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Saponinas de *Quillaja brasiliensis*: potencial imunoadjuvante e mecanismos celulares e moleculares de ação.

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Porto Alegre, setembro de 2015.

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Orientador: Prof. Dr. Paulo Michel Roehe

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Samuel Paulo Cibulski

SAPONINAS DE *QUILLAJA BRASILIENSIS*: POTENCIAL IMUNOADJUVANTE E MECANISMOS CELULARES E MOLECULARES DE AÇÃO.

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"A ciência, meu rapaz, é feita de erros, mas de erros benéficos, já que conduzem pouco a pouco à verdade".

Júlio Verne

Saponinas de *Quillaja brasiliensis*: potencial imunoadjuvante, mecanismos celulares e moleculares de ação.

RESUMO

A formulação de vacinas efetivas frequentemente requer a adição de adjuvantes capazes de otimizar as respostas imunes humoral e celular. Com o objetivo principal de contribuir para o desenvolvimento de novos adjuvantes, este trabalho foi desenvolvido buscando aprofundar o conhecimento do mecanismo de ação imunoadjuvante de preparações de saponinas de *Quillaja brasiliensis* e suas formulações em complexos imunoestimulantes do tipo ISCOM. Como a toxicidade das saponinas é um fator crítico para seu uso em preparações vacinais, inicialmente foram realizados ensaios visando comparar a toxicidade *in vitro* e *in vivo* de saponinas extraídas de *Quillaja brasiliensis* com saponinas de ação imunoestimulante reconhecidas, extraídas de *Quillaja saponaria* (Quil A). O potencial imunoadjuvante das saponinas solúveis de *Q. brasiliensis* foi avaliado utilizando preparações com dois antígenos: ovalbumina (OVA) e vírus da diarreia viral bovina (BVDV). Numa etapa seguinte, a atividade imunoadjuvante de ISCOMs preparados com saponinas de *Q. brasiliensis* foram avaliadas em duas vias de administração. O potencial imunomodulador dessas saponinas foi verificado em experimentos de recrutamento celular *in vivo* e expressão de genes relacionados ao sistema imune. Os resultados mostraram que saponinas de *Q. brasiliensis* são menos tóxicas que as de Quil A e apresentam atividade adjuvante similar, caracterizada por um perfil Th1/Th2 balanceado. *Q. brasiliensis* promoveu uma forte resposta imune celular do tipo Th1 caracterizada por uma robusta reação de hipersensibilidade celular tardia (DTH) e pela produção de IFN-γ e IL-2. A resposta imune induzida pelos ISCOMs produzidos a partir de saponinas de *Q. brasiliensis* foram superiores às respostas induzidas pelas saponinas solúveis. Os testes *in vivo* mostraram que as saponinas de *Q. brasiliensis* promovem um ambiente imunocompetente no local da inoculação e nos linfonodos drenantes. Esse ambiente foi caracterizado pelo intenso influxo celular (neutrófilos, células NK, células dendríticas, linfócitos T e B), além da expressão diferencial de genes relacionados à ativação do sistema imune. Em suma, os resultados mostraram que saponinas de *Q. brasiliensis* são seguras e seus potencial adjuvante foi equivalente a saponinas com ação imunoadjuvante conhecida de *Q. saponaria.*

Palavras-chave: saponinas; ISCOM; hemólise; adjuvante; recrutamento celular; ativação imune.

SAPONINS FROM QUILLAJA BRASILIENSIS: IMUNOADJUVANT ACTIVITY, CELLULAR AND MOLECULAR MECHANISMS OF ACTION

ABSTRACT

Effective vaccine formulations frequently require addition of adjuvants able to optimize the cellular and humoral immune responses. With the goal to contribute to the development of new classes of adjuvants, this work was developed in order to achieve deep knowledge on the imunoadjuvant mode of action for Quillaja brasiliensis saponins incorporated into immunostimulant complex (ISCOM). The toxicity of saponins is a critical factor for its usage as vaccine preparations. At first, in vivo and in vivo citoxicity assays were carried out to compare to the effects between saponins extracted from Quillaja brasiliensis and the immunostimulant saponins already known from Quillaja saponaria (Quil A). Imunoadjuvant potential of soluble saponins from Q. brasilienis was evaluated using preparations of two antigens: ovoalbumin (OVA) and bovine viral diarrhea (BVD). As a next step, imunoadjuvant activity of ISCOMS prepared with Q. brasiliensis saponins was evaluated using two routes of administration. The immunomodulatory potential of these saponins was tested during in vivo cell recruitment assays and gene expression related to immune system. Our results demonstrated that Q. brasilienis saponins are less toxic than those from Quil A and presenting similar adjuvant activity, characterized by a Th1/Th2 balance profile. Q.brasiliensis induced a strong Th1 cell-mediated immune responses indicated by a robust delayed type hypersensitivity (DTH) as well as IFN-y and IL-2 production. The immune response induced by ISCOMs from Q. brasiliensis saponins was higher than the one induced by soluble saponins. In vivo experiments indicated that saponins from Q. brasiliensis generate an immunocompetent environment at the injection site and draining lymph nodes. This environment was characterized by an intense cell influx (neutrophils, NK cells, dendritic cells, B and T cells) as well as differential gene expression related to immune system activation. In essence, the results showed that saponins from are safe and their adjuvant potential was equivalent to saponins with imunoadjuvant activity of Q. saponaria.

Keywords: saponins, ISCOMS, hemolysis, adjuvants, cell recruitment, immune activation.

LISTA DE ABREVIATURAS E SIGLAS

APC	células apresentadoras de antígeno
BVDV	vírus da diarreia viral bovina
CFA	adjuvante complete de Freund
CpG	dinocleotídeos não metilados
DAMP	*danger-associated molecular patterns*
DC	células dendríticas
DNA	ácido desoxiribonucléico
DTH	teste de hipersensibilidade tardia
IFA	adjuvante incomplete de Freund
IFN	interferon
IL	interleucina
IQA	complexos imunoestimulantes de Quil A
IQB-90	complexos imunoestimulantes de QB-90
ISCOM	complexos imunoestimulantes
LPS	lipopolissacarídeo
MF59®	adjuvante oleoso MF59
MHC	complexo de histocompatibilidade
NK	células *natural killer*
OVA	ovoalbumina
PAMP	*pathogen-associated molecular patterns*
PCR	reação em cadeia da polimerase
PRR	*Pattern recognition receptor*
QB-90	fração rica em saponinas de *Q. brasiliensis*
Quil A®	saponina comercial Quil A
RNA	ácido ribonucléico
Th1	*T-helper 1*
Th2	*T-helper 2*
TLR	*toll-like receptors*

TNF	fator de necrose tumoral

SUMÁRIO

1 INTRODUÇÃO

A formulação de vacinas efetivas frequentemente requer a adição de adjuvantes capazes de otimizar as respostas imune humoral e celular. Entretanto, um ponto importante no desenvolvimento de uma formulação vacinal, além da eficácia do adjuvante, é a avaliação de sua toxicidade, uma vez que frequentemente induzem efeitos secundários locais, como inflamação, dor, formação de granulomas ou indução de febre. Atualmente, os adjuvantes baseados em sais de alumínio continuam sendo os únicos licenciados para uso em vacinas humanas. Portanto, é clara a necessidade atual de desenvolver novos adjuvantes vacinais eficazes e com baixa toxicidade. Além disso, alguns dos adjuvantes que apresentam maior potencial são baseados em saponinas ou as suas formulações. Tais substâncias são glicosídeos compostos por uma aglicona ou sapogenina triterpênica ou esteróide, com uma ou mais cadeias de oligossacarídeos. As saponinas são adjuvantes de origem natural, presentes em muitas espécies vegetais, e apresentando grande variedade estrutural. Devido as suas características, as saponinas compartilham propriedades físico-químicas, incluindo sua tensoatividade associada a seu caráter anfifílico, o que explica também suas propriedades de superfície, atividade hemolítica e capacidade de formar complexos com moléculas de colesterol.

Em relação a seu uso como imunoadjuvantes, grande experiência tem sido acumulada no uso de saponinas, particularmente em vacinas de uso veterinário. Tal é o caso das saponinas triterpenóides isoladas das cascas da árvore chilena *Quillaja saponaria* Molina, que se destacam por apresentarem comprovada atividade imunoadjuvante (REED *et al.*, 2009). A importância atribuída a essa atividade é indicada por diversos estudos, incluindo seu emprego em vacinas contra o vírus influenza, *Escherichia coli*, vírus sincicial respiratório, vírus da imunodeficiência humana (HIV-1), sarampo, malária, leishmaniose e, ainda, para o tratamento de melanoma (FOON *et al.*, 2000; LECLERC, 2003; SANTOS *et al.*, 2007; REED *et al.*, 2009; DE COSTA *et al.*, 2011).

As saponinas de *Q. saponaria* movimentam um grande mercado mundial. Em 2003, a exportação de extrato de *Q. saponaria* do Chile foi de US$ 1.569.454 (www.prochile.cl). Além disso, o mercado mundial de vacinas é de aproximadamente US$ 1,2 bilhões (www.prochile.cl). Entretanto, a utilização comercial e experimental das cascas de *Q. saponaria* para a obtenção de saponinas tem levado à intensa

exploração das florestas chilenas, sendo necessárias cerca de 50.000 árvores/ano para suprir a demanda mundial.

Uma alternativa que diminuiria a pressão sobre a exploração comercial de *Q. saponaria*, além de favorecer uma exploração sustentável, é o uso de saponinas da árvore do sul do Brasil e do Uruguai *Quillaja brasiliensis* (A. St.-Hil. et Tul.) Mart. Nosso grupo purificou uma fração de saponinas de folhas dessa espécie, denominada QB-90 (KAUFFMANN *et al.*, 2004; FLECK *et al.*, 2012) que induziu uma resposta adjuvante similar às saponinas de *Q. saponaria* (como Quil A), demonstrando seu potencial como adjuvante de vacinas (FLECK *et al.*, 2006; SILVEIRA *et al.*, 2011).

No presente trabalho, a fração QB-90 e extrato aquoso solúvel (AE) de *Q. brasiliensis* foram avaliados quanto a seu potencial imunoadjuvante e sua toxicidade. Além disso, saponinas purificadas de *Q. brasiliensis* (QB-90) foram utilizadas para a formulação de nanopartículas denominadas complexos imunoestimulantes (ISCOMs). Essas nanopartículas, denominadas IQB-90, igualmente tiveram seu potencial imunoestimulante analisado em preparações vacinais. Complementando esses estudos, o recrutamento celular e a ativação de genes relacionados ao sistema imune de camundongos foram estudados através da administração de QB-90 e IQB-90, na ausência de antígenos.

2 REVISÃO BIBLIOGRÁFICA

2.1 Vacinas e imunização

As vacinas são ferramentas essenciais em saúde pública. Todos os anos, milhões de vidas são salvas devido à vacinação contra várias doenças infecciosas, incluindo sarampo, caxumba, difteria, *Haemophilus*, meningite, tétano, hepatite e coqueluche em crianças e adultos.

Tradicionalmente, as tecnologias de produção de vacinas utilizam três tipos de imunógenos: microrganismos vivos atenuados, microrganismos inativados e subunidades de microrganismos (as vacinas de subunidades). Com qualquer uma dessas preparações vacinais é possível obter uma boa proteção, baseada, principalmente, na geração de anticorpos (LECLERC, 2003; REED *et al.*, 2009)

Em diversos casos, para que possam induzir imunidade protetora, as vacinas requerem a adição de adjuvantes. Tais substâncias são compostos naturais ou sintéticos que têm sido utilizados em vacinas desde o início dos anos 1920 para melhorar ou modular a imunogenicidade de antígenos co-administrados. Os adjuvantes, descritos primeiramente por Ramon (1924), podem ser definidos como um *conjunto aditivos sumariamente heterogêneos, que aumentam a imunogenicidade dos antígenos (moléculas reconhecidas por anticorpos e/ou pelos receptores de células T), e modulam a resposta induzida pela imunização* (REED *et al.*, 2009). O uso de adjuvantes remonta às primeiras publicações de Freund (em 1942), que utilizou preparações que aumentavam a persistência do antígeno e demonstrou o efeito potencializador da resposta imune de extratos de micobactérias (KENNEY e EDELMAN, 2003; REED *et al.*, 2009). As novas vacinas de subunidades e vacinas geneticamente manipuladas têm perfis de segurança mais elevados. No entanto, a principal desvantagem destas novas vacinas é que elas são pouco imunogênicas e, portanto, requerem a adição de adjuvantes para induzir respostas imunes eficazes e sustentáveis.

Nos últimos anos, a pesquisa para o desenvolvimento de vacinas tem-se baseado na utilização de antígenos definidos e purificados, como proteínas (MAGGIOLI, ACOSTA, *et al.*, 2011; MAGGIOLI, SILVEIRA, *et al.*, 2011) e peptídeos recombinantes (LEROUX-ROELS, 2010). Desta forma, busca-se a geração de uma imunidade protetora utilizando preparações que não produzam reações adversas (como dor, ardor, eritema) desencadeadas pela administração de antígenos crus ou que possuem a capacidade de reverter-se a formas virulentas (no caso de vacinas atenuadas)

(LEROUX-ROELS, 2010), já que a pureza dos antígenos traz como consequência a redução da capacidade de induzir respostas imunes efetivas (O'HAGAN e DE GREGORIO, 2009).

Em termos gerais, o uso de adjuvantes tem permitido obter formulações vacinais mais efetivas, principalmente na geração de anticorpos (resposta humoral) (BALDRIDGE e WARD, 1997; O'HAGAN e DE GREGORIO, 2009; COFFMAN *et al.*, 2010). O desafio atual é a formulação de adjuvantes capazes de gerar proteção não somente através do estímulo à geração de imunidade humoral, mas também mediante a ativação de células T $CD8^+$ (linfócitos T citotóxicos), capazes de demonstrarem efeito adjuvante eficaz contra enfermidades geradas por patógenos intracelulares, bem como contra células tumorais. Atualmente, encontra-se disponível uma grande variedade de adjuvantes vacinais (Tabela 1), embora a maioria não esteja licenciada para uso em humanos.

Tabela 1. Substâncias utilizadas como adjuvantes em vacinas humanas e seu estado de licenciamento/testes em humanos.

Nome	Laboratório	Tipo de preparação	Exemplos de uso	Estado
Adjuvantes de primeira geração				
Sais de alumínio	Vários	Sais de alumínio	Várias	Licenciada
MF59	Novartis	Emulsão O/W	Gripe	Licenciada na EU
Lipossomas	Crucell	Vesículas lipídicas	HAV/Gripe	Licenciada na EU
Montanide	Vários	Emulsão W/O	Malária/Câncer	Fase III
PLG	Novartis	Micropartículas poliméricas	Vacinas de DNA	Fase I
Flagelina	Vaxinnate	Agonista de TLR	Gripe	Fase I
QS21	Antigenics	Saponina	Várias	Fase I, II e III
Adjuvantes de segunda geração (combinação de adjuvantes)				
ASO1	GSK	MPL + lipossomas + QS21	Malária/TB	Fase II
ASO2	GSK	MPL + emulsão O/W + QS21	Malária	Fase II
ASO3	GSK	Emulsão O/W + α-tocoferol	Gripe pandêmica	Licenciada na EU
ASO4	GSK	MPL + sais de alumínio	HBV/HPV	Licenciada na EU
RC-259	Dynamax	MPL sintético + sais de alumínio	HBV/HPV	Fase II
ISCOMs	CSL, Isconova	Saponinas + colesterol + fosfolipídeos	Várias	Fase I
IC31	Intercell	Peptídeos + oligonucleotídeos	TB	Fase I
CpG 7909	Coley/Pfizer/Novartis	Oligonucleotídeos + sais de alumínio + MF59	HBV/Malária/HCV	
ISS	Dynamax	Oligonucleotídeos + sais de alumínio	HBV	Fase I
MF59+MTP+PE	Chiron/Novartis	MDP + emulsão O/W	HIV/Gripe	Fase I

MDP (muranil di-peptídeo); MPL (monofosforil lipídico A; HAV, HBV, HCV: vírus da hepatite A, B e C, respectivamente. Tabela modificada de O'Hagan & de Gregório (O'HAGAN e DE GREGORIO, 2009).

2.2 Desenvolvimento de novas vacinas: antígenos e adjuvantes

O desenvolvimento de novas vacinas está fundamentalmente relacionado com a capacidade de identificar antígenos capazes de estimular uma resposta imune protetora. Por sua vez, especialmente quando tais antígenos são maus imunógenos, o êxito no desenvolvimento de vacinas está em grande parte relacionado com o adjuvante utilizado na formulação, que deve assegurar a estabilidade e imunogenicidade do antígeno (LECLERC, 2003; LIMA *et al.*, 2004; MBOW *et al.*, 2010). Dois são os maiores objetivos buscados na incorporação de adjuvantes em preparações vacinais. O primeiro está associado ao seu uso clínico (já que os adjuvantes permitem uma magnitude da resposta em populações que apresentam maior susceptibilidade a infecções – idosos e crianças), a diminuição das doses de antígeno e a redução do número de doses vacinais. O segundo está relacionado com a capacidade destas preparações de modular e dirigir a resposta imune, de acordo com o tipo de imunidade desejado (REED *et al.*, 2009; COFFMAN *et al.*, 2010; MASTELIC *et al.*, 2010). Para isso, a seleção de um bom adjuvante requer conhecimentos a respeito da natureza dos antígenos, bem como a respeito da resposta imune que se deseja estimular. Não obstante, as pesquisas envolvendo o desenvolvimento de novas vacinas são realizadas empiricamente, sem conhecimentos mais profundos a respeito de tais aspectos (BENDELAC e MEDZHITOV, 2002; LECLERC, 2003).

Em geral, os adjuvantes combinam duas propriedades; a primeira delas é o efeito de depósito, que assegura uma liberação lenta de antígeno evitando sua degradação; a segunda é o desenvolvimento de uma resposta inflamatória no sítio da inoculação a fim de estimular adequadamente mecanismos da imunidade inata, que também irão influenciar o tipo de resposta adaptativa gerada (COFFMAN *et al.*, 2010; TURVEY e BROIDE, 2010).

Os aspectos vinculados com a primeira propriedade (efeito de depósito) são essencialmente tecnológicos e têm-se desenvolvido por avanços nas áreas de tecnologia farmacêutica e físico-química. Os aspectos vinculados à segunda propriedade são essencialmente imunológicos; nos últimos anos, o conhecimento dos mecanismos de ação dos adjuvantes na ativação da resposta imune inata para a geração da resposta imune adaptativa teve um grande avanço (TURVEY e BROIDE, 2010). O conhecimento da base molecular destes mecanismos tem permitido entender o "efeito adjuvante" de numerosas preparações. Não obstante, ainda é necessário conhecer com maior profundidade que receptores da resposta imune inata são ativados pelos diferentes adjuvantes, além de conhecer que sinais convertem as células dendríticas (DC) e monócitos em células apresentadoras de antígenos (APCs) (REED *et al.*, 2009; MASTELIC *et al.*, 2010). O desafio atual é o preparo de uma "mistura

perfeita", com diferentes componentes que atuem sinergicamente e conduzam para a resposta imune desejada (GUY, 2007; PETROVSKY, 2008). Tanto para uso humano quanto para uso veterinário, é desejável que os adjuvantes sejam pouco tóxicos, rapidamente metabolizados, que produzam uma resposta humoral e/ou celular forte e duradoura. Além disso, devem ser estáveis, fáceis de fabricar, de baixo custo e aplicáveis a uma ampla gama de antígenos e vias de inoculação (O'HAGAN *et al.*, 2001; MARCIANI, 2003; SINGH e O'HAGAN, 2003; REED *et al.*, 2009).

2.3 Relação entre a imunidade inata, imunidade adquirida e adjuvantes

Os componentes da resposta imune inata (AKIRA *et al.*, 2006; TAKEUCHI e AKIRA, 2007) reconhecem especificamente padrões moleculares conservados (que são produtos essenciais na fisiologia dos microrganismos), conhecidos como PAMPs (*pathogen associated molecular patterns*) (TURVEY e BROIDE, 2010), e padrões associados ao dano celular ou DAMPs (*damage-associated molecular patterns*) (TAKEUCHI e AKIRA, 2007). Os PAMPs e os DAMPs constituem "sinais de perigo" que, em princípio, desencadeiam uma resposta inata que pode gerar um processo inflamatório (AKIRA *et al.*, 2006). Os principais mediadores são o fator de necrose tumoral α (TNF-α) e a interleucina 12 (IL-12) secretados, entre outras células, pelos macrófagos e pelas células dendríticas (DC) (MEDZHITOV e JANEWAY, 1997; MA, 2001). Os PAMPs incluem componentes bacterianos como lipopolissacarídeos (LPS), lipoproteínas, peptidoglicanos, flagelina, ácido micólico, dinucleotídeos não metilados (CpG), etc., e componentes de outros microrganismos como vírus e leveduras. Tanto os PAMPs como os DAMPs interagem com receptores de reconhecimento específicos, que coletivamente, se denominam PRRs (*pattern recognition receptors*) (TAKEUCHI e AKIRA, 2007). Os PRRs se agrupam em famílias muito diversas, como lectinas do tipo C, receptores citosólicos tipo NOD (*nucleotide oligomerization domain based-I-like receptors*), receptores induzidos pelo ácido retinóico e receptores do tipo *toll* ou TLRs (*toll-like receptors*) (MEDZHITOV e JANEWAY, 1997; BENDELAC e MEDZHITOV, 2002). A expressão de TLRs na superfície das células apresentadoras de antígenos (APCs), bem como nas DCs, tem um papel fundamental na iniciação da resposta imune inata (COFFMAN *et al.*, 2010; IWASAKI e MEDZHITOV, 2010).

Os PAMPs e os DAMPs induzem a maturação das DCs, o que se manifesta por um aumento da expressão de moléculas do complexo principal de histocompatibilidade do tipo II (MHC-II, *major histocompatibility complex*) e de moléculas de co-estimulação, assim como a indução de várias citocinas que promovem a diferenciação de linfócitos T virgens em

linfócitos T efetores e de memória. O conhecimento dos mecanismos moleculares desencadeados pelos PAMPs e DAMPs tem permitido compreender por que as vacinas fabricadas no passado (com microrganismos inteiros inativados ou seus subprodutos, como toxóides), eram capazes de induzir respostas potentes e prolongadas, sem a necessidade de adição de adjuvantes (PETROVSKY e AGUILAR, 2004; PETROVSKY, 2008; REED *et al.*, 2009). Igualmente, tornou-se possível compreender por que os antígenos purificados não são bons indutores do recrutamento e ativação das APCs, bem como por que a adição de adjuvantes na formulação da vacina com esse tipo de antígeno é essencial para garantir uma estimulação eficiente da produção de anticorpos e/ou linfócitos T citotóxicos (CTLs). As vacinas formuladas com DNA, vírus inativados e proteínas recombinantes, são exemplos de preparações que requerem adjuvantes para produzir respostas imunes potentes e prolongadas (LIMA *et al.*, 2004; O'HAGAN *et al.*, 2004; MAGGIOLI, ACOSTA, *et al.*, 2011).

2.4 Classificação dos adjuvantes

2.4.1 Classificação I: adjuvantes que promovem uma liberação controlada de antígeno e imunoestimuladores

Propõe dividir os adjuvantes em substâncias imunoestimuladoras e os que possuem um mecanismo de liberação controlada de antígeno (O'HAGAN *et al.*, 2001; O'HAGAN e SINGH, 2003; O'HAGAN e DE GREGORIO, 2009). Entre os imunoestimuladores, encontram-se agonistas de TLRs e NODs e também citocinas, quimiocinas, moléculas co-estimuladoras e compostos naturais como as saponinas (O'HAGAN *et al.*, 2001; DE GREGORIO *et al.*, 2009). Por outro lado, os que produzem uma liberação controlada de antígeno, denominados adjuvantes particulados, incluem os sais de alumínio, emulsões, micropartículas, lipossomas e os complexos imunoestimulantes (ISCOMs), aos quais são incorporados ou associados antígenos (COX e COULTER, 1997).

2.4.2 Classificação II: adjuvantes de primeira e segunda geração

É conhecido que o sistema dicotômico de classificação (visto acima) está amplamente superado, já que observações mostram que adjuvantes que possuem uma liberação controlada de antígeno também são imunostimuladores. Por esse motivo, O'Hagan e de Gregorio (2009) (O'HAGAN e DE GREGORIO, 2009), propuseram uma classificação que considera o número de adjuvantes utilizados na formulação: adjuvantes de primeira geração (um adjuvante) e de segunda geração (mais de um adjuvante).

Os adjuvantes de primeira geração incluem os sais de alumínio e as emulsões do tipo água em óleo (W/O) que são os adjuvantes que obtiveram maior êxito. Ambos baseiam sua ação na dispersão de partículas, nas quais estão associadas antígenos, e produzem um efeito persistente no sítio de inoculação (efeito depósito) (TRITTO *et al.*, 2009). As partículas poliméricas e os lipossomas também se integram nesse grupo (ALLISON e GREGORIADIS, 1974).

A formulação de adjuvantes de segunda geração (também conhecidos como sistemas de adjuvantes) (GARCON *et al.*, 2007) foi iniciada nos anos 70 com o agregado de outros componentes aos adjuvantes de primeira geração a fim de aumentar sua potência. O desenvolvimento dessa geração foi favorecido pelo uso de componentes sintéticos que ativam o sistema imune inato (como o MDP), que é constituído por componentes hidrossolúveis das paredes de micobactérias (ELLOUZ *et al.*, 1974). Nos sistemas adjuvantes se busca combinar as propriedades e efeitos dos diferentes tipos envolvidos na preparação (O'HAGAN e SINGH, 2003; LEROUX-ROELS, 2010).

2.5 Principais adjuvantes utilizados na preparação de vacinas

2.5.1 Sais minerais

Identificado originalmente em 1920 (BAYLOR *et al.*, 2002), o alumínio é o adjuvante mais utilizado na formulação de vacinas para humanos (BAYLOR *et al.*, 2002; AIMANIANDA *et al.*, 2009; TRITTO *et al.*, 2009; EXLEY *et al.*, 2010; MBOW *et al.*, 2010), embora seu mecanismo de ação não esteja bem esclarecido (PETRILLI, DOSTERT, *et al.*, 2007; PETRILLI, PAPIN, *et al.*, 2007; AIMANIANDA *et al.*, 2009). Possui propriedades físicas que favorecem a união dos antígenos mediante interações eletrostáticas estáveis, formando suspensões macroscópicas (BREWER, 2006). Tradicionalmente se propôs que os antígenos adsorvidos em alumínio são facilmente internalizados pelas APCs e que esse processo depende do tamanho do agregado (MOREFIELD *et al.*, 2005).

Os sais de alumínio são de baixo custo, seguros e aplicáveis a uma grande variedade de antígenos. Suas limitações estão associadas à baixa capacidade de estimular uma imunidade mediada por células (O'HAGAN *et al.*, 2001; REED *et al.*, 2009), e a capacidade de induzir a produção de IgE (relacionada a reações alérgicas em humanos) (NAGEL *et al.*, 1977; WALLS, 1977; NAGEL *et al.*, 1979; HAMMAD *et al.*, 2010) e a formação de granulomas no local da injeção. Ademais, seu uso como adjuvante apresenta alguns inconvenientes já que suas preparações são heterogêneas e as vacinas podem não ser efetivas

se congeladas, o que é um problema em países em desenvolvimento (LINDBLAD, 2004; REED *et al.*, 2009).

2.5.2 Adjuvantes oleosos

Entre os mais exitosos adjuvantes vacinais, se encontram os adjuvantes de Freund, conhecidos por sua potência e toxicidade. São formulações a base de óleos minerais e um tensoativo. Ao serem misturados com o antígeno em solução aquosa, formam emulsões de água em óleo (W/O). O adjuvante completo de Freund (CFA) contém uma suspensão de micobactérias mortas, diferentemente do adjuvante incompleto de Freund, que não as contém. A partir desses adjuvantes, se desenvolveram formulações alternativas a base de óleos biodegradáveis, como o esqualeno.

As emulsões do tipo W/O são formulações constituídas por gotas de água que contém antígeno (fase interna) envoltas por uma fase externa contínua e oleosa. Este tipo de emulsão retém antígeno no sítio de inoculações, permitindo sua liberação lenta e gradual (HERBERT, 1968). Devido a sua alta viscosidade, são difíceis de injetar. As injeções do tipo óleo-água (O/W) são caracterizadas por sua baixa viscosidade e boa tolerabilidade; não obstante, induzem respostas imunes pouco duradouras (AUCOUTURIER *et al.*, 2001). As gotas de óleo dessas emulsões O/W podem associar-se minimamente ou não associarem-se com o antígeno, que está localizado na fase aquosa (PODDA e DEL GIUDICE, 2003; JANSEN *et al.*, 2006).

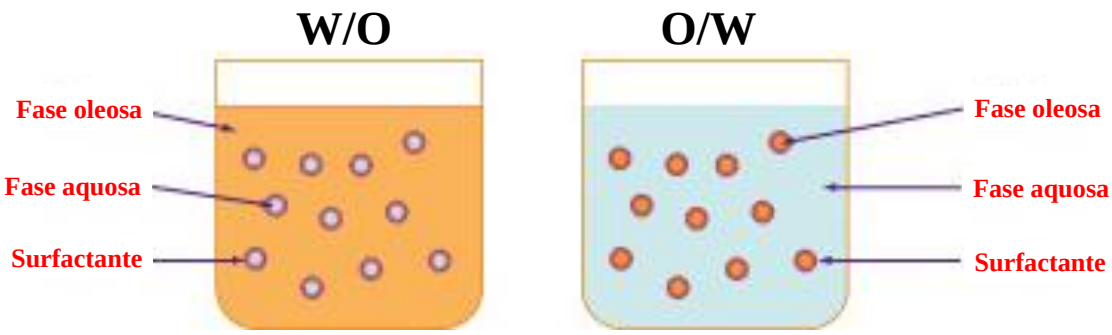


Figura 1. Dispersão das micropartículas em emulsões do tipo W/O (água/óleo) e O/W (óleo/água).

O MF59^{TM}, um dos adjuvantes utilizados em modelos animais com grande êxito e aprovado em mais de 20 países para uso em humanos, é uma emulsão do tipo O/W (O'HAGAN *et al.*, 2013). Tem sido utilizado na Europa em vacinas contra a gripe sazonal (WADMAN, 2005; SCHULTZE *et al.*, 2008) e em formulações que contém outros antígenos

virais, como HSV (Herpes simplex vírus) (STRAUS *et al.*, 1997), HBV (HEINEMAN *et al.*, 1999) e HIV (MCFARLAND *et al.*, 2001). Em geral, este adjuvante apresenta poucos efeitos colaterais e promove uma resposta de anticorpos superior a gerada por sais de alumínio.

Os óleos utilizados nestas formulações precisam ser biodegradáveis e os tensoativos próprios para uso em humanos. O processo utilizado na preparação das emulsões modifica sua qualidade, estabilidade e o tamanho das partículas na fase dispersa. Esse fato tem um efeito direto na imunogenicidade do antígeno administrado, necessitando-se de uma extensiva padronização do processo de fabricação das formulações para assegurar sua reprodutibilidade (PEREZ e HARANDI, 2008). As emulsões lipídicas estão sendo utilizadas também como sistemas de liberação para adjuvantes imunoestimuladores, como o MPL e a saponina QS-21 (O'HAGAN *et al.*, 2001).

2.5.4 Saponinas

Saponinas são compostos glicosídicos de origem natural, constituídos por uma porção oligossacarídica (hidrofílica) ligada através de um grupo hidroxila, carbonila ou ambos, a uma estrutura triterpênica ou esteroidal denominada aglicona (hidrofóbica) (OLESZEK, 2002), assim sendo classificadas como esteroidais ou triterpênicas (HARALAMPIDIS *et al.*, 2002; YENDO *et al.*, 2010).

Os oligossacarídeos (glicosídeos) são hidrofílicos e interatuam fortemente com moléculas do solvente em soluções aquosas. Por outro lado, as agliconas são lipofílicas, e ao associarem-se a outras agliconas, formam micelas. Por sua natureza anfipática, as saponinas são tensoativas (formam espuma e são emulsionantes) (PRICE *et al.*, 1987). O caráter anfipático das saponinas também determina sua capacidade de gerar micelas em meio aquoso (SIDHU e OAKENFULL, 1986). Por outro lado, tem-se reportado que as saponinas podem associar-se com esteróis gerando soluções coloidais de micelas mistas.

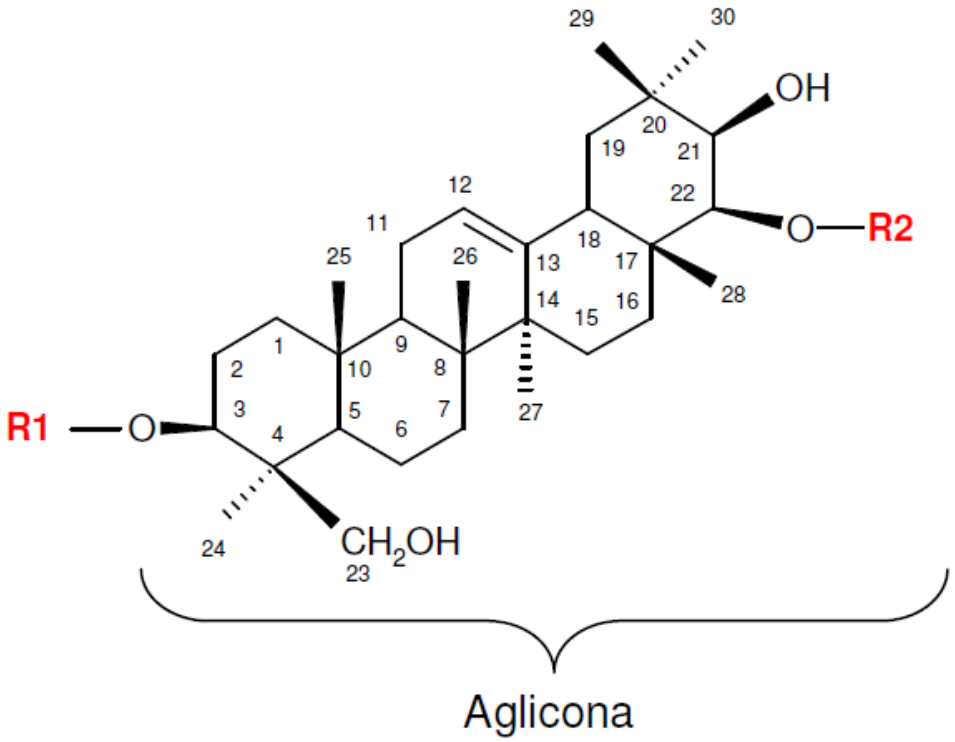


Figura 2. Estrutura modelo de uma saponina triterpênica. R1 e R2 representam os açúcares ligados à aglicona.

Têm-se atribuído às saponinas numerosos efeitos biológicos e farmacológicos, como: propriedades imunomoduladoras, antifúngicas, antitumorais, anti-inflamatórias, antivirais e hipocolesterolêmicas (YENDO *et al.*, 2010). O sabor das saponinas pode ser doce ou salgado e a maioria apresenta efeitos tóxicos, principalmente propriedades hemolíticas (ODA *et al.*, 2000).

2.5.4.1 Propriedades imunomoduladoras

Desde os anos 1930, sabe-se que extratos de *Quillaja saponaria* Molina (saponinas triterpênicas extraídas da árvore chilena "Quillay") possuem atividade adjuvante. Nos anos 1970, Dalsgaard obteve uma fração enriquecida de saponinas a partir do extrato de *Q. saponaria* que denominou Quil A, que estimula tanto a imunidade celular quanto a celular (DALSGAARD, 1974).

A Quil A é comercializada atualmente para uso veterinário, empregada, por exemplo, em vacinas contra o vírus da febre aftosa (DALSGAARD, 1974; DALSGAARD *et al.*, 1977). Em humanos, os resultados utilizando Quil A como adjuvante vacinal têm sido menos satisfatórios que nos modelos animais, devido a reações locais de dor e inflamação. Por esse motivo, existe uma grande busca de saponinas em plantas para avaliação de seu potencial

imunoadjuvante e sua toxicidade (SUN *et al.*, 2009; DE COSTA *et al.*, 2013). A Tabela 2 mostra alguns exemplos de saponinas avaliadas como adjuvantes.

Tabela 2. Saponinas avaliadas como adjuvantes de vacinas.

	Atividade hemolítica e citotóxica	**Características da resposta**	**Referência**
Achyranthes bidentata	Pouco hemolítica	Promove proliferação de esplenócitos e altos títulos de IgG, IgG1, IgG2b contra OVA	(SUN, 2006)
Anemone raddeana			(SUN *et al.*, 2008)
Astragalus membranaceus	Pouco hemolítica	Promove proliferação de esplenócitos e altos títulos de IgG, IgG1, IgG2b contra OVA; ativa macrófagos	(YANG *et al.*, 2005)
Chenopodium quinoa	Baixa citotoxicidade	Adjuvante de mucosas; aumenta os títulos de IgG, IgG1, IgG2b contra OVA e toxina colérica e OVA	(ESTRADA *et al.*, 1998; VERZA *et al.*, 2012)
Aesculus hippocastanum	Baixa citotoxicidade	Aumenta o nível de anticorpos contra OVA	(ODA *et al.*, 2000)
Glycyrrhiza	Pouco hemolítica	Promove proliferação de esplenócitos e altos títulos de IgG, IgG1, IgG2b contra OVA; promove a produção de IL-12 por macrófagos	(SUN e PAN, 2006)

Gynostemma pentaphyllum	Pouco hemolítica	Promove proliferação de esplenócitos e altos títulos de IgG, IgG1, IgG2b contra OVA; promove a produção de IL-12 por esplenócitos e IL-1 por macrófagos	(SUN e ZHENG, 2005)
Zizyphus joazeiro	Não hemolítica	Aumenta o nível de anticorpos contra OVA	(MATSUDA *et al.*, 1999)
Acacia concinna		Ativa células B e T; aumenta os níveis de IgG, IgG1, IgG2b contra OVA	(KUKHETPITAKWONG *et al.*, 2006)
Dolichos lablab		Induz a produção de IgG1, pouco IgG2a	(YOSHIKAWA *et al.*, 1998)
Periandra dulcis	Pouco hemolítica	Promove proliferação de esplenócitos e altos títulos de IgG, IgG1, IgG2b contra antígenos de *Leshmania donovani*	(SANTOS *et al.*, 1997)
Pulcherrima		Induz DTH, altos títulos de IgG2b	(NICO *et al.*, 2007)
Quillaja brasiliensis	Baixa citotoxicidade	Induz altos títulos de IgG, IgG1, IgG2a, IgG2b, IgG3 contra OVA, contra BoHV1, Raiva e poliovírus. Potente indutor de resposta celular	(FLECK *et al.*, 2006; SILVEIRA *et al.*, 2011; DE COSTA *et al.*, 2014)

Bupleurum chinense	Pouco hemolítica	Promove proliferação de esplenócitos e altos títulos de IgG, IgG1, IgG2b contra OVA	(USHIO e ABE, 1991)
Glycine max	Pouco hemolítica	Forte indutor de IgG1	(BOMFORD *et al.*, 1992)
Hedera taurica		Forte indutor humoral contra glicoproteínas do vírus HIV	(KRIVORUTCHENKO *et al.*, 1997)

A análise cromatográfica de Quil A (por cromatografia líquida de alta eficiência, HPLC) mostra que essa fração purificada é uma mistura complexa de saponinas (Figura 3 e Figura 4). Muitas dessas saponinas encontradas na Quil A já foram isoladas e caracterizadas. As saponinas que predominam em preparados de Quil A são: QS-7, QS-17, QS-18 e QS-21. Essas saponinas possuem de 7 a 9 resíduos monossacarídicos, incluindo ramnose, xilose, galactose, glicose e ácido 2,3-d-glicurônico. Esses sacarídeos estão distribuídos em duas cadeias oligossacarídicas e uma cadeia lateral lipofílica (KENSIL *et al.*, 1991; SOLTYSIK *et al.*, 1995).

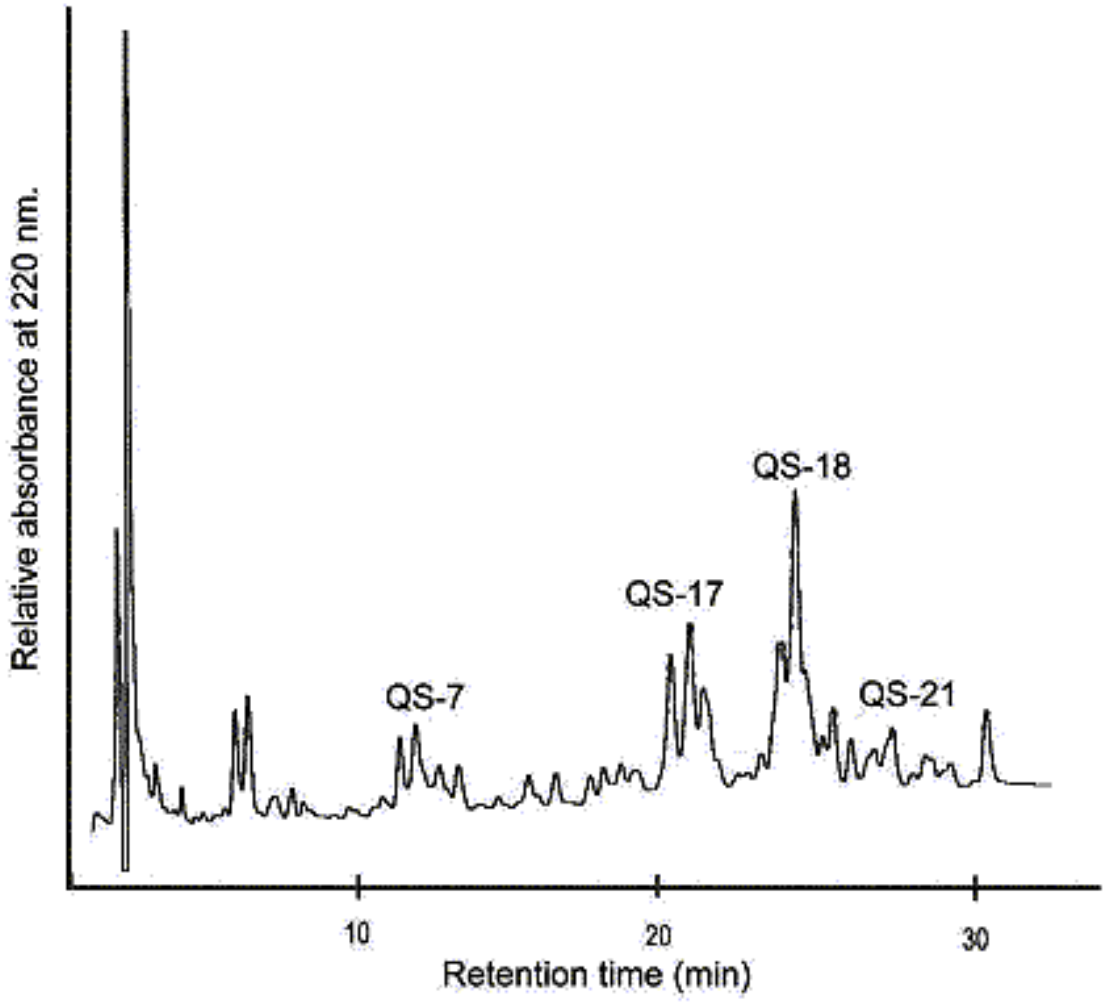


Figura 3. Perfil de cromatografia líquida de alta eficiência (HPLC) de Quil A. Adaptado de Kensil (1991) (KENSIL *et al.*, 1991).

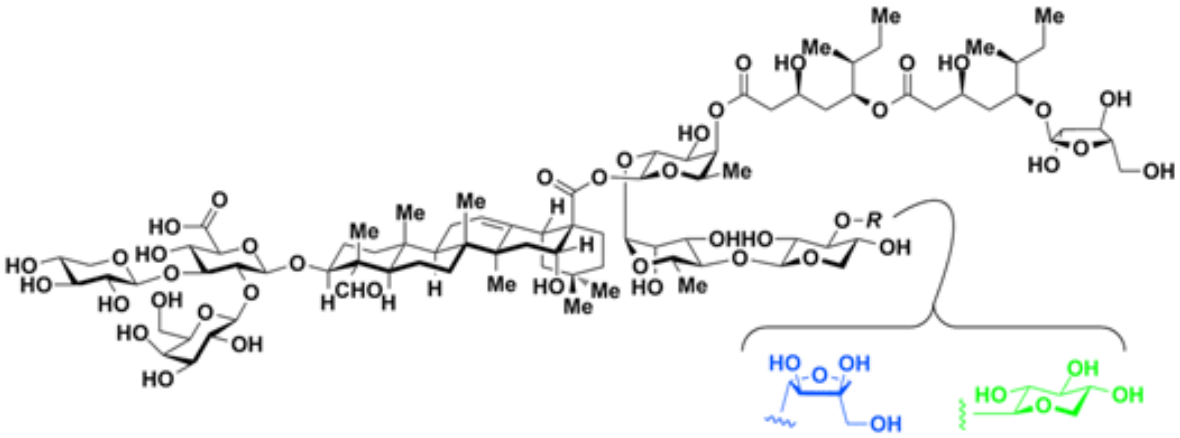


Figura 4. Estrutura da saponina QS-21 isolada de *Q. saponaria.*

Estudos recentes avaliando a toxicidade de Quil A em camundongos mostram que é letal a concentrações de aproximadamente 100 μg/dose (SUN *et al.*, 2010). As propriedades físicas dos isolados de Quil A são bastante similares; entretanto, as propriedades tóxicas variam significativamente. A saponina QS-18 é tóxica para camundongos numa concentração de 25 μg/dose, enquanto a QS-21 possui toxicidade apenas quando a dose supera os 500 μg. Por esse motivo, a QS-21 tem sido extensivamente empregada na formulação de diversas vacinas.

Vários estudos reportam que as saponinas de *Q. saponaria* polarizam a resposta imune para um perfil do tipo Th1, com produção de IL-2 e IFN-γ, estimulando a diferenciação de CTLs e a produção de anticorpos do isotipo IgG2a (TAKAHASHI *et al.*, 1990; VILLACRES-ERIKSSON *et al.*, 1992; KENSIL *et al.*, 1995; O'HAGAN *et al.*, 2001). Por esse motivo, existem numerosos trabalhos que aplicam as saponinas como adjuvantes para vacinas contra patógenos intracelulares e para vacinas terapêuticas contra o câncer (SUN *et al.*, 2009).

2.5.4.2 Efeitos tóxicos

Muitas saponinas causam hemólise *in vitro* (BANGHAM *et al.*, 1962). Sugere-se que a atividade hemolítica estaria relacionada com a afinidade da aglicona com o colesterol das membranas celulares (BANGHAM *et al.*, 1962). Devido ao seu caráter hidrofóbico, se postula que a aglicona pode intercalar-se com o colesterol das membranas, o que geraria poros que provocariam lise celular. O grau de atividade, ou afinidade pelo colesterol, pode depender da própria aglicona (TAKECHI e TANAKA, 1995) e/ou dos resíduos associados (oligossacarídeos

e/ou grupos acila) (SEGAL *et al.*, 1974; SEGAL e MILO-GOLDZWEIG, 1975; ODA *et al.*, 2000).

É importante lembrar que as propriedades hemolíticas e de citotoxicidade neo sempre coincidem. Nesse sentido, Gauthier e colaboradores, estudando saponinas triterpênicas, concluíram que existem saponinas hemolíticas com atividade citotóxica, embora algumas não apresentem atividade hemolítica e sejam citotóxicas (GAUTHIER *et al.*, 2009). As saponinas de Quil A, em particular a QS-21, são saponinas triterpênicas e apresentam grande atividade hemolítica (KENSIL *et al.*, 1991; ODA *et al.*, 2000; OLIVEIRA-FREITAS *et al.*, 2006; SUN *et al.*, 2010). Essa atividade hemolítica tem sido relacionada com as cadeias laterais (oligossacarídeos) unidos à aglicona (NICO *et al.*, 2007) e aos resíduos acilas (PRICE *et al.*, 1987). A presença do ácido graxo poderia favorecer a interação entre a saponina e o colesterol das membranas, promovendo a hemólise (JACOBSEN *et al.*, 1996), já que a remoção desse grupamento acila elimina a atividade hemolítica (OLIVEIRA-FREITAS *et al.*, 2006).

2.6 Mecanismo de ação dos adjuvantes vacinais

O objetivo da vacinação é a indução de imunidade protetora. Entretanto, em algumas vacinas este objetivo só pode ser alcançado pela adição de adjuvantes. Diversas classes de compostos foram avaliados como adjuvantes, incluindo sais minerais, produtos microbianos, emulsões, saponinas, citocinas, polímeros, micropartículas e lipossomas (GUY, 2007). Com base em seus mecanismos de ação propostos, adjuvantes de vacinas têm sido divididos em sistemas de entrega (*delivery systems*) e adjuvantes imunoestimulantes (O'HAGAN e SINGH, 2003). Em geral, os sistemas de entrega foram previamente pensados para atuar através da formação de um depósito (*depot*) enquanto adjuvantes imunoestimuladores buscam ativar células do sistema imune inato. No entanto, esta classificação não é mais adequada uma vez que evidências surgiram de que alguns sistemas de entrega pode ativar a imunidade inata.

Apesar da ampla utilização de adjuvantes de vacinas em milhares de milhões de doses em vacinas humanas e animais, os mecanismos de ação pelo qual eles potenciam respostas imunológicas não estão bem caracterizados. No entanto, os recentes avanços na pesquisa de imunobiológicos têm desvendado vários mecanismos pelos quais os adjuvantes atuam. Evidências recentes sugerem que os adjuvantes empregam um ou mais mecanismos para induzir respostas imunes (Figura 5) (HOEBE *et al.*, 2004; FRASER *et al.*, 2007; O'HAGAN e DE GREGORIO, 2009; AWATE *et al.*, 2013) entre eles:

1) Liberação sustentada do antígeno no local da injeção (efeito de depósito): a formação de um depósito é, talvez, o mecanismo mais antigo e mais amplamente reconhecido de ação de adjuvantes. O aprisionamento de antígeno e libertação lenta no local da injeção garante constante estimulação do sistema imune para a produção de títulos elevados de anticorpos (SISKIND e BENACERRAF, 1969). O efeito de depósito foi considerado um mecanismo clássico de ação de muitos adjuvantes. Vários adjuvantes, tais como suspensões de sais de alumínio, emulsões de água-em-óleo agem dessa forma, gerando depósitos e promovem títulos elevados e prolongados de anticorpos (HERBERT, 1968).

2) Recrutamento celular: estudos sobre os mecanismos de adjuvantes têm-se centrado sobre o recrutamento de células imunitárias inatas no local da injeção. Adjuvantes particulados parecem criar um ambiente de pró-inflamatório no sítio da inoculação para recrutar células imunes (GOTO e AKAMA, 1982). Apesar de vários adjuvantes recrutarem células imunes para o sítio de inoculação da vacina (bem como seus gânglios drenantes), a relação entre estas células recrutadas e indução de respostas imunes não é muito clara. Estudos de depleção sugerem que a função de células imunitárias inatas recrutadas no local da injeção é redundante na geração de respostas adaptativas (MCKEE *et al.*, 2009; CALABRO *et al.*, 2011). A injeção de adjuvantes muitas vezes leva ao recrutamento de uma variedade de populações de células e, devido à elevada redundância no sistema imunitário, outras células recrutadas podem compensar as células que experimentalmente foram depletadas. Por isso, mais estudos são necessários para investigar a relação detalhada entre as células imunes recrutadas e atividade adjuvante.

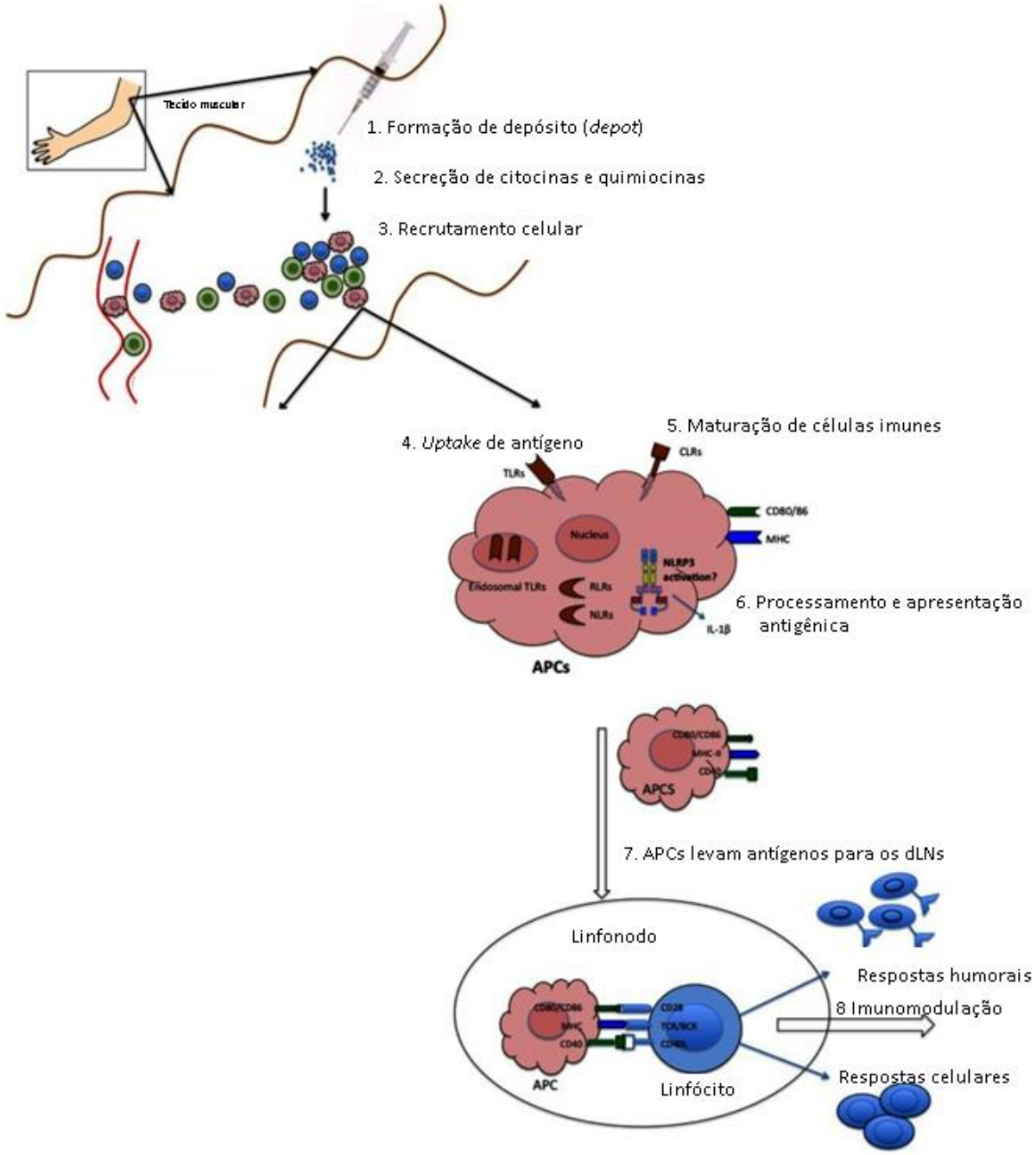
3) Aumento da captura de antígeno e a apresentação para APCs: a apresentação eficiente de antígeno pelo complexo principal de histocompatibilidade (MHC) nas APCs é importante para a indução da resposta imunitária adaptativa. Embora o papel da apresentação do antígeno, induzida por adjuvante no desenvolvimento da imunidade adaptativa, não ser claramente estabelecido, antígenos apresentados à APCs na forma de partículas (como os conjugados com alumínio ou na forma de ISCOMs) são melhor internalizados (UTO *et al.*, 2013; JOSHI *et al.*, 2014).

4) Ativação e maturação de APCs (aumento de MHC de classe II e de moléculas co-estimuladoras) e migração para a drenagem dos gânglios linfáticos: a ativação de DCs é essencial para a indução de respostas imunitárias adaptativas. O aumento da expressão de MHC classe II,

do marcador de ativação CD86 e do marcador de maturação CD83 leva a um aumento da capacidade de APCs para induzir ativação e diferenciação de células T (COYLE e GUTIERREZ-RAMOS, 2001). Em geral, adjuvantes que possuem a capacidade de estimular a ativação e maturação de DCs promovem uma eficiente ativação de células T, gerando uma forte resposta celular.

5) Ativação de inflamassomas: células imunes inatas expressam vários receptores de reconhecimento de patógenos (PRRs) para identificar agentes infecciosos. Muitos adjuvantes sinalizam via PRRs ou agem como ligantes para os receptores da imunidade inata. Em contraste com os agonistas de TLR, adjuvantes particulados não são reconhecidos por PRRs específicos, mas ainda assim induzem respostas adaptativas. A hipótese do "*perigo*" foi discutida pela primeira vez por Matzinger, que propôs que, além da discriminação "*self/non-self*" contra as infecções, sinais de perigo de células danificadas podem desencadear a ativação do sistema imune (MATZINGER, 1994). As moléculas associadas aos danos nos tecidos, tais como o ácido úrico, espécies reativas de oxigênio, nucleotídeos, trifosfato de adenosina (ATP), espécies reativas de oxigênio e citocinas são libertados no sítio da injeção (SHI *et al.*, 2003).

6) Imunomodulação/*priming* de células T ou células B: diferentes adjuvantes induzem notavelmente diferentes tipos de respostas adaptativas. A maioria dos agonistas para TLRs endossomais como TLR3, TLR7, TLR8, e TLR9 (Poly I:C, imiquimods, CpG, MPL) promovem o desenvolvimento de respostas imunes tipo Th1. Quil A e suas saponinas derivadas (como a QS-21) não apenas estimulam a produção de citocinas Th1 (IL-2 e IFN-γ), mas também induzem a produção de linfócitos T citotóxicos (CTLs) (TAKAHASHI *et al.*, 1990; KENSIL *et al.*, 1995; SUN *et al.*, 2009). No entanto, a alta toxicidade causada por Quil A faz com que esta saponina seja não adequada para utilização em vacinas humanas (WAITE *et al.*, 2001). Os ISCOMs induzem fortes respostas celulares T $CD8^+$ por meio de apresentação cruzada de antígenos em DCs (SCHNURR *et al.*, 2009). Além disso, desencadeiam a ativação das DCs e induzem a expressão de moléculas de MHC de classe II para a apresentação de antígenos, desencadeando respostas do tipo Th1 (SCHNURR *et al.*, 2009; DUEWELL *et al.*, 2011). Em geral, estes estudos indicam que há um enorme potencial para exploração de vários adjuvantes de forma isolada ou em combinação para induzir uma resposta mediada por anticorpos e células.



126

127 **Figura 5.** Mecanismo de ação dos adjuvantes. Adaptado de Awate et al. (AWATE *et al.*, 2013).

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3 OBJETIVOS

3.1 Objetivo geral

Aprofundar o conhecimento do mecanismo de ação de saponinas de *Quillaja brasiliensis* e suas formulações em nanopartículas tipo ISCOM, bem como suas propriedades imunoadjuvantes.

3.2 Objetivos específicos

1. Avaliar o potencial imunoadjuvante de saponinas de *Q. brasiliensis* em modelo de vacina viral;
2. Avaliar o potencial imunoadjuvante de nanopartículas tipo ISCOM formuladas com saponinas de *Quillaja brasiliensis*;
3. Avaliar o recrutamento celular e a ativação de genes relacionados ao sistema imune pela administração de QB-90 e IQB-90;
4. Determinar a toxicidade *in vitro* e *in vivo* de saponinas extraídas de *Q. brasiliensis*.

4 CAPÍTULO 3: “Cell Recruitment and Immune Related Genes Activation by Saponins and ISCOMs from *Quillaja brasiliensis* in Mice”.

Cell Recruitment and Immune Related Genes Activation by Saponins and ISCOMs from *Quillaja brasiliensis* in Mice

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Abstract

The lack of understanding the immunological mechanism of action of adjuvants has limited the rational development of adjuvants for vaccines. Saponin-based adjuvants are used to enhance humoral and cellular immune responses towards vaccine antigens, although it is not yet known how they mediate their stimulatory effects. Furthermore, the immune stimulating complex (ISCOM) as a saponin-based particulate delivery system with known immune stimulating activity and reduced toxicity. Our group has successfully formulated ISCOMs from *Quillaja brasiliensis* saponins (IQB-90) and reported the potential of IQB-90 to induce high levels of humoral and cellular immune responses. However the mechanisms that mediate its adjuvant activity have not been investigated. The goal of the present study was to elucidate some issues about the immune stimulatory properties of QB-90 and IQB-90. BALB/c mice were subcutaneously injected once in the lateral tail with QB-90 (10 µg), IQB-90 (2.5 µg) or Quil A® (10 µg) resulting in recruitment of leukocytes to spleen and draining lymph nodes (dLNs) at 24 and 48h post treatment. Flow cytometry analysis showed that QB-90 as well as IQB-90 adjuvant induced significant recruitment of neutrophils, dendritic cells (DCs), NK, B and T cells at the spleen and dLNs. A number of genes encoding mainly cytokines and chemokines were upregulated at draining lymph nodes. These observations suggest that the generation of an "immunocompetent environment" at the dLNs and recruitment of distinct immune cells to the spleen and dLNs may be an important mechanism which saponins potentiates immune responses to antigens.

Keywords: saponins; ISCOM; cell recruitment; immune-related genes; draining lymph nodes.

Introduction

Adjuvants have been used in veterinary and human vaccines for almost a century, although very few are licensed for human use, mainly because of their toxicity, adverse side effects and the lack of understanding their mechanism of action. Triterpenoid saponins called Quil A® extracted from *Quillaja saponaria* Molina have a long usage record as adjuvants (Dalsgaard, 1974; de Costa et al., 2011). These compounds of vegetal origin trigger strong immune responses characterized by high and powerful antibodies titers as well as generation of Th1/Th2 $CD4^+$cell response and $CD8^+$ cytotoxic T-cell activation (Sun et al., 2009; Takahashi et al., 1990). However, in spite of its recognized adjuvant potential, the use of Quil A® in human vaccines has been restricted due to undesirable side effects, including local reactions, haemolytic activity and even systemic toxicity (Kensil et al., 1991; Sun et al., 2009). In order to reduce the haemolytic effect that characterize these potent adjuvant, molecules have been included in several colloidal formulations which act as antigen delivery systems. In particular, immunostimulating complex (ISCOM) described for Morein et al. (Morein et al., 1984) are self-assembled structures comprised of biomolecular components as Quil A® saponins, cholesterol, phospholipid and hydrophobic antigen (Brito and O'Hagan, 2014; O'Hagan and Fox, 2015). Then it was shown that ISCOM-like structures could also form in the absence of immunogen and called these structures ISCOM matrix which is the origin of the ISCOMATRIX adjuvant (Maraskovsky et al., 2009; Pearse and Drane, 2005).

These formulated adjuvants are effective antigen delivery system with powerful immunostimulating activity and reduced toxicity tested in a variety of experimental animal models (Maraskovsky et al., 2009; Morelli et al., 2012; Sjolander et al., 2001). In all cases, these vaccines have been shown to be safe and well tolerated as well as immunogenic, generating both

antibody (Ab) and $CD4^+$ and $CD8^+$ T cell responses making this adjuvant suitable for use in both prophylactic and therapeutic vaccines (Drane et al., 2007; Maraskovsky et al., 2009; Smith et al., 2015).

Over the last decade, our group has been working on the isolation, toxicity and immunological activities of saponins from *Quillaja brasiliensis* leaves (A. St.-Hil. et Tul.) Mart. a tree native found in Southern Brazil and Uruguay. In particular, in one saponin fraction, named QB-90, which was found to have similarities with Quil A (Fleck et al., 2006). Furthermore, we have shown that QB-90 presents low toxicity when subcutaneously administered into mice and strongly potentiates the humoral and cellular immune response against viral antigens (de Costa et al., 2014; Fleck et al., 2006; Silveira et al., 2011a). Elsewhere, we built up ISCOMs with QB90, cholesterol, phospholipid and ovalbumin (OVA) as antigen that we call IQB-90. These ISCOMs promoted similar immune responses as ISCOMs formulated with Quil A.

The mechanisms of action of immune stimulatory saponin-based adjuvants are not well understood (Reed et al., 2013). In this sense, the effort of researchers in understanding the importance of saponin activity focusing on mechanisms of action is relevant. In the present study, we investigated some issues about the immune cell recruitment at dLNs and immune genes regulation by *Quillaja brasiliensis* saponins and ISCOMs built up by QB-90 in mice without antigen.

Material and methods

Ethics statement

Animal manipulation were performed in accordance with CHEA guidelines (Comisión Honoraria de Experimentación Animal) and were approved either by the Uruguayan University Research Ethics Committee (approval number 070153-000531-13) and Ethical commission on animal experimentation (CEUA) in the "Instituto de Pesquisas Veterinárias Desidério Finamor" (IPVDF). Animals were appropriately housed with controlled temperature (22 ± 2 ºC) and humidity in a 12/12 h light/dark cycle, with food and water *ad libitum*.

Saponin-derived adjuvants

QB-90, a purified saponin fraction from *Quillaja brasiliensis* leaves (Fleck et al., 2006; Kauffmann et al., 2004), ISCOMs derived from QB-90 (IQB-90) and Quil A® (QA) (Brenntag, Denmark) were used in this study. *Quillaja brasiliensis* leaves (A. St.-Hil. etTul.) Mart. was collected in Parque Battle, Montevideo, Uruguay. Saponins extraction and purification steps were carried out as previously described (Fleck et al., 2006; Kauffmann et al., 2004). IQB-90 were prepared by ethanol injection technique (Lendemans et al., 2005) and modified by Quirici et al. 2013. Formation of ISCOMs-like particles was confirmed by transmission electron microscopy (TEM).

Viral antigen preparation and mice immunization

BVDV (EVI001/94 cytopathogenic isolate) was multiplied in MDBK monolayers (Madin Darby Bovine Kidney cells; originally ATCC CCL-22) according to standard protocols. The viral suspension was inactivated with binary ethylenimine (BEI) as described previously (Bahnemann et al., 1974). The median tissue culture infectious doses ($TCID_{50}$) before

inactivation was $10^{7.5}$ $TCID_{50}$/mL. The suspension of inactivated virus (to which we refer as BVDV) was used as antigen for adjuvant testing for all assays.

Female Rockfeller mice (n=5) of the CF-1 breed (5-6 weeks old) were purchased from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, RS, Brazil). These mice were subcutaneously inoculated (100 µL) in the hind limb twice at two weeks intervals with BVDV antigen alone or with adjuvant QB-90 (50 µg). Saponins adjuvant were injected 48 or 24 h before or 24 or 48 h after antigen injection or at the same time but in the opposite limb site. Additionally at zero hour, the animals were inoculated with formulated (adjuvanted vaccine) or unformulated (two shots; one for antigen and after 5 minutes, one for adjuvant) (Table 1). Mice were bled prior to inoculations (on days 0) and 2 weeks after the second immunization (day 28); sera were kept frozen until processing.

Immunoassays for antibodies and delayed type hypersensitivity

Anti-BVDV IgG (total), IgG1 and IgG2a were determined for each serum samples by ELISA, carried out essentially as previously described (Silveira et al., 2011b) using as antigen the BVDV suspension used for mouse immunization. Antibody titres were expressed in OD_{492} nm.

DTH responses were evaluated in immunized mice on day 28. It was determined by injecting mice intradermally, in the right hind footpad, with 10 µl of BVDV antigen used for immunization, measuring the footpad thickness with a calliper, both 24 h before and 24 h after injection. The BVDV-specific response of each animal was calculated based on values of its injected footpad minus the average of the basal swelling (Silveira et al., 2011b).

Cell isolation from spleen and draining lymph node (dLNs)

Eight weeks old female BALB/c mice were purchased from DILAVE (Ministerio de Agricultura y Pesca, Uruguay) and kept at the Instituto de Higiene, (Facultad de Medicina, Uruguay). Mice were inoculated subcutaneously (s.c.) (n=15 per group) at the base of the tail with 25 µL of QB-90 (10 µg) or IQB-90 (2.5 µg) or Quil A (10 µg). Control mice received 25 µL of saline, pH 7.4. Spleen and draining lymph nodes (dLNs, inguinal) were collected 24 and 48 h post inoculation (p.i.) (5 animals per group). The remained 5 animals were used for gene expression studies. Thus, dLN were collected 24 h post saponin administration and keep in RNAlater (Ambiom) solution at -80 °C.

Spleen and dLNs were collected in ice cold PBS and processed to single cell suspensions by mechanical disaggregation and then filtered through 100 µm cell strainer (BD) to obtain a single cell suspension. Cells from two dLNs were pooled. Splenocytes were incubated in red blood cell lysis buffer for 2 min, washed with PBS containing 2% FBS and passed through a 100 µm cell strainer. Cells were suspended in staining buffer (PBS, pH 7.4, 0.5% BSA, 2 m M EDTA and 0.1% sodium azide). The cell numbers for each sample was counted using automated apparatus (Countess® Automated Cell Counter, Life technologies™).

Flow cytometry analysis

Cell suspensions were prepared as described above and incubated for 20 min at 4 °C with rat serum (10% in FACS buffer). Cells were then transferred to a 96-well microtiter plate and incubated with antibodies for 30 min at 4 °C (5×10^5 cells, 100 µl/well). Antibodies used were anti-mouse CD3:PE (145-2C11), CD4:APC-Cy7 (RM4-5), CD8:PerCP(53-6.7), CD19:FITC (DX5), CD49:FITC (H1.2F3), MHC-II:FITC (I-A/I-E, 2G9), Gr-1:PE-Cy7 (RB6-8C5), CD11c:APC. All staining procedures were conducted on ice and reagents were purchased from

Life Technologies. Cell populations were analyzed using a FACScanto II flow cytometer (BD Biosciences). Retrieved data was analyzed using the FlowJo 7.6.2 software.

RNA extraction and immune-related gene expression

Changes in mouse immune-related gene expression in dLN from QB-90 and IQB-90 administration were detected using a TaqMan® Mouse Immune Array with the 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). For these studies, RNA was isolated from dLN at 24 h post adjuvant administration (or mock administration, with saline). Each experimental sample represents five pooled dLN collected from mice. Prior to RNA isolation, dLN were homogenized in 1 mL TRIzol. RNA was further purified using Pure Link RNA mini Kit (Life Technologies). Fold change was calculated using the Pfaffl method ($2^{-\Delta\Delta Ct}$) with dLN from QB-90 and IQB-90 and being compared with saline mock group (Pfaffl, 2001).

Statistical analysis

Data were expressed as mean ± SEM and examined for statistical significance on ANOVA with Dunnet post test (GraphPad Prism 5.01 for Windows, GraphPad Software, San Diego, California, USA). Statistical significance was assigned at a p value of ≤ 0.05.

Results

QB90 saponins promotes a transient 'immunocompetent environment' at the injection site and its immunoadjuvant activity independs of antigen ligation

In order to demonstrate that the mechanism of QB-90 action is independent of the binding with the antigen, the antigen and adjuvant portions of the vaccine were injected into proximal, as well as in distal sites at different times. The results in Figure 1 show that QB-90 induces a strong immune response characterized for the similar high antigen-specific antibody titers (IgG, IgG1 and IgG2a) and a robust DTH reaction when the adjuvant saponins were formulated or unformulated administered up to 24 h before the antigen (BVDV). However, if the adjuvant was administered before (48 h) or later (24 and 48 h) there was no adjuvant effect (data not shown).

Summing up, our results showed that QB-90 inoculated before the antigen works as a potent adjuvant with the ability to stimulate the immune response at systemic level promoting a transient 'immunocompetent environment' that could be exploited by co-administration of antigen. These results suggest that QB-90 action mechanism is not dependent on antigen binding.

Increased cellularity in dLNs and spleen after QB-90 or IQB-90 administration

Following s.c. injection with QB-90, IQB-90 or Quil A, single-cell suspensions were prepared from spleen and dLNs and analyzed after 24 and 48 h. In spleen from QB-90 and IQB-90-treated mice, the cells number were significantly increased compared to the saline control at 24 h and 48 h ($P<0.001$, Figure 2A). In dLNs from QB-90 and IQB-90-treated mice, a significant increase on cell numbers were detected in 24 h ($P<0.001$), but only IQB-90 at 48 h maintained this increase ($P<0.01$) (Figure 2A and 2B). However, within the group inoculated with Quil A

353 did not revealed any detectable rise neither in spleen cell numbers nor in dLNs cell numbers
354 (Figure 2A and 2B). After 48 h spleen from QB-90 and IQB-90-inoculated mice contained
355 significantly higher cell numbers than 24h p.i. On the other side, a decrease in dLN cell numbers
356 was observed.

357 *QB-90 and IQB-90 recruits and activates immune cells in spleen and dLNs*

358 In spleen, neutrophil ($Gr1^{high}$) cells, NK ($CD49^{+}CD3^{-}$), B cells ($CD19^{+}$) and T cells
359 populations identified as $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ were higher in QB-90 and IQB-90-
360 inoculated mice compared to saline group 24 and 48 h post injection (Figure 3A-F). Moreover,
361 the DCs populations, identified as CD11c expressing MHC class II, significantly increased in
362 IQB-90-treated mice only 48 h post injection ($P \leq 0.05$, Figure 3C). Surprisingly, Quil A-
363 inoculated mice showed a significant increase in neutrophil, NK, B cells and $CD4^{+}$ and $CD8^{+}$ T
364 cells populations only at 48 h p.i. ($P \leq 0.05$, Figure 3A-F).

365 In dLNs from QB-90 and IQB-90-inoculated groups, neutrophil, NK, DC, B cells and
366 CD4 and CD8 T cells were significantly increased at 24 h p.i. (Figure 4A-F), with exception in
367 NK cells from IQB-90 group. Regarding Quil A, DC at 24 h and CD8 T cells at 48 h
368 increased significantly when compared with the saline group.

369 *Analysis of gene expression in the draining lymph node*

370 The expression of ninety two genes related to immune function in draining lymph nodes
371 was analyzed by qPCR (supplemental Table 1). In QB-90-treated group, 44 genes were
372 differentially expressed (as compared with saline-treated group) whereas in IQB-90-treated mice
373 23 genes were differentially expressed (Figure 5 and 6). The most prominent host gene
374 alterations only present in QB-90-treated mice involves up regulation of Ccr7, Cd28, Il12b, Il1b,

Nos2, Prf1 and Lif and down regulation of Il7 and Fn1. In IQB-90 treated mice, only four genes were different from those activated by both treatments: Il2 and Il4 were down regulated and Il9 and Lta were up regulated (Figure 6). Nineteen genes were found differentially expressed in both groups (Table 1).

Three chemokine genes were up regulated: Ccl2, a chemotactic factor that attracts monocytes, Ccl3, with chemotactic activity for eosinophils and Cxcl10, which participate in T-cell effector function and T-cell development. Csf2, Ctla4, Ifng, Il10, Il15, Il5, Il6 and Fasl were up regulated, however the most up regulated cytokine gene was Cxcl11. This cytokine is a chemotactic factor for interleukin-activated T-cells, but not for unstimulated T-cells, neutrophils or monocytes. Six other genes were up-regulated: Fas, Edn1, Ptgs2, Socs1, Stat1 and Gzmb. One gene was down regulated in both treatments: Lpr2. The function of this gene is not well understood.

Discussion

Adjuvants are used in many vaccines, but their mechanisms of action are not fully understood. However, recent insights into the innate immune system and its importance in initiating the adaptive immune response have sparked the rational design and development of the next generation of adjuvants (De Gregorio et al., 2013; O'Hagan and Fox, 2015). In this regard, the adjuvant potential of natural products and saponins, in particular, has been largely explored. Thus, saponins and saponins-based formulations as ISCOM or ISCOMATRIX have been studied for their potential to trigger powerful immune responses, but their mode of action as adjuvants is not well understood (Morelli et al., 2012).

Results showed that QB-90 when first administered, up to 24 h before the antigen, the adjuvant effect was maintained, as long as the antigen was administered at the same site. These results show that QB-90 promote an "immunocompetent environment". However, when the antigen was administered before the adjuvant, there was no adjuvant effect. Similar results have been described for MF59®, an oil-in-water emulsion adjuvant. In this report the authors suggested that MF59 droplets activate the immune system in the absence of antigen and that the activation persist for at least 24 h (Ott et al., 1995). In conclusion, our results suggest that QB-90 is a potent adjuvant with the ability to stimulate the immune response and promotes a transient 'immunocompetent environment' like a MF59 that could be exploited by co administration of antigen.

In this study we also show that spleen from QB-90 and IQB-90-inoculated mice were higher and had significantly increased cellularity compared to control group, indicating a high cell recruitment in analyzed sites. Similar effects were not seen for Quil A although shares similarities in promoting effective and specific immune responses (de Costa et al., 2014; Silveira

et al., 2011a). With regard to the QB-90-inoculated mice, this was the only group that shows significantly differences between 24 and 48 h post treatment. In the spleen at 48 h post treatment QB-90-inoculated mice had significantly increased cellularity compared at the same 24 h, however in the dLNs the significantly differences was evidenced at 24 h p.i. for IQB-90 and Quil A, in contrast, did not induce differential cell recruitment 24 or 48 h p.i.

Further supported by flow cytometry data, we show that QB-9O as well as IQB-90 administration results in immune stimulation with recruitment of neutrophil, NK cells, B-cells and T-cells and DCs after 24-48 h in spleen and dLNs. These results are in agreement with previous findings with a similar adjuvant where recruitment of these cells populations to spleen and dLNs (Magnusson et al., 2013; Reimer et al., 2012). In line with this observation, it has further been shown that first sign of immune cell reaction observed in mice inoculated with ISCOMATRIX or ISCOM vaccines is an increase in the number of NK cells, B-cells, T-cells, DCs and granulocytes detected within the lymph nodes at 6 h and peaking by 24–48 h following vaccination (Morelli et al., 2012). This is a transient and reversible efflux of cells, declining to normal levels by 72 h post-vaccination (Wilson *et al.*, 2011; Duewell*et al.*, 2011).

Moreover it has been shown that saponins in solution have a potent ability to produce the migration of neutrophils to the injection site, and the presence of these cells was persistent at different time points (Vitoriano-Souza et al., 2012). At the injection site, neutrophils attract other immune cells by producing increased amounts of chemokines and transport antigens to the draining lymph nodes (Calabro et al., 2011; Moreletal., 2011). In this regard, neutrophils are the most abundant cellular component of the host immune system and primary mediators of the innate immune response to invading microorganisms. The ability of neutrophils to rapidly kill invading microbes is indispensable for maintaining host health (Kobayashi and DeLeo, 2009).

However, the role of neutrophils in adjuvant activity is not completely clear. These cell populations may play an important role as the vehicle for transport vaccine antigen into the draining lymph nodes for further influence the activation of different leukocyte types including NK cells, B cells, and DCs (Schuster et al., 2013).

ISCOMATRIX vaccines rapidly traffic to the dLN within the first 2 h after injection in mice, loading lymph node-resident DCs and other antigen presenting cells (APCs) (Wilson et al., 2012). Another work emphasized that DCs and APCs at the injection site will also traffic captured ISCOMATRIX vaccine from the injection site into the dLN, but their contribution occurs later (24–48 h) (Morelli et al., 2012). Together, these two waves of vaccine antigen trafficking (direct trafficking of adjuvant and via peripheral APC-transport) result in prolonged antigen presentation. This is in contrast to other adjuvant systems that predominantly remain within the injection site and require uptake locally by innate immune effectors or APCs such as CD11b+ monocytes for transportation to the dLN (Mosca et al., 2008). In the present study we observed that DC expressing MHCII was increased in spleen and dLNs after QB-90 or IQB-90 administration at 24 and 48 h post treatment. Moreover, in the spleen these cell populations show significantly increasing at 48 h with respect to 24 h post treatment.

ISCOMs and ISCOMATRIX enhance antigen uptake and prolong retention by DCs in draining lymph nodes, induce activation of DCs, and lead to strong antibody and T cell responses (Duewell et al., 2011; Maraskovsky et al., 2009). Interestingly, our results show that QB-90 and ImQB-90-treated mice were also capable of inducing B- and T-cell ($CD4^+$ and $CD8^+$). These feeding agree with previously publish data showing the capacity of saponins, ISCOMs as well as ISCOMATIRX adjuvant vaccine to stimulates robust and detectable B-, $CD4^+$ and $CD8^+$ T-cell response .

NK cells increased in spleen and dLNs after QB-90 and IQMB-90-treatment. NK cells are also a major source of the type 1 cytokine IFN-γ, and other cytokines and chemokines. Production of these soluble factors by NK cells in early innate immune responses significantly influences the recruitment and function of other hematopoietic cells. For example, NK cells enhance antigen-specific T-cell responses under conditions. Also NK cells are central players in a regulatory crosstalk network with dendritic cells and neutrophils to promote or restrain immune responses (Campbell and Hasegawa, 2013).

As it's was detailed above our studies showed that QB-90 trigger a strong immune response characterized by an balanced IgG1/IgG2a-antibody response, Th1 cytokine profile and a robust DTH reaction (de Costa et al., 2014; Silveira et al., 2011a). In the present study, we show that QB-90- and IQB-90-treatment results in a significantly increased cellularity compared to the QA saponins adjuvants, indicating an immune activation in the spleen and dLNs. The numbers of cells in spleen and dLNs increased after 24- and 48h in IQB-90-treated mice relative to control group. Although the same results were obtained with QB-90-treated mice in spleen, in dLNs the significantly increase was only detected at 24h. This finding was supported by flow cytometry data, showing increased levels of neutrophil, macrophages and lymphocyte populations.

In summary, early immunostimulatory properties by QB-90 and IQB-90 were demonstrated. QB-90 and IQB-90 promoted increase in neutrophils, NK cells, DCs, B-cells and T-cells after 24- and 48 h following administration, resulted in a local transient immune response with recruitment and activation of central immune cells. Moreover, immune-related genes, mainly cytokines and chemokines, were overexpressed in dLNs.

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577 **Table 1.** Genes differentially expressed over two treatments: QB-90 and IQB-90.

Gene symbol	Gene name	Group	QB-90	IQB-90
Ccl2	chemokine (C-C motif) ligand 2	Chemokine	17,2	12,5
Ccl3	chemokine (C-C motif) ligand 3		8,7	2,6
Cxcl10	chemokine (C-X-C motif) ligand 10		15,2	9,2
Cxcl11	chemokine (C-X-C motif) ligand 11	Cytokine	69,8	32,4
Csf2	colony stimulating factor 2 (granulocyte-macrophage)		10,3	2,5
Ctla4	cytotoxic T-lymphocyte-associated protein 4		5,1	2,6
Ifng	interferon gamma		20,4	11,3
Il10	interleukin 10		3,9	3,9
Il15	interleukin 15		3,4	2,7
Il5	interleukin 5		47,2	100,7
Il6	interleukin 6		22,9	28,3
Fasl	Fas ligand (TNF superfamily, member 6)		7,9	2,5
Fas	Fas (TNF receptor superfamily member 6)	Cytokine receptor	2,3	2,2
Edn1	endothelin 1	Peptide hormone	2,2	2,2
Ptgs2	prostaglandin-endoperoxide synthase 2	Synthase	16,6	8,2
Socs1	suppressor of cytokine signaling 1	Kinase modulator	9,2	5,1
Stat1	signal transducer and activator of transcription 1	Transcription factor	6,7	3,9
Gzmb	granzyme B	Serine protease	42,5	10,8
Lrp2	low density lipoprotein receptor-related protein 2	Unclassified	-13,1	-2,0

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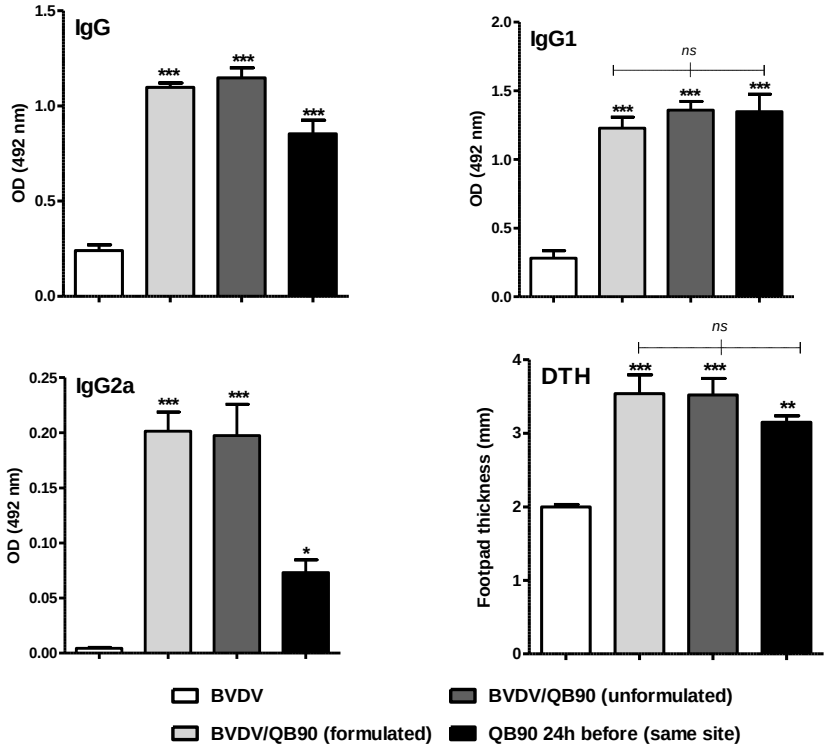
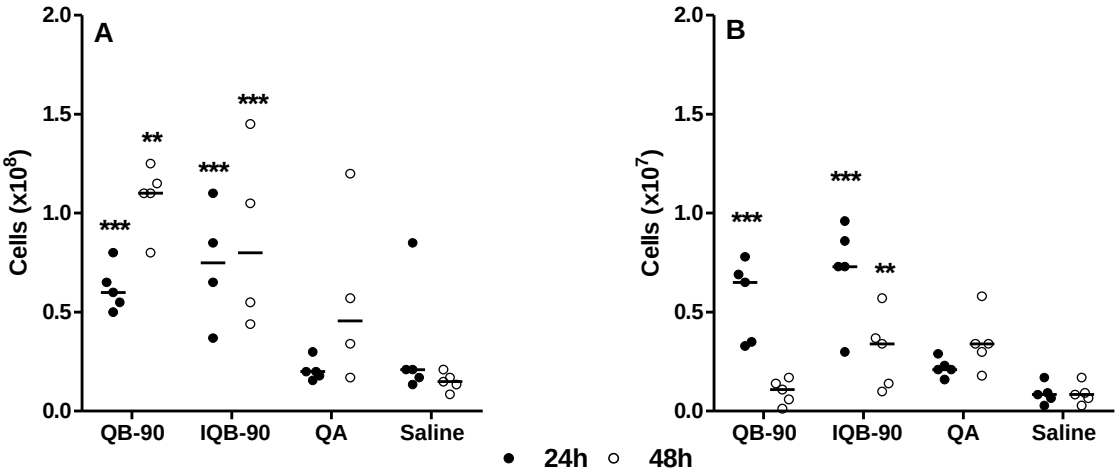


582

583 **Figure 1.** QB-90 activity is not dependent on antigen absorption. Female CF1 mice (n=5) were
584 immunized s.c. in the hind limb twice at two weeks intervals with BVDV antigen alone or with
585 QB90 (50 µg). Adjuvant were injected 48, 24 h before or 24, 48 h after antigen injection or at the
586 same time, but in the opposite limb side. Additionally, at zero hour the animals were inoculated
587 with formulated and unformulated vaccine. Serum titres of anti-BVDV total IgG, IgG1 and
588 IgG2a isotypes are expressed as the mean value ± SEM of OD at 492 nm. DTH responses were
589 expressed as the mean value of footpad thickness ± SEM. Significant differences are indicated:
590 *(P<0.05), **(P<0.01) and ***(P<0.001), with the group immunized with only antigen.
591



592

593 **Figure 2.** Cell numbers in spleen and dLNs after adjuvants treatment. QB-90 (10 µg), IQB-90
594 (2.5 µg) or Quil A (10 µg) were injected subcutaneously at the base of the tail. After 24 and 48h
595 cells from spleen (A) and dLNs (B) were prepared and total cell counts were performed. The
596 relative difference in cell numbers among QB-90, IQB-90, Quil A and saline-inoculated mice are
597 presented. Median values (n=4-5) are shown. Significant differences between adjuvants and
598 saline treated mice was performed using ANOVA with Dunnet post test are indicated with
599 **($P \leq 0.01$); ***($P \leq 0.001$).
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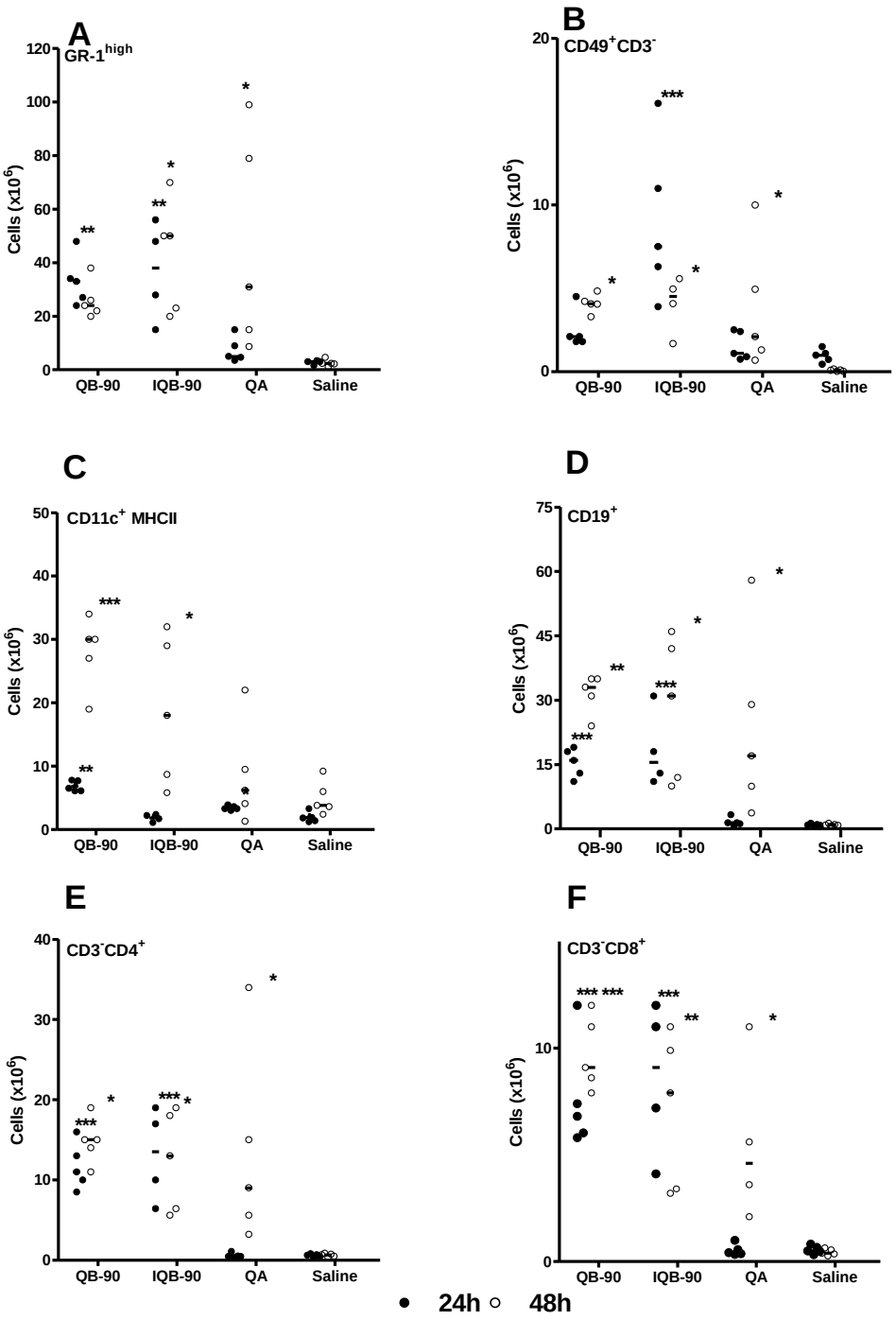


Figure 3. Cell populations in spleen after saponin-based adjuvants inoculation. QB-90 (10 µg), IQB-90 (2.5 µg) or Quil A (10 µg) were subcutaneously injected at the base of the tail and 24 and 48 h post treatment cells were analyzed by flow cytometry. Median values (n = 4-5) are shown. Significant differences between adjuvants and saline treated mice using ANOVA with Dunnet post test are indicated with *($P \leq 0.05$); **($P \leq 0.01$); ***($P \leq 0.001$).

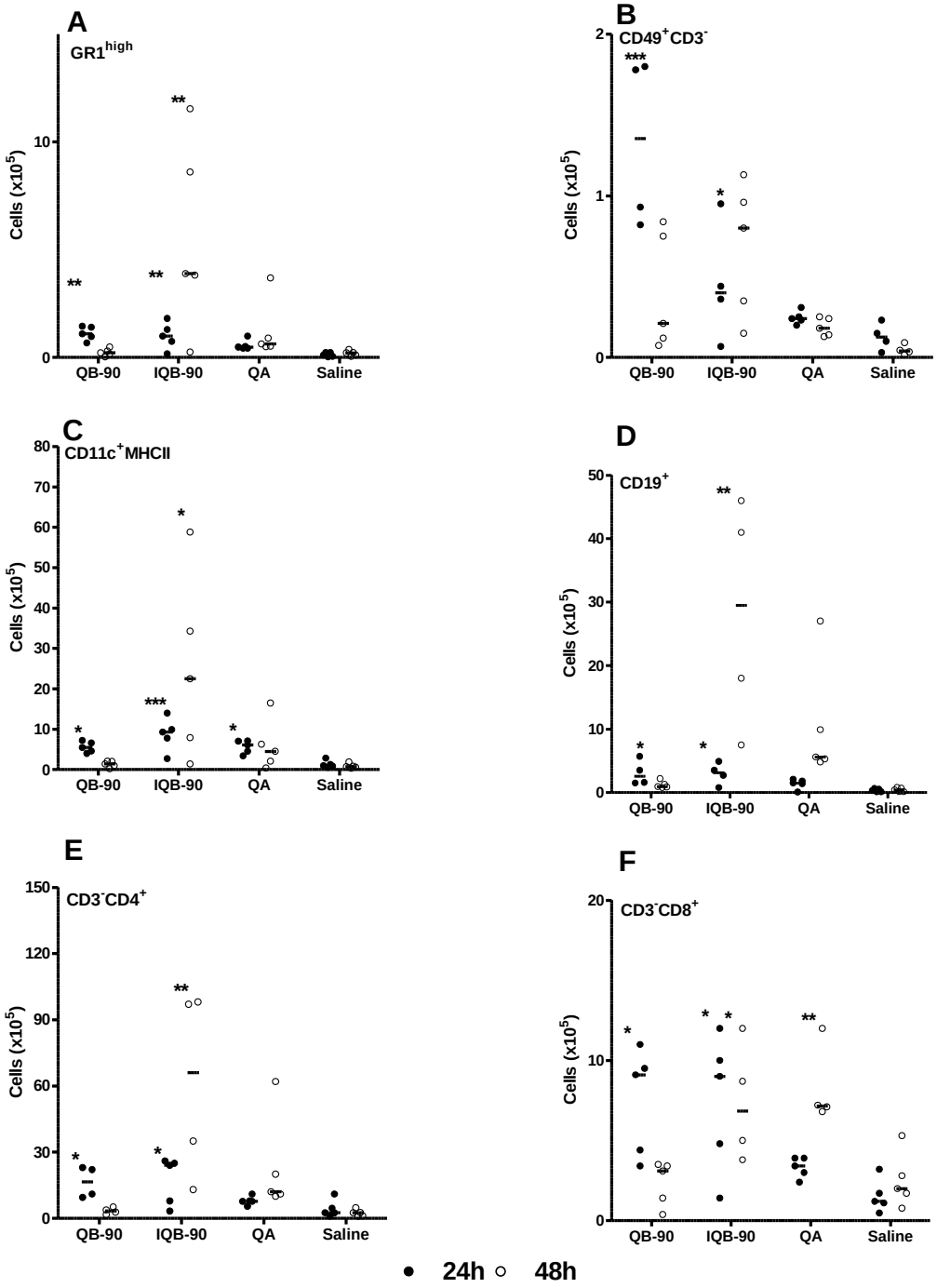
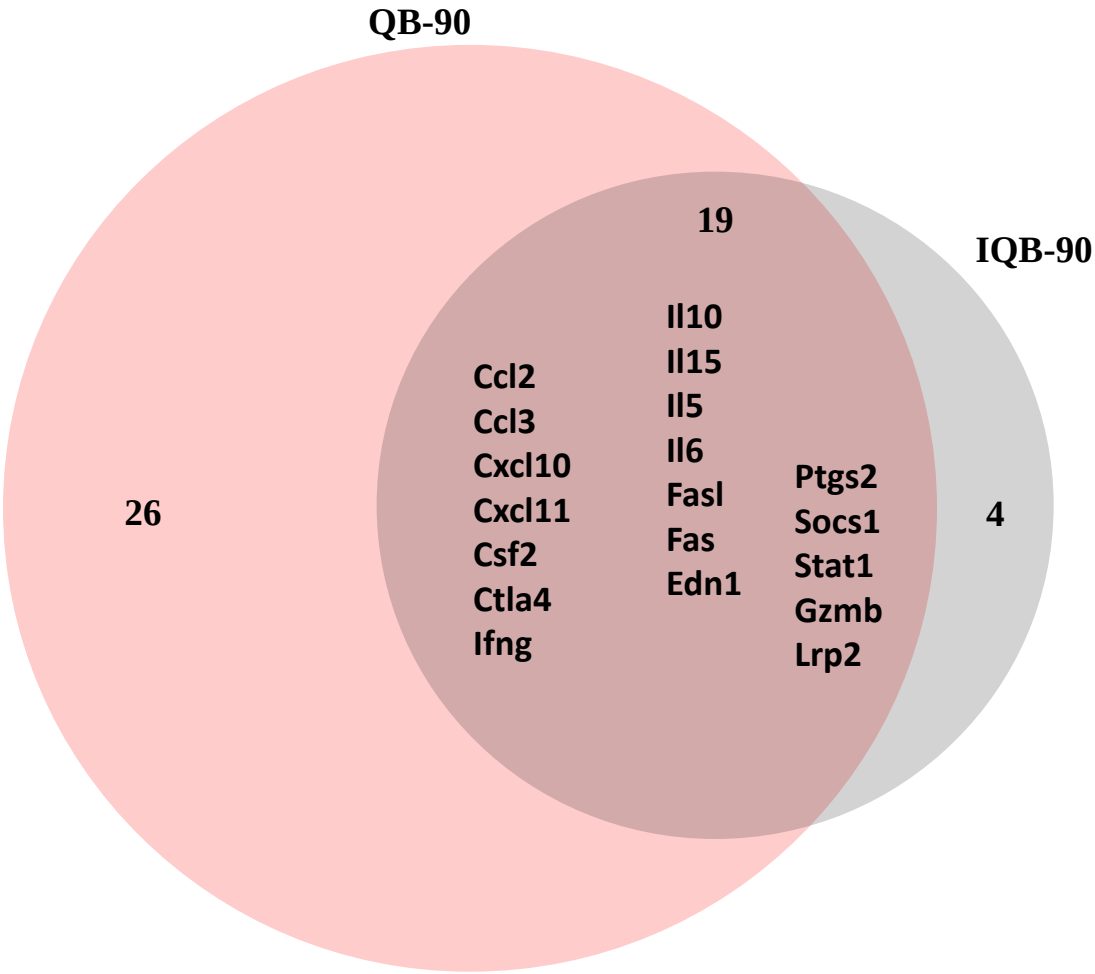


Figure 4. Cell populations in dLNs after saponin-based adjuvants inoculation. QB-90 (10 µg), IQB-90 (2.5 µg) or Quil A (10 µg) were subcutaneously injected at the base of the tail and 24 and 48 h post treatment, the cells were analyzed by flow cytometry. Median values (n = 4-5) are shown. Significant differences between adjuvants and saline treated mice using ANOVA with Dunnet post test are indicated with *($P \leq 0.05$); **($P \leq 0.01$); ***($P \leq 0.001$).



616

617 **Figure 5.** Venn diagram of genes differentially expressed with a fold change ≥2 in QB-90 and
618 IQB-90-treated groups compared to saline treated group.

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	QB-90	IQB-90		QB-90	IQB-90		QB-90	IQB-90		QB-90	IQB-90		QB-90	IQB-90
Agtr2			**Cd40lg**	*		**H2-Ea**			**Il5**	***	***	**Stat1**	**	*
Bax			**Cd68**			**H2-Eb1**			**Il6**	***	***	**Stat3**	*	
Bcl2			**Cd80**	*		**Hmox1**			**Il7**	*		**Stat4**	*	
Bcl2l1			**Cd86**	*		**Icos**			**Il9**		**	**Stat6**		
C3			**Cd8a**	*		**Ifng**	***	***	**Lrp2**	***	*	**Tbx21**	*	
Ccl19	*		**Csf1**			**Ikbkb**			**Lta**		*	**Tgfb1**		
Ccl2	***	***	**Csf2**	***	*	**Il10**	*	*	**Nfkb1**			**Tnf**	*	
Ccl3	**	*	**Csf3**			**Il12a**			**Nfkb2**			**Tnfrsf18**	*	
Ccl5			**Ctla4**	**	*	**Il12b**	*		**Nos2**	*		**Vcam1**		
Ccr2			**Cxcl10**	***	**	**Il13**			**Prf1**	**		**Vegfa**		
Ccr4			**Cxcl11**	***	***	**Il15**	*	*	**Ptgs2**	***	**	**Ace**		
Ccr7	*		**Cxcr3**			**Il17a**			**Ptprc**			**Icam1**	*	
Cd19			**Cyp1a2**			**Il18**			**Sele**			**Lif**	*	
Cd28	*		**Cyp7a1**			**Il1a**			**Selp**			**Ly96**		
Cd34			**Edn1**	*	*	**Il1b**	*		**Ski**			**Nfatc3**		
Cd38			**Fas**	*	*	**Il2**		*	**Smad3**			**Nfatc4**		
Cd3e	*		**Fasl**	**	*	**Il2ra**	*		**Smad7**	*				
Cd4			**Fn1**	*		**Il3**			**Socs1**	**	**			
Cd40	*		**Gzmb**	***	***	**Il4**		**	**Socs2**	*				

Figure 6. Analysis of the gene expression of immune related genes in the draining iliac lymph node 24 h post injection of QB-90 and IQB-90, as determined by quantitative PCR. Red boxes: up regulation; blue boxes: down regulation. * refers to twofold variations (≥ or ≤) 2; **fivefold ≥ 5; ***tenfold ≥ 10.

Supplemental Table 1.

Gene name	Category	Group	Gene Symbol	QB-90	IQB-90
angiotensin II receptor, type 2	Receptor	G-protein coupled receptor	Agtr2	1,00	-1,33
Bcl2-associated X protein	Miscellaneous function	Other miscellaneous function protein	Bax	1,55	1,08
B-cell leukemia/lymphoma 2	Miscellaneous function	Other miscellaneous function protein	Bcl2	1,37	1,10
Bcl2-like 1	Miscellaneous function	Other miscellaneous function protein	Bcl2l1	1,18	1,32
complement component 3	Select regulatory molecule	Protease inhibitor	C3	1,17	-1,14
chemokine (C-C motif) ligand 19	Signaling molecule	Chemokine	Ccl19	2,46	1,08
chemokine (C-C motif) ligand 2	Signaling molecule	Chemokine	Ccl2	17,22	12,49
chemokine (C-C motif) ligand 3	Signaling molecule	Chemokine	Ccl3	8,67	2,60
chemokine (C-C motif) ligand 5	Signaling molecule	Chemokine	Ccl5	1,55	-1,50
chemokine (C-C motif) receptor 2	Receptor	G-protein coupled receptor	Ccr2	1,52	1,40
chemokine (C-C motif) receptor 4	Receptor	G-protein coupled receptor	Ccr4	0,93	-1,37
chemokine (C-C motif) receptor 7	Receptor	G-protein coupled receptor	Ccr7	3,99	1,30
CD19 antigen	Defense/immunity protein	Immunoglobulin receptor family member	Cd19	1,27	-1,60
CD28 antigen	Defense/immunity protein	Immunoglobulin receptor family member	Cd28	3,44	1,61
CD34 antigen	Cell adhesion molecule	Cell adhesion molecule	Cd34	-1,34	1,02
CD38 antigen	Hydrolase	Glycosidase	Cd38	1,31	-1,20
CD3 antigen, epsilon polypeptide	Defense/immunity protein	Immunoglobulin receptor family member	Cd3e	2,82	-1,06
CD4 antigen	Defense/immunity	Immunoglobulin receptor	Cd4	1,86	-1,18

	protein	family member			
CD40 antigen	Receptor	Cytokine receptor	Cd40	2,61	1,20
CD40 ligand	Signaling molecule	Cytokine	Cd40lg	2,26	1,00
CD68 antigen	Molecular function unclassified	Molecular function unclassified	Cd68	0,98	-1,12
CD80 antigen	Signaling molecule	Membrane-bound signaling molecule	Cd80	2,61	1,71
CD86 antigen	Signaling molecule	Membrane-bound signaling molecule	Cd86	2,58	1,50
CD8 antigen, alpha chain	Defense/immunity protein	Immunoglobulin receptor family member	Cd8a	2,64	1,15
colony stimulating factor 1 (macrophage)	Signaling molecule	Cytokine	Csf1	1,37	1,73
colony stimulating factor 2 (granulocyte-macrophage)	Signaling molecule	Cytokine	Csf2	10,29	2,52
colony stimulating factor 3 (granulocyte)	Signaling molecule	Cytokine	Csf3	1,77	-1,09
cytotoxic T-lymphocyte-associated protein 4	Signaling molecule	Cytokine	Ctla4	5,14	2,62
chemokine (C-X-C motif) ligand 10	Signaling molecule	Chemokine	Cxcl10	15,20	9,20
chemokine (C-X-C motif) ligand 11	Signaling molecule	Cytokine	Cxcl11	69,82	32,39
chemokine (C-X-C motif) receptor 3	Receptor	G-protein coupled receptor	Cxcr3	1,05	-1,71
cytochrome P450, family 1, subfamily a, polypeptide 2	Oxidoreductase	Oxygenase	Cyp1a2	1,00	1,00
cytochrome P450, family 7, subfamily a, polypeptide 1	Oxidoreductase	Oxygenase	Cyp7a1	1,00	1,00
endothelin 1	Signaling molecule	Peptide hormone	Edn1	2,16	2,15
Fas (TNF receptor superfamily member 6)	Receptor	Cytokine receptor	Fas	2,34	2,15
Fas ligand (TNF superfamily, member 6)	Signaling molecule	Cytokine	Fasl	7,95	2,50
fibronectin 1	Extracellular matrix	Extracellular matrix linker protein	Fn1	-2,59	1,57
granzyme B	Protease	Serine protease	Gzmb	42,54	10,78
histocompatibility 2, class II antigen E alpha	Defense/immunity protein	Major histocompatibility complex antigen	H2-Ea	1,18	0,88

histocompatibility 2, class II antigen E beta	Defense/immunity protein	Major histocompatibility complex antigen	H2-Eb1	1,00	0,65
heme oxygenase (decycling) 1	Oxidoreductase	Oxygenase	Hmox1	-1,17	1,34
inducible T-cell co-stimulator	Defense/immunity protein	Immunoglobulin receptor family member	Icos	1,62	1,07
interferon gamma	Signaling molecule	Cytokine	Ifng	20,37	11,26
inhibitor of kappaB kinase beta	Kinase	Protein kinase	Ikbkb	1,32	1,18
interleukin 10	Signaling molecule	Cytokine	Il10	3,93	3,89
interleukin 12a	Signaling molecule	Cytokine	Il12a	1,42	0,64
interleukin 12b	Signaling molecule	Cytokine	Il12b	3,77	1,61
interleukin 13	Signaling molecule	Cytokine	Il13	1,19	-1,64
interleukin 15	Signaling molecule	Cytokine	Il15	3,40	2,72
interleukin 17A	Signaling molecule	Cytokine	Il17a	1,00	1,00
interleukin 18	Signaling molecule	Cytokine	Il18	1,03	1,01
interleukin 1 alpha	Signaling molecule	Cytokine	Il1a	1,64	1,02
interleukin 1 beta	Signaling molecule	Cytokine	Il1b	3,05	1,82
interleukin 2	Signaling molecule	Cytokine	Il2	1,04	-2,32
interleukin 2 receptor, alpha chain	Receptor	Cytokine receptor	Il2ra	2,02	1,03
interleukin 3	Signaling molecule	Cytokine	Il3	1,19	1,28
interleukin 4	Signaling molecule	Cytokine	Il4	-1,39	-8,83
interleukin 5	Signaling molecule	Cytokine	Il5	47,21	100,65
interleukin 6	Signaling molecule	Cytokine	Il6	22,90	28,31
interleukin 7	Signaling molecule	Cytokine	Il7	-4,15	-1,16
interleukin 9	Signaling molecule	Cytokine	Il9	1,32	6,88
low density lipoprotein receptor-related protein 2	Molecular function unclassified	Molecular function unclassified	Lrp2	-13,10	-2,03
lymphotoxin A	Signaling molecule	Cytokine	Lta	1,39	4,95
nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	Transcription factor	Other transcription factor	Nfkb1	0,81	1,26
nuclear factor of kappa light polypeptide gene	Transcription factor	Other transcription factor	Nfkb2	1,91	0,95

enhancer in B-cells 2, p49/p100					
nitric oxide synthase 2, inducible, macrophage	Synthase and synthetase	Synthase	Nos2	3,00	1,53
perforin 1 (pore forming protein)	Defense/immunity protein	Other defense and immunity protein	Prf1	5,11	1,72
prostaglandin-endoperoxide synthase 2	Synthase and synthetase	Synthase	Ptgs2	16,63	8,22
protein tyrosine phosphatase, receptor type, C	Receptor	Other receptor	Ptprc	1,94	1,31
selectin, endothelial cell	Cell adhesion molecule	Other cell adhesion molecule	Sele	-1,15	1,17
selectin, platelet	Cell adhesion molecule	Other cell adhesion molecule	Selp	1,17	1,49
ski sarcoma viral oncogene homolog (avian)	Transcription factor	Other transcription factor	Ski	0,95	-1,26
MAD homolog 3 (Drosophila)	Transcription factor	Other transcription factor	Smad3	1,40	1,41
MAD homolog 7 (Drosophila)	Transcription factor	Other transcription factor	Smad7	2,05	0,98
suppressor of cytokine signaling 1	Select regulatory molecule	Kinase modulator	Socs1	9,20	5,12
suppressor of cytokine signaling 2	Signaling molecule	Other signaling molecule	Socs2	2,00	1,50
signal transducer and activator of transcription 1	Transcription factor	Other transcription factor	Stat1	6,69	3,87
signal transducer and activator of transcription 3	Transcription factor	Other transcription factor	Stat3	2,61	1,93
signal transducer and activator of transcription 4	Transcription factor	Other transcription factor	Stat4	2,84	1,63
signal transducer and activator of transcription 6	Transcription factor	Other transcription factor	Stat6	1,78	0,97
T-box 21	Transcription factor	Other transcription factor	Tbx21	2,71	-1,09
transforming growth factor, beta 1	Signaling molecule	Cytokine	Tgfb1	1,44	-1,38
tumor necrosis factor	Signaling molecule	Cytokine	Tnf	2,77	1,13
tumor necrosis factor receptor superfamily, member 18	Receptor	Receptor	Tnfrsf18	2,65	1,19
vascular cell adhesion molecule 1	Cell adhesion molecule	Cam family adhesion molecule	Vcam1	1,60	1,05
vascular endothelial growth factor A	Signaling molecule	Growth factor	Vegfa	-1,45	0,96
angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	Protease	Metalloprotease	Ace	-1,30	1,51

intercellular adhesion molecule	Cell adhesion molecule	Cam family adhesion molecule	Icam1	2,15	1,48
leukemia inhibitory factor	Signaling molecule	Cytokine	Lif	2,98	1,53
lymphocyte antigen 96	Miscellaneous function	Transmembrane receptor regulatory/adaptor protein	Ly96	0,97	1,01
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	Transcription factor	Other transcription factor	Nfatc3	1,72	1,11
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Molecular function unclassified	Molecular function unclassified	Nfatc4	-1,63	-1,10

5 CAPÍTULO 2: "*Quillaja brasiliensis* saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice".

Quillaja brasiliensis saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice.

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Abstract

Triterpenoid saponins extracted from *Quillaja saponaria* Molina have a long history of usage as vaccine adjuvants, from which the purified fraction "Quil A®" is the major – as well as the most studied - representative. A similar saponin fraction has been extracted from *Quillaja brasiliensis* leaves, named QB-90, which was demonstrated to possess adjuvant activity at levels comparable to Quil A® yet with decreased toxicity. The aim of this study was evaluate the adjuvant potential of QB-90, as well as a semi-purified aqueous extract (AE) of *Q. brasilienis* in a bovine viral diarrhea virus (BVDV) vaccine in mice. The animals were immunized twice (day 0 and day 14) either with BVDV antigen plus QB-90 or AE and its immune stimulating capacity compared to that induced by an oil-adjuvanted vaccine. Humoral and cellular immunity were evaluated two weeks after boosting. Antibodies were measured by indirect ELISA and cellular immunity by delayed type hypersensitivity (DTH), lymphoproliferation, cytokine release and single cell IFN-γ production. Serum levels of anti-BVDV IgG, IgG1 and IgG2b were significantly increased in the QB-90- and AE-adjuvanted vaccines in comparison to the results obtained with the oil-adjuvanted vaccine. A robust DTH response was observed in mice immunized with QB-90, as well as increased splenocyte proliferation and high levels of Th1-type cytokines. The QB-90 adjuvanted vaccine was also shown to induce production of IFN-γ by CD4- and CD8-T lymphocytes. The AE, despite stimulating humoral responses, did not stimulate significantly cellular immune responses. These findings reveal that QB-90, when employed as an adjuvant to BVDV antigens, elicits robust cellular and humoral immune responses in mice.

Keywords: vaccine; adjuvant; *Quillaja brasiliensis*; QB-90; BVDV; immune response; cellular immunity.

Introduction

Bovine viral diarrhea (BVD) is a viral disease with major significance in cattle, where it may be responsible for huge economic losses. Acute BVDV infections are common, usually resulting in mild disease characterized by fever, increased respiratory rate, diarrhea and a reduction of white blood cells. Although animals generally recover, the effect of BVDV on the immune system reduces the host's resistance to diseases. In addition, BVDV infections in pregnant cows can give rise to abortions, malformations, calves with deficits in sizes or weight gain, size and persistently infected calves, which represent a major source for virus dissemination in herds.

Vaccination, aims to mimic the development of acquired immunity by inoculation of immunogenic components of a particular pathogen or closely related microorganisms (Meeusen et al., 2007). Nonliving vaccine antigens, especially purified or recombinant subunit vaccines, are often poorly immunogenic and require additional components to help stimulate protective immunity based on antibodies and effector T cell functions (O'Hagan and Fox, 2015; Reed et al., 2013). An appropriate adjuvant, capable of inducing the adequate type of immune response to antigens, boosting the immune system for both humoral and cellular immune response, would be highly advantageous to the vaccine industry (Sun et al., 2009). Thus, there is a broadly recognized need for the development of new adjuvants to improve immunogenicity.

Triterpenoid saponins extracted from *Quillaja saponaria* Molina have a long usage record as adjuvants in veterinary vaccines (Sparg et al., 2004; Sun et al., 2009). Furthermore, saponins of *Quillaja brasiliensis* (Quillajaceae), a native tree from South America, have been evaluated to enhance immune responses to antigens (de Costa et al., 2014; Fleck et al., 2006; Silveira et al., 2011). It has further been demonstrated that QB-90 saponin fraction and aqueous extract (AE) of the Brazilian species were able to stimulate both humoral and cellular

immune responses against viral antigens to levels comparable to those induced by *Q. saponaria* saponins (de Costa et al., 2014; Fleck et al., 2006; Silveira et al., 2011)

The aim of this study was to demonstrate that QB-90 and AE from *Q. brasiliensis* formulated with BVDV antigen elicited robust and balanced immune responses in mice.

Material and methods

Ethics statement

All experiments were performed in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series – No. 170 revised 2005) and the procedures of the Brazilian College of Animal Experimentation (COBEA). The project was approved by the Ethics Commission on Animal Experimentation (CEUA) of FEPAGRO Animal Health, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF).

Vaccine adjuvants

Q. brasiliensis leaves were collected from adult plants naturally growing near Canguçu, RS, Brazil. AE Aqueous extract (AE) from *Q. brasiliensis* were extracted from air-dried powdered leaves in distilled water (1:10, w/v) for 8 h, filtered, partitioned with ethyl acetate and lyophilized. The AE was then submitted to further purification through reverse-phase chromatography to obtain fraction QB-90, as described in details in previous work (Fleck et al., 2006). Quil A was purchased from Brenntag Biosector (Denmark). Alum was obtained from Omega Produtos Quimicos Ltda. (Brazil). Incomplete Freund's Adjuvant (IFA) were purchased from Sigma (USA).

Antigen production

MDBK (Madin-Darby bovine kidney cells) were obtained from ATCC (originally CCL-22^{TM}) and cultured at 37 ºC in a 5% CO_2 incubator in Eagle's minimal essential medium (E-MEM; Gibco) supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 IU/mL; streptomycin 100 µg/mL). Cells were subcultured every 3-4 days following standard procedures. For virus multiplication, an autochthonous cytopathogenic BVDV1 isolate (EVI001/94) was inoculated onto nearly confluent monolayers of MDBK cells at a multiplicity of infection of 0.01. When cytopathic effect was evident in about 90% of monolayers, cells and supernatants were harvested and frozen at -80 ºC. Subsequently the virus containing suspension was thawed, clarified by low speed centrifugation (1500 × g for 10 min) and used as virus stock. Titers obtained were around $10^{8.3}$ 50% tissue culture infectious doses per mL ($TCID_{50}$) before inactivation with binary ethylenimine (BEI). The inactivated virus suspension was used as antigen for vaccine formulations.

Experimental design

Female Swiss mice (6-7 weeks of age) were purchased from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, Brazil). Mice were acclimatized one week prior to use under controlled temperature (22 ± 2 ºC) and humidity, with a cycle of 12/12 h day/night and fed with standard pelleted food and tap water ad libitum.

Mice were divided into ten groups, seven mice each. Animals were immunized subcutaneously on the hind neck with 150 µL of BVDV antigen in the presence of 50 µL of QB-90 (containing 100, 50 or 10 µg per dose); AE (containing 400, 200 or 100 µg per dose); Quil A (50 µg per dose); alum (100 µg per dose); incomplete Freund's adjuvant (IFA, 150 µL of antigen emulsified with 150 µL of oil) or without adjuvant (antigen only). The formulations of BVDV vaccines were filtered through 0.22 µm (Millipore) and kept at 4 ºC until use. A boosting injection was given 2 weeks later (day 14). Blood were collected on

days 0, 14 and 28 post-inoculation of the first dose of vaccine via tail vein and kept frozen until processed for determination of specific antibody in immunoassays.

Immunoassays for antibodies

Anti-BVDV IgG (total), IgG1 and IgG2a were determined in each serum sample by an indirect ELISA. ELISA plates (Nunc) were coated with the same BVDV antigen used for preparation of the vaccines above and diluted (1:100, v/v) in PBS (pH 7.2) for 18 h at 4 ºC. After adsorption, plates were washed two times with 200 µL of PBS containing 0.05% Tween-20 (PBS-T), filled with 180 µl of PBS-T containing 5% of non-fat dry milk and left standing for 60 min at 37 ºC. Subsequently, the plates were washed with PBS-T twice. Sera were appropriately diluted in PBS-T and added to duplicate wells. After 1 h at 37 ºC, plates were washed three times with PBS-T and incubated with adequate dilutions of peroxidase conjugated anti-mouse IgG, anti-mouse IgG1or anti-mouse IgG2a (Sigma®) for 1h at 37 ºC. After washing, 100 µl of OPD (ortho-phenylenediamine, Sigma®) with 0.03% of H2O2 were added to each well. After 30 min of incubation in the dark at room temperature, the reaction was stopped with the addition of 1M HCl (25 µl/well). Optical density was determined in a microplate reader set to 492 nm. Data were expressed as the mean OD value of the samples minus the mean OD recorded in control wells.

Delayed type hypersensitivity (DTH) assay

DTH responses were evaluated in three animals of each group on day 28 post inoculation of the experimental vaccines. The assay was performed by intradermal (ID) injections of 10 µL of the same BVDV antigen used for the preparation of the vaccines, in the right hind footpad. The thickness of the footpad was measured with a caliper at 24 h before and 24 h after ID injections. The BVDV-specific response of each animal was calculated based on values of its injected footpad minus the average of the basal swelling (Silveira et al., 2011).

Splenocyte proliferation assay

Spleens were collected 28 days after the second immunization under aseptic conditions, immersed in RPMI 1640 medium (Invitrogen), minced, and mechanically dissociated to obtain a homogeneous cell suspension. Erythrocytes were lysed with ACK lysis buffer. After centrifugation (380 x g at 4 °C for 10 min), pelleted cells were washed three times in RPMI 1640 and resuspended in the same medium supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. By trypan blue dye exclusion, cell counting revealed >95% viability. Splenocytes were seeded at 2.5×10^6 cell/mL in 100 μL of complete medium into each well of a 96-well flat-bottom microtiter plate (Nunc). Subsequently, 100 μL of BVDV antigen or medium only was added. Plates were then incubated at 37 °C in a humid atmosphere with 5% CO_2. After 68 h, 50 μL of MTT (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan Sigma) solution (2 mg/mL) was added to each well and incubated for 4 h. The plates were centrifuged at 1400 x g for 5 min and the untransformed MTT was removed carefully by pipetting. Next, a dimethyl sulfoxide (DMSO) solution (192 μL of DMSO; 8 μL of 1 N HCl) was added to wells in volumes of 100 μL. After 15 min of incubation, the absorbance was measured in an ELISA reader at 550 nm with wavelength reference fixed at 620 nm. The stimulation index (SI) was calculated as the ratio of Abs of mitogen-stimulated cultures and the absorbance of non-mitogen-stimulated cultures.

In order to evaluate $CD8^+$ T-cell proliferation, splenocytes (10^6 cells) were labeled with 1 μM of CFSE (carboxyfluorescein succinimidyl ester, CellTrace™, Life Technologies), cultured in 24-well plates for 3 days as above. Then, cells were washed two times with PBS/FBS and stained with anti-CD8 (CD8a Rat Anti-Mouse mAb, PE conjugate, Molecular Probes). The cells were analyzed on a FACScanto II (BD). $CD8^+$ proliferation was expressed

as the ratioof divided daughter cells to total lymphocytes, expressed as a percentage, in analogy to calculation of a stimulation index in MTT proliferation assays.

Cytokine quantification by cytometric bead array (CBA)

Splenocytes ($5x10^5$ cells) were pulse for 3 days with BVDV antigen (as described above) and supernatants harvested. Cytokines were measured by mouse BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit in BD FACScanto II. The theoretical limit of detection of the analyzed cytokines is 0.1 pg/mL to IL-2, 0.03 pg/mL to IL-4, 1.4 pg/mL to IL-6, 0.5 pg/mL to IFN-γ, 0.9 pg/mL to TNF, 0.8 pg/mL to IL-17 and 16.8 pg/mL to IL-10.

Measure of $CD4^+/IFN\text{-}\gamma^+$ and $CD8^+/IFN\text{-}\gamma^+$ cells

Splenocytes ($1x10^6$ cells) were pulsed for 18 hours with BVDV antigen (as described above). After 13 hours of culture, brefeldin A (GolgiPlug™, Pharmingen) was added to medium as previously described (de Souza et al., 2007). Following incubation, cells were washed twice in PBS supplemented with 2% of FBS and stained with anti-CD8and anti-CD4 (FITC conjugate, Molecular Probes), independently. Next, the cells were washed, fixed with 4% paraformaldehyde and permeabilized with saponin (Sigma). Then, the cells were intracellularly stained for IFN-γ (PE conjugate, Molecular Probes). FACS analysis was performed on a BD FACScantoII flow cytometer and the results were analyzed using FlowJo (Tree Star) software. The percentage of virus specific IFN-γ synthesis was calculated according to the formula (% of cells synthetizing IFN-γ after virus stimulation)-(% of cells synthetizing IFN-γ after culture in medium alone).

Statistical analysis

GraphPad Prism 5.0 was used for drawing graphs and statistical analysis. Significant differences between experimental and control groups were analyzed by one-way ANOVA, using Dunnett's post-test. Differences were considered statistically significant when $P \leq 0.05$.

Results and discussion

QB-90 saponins and AE from Q. brasiliensis have lower haemolytic activity than Quil A

Figure 1 shows the haemolytic activities of AE, QB-90 and Quil A. Their respective HD_{50} values were 1214.04 μg/mL, 90.89 μg/mL and 27.99 μg/mL. As compared to the high hemolytic activity displayed by Quil A saponins, the AE and QB-90 saponins fraction were significantly less hemolytic than Quil A (Figure 1).

Whilst the mechanism of action of saponins as adjuvants will be important to decipher, the critical attributes for prospective animal and human vaccine adjuvants are efficacy, safety and tolerability. Reassuringly, there was no evidence of any local or systemic toxicity from multiple injections of *Quillaja brasiliensis* saponins adjuvant with BVDV antigen. This is consistent with data from our previous experiments that confirmed that QB-90, as well AE, were safe and well tolerated in mice (de Costa et al., 2014; Silveira et al., 2011).

QB-90 saponins and AE as adjuvants enhance anti-BVDV antibody responses in mice

To evaluate the humoral immune responses, serum levels of BVDV antigen-specific IgG and its isotypes, IgG1 and IgG2a from all the vaccinated groups were assessed by indirect ELISA. Following immunization, BVDV specific IgG as well as IgG1 and IgG2a were elevated in all groups inoculated with QB-90-adjuvanted preparations (Figure 2). Groups treated with 400 and 200 μg of AE, also showed a statistically significant increase in antibodies levels. However, the group treated with 100 μg of AE remained at background levels of control group (BVDV antigen plus saline) (Figure 2). A dose-dependent antibody fashion was evident in QB-90 and AE and groups. Mice immunized with the largest dose of QB-90 and AE displayed the highest antibodies levels, superior (in QB-90) and similar (in AE) to IFA-adjuvanted group.

The search of new immunoadjuvants based on plant-derived saponins has increased in recent years in search for safer and more effective vaccines for animals and humans (de Costa et al., 2011). Semi purified extract from *Q. brasiliensis*, as well as QB-90 fraction of saponins increased anti-BVDV total IgG levels through elevation of IgG1, a Th2 antibody isotype, with contribution of IgG2a, a Th1 isotype. These results are in agreement with those previously obtained when viral antigens were co-administered with QB-90 saponins (de Costa et al., 2014; Fleck et al., 2006; Silveira et al., 2011).

QB-90 and AE increases anti-BVDV cellular responses when used as adjuvants

Delayed-type hypersensitivity (DTH) is a useful approach for evaluating cell-mediated immune responses associated with Th1 reactivity. Immunized mice were subjected to detection of BVDV-specific DTH reaction using the footpad swelling response two weeks after boosting. Figure 3 summarizes the results of the DTH assay in the different groups of mice. A significant DTH response was observed in mice immunized with QB-90 (100 and 50 μg), AE (400 and 200 μg), Quil A and IFA-adjuvated preparations. We verified a dose-dependency of the DTH reaction in the QB-90-adjuvanted and AE-adjuvanted groups (likewise verified with antibody assays). Adjuvanted preparation with 100 μg of QB-90 and 400 ug of AE proved superior to Quil A and IFA on generation of DTH responses. QB-90 50 μg and Quil A showed no significant difference. Taking into account that a positive DTH reaction is attributed to memory Th1 $CD4^+$ T cells (Cher and Mosmann, 1987), these results indicate that, similar to Quil A, AE and QB-90 is capable of stimulating the generation of Th1 cells against the administered antigen.

To assess whether AE and QB-90 saponins adjuvant enhances anti-BVDV T-cell responses, splenocytes were isolated from mice 28 days post-BVDV immunization and cultured with BVDV antigen for 3 days in a standard MTT and CFSE proliferation assays. The effects of AE (400 μg) and QB-90 (100 μg) as well as IFA on splenocyte proliferation in

response to BVDV stimulation are shown in Figure 4. Compared with the control (saline plus antigen), mice receiving QB-90-adjuvanted vaccine had significantly higher BVDV-specific cell proliferation compared to mice immunized with BVDV alone ($P \leq 0.01$) (Figure 4A). Furthermore, mice receiving BVDV with QB-90 had significantly higher BVDV-specific $CD8^+$ T-cell proliferation compared to mice immunized with BVDV alone ($P \leq 0.001$) (~3 fold increase, Figure 4B).

Th1 cells produce IFN-γ, IL-2, and lymphotoxins, playing a critical role in directing cell-mediated immune responses, which are important for clearance of intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which are important for humoral responses (Crotty, 2015; Marciani, 2014). To better characterize the cytokine profile induced by immunization of BVDV with AE and QB-90, splenocytes from immunized animals were re-stimulated with BVDV *in vitro* for 3 days and supernatants measured using BD Th1/Th2/Th17 bead array (Figure 5). The strong induction of Th1 antibodies by QB-90 saponins was also observed on the Th1 cytokine levels. QB-90 adjuvanted vaccine induces high levels of IFN-γ ($P \leq 0.01$), TNF ($P \leq 0.001$) and IL-2 ($P \leq 0.01$) (Th1 induced cytokines). The results were in agreement with the tendency of elevated IgG2a antibody level in mice vaccinated with QB-90. Moreover, QB-90 significantly enhances the production of IL-10 (suppressive cytokine) ($P \leq 0.05$), and IL-17 (Th17 cytokine) ($P \leq 0.05$). Th2 induced cytokines (IL-4 and IL-6) do not have a significant increase in animals immunized with QB-90, suggesting a Th1 bias. Unlike the group immunized with QB-90, the group immunized with BVDV combined with AE showed no increase in the production of any examined cytokine ($P \geq 0.05$).

Another important adjuvant attribute, particularly for therapeutic vaccines, is the ability to induce robust T-cell responses (Kamphorst et al., 2015). The induction of memory $CD8^+$ T-cells by exogenous antigen requires antigen cross-presentation, normally a feature

restricted to dendritic cells (Heath and Carbone, 1999). The strong anti-BVDV $CD8^+$ T-cell recall responses seen in BVDV plus QB-90 immunized animals perhaps indicates that QB-90 enhances antigen cross-presentation possibly by a chemotactic effect that recruits dendritic cells to the site of immunization.

An important natural mechanism for elimination of virus in vivo is an antigen-specific cell-mediated immunity. $CD8^+$ T cells recognize viral antigens in the form of short peptides presented by MHC class I molecules on the surface of virus-infected cells. The recognition of these viral peptides in the context of MHC molecules by $CD8^+$ T cells can trigger the specific lysis of virally infected cells (Kagi et al., 1995) or noncytopathic intracellular inactivation of virus mediated by cytokines such as gamma interferon and tumor necrosis factor α (Guidotti and Chisari, 2000; Mosmann et al., 1997).

To investigate IFN-γ production by T cells, splenocytes were re-stimulated with BVDV and the frequency of IFN-γ^+ $CD8^+$ and $CD4^+$ T cells was analyzed by flow cytometry. Mice immunized with unadjuvanted BVDV were used as controls. Frequencies of IFN-γ^+ $CD4^+$ T cells and IFN-γ^+ $CD8^+$ T cells were significantly higher in mice immunized with the QB-90-adjuvanted vaccine than the group immunized with no adjuvant. BVDV in combination with QB-90 than in mice immunized with BVDV alone (Figure 6). The QB-90-adjuvanted vaccine promoted a 14-fold increase in IFN-γ^+ $CD4^+$ T cells and a 25-fold increase in IFN-γ^+ $CD8^+$ T cells when compared with mice received unadjuvanted vaccine. These findings indicate that the QB-90-adjuvanted vaccine is capable of inducing antigen-specific activation of $CD8^+$ and $CD4^+$ T cells.

Another relevant issue is the fact that the large scale use of *Q. saponaria* bark saponins may compromise sustainable production of this non wood-forest product. Consequently, the easily renewable use of bioactive saponins from leaves with equal power of immune potentiation assumes even more importance. The availability of an alternative source of

saponins such as *Q. brasiliensis* may prove helpful to contribute to the sustainable/renewable culturing of *Q. saponaria* (de Costa et al., 2011, 2013; Yendo et al., 2010).

The findings reported here suggest that QB-90 in particular seems to be an interesting alternative to the usage as adjuvant for vaccines. Additional testing would unavoidably require evaluation of the QB-90-adjuvanted vaccine on cattle, the actual target host, for BVDV prophylaxis. Considered globally, our results demonstrate that the BVDV vaccine adjuvanted with *Quillaja brasiliensis* saponins induces strong humoral and cellular immune responses in mice.

Competing interests

None of the authors has any potential financial conflict of interest related to this manuscript.

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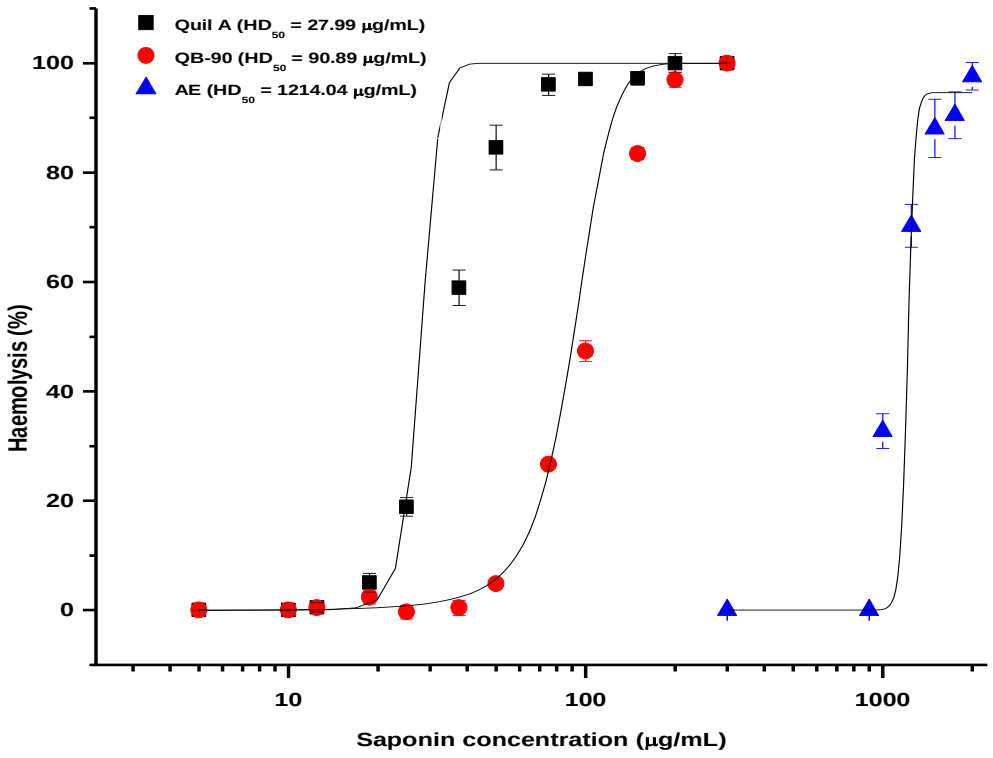

Figure 1. *In vitro* toxicity of QB-90, AE and Quil A. Haemolytic activity expressed as percent haemolysis referred to saline and *Q. saponaria* saponins (250 µg/mL), which were used as 0% and 100%, respectively. Results are presented as the mean value ± S.D (n = 4).

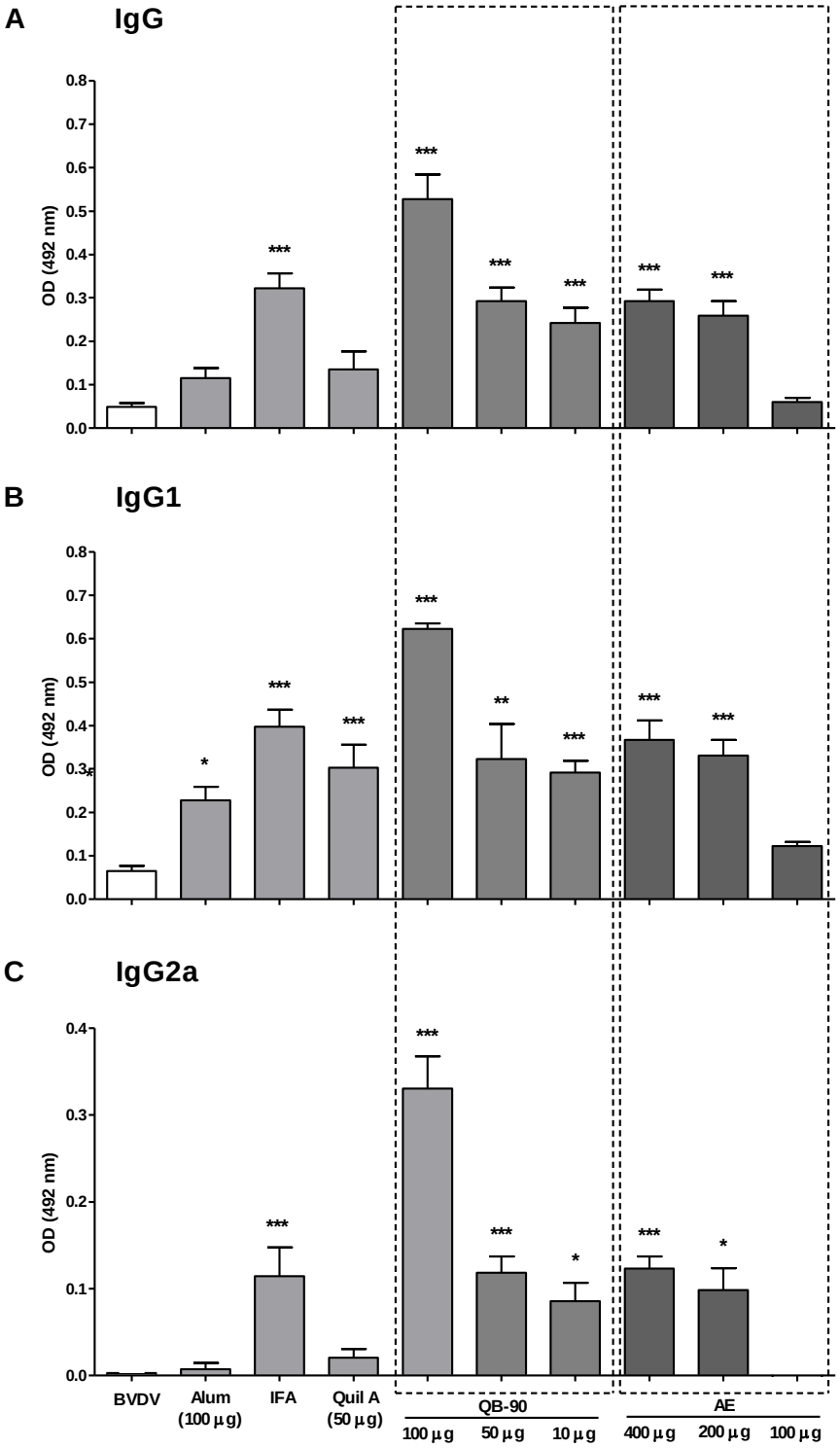

Figure 2. *Quillaja brasiliensis* AE and QB-90 enhances anti-BVDV antibody titers. Adult female CF1 mice were immunized twice s.c. at a 2-week interval with BVDV antigen alone (empty bar) or together with QB-90 (light gray bars) or AE (gray bars. Blood samples were collected 2 weeks after the second immunization and anti-BVDV total IgG, IgG1, IgG2a measured by ELISA. Significant differences are indicated: *($P \leq 0.05$), **($P \leq 0.01$) and ***($P \leq 0.001$), with the group immunized with no adjuvant.

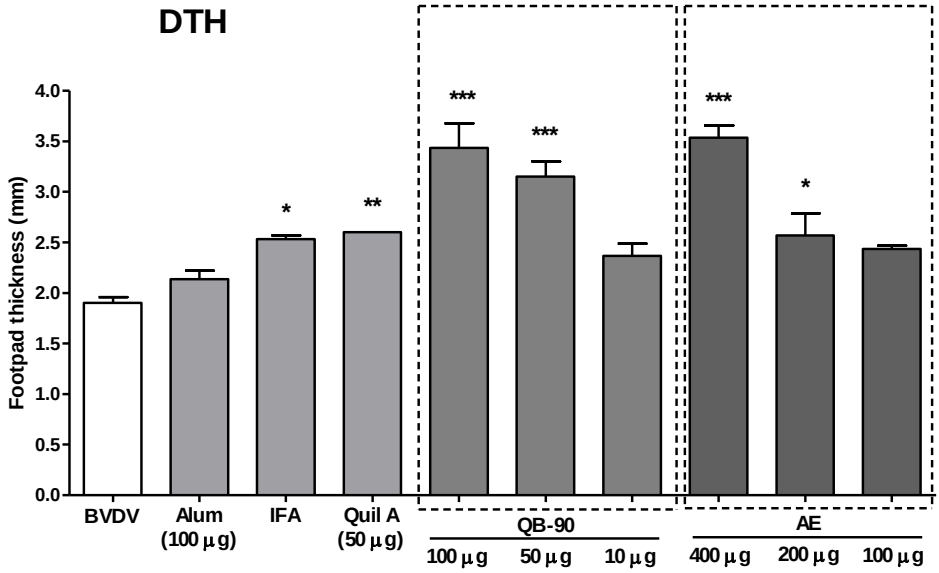

Figure 3. Delayed type hypersensitivity (DTH) in immunized mice. The assay was carried out 2 weeks after the second immunization of mice with the BVDV preparation either with no adjuvant or formulated with QB-90, AE, and others adjuvants Mice injected only with saline plus antigen was used as a control group (BVDV). The DTH response is expressed as the mean value ± S.D. (n=3); significant differences are indicated: *($P \leq 0.05$), **($P \leq 0.01$) and ***($P \leq 0.001$), with the group immunized with no adjuvant.

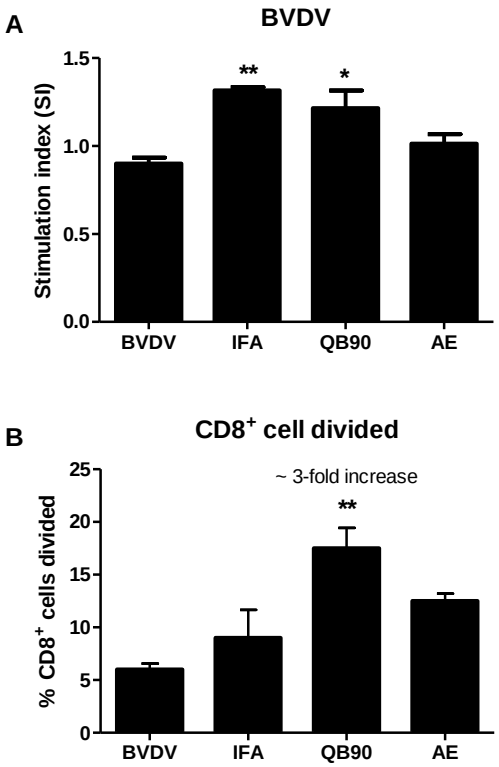

Figure 4. Splenocyte proliferation assays. Mice (*n*=6/group) were s.c. immunized with BVDV plus saline, or adjuvanted with 100 µg of QB-90 (100µg), 400 µg of AE (400 µg), IFA or unadjuvanted vaccine. Splenocytes were prepared two weeks after boosting and cultured with BVDV antigen or RPMI 1640 medium during 68 hours. Splenocyte proliferation was measured by the MTT method (refer to text for methods), and is shown as a stimulation index (SI) (A). $CD8^+$ T-cell proliferation was measured by culturing CFSE-labeled splenocytes with BVDV antigen for 3 days (B). The values are presented as means ± SD. Significant differences are indicated: *($P \leq 0.05$), **($P \leq 0.01$) and ***($P \leq 0.001$), with the group immunized with unadjuvanted vaccine.

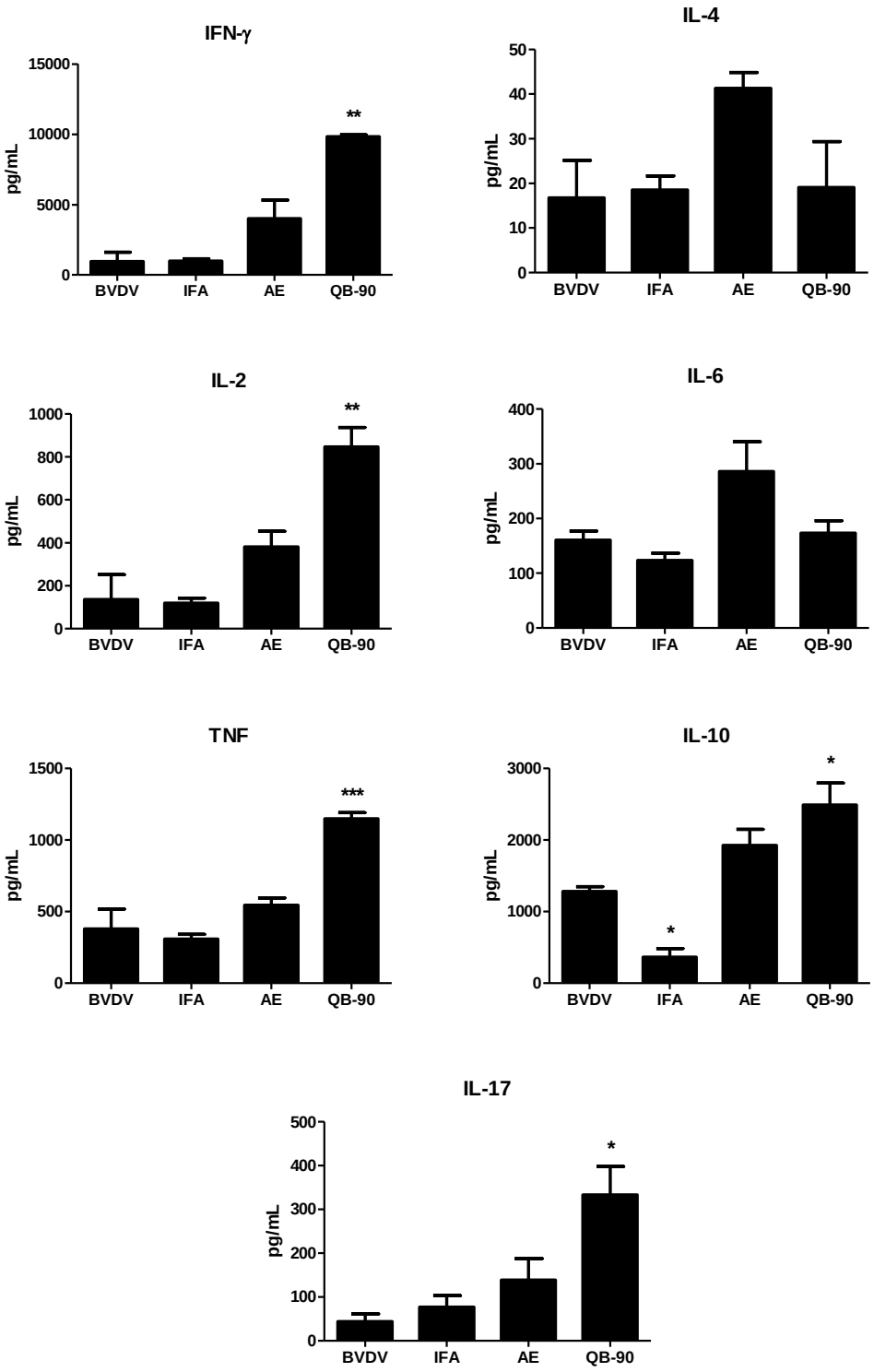

Figure 5. Th1, Th2 and Th17 cytokine profile. Spleens were collected from mice (n=3) immunized with BVDV alone or with IFA, AE or QB-90, and cultured with BVDV antigen for three days. Cytokines in the supernatant were quantitated by cytometric bead array and present by mean ± S.E.M. Significant differences are indicated: *($P \leq 0.05$), **($P \leq 0.01$) and ***($P \leq 0.001$), with the group immunized with unadjuvanted vaccine.

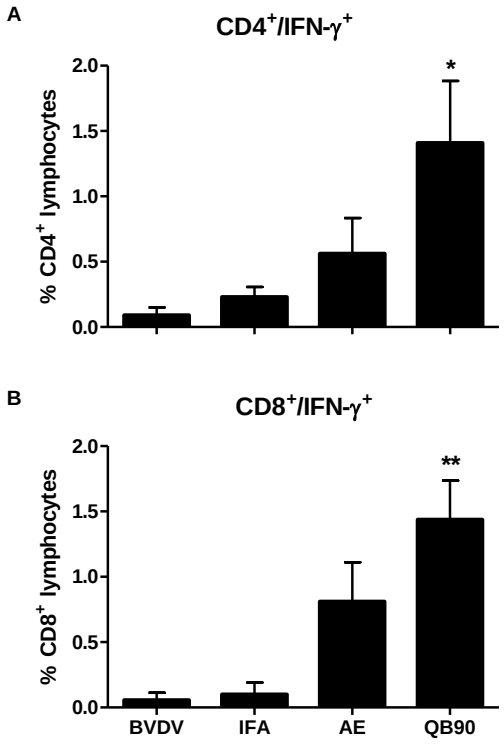

Figure 6. QB-90 induces antigen-specific IFN-γ production in $CD4^+$and $CD8^+$T cells. Mice (*n*=3) were immunized with unadjuvanted antigen (BVDV) or antigen plus IFA, AE (400μg) or antigen plus QB-90 (100 μg). Mice were euthanized on day 28 after first immunization to collect spleens. Splenocytes ($1x10^6$cells) were re-stimulated with BVDV for 18 h and intracellular production of IFN-γ by $CD4^+$ (A) and $CD8^+$T cells (B) was analyzed by flow cytometry. Statistical analysis was done by one-way ANOVA and the differences between the treatments were compared by Tukey's multiple-comparison test. Significant differences are indicated: *($P\leq0.05$), **($P\leq0.01$) and ***($P\leq 0.001$), with the group immunized with no adjuvant.

6 CAPÍTULO 3: "ISCOMs from *Quillaja brasiliensis* saponins: low-toxicity, improved antigen uptake and enhancement of antibody and cellular immune responses".

ISCOMs from *Quillaja brasiliensis* saponins: low-toxicity, improved antigen uptake and enhancement of antibody and cellular immune responses.

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Abstract

In the last decades, significant efforts have been dedicated to the search for novel vaccine adjuvants. In this regard, saponins and its formulations as "immunostimulating complexes" (ISCOMs) have shown to be capable of stimulating potent humoral and cellular immune responses, enhanced cytokine production and activation of cytotoxic T cells. Here, a report is presented on the immunological activity of ISCOMs formulated with a saponin fraction extracted from *Quillaja brasiliensis* (QB-90 fraction) as an alternative to classical ISCOMs based on Quil A® (IQA). The ISCOMs prepared with QB-90, named IQB-90, typically consist of 40-50 nm, spherical, cage-like particles, built up by QB-90, cholesterol, phospholipids and antigen (ovalbumin, OVA). These nanoparticles were efficiently uptaken *in vitro* by murine bone marrow-derived dendritic cells. Subcutaneously inoculated IQB-90 induced strong serum antibody responses encompassing specific IgG1 and IgG2a, robust DTH reactions, significant T cell proliferation and increases in Th1 (IFN-γ and IL-2) cytokine responses measured in immunized mice. Intranasally delivered IQB-90 elicited serum IgG and IgG1, and mucosal IgA responses at distal systemic sites (nasal passages, large intestine and vaginal lumen). These results indicate that IQB-90 is a promising alternative as vaccine adjuvants, capable of enhancing cellular and humoral immunity.

Keywords: *Quillaja brasiliensis*, ISCOMs, uptake, subcutaneously, intranasally delivered, humoral and cell responses

1. Introduction

Infectious diseases are major causes of morbidity and mortality worldwide, especially in poor and developing countries. Amongst a plethora of preventive measures available in attempting to reduce such burden, vaccines stand out as a highly efficacious and cost effective tools, which have been successfully used in the control or eradication of some of the most impacting infectious diseases in humans and animals (Rappuoli, 2011).

Subunit vaccines, whose immunogenicity relies essentially on a particular protein or peptide, are generally less immunogenic than vaccines based on live attenuated or whole inactivated microorganisms. Therefore, subunit vaccines usually require addition of adjuvants in order to improve its immunogenicity These have been used in order to either induce more rapid and robust immune responses that correlate with increased protection or to allow dose sparing in the context of antigens which can be in limited supply or problematic to manufacture (Morelli et al., 2012).

A crucial aspect addressing the challenges in vaccine development is antigen delivery, which encompasses administration of drugs to specific sites of the body, as well as the delivery of the antigen provide the necessary signals for activation and maturation of relevant antigen presenting cells (APCs) (Brito et al., 2013; O'Hagan and De Gregorio, 2009). Most pathogens invade the host or establish infection at mucosal surfaces. In this regard, antigen delivery in mucosal surfaces may mimicry natural infection and induce local and remote specific immune responses (Harandi et al., 2010).

Most vaccine adjuvants in clinical use for human vaccines, of which alum compounds are the main representatives, induce protection by enhancing antibody responses. However, for some infections, particularly those caused by intracellular pathogens, specific antibodies are not sufficient to induce protection. In such cases, stimulation of antigen-specific $CD4^+$ or

$CD8^+$ T-cell is not only required but essential for eliciting protective responses (Garcon et al., 2013).

Many natural products with potential for use as adjuvants are currently under investigation (Rey-Ladino et al., 2011; Rosales-Mendoza and Salazar-Gonzalez, 2014). In veterinary medicine, triterpenoid saponins extracted from *Quillaja saponaria* Molina have a long usage history as vaccine adjuvants. In fact, a partially purified mixture of saponins from *Q. saponaria*, named Quil A® (Kensil et al., 1991), is the most widely used (yet the most studied) saponin-based vaccine adjuvant. However, Quil A® use in human vaccines has been restricted due to undesirable side effects such as local reactions, haemolytic activity and occasional events of systemic toxicity (Kensil et al., 1991; O'Hagan and Fox, 2015). A similar saponin fraction has been extracted from *Quillaja brasiliensis* leaves, named QB-90 (Kauffmann et al., 2004), which was found to possess adjuvant potential into levels comparable to those of Quil A®. In addition, QB-90 was less toxic than Quil A® (de Costa et al., 2014; Fleck et al., 2006; Silveira et al., 2011a). Both QB-90 and Quil A® showed similar patterns of antibody induction (IgG and subclasses) and stimulation of cellular immunity by generation of Th1 responses (de Costa et al., 2014; Silveira et al., 2011a; Villacres-Eriksson et al., 1997).

An improvement to the use of saponins as adjuvants was introduced by the development of immune stimulating complexes (ISCOMs). These have been used as antigen delivery systems that proved to exert powerful immune stimulating activities, yet displaying reduced toxicity in several animal models (Lovgren Bengtsson et al., 2011; Sun et al., 2009b). Physicochemical properties of ISCOMs include the cage-like structures with about 40 nm in diameter, composed by aggregates of an antigen (usually proteic), cholesterol, phospholipids and saponins from *Quillaja saponaria*. ISCOMs were shown to up-regulate both Th1- and Th2-like immune responses as well as to stimulate strong humoral responses (IgG1, IgG2b

and IgG2a) with cytotoxic T cell induction (Lovgren Bengtsson et al., 2011; Sjolander et al., 2001). In view of these findings, ISCOMs are considered promising innate immune cell-stimulating adjuvants (Morelli et al., 2012; Zhao et al., 2014).

In this study, for the very first time, ISCOM formulations were constructed by replacing the Quil A® component by QB-90. This formulation was assessed on its adjuvant capacity using a model protein antigen. Safety and efficiency analyses were performed and its use as an effective alternative to Quil A®-based ISCOMs is then suggested.

2. Material and methods

2.1. Adjuvant and vaccine preparation

Quillaja brasiliensis (A. St.-Hil. et Tul) Mart. leaves were collected in Parque Battle (Montevideo, Uruguay). Extraction and purification of saponins were carried out as previously described (Kauffmann et al., 2004). OVA-ISCOMs (ISCOMs with ovoalbumin) were prepared by the modified ethanol injection technique (Quirici et al., 2013). Briefly, OVA (Sigma, USA) solution (1 mg/mL in TBS, pH 7.4) was added either to a mixture of *Quillaja brasiliensis* saponins fraction (QB-90, 1 mg/mL) or to Quil A® (QA) (Brenntag, Denmark) (1 mg/mL). Ethanol-dissolved cholesterol (Sigma, USA) and di-palmitoyl phosphatidyl choline (Avanti Polar Lipids, USA) were immediately injected into the mixture, which were finally stirred during 48 hours at 4 °C. After that procedure, ISCOMs derived from QB-90 (IQB-90) and ISCOMs from QA (IQA) were obtained.

2.2. Transmission electron microscopy (TEM)

An aliquot (10 μL) of an aqueous solution of both ISCOMs formulations was placed on formvar carbon grids (200 mesh) and negatively stained with 2% phosphotungstic acid (pH 7.2) for 2 min at room temperature and air dried. The samples were examined with a JEOL (JEMM 10.10) transmission electron microscope (JEOL, Japan) operated at an 80 kV accelerating voltage.

2.3. In vitro and in vivo toxicity assays

Haemolytic activities of QA, QB-90 and IQB-90 were assessed as previously described (Silveira et al., 2011b), over a range of 10-200 µg/mL and using a concentration of 0.5% rabbit red blood cells. Saline and *Q. saponaria* saponins (250 µg/mL) were used for 0% and 100% haemolysis, respectively. Samples were tested in triplicates. Haemolytic activity was expressed as the sample concentration producing 50% of the maximum haemolysis (HD_{50}).

Cytotoxicity on VERO cells (ATCC CCL-81) was determined through MTT assay and by determination of lactate dehydrogenase (LDH) release into the supernatant media with samples of QA and QB-90. Briefly, cells were cultured in Eagle's minimal essential medium (E-MEM) supplemented with 10% foetal bovine serum (FBS, GIBCO) and antibiotics (penicillin 100 UI/mL; streptomycin 100 µg/mL) (E-MEM/FBS) at $4.0x10^4$ per well on 96-well microplates and incubated for 18 h at 37 °C in a humid atmosphere with 5% CO_2. Afterwards, the medium was removed and cells were further incubated for 24 h with 100 µL/well of E-MEM/FBS containing different concentrations of either QA or QB-90. For MTT assays, 50 µL/well of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; Invitrogen, USA) at 2 mg/mL was added. Cells were incubated during 4 h at 37 °C. After centrifugation (1400 × g for 5 min), supernatants were removed and 100 µL/well of dimethyl sulfoxide (DMSO) were added. Optical density (OD) was measured in a microplate reader (Dynex MMXII, USA) at 570 nm. Results were expressed as the percentage of each cell treatment OD related to the OD of untreated cells. General cytotoxicity was reported as the concentrations that decreased viability by 50%. LDH released from damaged cells into culture media was quantified with the aid of a LDH assay kit (Labtest, Brazil). Survival ratio was determined by comparing the absorbance in test wells with those of positive (complete cell destruction) and negative (spontaneous cell destruction) control wells. Values were expressed

as the highest dilution of sample concentration which caused 50% LDH activity release compared to positive controls (EC_{50}).

Acute toxicity was evaluated as previously described (Sun et al., 2010) with some modifications. Briefly, groups of CD-1 male mice (8 weeks of age, n=5) were given a subcutaneous administration of 100 μL of QB-90 or QA in PBS (31.25, 62.5 and 125 μg/dose) on the scapular region. Mice were monitored during 3 days in search for signs of toxicity (lethality, local swelling, loss of hair, and piloerection). Control mice were inoculated with 100 μL of PBS.

2.4. Antigen uptake by murine bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were obtained by differentiation of bone marrow precursors from 8-10 weeks old C57Bl/6 mice (Lutz et al., 1999). Briefly, femurs and tibia were flushed out and the cells were cultured on Petri plates in RPMI 1640 culture medium (Gibco), complemented with 10% FBS (Gibco), 50 μM 2-mercaptoethanol (Sigma), 1% HEPES (Sigma), 1% sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco) and 20 ng/mL of recombinant mouse GM-CSF (PeproTech). Medium was replaced on day 3 and BMDCs were obtained on day 10. Phenotype was routinely checked and 90-95% cells were $CD11c^{+}$.

Antigen (Ag) uptake was performed according to protocols already reported (West et al., 2004). Briefly, BMDCs (2×10^{5}) were incubated with 2 μg of FITC-labeled OVA (OVA:FITC, Molecular Probes®) in 200 μL of RPMI media for 120 min at 37 °C in the presence of 10 μg/mL of QB-90, vehicle or IQB-90 formulated with 2 μg of OVA:FITC. One parallel experiment was performed on ice to inhibit intracellular uptake (cell surface binding controls). Then, cells were washed twice with ice-cold PBS, and resuspended in FACS buffer

(PBS with 2% FBS and 0.1% sodium azide). Cell staining was performed with PE-Cy7-conjugated anti-mouse CD11c (clone HL3, BD) and flow cytometry was performed on a FACS Canto II (BD) cytometer. Fluorescence values were reported as mean fluorescence intensity (MFI) of FITC in $CD11c^+$ cells.

For fluorescence microscopy, $1.0x10^6$ BMDCs were plated on 24-well plates (Costar) and after 18 hours of culture, OVA:FITC alone, with QB-90 or formulated as IQB-90 was added. After 120 min, cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde and then stained with DAPI (4′,6-Diamidino-2-Phenylindole, Dilactate, Invitrogen) for 10 min at 37 °C. Fluorescence microscopy was carried out with a Spot insight camera (model no. 3.1.0; Diagnostic Instruments Inc, Sterling Heights, MI) mounted over an Axiovert S100 microscope (Zeiss, Göttingen, Germany). Image acquisition was performed with Meta Imaging Series 6.1 software (Universal Imaging Corporation, Downington, PA).

2.5. Immunizations and sample collection

Experimental work involving animals was carried out following international guides on use and care of laboratory animals. Protocols were performed in accordance with CHEA guidelines (Comisión Honoraria de Experimentación Animal) and were approved by the Uruguayan University Research Ethics Committee (approval number 070153-000531-13). Animals were properly housed under controlled temperature (22 ± 2 °C) and humidity in a 12/12 h light/dark cycle, with food and water *ad libitum.*

Female Rockfeller mice of the CF-1 breed (5-6 weeks old) were purchased from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, RS, Brazil) and were immunized on days 0 and 14 with OVA through subcutaneous (s.c.) or intranasal (i.n.)

routes. Subcutaneous immunizations (*n* = 6) (in the hind neck) were performed with 10 µg/dose of IQB-90, IQA or OVA alone (unadjuvanted). For intranasal immunization, mice (*n* = 7) were anesthetized with ketamine-xylazine and received 2 µg of antigen per dose. Bleedings were performed immediately before inoculations (days 0 and 14) and 2 weeks after the second immunization (day 28). Sera were stored at -20 °C.

The samples, including nasal and vaginal washes as well as feces, were collected from euthanized animals on 28 day post priming. Fecal samples were obtained from 3-4 freshly voided pellets from each animal, which were weighed and collected into a 15 mL conical tube. A 10 x volume (per gram of wet feces) of extraction buffer (PBS with 5% FBS and 0.02% sodium azide) was added to each tube, which were vigorously vortexed and centrifuged at 16,000 × *g* for 20 min. Supernatants were collected and stored at -70 °C.

For nasal washes, a 1 cm incision was performed parallel to the trachea through the skin, and a midline incision was made on the ventral aspect of the trachea slightly superior to the thoracic inlet with a scalpel. A 25G needle was tied at the top of the trachea and 0.5 mL of PBS was slowly injected. Nasal washes were collected through the nostrils and stored at -70 °C. Vaginal washes were performed with a micropipette. PBS (75 µL) were flushed 10 times through vaginas, and samples were centrifuged 5 min at 16.000 x *g*. Supernatants were stored at -70 °C.

2.6. Splenocyte proliferation assay

Six-week-old female Rockfeller mice were divided into 5 groups, each consisting of six mice. Animals were immunized subcutaneously with OVA 10 μg alone or with OVA 10 μg dissolved in saline containing QB-90 (10 μg), Quil A (10 μg), IQB-90 (10 μg) and IQA (10 μg) on day 0. Saline-treated animals were included as controls. A boosting injection was given 2 weeks later.

Spleens were collected 28 days after the second immunization under aseptic conditions, immersed in RPMI 1640 medium (Gibco), minced, and mechanically dissociated to obtain a homogeneous cell suspension. Erythrocytes were lysed with ACK (Ammonium-Chloride-Potassium) lysis buffer. After centrifugation (380 x g at 4 °C for 10 min), pelleted cells were washed three times in RPMI 1640 and resuspended in the same medium supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (RPMI complete media). By trypan blue dye exclusion, cells counting revealed >95% viability. Splenocytes were seeded at 2.5×10^6 cells/mL in 100 μL of RPMI complete medium into each well of a 96-well flat-bottom microtiter plate (Nunc). Subsequently, 100 μL OVA (10 μg/mL, Sigma) or medium only was added. Plates were then incubated at 37 °C in a humid atmosphere with 5% CO_2. After 68 h, 50 μL of MTT (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan Sigma) solution (2 mg/mL) was added to each well and incubated for 4 h. The plates were centrifuged at 1400 x g for 5 min and the untransformed MTT was removed carefully by pipetting. Next, a DMSO solution (192 μL of DMSO with 8 μL of 1 N HCl) was added to wells in volumes of 100 μL. After 15 min of incubation, the absorbance was measured in an ELISA reader at 550 nm with wavelength reference fixed at 620 nm. The stimulation index (SI) was calculated as the absorbance ratio of mitogen-stimulated cultures and the non-mitogen-stimulated cultures.

2.7. Quantification of cytokine levels in spleen culture supernatants.

Spleens were aseptically removed, rinsed and mechanically disrupted in RPMI media and isolated spleen cells were pelleted and incubated in RBC lysis solution. After washing with RPMI, splenocytes ($5x10^5$ cells) were re-stimulated for 3 days with OVA antigen (10 µg/mL). The supernatants were harvested and IL-2 and IFN-γ cytokines were measured by ELISA (Novex, USA).

2.8. Determination of antigen-specific antibodies

Anti-OVA IgG, IgG1 and IgG2a antibodies were determined by ELISA as described (Silveira et al., 2011b). Briefly, ELISA plates (Greiner Bio-One, Germany) were coated with OVA (5 µg/mL) in acetate buffer (100 µL/well, pH 5.0) overnight at 4 °C. Then, plates were washed three times with PBS containing 0.05% Tween® 20 (Sigma, USA) (PBS-T20) and blocked with 1% Tween® 20 in PBS at 37 °C for 2 h. Appropriately diluted samples in PBS-T20 were added in duplicate (100 µL/well) and incubated for 1 h at 37 °C. After washing three times with PBS-T20, HRP-conjugated anti-mouse IgG (Sigma, USA), IgG1 (Invitrogen, USA) and IgG2a (Invitrogen, USA) diluted in PBS-T20 (1:5000, 1:5000 and 1:2000, respectively) were added to each well (100 µL/well) and plates were incubated for 1 h at 37 °C. After five washings, 100 µL of OPD (*ortho*-phenylenediamine; Sigma, USA) with 0.003% H_2O_2 were added to wells, and plates were further incubated for 30 min at 25 °C. Reactions were stopped with 30 µL/well of 1N HCl. Optical densities (OD) were measured in an ELISA plate reader (Anthos 2020) at 492 nm. A pool of positive sera was used as standard curve, and antibody titers were expressed in arbitrary units per mL (AU/mL).

Anti-OVA IgA determinations were similarly performed. After incubation of samples for 1 hour at 37 °C, plates were washed five times and 100 µL/well of goat anti-mouse IgA antibodies were added to each well (1:4000 dilution; Sigma, USA). After incubation at 37 °C for 1 hour and five washings, 100 µL/well of HRP-conjugated anti-goat antibodies (1:5000, Zimed, USA) were added and incubated for 1 h at 37 °C and reveled as above. OVA-specific IgA titers were expressed as OD values for samples diluted 1:10 (fecal, vaginal and nasal samples).

2.9. Delayed-type hypersensitivity (DTH) assay

Delayed type hypersensitivity (DTH) responses were tested 28 days post-priming. Briefly, mice were intradermally injected with 1 µg of OVA in one footpad of the hind limb. Thickness of the injected footpads was measured 24 h later with a caliper. Swelling in mice inoculated with saline revealed basal conditions. OVA-specific DTH responses in each animal were determined as the thickness of injected footpad minus average basal swelling.

2.10. Statistical analyses

Statistical significance was assessed by one-way-ANOVA with Dunnet's post test correction (GraphPad Prism 5.01, GraphPad Software, USA). Significance was assigned at *p*-value <0.05.

3. Results

3.1. Formulation of ISCOMs with Quillaja brasiliensis saponins

Purified fraction of saponins from *Quillaja brasiliensis* (QB-90) and OVA were combined with cholesterol and phospholipid under controlled conditions to obtain IQB-90, which formed cage-like structures. IQB-90 structures were confirmed by transmission electron microscopy (TEM), where the average diameter of the cage-like structures was about 47 nm (overall range: 40-50 nm) (Figure 1).

3.2. QB-90 showed lower toxicity than QA in vitro and in vivo

Haemolytic activities of QB-90 and QA showed HD_{50} values of 88.83 ± 0.16 µg/mL and 40.43 ± 0.10 µg/mL, respectively. No significant hemolytic activity was observed at the concentration used in the vaccine formulations (i.e. 10 µg/mL) (Figure 2A). Interestingly, no haemolytic activity was determined for ISCOMs formulated with QB-90 in any tested concentration (Figure 2A).

Similar results were obtained from the cytotoxicity assays (Figure 2B and 2C). Results shown in Figure 2B revealed that QA was more toxic to VERO cells (EC_{50} = 50.6 ± 0.38 µg/mL) than QB-90 (EC_{50} = 70.8 ± 0.03 µg/mL). Indeed, at 50 µg/mL, more than 90% of cells exposed to QB-90 were viable, whereas cell viability of QA-exposed cells was only 55% ($P \leq 0.001$).

The LDH assay (Figure 2C) revealed that QA promoted a more extensive cytoplasmic content release than QB-90 (EC_{50} values of 56.59 ± 2.37 µg/mL and 72.76 ± 3.93 µg/mL for QA and QB-90, respectively). In fact, VERO cells exposed to 50 µg/mL of QA released

approximately 40% of LDH, whereas those exposed to QB-90 released similar levels of LDH as unexposed control cells ($P \leq 0.001$).

Finally, acute toxicity assay at the lowest dose tested (31.25 μg) showed no lethality or signs of local toxicity (local swelling, loss of hair and piloerection) within the mice group inoculated with QB-90, but 40% lethality in the group treated with QA (Table 1). Moreover, at the highest dose tested (125 μg) lethality showed to be 60% and 100% in mice inoculated with QB-90 and QA, respectively (Table 1). Summing up, the results presented here provide evidence that QB-90 is significantly less toxic than QA, both *in vivo* and *in vitro*, suggesting that QB-90 could be used as an alternative to QA.

3.3. BMDCs take up IQB-90 more efficiently than OVA formulated with soluble QB-90

In order to determine whether IQB-90 enhances antigen internalization, the uptake of OVA:FITC by BMDCs *in vitro* in the presence of soluble QB-90 or IQB-90 was analyzed (Figure 3). Results in Figure 3A showed that BMDCs internalized OVA:FITC at the same extent either in the presence or in absence of soluble QB-90 (10 μg/mL) ($P \geq 0.05$). However, OVA:FITC as IQB-90 was more efficiently internalized by BMDCs than OVA:FITC in the presence or absence of soluble QB-90 ($P < 0.001$). In order to confirm that detected fluorescence in BMDCs was due to internalization of OVA:FITC and not an artifact of OVA:FITC binding to cell surface, similar experiments were performed at 4 °C showing a FITC signal abrogation independently of the test conditions. Interestingly, similar results were obtained by fluorescence microscopy (Figure 3B). Therefore, the results presented here showed that OVA uptake by BMDCs is largely more efficient when formulated as IQB-90 than a mixture with soluble QB-90.

3.4. Subcutaneous administration of QB-90 or IQB-90-adjuvanted vaccine significantly increases humoral and cellular responses in mice

Immune-stimulating activities of OVA formulated in ISCOMs from QB-90 or QA were studied in immunized mice, both at the humoral (i.e. antibody) and cellular (i.e. DTH and proliferation assay) levels. Administration of OVA formulated with soluble saponins was also analyzed. Modulation of antibody responses (Figure 4A and B) show that anti-OVA IgG as well as IgG1 levels were significantly enhanced in respect to control group ($P<0.001$), independently of the saponin (QB-90 or QA) or the formulation type (ISCOMs or soluble). However, anti-OVA IgG2a levels were only increased in mice immunized with ISCOM formulations with either IQB-90 or IQA ($P<0.001$) (Figure 4C).

In order to determine immune-stimulating activity on cellular immune responses, we evaluated the induction of *in vivo* DTH reactions and *in vitro* cell proliferation in response to OVA. Significant DTH reactions were observed in mice immunized with both ISCOM formulations (IQB-90 and IQA) and with soluble QA, while no DTH reactions were observed for soluble QB-90 (Figure 5A). On the other hand, animals vaccinated with QB-90 (10 µg) or QA (10 µg) saponins significantly enhanced the proliferative responses ($P<0.05$, $P<00.1$, respectively). The highest proliferation was also observed in the groups vaccinated with IQB-90 and IQA ($P<0.01$) which was not significantly different between them (Figure 5B). Altogether, results showed that IQB-90 induces strong humoral and cellular immune responses. Interestingly, Th1 and Th2 modulation of antibody responses seem to be induced by IQB-90.

The profile of major Th1 cytokines (IFN-γ and IL-2) was obtained from antigen stimulated and unstimulated splenocytes cultures. Spleen cells from OVA, QB-90, IQB-90, QA and IQA-treated mice were cultured *in vitro* in presence of antigen and cytokine

production was measured in the supernatant after 3 days of culture using ELISA. Mice immunized with either IQB-90 or IQA presented a similar significantly increase of IFN-γ ($P<0.01$ and $P<0.05$ respectively) and IL-2 ($P<0.05$) cytokines levels in the supernatant than those obtained for the control group (Figure 6).

3.5. Intranasal immunization with IQB-90 induces serum and mucosal specific antibody responses

Results in Figure 7 illustrate the serum levels of OVA-specific IgG, IgG1 and IgA in i.n. immunized mice determined 2 weeks after the last immunization. Delivery of IQB-90 and IQA by intranasal route induced significant serum levels of OVA-specific IgG, IgG1 and IgA ($P<0.001$). However, no significant increases in specific IgG2a antibodies and DTH reactions were observed ($P\geq0.05$). Interestingly, no antibody induction was observed after i.n. administration of OVA formulated with either soluble QB-90 or QA (Figure 7). Results on production of specific antibodies at mucosal sites 2 weeks after i.n. immunizations are shown in Figure 8. While administration of IQA induced significant OVA-specific sIgA responses only at the nasal level, IQB-90 enhanced specific sIgA responses at several distal mucosal sites (Figure 8). Once again, no antibody production at mucosal sites was observed after i.n. administration of OVA formulated with either soluble QB-90 or QA. In summary, these results indicate that intranasal delivery of IQB-90 induces significant specific antibody responses both at the systemic and mucosal level, even at distal mucosal sites.

4. Discussion

Adjuvants are defined as any substance usually added to vaccine antigens in order to enhance and/or modulate their immunogenicity. Remarkably, saponins have been shown to activate the mammalian immune system, turning them into interesting substances in the field of potential vaccine adjuvants (Sun et al., 2009a). Quil A® have been widely used as adjuvants in veterinary vaccines (Dalsgaard, 1978) and have the exclusive capacity of stimulating Th1 immune responses as well as inducing production of CTLs against exogenous antigens (Mastelic et al., 2010; Ragupathi et al., 2011). This fact makes them ideal for subunit vaccines and vaccines directed against intracellular pathogens or cancer (Brito and O'Hagan, 2014; O'Hagan and Fox, 2015).

In the present study a saponin fraction from *Q. brasiliensis* known as QB-90 (Kauffmann et al., 2004) showed lower toxic effects than QA *in vitro* and *in vivo*. In this regard, haemolytic activity and cytotoxicity on VERO cells of QB-90 were lower than QA saponins, a fact previously reported (Silveira et al., 2011a). Here, complementary toxicity assays were performed and showed that QB-90 induced-release of LDH is lower than QA. Consistent results were obtained *in vivo* through acute toxicity assays, which showed no lethality or signs of local toxicity in mice after s.c. administration of 31.25 μg of QB-90 while 40% lethality with the same dose of QA. Therefore, as reported elsewhere (Kauffmann et al., 2004; Silveira et al., 2011a) this work reinforces that saponins in QB-90 show significantly less toxicity. Saponins have high-affinity for cholesterol, and consequently tend to form pores in mammalian cell membranes (Gauthier et al., 2009). Concerning this matter, ISCOMs – as well as other micellar formulations built up by saponins and cholesterol – prevent interactions between saponins and membranes, thus preventing haemolysis. In this work we were able to show that IQB-90 displays no haemolysis in comparison with soluble QA or QB-90.

The initiation of an immune response requires participation of antigen-presenting cells (APCs) such as dendritic cells (DCs), which internalize, process, and present Ag-derived peptides to $CD4^+$ and/or $CD8^+$ T cells. Particulate formulations of saponins – like Matrix-M™ and ISCOMs – are known to enhance cell trafficking and activate innate immune cells (Reimer et al., 2012; Zhao et al., 2014). BMDCs were evaluated for their capacity of uptaking FITC-labelled OVA formulated with IQB-90 or mixed with soluble QB-90. OVA:FITC was taken up more efficiently by BMDCs as IQB-90 than mixed with soluble QB-90, supporting the idea that saponin-based formulations as ISCOMs represent the best choice for activation of innate immunity (Maraskovsky et al., 2009; Sun et al., 2009b). Likewise, ISCOMs as well as Quil A® formulations are known to induce potent Th1 and Th2 responses, to activate CTLs and to enhance antibody responses including high levels of IgG1, IgG2b and IgG2a (Ragupathi et al., 2011; Sun et al., 2009b). Here, the effects of IQB-90 and QB-90 on humoral and cellular immune responses were analyzed. The results showed that ISCOMs (IQB-90 and IQA) and soluble saponins (QB-90 and QA) promoted significant Ag-specific splenocyte proliferation and strong DTH reactions. Regarding humoral responses, ISCOMs, as well as saponin formulations, efficiently enhanced the systemic production of Ag-specific antibodies after s.c. immunizations. Remarkably, ISCOMs – unlike soluble saponin formulations – were able to induce significant titers of serum OVA-specific IgG2a. The results presented here indicated that IQB-90 is an efficient and potent modulator of T and B cells functions, a fact previously reported for ISCOMs containing Quil A® (Coffman et al., 2010; Sjolander et al., 1997; Sun et al., 2009b). Furthermore, the immune potentiation of *Q. brasiliensis* saponins in a OVA antigen model are in agreement with other reports (de Costa et al., 2014; Silveira et al., 2011a). The Th1 immune response is characterized by production of the cytokines IL-2, TNF-β and IFN-γ, and an enhanced production of IgG2a, IgG2b and IgG3 in mice. On the other hand, the Th2 subset produces cytokines, such as IL-4, IL- 5 and IL-10, and stimulates

the production of IgG1 and secretory IgA (Sun et al., 2009b; Villacres-Eriksson et al., 1997). Despite potent antibody responses and the DTH reaction stimulated by saponins formulations (QB-90 and QA), the only re-stimulation effect was only significant for IQA and IQB90 formulation (Figure 6). However, others have shown that splenocytes from mice vaccinated with QB-90 saponins formulations vaccine adjuvanted with bovine herpesvirus 5 or poliovirus antigen respond with increased production of Th1 cytokines upon in vitro re-stimulation compared to non-adjuvanted vaccine (de Costa et al., 2014; Silveira et al., 2011a). Here our results show that ISCOMs preparations were more efficient than saponins formulations vaccine in eliciting cytokines responses.

Induction of both systemic and mucosal antibody responses is a desirable feature of the intranasal delivery of vaccines. This route offers the advantages of being easier to administer and thus not requiring trained healthcare workers. Besides, it has the major potential of inducing immunity at the portal of entry of many pathogens (e.g. mucosal sites) (Davitt and Lavelle, 2015; Sjolander et al., 2001). Therefore, immune responses from i.n. delivered ISCOMs and saponin formulations interestingly differ from s.c. immunizations. The i.n. delivery of ISCOMs significantly increased OVA immunogenicity, as seen through the induction of serum specific IgG, IgG1 and IgA antibodies. However, neither systemic IgG2a responses nor DTH reactions were observed after i.n. delivery. These results partially differed from those reported by Hu et al. (Hu et al., 1998), who showed that s.c and i.n. immunizations of respiratory syncytial virus envelope protein in ISCOMs formulated with *Q. saponaria* saponins induced similar specific IgG2a levels in serum. As a consequence, it has been suggested that immunization route and antigen modulate the type of immune response elicited by an adjuvant. Indeed, in several reports it has been demonstrated that subcutaneous administration of ISCOMs induces prominent Th1 responses, while intranasal deliveries bias the response towards a Th2 profile (Morelli et al., 2012; Sjolander et al., 1998). Remarkably,

i.n. immunization with IQB-90 induced local (nasal) as well as distal (vaginal and faeces) mucosal production of OVA-specific IgA.

Finally, this is the first work that reports the formulation of ISCOMs with non-*Q. saponaria* saponins. QB-90 and IQB-90 are significantly less toxic than Quil A®, both *in vivo* and *in vitro*. Additionally, it was observed that Ag uptake by BMDCs is largely more efficient when it is formulated as IQB-90 than soluble saponins. Subcutaneously delivered IQB-90 induces strong humoral (Th1 and Th2-types) and cellular immune responses. Interestingly, the i.n. delivery of IQB-90 induces significant specific antibody responses both at the systemic and mucosal level – even at distal mucosal sites – without evidence of cellular responses. Overall, the properties of IQB-90 reported demonstrate their potential as candidate for further development of prophylactic and therapeutic vaccines, as an alternative to the classic ISCOMs derived from Quil A®.

Acknowledgements

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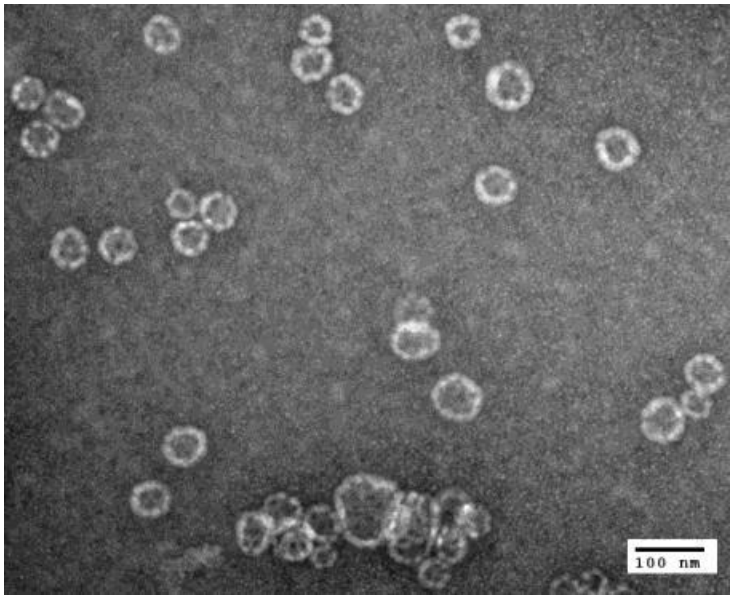

Figure 1. Transmission electron microscopy (TEM) of IQB-90. Microphotography of IQB-90 prepared with purified fraction of saponins from *Quillaja brasiliensis* (QB-90) and OVA by the ethanol injection technique. QB-90 or QA formulation with 3:2:5 relative proportions of saponins:cholesterol:phosphatidylcholine rendered mostly ISCOM particles with an average diameter of 47 nm (overall range of 40-50 nm).

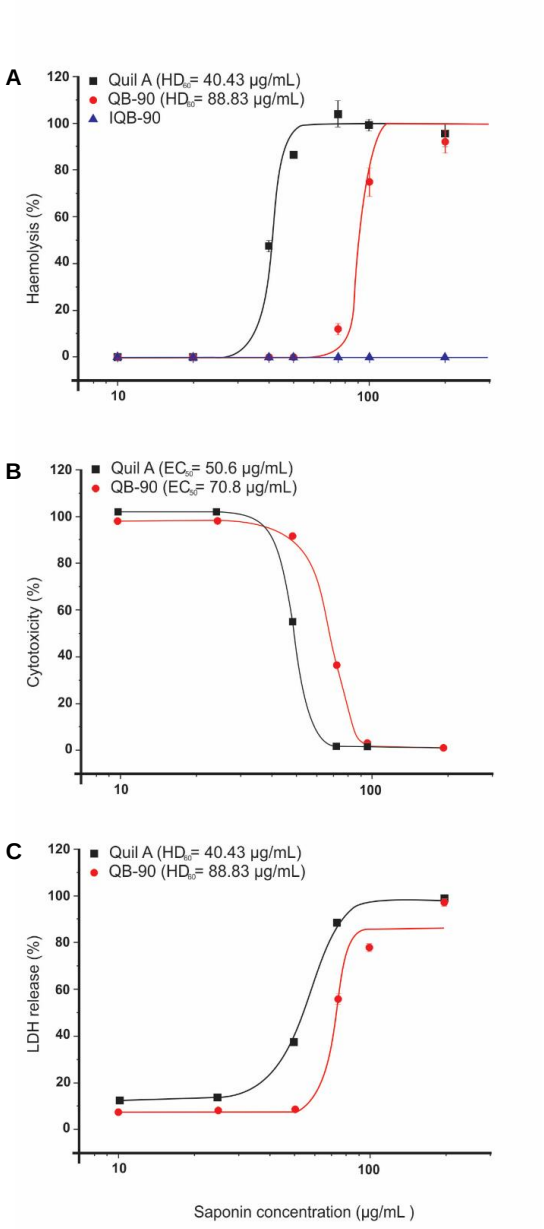

Figure 2. *In vitro* toxicity assays. (A) Haemolytic activity of QB-90, QA and IQB-90. Haemolysis was expressed as percent referred to saline and *Q. saponaria* saponins (250 µg/mL), which were used as 0% and 100% of haemolysis, respectively. (B) Cytotoxicity of QB-90 and QA on VERO cells. Cell viability was measured by MTT 24 h after treatment with the indicated saponin concentrations. (C) LDH release. QB-90 and QA on VERO cells was measured 24 h after treatment with the indicated saponin concentrations. Results are presented as the mean value ± SD (A) and ± SEM (B and C).

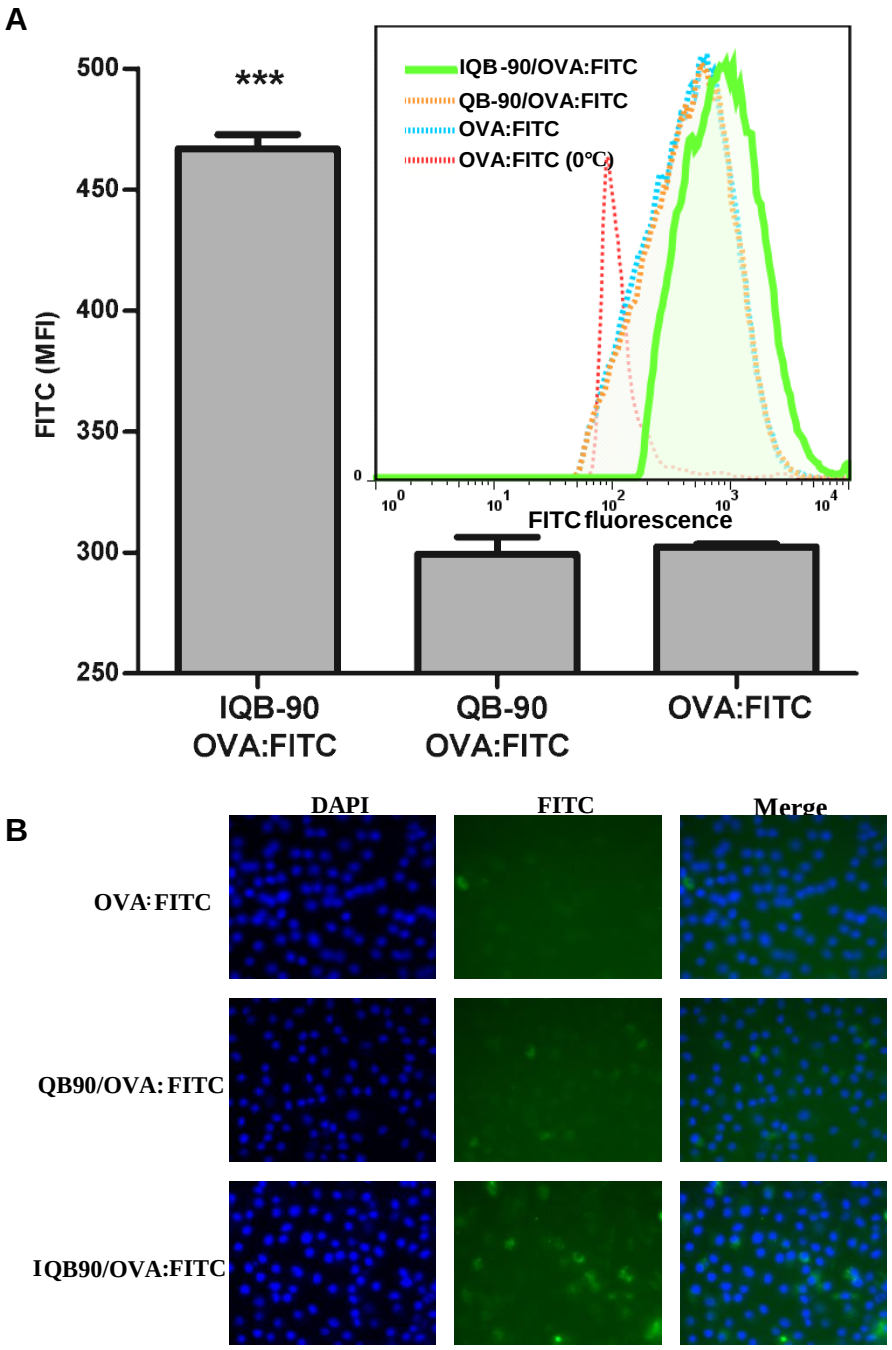

Figure 3. Uptake of model antigen (OVA) by BMDCs. (A) BMDCs were loaded with 2 µg of fluorescein isothiocyanate (FITC)-conjugated OVA (OVA:FITC) in the presence of 10 µg/mL of QB-90, vehicle or as IQB-90 and their uptake was detected by flow cytometry after 120 min. The mean fluorescence increase (MFI) ± standard deviation of three experiments is shown. (B) BMDCs were treated as in (A) and examined by fluorescence microscopy (600X). Data is shown as mean ± SEM and one-way ANOVA with Dunnet´s post-test was used to investigate significant statistical differences (***P <0.001).

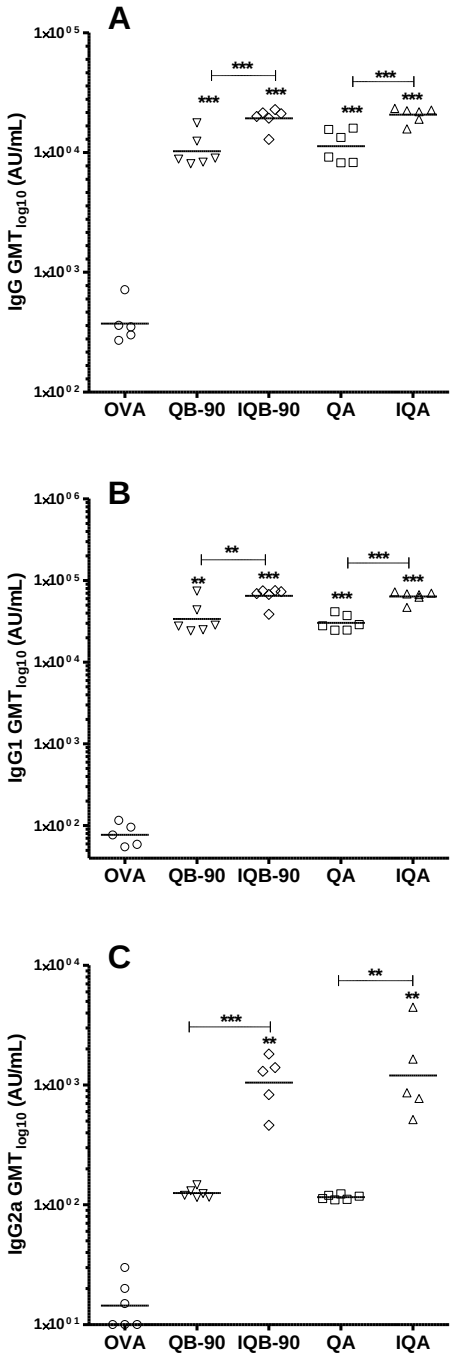

Figure 4. **OVA-specific antibodies response of subcutaneously immunized mice.** Serum titres of anti-OVA total IgG (A), IgG1 (B) and IgG2a (C) 2 weeks after the second immunization. Data is shown as geometric mean titres (*n*=5-6). One-way ANOVA with Dunnet´s post-test was used to investigate significant statistical differences (***P*<0.01 and ****P*<0.001).

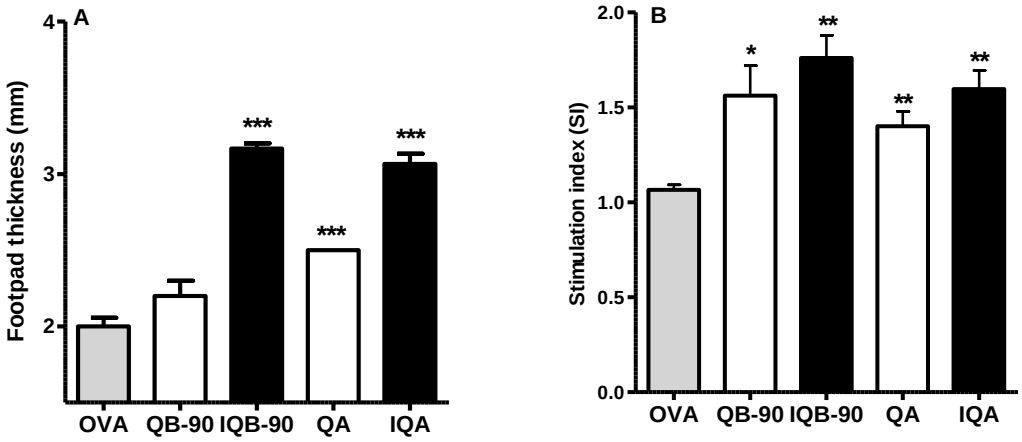

Figure 5. **Evaluation of delayed type hypersensitivity (DTH) and splenocyte proliferation responses in OVA-immunized mice**. (A) DTH; (B) splenocyte proliferation assay. Data is shown as mean ± SEM (*n*=3 or 6 mice, DTH reaction or cell proliferation assay, respectively). One-way ANOVA with Dunnet´s post-test was used to investigate any significant statistical differences (**P*<0.05, ***P*<0.01 and ****P*<0.001).

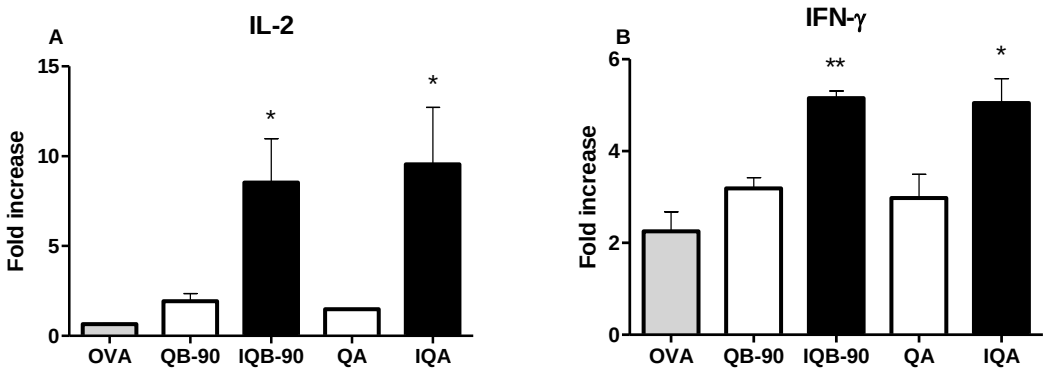

Figure 6. Analysis of IL-2 and IFN-γ patterns in immunized mice. Mice were immunized with OVA alone or adjuvanted with QB-90, IQB-90, QA or IQA and a booster immunization was administered at day 14. Splenocytes were prepared 2 weeks after second antigen dose. The cell supernatant was analyzed after 72 h of stimulation with OVA antigen for IFN-γ and IL-2 using ELISA (*n*=3). Data is shown as mean ± SEM and one-way ANOVA with Dunnet´s post-test was used to investigate any significant statistical differences (**P*<0.05 and ***P*<0.01).

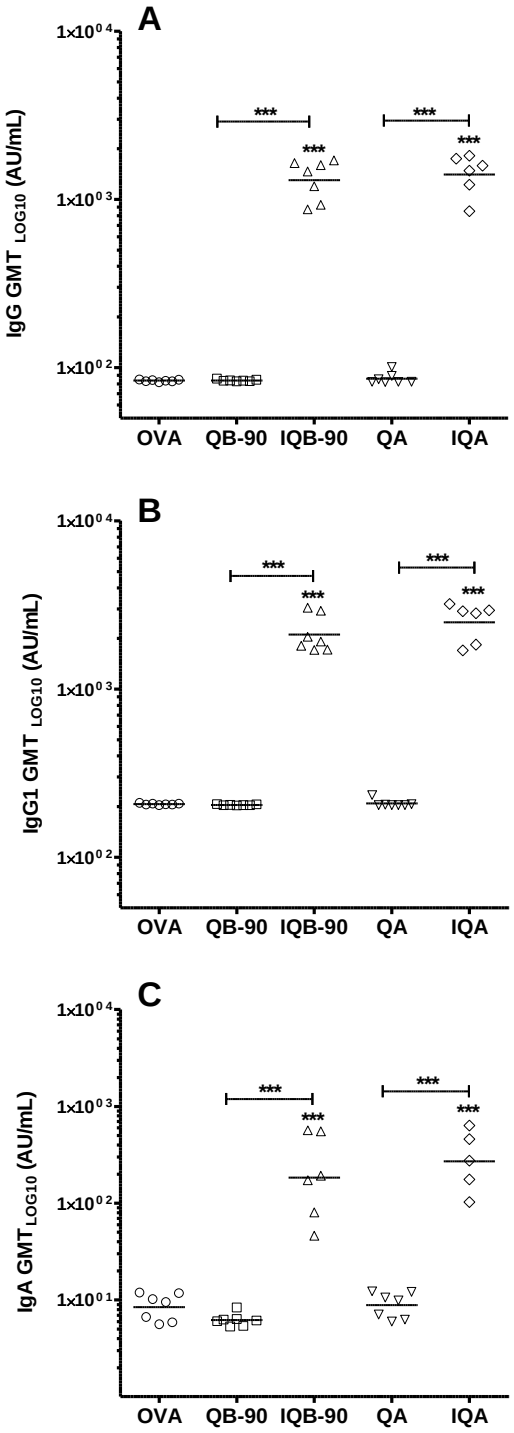

Figure 7. **OVA-specific serum antibodies response from intranasal immunized mice**. Serum titres of anti-OVA IgG (A), IgG1 (B) and IgA (C) 2 weeks after the second immunization administered either with no adjuvant or formulated with QB-90, IQB-90, QA or IQA. Data is shown as geometric mean (*n*=6-7) and one-way ANOVA with Dunnet´s post-test was used to investigate significant statistical difference (***P <0.001).

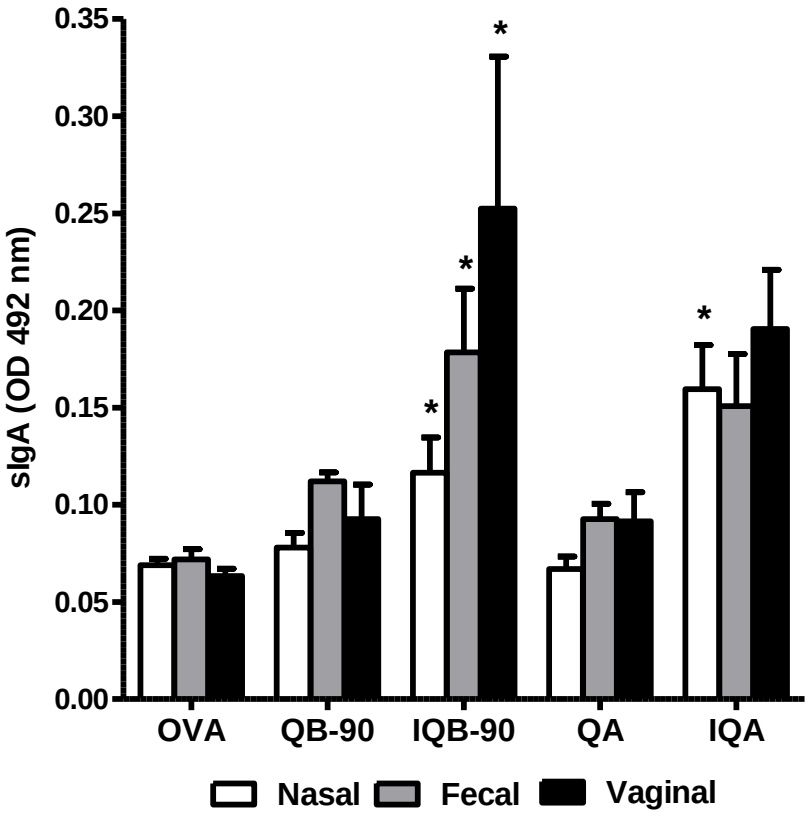

Figure 8. **OVA-specific antibodies response of intranasal immunized mice in mucosal sites**. Mucosal titres of anti-OVA sIgA in nasal, fecal and vaginal mucosae 2 weeks after the second immunization. Data is shown as media ±SEM (*n*=6-7) and one-way ANOVA with Dunnet´s post-test was used to investigate any significant statistical differences (**P*<0.05).

7 DISCUSSÃO GERAL

As saponinas integram uma classe de metabólitos secundários estruturalmente diversa e amplamente distribuída entre as plantas. Estas são amplamente utilizadas nos setores alimentício, têxtil e cosmético, além de atividades farmacológicas, como antiplaquetária, hipocolesterolêmica, antitumoral, antiviral, imunoadjuvante, anti-inflamatória, antibacteriana, inseticida, fungicida e leishmanicida (YENDO *et al.*, 2010).

Desde os anos 1930, extratos de *Quillaja saponaria* são utilizados como adjuvantes vacinais. Nos anos 1970, Dalsgaard obteve uma fração enriquecida de saponinas a partir do extrato de *Q. saponaria,* denominada Quil A, que foi demonstrada estimular tanto a imunidade celular quanto a celular. A fração Quil A foi e ainda é aplicada, principalmente para uso veterinário, pois, em humanos, resultados utilizando Quil A como adjuvante vacinal tem sido menos satisfatórios, devido a reações locais de dor e inflamação. Por esse motivo, existe uma grande busca de saponinas em plantas para avaliação de seu potencial imunoadjuvante e sua toxicidade.

Quillaja brasiliensis apresenta em suas folhas saponinas estrutural e funcionalmente semelhantes às saponinas encontradas nas cascas da espécie chilena (KAUFFMANN *et al.*, 2004; FLECK *et al.*, 2006). Estudos demonstraram a possibilidade de utilização de saponinas de folhas de *Q. brasiliensis* como alternativa à utilização de saponinas extraídas de cascas de *Quillaja saponaria*. Como as folhas são uma fonte de matéria-prima mais prontamente renovável, a exploração autossustentável pode ser realizada, não havendo necessidade da derrubada de árvores.

No capítulo 1, foram abordados temas referentes ao mecanismo de ação das saponinas de *Q. brasiliensis* (QB-90) bem como formulações nanoparticuladas tipo ISCOM (IQB-90). Primeiramente, a necessidade de associação física com o antígeno para a potencialização da resposta imune foi verificada. Para isso, camundongos foram imunizados com a vacina formulada; ou não formulada: com uma dose de antígeno e cinco minutos após com uma dose de QB-90; ou ainda, administrando o adjuvante 24 ou 48 horas antes ou depois do antígeno, no mesmo sítio de inoculação do adjuvante ou em sítios distais daqueles em que o adjuvante foi administrado.

Esse experimento permitiu verificar que a ação adjuvante da QB-90 independe da ligação física com o antígeno, já que animais imunizados com a vacina formulada ou não formulada tiveram uma resposta imune equivalente. Além disso, o potencial imunoadjuvante

da QB-90 foi mantido nos animais que foram sensibilizados com saponina 24 horas antes da administração do antígeno no mesmo local de inoculação do adjuvante. Esse resultado sugere a criação de um ambiente imunocompetente pela administração prévia de QB-90. Esse ambiente aparenta ser transitório, já que nos animais sensibilizados com estas saponinas 48 horas antes da administração do antígeno no mesmo sítio de inoculação do adjuvante, não foi observado potencial imunoadjuvante. Igualmente, animais sensibilizados com saponinas após a administração do antígeno (24 ou 48 horas), ou animais que tiveram a aplicação do antígeno em um sítio distal ao da aplicação do adjuvante, não apresentaram incremento da resposta imune, mostrando que o potencial adjuvante da QB-90 é restrito ao local de inoculação.

Buscando complementar essas observações, foram analisados linfonodos drenantes (dLNs) de animais sensibilizados com QB-90, IQB-90 e a saponina comercial Quil A®. QB-90 (10 μg), IQB-90 (correspondente a 2,5 μg de saponina em massa), Quil A (10 μg) e salina (veículo) foram inoculados por via subcutânea na lateral da cauda. Após 24 ou 48 horas, os dLNs e o baço desses animais foram removidos.

O número de células recuperadas no baço dos animais inoculados com QB-90 e IQB-90 aumentou significativamente em 24 e 48 horas em relação ao controle não tratado, mostrando intensa mobilização de células para esse órgão. Quanto à mobilização de células para os dLNs, houve um aumento significativo para QB-90 e IQB-90 em 24 horas e somente para IQB-90 em 48 horas, mostrando que esse influxo celular é transiente. No baço de animais tratados com QB-90 houve um influxo significativo de neutrófilos e células NK em 48 horas. O número de células dendríticas, linfócitos B e T $CD4^+$ e $CD8^+$ foram significativamente aumentados nos dois tempos analisados. Um perfil semelhante foi observado em animais tratados com IQB-90.

Nos dLNs, neutrófilos foram recrutados de forma significativa após a administração de QB-90 e IQB-90 às 24 horas após inoculação. Após 48 horas, o nível de neutrófilos encontrou-se semelhante aos níveis de animais tratados com salina. O mesmo padrão foi observado para células NK. Células dendríticas e linfócitos B foram encontrados em número aumentado nos dois tratamentos nas primeiras 24 horas. IQB-90 manteve esse aumento significativo nas 48 horas. Linfócitos T $CD4^+$ e $CD8^+$ sofreram um influxo significativo nas primeiras 24 horas em ambos os tratamentos, sendo que IQB-90 manteve esse influxo em 48 horas. Esses resultados de recrutamento de células nos linfonodos drenantes corroboram o padrão de resultados que mostram a criação de um ambiente imunocompetente por saponinas presentes na preparação QB-90.

A fim de caracterizar a expressão genes relacionados ao sistema imune, RNA de dLNs de animais sensibilizados com QB-90 e IQB-90 foram purificados e analisados por qPCR. QB-90 promoveu a expressão diferencial de 44 genes, enquanto os ISCOMs derivados de QB-90 promoveram a expressão diferencial de 23 genes. Dezenove genes foram diferencialmente expressos em ambos os tratamentos.

Os genes *Ccl2* (participa do recrutamento de monócitos, células T de memória e células dendríticas), *Cxcl10* (atração de monócitos/macrófagos, células T, células NK e células dendríticas), *Cxcl11* (recruta células T ativadas), *Ifng* (ativa macrófagos e induz a expreção de MHC de classe I), *Il5* (expansão de células B), *Il6* (proteína de fase aguda, envolvimento com febre), *Ptgs2* (síntese de prostaglandinas) e *Socs2* (indutor da síntese de diversas citocinas, incluindo IFN-γ) foram os que tiveram uma maior expressão em relação ao controle (salina). O gene *Lrp2* (envolvido no transporte de lipídeos e metabolismo necrótico) foi o único a ter sua expressão diminuída em ambos os tratamentos.

No capítulo 2, o potencial imunoadjuvante de QB-90 e o extrato aquoso (AE) semipurificado de *Q. brasiliensis* foi avaliado em sua capacidade adjuvante quando adicionados a um antígeno viral (BVDV). Neste trabalho foi demonstrada a dose-dependência do adjuvante QB-90 e do AE em modelo murino. A geração da resposta imune celular e humoral foi comparada com adjuvantes como o hidróxido de alumínio e o adjuvante incompleto de Freund. Em acordo com os resultados obtidos nos experimentos do Capítulo 1, saponinas de QB-90 e AE foram capazes de aumentar os níveis de IgG total, IgG1 e IgG2a anti-BVDV. Os animais imunizados com esses adjuvantes apresentaram uma resposta DTH positiva. Além disso, quando imunizados com QB-90, apresentaram linfoproliferação estatisticamente superior aos animais imunizados somente com o antígeno viral, proliferação de linfócitos T $CD8^+$ e produção de interferon gama por linfócitos T $CD4^+$ e $CD8^+$. Após reestimulação antigênica *in vitro*, esplenócitos de animais vacinados com QB-90 produziram quantidades significativas de citocinas relacionadas com a resposta Th1. Esses resultados reforçam os encontrados anteriormente por nosso grupo de pesquisa, que mostram que saponinas de *Quillaja bralilliensis* potencializam as respostas imunes e polarizam essa resposta para o tipo Th2 Th1 (FLECK *et al.*, 2006; SILVEIRA *et al.*, 2011; DE COSTA *et al.*, 2014).

No capítulo 3 dessa tese, foi avaliada a toxicidade *in vitro* e *in vivo* de uma fração purificada de saponinas extraídas de *Quillaja brasiliensis* (QB-90). Para os testes *in vitro*, foram utilizados três ensaios tipicamente utilizados na avaliação de citotoxicidade: hemólise em eritrócitos de coelhos, citotoxicidade em células VERO (rim de macaco verde africano) e

liberação de lactato desidrogenase (LDH) em células VERO. A toxicidade induzida por QB-90 foi comparada com a toxicidade induzida por Quil A (porção purificada de saponinas de *Quillaja saponaria*). Os níveis de toxicidade induzidos por QB-90 nos três ensaios realizados foram significativemente menores do que aqueles induzidos pelas saponinas presentes em Quil A®. Esses resultados confirmam os dados obtidos por outros autores – que igualmente demonstraram que saponinas extraídas da espécie sul americana são menos tóxicas que as de *Q. saponaria* (FLECK *et al.*, 2006; SILVEIRA *et al.*, 2011). Como demonstrado no experimento de liberação de LDH, foi observado que QB-90 possui alta afinidade por membranas celulares e, em altas concentrações, forma poros na membrana plasmática extravasando o conteúdo intracelular para o meio extracelular – assim, induzindo a morte da célula. Os resultados dos experimentos *in vitro* foram confirmados em um teste de toxicidade *in vivo*, mostrando que saponinas de *Quillaja brasiliensis* são menos tóxicas que saponinas de *Quillaja saponaria*.

Posteriormente, foi demonstrada a capacidade de formação de complexos imunoestimulantes (nanopartículas do tipo *immunostimulating complexes* ou ISCOMs) a partir de QB-90 (IQB-90). Essas nanopartículas foram avaliadas por microscopia eletrônica de transmissão, apresentando estrutura e tamanho similares aos ISCOMs produzidos com saponinas de *Q. saponaria*. Tais nanopartículas se apresentaram em forma de gaiola, com aproximadamente 40 nm de diâmetro (Anexo A). Os IQB-90 foram avaliados quanto à capacidade hemolítica quando adicionados a eritrócitos de coelho. Surpreendentemente, nenhum grau de hemólise foi verificado. Isso se deve, provavelmente, pela interação das saponinas com os lipídeos presentes no meio para a formação dos nanocomplexos (especialmente o colesterol).

A capacidade de captura de antígeno em células dendríticas foi avaliada por citometria de fluxo. Para tanto, ovalbumina (OVA) conjugada ao fluoróforo isotiocianato de fluoresceína (OVA:FITC) foi utilizado como modelo de antígeno. Os ISCOMs contendo o antígeno OVA:FITC foram produzidos e administrados à células dendríticas *in vitro*, e comparado com OVA:FITC contendo QB-90, ou seja, a suspensão de saponinas solúveis. Os resultados mostraram que OVA:FITC na forma de ISCOMs foram melhor captadas pelas células dendríticas do que OVA:FITC contendo saponinas solúveis. Esta última teve sua integração comparável aos níveis basais de integração de OVA:FITC. Esses resultados foram confirmados por microscopia de fluorescência, que evidenciou claramente uma maior captação de antígenos marcados (OVA:FITC) à cultura de células dendríticas. Esses

resultados mostram que antígenos na forma de ISCOMs (particulado) são internalizados de uma forma mais eficiente que antígenos solúveis.

Os IQB-90, bem como saponinas solúveis (QB-90) foram avaliadas em modelo murino quanto à capacidade imunoadjuvante, utilizando OVA como antígeno. Como controles, foram utilizados ISCOMs derivados de Quil A e a saponina Quil A como controle para saponina solúvel. Os resultados mostraram que as frações solúveis QB-90 e Quil A, bem como seus ISCOMs, potencializaram a resposta imune humoral para OVA nos animais vacinados pela via subcutânea. Entretanto, as nanopartículas do tipo ISCOM tiveram resultados estatisticamente superiores quando comparados às saponinas solúveis em sua capacidade de indução de anticorpos anti-OVA IgG total, IgG1 e IgG2a. Além disso, os ISCOMs de QB-90 e Quil A promoveram uma forte resposta imune celular, caracterizada por intensa reação de hipersensibilidade tardia (DTH), linfoproliferação após reestimulação antigênica *in vitro*, além de produção de interleucina 2 e interferon gama.

A ativação da imunidade de mucosas é crucial para a proteção contra patógenos que acessam o hospedeiro por essa via. Quanto à capacidade de gerar uma resposta imune a partir de imunização intranasal, ISCOMs de QB-90 bem como ISCOMs de Quil A, potencializaram os níveis de IgG total e IgG1 anti-OVA. Nenhum incremento nos níveis de IgG2a e resposta do tipo DTH foi verificado, evidenciando uma clara resposta do tipo Th1. Formulações contendo saponinas solúveis não ofereceram nenhum aumento nos níveis de anticorpos anti-OVA.

Os resultados obtidos revelaram uma menor toxicidade da QB-90 quando comparadas a Quil A. Além disso, nestes experimentos foi possível verificar a formação de ISCOMs a partir de saponinas de *Quillaja brasiliensis* (QB-90), os quais foram melhor internalizados por células dendríticas *in vitro* e induziram uma forte resposta imune humoral e celular. A resposta induzida por essas formulações não diferiu de forma significativa das respostas induzidas com as formulações contendo saponinas de *Quillaja saponaria*.

Em suma, os resultados desta tese expandem significativamente o entendimento do potencial imunoadjuvante de saponinas isoladas da espécie nativa *Quillaja brasiliensis*. Os resultados obtidos reforçam o uso de saponinas da espécie como alternativa às saponinas de *Quillaja saponaria*, em especial a Quil A.

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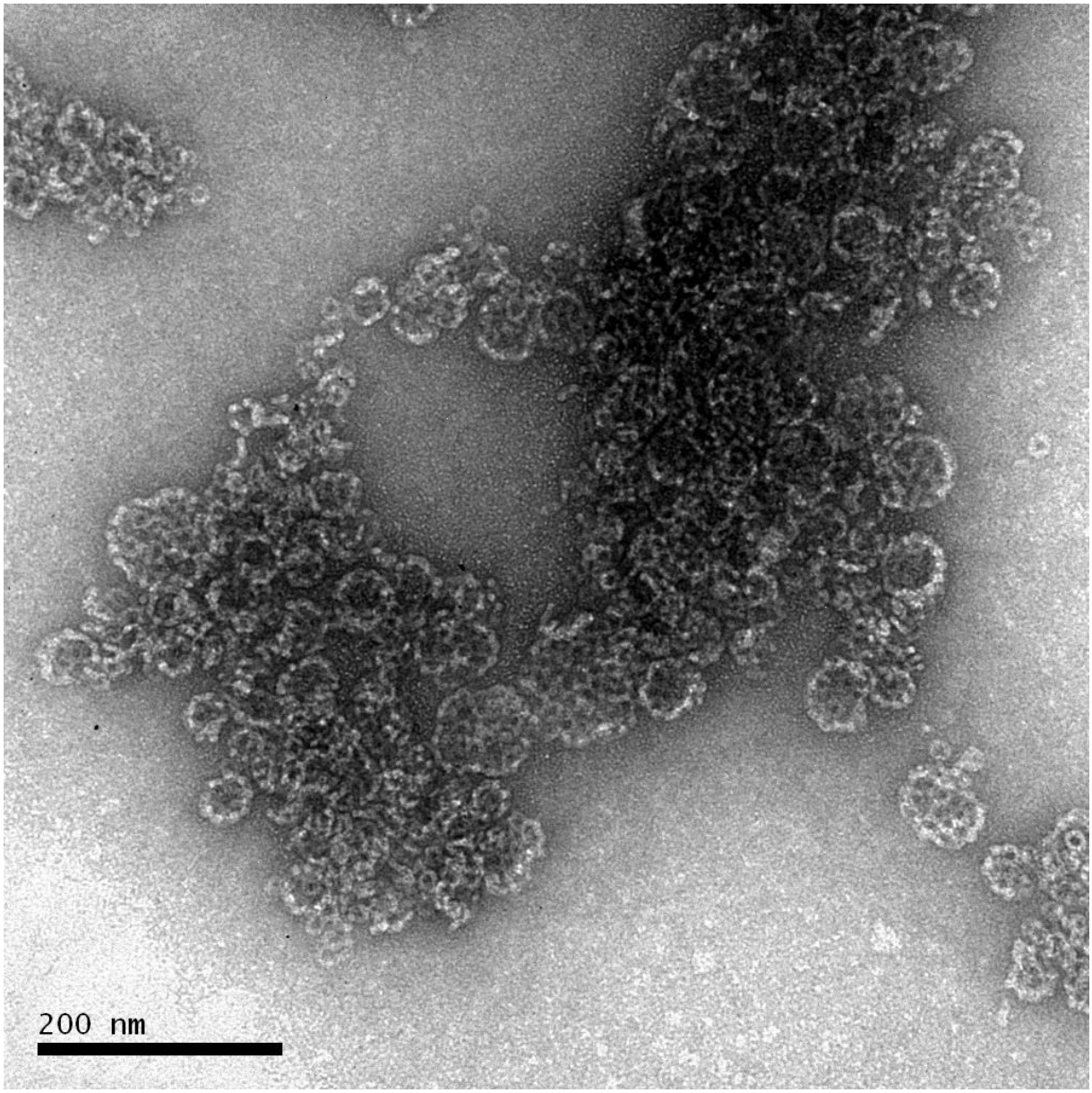
APÊNDICES

APÊNDICE A - Estruturas do tipo ISCOM formadas por saponinas de *Quillaja brasiliensis.*



APÊNDICE B - Indivíduo nativo de *Q. brasiliensis*, localizado em Canguçu, RS.
(A) folha. (B) flores. (C). Frutos verdes. (D) Fruto maduro.