a Thermal Cycler (BioRad T1009). An annealing temperature of 58.2°C was used for primers pairs 1-2, 3-4 and 1-4 (Table 1). For PCR conditions we followed manufacturer instructions with 2 min of extension and 35 cycles. Amplicons were separated in agarose gel electrophoresis, stained and photographed in a Documentation

system. Then the bands of interest were cut and purified by NucleoSpin® Gel and PCR Clean Up Kit (Qiagen) following instructions of the manufacturer. The purity, quality and quantity of DNA or RNA samples were evaluated by Nanodrop 2000 spectrophotometer. Purified amplicons were sequenced at the sequencing laboratory from The University of Arizona Genetics Core. Sequences were analyzed with MEGA, Chromas and SnapGene software.

3.5. Identification of homologues in different *Giardia* genotypes

Alignment of VSP-5G8 aminoacid and nucleotide sequences was done using BLAST, ClustalW was used to determine conserved regions with other VSP reported for different isolated and genotypes of *Giardia*.

3.6. T-cell epitope prediction for VSP-5G8 sequence

G. lamblia VSP-5G8 sequence was analyzed to predict T-cell epitopes with SYFPEITHI algorithm which define the ligation strength of the epitopes to the MHC-II type I-Ak; defined for the mice strain used in our study. Higher scores indicated the most probable recognized epitopes (15 mers).

The identification of conserved epitopes was made contrasting epitopes to VSP-5G8 homologue aminoacid sequences. We established the following criterion for conserved epitopes annotation. (+) Conserved at least anchoring residues at P1 and P9 plus two or three more aminoacids. (++) Conserved at least three anchoring residues (P1, P4, P6 or P9) with a total of 7-8 homologue aminoacids. (+++) Conserved at least three anchoring residues with a total of 9-13 homologue aminoacids. (++++) Conserved the four anchoring residues with a total of 14-15 homologue aminoacids.

3.7. Mice infection

The inoculum for infection was prepared by washing *in vitro*-cultivated trophozoites of the strains GS/M-83-H7 or GS/M-83-H7-5G8 (+) three times with ice-cold sterile phosphate saline solution (PBS), cells were counted and then pelleted by centrifugation. Three groups were used. Group 1 was considered the negative control, they received 200 μL of PBS at the time of infection. Groups 2 and 3 received five millions of trophozoites GS or GS-5G8(+) resuspended in 200 μL of sterile PBS and administered by using a sterile animal feeding needle for peroral inoculation. Primary infection occurred on day

0, while the second challenge took place on day 42. The infected mice were bled from the tail vein weekly for 6 weeks after primary infection and again for 6 weeks after secondary challenge, the serum was recovered and stored at -80 °C for future analysis.

3.8. Evaluation of humoral immune response to surface antigens of *G. lamblia* trophozoites

For cell surface staining, confluent cultures of trophozoites GS/M-83-H7 or GS/M-83-H7-5G8 (+) were used. Cells were washed twice with ice-cold PBS. Then, $5x10^6$ cells were incubated with serum from infected or reinfected mice (1:20) or pre-infection mice serum (1:20) diluted in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) [D5F] with 0.05% NaN_3 for 1 h at 4 °C. After two washes with cold PBS, the trophozoites were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:200 with D5F-0.05% NaN_3) (Sigma, St. Louis, MO, USA) for 1 h at 4 °C. Then, the trophozoites were washed two times with cold PBS and fixed for 15 min at RT with 1% paraformaldehyde (PFA) (Sigma, St. Louis MO, USA) in D5F-0.05% NaN_3. At least 10,000 trophozoites were acquired using fluorescence-activated cell sorting (FACS) (Canto II FACS, Becton Dickinson, CA, USA). The fixation procedure did not alter the antigenicity of the *Giardia* proteins.

4. RESULTS

4.1. VSP-5G8 is one polypeptide chain expressed by one gene.

In this work, we performed a series of experiments to determine if VSP-5G8 is expressed as only one polypeptide chain codified by one gene. To identify the genomic localization of VSP-5G8 we contrasted the reported nucleotide sequences of identified products 28 kDa (GSB_151175) and 38 kDa (GSB_155403) against the genome sequence reported for the GS strain. The genomic analysis showed that nucleotide sequences of the 28 and 38 kDa products are contiguous and even overlapping. We found that the nucleotide sequence of the 28 kDa product was localized in the contig AHHH01000109 with 100% of coverage and identity and next to this was found a region which coincides with the 38 kDa product sequence in a 100% of coverage and 96% of identity. With this information we constructed the theoretical gene sequence of

VSP-5G8 (1824 bp). Alignment analysis of theoretical gene to *Giardia* GS_B genome indicated an identity of 98%. The observed point nucleotide differences (2%) has no significant impact in the traduced product except for a deletion of a Guanine at 801 position (DelG801). If the gene presents a guanine at position 801 (from the start AUG), it generates an ORF (open reading frame) that translates to a protein of 607 aminoacids. The calculated molecular mass of this theoretical gene is 61 kDa, which coincide with the expected mass for VSP-5G8 protein. In contrast, if DelG801 is present, it generates an early STOP codon and translates into a product of 28 kDa, a truncated version of VSP-5G8.

With the aim of confirm the obtained data from bioinformatics analysis, we designed specific oligonucleotides to amplify *vsp-5g8* gene (Table 1). PCR reactions with gDNA and cDNA of *Giardia* GS/M83-H7 and GS/M83-H7-5G8 (+) strains revealed, among others, a product of 1824 bp that corresponded to the size expected for the complete gene *vsp-5g8* (Figure 1a). Additionally we amplified fragments of 28 and 38 kDa from cDNA samples of two *Giardia* strains (Figure 1b). This result suggests that *vsp-5g8* gene is present and expressed in both *Giardia* strains. We observed additional bands to the expected products specifically in GS gDNA amplification. That suggests some differences in the strain sequences. However, reamplification of the agarose purified 1800 bp product revealed the same subproducts, indicating that the smaller additional bands are product of oligonucleotide alignment intra *vsp-5g8* gene (data not shown).

In order to elucidate the nucleotide sequence of *vsp-5g8*, amplicons of 1800 bp, 1140 and 816 bp corresponding to complete gene, 38 kDa and 28 kDa fragments from cDNA of GS and GS-5G8 (+) *Giardia* strains, those were purified from agarose gel and sequenced. The analysis of the nucleotide sequences revealed a product of 1729 bp with 95% of cover and 99% of identity with the theoretical gene of VSP-5G8 (Figure 2a). In addition to that, we found a G or a C in position 801, that encode a Pro and generates an ORF whose the translated product consist of 576 aminoacids. This result coincide with the 95% of the expected sequence for VSP-5G8 except for an aminoacid change because a nucleotide transition T963C resulting in a change of a Tyr by an His at Pos321 (Figure 2a). However, this aminoacid change is not included in the recognized peptides by

MS/MS analysis previously described and all of the identified peptides are found in the translated sequence of the *vsp-5g8* gene (data not shown). Thus, the nucleotide product obtained correlate in length and sequence with previous biochemical analysis. These results indicate that VSP-5G8 is expressed as one polypeptide chain as a product of a single gene.

4.2. The antigen VSP-5G8 contains conserved regions found in VSPs of different *Giardia* genotypes.

In this analysis we determined whether VSP-5G8 has conserved regions similar to other VSP of different isolates and genotypes of *Giardia* using BLASTp algorithm. More than 30 proteins from A, A2, B and E genotypes with considerable homology and coverage to VSP-5G8 were found (Table 2). The most similar sequences to VSP-5G8 are GSB_151175 and GSB_155403, corresponding to the 28 and 38 kDa products previously reported. Additionally, we observed one aminoacid sequence that covers 100% of VSP-5G8 with 97% identity (QR46_3107). This transcript belongs to isolate BAH15c1from genotype B and it has also the same length suggesting they could be the same protein. This analysis indicates that VSP-5G8 not only has conserved regions to other VSP but also could be expressed in other isolates of *Giardia*.

4.3. The antigen VSP-5G8 contains regions with immunogenic potential conserved in VSPs of different *Giardia* genotypes.

In previous works we have demonstrated the immunogenic features of VSP-5G8. To elucidate the potential regions with high probability to be recognized by T cells we performed an *in silico* prediction of epitopes. We used the SYFPEITHI algorithm to predict most probably epitopes to interact with T cells by MHC-II (I-A^k). In table 3 we show the ten most probably recognized epitopes. Several of the potential epitopes with high score value are conserved specially in VSP from B genotype isolates. We also observed that epitopes 7 and 8 are conserved among VSPs from different *Giardia* genotypes at least at the anchoring residues (P1, P4, P6, P8 of the MHC-II) (Table 4, Figure 3). This suggests potential sites for cross reactivity by host defenses.

4.4. **VSP-5G8 is an immunogenic protein and its expression determines immunogenicity of the *Giardia* strain.**

With the aim to analyze how the expression of VSP-5G8 contribute or affect the ability of GS or GS-5G8(+) strains to be recognized and activate the host immune system, an infection assay in C3H-HeJ mice was performed. Mice were infected with both strains, and re infected six weeks later. Six week post-infection (PI) and six week post-reinfection (PRI) serum were used in a FACS assay to analyze the antigen recognition pattern of GS or GS-5G8(+) trophozoites. Both strains of *G. lamblia* induced specific IgG antibodies against parasite surface antigens. However the pattern of antigenic recognition was different. Antibodies recognized 66-95% of GS-5G8(+) trophozoites. In contrast, these antibodies recognized only 23-32% of the GS trophozoite population (Figure 4). These results indicate that GS and GS-5G8 (+) are different strains and have a different antigen profile; thus, the recognition of both strains by antibodies derived from infected mice is not the same. In addition, the expression of VSP-5G8 by a small percentage (5-6%) of trophozoites GS (Figure 4) is sufficient to trigger a humoral response and recognize almost all the GS-5G8 (+) trophozoites.

5. DISCUSSION

Giardia lamblia VSPs are proteins with cysteine rich motifs, a variable amino-terminal region and a conserved carboxi-terminal with the cytoplasmatic tail CRGKA (Nash et al. 1992; Luján et al. 2011). The VSP repertoire differs between *Giardia* isolates, GS_B has more than 270 predicted VSP genes (Morrison et al. 2007; Adam et al. 2013), but only is expressed on the trophozoite surface at the time (Nash 1997; Luján et al. 2011). In a previous report we suggested that the immunogenic protein VSP-5G8 is constituted by one polypeptide chain and localize *vsp-5g8* gene (1824 bp) in the contig AHHH01000109 of *Giardia* GS_B genome (Quintero-Vargas Unnpublished). In this study, we confirmed that results by sequencing the *vsp-5g8* gene. We obtained 95% of the expected sequence length with 99% of homology. The deduced aminoacid sequence of *vsp-5g8* gene is consistent with molecular mass and sequence of MS/MS peptides found in previous analysis. These results evidence that the polymorphism described (G

or C at 801) can determine the differential expression of VSP-5G8 that we typically observed in previous works (Quintero et al. 2013; Quintero-Vargas Unnpublished). Approximately 5% of GS trophozoites present the polymorphism, so they express the complete 5G8 (~60) in the surface, whereas the other 95% of trophozoites express separately a truncated form of 5G8 of 28 kDa and a 38 kDa product that are not recognized by the monoclonal antibody specific for the complete VSP-5G8 form (Figure 5).

Recent studies indicate that *Giardia* genotype B has genetic diversity between and within isolates (Caccio et al. 2008; Sprong et al. 2009; Wielinga et al. 2011; Adam et al. 2013; Wielinga et al. 2015). Since *Giardia* is a tetraploid organism with two diploid nuclei, it is expected to accumulate sequence divergences (Franzen et al. 2009). However, genotype B is reported to have 50 times more allelic sequence heterozygosity (ASH) than genotype B (WB) (Ankarklev et al. 2012; Wielinga et al. 2015). This ASH occur at the single cell level, which complicates sequencing chromatograms interpretations (Ankarklev et al. 2012). In this analysis we observed some polymorphic differences in sequence of *vsp-5g8* gene. However, the homology analysis of VSP-5G8 antigen showed that this antigen has regions conserved among other VSP from different isolates and genotypes. It is noteworthy that a sequence almost identical to VSP-5G8 was expressed in other B genotype isolate (Wielinga et al. 2015). In addition, we determined T cell epitopes of VSP-5G8 that are conserved mainly in B genotype but also shares some aminoacids important for cleavage to MHC-II ($I\text{-}A^k$) (Fremont et al. 1998; Velazquez et al. 2001). These results indicate that our 60kDa VSP can be expressed in other isolates from B genotype or at least shares in its sequence potential sites for host immunological recognition.

Antigenic variation of *Giardia* has been proposed as a mechanism to immune system evasion (Luján et al. 2011). Total clearance of a VSP type during an infection has been associated to the establishment of host humoral immune responses (Gottstein et al. 1991; Nash 2002; Luján et al. 2011). In fact, antibodies released by the host could select the expression of certain VSPs (Nash 2002). In this study, we evaluated whether the expression of VSP-5G8 contribute or affect the ability of the *Giardia* strain to be recognized and activate the host immune system. We performed crossreaction of

infected mice serum and *Giardia* GS or GS-5G8 (+) trophozoites. The results indicated that both strains are recognized different by antibodies produced by an infected host. The immune response induced is mainly directed but not limited to VSP-5G8. However, even when only a small percentage of GS population express VSP-5G8 in surface, it is sufficient to induce in host an immune humoral response. These observations suggest that the expression of VSP-5G8 determine the immunogenicity of a *Giardia* strain. In addition, since VSP-5G8 is significanttly conserved at epitopes among *Giardia* genotypes, it suggests potential sites for cross immune reaction and a role of this antigen in sensibilizing the immune system to recognize other VSP or no VSP *Giardia* proteins.

Summarizing, in this work we determined the molecular characteristics of the VSP-5G8 *Giardia* protein. This antigen consist of only one polypeptide chain of 61 kDa codified by a 1824 bp *vsp-5g8* gene in *Giardia* GS/M83-H7 (ATCC 50581), but also it is expressed in the human isolate BAH15c1. VSP-5G8 analysis showed regions with immunological relevance conserved among other genotypes of *Giardia*. In addition, the expression of this antigen could mediate the immune response in host and sensibilize to recognition of other antigens of *Giardia*. Taken together, these results indicate that VSP-5G8 is a strong immunogenic protein which expression even at a small level in trophozoite populations induce an immune response against this and other *Giardia* antigens. However, more experiments are needed to determine if the immunological mechanisms activated by 5G8 are able to protect against future *Giardia* challenges, as well as elucidate the role of this antigen in host-parasite interaction.

6. DISCLOSURES

The authors declare that there is not conflict of interest.

7. FUNDING

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Table 1. Primers used for *vsp-5g8* gene amplification

#	Primer name	Sequence 5´-3´	Nt	GC%	Tm °C
1	5G8_28F	5´-ATGTTGAATAAATTTGCCCTTGTAGC-3´	26	34.6	54.7
2	5G8_28R	5´-CTATGCACTGGCCGGTGG-3´	18	66.7	58.9
3	5G8_38F	5´-ATGTACCTGAAGAAGAATACTGGTAGTG-3´	28	39.3	55.9
4	5G8_38R	5´-TCACGCCTTGCCCCTG-3´	16	68.8	58.6

To amplify VSP-5G8 complete gene we used primers 1 and 4.

Table 2. The VSP-5G8 have conserved regions with other VSPs among *Giardia* genotypes

#	Name*	GeneBank number**	Isolate	Genotype	Protein size (aa)	Cover of 5G8 (%)	Identity with 5G8 (%)	Reference
1	GSB_151175	ESU41816.1	GS_B	B	271	45	100	Adam R.D. 2013
2	GSB_155403	ESU39941.1	GS_B	B	379	62	99	Adam R.D. 2013
3	QR46_3107	KWX12887.1	BAH15c1	B	607	100	97	Wielinga C. 2015
4	QR46_2660	KWX13376.1	BAH15c1	B	352	57	76	Wielinga C. 2015
5	GSB_153033	ESU41143.1	GS_B	B	732	89	70	Adam R.D. 2013
6	GSB_154953	ESU40281.1	GS_B	B	679	100	63	Adam R.D. 2013
7	GSB_152640	ESU41738.1	GS_B	B	683	81	60	Adam R.D. 2013
8	GSB_153115	ESU40784.1	GS_B	B	646	100	55	Adam R.D. 2013
9	GSB_153790	ESU41862.1	GS_B	B	646	100	54	Adam R.D. 2013
10	DHA2_154068	ESU34840.1	DH	A2	652	81	52	Adam R.D. 2013
11	VSP-5	EDO82404.1	WB	A	713	80	51	Morrison,H.G. 2007
12	TSA4	gi|136441|	WB	A	713	80	51	Gillin,F.D. 1990
13	VSP-44	EDO80490.1	WB	A	732	80	50	Morrison,H.G. 2007
14	GLP15_2640	EFO61461.1	P15	E	745	82	48	Jerlstrom-Hultqvist,J. 2010
15	DHA2_150066	ESU39141.1	DH	A2	747	81	48	Adam R.D. 2013
16	VSP-92	EDO78978.1	WB	A	852	84	47	Morrison,H.G. 2007
17	VSP-186	EDO76439.1	WB	A	733	81	47	Morrison,H.G. 2007
18	VSP-54	EDO80151.1	WB	A	682	100	46	Morrison,H.G. 2007
19	DHA2_150893	ESU38743.1	DH	A2	702	100	46	Adam R.D. 2013
20	DHA2_153020	ESU35330.1	DH	A2	608	100	46	Adam R.D. 2013
21	TS11	gi|549130|	WB	A	667	99	45	Ey,P.L. 1993
22	VSP-116	EDO78106.1	WB	A	699	99	44	Morrison,H.G. 2007
23	WB/9B10-B	AAK97086.1	WB	A	667	100	43	Carranza,P.G. 2002
24	VSP-45	EDO80509.1	WB	A	727	100	43	Morrison,H.G. 2007
25	GLP15_2	EFO65462.1	P15	E	654	100	43	Jerlstrom-Hultqvist,J. 2010
26	GLP15_2823	EFO62683.1	P15	E	636	100	43	Jerlstrom-Hultqvist,J. 2010
27	GLP15_625	EFO62362.1	P15	E	682	99	42	Jerlstrom-Hultqvist,J. 2010
28	DHA2_151647	ESU39452.1	DH	A2	741	100	42	Adam R.D. 2013
29	GLP15_2839	EFO64161.1	P15	E	693	100	40	Jerlstrom-Hultqvist,J. 2010
30	VS4A1	gi|11387318|	WB	A	687	99	30	Papanastasiou, P. 1997

* All of the proteins analyzed are members of the VSP family. ** Accession number are shown.

Table 3. T cell epitopes predicted for VSP-5G8 sequence

					P1			P4		P6			P9				
#	**Pos**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**0**	**1**	**2**	**3**	**4**	**5**	**Score**
1	67-81	T	K	Q	**D**	T	Q	**N**	G	**T**	C	K	**S**	C	A	A	26
2	95-109	Q	S	P	**G**	S	L	**I**	C	**Q**	T	A	**S**	N	T	D	22
3	186-200	Y	Y	T	**D**	D	T	**S**	S	**E**	P	N	**G**	K	T	C	20
4	114-128	T	C	K	**D**	G	Y	**F**	T	**V**	S	D	**A**	T	A	T	18
5	121-135	T	V	S	**D**	A	T	**A**	T	**Q**	D	S	**C**	V	A	C	18
6	182-196	A	A	K	**D**	Y	Y	**T**	D	**D**	T	S	**S**	E	P	N	18
7	308-322	L	C	A	**D**	G	F	**Y**	G	**S**	S	C	**S**	K	C	H	18
8	370-384	K	A	C	**D**	L	T	**I**	D	**G**	T	K	**Y**	C	S	A	18
9	533-547	D	K	S	**D**	G	S	**I**	A	**G**	V	K	**D**	C	L	S	18
10	541-555	G	V	K	**D**	C	L	**S**	C	**A**	A	P	**S**	G	S	T	18

We used SYFPEITHI algorithm to determine the most probable epitopes (15mers) of VSP-5G8 to be recognized by T cells in the context of MHC-II ($I\text{-}A^k$).

Table 4. T cell epitopes of VSP-5G8 are conserved in other VSP among different *Giardia* isolates and genotypes.

#	Name	Isolate	Genotype	VSP-5G8 epitopes									
				E 1	E 2	E 3	E 4	E 5	E 6	E 7	E 8	E 9	E 10
1	GSB_151175	GS_B	B	++++	++++	++++	++++	++++	++++				
2	GSB_155403	GS_B	B							++++	++++	++++	++++
3	QR46_3107	BAH15c1	B	++++	++++	++++	++++	+++	++++	+++	++++	++++	++++
4	QR46_2660	BAH15c1	B							+++	+++	++++	++++
5	GSB_153033	GS_B	B	+++		++++			++++	++++	++		++
6	GSB_154953	GS_B	B			+++	++++	++++	++	+++	+++	+++	+++
7	GSB_152640	GS_B	B							+++	++		+++
8	GSB_153115	GS_B	B		++					+++			
9	GSB_153790	GS_B	B		++								
10	DHA2_154068	DH	A2								++		+
11	VSP-5	WB	A								++		
12	TSA4	WB	A								++		
13	VSP-44	WB	A					+			++		
14	GLP15_2640	P15	E			++				+++	++		
15	DHA2_150066	DH	A2			++					++		
16	VSP-92	WB	A								+++		
17	VSP-186	WB	A					++		+			
18	VSP-54	WB	A		+			+			++		+
19	DHA2_150893	DH	A2		++			+			++		+
20	DHA2_153020	DH	A2								++	+	
21	TS11	WB	A		+						++		+
22	VSP-116	WB	A							+++	++		
23	WB/9B10-B	WB	A								+++		+
24	VSP-45	WB	A						+		++		
25	GLP15_2	P15	E								++	++	
26	GLP15_2823	P15	E	++				++		++			
27	GLP15_625	P15	E		+			++			++	++	

28	DHA2_151647	DH	A2				+			
29	GLP15_2839	P15	E						+++	++
30	VS4A1	WB	A	+	++					

We used SYFPEITHI algorithm to determine the most probable epitopes (15mers) of VSP-5G8 to be recognized by T cells in the context of MHC-II (I-A^k). (+) Conserved at least anchoring residues at P1 and P9 plus two or three more aminoacids. (++) Conserved at least three anchoring residues (P1, P4, P6 or P9) with a total of 7-8 homologue aminoacids. (+++) Conserved at least three anchoring residues with a total of 9-13 homologue aminoacids. (++++) Conserved the four anchoring residues with a total of 14-15 homologue aminoacids.

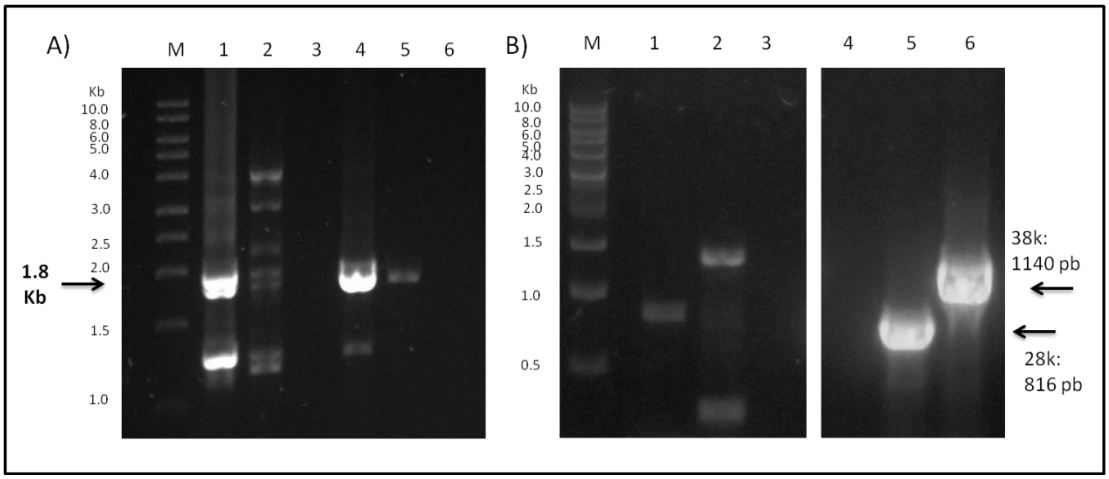


Figure 1. PCR amplification of 5G8 from gDNA and cDNA of *Giardia* strains. Specific primers were used to amplify complete gene or fragments from genomic DNA (gDNA) and complementary DNA (cDNA) of *Giardia* strains GS/M83-H7 and GS/M83-H7-5G8 (+). A) Comparison of 5G8 amplification from gDNA (1, 2, 3) and cDNA (4, 5, 6) from GS (2, 5) or GS-5G8 (+) (1, 4), reaction negative controls (3,6), (M) 1 Kb DNA ladder. B) Fragments 28K and 38K amplification from GS cDNA (1, 2) or GS-5G8 (+) cDNA (5, 6), reaction negative controls (3,4).

>*vsp-5g8*

ATGTTGAATAAATTTGCCCTTGTAGCCGTAATTCTCCAGATCGCCCGGGCCGCCTGCACGCCAGGCACTGAAACTACTAACTGCAAGGATGGCGCATGCA
ACGTCCAGATTGGCGGTGAGACATACTGCTCGCAGTGCTACACAACAAGCGAAGCACCAGTTGATGGGGTATGCACAGCAAGTACTGATTCTAAATGCAC
AAAACAGGATACCCAAAATGGCACTTGTAAATCCTGCGCAGCGAACTACTTCCTCTTTAAGGGAGGGTGCTATCAAATCGGACAATCTCCCGGTAGTTTG
ATTTGTCAAACTGCAAGCAACACCGATGGAATCTGTCAAACTTGTAAGGATGGCTACTTCACGGTTTCAGACGCTACTGCTACTCAGGACTCCTGTGTTG
CCTGCGGGGACGAGAACTGCGCAACGTGCACAGTAGGAGCAGAACAGCAGAAGTGCAGCAAGTGCAAGGCCGACGGCAAGATGTACCTGAAGAAGAATAC
TGGTAGTGAGACAGGCACCTGCGTCACTGCCGACGAGTGCACAGCAGCCAAGGATTATTACACAGACGATACCAGCAGTGAACCAAATGGTAAGACGTGC
AAGGCGTGCAGCGCAAAGGTGGAGAACTGTGCTTCCTGCAGCAGTGAAGGAGCCTGCCAGAAGTGCGCCTCTGGCTTTGTATTGGAGGGATCGAACTGCG
TCAAGAGCGATTGCAGTACCGAGAACTGCAAGACGTGCACCAACCCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCACCCC
GACCGGCCAGTGCATAGACGAGTGCGTAACTATCTCCGGCTACTACGGAACAGCTGATGGCAAGTGCAAGAAATGCGAAGTTGCTAACTGCGTCGTCTGC
GGGGCAACTGGAGCGTGCGATCTTTGCGCTGACGGCTTCTACGGTTCGAGCTGCTCTAAGTGCCACGAGAGCTGCAAGAATTGCAAAGGAGCTACTGCCA
GCGACTGCACAGCGTGCCCTGCTGGGAGGGCGCTCATCTACGGAGATGATCCCACCAAGGGCACGTGCGGAGAGGGTTGCACGACAGGCACAGGGAAAGG
TGCATGTAAGGCATGCGATTTAACGATCGACGGGACGAAGTACTGCTCTGCCTGTGACACGGCTACTGAATATCCGCAGAACGGCGTGTGTGCGACGCCG
ACTGCACGTGCCTCCTCGTGTCAGAGCCAAAATGTTGCTAGTGGTGCGTGCAATACTTGTGAAAATGGCTTCTTCAAGATGAACGGGGGCTGCTACTCCA
CCAGCCAGCTCCCGGGGTCGACGGTGTGCGTCACTGCGCCGACCGGAGGAACATGCACCAAATCAGAGCCGGGCTACAATGTGAATAGCGGCACCCTTGT
AACCTGTGGTGTTGGCTGCGCTGAGTGTACCAACTCTGATTCATGTACTACATGTGCATCTGGATATGTCAAGCTCACTAGTGCTGCCACTTGCACAAAG
TGCGACGCTGGGTGTGCCACGTGTACAACTGCTGCCTCAACCTGCAGCACCTGCGCCGACGGGTACTACCTCTCTAACAGCAAGTGCATCGCGTGCGACA
AAAGCGATGGCAGCATCGCCGGCGTCAAAGACTGCCTGAGCTGCGCGGCTCCATCTGGCAGCACTGGCCCTGTCCTCTGCTACCTCGTGAGGGACAGCGC
CTCGGTCAACAAGGGCGGCCTCAGCAGCGGGGCGATCGCGGGGATCTCCGTCGCAGTAATAGTCGTTGTCGGGGGCCTCGTCGGCTTCCTCTGCTGGTGG
TTCATCTGCAGGGGCAAGGCGTGA

[redacted]

>VSP-5G8

MLNKFALVAVILQIARAACTPGTETTNCKDGACNVQIGGETYCSQCYTTSEAPVDGVCTASTDSKCTKQDTQNGTCKSCAANYFLFKGGCYQIGQSPGSL
ICQTASNTDGICQTCKDGYFTVSDATATQDSCVACGDENCATCTVGAEQQKCSKCKADGKMYLKKNTGSETGTCVTADECTAAKDYYTDDTSSEPNGKTC
KACSAKVENCASCSSEGACQKCASGFVLEGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTPTGQCIDECVTISGYYGTADGKCKKCEVANCVVC
GATGACDLCADGFYGSSCSKCHESCKNCKGATASDCTACPAGRALIYGDDPTKGTCGEGCTTGTGKGACKACDLTIDGTKYCSACDTATEYPQNGVCATP
TARASSCQSQNVASGACNTCENGFFKMNGGCYSTSQLPGSTVCVTAPTGGTCTKSEPGYNVNSGTLVTCGVGCAECTNSDSCTTCASGYVKLTSAATCTK
CDAGCATCTTAASTCSTCADGYYLSNSKCIACDKSDGSIAGVKDCLSCAAPSGSTGPVLCYLVRDSASVNKGGLSSGAIAGISVAVIVVVGGLVGFLCWW
FICRGKA

Figure 2. Nucleotide and aminoacidic 5G8 sequence. The antigen 5G8 was amplified from GS and GS-5G8(+) cDNA and sequenced. A) Nucleotide sequence for 5G8 antigen. B) Deduced aminoacid sequence (ExPASy translate).

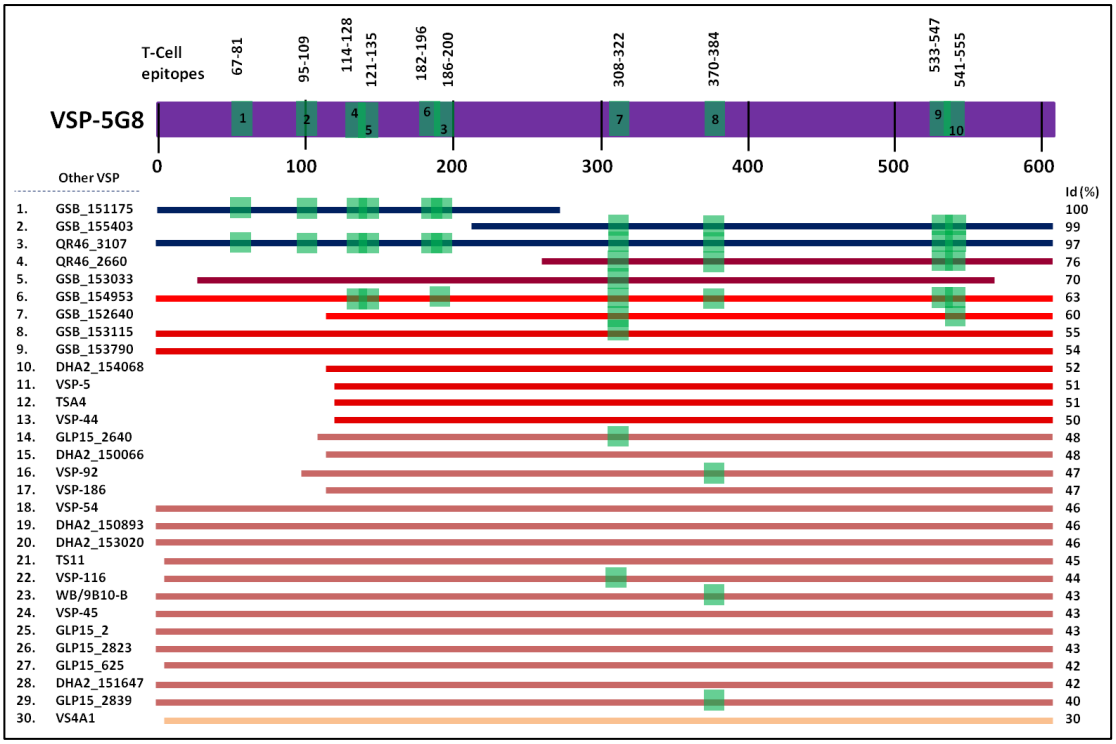


Figure 3. T cell epitopes of VSP-5G8 are conserved in other VSP among different *Giardia* isolates and genotypes. The homology analysis of VSP-5G8 (purple) antigen by BLASTp algorithm showed that this antigen has regions conserved among other VSP from different isolates and genotypes. Ten of the most probable T cell epitopes (15 mers) predicted by SYFPHEITY were seek in conserved regions of other VSPs. Genotype B VSPs conserve almost all the epitopes analyzed (green), but epitopes 7 and 8 are almost seen conserved in other *Giardia* genotypes.

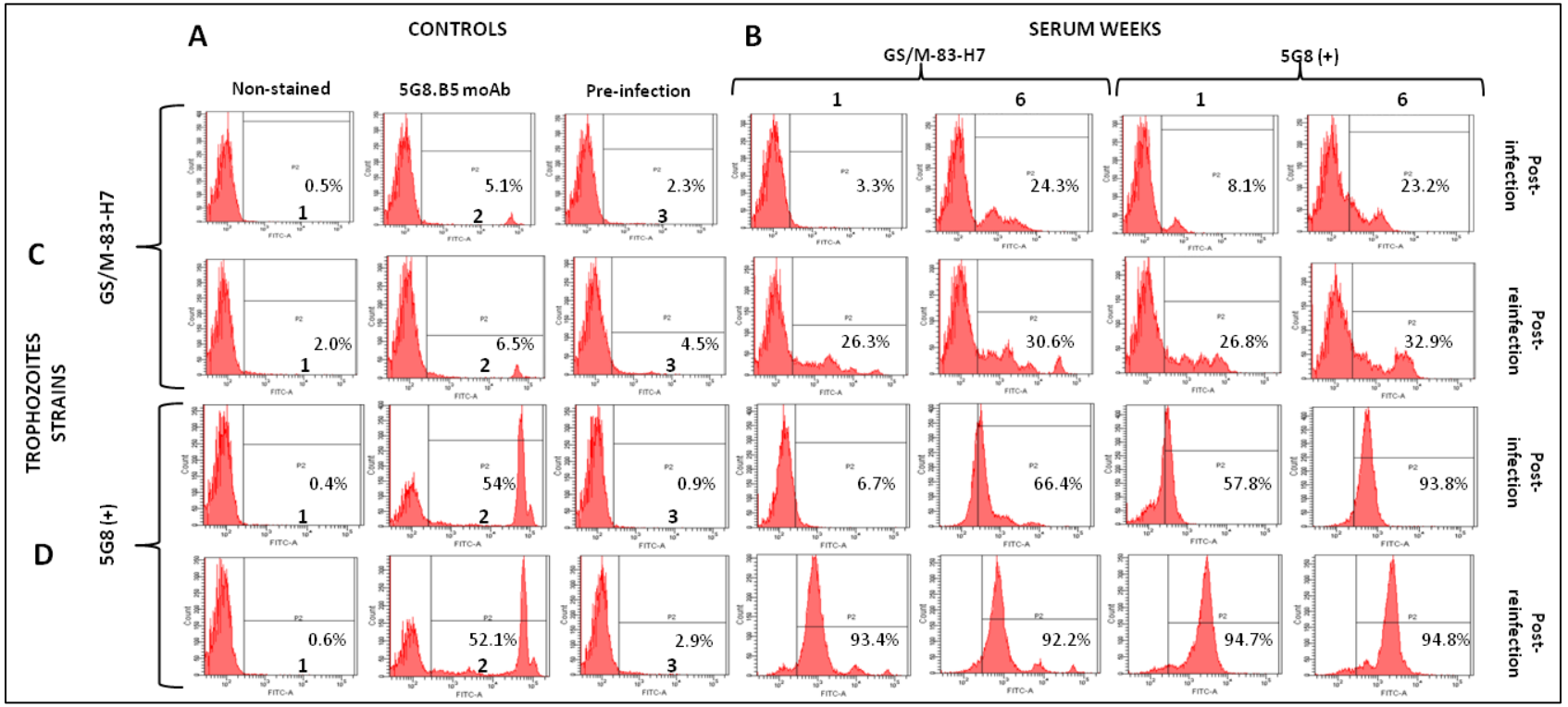


Figure 4: GS/M-83-H7 and 5G8 (+) *G. lamblia* strains are differentially recognized by serum from infected and re-infected mice. (A) Control panel: Non-stained control cells (1), Monoclonal antibody 5G8.B5 (2) and Pre-infection serum (3). (B) Serum panel: 1st and 6th weeks (PI) and (PRI) from infected mice with: (C) GS/M-83-H7 and (D) 5G8 (+) strains. The blue squares selection compares the difference in surface antigen recognition between GS/M-83-H7 infected mice at 6th week (PI) and (PRI) The red squares show the main differences on surface antigen recognition between 5G8 (+) and GS/M-83-H7 *G. lamblia* infected mice.

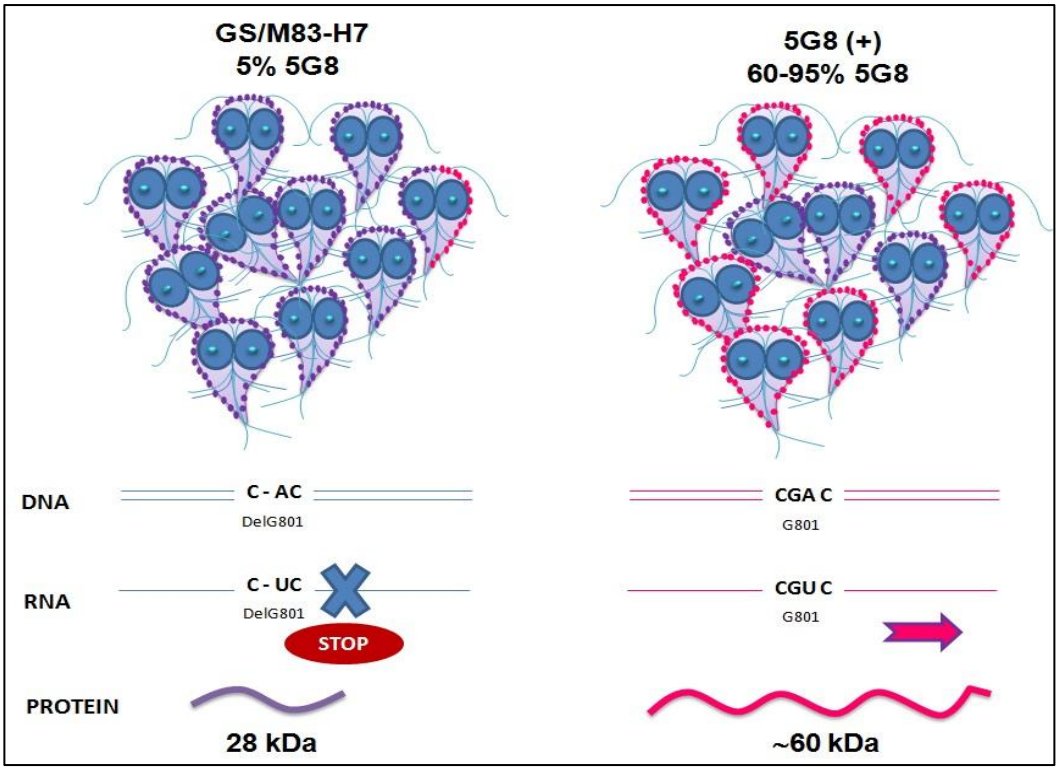


Figure 5. *G. lamblia* polymorphisms determine expression of the VSP-5G8 antigen in trophozoites. Approximately 5% of *G. lamblia* GS/M83-H7 trophozoites express VSP-5G8 in surface because the polymorphism InsG801, this results in generation of a complete antigen about 60 kDa, whereas the other 95% of trophozoites express separately a truncated form of VSP-5G8 of 28 kDa and separately a 38 kDa product that are not recognized by the monoclonal antibody specific for the complete VSP-5G8 form (Figure 5).

ANEXOS

ANEXO 1. Secuencias de BIP

1.1 Secuencia aminoacídica de BIP *Giardia intestinalis* ATCC 50581

GenBank: EES99538.1

>gi|253742871|gb|EES99538.1| Bip [*Giardia intestinalis* ATCC 50581]

MTSSRVNQKEYTKIKMIALVFAALALAETIIGIDLGTTYSCVAVSRAGQVEIIPNELGA
RVTPSYVAFTADGERLVGDAAKNYAPISPENTIFDVKRLIGRKFDDPEVQKDMKLLPYK
VINKDGRPFVQLSGTNLPKELQXKIMSPEEISAMVLTKMKTIAEDYLGEKITKAVVTVP
AYFSDSQRSATKDAGRIAGLDVVRIINEPTSSSIAYGLDKKTQETSGKAKNILVFDCGG
GTHDVSILSVDSGVFEVLATAGNTHLGGEDFDRRLLDHFIAIFKKKNNIDLSITNTGDK
AKDMAVKKAISRLRREIEAGKRQLSTASSVQIVVDSLIDGVDFSESLTRAKFEELNIDL
FKKSIKPVEQVLRDAKLKTTDIDEVVLVGGSTRIPKIRQLLQDYFNGKALNKDINADEA
VAWGAAVQASILSGAKDHDVLLIDVTPLTLGIETQGGIMTPLIERNSYIPVKKSKIFST
VQDQQTMVKIQVYEGERSMVKDNNLLGNFDLNDIPPAPRGTPQIEVTFEIDSNGILTVS
AVEKSSGKEESITIKNDRGRLSEDEINRLVREAEEFAEEDKINRERAEARNAFETIVSI
TTTQTTADKEGNIVDKISSDDLEKVKEAIKEAQDWLRDNTDASKEEIEEEKSKFEKVVQ
PILGENFGRSASAGGSGPEYDYAEKDEL

1.2 Secuencia aminoacídica BIP *Giardia intestinalis* ATCC 50581

GenBank: EES99538.1 (MODIFICADA)

gi|253742871|gb|EES99538.1| Bip [*Giardia intestinalis* ATCC 50581] **-46aa´s del N-terminal (Soltys et. al.) + Poli-Histidinas (6) + secuencia sensible a trombina (6aa´s).**

Aminoácido 107 X→N (con base en ATCC 50803)

> BIP mod

M**HHHHHH****LVPRGS**GQVEIIPNELGARVTPSYVAFTADGERLVGDAAKNYAPISPENTIF
DVKRLIGRKFDDPEVQKDMKLLPYKVINKDGRPFVQLSGTNLPKELQ**N**KIMSPEEISAM
VLTKMKTIAEDYLGEKITKAVVTVPAYFSDSQRSATKDAGRIAGLDVVRIINEPTSSSI
AYGLDKKTQETSGKAKNILVFDCGGGTHDVSILSVDSGVFEVLATAGNTHLGGEDFDRR
LLDHFIAIFKKKNNIDLSITNTGDKAKDMAVKKAISRLRREIEAGKRQLSTASSVQIVV
DSLIDGVDFSESLTRAKFEELNIDLFKKSIKPVEQVLRDAKLKTTDIDEVVLVGGSTRI
PKIRQLLQDYFNGKALNKDINADEAVAWGAAVQASILSGAKDHDVLLIDVTPLTLGIET
QGGIMTPLIERNSYIPVKKSKIFSTVQDQQTMVKIQVYEGERSMVKDNNLLGNFDLNDI
PPAPRGTPQIEVTFEIDSNGILTVSAVEKSSGKEESITIKNDRGRLSEDEINRLVREAE
EFAEEDKINRERAEARNAFETIVSITTTQTTADKEGNIVDKISSDDLEKVKEAIKEAQD
WLRDNTDASKEEIEEEKSKFEKVVQPILGENFGRSASAGGSGPEYDYAEKDEL

Masa molecular deducida 70.97 KDa

1. 3 Secuencia de nucleotídica de BIP de *G. lamblia* optimizada para su expresión en *E. coli* (DNA 2.0).

>BiP_OptGl

AGGAGGTAAAACATATGCATCATCATCACCACCATCTCGTACCACGCGGTTCGGGTCAA
GTAGAAATCATCCCAAACGAACTGGGTGCCCGTGTGACCCCGTCTTACGTTGCATTTAC
GGCGGATGGCGAGCGTCTGGTGGGTGACGCGGCAAAGAACTATGCGCCGATTAGCCCGG
AGAATACGATTTTCGACGTGAAGCGCCTGATCGGCCGTAAGTTCGATGATCCGGAAGTG
CAGAAAGATATGAAACTGCTGCCGTATAAAGTCATTAACAAGGATGGTCGCCCGTTTGT
TCAGCTGAGCGGTACCAATTTGCCGAAAGAGCTGCAGAACAAGATCATGAGCCCGGAAG
AGATCAGCGCGATGGTTTTGACCAAAATGAAAACGATTGCAGAGGATTACTTGGGTGAG
AAGATTACGAAAGCCGTGGTTACCGTGCCGGCATATTTCAGCGACAGCCAACGCTCCGC
AACGAAAGATGCTGGTCGTATCGCGGGTCTGGACGTTGTCCGTATCATTAACGAGCCGA
CCAGCAGCAGCATCGCATACGGCCTGGATAAGAAAACCCAGGAAACCAGCGGTAAAGCG
AAGAATATCCTGGTTTTCGATTGCGGCGGTGGCACCCACGATGTCAGCATTCTGAGCGT
CGATAGCGGCGTCTTTGAAGTTCTGGCTACCGCCGGTAATACCCACCTGGGTGGCGAGG
ACTTCGACCGCCGTCTGTTGGACCACTTTATCGCTATTTTCAAAAAGAAAAACAACATC
GACCTGAGCATTACCAACACGGGCGACAAGGCTAAGGATATGGCGGTGAAGAAGGCGAT
CAGCCGCCTGCGTCGTGAGATCGAGGCTGGTAAGCGCCAACTGAGCACTGCAAGCAGCG
TTCAGATTGTGGTCGATTCCCTGATCGACGGTGTTGACTTCAGCGAGTCCCTGACGCGT
GCGAAGTTTGAAGAACTGAACATTGATCTGTTTAAGAAAAGCATTAAGCCGGTTGAACA
AGTTCTGCGCGACGCGAAGTTGAAAACCACTGATATCGACGAGGTCGTGCTGGTTGGTG
GCAGCACGCGTATCCCGAAGATTCGCCAACTGCTGCAAGATTACTTTAATGGCAAAGCC
CTGAACAAAGACATTAACGCGGACGAGGCCGTCGCGTGGGGTGCGGCCGTTCAAGCAAG
CATCCTGAGCGGTGCGAAGGATCACGACGTCCTGCTGATTGATGTGACGCCGCTGACCC
TGGGCATTGAAACGCAGGGTGGTATCATGACCCCTCTGATTGAGCGTAATTCCTACATT
CCGGTCAAAAAGAGCAAGATTTTCTCTACCGTGCAAGACCAGCAGACGATGGTCAAGAT
CCAGGTGTATGAGGGTGAGCGTAGCATGGTTAAAGACAATAATCTGCTGGGTAACTTCG
ACTTGAATGATATTCCGCCTGCCCCACGCGGTACCCCGCAGATTGAGGTCACTTTCGAG
ATCGACTCTAACGGCATCCTGACGGTGTCCGCGGTTGAAAAGAGCTCGGGCAAAGAAGA
GAGCATCACCATCAAAAACGACCGTGGCCGTTTGAGCGAGGACGAGATTAATCGTCTGG
TTCGTGAGGCGGAAGAGTTTGCGGAAGAAGATAAGATCAACCGTGAGCGCGCAGAAGCT
CGTAATGCCTTCGAAACCATTGTCTCCATTACCACCACGCAGACGACCGCAGACAAAGA
AGGTAACATCGTGGACAAGATCAGCAGCGACGATTTGGAGAAGGTTAAAGAGGCGATTA
AAGAAGCGCAAGACTGGCTGCGTGATAATACCGACGCGTCTAAAGAAGAAATCGAAGAA
GAAAAATCTAAATTCGAGAAAGTGGTGCAGCCGATTCTGGGCGAGAATTTTGGCCGTAG
CGCATCTGCGGGTGGTAGCGGTCCGGAGTACGATTATGCCGAGAAAGATGAGCTGTAAC
TCGAG

Tamaño: 1952 pb

ANEXO 2. Plásmido de expresión

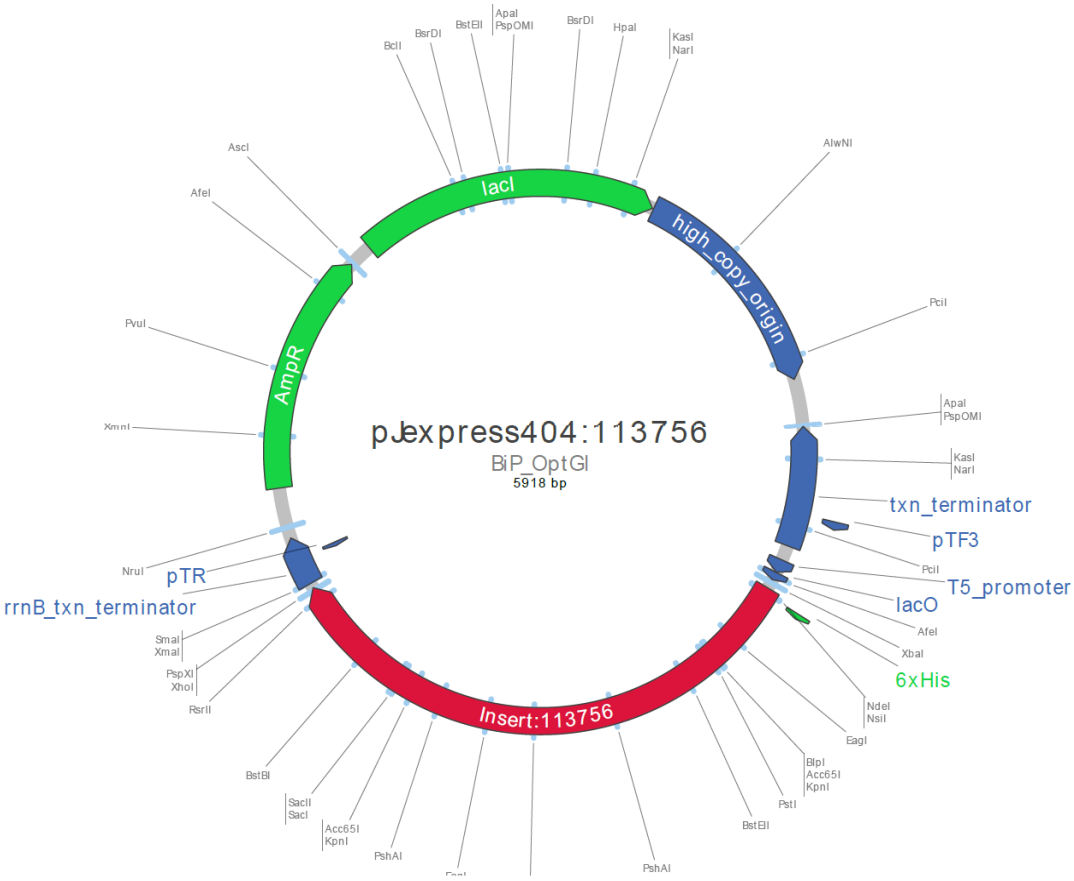


Figura A2. Mapa del plásmido de expresión pJexpress404/BIP. El plásmido comercial contiene un gen de resistencia a ampicilina (*AmpR*), el promotor del fago T5, el represor *lac*, y el inserto de interés (1932 pb), correspondiente a la secuencia nucleotídica de BIP, la cual fue optimizada para su expresión en *E. coli*. El inserto de interés codifica para una BIP modificada que incluye una secuencia de 6His en el N-terminal, una secuencia sensible a trombina adyacente a las 6His y la deleción de la región transmembranal (47 aminoácidos en el N-terminal). El tamaño total del vector es de 5918 pb.

ANEXO 3. Sobreexpresión de BIP

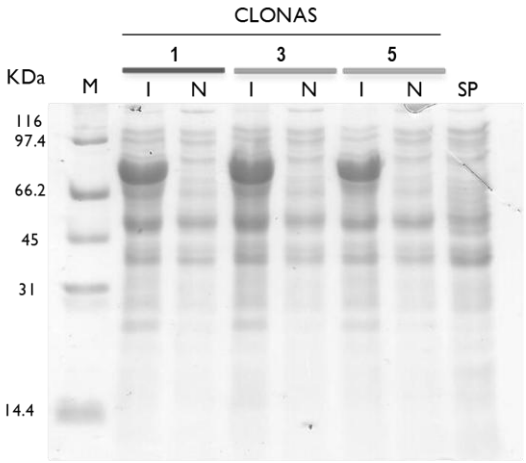


Figura A3.1. Sobre-expresión de BIPr en *E. coli*. Electroforesis en gel de poliacrilamida al 12% condiciones desnaturalizantes. El plásmido de expresión pJexpress404/BIP fue empleado para transformar bacterias *E. coli Rosetta gami* competentes (pre-tratadas con $CaCl_2$). Las clonas positivas fueron seleccionadas por su resistencia al antibiótico ampicilina (100 μg/mL) en agar LB. Se seleccionaron tres clonas para sobreexpresión con IPTG (1mM) 37ºC y 6 horas de incubación. Se observa una banda proteica de 71 kDa correspondiente a BIP en las muestras de cultivos inducidos. En cada carril se cargaron 5μL de cultivo bacteriano. M: marcador de peso molecular broad-range, I: inducción con IPTG, N: no inducción, SP: clona sin el plásmido de expresión.

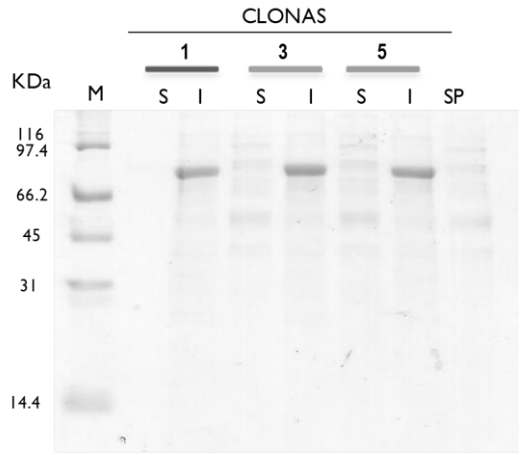


Figura A3.2. Ensayo de solubilidad de BIPr. Análisis de tres clonas positivas para la sobre-expresión de BIPr. Los cultivos inducidos a (37°C, 6h, IPTG de 1mM) fueron sometidos a lisis con lisozima (2mg/mL) y sonicación (6 ciclos 10s 20% amplitud) y separados por centrifugación, el sobrenadante representa la fracción soluble (S) y el botón celular la fracción insoluble (I), este fue resuspendido en una cantidad proporcional de volumen con respecto a su masa. Bajo estas condiciones, la proteína BIPr se recupera solo en la fracción insoluble en las tres clonas probadas. M: marcador de peso molecular, SP: bacterias no transformadas.

ANEXO 4. Optimización de la sobreexpresión de BIP

La optimización se realizó en dos etapas, en una etapa inicial, se analizó la expresión por electroforesis, en la cual se determinó que se obtenía mayor cantidad de BIPr soluble en medio TB durante 6h de inducción con 0.5 mM de IPTG a 37 °C (Figura A4.1). En la segunda etapa se contaba ya con anticuerpos policlonales anti-BIP, por lo que se por lo que se pudo obtener mayor resolución mediante Western blotting (Figura A4.2). Se determinó que a 4h de inducción con 0.5 mM de IPTG a 37 °C se obtiene suficiente proteína soluble, pero ésta sufre menor degradación a 25 °C de 18 a 30 horas de inducción.

Tabla A4. Condiciones de sobreexpresión de BIP evaluadas.

Medio LB	37 °C				25 °C			
[IPTG] / horas	4	6	8	18	4	6	8	18
0.2 mM								
0.5 mM								

Medio TB	37 °C				25 °C										16 °C								
[IPTG] / horas	4	6	8	18	4	6	8	18	24	30	40	48	55	64	4	12	18	24	30	40	48	55	64
0.2 mM																							
0.5 mM																							

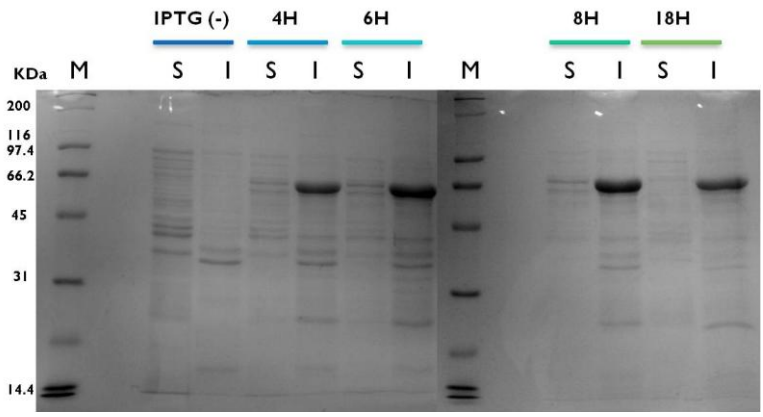


Figura A4.1. Optimización de la sobreexpresión de BIPr primera etapa. Evaluación por comparación en electroforesis en gel de poliacrilamida. Las condiciones seleccionadas de inducción con IPTG 0.5mM en medio de cultivo Terrific broth (TB) incubación a 37 °C durante 6h. M: marcador de peso molecular, S: fracción protéica soluble, I: fracción protéica insoluble.

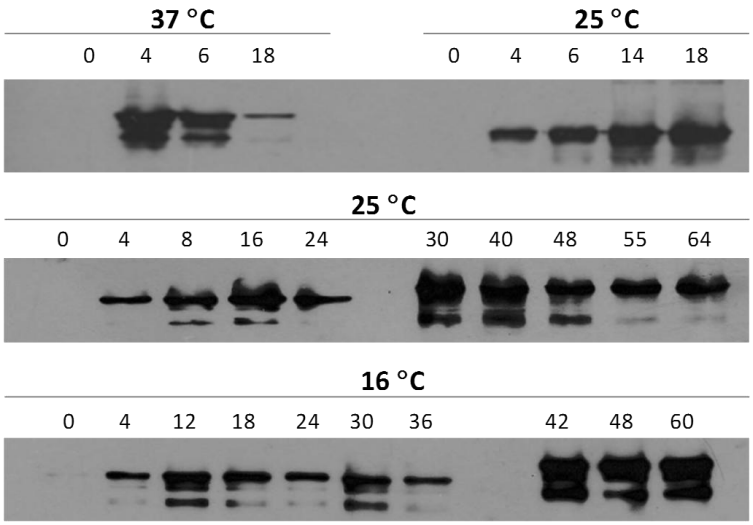


Figura A4.2. Optimización de la sobreexpresión de BIPr segunda etapa. Evaluación por comparación en Western blotting. En cada carril se colocaron 15 μL de fracción soluble la condición correspondiente. Para detectar BIP, los geles se transfirieron a una membrana de nitrocelulosa y éstas se incubaron con anticuerpos policlonales (1:5000, 5ta semana post-inmunización) y se reveló por quimioluminiscencia. Se observó que la condición que presenta mejor rendimiento y preservación de la proteína es a 25 °C entre 18 a 30 h de inducción. M: marcador de peso molecular, S: fracción protéica soluble, I: fracción protéica insoluble.

ANEXO 5. Purificación de BIP

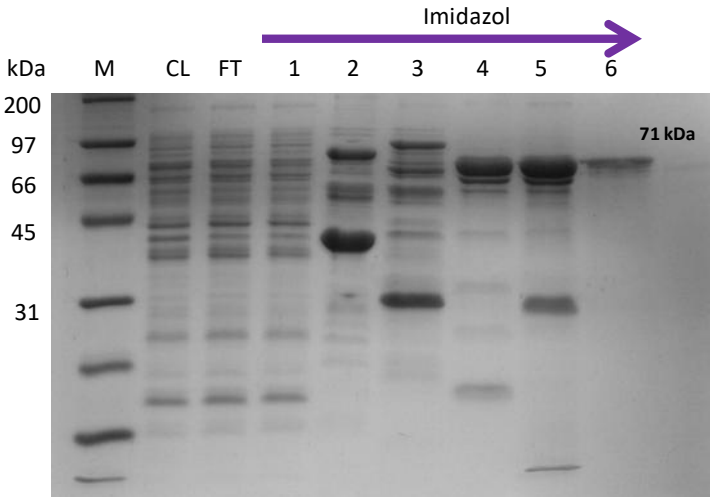


Figura A1. Perfil electroforético de BIPr eluída con imidazol a diferentes concentraciones. Electroforesis SDS-PAGE 12 % de las principales fracciones de la purificacion de BIPr. Teñidas con azul de Coomassie. Cada carril contiene 20 uL de la fracción correspondiente. CL: Lisado celular (1-6): Fracciones eluídas con (5, 20, 50, 100, 200, 500 mM de imidazol, M: Marcador de peso molecular.

ANEXO 6. Generación de anticuerpos policlonales anti-BIP

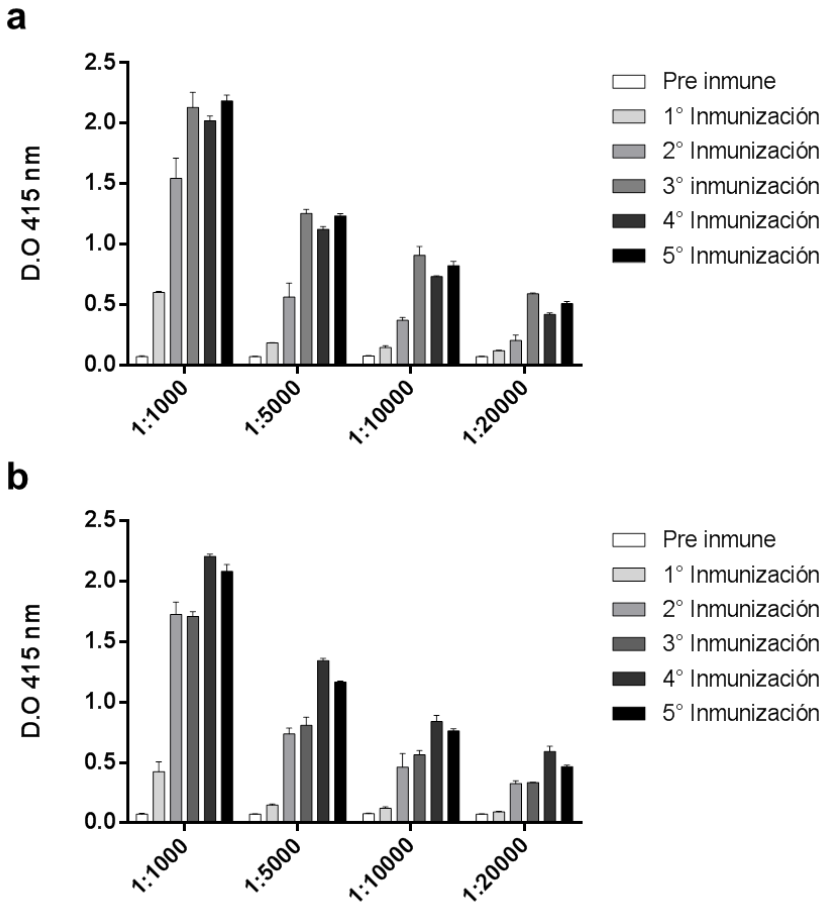
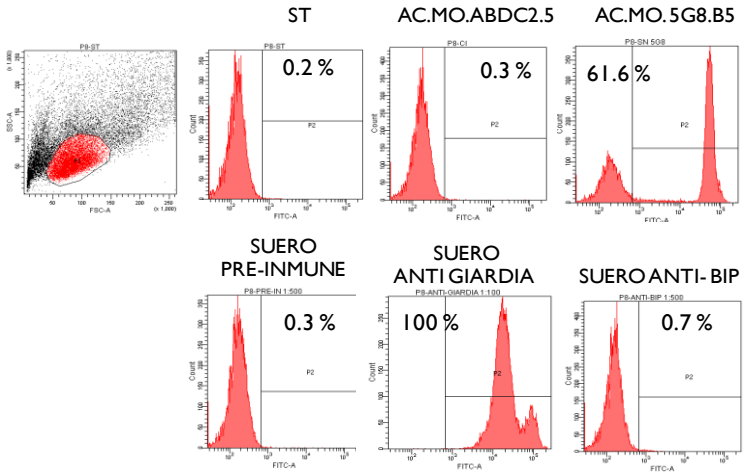


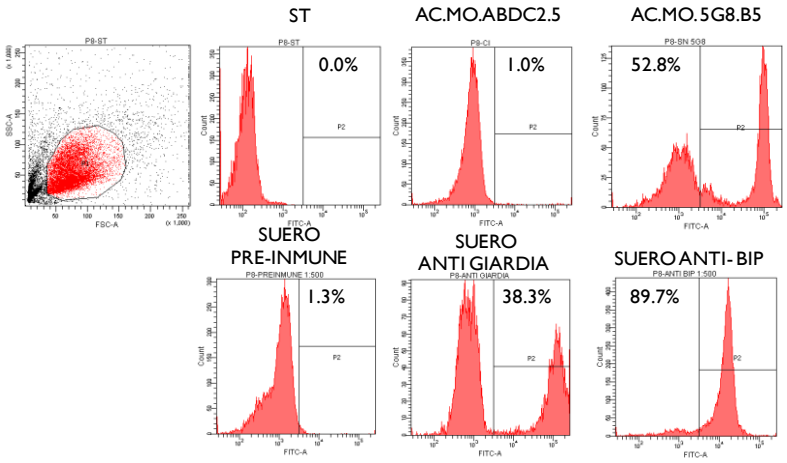
Figura A2. Determinación de titulo de anticuerpos anti-BIP en suero de dos ratones inmunizados mediante ELISA indirecto. Los títulos más altos de anticuerpos policlonales se obtuvieron a partir de la 5ª semana posterior a la inmunización. Se evaluaron muestras de sueros recolectadas de los ratones a y b, 7 días posteriores a cada inmunización. Datos mostradas en el eje X, representan dilución correspondiente de cada uno de los sueros.

ANEXO 7. Análisis de expresión de antígenos de *Giardia* por citometría de flujo

a) *Giardia lamblia* GS/M83-H7-5G8 (+)



b) *Giardia lamblia* GS/M83-H7-5G8 (+)



c) *Giardia lamblia* GS/M83-H7

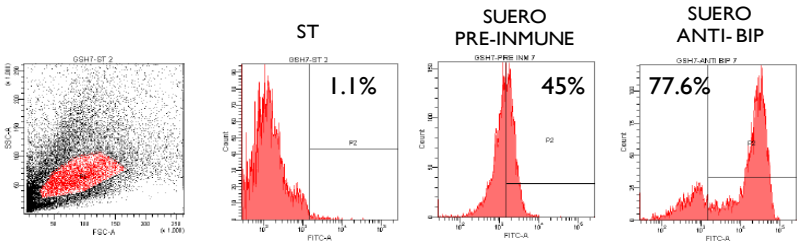


Figura A7. Perfil de expresión de antígenos de Giardia. a) Tinción superficial de trofozoítos de *Giardia lamblia*. b y c) Tinción intracelular de trofozoítos de *Giardia lamblia*

ANEXO 8. Análisis de respuesta inmune humoral contra BIP

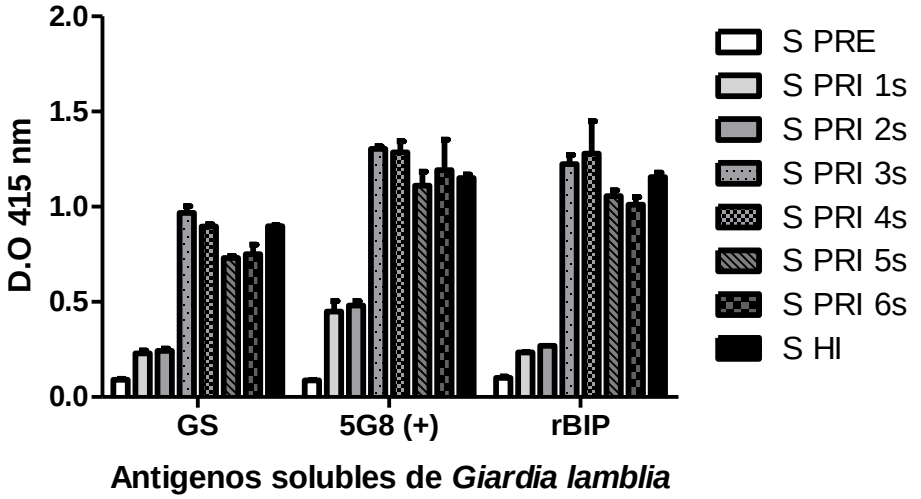


Figura A8. El antígeno BIP es reconocido por sueros de ratones re-infectados. Mediante un ensayo de ELISA se determinó que los ratones reinfectados con *Giardia* GS/M83-H7-5G8 (+) presentan altos títulos de anticuerpos IgG contra BIPr así como también contra antígenos solubles de ambas cepas de *Giardia*. Los resultados son más evidentes a partir de la tercera semana post-reinfección (S PRI 3s). S PRE: Suero preinmune, S PRI 1s-6s: suero post-reinfección de la 1ra a la 6ta semana, S HI: Suero hiperinmune.

ANEXO 9. Análisis de respuesta inmune celular contra BIP

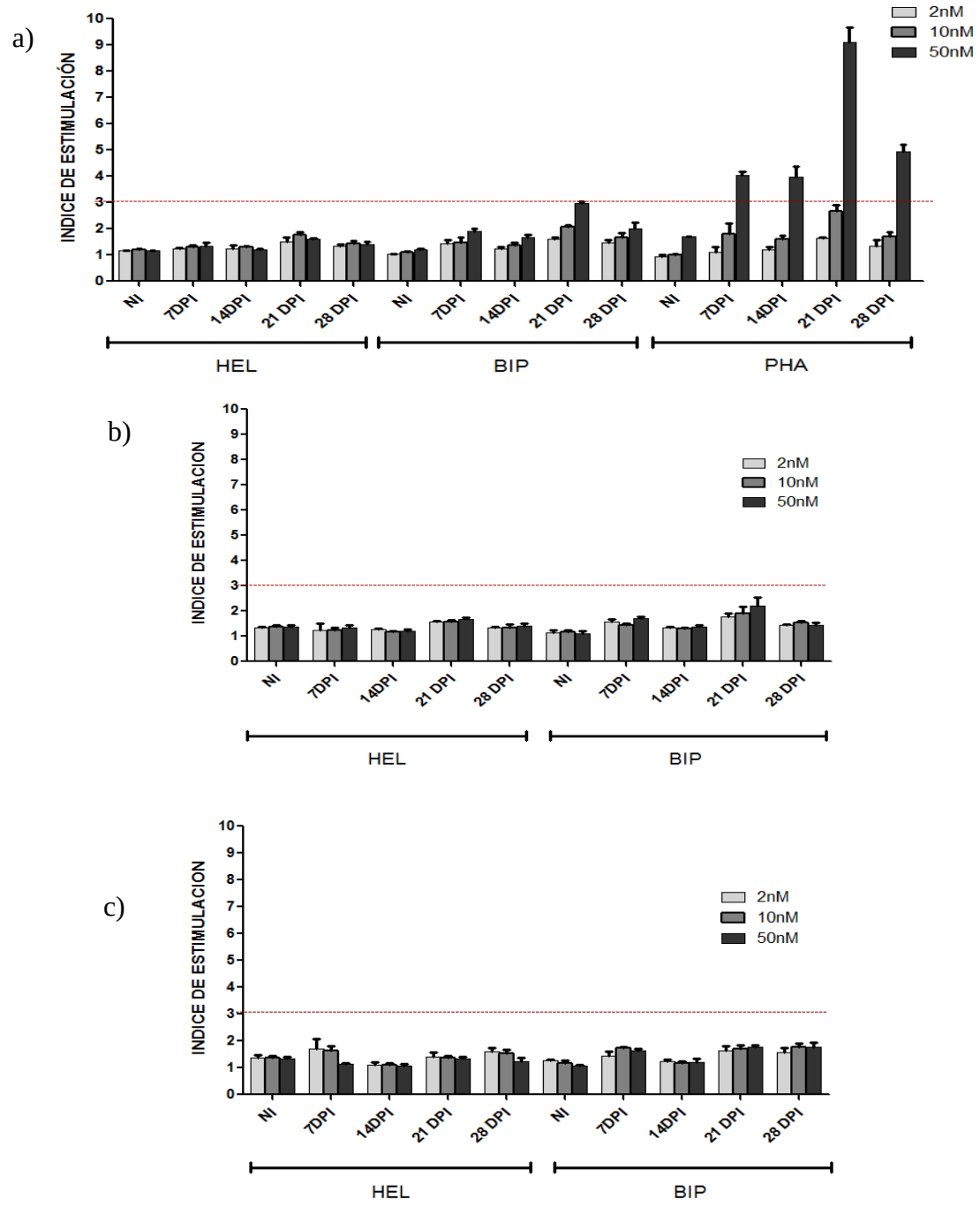


Figura A9. BIP no induce la proliferación de esplenocitos de ratones infectados. La inducción de la respuesta inmune celular se evaluó considerando la proliferación de esplenocitos de ratones previamente infectados ante el estimulo del antígeno. Se evaluaron tres dosis del antígeno (2, 10 y 50 nM) y se midió la respuesta a las a) 72, b) 96 y c) 120h post-tratamiento.

ANEXO 10. Predicción de epítopes de BIP y 5G8

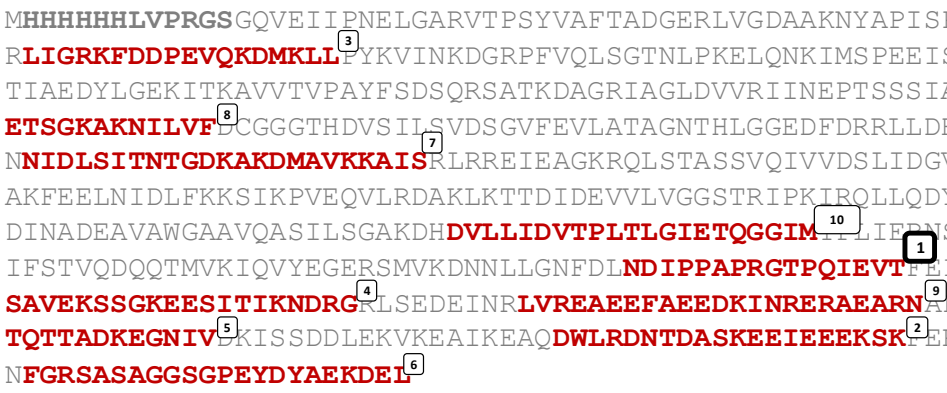


Figura A10.1. Análisis de epítopes de células B en la secuencia de BIP. Se emplearon los algoritmos Bcpred, FBCPred, AAP, ABCPred, BcePred , Bepipred y Antigenicity para determinar los 10 epítopes de células B con mayor probabilidad de ser reconocidos.

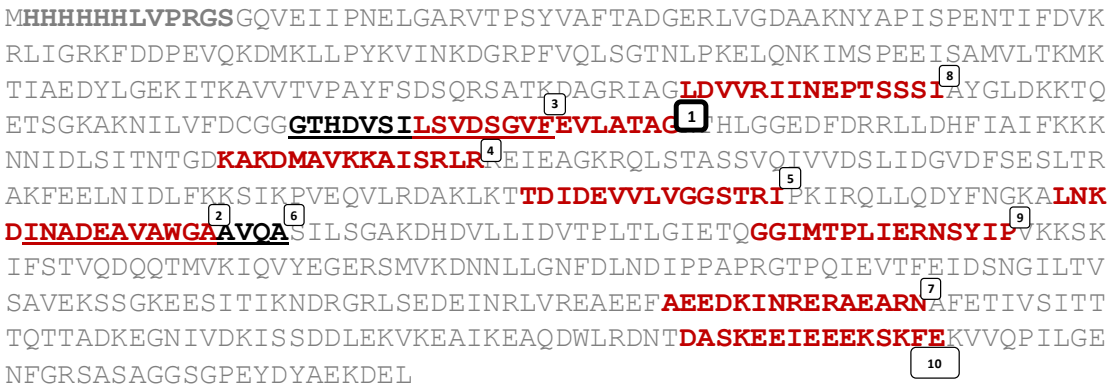


Figura A10.1. Análisis de epítopes de células T en la secuencia de BIP. Se empleó el algoritmo SYFPEITHI para determinar los 10 epítopes con mayor probabilidad de interactuar con el MHC-II I-A^k.

Figura A10.1. Análisis de epítopes de células T en la secuencia de BIP. Se empleó el algoritmo SYFPEITHI para determinar los 10 epítopes con mayor probabilidad de interactuar con el MHC-II I-A^k.

ANEXO 11. Secuencias del análisis de identificación del antígeno 5G8

Aminoácidos

11. 1. Proteína parcial de 28 kDa

MLNKFALVAVILQIARAACTPGTETTNCKDGACNVQIGGETYCSQCYTTSEAPVDGVCTASTDSKCTKQDTQNGTCKSCAANYFLFKGG
CYQIGQSPGSLICQTASNTDGICQTCKDGYFTVSDATATQDSCVACGDENCATCTVGAEQQKCSKCKADGKMYLKKNTGSETGTCVTAD
ECTAAKDYYTDDTSSEPNGKTCKACSAKVENCASCSSEGACQKCASGFVLEGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTP
PASA

Tamaño: 271 aminoácidos

11. 2. Proteína parcial de 38 kDa

EGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTPTGQCIDECVTISGYYGTADGKCKKCEVANCVVCGATGACDLCADGFYGSS
CSKCYESCKNCKGATASDCTACPAGRALIYGDDPTKGTCGEGCTTGTGKGACKACDLTIDGTKYCSACDTATEYPQNGVCATPTARASS
CQSQNVASGACNTCENGFFKMNGGCYSTSQLPGSTVCVTAPTGGTCTKSEPGYNVNSGTLVTCGVGCAECTNSDSCTTCASGYVKLTSA
ATCTKCDAGCATCTTAASTCSTCADGYYLSNSKCIACDKSDGSIAGVKDCLSCAAPSGSTGPVLCYLVRDSASVNKGGLSSGAIAGISV
AVIVVVGGLVGFLCWWFICRGKA

Tamaño: 379 aminoácidos

11.3. Secuencia teórica de la proteína 5G8, constructo 61 kDa.

MLNKFALVAVILQIARAACTPGTETTNCKDGACNVQIGGETYCSQCYTTSEAPVDGVCTASTDSKCTKQDTQNGTCKSCAANYFLFKGG
CYQIGQSPGSLICQTASNTDGICQTCKDGYFTVSDATATQDSCVACGDENCATCTVGAEQQKCSKCKADGKMYLKKNTGSETGTCVTAD
ECTAAKDYYTDDTSSEPNGKTCKACSAKVENCASCSSEGACQKCASGFVLEGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTP
TGQCIDECVTISGYYGTADGKCKKCEVANCVVCGATGACDLCADGFYGSSCSKCYESCKNCKGATASDCTACPAGRALIYGDDPTKGTC
GEGCTTGTGKGACKACDLTIDGTKYCSACDTATEYPQNGVCATPTARASSCQSQNVASGACNTCENGFFKMNGGCYSTSQLPGSTVCVT
APTGGTCTKSEPGYNVNSGTLVTCGVGCAECTNSDSCTTCASGYVKLTSAATCTKCDAGCATCTTAASTCSTCADGYYLSNSKCIACDK
SDGSIAGVKDCLSCAAPSGSTGPVLCYLVRDSASVNKGGLSSGAIAGISVAVIVVVGGLVGFLCWWFICRGKA

Tamaño: 607 aminoácidos

Nucleótidos

11.4. Proteína parcial de 28 kDa

GSB_151175 /Variant-specific surface protein /*Giardia intestinalis (Giardia lamblia)*
UniProtKB - V6TSX5 (V6TSX5_GIAIN)

ATGTTGAATAAATTTGCCCTTGTAGCCGTAATTCTCCAGATCGCCCGGGCCGCCTGCACGCCAGGCACTGAAACTACTAACTGCAAGGA
TGGCGCATGCAACGTCCAGATTGGCGGTGAGACATACTGCTCGCAGTGCTACACAACAAGCGAAGCACCAGTTGATGGGGTATGCACAG
CAAGTACTGATTCTAAATGCACAAAACAGGATACCCAAAATGGCACTTGTAAATCCTGCGCAGCGAACTACTTCCTCTTTAAGGGAGGG
TGCTATCAAATCGGACAATCTCCCGGTAGTTTGATTTGTCAAACTGCAAGCAACACCGATGGAATCTGTCAAACTTGTAAGGATGGCTA
CTTCACGGTTTCAGACGCTACTGCTACTCAGGACTCCTGTGTTGCCTGCGGGGACGAGAACTGCGCAACGTGCACAGTAGGAGCAGAAC
AGCAGAAGTGCAGCAAGTGCAAGGCCGACGGCAAGATGTACCTGAAGAAGAATACTGGTAGTGAGACAGGCACCTGCGTCACTGCCGAC
GAGTGCACAGCAGCCAAGGATTATTACACAGACGATACCAGCAGTGAACCAAATGGTAAGACGTGCAAGGCGTGCAGCGCAAAGGTGGA
GAACTGTGCTTCCTGCAGCAGTGAAGGAGCCTGCCAGAAGTGCGCCTCTGGCTTTGTATTGGAGGGATCGAACTGCGTCAAGAGCGATT
GCAGTACCGAGAACTGCAAGACGTGCACCAACCCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCAC**CCCA**
CCGGCCAGTGCATAG

Tamaño: 816 pb

11.5. Proteína parcial de 38 kDa

GSB_155403/Variant-specific surface protein/*Giardia intestinalis (Giardia lamblia)*
UniProtKB - V6TMH9 (V6TMH9_GIAIN)

GAGGGATCGAACTGCGTCAAGAGCGATTGCAGTACCGAGAACTGCAAGACGTGCACCAACCCGAAGGCAGCCAACGAGGCCTGCACAGC
GTGTGTTACTGGCATGTTTCTCA**CCCCGAC**CGGCCAGTGCATAGACGAGTGCGTAACTATCTCCGGCTACTACGGAACAGCTGATGGCA
AGTGCAAGAAATGCGAAGTTGCTAACTGCGTCGTCTGCGGGGCAACTGGAGCGTGCGATCTTTGCGCTGACGGCTTCTACGGTTCGAGC
TGCTCTAAGTGCTACGAGAGCTGCAAGAATTGCAAAGGAGCTACTGCCAGCGACTGCACAGCGTGCCCTGCTGGGAGGGCGCTCATCTA
CGGAGATGAT**CCCACC**AAGGGCACGTGCGGAGAGGGTTGCACGACAGGCACAGGGAAAGGTGCATGTAAGGCATGCGATTTAACGATCG
ACGGGACGAAGTACTGCTCTGCCTGTGACACGGCTACTGAATATCCGCAGAACGGCGTGTGTGCGACGCCGACTGCACGTGCCTCCTCG
TGTCAGAGCCAAAATGTTGCTAGTGGTGCGTGCAATACTTGTGAAAATGGCTTCTTCAAGATGAACGGGGGCTGCTACTCCACCAGCCA
GCTCCCGGGGTCGACGGTGTGCGTCACTGCGCCGACCGGAGGAACATGCACCAAATCAGAGCCGGGCTACAATGTGAATAGCGGCACCC
TTGTAACCTGTGGTGTTGGCTGCGCTGAGTGTACCAACTCTGATTCATGTACTACATGTGCATCTGGATATGTCAAGCTCACTAGTGCT
GCCACTTGCACAAAGTGCGACGCTGGGTGTGCCACGTGTACAACTGCTGCCTCAACCTGCAGCACCTGCGCCGACGGGTACTACCTCTC
TAACAGCAAGTGCATCGCGTGCGACAAAAGCGATGGCAGCATCGCCGGCGTCAAAGACTGCCTGAGCTGCGCGGCTCCATCTGGCAGCA
CTGGCCCTGTCCTCTGCTACCTCGTGAGGGACAGCGCCTCGGTCAACAAGGGCGGCCTCAGCAGCGGGGCGATCGCGGGGATCTCCGTC
GCAGTAATAGTCGTTGTCGGGGGCCTCGTCGGCTTCCTCTGCTGGTGGTTCATCTGCAGGGGCAAGGCGTGA

Tamaño: 1140 pb

11.6. Secuencia teórica de 5G8, constructo (61 kDa).

>VSP-5G8 Theoretical
ATGTTGAATAAATTTGCCCTTGTAGCCGTAATTCTCCAGATCGCCCGGGCCGCCTGCACGCCAGGCACTGAAACTACTAACTGCAAGGA
TGGCGCATGCAACGTCCAGATTGGCGGTGAGACATACTGCTCGCAGTGCTACACAACAAGCGAAGCACCAGTTGATGGGGTATGCACAG
CAAGTACTGATTCTAAATGCACAAAACAGGATACCCAAAATGGCACTTGTAAATCCTGCGCAGCGAACTACTTCCTCTTTAAGGGAGGG
TGCTATCAAATCGGACAATCTCCCGGTAGTTTGATTTGTCAAACTGCAAGCAACACCGATGGAATCTGTCAAACTTGTAAGGATGGCTA
CTTCACGGTTTCAGACGCTACTGCTACTCAGGACTCCTGTGTTGCCTGCGGGGACGAGAACTGCGCAACGTGCACAGTAGGAGCAGAAC
AGCAGAAGTGCAGCAAGTGCAAGGCCGACGGCAAGATGTACCTGAAGAAGAATACTGGTAGTGAGACAGGCACCTGCGTCACTGCCGAC
GAGTGCACAGCAGCCAAGGATTATTACACAGACGATACCAGCAGTGAACCAAATGGTAAGACGTGCAAGGCGTGCAGCGCAAAGGTGGA
GAACTGTGCTTCCTGCAGCAGTGAAGGAGCCTGCCAGAAGTGCGCCTCTGGCTTTGTATTGGAGGGATCGAACTGCGTCAAGAGCGATT
GCAGTACCGAGAACTGCAAGACGTGCACCAACCCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCACCC**CG**
ACCGGCCAGTGCATAGACGAGTGCGTAACTATCTCCGGCTACTACGGAACAGCTGATGGCAAGTGCAAGAAATGCGAAGTTGCTAACTG
CGTCGTCTGCGGGGCAACTGGAGCGTGCGATCTTTGCGCTGACGGCTTCTACGGTTCGAGCTGCTCTAAGTGCTACGAGAGCTGCAAGA
ATTGCAAAGGAGCTACTGCCAGCGACTGCACAGCGTGCCCTGCTGGGAGGGCGCTCATCTACGGAGATGATCCCACCAAGGGCACGTGC
GGAGAGGGTTGCACGACAGGCACAGGGAAAGGTGCATGTAAGGCATGCGATTTAACGATCGACGGGACGAAGTACTGCTCTGCCTGTGA
CACGGCTACTGAATATCCGCAGAACGGCGTGTGTGCGACGCCGACTGCACGTGCCTCCTCGTGTCAGAGCCAAAATGTTGCTAGTGGTG
CGTGCAATACTTGTGAAAATGGCTTCTTCAAGATGAACGGGGGCTGCTACTCCACCAGCCAGCTCCCGGGGTCGACGGTGTGCGTCACT
GCGCCGACCGGAGGAACATGCACCAAATCAGAGCCGGGCTACAATGTGAATAGCGGCACCCTTGTAACCTGTGGTGTTGGCTGCGCTGA
GTGTACCAACTCTGATTCATGTACTACATGTGCATCTGGATATGTCAAGCTCACTAGTGCTGCCACTTGCACAAAGTGCGACGCTGGGT
GTGCCACGTGTACAACTGCTGCCTCAACCTGCAGCACCTGCGCCGACGGGTACTACCTCTCTAACAGCAAGTGCATCGCGTGCGACAAA
AGCGATGGCAGCATCGCCGGCGTCAAAGACTGCCTGAGCTGCGCGGCTCCATCTGGCAGCACTGGCCCTGTCCTCTGCTACCTCGTGAG
GGACAGCGCCTCGGTCAACAAGGGCGGCCTCAGCAGCGGGGCGATCGCGGGGATCTCCGTCGCAGTAATAGTCGTTGTCGGGGGCCTCG
TCGGCTTCCTCTGCTGGTGGTTCATCTGCAGGGGCAAGGCGTGA

Tamaño: 1824 pb

11.6. Producto secuenciado gen *vsp-5g8*.

>*vsp-5g8* Secuenciado
ATGTTGAATAAATTTGCCCTTGTAGCCGTAATTCTCCAGATCGCCCGGGCCGCCTGCACGCCAGGCACTGAAACTACTAACTGCAAGGA
TGGCGCATGCAACGTCCAGATTGGCGGTGAGACATACTGCTCGCAGTGCTACACAACAAGCGAAGCACCAGTTGATGGGGTATGCACAG
CAAGTACTGATTCTAAATGCACAAAACAGGATACCCAAAATGGCACTTGTAAATCCTGCGCAGCGAACTACTTCCTCTTTAAGGGAGGG
TGCTATCAAATCGGACAATCTCCCGGTAGTTTGATTTGTCAAACTGCAAGCAACACCGATGGAATCTGTCAAACTTGTAAGGATGGCTA
CTTCACGGTTTCAGACGCTACTGCTACTCAGGACTCCTGTGTTGCCTGCGGGGACGAGAACTGCGCAACGTGCACAGTAGGAGCAGAAC
AGCAGAAGTGCAGCAAGTGCAAGGCCGACGGCAAGATGTACCTGAAGAAGAATACTGGTAGTGAGACAGGCACCTGCGTCACTGCCGAC
GAGTGCACAGCAGCCAAGGATTATTACACAGACGATACCAGCAGTGAACCAAATGGTAAGACGTGCAAGGCGTGCAGCGCAAAGGTGGA
GAACTGTGCTTCCTGCAGCAGTGAAGGAGCCTGCCAGAAGTGCGCCTCTGGCTTTGTATTGGAGGGATCGAACTGCGTCAAGAGCGATT
GCAGTACCGAGAACTGCAAGACGTGCACCAACCCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCACCC**CG**
ACCGGCCAGTGCATAGACGAGTGCGTAACTATCTCCGGCTACTACGGAACAGCTGATGGCAAGTGCAAGAAATGCGAAGTTGCTAACTG
CGTCGTCTGCGGGGCAACTGGAGCGTGCGATCTTTGCGCTGACGGCTTCTACGGTTCGAGCTGCTCTAAGTGCCACGAGAGCTGCAAGA
ATTGCAAAGGAGCTACTGCCAGCGACTGCACAGCGTGCCCTGCTGGGAGGGCGCTCATCTACGGAGATGAT**CCCACC**AAGGGCACGTGC
GGAGAGGGTTGCACGACAGGCACAGGGAAAGGTGCATGTAAGGCATGCGATTTAACGATCGACGGGACGAAGTACTGCTCTGCCTGTGA
CACGGCTACTGAATATCCGCAGAACGGCGTGTGTGCGACGCCGACTGCACGTGCCTCCTCGTGTCAGAGCCAAAATGTTGCTAGTGGTG
CGTGCAATACTTGTGAAAATGGCTTCTTCAAGATGAACGGGGGCTGCTACTCCACCAGCCAGCTCCCGGGGTCGACGGTGTGCGTCACT
GCGCCGACCGGAGGAACATGCACCAAATCAGAGCCGGGCTACAATGTGAATAGCGGCACCCTTGTAACCTGTGGTGTTGGCTGCGCTGA
GTGTACCAACTCTGATTCATGTACTACATGTGCATCTGGATATGTCAAGCTCACTAGTGCTGCCACTTGCACAAAGTGCGACGCTGGGT
GTGCCACGTGTACAACTGCTGCCTCAACCTGCAGCACCTGCGCCGACGGGTACTACCTCTCTAACAGCAAGTGCATCGCGTGCGACAAA
AGCGATGGCAGCATCGCCGGCGTCAAAGACTGCCTGAGCTGCGCGGCTCCATCTGGCAGCACTGGCCCTGTCCTCTGCTACCTCGTGAG
GGACAGCGCCTCGGTCAACAAGGGCGGCCTCAGCAGCGGGGCGATCGCGGGGATCTCCGTCGCAGTAATAGTCGTGTCGGGGGCCTCGT
CGGCTTCCTCTGCTGGTGGTTCATCTGCAGGGGCAAGGCGTGA

Anotaciones: Secuencia obtenida (negro), lugar de alineamiento de primers (subrayado).

11.6. Secuencia aminoacídica deducida parcial del gen *vsp-5g8*.

>VSP-5G8
MLNKFALVAVILQIARAACTPGTETTNCKDGACNVQIGGETYCSQCYTTSEAPVDGVCTASTDSKCTKQDTQNGTCKSCAANYFLFKGG
CYQIGQSPGSLICQTASNTDGICQTCKDGYFTVSDATATQDSCVACGDENCATCTVGAEQQKCSKCKADGKMYLKKNTGSETGTCVTAD
ECTAAKDYYTDDTSSEPNGKTCKACSAKVENCASCSSEGACQKCASGFVLEGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTP
TGQCIDECVTISGYYGTADGKCKKCEVANCVVCGATGACDLCADGFYGSSCSKCHESCKNCKGATASDCTACPAGRALIYGDDPTKGTC
GEGCTTGTGKGACKACDLTIDGTKYCSACDTATEYPQNGVCATPTARASSCQSQNVASGACNTCENGFFKMNGGCYSTSQLPGSTVCVT
APTGGTCTKSEPGYNVNSGTLVTCGVGCAECTNSDSCTTCASGYVKLTSAATCTKCDAGCATCTTAASTCSTCADGYYLSNSKCIACDK
SDGSIAGVKDCLSCAAPSGSTGPVLCYLVRDSASVNKGGLSSGAIAGISVAVIVVVGGLVGFLCWWFICRGKA

Anotaciones: Secuencia deducida de la secuencia nucleotídica obtenida de *vsp-5G8* (negro), diferencia con el constructo teórico (rojo).

ANEXO 12. Análisis de secuencias. Alineamientos

12.1. Alineamiento de proteínas parciales de 28 kDa y 38 kDa (BLASTp).

Score	Expect	Method	Identities	Positives	Gaps
80.1 bits(196)	1e-21	Compositional matrix adjust.	39/39(100%)	39/39(100%)	0/39(0%)

```
Query   229     EGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTP             267
                EGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTP
Sbjct   1       EGSNCVKSDCSTENCKTCTNPKAANEACTACVTGMFLTP             39
```

12.2. Alineamiento de la secuencias nucleotídica de la proteína parcial de 28 kDa con el Genoma de *Giardia lamblia* GS_B.

```
AHHH01000109 | organism=Giardia_Assemblage_B_isolate_GS_B | version=2013-11-25
| length=40569 | SO=contig
Length=40569
Score = 1472 bits (1632), Expect = 0.0 Identities = 816/816 (100%), Gaps = 0/816 (0%)
Strand = Plus/Plus

Query  1      ATGTTGAATAAATTTGCCCTTGTAGCCGTAATTCTCCAGATCGCCCGGGCCGCCTGCACG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38377  ATGTTGAATAAATTTGCCCTTGTAGCCGTAATTCTCCAGATCGCCCGGGCCGCCTGCACG  38436

Query  61     CCAGGCACTGAAACTACTAACTGCAAGGATGGCGCATGCAACGTCCAGATTGGCGGTGAG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38437  CCAGGCACTGAAACTACTAACTGCAAGGATGGCGCATGCAACGTCCAGATTGGCGGTGAG  38496

Query  121    ACATACTGCTCGCAGTGCTACACAACAAGCGAAGCACCAGTTGATGGGGTATGCACAGCA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38497  ACATACTGCTCGCAGTGCTACACAACAAGCGAAGCACCAGTTGATGGGGTATGCACAGCA  38556

Query  181    AGTACTGATTCTAAATGCACAAAACAGGATACCCAAAATGGCACTTGTAAATCCTGCGCA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38557  AGTACTGATTCTAAATGCACAAAACAGGATACCCAAAATGGCACTTGTAAATCCTGCGCA  38616

Query  241    GCGAACTACTTCCTCTTTAAGGGAGGGTGCTATCAAATCGGACAATCTCCCGGTAGTTTG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38617  GCGAACTACTTCCTCTTTAAGGGAGGGTGCTATCAAATCGGACAATCTCCCGGTAGTTTG  38676

Query  301    ATTTGTCAAACTGCAAGCAACACCGATGGAATCTGTCAAACTTGTAAGGATGGCTACTTC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38677  ATTTGTCAAACTGCAAGCAACACCGATGGAATCTGTCAAACTTGTAAGGATGGCTACTTC  38736

Query  361    ACGGTTTCAGACGCTACTGCTACTCAGGACTCCTGTGTTGCCTGCGGGGACGAGAACTGC  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38737  ACGGTTTCAGACGCTACTGCTACTCAGGACTCCTGTGTTGCCTGCGGGGACGAGAACTGC  38796

Query  421    GCAACGTGCACAGTAGGAGCAGAACAGCAGAAGTGCAGCAAGTGCAAGGCCGACGGCAAG  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38797  GCAACGTGCACAGTAGGAGCAGAACAGCAGAAGTGCAGCAAGTGCAAGGCCGACGGCAAG  38856

Query  481    ATGTACCTGAAGAAGAATACTGGTAGTGAGACAGGCACCTGCGTCACTGCCGACGAGTGC  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38857  ATGTACCTGAAGAAGAATACTGGTAGTGAGACAGGCACCTGCGTCACTGCCGACGAGTGC  38916

Query  541    ACAGCAGCCAAGGATTATTACACAGACGATACCAGCAGTGAACCAAATGGTAAGACGTGC  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38917  ACAGCAGCCAAGGATTATTACACAGACGATACCAGCAGTGAACCAAATGGTAAGACGTGC  38976

Query  601    AAGGCGTGCAGCGCAAAGGTGGAGAACTGTGCTTCCTGCAGCAGTGAAGGAGCCTGCCAG  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38977  AAGGCGTGCAGCGCAAAGGTGGAGAACTGTGCTTCCTGCAGCAGTGAAGGAGCCTGCCAG  39036
```

```
Query  661    AAGTGCGCCTCTGGCTTTGTATTGGAGGGATCGAACTGCGTCAAGAGCGATTGCAGTACC  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  39037  AAGTGCGCCTCTGGCTTTGTATTGGAGGGATCGAACTGCGTCAAGAGCGATTGCAGTACC  39096

Query  721    GAGAACTGCAAGACGTGCACCAACCCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTT  780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  39097  GAGAACTGCAAGACGTGCACCAACCCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTT  39156

Query  781    ACTGGCATGTTTCTCACCCCACCGGCCAGTGCATAG  816
              |||||||||||||||||||||||||||||||||||
Sbjct  39157  ACTGGCATGTTTCTCACCCCACCGGCCAGTGCATAG  39192
```

12.3. Alineamiento de proteína parcial de 38 kDa con el Genoma de *Giardia lamblia* GS_B.

```
> AHHH01000398 | organism=Giardia_Assemblage_B_isolate_GS_B | version=2013-1125
| length=3083 | SO=contig
Length=3083
Score = 2057 bits (2280), Expect = 0.0 Identities = 1140/1140 (100%), Gaps = 0/1140 (0%)
Strand = Plus/Plus

Query  1     GAGGGATCGAACTGCGTCAAGAGCGATTGCAGTACCGAGAACTGCAAGACGTGCACCAAC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2     GAGGGATCGAACTGCGTCAAGAGCGATTGCAGTACCGAGAACTGCAAGACGTGCACCAAC  61

Query  61    CCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCACCCCGACC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62    CCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCACCCCGACC  121

Query  121   GGCCAGTGCATAGACGAGTGCGTAACTATCTCCGGCTACTACGGAACAGCTGATGGCAAG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122   GGCCAGTGCATAGACGAGTGCGTAACTATCTCCGGCTACTACGGAACAGCTGATGGCAAG  181

Query  181   TGCAAGAAATGCGAAGTTGCTAACTGCGTCGTCTGCGGGGCAACTGGAGCGTGCGATCTT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182   TGCAAGAAATGCGAAGTTGCTAACTGCGTCGTCTGCGGGGCAACTGGAGCGTGCGATCTT  241

Query  241    TGCGCTGACGGCTTCTACGGTTCGAGCTGCTCTAAGTGCTACGAGAGCTGCAAGAATTGC  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242   TGCGCTGACGGCTTCTACGGTTCGAGCTGCTCTAAGTGCTACGAGAGCTGCAAGAATTGC  301

Query  301   AAAGGAGCTACTGCCAGCGACTGCACAGCGTGCCCTGCTGGGAGGGCGCTCATCTACGGA  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302   AAAGGAGCTACTGCCAGCGACTGCACAGCGTGCCCTGCTGGGAGGGCGCTCATCTACGGA  361

Query  361   GATGATCCCACCAAGGGCACGTGCGGAGAGGGTTGCACGACAGGCACAGGGAAAGGTGCA  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362   GATGATCCCACCAAGGGCACGTGCGGAGAGGGTTGCACGACAGGCACAGGGAAAGGTGCA  421

Query  421   TGTAAGGCATGCGATTTAACGATCGACGGGACGAAGTACTGCTCTGCCTGTGACACGGCT  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422   TGTAAGGCATGCGATTTAACGATCGACGGGACGAAGTACTGCTCTGCCTGTGACACGGCT  481

Query  481   ACTGAATATCCGCAGAACGGCGTGTGTGCGACGCCGACTGCACGTGCCTCCTCGTGTCAG  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482   ACTGAATATCCGCAGAACGGCGTGTGTGCGACGCCGACTGCACGTGCCTCCTCGTGTCAG  541

Query  541   AGCCAAAATGTTGCTAGTGGTGCGTGCAATACTTGTGAAAATGGCTTCTTCAAGATGAAC  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542   AGCCAAAATGTTGCTAGTGGTGCGTGCAATACTTGTGAAAATGGCTTCTTCAAGATGAAC  601

Query  601   GGGGGCTGCTACTCCACCAGCCAGCTCCCGGGGTCGACGGTGTGCGTCACTGCGCCGACC  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602   GGGGGCTGCTACTCCACCAGCCAGCTCCCGGGGTCGACGGTGTGCGTCACTGCGCCGACC  661

Query  661   GGAGGAACATGCACCAAATCAGAGCCGGGCTACAATGTGAATAGCGGCACCCTTGTAACC  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662   GGAGGAACATGCACCAAATCAGAGCCGGGCTACAATGTGAATAGCGGCACCCTTGTAACC  721
```

```
Query  721   TGTGGTGTTGGCTGCGCTGAGTGTACCAACTCTGATTCATGTACTACATGTGCATCTGGA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722   TGTGGTGTTGGCTGCGCTGAGTGTACCAACTCTGATTCATGTACTACATGTGCATCTGGA  781

Query  781   TATGTCAAGCTCACTAGTGCTGCCACTTGCACAAAGTGCGACGCTGGGTGTGCCACGTGT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782   TATGTCAAGCTCACTAGTGCTGCCACTTGCACAAAGTGCGACGCTGGGTGTGCCACGTGT  841

Query  841   ACAACTGCTGCCTCAACCTGCAGCACCTGCGCCGACGGGTACTACCTCTCTAACAGCAAG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  842   ACAACTGCTGCCTCAACCTGCAGCACCTGCGCCGACGGGTACTACCTCTCTAACAGCAAG  901

Query  901   TGCATCGCGTGCGACAAAAGCGATGGCAGCATCGCCGGCGTCAAAGACTGCCTGAGCTGC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  902   TGCATCGCGTGCGACAAAAGCGATGGCAGCATCGCCGGCGTCAAAGACTGCCTGAGCTGC  961

Query  961   GCGGCTCCATCTGGCAGCACTGGCCCTGTCCTCTGCTACCTCGTGAGGGACAGCGCCTCG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  962   GCGGCTCCATCTGGCAGCACTGGCCCTGTCCTCTGCTACCTCGTGAGGGACAGCGCCTCG  1021

Query  1021  GTCAACAAGGGCGGCCTCAGCAGCGGGGCGATCGCGGGGATCTCCGTCGCAGTAATAGTC  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1022  GTCAACAAGGGCGGCCTCAGCAGCGGGGCGATCGCGGGGATCTCCGTCGCAGTAATAGTC  1081

Query  1081  GTTGTCGGGGGCCTCGTCGGCTTCCTCTGCTGGTGGTTCATCTGCAGGGGCAAGGCGTGA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1082  GTTGTCGGGGGCCTCGTCGGCTTCCTCTGCTGGTGGTTCATCTGCAGGGGCAAGGCGTGA  1141
```

AHHH01000109 | organism=Giardia_Assemblage_B_isolate_GS_B | version=2013-11-25 | length=40569 | SO=contig
Length=40569
Score = 1855 bits (2056), Expect = 0.0 Identities = 1096/1140 (96%), Gaps = 1/1140 (0%)
Strand = Plus/Plus

```
Query  1      GAGGGATCGAACTGCGTCAAGAGCGATTGCAGTACCGAGAACTGCAAGACGTGCACCAAC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  39061  GAGGGATCGAACTGCGTCAAGAGCGATTGCAGTACCGAGAACTGCAAGACGTGCACCAAC  39120

Query  61     CCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCACCCCGACC  120
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  39121  CCGAAGGCAGCCAACGAGGCCTGCACAGCGTGTGTTACTGGCATGTTTCTCACCCC-ACC  39179

Query  121    GGCCAGTGCATAGACGAGTGCGTAACTATCTCCGGCTACTACGGAACAGCTGATGGCAAG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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14.5. Alineamiento del constructo teórico 61 kDa (5G8) con Genoma de *Giardia lamblia* GS_B.

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> AHHH01000109 | organism=Giardia_Assemblage_B_isolate_GS_B | version=2013-11-25
| length=40569 | SO=contig
Length=40569
Score = 3088 bits (3424),  Expect = 0.0 Identities = 1780/1824 (98%), Gaps = 1/1824 (0%)
Strand = Plus/Plus

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Query  1801   TTCATCTGCAGGGGCAAGGCGTGA  1824
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Sbjct  40176  TTCGTCTGCCGCGGAAAGGCGTGA  40199
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ANEXO A. Medios de cultivo, buffers y soluciones

1.1 Medio Luria Bertani (LB) para cultivo de bacterias.

Medio liquido

Componentes	Cantidad para 1L
Triptona	10 g
Extracto de levadura	5 g
NaCl	10 g

Medio sólido

Componentes	Cantidad para 1L
Triptona	10 g
Extracto de levadura	5 g
NaCl	10 g
Agar	15 g

Una vez disueltos los componentes en polvo, aforar a 1L con agua ultrapura y esterilizar por autoclave (20 min 15 psi en ciclo líquido). Una vez temperado, almacenar en refrigeración 4 °C hasta su uso. En caso de requerir la adición de un antibiótico deberá añadirse hasta temperarse el medio correspondiente, en el caso del medio sólido será añadida antes de dispensar en placas petri.

1.2 Medio Terrific Broth (TB) para cultivo de bacterias.

Medio liquido

Componentes	Cantidad para 1L
Triptona	12 g
Extracto de levadura	24 g
Glicerol	4 mL

Una vez disueltos los componentes, aforar a 900 mL con agua ultrapura y esterilizar por autoclave (20 min 15 psi en ciclo líquido). Dejar enfriar el medio a 60 °C o menos y añadir justo antes de usar 100mL de una solución reguladora estéril.

Solución reguladora

Componentes	Cantidad para 100 mL
KH_2PO_4	2. 31 g (0.17 M)
K_2HPO_4	12. 54 g (0.72 M)

Una vez disueltas las sales ajustar el volumen a 100 mL con agua ultrapura y esterilizar por autoclave (20 min 15 psi en ciclo líquido).

1.3 Medio TYI-S-33 para cultivo de *Giardia*.

Componentes	Cantidad para 1L
Tripticasa	20 g
Extracto de Levadura	10 g
Dextrosa	10 g
NaCl	2 g
L-Cisteína	2 g
Fosfato de sodio dibásico	1 g
Bilis	0.6 g
Ácido ascórbico	0.1 g
Citrato férrico amoniacal	0.023 g
Suero NBCS	10% (v/v)
Ceftriaxona	100 μg/mL

Una vez disueltos los componentes en polvo, ajustar el pH a 6.9 con NaOH. Añadir el antibiótico y suero de ternera recién nacida (NBCS) en esterilidad. Aforar al volumen final de 1L con agua ultrapura y esterilizar por filtración (0.45 μm). Almacenar en refrigeración 4 °C hasta su uso.

1.4 Medio Mínimo Esencial Modificado por Dulbecco (DMEM) para cultivo de células eucariotas.

Componentes	Cantidad para 1L
DMEM	13. 37 g
L-Arginina	0.116 g
L-Asparagina anhidrido	0.036 g
$NaHCO_3$	2 g
Piruvato de Sodio	10 mL
L-Glutamina	7.5 mL
Penicilina	10 mL
HEPES	2.38 g
β-Mercaptoetanol	3.7 g
SFB (D5F)	5% (v/v)
SFB (D10F)	10% (v/v)

Una vez disueltos los componentes en polvo, se ajustó el pH de 7.2 - 7.4. Se añadieron los componentes líquidos en esterilidad. Se aforó a 1L con agua ultrapura y se esterilizó por filtración (0.45 μm). Se almacenó en refrigeración 4 °C hasta su uso.

1.5. Buffer de fosfatos (PBS) 10x

NaH2PO4	1.9 g
Na2HPO4	12.0 g
NaCl	85.0 g

Disolver en 800mL de agua deionizada y ajustar pH a 7.2, aforar a 1000mL con agua deionizada y esterilizar por autoclave.

1.6. Buffer TBE (10x) para electroforesis de DNA

Trisma base	54 g
Acido bórico	27.5
EDTA 0.5M pH=8.0	20 mL

1.6. Acrilamida/Bisacrilamida 30 %

Acrilamida	30 g
N,N-metilen-bisacrilamida	0.8 g

Mezclar en 100 mL de agua ultrapura. Filtrar la solución con un filtro de 0.45 µm . Almacenar en frasco color ambar a 4 °C. Descartar el reactivo después de 30 días para evitar la hidrólisis a ácido acrílico y amoníaco.
NOTA: La acrilamida monomérica en neurotóxica por lo cual se debe usar mascarilla y guantes durante su preparación.

1.7. Trizma-base/SDS, pH 6.8 4X (Trizma-base 0.5M conteniendo SDS al 0.4 %)

Trizma-base	30 g
Agua ultrapura	40 mL

Ajustar el pH a 6.8 con con HCl 1N, aforar a 100 mL. Filtrar con un filtro de 0.45 µm de diámetro de poro y adicionar 0.4 g de SDS. Almacenar a 4 °C.

1.8. Trizma-base/SDS, pH 8.8 4X (Trizma-base 1.5M conteniendo SDS al 0.4 %)

Trizma-base	91 g
Agua ultrapura	100 mL

Ajustar el pH a 8.8 con HCl 1N, aforar a 500 mL. Filtrar con un filtro de 0.45 µm de diámetro de poro y adicionar 2 g de SDS. Almacenar a 4 °C.

1.9. Persulfato de amonio (PSA) al 10 %

$(NH_4)_2S_2O_8$	10 µg
Agua ultrapura	100 µL

Buffer de corrida 1X, pH 8.3

Glicina	72 µg
Trizma-base	15.1 g
SDS	5 g

Disolver el agua ultrapura y aforar a 1 L. para obtener la solución de trabajo 1X diluir 1:5 la solución stock con agua ultrapura y ajustar el pH a 8.3. Almacenar a 4 °C.

1.10 Solución reguladora de muestra (“buffer” muestra) 2X.
En un pequeño volumen de agua ultrapura disolver 0.38 g de Trizma-base. Ajustar pH a 6.8 y adicionar los siguientes reactivos en campana ventilada:

SDS	1 g (0.05 %)
Glicerol	5 mL (20 %)
2-Mercaptoestanol	2.5 mL (0.05 %)
Azul de bromofenol	0.5 mg

Aforar a 25 mL con agua ultrapura y almacenar en congelación.

1.11. Solución Azul de Coomassie para tinción de geles de poliacrilamida

Metanol	50 % (v/v)
Azul de Coomassie brillante R-250	0.05 % (v/v)
Ácido acético	10 % (v/v)
Agua ultrapura	40 % (v/v)

1. 12. Solución Desteñidora I

Metanol	50 % (v/v)
Ácido acético	10 % (v/v)
Agua ultrapura	40 % (v/v)

1. 13. Solución Desteñidora II

Metanol	5 % (v/v)
Ácido acético	7 % (v/v)
Agua ultrapura	88 % (v/v)

1. 14. Buffer de lisis para aislamiento de esplenocitos

NH_4Cl	150 mM (4.02 g)
$KHCO_3$	10 mM (0.1 g)
$Na_2EDTA \cdot 2\ H_2O$	0.1 mM (18.6 mg)

Se puede sustituir por 100 µL de EDTA 0.5 M, pH 8. Aforar a 500 mL con agua MiliQ y ajustar pH entre 7.2 y 7.4. Esterilizar por filtración. Almacenar a 4 °C, Descartar después de un mes.

1. 15. Buffers de lisis y elución para purificación de proteína BIP:

Buffer de lisis

Fosfato de sodio (NaH_2PO_4)	50 mM
NaCl	300 mM
Imidazol	5 mM

NOTA: dependiendo de la proteína, el imidazol puede empezar desde 5mM

Buffer base

Fosfato de sodio dibásico	20 mM
NaCl	0.5 M

Depende de la solución agregar la cantidad necesaria para 30 mM, 50 mM, 80 mM, 100 mM, 180 mM y 500 mM de imidazol. Todas a pH= 7.4.

1. 16. Solución reguladora de citratos para ELISA

Ácido cítrico	3.2 g
KH_2PO_4	3.2 g

Adicionar 200 mL de agua desionizada, ajustar pH a 4.2 con NaOH. Aforar a 250 mL con agua desionizada

1. 17. Solución reguladora de carbonatos para ELISA

Na_2CO_3	0.795 g
$NaHCO_3$	1.465 g

Adicionar agua desionizada hasta un volumen de 400 mL, ajustar pH a 9.6. Aforar a 500 mL con agua desionizada.

1. 18. Solución de lavado para inmunoensayos

PBS 1X/Tween-20 (0.05 %). A 1 L de PBS 1X, adicionar 0.5 mL de Tween-20

1. 19. Solución de bloqueo para inmunoensayos

PBS 1X/BSA 1 %. Disolver 0.1 g de BSA en 10 mL de PBS 1X.

1. 20. Solución ABTS para ELISA

ABTS	0.5 mg/mL
Solución reguladora de citratos	250 mL

1. 21. Solución reveladora para ELISA

A 10 mL de ABTS adicionar 10 µL de H_2O_2 al 30 %

1.22. Soluciones para extracción de DNA plasmídico

Solución I: Glucosa 50 mM, Tris-Cl 25 mM (pH 8) y EDTA 10 mM (pH 8). Esterilizar por autoclave 15 min. y almacenar a 4 °C.

Concentración Final	Mezcla de reactivos
50 mM glucosa	0.9 g de glucosa
Tris-HCl 25mM	2.5 mL de Tris-HCl 1M (pH 8)
EDTA 10 mM	5.0 mL de EDTA 0.2M
pH 8.0	Aforar a 100 mL con H_2O destilada

Solución II: Mezclar NaOH 0.4N (1.6 g/100 mL) y SDS 2% (2 g/100 mL) en proporción 1:1 (v/v). Esta solución se esteriliza en autoclave 15 min. y se almacena a temperatura ambiente.

Solución III: Mezclar 60 mL de acetato de potasio 5M (29.445g en 60 mL de H_20), 11.5 mL de ácido acético glacial y 28.5 mL de agua, pH final 4.8. Almacenar la solución a 4° C y transferir a hielo justamente antes de usarse.

ANEXO B. Protocolos

B1. Generación de bacterias calciocompetentes y transformación

1. Crecer un cultivo de *E. coli* a 37 °C con agitación vigorosa (190-200 rpm) en medio LB hasta alcanzar saturación (*overnight*).
2. Diluir el inóculo al 1% medio, crecer a 37 °C con agitación vigorosa (190-200 rpm) hasta alcanzar una OD_{600} de 0.5 a 0.6 (fase logarítmica tardía).
3. Incubar las bacterias durante 10 a 15 minutos en agua- hielo.
4. Centrifugar a 5000 rpm durante 3 minutos a 4°C.
5. Resuspender cuidadosamente el *pellet* en 100 ml de $CaCl_2$ 100mM.
6. Incubar la suspensión durante 30 minutos en agua- hielo.
7. Centrifugar a 3000 rpm durante 5 minutos a 4°C.
8. Resuspender cuidadosamente el *pellet* en 1 ml de solución de $CaCl_2$ 100mM, y 30% de glicerol (solo si se van a congelar).
9. Fraccionar en alícuotas de 50 a 100 µl en tubos *Eppendorf*.
10. Conservar a -80°C hasta su utilización.

Transformación de bacterias

1. Tomar 50 µl de bacterias competentes e incubarlas en baño de agua- hielo durante 10 minutos.
2. Agregar el plásmido a transformar (entre 5 y 20 ng).
3. Incubar 30 minutos en agua- hielo.
4. Shock termico: 42°C 90 segundos. Agitar levemente.
5. Colocar durante 10 minutos en agua- hielo.
6. Agregar 1mL de medio LB sin antibiótico e incubar a 37°C (preferentemente con agitación) durante 1h.
7. Sembrar en placa con medio agar LB y antibiótico 50 µl de la suspensión bacteriana e incubar a 37°C de 12 a 16 horas observar crecimiento de colonias.
8. Seleccionar algunas clonas y crecer en medio liquido LB con antibiótico.
9. Realizar extracción de DNA plasmídico para caracterizar.

B2. Extracción de DNA plasmídico. Minipreps

1. Crecer colonias en tubos con 4 mL de medio selectivo (*LB* con antibiótico), a 37°C y en agitación.
2. Empastillar 3 mL de muestra en un tubo de 2 mL por centrifugación a 10,000 r.p.m. durante 5 minutos.
3. Resuspender bien en 100 µL de solución I fría con vortex vigoroso.
4. Agregar 200 µL de solución II (recién preparada), mezclar por inversión 5 veces (No vortex) y posteriormente incubar de 5 a 7 minutos en hielo.
5. Agregar 150 µL de solución III fría, mezclar y posteriormente incubar de 3 a 5 minutos en hielo.
6. Centrifugar 5 minutos a 14,000 r.p.m. a 4°C y transferir sobrenadante a un tubo nuevo.
7. Agregar ½ volúmen de fenol saturado y ½ volúmen de SEVAG, mezclar vigorosamente por vortex. Centrifugar durante 2 minutos a 14,000 r.p.m. a 4ºC. Extraer la fase acuosa (superior) y transferir a un tubo nuevo (paso opcional).

8. Agregar 2 volúmenes de etanol al 100% (frío), mezcle con vortex, deje reposar 2 minutos a T.A., centrifugue durante 5 min. a 14,000 r.p.m. a 4°C y decante el sobrenadante.
9. Agregar 1 mL de etanol al 70 % (frío), agitar suavemente por inversión para lavar.
10. Recobrar el DNA por centrifugación durante 5 min. a 14,000 r.p.m. a 4ºC.
11. Decantar *(tener cuidado de no perder la pastilla, a veces ésta no se adhiere fuertemente)*, dar un "spin" y eliminar el remanente con una micropipeta.
12. Almacenar el tubo abierto a T.A. hasta que el etanol se evapore (5-10 minutos).
13. Agregar 50 μL de TE 1X con RNAsa pancreática (20 μg/mL) libre de DNAsa.
14. Mezclar gentilmente, con vortex, la solución por algunos segundos y dejar incubando a 37°C toda la noche. Pegar una etiqueta con la información requerida y almacenar a -20°C.

B3. Sobreexpresión de BIP recombinante (*E. coli Rosetta gami*)

1. Se cultivó un pre-inoculo de cada clona durante toda la noche en 5mL de medio LB con ampicilina (100 μg/mL) en agitación a 220 rpm.
2. Posteriormente se tomo una alícuota de cada pre-inóculo y se hizo una dilución de 1:50 en un volumen final de 50 mL de medio LB con ampicilina, los cultivos se incubaron en agitación a 220 rpm y 37°C hasta que alcanzaran una DO_{600} de 0.6.
3. Una vez alcanzada la densidad óptica, se tomó una alícuota de 1mL (t0, sin inducción) y se centrifugó a 3000g durante 20 min a 4 °C, se conservó el botón celular y se congeló a -20 °C hasta su uso.
4. Se añadió el inductor IPTG a una concentración de 1mM a cada cultivo y se incubó en agitación 220 rpm durante 6 horas y a 37°C.
5. Los cultivos se centrifugaron a 3000g durante 20 min a 4 °C, se conservó el botón celular. Estas se resuspendieron en una cantidad de buffer de lisis proporcional al tamaño del botón celular. (1 μg de pellet -- 4 μL buffer de lisis).

Ensayo de solubilidad de proteínas sobre-expresadas

1. Se cultivó un pre-inoculo de cada clona durante toda la noche en 5mL de medio LB con ampicilina (100 μg/mL) en agitación a 220 rpm.
2. Posteriormente se tomo una alícuota de cada pre-inóculo y se hizo una dilución de 1:100 en un volumen final de 1L de medio LB con ampicilina, los cultivos se incubaron en agitación a 220 rpm y 37°C hasta que alcanzaran una DO_{600} de 0.6.
3. Una vez alcanzada la densidad óptica, se tomó una alícuota de 10 mL (t0, sin inducción) y se centrifugó a 3000g durante 20 min a 4 °C, se conservó el botón celular y se congeló a -20 °C hasta su uso.
4. Se añadió el inductor IPTG a una concentración de 1mM a cada cultivo y se incubó en agitación 220 rpm durante 6 horas y a 37°C.
5. Los cultivos se centrifugaron a 3000g durante 20 min a 4 °C, se conservó el botón celular.
6. Se llevo a cabo la lisis celular enzimática con lisozima (2μg/mL), se incubo durante 30 minutos en hielo.
7. Posteriormente se reallizó lisis mecánica sonicando (sonicador con punta) seis ciclos de 1 min cada uno e incubando en hielo entre cada ciclo.

8. Las fracciones se separaron centrifugando a 3000g 30 min a 4°C.
9. Los botones celulares fueron lavados dos veces con PBS (10mL) y entre cada lavado se resuspendió el botón celular y se centrifugaron a 3000g 30 min a 4°C.
10. Los lisados celulares se congelaron a -20º C hasta su uso.
11. Posteriormente se resuspendieron en una cantidad de buffer de lisis proporcional al tamaño del botón celular. (1 μg de pellet -- 4 μL buffer de lisis).

Conservar en hielo mientras se prepara la columna cromatografía. Una vez resuspendido el lisado, usar inmediatamente.

B4. Purificación de la proteína BIPr

1. Pesar la pastilla de bacterias
2. Resuspenderla en solución de unión con imidazol (30 mM). Por cada 1 mg de pastilla, 3 μL de solución de unión, o por cada 1 μL, 4 μL de solución de unión.
3. Agregar inhibidor de proteasas [4-(2-amonoetil) fluoruro benceno sulfonilo (AEBSF)] 23 mM, pepstatina A 0.3 mM, E-64 0.3 mM, bestatina 2 mM, y EDTA sódico 100 mM (Sigma, St. Louis, MO, USA). (Tener cuidado con las concentraciones de EDTA, teniendo en cuenta la estabilidad de la columna usada). Por cada 4 g de pastilla, agregar 1 mL de inhibidor ya reconstituido como lo indica el fabricante.
4. Agregar lisozima (L6876, Sigma, St. Louis, MO, USA) a una concentración final 2 mg/mL.
5. Agitar la solución en hielo durante 30 minutos.
6. Sonicar por 6 ciclos de 10 segundos, con lapsos de 10 segundos entre ciclo, con una amplitud de 20 %.
7. Centrifugar durante 30 minutos a 5000 g (recomendable 10 000 g) a 4 °C.
8. Filtrar el sobrenadante al vacío mediante un filtro de 0.45 μm.
9. Hacer pasar por la columna pre empaquetada His trap HP a un flujo entre 0.25 a 1 mL/minuto. (Cuidar que no entren burbujas a la columna).
10. Comenzar con el proceso de elución con las diferentes soluciones de elución con imidazol (30 mM, 50 mM, 80 mM, 100 mM, 180 mM y 500 mM). 20 volúmenes de cada solución en un flujo de 1 mL/minuto. Recolectar fracciones de 1 mL.

B5. Electroforesis en geles de poliacrilamida en condiciones ligeramente desnaturalizantes y reductoras (SDS-PAGE)

1. Ensamblar los vidrios en el equipo para geles de 0.75 mm.
2. Preparar el gel de separación al 12 %:

Acrilamida/Bisacrilamida 30 %/ 8 %	6 mL
Buffer Tris 4X/SDS pH 8.8	3.75 mL
Agua ultrapura	5.25 mL
Persulfato de amonio	0.08 mL
TEMED	0.18 mL

3. Mezclar bien y colocar la solución en los vidrios, adicionar alcohol etílico al 70 % y dejar polimerizar a temperatura ambiente.
4. Preparar el gel concentrador al 12 %:

Acrilamida/Bisacrilamida 30 %/ 8 %	0.65 mL

Buffer Tris 4X/SDS pH 6.8	1.25 mL
Agua ultrapura	3.05 mL
Persulfato de amonio	0.07 mL
TEMED	0.01 mL

5. Mezclar bien y colocar la solución en los vidrios, colocar el peine para crear los pocillos y dejar polimerizar a temperatura ambiente.

B6. Generación de anticuerpos policlonales

1. Realizar una electroforesis de la proteína de interés, en un gel preparativo de 0.75 mm a 1.5 mm de grosor.
2. Someter el gel en tinción con Azul de Comassie y desteñir.
3. Cortar la banda del gel, y macerarla con PBS 1X.
4. Para la inmunización inicial, administrar 10 µg de proteína por vía intraperitoneal con ayuda de una jeringa (200 µL). El antígeno está en presencia de Adyuvante completo de Freunds (200 µL de antígeno + 200 µL de adyuvante, Mezclar con vórtex antes de administrar el antígeno). Para las inmunizaciones siguientes (5 a 6 semanas), repetir el procedimiento del punto anterior. Sustituir por Adyuvante Incompleto de Freunds.

Antes de cada inmunización, obtener muestras de suero:

1. Limpiar la punta de la cola de ratones con una torunda impregnada de etanol al 70 % y permitir secar.
2. Con una navaja de un filo, cortar la punta de la cola y recoger las gotas de sangre, aproximadamente 300 µL, en un microtubo eppendord.
3. Dejar reposar las muestras de sangre por 30 minutos y posteriormente centrifugarlas en una microcentrífuga (Labnet Spectrafuge 7M) a 1000 xg por 10 minutos.

B7. Ensayo Inmunoadsorbente Ligado a Enzima (ELISA) Indirecto

1. Adsorber a los pozos de la placa de ELISA, 0.25 µg de la proteína purificada en 50 µL de solución reguladora de carbonatos pH 9.6 por pozo.
2. Incubar por una hora a 37^{o}C o toda la noche a 4^{o}C.
3. Lavar cada pozo de la placa con PBS 1X-Tween 20 (0.05 %), 5 veces.
4. Llenar cada pozo con 50 µL de solución bloqueadora (PBS 1X-BSA 1 %), incubar una hora a temperatura ambiente y en constante rotación.
5. Lavar cada pozo de la placa con PBS 1X pH 7.2-Tween 20 (0.05 %), 5 veces.
6. Adicionar 50 µL del primer anticuerpo (suero de inmunización) en diluciones seriadas de 1:1000 hasta 1:20 000 e incubar una hora a temperatura ambiente y en constante rotación.
7. Lavar cada pozo de la placa con PBS 1X pH 7.2-Tween 20 (0.05 %), 5 veces.
8. Adicionar 50 µL del segundo anticuerpo (cabra anti-IgG de ratón conjugado a peroxidasa) diluido 1:1000 en PBS 1X, incubar una hora a temperatura ambiente y en constante rotación.
9. Lavar cada pozo de la placa con PBS 1X pH 7.2-Tween 20 (0.05 %), 5 veces.
10. Adicionar 50 µL de solución reveladora (ABTS + H_2O_2 al 30 %).

11. Incubar por 15 minutos a temperatura ambiente.
12. Medir la densidad óptica a 415 nm en el lector de microplaca.

B8. Electrotransferencia e inmunodetección (Western-Blotting)

1. Colocar el gel de poliacrilamida realizado durante la electroforesis, membrana de nitrocelulosa y dos cojinetes (BIO-RAD) en solución de transferencia 1X, pH 8.3 por 15 minutos.
2. Ordenar de la siguiente manera: cojinete (Extra thick blot paper Filter paper Bio-Rad Laboratories Inc, EEUU), membrana de nitrocelulosa de 0.45 μm (Bio-Rad Laboratories Inc, EEUU, gel de poliacrilamida y cojinete, eliminando la mayor cantidad de burbujas con la ayuda de un tubo de ensaye.
3. Electrotransferir en sistema semiseco (Semi-Dry Blotting System, IMM-1-A, Serie 11798) por 20 minutos a 15 V constantes.
4. Una vez terminada la transferencia la membrana se puede almacenar a -80 °C envuelta en papel aluminio.
5. Bloquear los sitios libres de la membrana con leche Svelty al 5 % en PBS-BSA 1% por 1 hora.
6. Lavar membranas con PBS para remover el exceso de solución de bloqueo.
7. Agregar primer anticuerpo y dejar incubarse por 1 hora en agitación constante.
8. Enjuagar las membranas con PBS 1X y realizar 5 lavados de 5 minutos en agitación constante con PBS 1X 0.05 % Tween 20.
9. Agregar el segundo anticuerpo (cabra anti-ratón anti-IgG peroxidasa conjugada) en una dilución 1:75000 con PBS 1X-BSA 0.01 % e incubar durante una hora en agitación constante, posteriormente enjuagar membrana suavemente y realizar 5 lavados como se mencionan en el paso 4.
10. Quitar el exceso de solución de lavado con PBS 1X y remover el exceso manteniendo húmeda la membrana.
11. Mezclar la solución de Luminol y peróxido de hidrogeno en dilución 1:2 (Super Signal, Best Pico, Chemiluminescent Substrat, prod 34080, Lot No. EB60813, Pierce, Thermo Scientific).
12. Cubrir la membrana de nitrocelulosa con la solución Luminol-peróxido e incubar por 10 minutos manteniendo la membrana húmeda.
13. Montar la membrana de nitrocelulosa en un casete de Revelado (Hypercassette, Amersham Biosciences, UK).
14. En oscuridad, exponer la membrana dentro del casete por 30 segundos a una película fotográfica (Fuji Medical X-RAY Film, 100NIF, FUJIFILM Corporation, Tokio, Japón).
15. Revelar reacción en oscuridad:
    a. Enjuagar la película fotográfica por 10 segundos en solución Reveladora (GBX Developer and Replenisher, Carestream Health Inc).
    b. Enjuagar la película fotográfica por 10 segundos en agua.
    c. Enjuagar la película fotográfica por 10 segundos en solución fijadora (GBX Fixer and Replenisher, Carestream Health Inc.).
    d. Enjuagar la película fotográfica por 10 segundos en agua.

B9. Cultivo de G *lamblia*

1. Adicionar aproximadamente 7.5 mL de medio TYI-S-33 suplementado l 10 % con NBCS en un tubo de ensayo 13 x 100 estéril.
2. Adicionar 0.5 mL de cultivo de *G. lamblia* en confluencia ($1x10^6$ trozoítos/mL)
3. Realizar cambio de medio o subcultivos en condiciones completamente estériles, en un campana de flujo laminar Bioseguridad II.

B10. Obtención de extracto de proteínas solubles de *G. lamblia*

1. Colocar en agua hielo (10 minutos) los tubos que contengan los cultivos de trozoítos de *G. lamblia.*
2. Centrifugar a 800 g por 10 minutos a 4 °C (Fisher Scientific, MARATON 3000 R) y lavar 3 veces con PBS estéril pH 7.2.
3. Resuspender en 1.4 mL de PBS pH 7.2.
4. Obtener la concentración celular por un hematocitometro (dilución1:200) y una dilución 1:2 con azul de tripano para en conteo.
5. Adicionar 5 µL de inhibidor de proteasas (Sigma,St. Louis, MO,USA) y lisar las células sometiendo el microtubo a 3 ciclos de congelación y descongelación (congelador -80 °C. Thermo Fisher Scientific modelo 703) hasta que la suspensión esté completamente congelada (aproximadamente 45 minutos).
6. Retirar del congelador y dejar descongelar a temperatura ambiente (aproximadamente 15 minutos).
7. Sonicar por 3 ciclos de 20 segundos a una amplitud de 10 % (Brandon Sonifier 250, Shelton, CT, USA) manteniendo el microtubo con el lisado de trofozoítos frío durante el proceso.
8. Centrifugar a 10 000 g a 4 °C por 20 minutos, recuperar el sobrenadante el cual contiene la mezcla heterogénea de proteínas solubles de *G. lamblia*. Almacenar a -80 °C hasta su uso.

B11. Infección de Animales de Experimentación

1. Someter a los ratones a infectar a 6 o 9 horas de ayuno, con el fin de favorecer la infección.
2. Mantener el cultivo de trofozoítos de *G. lamblia* por 20 minutos en agua-hielo para liberar las células adheridas a la pared del tubo.
3. Lavar los trofozoítos 3 veces en PBS 1X, usando centrífuga (Thermo IEC Marathon 3000R, Fisher Scientific) a 1800 rpm por 5 minutos a 4^oC.
4. Disolver pastilla en 1.4 mL de PBS 1X frío y estéril.
5. Para su cuantificación en hemacitómetro, tomar una alícuota y hacer dilución adecuada con PBS 1X y azul de tripano.
6. Después del último lavado, resuspender la pastilla de trofozoítos en PBS 1X y ajustar la concentración a $5x10^6$ de trofozoítos en 200 µL de PBS 1X.
7. Con ayuda de una jeringa para alimentación forzada, inocular por vía oral, 200 µL de la suspensión de trofozoítos a temperatura ambiente.

Nota: mantener trofozoítos a 4^oC durante toda su manipulación y antes de inocular.

B12. Tinción Superficial e Intracelular de Trofozoítos de *G. lamblia*

Tinción Intracelular:

1. Fijar trofozoítos de G. lamblia por 30 minutos con 1 % de paraformaldehído.
2. Lavar (2 veces) con PBS frío, los trofozoítos se permeabilizaron por 5 minutos con 0.1 % de Triton X-100 a temperatura ambiente.

Tinción Superficial:

1. Para la tinción superficial los trofozoítos fueron no permeabilizados.
2. Lavar (2 veces) con PBS frío.
3. Incubar a 4 °C por 1 hora con el AcMo 5G8.B5 (1 µg/mL) o con el control de isotipo aBDC.2 (IgG2b, 1 µg/mL).
4. Lavar 2 veces con PBS frío.
5. Incubar los trofozoítos por 1 hora a 4 °C con el anticuerpo anti-ratón IgG de cabra conjugado con FITC (diluido a 1:200 con 0.05 % de NaN3-DMEM).
6. Lavar 2 veces con PBS frío.
7. Analizar la expresión de la proteína 5G8 por medio de un citómetro de flujo Becton Dickinson (Canto II FACS, CA, USA).

B13. Evaluación de la respuesta inmune celular por MTT

Aislamiento de esplenocitos con solución de lisis

1. Utilizar material y reactivos estériles
2. En una placa de Petri con 5 mL de medio de cultivo, macerar el tejido con el pie de una jeringa
3. Filtrar la suspensión celular por organza en un tubo de 50 mL
4. Lavar la placa con 15 mL más de medio de cultivo y filtrarlo por organza en el mismo tubo
5. Centrifugar a 1500 rpm (500 xg) por 7 minutos a 4 °C
6. Descartar el sobrenadante, no decantar. Utilizar una pipeta
7. Resuspender suavemente el botón celular en aproximadamente 500 µL de PBS,
8. Agregar solución de lisis (ACK) a temperatura ambiente (25 °C) 5 mL/bazo
9. Agitar en Vortex® a potencia baja (4 o 5) por 2 segundos
10. Detener reacción de lisis a los 2 minutos después de agregar ACK con DMEM frio llevando a un volumen de 25 mL
11. Centrifugar a 1500 rpm (500 xg) por 7 minutos a 4 °C
12. Descartar el sobrenadante, no decantar. Utilizar una pipeta
13. Resuspender el botón celular en DMEM frio. Utilizar un volumen pequeño (1 mL aproximadamente) para desprender el botón celular y después llevar a un volumen de 25 mL
14. Filtrar por organza
15. Centrifugar a 1500 rpm (500 xg) por 7 minutos a 4 °C
16. Descartar el sobrenadante, no decantar. Utilizar una pipeta
17. Resuspender en 5 mL de D10F frio y contar células en cámara de Neubauer

Ajustar células a la concentración deseada para el experimento en D10F.

Ensayo de reducción del Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT)

1. Agregar 15 μL de MTT 5 mg/mL a cada pozo (150 μL cultivo)(10 μL MTT por cada 100 μL de cultivo celular en el pozo).
2. Incubar a 37 °C por 4 horas.
3. Agregar 150 μL de Isopropanol-HCl.
4. Disolver completamente los cristales de formazán.
5. Leer en lector de microplaca a 530 nm y 630 de referencia.

B13. Extracción de ácidos nucléicos de *Giardia*

Se emplearon cultivos al 80% de confluencia ($10x10^6$ trofozoitos). El DNA se extrajo con el kit QIAmp® DNA Mini Kit (Qiagen) y las muestras de RNA usando RNeasy® Mini Kit (Qiagen).

Extracción de DNA

1. Colocar en un tubo 1.4 mL del amortiguador de lisis (ASL).
2. Adicionar 200 μL de suspensión celular.
3. Aplicar 3-5 cambios de temperatura de 80-96 °C.
4. Mezclar en vortex por 1 min, centrifugar 16000 g/ 1 min T.A.
5. Pasar 1.5 mL del sobrenadante a otro tubo, agregar 15 μL de proteinasa K
6. Adicionar 200 μL del sobrenadante del paso anterior y agregar 200 μL del amortiguador AL, mezclar en vortex 15 seg.
7. Calentar a 70 °C 10 min y centrifugar 1 min a 16000 g y posteriormente adicionar 200 μL de etanol absoluto, mezclar en vortex y centrifugar 1 min a 16000 g.
8. Pasar sobrenadante a una columna QIAmp spin y centrifugar 1 min a 16000 g.
9. Cambiar la columna a otro tubo de colección y adicionar a la columna 500 μL del amortiguador AW1, centrifugar 1 min a 16000 g.
10. Cambiar la columna a otro tubo de colección y adicionar a la columna 500 μL de AW2, centrifugar 3 min a 16000 g.
11. Cambiar la columna a otro tubo y adicionar 200 μL del amortiguador de elución AE incubar 1 min a T.A. centrifugar 1 min a 16000g para eliminar la columna y guardar el DNA a -20 °C.

Extracción de RNA (Trizol reagent)

1. Adicionar 0.75 mL de trizol por cada 5-10 $x10^6$ células en 250 μL
2. Incubar la muestra homogenizada por 5 min. A T.A.
3. Añadir 0.2 mL de cloroformo por 1 mL de trizol,
4. Incubar 2-3 min a T.A.
5. Centrifugar a 12 000g 15 min a 4 °C.
6. Remover fase acuosa y pasar a otro tubo.
7. Añadir 0.5 mL de isopropanol al 10% a la fase acuosa por cada 1 ml de trizol.

8. Incubar a T.A. por 10 min.
9. Centrifugar a 12 000 g durante 10 min a 4 °C
10. Remover sobrenadante y lavar el botón de RNA con 1 mL de etanol abs.
11. Agitar por vortex cuidadosamente y centrifugar a 7500 g 5 min °C
12. Secar el botón de RNA por 30 min tubo abierto
13. Resuspender en 30 μL de agua -DEPC.

Se evaluó la pureza, calidad y concentración de las muestras por espectrofotometría en Nanodrop 2000. Las muestras de RNA se guardaron a -80 °C hasta su uso. Para generar cDNA, se empleó el kit QuantiTect® Reverse Transcription Kit (Qiagen).

B14. Amplificación del gen *vsp-5g8* por PCR

Se empleó el kit de Qiagen Taq DNA polimerasa.
Descongelar los reactivos a utilizar y mantener en hielo.
Preparar mezcla de reacción según la tabla siguiente.

Tabla B14.1. Mezcla de reacción

Componente	Volumen recomendado	Concentración final recomendada	Concentración final empleada
10x PCR Buffer	10 μL	1x	1x
dNTP (10 mM)	2 μL	200 μM	200 μM
Primer A	Variable	0.1-0.5 μM	0.25 μM
Primer B	Variable	0.1- 0.5 μM	0.25 μM
Taq DNA pol	0.5 μL	2.5 unidades / rx	2.5 unidades / rx
Agua	Variable	--	--
Buffer Q 5x	20 μL	1x	1x
DNA	Variable	≤ 1μg/reacción	90 -100ng/rx
Vf	100 μL		

Tabla B14.2. Condiciones de amplificación

Paso	Tiempo	Temperatura	Comentario
Desnaturalización inicial	3 min	94 °C	
Desnaturalización	30 seg	94 °C	
Alineamiento	30 sec	58.2 °C	
Extensión	2 min	72 °C	1 min / 1 kb
Numero de ciclos	35		
Extensión final	10 min	72 °C	