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Tese

Adjuvantes: impacto na eficácia de vacina de subunidade contra leptospirose

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ADJUVANTES: IMPACTO NA EFICÁCIA DE VACINA DE SUBUNIDADE CONTRA LEPTOSPIROSE

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“Inteligência e imaginação são essenciais para a ciência. Mas somente o trabalho organizado leva a ciência aos seus resultados”.

Albert Einstein

RESUMO

BACELO, Kátia Leston. **Adjuvantes: impacto na eficácia de vacina de subunidade contra leptospirose.** 2013. 108 f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O maior desafio para o desenvolvimento de vacinas baseadas em subunidades proteicas, recombinantes ou purificadas e peptídeos sintéticos, reside no fato destas serem pouco imunogênicas e mobilizarem uma resposta imunoprotetora insuficiente. Adjuvantes são utilizados associados a estas subunidades com o intuito de amplificar e direcionar a resposta imune induzida. Na atualidade, existe uma grande gama de compostos que demostram ação adjuvante, contudo, poucos são aprovados para uso humano, e estes muitas vezes falham em induzir resposta imune adequada contra determinado agente patogênico. Assim, existe a necessidade do desenvolvimento de novos adjuvantes que sejam seguros, efetivos e representem uma alternativa aos atualmente disponíveis. No presente estudo, utilizamos como antígeno modelo a porção não-idêntica da proteína LigA *(Leptospiral immunoglobulin-like protein A*) de *Leptospira* spp., uma proteína de membrana externa de grande interesse como mediadora de mecanismos de patogenicidade, utilizada em sorodiagnóstico e em vacinas experimentais. Esta proteína foi associada a diferentes adjuvantes, e as formulações testadas quanto ao seu potencial imunoprotetor em hamsters desafiados com cepa virulenta de *Leptospira interrogans* sorovar Copenhageni. Para isso, a proteína LigAni foi produzida em sua forma recombinante (rLigAni) utilizando *Escherichia coli* como sistema de expressão e associada ao polissacarídeo xantana, em suas variantes xantana pruni cepas 106 (X1) e 101 (X2), xantana comercial, e também ao oligodinucleotídeo CpG (CpG ODN), nanotubos de carbono (CNTs) e hidróxido de alumínio (Alhydrogel). Formulações contendo rLigAni associada ao polissacarídeo xantana e aos CNTs induziram títulos de anticorpos IgG significativos e comparáveis aos induzidos quando a proteína foi associada ao Alhydrogel. Proteção contra o desafio letal foi observada em 100%, 100%, 67% e 50% dos hamsters imunizados com rLigAni-X1, rLigAni-CpG-X1, rLigAni-Alhydrogel e rLigAni-X2, respectivamente (Fisher test $P < 0,05$). As preparações contendo rLigAni associada aos CNTs, embora tenham induzido resposta de anticorpos, falharam em conferir imunoproteção. Adicionalmente, os adjuvantes xantana e CNTs não se mostraram tóxicos em células de ovário de hamster Chinês (CHO), *in vitro*. Os resultados desse estudo apontam a xantana como um novo adjuvante para vacinas de subunidade contra leptospirose, apresentando a propriedade de potencializar a resposta imune contra o antígeno, além de biocompatibilidade e a possibilidade de redução no número de doses requeridas para proteção.

Palavras chaves: Adjuvantes. Xantana. Vacinas de subunidade. Nanotubos de carbono. Leptospirose.

ABSTRACT

BACELO, Kátia Leston. **Adjuvants: impact on the effectiveness in subunit vaccine against leptospirosis.** 2013. 108 f. Tese (Doutorado) **–** Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A major challenge for the development of vaccines based on purified or recombinant protein subunits, and synthetic peptides resides in the fact that these are poorly immunogenic and mobilize insufficient immunoprotective response. Adjuvants are often used in association with these subunits in order to amplify and direct the immune response induced. Nowadays, there is a wide range of compounds that demonstrate adjuvant activity, however, few are approved for human use, and these often fail to induce appropriate immune response against a particular pathogen. Thus, there is a need to develop new adjuvants that are safe, effective and represent an alternative to currently available. In the present study, we used as a model antigen the non- identical portion of the *Leptospira* LigA protein (*Leptospiral immunoglobulin-like protein A*), an outer membrane protein of great interest as a mediator pathogenic mechanisms, used in serological diagnosis and as experimental vaccines. This antigen was formulated with various adjuvants, and the formulations tested for their immunoprotective potential in hamsters, challenged with a virulent strain of *L. interrogans* serovar Copenhageni. For this, the LigAni protein was produced in recombinant form (rLigAni) using *Escherichia coli* as the expression system and associated to xanthan polysaccharide, in its variants xanthan pruni strains 106 (X1) and 101 (X2), commercial xanthan and also to oligodinucleotídeo CpG (CpG ODN), carbon nanotubes (CNTs) and aluminum hydroxide (Alhydrogel). Formulations containing rLigAni associated with xanthan polysaccharide and CNTs induced significant IgG antibody titers, comparable to that induced when the protein was associated with Alhydrogel. Protection against lethal challenge was observed in 100%, 100%, 67% and 50% of the hamsters immunized with rLigAni-X1, rLigAni-CpG-X1, rLigAni-Alhydrogel and rLigAni-X2, respectively (Fisher test $P < 0.05$). The preparations containing rLigAni associated with CNTs, although induced an antibody response, failed to confer immunoprotection. Additionally, xanthan and CNTs adjuvants were not toxic to Chinese hamster ovary (CHO) cells, *in vitro*. The results of this study indicate xanthan as a new adjuvant for subunit vaccines against leptospirosis, presenting the ability to potentiate the immune response against the antigen, besides biocompatibility and the possibility of reduction of number of doses required for protection.

Keywords: Adjuvant. Xanthan. Subunit vaccines. Carbon nanotubes. Leptospirosis.

SUMÁRIO

1 INTRODUÇÃO

A vacinação é uma estratégia de controle de doenças infecciosas, introduzida há mais de 200 anos, que tem se mostrado extremamente importante na redução da morbidade e mortalidade dessas patologias, sendo considerada como uma das mais bem sucedidas intervenções médicas nessa área (DE et al., 2013; HILLEMAN, 2000). Programas de vacinações têm erradicado diversas patologias, como varíola, difteria, poliomielite e tétano neonatal, na maioria dos países desenvolvidos e em desenvolvimento (NANDEDKAR, 2009). Desde 1973, o Brasil dispõe de um Programa Nacional de Imunizações (PNI), que visa integrar ações de imunização realizadas no país. A finalidade da vacinação é gerar uma resposta imune protetora, de suficiente intensidade e duração, para prevenir ou atenuar a virulência de organismos patogênicos (PETROVSKY; COOPER, 2011). Uma grande proporção de vacinas licenciadas é baseada em organismos vivos, e apesar da sua forte potência, esses sistemas vivos têm reações adversas que variam desde reações anafiláticas até encefalite, doença associada à vacina e até morte (HUANG et al., 2004; PERRIE et al., 2007).

Contudo, o desenvolvimento de novas, seguras e efetivas vacinas, não é um processo fácil, especialmente considerando-se os antígenos obtidos a partir de tecnologias moleculares recombinantes (MALLAPRAGADA; NARASIMHAN, 2008). Proteínas altamente purificadas ou peptídios sintéticos constituem vacinas seguras, reduzindo a ocorrência de efeitos adversos decorrentes da sua utilização. Entretanto, desafortunadamente, a falta de características de um patógeno original, como a habilidade de replicação e produção de altos níveis de antígenos e componentes imunoestimulatórios,resultam na baixa imunogenicidade dessas

preparações, o que torna a sua efetiva implementação limitada quando administrados sem adjuvantes (KAZZAZ et al., 2006; PERRIE et al., 2008; SHARP et al., 2009; VANGALA et al., 2006).

A resposta imune inata promove uma primeira linha de defesa necessária para debelar a infecção em virtude da relativa lentidão da resposta imune adaptativa (MEDZHITOV; JANEWAY, 1997). Entre seus muitos efeitos, promove um rápido aumento de citocinas inflamatórias e a ativação das células apresentadoras de antígeno (APCs), como macrófagos e células dendríticas. Esta resposta não específica permite condicionar o sistema imune ao subsequente desenvolvimento de uma resposta adaptativa específica ao antígeno invasor (PASHINE et al., 2005). Vacinas tradicionais, contendo microrganismos mortos ou vivos atenuados, contêm uma série de componentes, como o DNA bacteriano e lipolissacarídeos, que são conhecidos como padrões moleculares associados ao patógeno (PAMPs) e são extremamente importantes no desencadeamento da resposta imune (SINGH; O'HAGAN, 2003). Quando reconhecidos por receptores específicos (PRRs), incluindo os receptores Toll-like (TLR) presentes nas células dendríticas, uma resposta inata é rapidamente induzida (HEEGAARD et al., 2011). Entretanto, esses componentes têm sido eliminados da nova geração de vacinas, particularmente aquelas baseadas em proteínas recombinantes purificadas, peptídeos sintéticos e DNA plasmidial, o que leva à necessidade premente da utilização de potentes adjuvantes, bem como à busca por novos produtos com esse potencial (FOGED, 2011; LIMA et al., 2004).

Neste contexto, testamos preparações vacinais com o antígeno LigANI de *Leptospira interrogans*, uma adesina pertencente a superfamília *Leptospiral Immunoglobulin-like* (Lig) já caracterizada pelo nosso grupo (SEIXAS et al., 2007; SILVA et al., 2007). O objetivo geral do trabalho foi avaliar a ação de diferentes adjuvantes quando utilizados juntamente com o antígeno recombinante, na indução de uma resposta imune protetora contra leptospirose. Para isso, traçamos os seguintes objetivos específicos: (i) Preparar as vacinas recombinantes de subunidade com o antígeno LigANI de *Leptospira*; (ii) Formular as combinações antígeno-adjuvante (polissacarídeo bacteriano, CpG ODN e nanotubos de carbono); (iii) Avaliar os níveis de anticorpos induzidos pelas vacinas através de ELISA; (iv)

Avaliar o potencial imunoprotetor conferido pelas vacinas recombinantes através de teste de desafio.

Os dados gerados nesta tese estão apresentados na forma de artigos científicos. O artigo 1 compara o polissacarídeo xantana produzido por *Xanthomonas arboricola* pv pruni (strain 106), hidróxido de alumínio (alhydrogel) e CpG ODN como adjuvantes em uma vacina de subunidade recombinante contendo a proteína LigA de leptospira. O uso da xantana se monstrou uma nova alternativa eficaz para reforçar a imunogenicidade de vacinas contra leptospirose. Esse trabalho foi submetido ao periódico **International Journal of Macromolecules**. Na sequência, o artigo 2 descreve a utilização de nanotubos de carbono com paredes múltiplas (MWCNT) como adjuvante em uma vacina recombinate contra leptospirose utilizando o mesmo antígeno avaliado no trabalho anterior. O uso dos MWCNT mostrou ser capaz de promover um incremento na geração de anticorpos IgG específicos contra a proteína. Esse trabalho está formatado segundo as normas do periódico **International Journal of Pharmaceutics**. Como prosseguimento do estudo apresentado no primeiro artigo, avaliamos o potencial adjuvante de outras duas xantanas, a xantana pruni 101 e uma xantana comercial. Também avaliamos a possibilidade da utilização da xantana pruni 106, utilizada com sucesso no primeiro artigo, em uma única dose e também com percentual menor na preparação. Este trabalho originou o artigo 3 desta tese, que está formatado segundo as normas do periódico **Carbohydrate Polymers**.

Em função do ineditismo do uso da xantana como adjuvante em vacinas de subunidade recombinantes, como produto adicional dessa tese, foi encaminhado ao Instituto Nacional da Propriedade Industrial (INPI), um depósito de pedido de Patente de Invenção denominada: “XANTANA COMO ADJUVANTE EM VACINA DE SUBUNIDADE RECOMBINANTE” (Número do registro: PI1020120218100).

2 REVISÃO DE LITERATURA

Adjuvantes imunológicos foram originalmente descritos como “substâncias usadas em associação com antígenos específicos que induzem maior imunidade do que a produzida pelo antígeno utilizado isoladamente” (RAMON, 1924). A palavra adjuvante deriva de *adjuvare*, do latim “socorrer” ou “reforçar”. Portanto, são substâncias que tem a habilidade de potencializar o efeito imunogênico de um determinado antígeno, preferencialmente com pequeno ou nenhum efeito colateral.

Adjuvantes podem ser utilizados com múltiplos propósitos: para aumentar a imunogenicidade, diminuir a dose necessária de antígeno, acelerar a resposta imune, reduzir o número de imunizações necessárias, aumentar a duração da proteção, ou ainda, para melhorar a eficácia da imunização em respondedores fracos, como neonatos e idosos (PETROVSKY; AGUILAR, 2004). Uma extensa família de moléculas e substâncias, incluindo sais minerais, produtos microbianos, emulsões, saponinas, citocinas, polímeros, micro e nanopartículas, e lipossomas, têm sido avaliadas quanto a sua capacidade adjuvante, muitas vezes de forma empírica, sem o conhecimento dos mecanismos de ação específicos (AWATE et al., 2013; GARLAPATI et al., 2009; VOGEL; POWELL, 1995). No entanto, a maioria delas não foi aprovada para uso rotineiro em vacinas em função da sua toxicidade.

Baseado no mecanismo de ação proposto, os adjuvantes podem ser divididos em adjuvantes imunoestimulatórios e sistemas de veiculação de vacinas (SCHWENDENER et al., 2010; SINGH; O'HAGAN, 2003). No primeiro grupo encontram-se os sais de alumínio, os quais têm sido utilizado com sucesso por muitos anos como adjuvantes em vacinas licenciadas. Em geral, esses adjuvantes ativam células do sistema imune inato (PASHINE et al., 2005). Os sistemas de veiculação de vacinas são geralmente compostos de partículas de dimensões comparáveis a patógenos, como bactérias ou vírus (PERRIE et al., 2008;

SCHWENDENER et al., 2010), primeiramente descritos como formadores de depósito do antígeno, embora atualmente existam evidências que alguns desses adjuvantes possam ativar a resposta imune inata (AWATE et al., 2013). O encapsulamento pode aumentar a veiculação e aumentar a imunogenicidade da formulação devido ao aumento da proteção contra degradação e reconhecimento pelas células apresentadoras de antígenos (MALYALA et al., 2008).

Adjuvantes podem atuar pelo emprego de um ou vários mecanismos de ação como: liberação controlada do antígeno no sítio de infecção (efeito depósito), estímulo na produção de citocinas e quimiocinas, recrutamento de células relacionadas à resposta imune no sítio de injeção, aumento do ingresso do antígeno nas APCs, ativação e maturação das APCs, com aumento de expressão do complexo maior de histocompatibilidade (MHC) de classe II e de moléculas co-estimulatórias, e ativação de inflamossomos (AWATE et al., 2013; COX; COULTER, 1997; DE et al., 2013).

Apesar de não haver um adjuvante universal, as emulsões oleosas e os sais de alumínio são os adjuvantes mais comumente utilizados em vacinas de uso veterinário e humano, ainda que emulsões do tipo óleo em água, como o MF59, e agonistas de receptores “toll-like”, como o AS04, venham sendo utilizadas em algumas preparações (AUCOUTURIER et al., 2001; DE et al., 2013).

2.1 Sais de alumínio

Os sais de alumínio, que incluem fosfato de alumínio, hidróxido de alumínio e hidróxido-fosfato de alumínio, comumente denominados “alum”, têm sido amplamente utilizados em vacinas humanas, por quase noventa anos. O primeiro estudo em modelos animais foi publicado em 1926 (GLENNY, 1926). Esta classe de adjuvantes é componente de várias vacinas virais e bacterianas, como as vacinas contra difteria, tétano e coqueluche, vacinas contra os vírus das hepatites A e B, raiva, entre outras.

Os antígenos vacinais são adsorvidos nas partículas de alumínio, o que resulta em um aumento da estabilidade do antígeno e fez supor inicialmente que o alumínio criasse um depósito *in situ*, que permitiria a liberação prolongada do antígeno e consequente aumento da exposição deste ao sistema imune (HEM;

WHITE, 1995). Contudo, ensaios posteriores puseram em dúvida o modo de ação desse adjuvante. Gupta e colaboradores mostraram que após injeção intramuscular, a maioria do antígeno se difundia do local de injeção em algumas horas (GUPTA et al., 1996). Corroborando com esse achado, foi também demonstrado que a administração do antígeno adsorvido ao alumínio não aumentava a meia vida deste *in situ* (HEM, HOGENESCH, 2007). Praticamente excluindo a teoria da formação de depósito como mecanismo de adjuvanticidade do alumínio, recentemente foi demonstrado que o ingresso nas células apresentadoras de antígenos do antígeno adsorvido ao alumínio ou a um imunomodulador como o CpG, não diferia e que ainda, notavelmente, a remoção do sítio de injeção e por conseguinte o suposto depósito de alumínio associado, não ocasionava efeito expressivo na resposta específica de células T e B (HUTCHISON et al., 2012).

Formulações vacinais particuladas, a exemplo do que ocorre com antígenos adsorvidos ao alumínio, são em geral mais prontamente internalizadas pelas células apresentadoras de antígenos (APCs) do que antígenos solúveis (DE et al., 2013). O mecanismo pelo qual o ingresso do antígeno é facilitado pelos sais de alumínino não está claro, mas estudo recente propõe que o ingresso de antígeno ocorra na ausência do ingresso de alumínio nas células dendríticas. Nesse estudo Flach e colaboradores (FLACH et al., 2011) demonstraram que o alumínio é capaz de se ligar aos lipídios de membrana das células dendríticas com substancial força e não a receptores específicos nessa membrana. Essa ligação promove uma alteração na estrutura dos lipídios de membrana que envolve a agregação dos motivos ativadores baseados nos imunoreceptores de tirosina (ITAMs), e subsequente resposta fagocítica mediada por tirosina quinase esplênica (Syk) e fosfoinositol-3-quinase (PI3K). Dessa forma o alumínio não ingressaria na célula, ao invés disso, entregaria o antígeno adsorvido através da membrana plasmática. As células dendríticas ativadas pelo alum, segundo os autores, desenvolvem uma forte afinidade pelas células T CD4, medida por ICAM-1 e LFA-1, sendo esse mecanismo essencial para a adjuvanticidade dos sais de alumínio. Esses resultados se opõem a estudo prévio utilizando microscopia confocal, que mostra que o alumínio é internalizado pelas APCs (HORNUNG et al., 2008).

Tipicamente o alumínio induz resposta imune do tipo Th2, entretanto falha em produzir resposta imune celular do tipo Th1. O mecanismo pelo qual esse

adjuvante estimula predominantemente resposta Th2 não é bem entendido, no entanto, foi demonstrado que ao contrário do que ocorre com os agonistas de receptores “Toll-like” (agonistas TLR), que requerem moléculas adaptadoras MyD88 e TRIF, o efeito adjuvante do alumínio não é diminuído na ausência dessas proteínas, sugerindo que o alumínio não sinaliza de maneira dependente de TLR (GAVIN et al., 2006). Formulações contendo alumínio como adjuvante estimulam a ativação do inflamossomo, envolvendo a sinalização mediada por NLRP3 e caspase-1 e consequente liberação de IL-1 beta e IL-18 pelas células dendríticas (DCs) (SOKOLOVSKA et al., 2007). As DCs ativadas desse modo estimulam as células T CD4(+) a secretarem IL-4 e IL-5, direcionando para uma resposta Th2. Por outro lado, o alumínio pode indiretamente promover a liberação de certas moléculas pelas células as quais podem auxiliar na atividade adjuvante. Exemplo disso é a estimulação na produção de ácido úrico, o qual é produzido normalmente como um padrão molecular associado ao perigo (DAMP) após injúria celular (KOOL et al., 2008). O ácido úrico liberado é então internalizado pelas APCs, e as ativa via inflamossomo, promovendo um efeito imunoestimulatório secundário em resposta à vacinação com vacinas contendo alumínio.

O efeito imunoestimulatório do alumínio, responsável por suaimunogenicidade, é amplo e parece envolver múltiplas vias, cuja elucidação requer mais estudos. Embora o alumínio não induza a produção de interferon gama e linfócitos T citotóxicos, requerida para debelar infecções virais intracelulares (KAZZAZ et al., 2006) e tenha sido associado com reações locais severas, como eritema, nódulos subcutâneos e hipersensibilidade de contato (PERRIE et al., 2008), esse adjuvante tornou-se referência em estudos que avaliam novas substâncias com potencial adjuvante, uma vez que é regularmente utilizado em vacinas humanas, com bom histórico de segurança.

2.2 Agonistas de receptores “Toll-like”

Em adição ao alumínio e as emulsões óleo em água, vários outros adjuvantes tem sido avaliados em ensaios clínicos em humanos em vacinas contra o vírus da hepatite B (HBV), papiloma vírus humano (HPV), malária, influenza,

câncer, entre outras patologias. Muitos desses adjuvantes são conhecidos elementos alvo sinalizadores de vias da resposta imune inata, reconhecidos por receptores de reconhecimento de patógenos (PRRs), em particular ativadores de receptores TLR (DE et al., 2013).

A primeira classe de PRRs celulares identificados foram os TLRs. Receptores toll foram definidos pela primeira vez na mosca da fruta, e foram localizados na membrana celular externa e de endossomos (TAKEDA; AKIRA, 2005). Outros PRRs, no entanto, como os receptores semelhantes aos *domínios* de *ligação de nucleotídeos* e *oligomerização* (NOD-like) (NLRs), e receptores semelhantes aos induzidos por ácido retinóico (RIG-I-like), entre outros, também estão presentes nas células do hospedeiro (DE et al., 2013).

O sistema imune inato é a primeira linha de desfesa, e está envolvido na detecção inicial e remoção de patógenos do organismo. Diferente das reações do sistema imune adquirido, a resposta inata é ativada em minutos. O desencadeamento dessa resposta se dá através da ativação de PRRs que reconhecem estruturas microbianas conservadas nos patógenos, frequentemente referidas como padrões moleculares associados ao patógeno (PAMPs). Esses PAMPs, em geral, desempenham uma função crítica na vida do microrganismo e incluem lipopolissacarídeos (LPS), peptideoglicano (PGN), flagelina e ácidos nucleicos (FRANCHI et al., 2009).

A ligação de um PAMP, como um polissacarídeo, aos TLRs pode induzir a transcrição, síntese e secreção de citocinas como IL-8, IFN-γ e IL-12 (JANEWAY; MEDZHITOV, 2002), bem como a produção intracelular de pro-IL-1β e pro-IL-18, mas não sua secreção. Um segundo “sinal de perigo”, liberado por células do hospedeiro em stress ou infectadas, ou detectado como um PAMP no citosol, pode estimular a montagem de um inflamossomo que ativa uma protease, a caspase-1. A Caspase-1 por sua vez, é responsável pelo processamento e secreção de IL-1β e IL-18, propiciando dessa forma um elo importante entre as respostas imune inata e adquirida (ABDUL-SATER et al., 2009).

Vacinas que contêm vírus ou bactérias mortos ou atenuados incluem naturalmente componentes que podem ser reconhecidos por TLRs. Este é o caso, por exemplo, da vacina contra a febre amarela, que é baseada em vírus atenuado e é capaz de interagir com pelo menos quatro TLRs (2, 7, 8 e 9) nas células

dendríticas do hospedeiro (QUEREC et al., 2006). Dessa forma, agonistas TLRs e outros PRRs são alvos atrativos como adjuvantes vacinais. Dois desses agonistas, TLR-4 e TLR-9, são sucintamente descritos abaixo.

TLR-4 é um PRR presente na superfície celular de células dendríticas e monócitos, que reconhece vários PAMPs, incluindo lipopolissacarídeo (LPS) de bactérias Gram negativas e ácido lipoteicóico de bactérias Gram positivas. Normalmente o LPS é tóxico e não apropriado para ser utilizado em vacinas de subunidade, em virtude de sua alta efetividade em estimular receptores TLR-4, o que induz a um processo inflamatório excessivo e tóxico (MCKEE et al., 2007). No entanto, a hidrólise do lipídio A, bioativo do LPS, resulta em uma molécula chamada 3-O-desacetil-4'-monofosforil lipídio A (MPL), uma substância com reduzida toxicidade comparada com o lipídio A (~1000 vezes menor que LPS), mas que retem a capacidade de ligação ao PRRs TLR-4 (DE et al., 2013).

O adjuvante conhecido como AS04 (adjuvante system 04), combina o MPL e sais de alumínio e, como consequência, combina uma resposta protetora humoral, bem como é eficiente em promover uma resposta celular Th1. AS04 é atualmente utilizado com sucesso, como adjuvante, em vacinas licenciadas contra a hepatite B e contra o HPV (DIDIERLAURENT et al., 2009).

Outras substâncias têm sido demonstradas como agonistas TLR-4. Esse é o caso da goma xantana (TAKEUCHI et al., 2009) e do angelan, um polissacarídeo ácido isolado de *Angelica gigas,* que foi capaz de incrementar a maturação e migração de células dendríticas (DC), via TLR-4, sendo essa propriedade avaliada na imunoterapia baseada em DCs contra o câncer (KIM et al., 2011).

TLR-9 é um PRR pertencente a subfamília de TLRs, formado por TLR-3, TLR-7, TLR-8 e TLR-9, que reconhecem ácidos nucleicos. Localizado no endossomo, é capaz de reconhecer DNA contendo resíduos de oligodinucleotídeo (ODN) citosina-guanosina (CG) os quais são sequências não metiladas de DNA bacteriano, denominados motivos CG, responsáveis por propriedades estimuladoras do sistema imune (KRIEG, 2002). Esses motivos estão presentes, em maior frequência (1:16), no DNA bacteriano, enquanto que em vertebrados, estão suprimidos (1:60) e seletivamente metilados (KRIEG; WAGNER, 2000). Os oligodinucleotídeos CG sintéticos ligados por pontes de fosforotioato (CpG ODNs), mimetizam o efeito estimulatório do DNA microbiano, sendo potentes adjuvantes

que aceleram e impulsionam uma resposta imune antígeno específica, facilitando a erradicação de doenças infecciosas (KLINMAN et al., 2009; LIU et al., 2012).

A evidência de que motivos CpG estimulavam respostas imunes em mamíferos foi corroborada por estudos com CpG ODN sintéticos em camundongos (DAVIS et al., 1998; KRIEG; WAGNER, 2000). Os CpG ODNs estimulam diretamente um pequeno grupo de células competentes do sistema imune, incluindo linfócitos B, células dendríticas, macrófagos e monócitos (HARTMANN; KRIEG, 1999; STACEY et al., 1996) via ativação de TLR9 (HUBBELL et al., 2009).

Existem vários tipos desses motivos CpG, todos dependentes de TLR-9, mas com diferenças quantitativas e qualitativas na resposta imune que desencadeiam. A atividade do CpG ODN é determinada pelo seu comprimento, número de motivos CpG e pelo espaçamento, posição e bases que rodeiam o motivo (LIU et al., 2012). CpG ODNs podem ser divididos em Classe A (também conhecido como tipo D), Classe B (tipo K) e classe C (GRAY et al., 2007). Em contraste com os de classe A, os ODNs de classe C possuem pontes fosforotioato em todos dinucleotídeos CG, característico dos CpG ODNs de classe B, e não contêm espaçadores poli-G, por outro lado contêm sequências palindrômicas combinadas aos motivos CG, semelhante aos ODNs de classe A (KRIEG, 2012; VOLLMER et al., 2004). Um representante característico da classe B é o ODN-1826 que contém 18-24 pares de base em comprimento (5′-TCC ATG ACG TTC CTG ACG TT-3′), enquanto a classe C pode ser exemplificada pelo ODN-M362 (5'-TCG TCG TCG TTC GAA CGA CGT TGA T).

Mais recentemente foi descrita uma nova classe de CpG ODNs, chamada classe P, que combina as características preferenciais das outras classes, propiciando um substancial incremento na produção de interferon e citocinas. Essa classe contém duas sequências palindrômicas, o que permite que sejam formados concatâmeros com unidades multiméricas, onde cada molécula é ligada através do pareamento de “Watson-Crick” a uma segunda e a uma terceira palíndrome (SAMULOWITZ et al., 2010). CpG ODNs de classe B estimulam de maneira acentuada a ativação de células “Natural Killer” (NK) e produção de IgM e IL-6 por linfócitos B. Uma maior estimulação de células NK e produção de IFN-alfa por células dendríticas plasmocitoides é atingida com CpG ODN de classe A, no entanto essa classe é falha na indução de células B. A classe C de CpG ODNs combina o

efeito imune induzido pelas classes A e B, isto é, promovem forte estimulação de células B e NK e produção de IFN-alfa (VOLLMER et al., 2004).

Os ODNs CpG exibem diferenças específicas de acordo com a espécie, o que tem dificultado o desenvolvimento dessa classe de adjuvante. Por exemplo, CpG-ODN contendo motivos GTCGTT preferencialmente ativam células humanas, enquanto CpG-ODN com motivos GACGTT demonstram maior atividade em células de camundongos (LIU et al., 2012). A indução da resposta imune pelos agonistas TLR-9 se dá via ativação das moléculas adaptadoras MyD88, IRAK e TRAF-6 levando ao recrutamento de fatores transcricionais, o que se reflete num aumento de expressão de IL-1, IL-6, IL-12, IL-18, e TNF-alfa (AWATE et al., 2013).

Agonistas TLR-9 têm sido avaliados em vacinas contra HBV, Influenza, câncer e tuberculose, com ensaios clínicos em humanos já realizados, embora seu uso ainda não tenha sido aprovado pelos órgãos reguladores (DE et al., 2013). Em bovinos, a utilização de vacinas de subunidade recombinante contra herpes vírus tipo 1 (IOANNOU et al., 2002; RANKIN et al., 2002) e peptídeo sintético de *Mycobacterium bovis* (VORDERMEIER et al., 2005), associadas ao CpG ODN mostraram resultados promissores demonstrado a utilidade dos ODN CpG na adjuvanticidade.

2.3 Nanopartículas como adjuvantes

Nanopartículas são geralmente partículas com diâmetro variando entre 1 e 100 nm. O seu pequeno tamanho possibilita facilmente seu ingresso nas células, e são geralmente biocompatíveis (BURGESS, 2009). O efeito das nanopartículas nas células imunes pode beneficiar o tratamento de desordens mediadas por respostas imunes não desejadas, e reforçar a resposta imune à antígenos fracos. Nanovacinas oferecem diversas vantagens potenciais, incluindo entrega do antígeno no sítio específico, incremento na biodisponibilidade do antígeno e redução no perfil de eventos adversos (ZOLNIK et al., 2010). As propriedades imunoestimulatórias de nanopartículas permitem seu uso como adjuvantes ou carreadores de antígenos vacinais. Exemplos de algumas das principais nanopartículas para o desenvolvimento de nanovacinas incluem: nanopartículas

metálicas relativamente inertes como o ouro coloidal, com 10 a 50 nm, nas quais o antígeno é conjugado ou associado; micelas compostas de polímeros anfifílicos que formam estruturas contendo 5 a 100 nm de diâmetro em solução aquosa; lipossomas (20 nm a 1μm) que são estruturas compostas de um núcleo aquoso completamente encapsulado por uma bicamada lipídica; e nanotubos de carbono (2 a 100nm) compostos de anéis de benzeno formando um cilindro de carbono (ZAMAN et al., 2013).

Ainda que o exato mecanismo de adjuvanticidade das nanopartículas não estar claro, é sugerido que elas possam incrementar o ingresso do antígeno e/ou estimular as APCs induzindo uma resposta celular e humoral. Nanopartículas utilizadas em formulações vacinais tendem a ter tamanho comparável à patógenos reconhecidos pelo sistema imune (ELAMANCHILI et al., 2007; XIANG et al., 2008).

Os nanotubos de carbono, descobertos em 1991, apresentam um grande potencial na área biomédica devido à propriedade de biocompatibilidade, principalmente nas áreas de engenharia de tecidos e transfecção genética (FILHO et al., 2007). Nanotubos de carbono (CNTs) podem ser de parede simples (single walled SWCNT), parede dupla (double walled - DWCNT) ou de parede múltipla (multiwalled- MWCNT) na depência do processo pelo qual são produzidos. Em geral, CNTs têm difícil processamento em virtude da falta de solubilidade em muitos solventes, o que pode ser minimizado pela funcionalização química de sua parede externa. A ligação de grupamentos de forma covalente ou não covalente a essas nanopartículas gera os chamados CTNs funcionalizados (f-CTNs), e tem sido utilizada com sucesso para amenizar a dificuldade de seu processamento (NIYOGI et al., 2002; PANTAROTTO et al., 2003a). Um exemplo é a funcionalização de MWCNT com grupamentos carboxil para melhorar sua dispersão em água (ZHAO et al., 2010).

Os CNTs podem ser ligados a proteínas por várias interações fracas como empilhamento π–π, interações hidrofóbicas e eletrostáticas, além de ligações covalentes (ZUO et al., 2012). Isso possibilita uma aplicação para os f-CNTs como carreadores de peptídeos vacinais de antígenos bacterianos, virais e parasitários ao sistema imune (BIANCO et al., 2005; KAM et al., 2006; MARCATO; DURAN, 2008; PANTAROTTO et al., 2003a; PANTAROTTO et al., 2003b).

2.4 Polímeros naturais como adjuvantes

Recentemente, alguns polímeros têm se mostrado candidatos promissores na substituição de adjuvantes convencionais pelas suas propriedades de biocompatibilidade e biodegradabilidade. Esses polímeros, juntamente com o antígeno, podem ativar de maneira efetiva a resposta imune, tornando-se, portanto, adjuvantes vacinais de interesse para a indústria farmacêutica (SHAKYA; NANDAKUMAR, 2013). O esquema de vacinação convencional utilizando múltiplas doses pode acarretar sérias dificuldades, principalmente em países em desenvolvimento. De uma maneira bastante interessante, polímeros nas formulações vacinais podem incrementar a veiculação de antígenos e dessa forma reduzir as doses de reforço requeridas para uma resposta imune apropriada.

Vários polímeros biodegradáveis naturais ou sintéticos têm sido avaliados no aumento da imunogenicidade de antígenos fracos ou em imunoterapia para o câncer. Dentre eles, podem ser citados o dextran (BACHELDER et al., 2010), o lentinano na vacina contra a doença de Newcastle (GUO et al., 2009), quitosana em imunização contra hepatite B (PREGO et al., 2010), copolímero de ácidos glicólico e lático, avaliado em vacinas contra malária (MOON et al., 2012), hepatite B (SAINI et al., 2011) e leptospirose (FAISAL et al., 2009), o polissacarídeo extraído do pólen de Taishan pinus massoniana (TPPPS) (CUI et al., 2013) e os polissacarídeos angelan (KIM et al., 2011) e xantana (TAKEUCHI et al., 2009) em terapias antitumorais.

Produtos naturais são, portanto, uma fonte importante de muitos produtos farmacêuticos na atualidade e são uma fonte potencial de agentes imunomoduladores (NEWMAN; CRAGG, 2007; REY-LADINO et al., 2011). Os obstáculos que um novo adjuvante enfrenta para que seja aprovado pelas agências regulatórias podem ser significativamente reduzidos se for constituído de um composto com segurança e tolerabilidade ao homem já conhecida. Numerosos polissacarídeos originados de plantas e microrganismos, alguns já exemplificados acima, têm sido testados pelo seu poder adjuvante e têm a vantagem, com raras exceções, de apresentarem alta biocompatibilidade e baixa toxicidade (PETROVSKY; COOPER, 2011; REY-LADINO et al., 2011).

Carboidratos são facilmente metabolizados ou excretados, com pequeno risco de geração de metabólitos tóxicos ou de reações inflamatórias tardias no local

de aplicação, como a que ocorre com os sais de alumínio em uma condição conhecida como miofascite macrofágica. Essa enfermidade cursa com hipotonia e atraso motor e psicomotor, reportada principalmente em adultos na França, mas também podem ocorrer em crianças (RIVAS et al., 2005). Adjuvantes vacinais à base de carboidratos podem ser bem distintos, com cada um deles apresentando suas próprias características físico-químicas e atributos imunológicos e de comportamento, promovendo uma ampla gama de opções no desenvolvimento de vacinas (PETROVSKY; COOPER, 2011).

Glicanas são polissacarídeos derivados de plantas ou microrganismos, constituídos de unidades repetidas de D- glicose unidas por ligações glicosídicas, com várias conformações alternativas. Alfa – glicanas incluem dextran (α-1,6-glicana), glicogênio (α-1,4- e α-1,6-glicana), pululana (α-1,4- e α-1,6-glicana) e amido (α-1,4- e α-1,6-glicana). β-glicanas incluem celulose (β-1,4-glicana), curdulana (β-1,3-glicana), laminarina (β-1,3- e β-1,6-glicana), lentinano (purificado β-1,6:β-1,3-glicano obtido do cogumelo *Lentinus edodes*), zimosana (β-1,3-glicana de *Saccharomyces*), xantana (β-1,4-glicana), entre outros (PETROVSKY; COOPER, 2011; RODD et al., 2000). Cada tipo e fonte de glicanas apresenta uma variedade em termos de qualidade e pureza e pode conter uma mistura de diferentes estruturas de polímeros com ramificações e tamanhos de cadeias diversos.

Glicanas podem reforçar tanto a imunidade humoral quanto celular. A maioria dos adjuvantes a base de carboidratos, como a zimosana, manana, Muramildipeptide (MDP; originário de peptideoglicano de micobactéria), atuam através da ligação a receptores específicos da resposta imune inata que reconhecem PAMPS. Esses receptores incluem os TLRs, NOD2 e C-type lectins (como as lectinas ligadoras de manana, receptores β-glicano e Dectin-1), cuja ligação resulta na ativação de NF-κB e produção de citocinas inflamatórias, incluindo TNF-α e IL-1 (HUANG et al., 2010; REY-LADINO et al., 2011). As citocinas produzidas a partir da ligação PAMP-receptor podem atuar como moléculas co-estimulatórias, para reforçar uma resposta específica de linfócitos T e B. A adjuvanticidade dos carboidratos pode também ser explicada pelo aumento de expressão de moléculas co-estimulatórias, incluindo MHC de classe I e II, CD40, CD80 e CD86 nas APCs (LEE et al., 2001).

Compostos adjuvantes polissacarídicos raramente se ligam exclusivamente a um único receptor, com alguns se ligando a quatro ou mais receptores (PETROVSKY; COOPER, 2011), fazendo com que a elucidação do mecanismo de ação adjuvante de um carboidrato seja complexa. Carboidratos como o MDP e lipolissacarídeos de membranas são capazes também de ativar a via do complemento, gerando componentes como C3a, C3b, C3d, C5a e C5b que atuam como opsoninas e quimiocinas e dessa forma contribuem também para a atividade adjuvante (BOHANA-KASHTAN et al., 2004; KAWASAKI et al., 1987). Da mesma forma que o alumínio, a ação adjuvante da zimosana e da manana também se dá pela ligação ao receptor NALP3 e consequente ativação do inflamossomo e da caspase-1 e clivagem de pro-IL-1 em IL-1 (LAMKANFI et al., 2009).

O excesso de sinais inflamatórios, por outro lado, é em grande parte responsável pela toxicidade de adjuvantes que atuam via ativação de NF-κB e do inflamossomo, o que pode gerar desde reatogenicidade local com manifestações de dor e inflamação, até manifestações sistêmicas como linfadenopatia, esplenomegalia, náusea, vômitos e diarreia, febre, mialgia, fadiga, dor de cabeça, artrite, autoimunidade e anafilaxia, entre outros (PETROVSKY, 2008). No entanto, como mencionado anteriormente, os polímeros naturais são em geral biocompatíveis e compartilham uma característica bastante interessante, a biodegradabilidade.

Quimicamente os carboidratos podem ter uma estrutura complexa o que pode dificultar a transposição da fabricação de pequena para grande escala, principalmente quando se compara com adjuvantes com estruturas bastante simples como o hidróxido de alumínio. Felizmente uma característica de polissacarídeos como glicanos é que, usualmente eles podem ser purificados a um produto uniforme apesar da complexidade de sua estrutura química (PETROVSKY; COOPER, 2011).

A goma xantana, uma β-glicana produzida por bactérias pertencentes ao gênero *Xanthomonas* sp., é um polímero polissacarídico constituído quimicamente de unidades de β-D-glicose, unidas por ligação 1-4, formando uma cadeia principal celulósica. Na posição C(3) de cada resíduo alternado de glicose existe uma cadeia lateral trissacarídica contendo unidades de β-D-manose-1,4-β-D-ácido glicurônico-1,2-α-D-manose. Um resíduo de ácido pirúvico é ligado na posição 4 e 6, entre 31-56% na β-D-manose terminal, podendo apresentar ainda na posição C(6) da α-D-

manose interna grupos O-acetil (ROSS-MURPHY et al., 1983; SHATWELL et al., 1990).

Esse polissacarídeo desperta grande interesse para as indústrias de alimentos, farmacêuticas e de petróleo devido as suas propriedades de emulsificação, suspensão, estabilização e floculação (LIMA, 2001). A goma xantana apresenta capacidade de formar soluções viscosas e géis hidrossolúveis o que lhe fornece propriedades reológicas únicas (LUVIELMO; SCAMPARINI, 2009).

As propriedades adjuvantes intrínsecas da goma xantana como ativador murino de linfócitos foram originalmente descritas na década de 1980, mas permaneceram inexploradas nas décadas seguintes (ISHIZAKA et al., 1983). No entanto, recentemente, a xantana foi apontada como contendo propriedades antitumorais (TAKEUCHI et al., 2009), e foi utilizada com sucesso em formulações bioadesivas em vacinas nasais contra o vírus influenza (BERTRAM et al., 2010; CHIOU et al., 2009) sem, no entanto, ter sido avaliada ainda a sua propriedade adjuvante em reforçar a imunogenicidade de antígenos recombinantes, sabidamente pouco imunogênicos.

2.5 Leptospirose e vacinas de subunidade recombinante contra leptospirose

A leptospirose é uma doença zoonótica de distribuição mundial, causada pela infecção com espécies patogênicas de *Leptospira*, uma espiroqueta pertencente à família *Leptospiraceae*. Com 500.000 casos reportados anulamente, é considerada um problema de saúde pública (BHARTI et al., 2003; LEVETT, 2001;WHO, 2003). A epidemiologia da doença está associada ao contato humano com animais reservatórios, principalmente roedores, ou ainda com o meio ambiente contaminado com a urina desses animais (BHARTI et al., 2003; FAINE et al., 1999). Foi inicialmente reconhecida como uma doença ocupacional (LEVETT, 2001), no entanto tem sido cada vez mais associada com as condições sanitárias da população, sendo frequentemente relacionada em áreas urbanas com extrema pobreza e a estações chuvosas e enchentes (KO et al., 1999; REIS et al., 2008). É

mais incidente em regiões tropicais, provavelmente pelo favorecimento da sobrevida da *Leptospira* nas condições climáticas desses locais. A doença é sazonal, com pico de incidência nos períodos de maior densidade pluviométrica (BHARTI et al., 2003; VINETZ, 2001). Em países desenvolvidos a doença vem sendo relacionada à prática de esportes aquáticos ou ocupacional (HAAKE et al., 2002).

A maioria dos casos de infecção por *Leptospira* apresenta-se subclínico ou brando, e usualmente se apresenta como uma doença febril não específica, com sinais e sintomas similares à dengue e a doenças por influenza, entre outras (LEVETT, 2001). Entretanto, se não diagnosticada e tratada precocemente, a leptospirose pode progredir para uma doença mais severa, carcterizada por disfunção hepática, renal e pulmonar ou manifestações hemorrágicas (GOUVEIA et al., 2008). Medidas de vigilância são úteis na detecção precoce de surtos de infecção, porém, a vacinação é a medida de prevenção mais factível. Não obstante, vacinas contra leptospirosis têm sido adotadas apenas regionalmente para uso humano, em alguns países como China e França. Barreiras para o desenvolvimento de vacinas incluem a falta de conhecimento de sorovares localmente circulantes, avaliação de segurança e eficácia das preparações e custo (GUERRA, 2013).

Preparações vacinais contendo bactérias inteiras inativadas pelo calor, bacterinas, são efetivas em conferir proteção, mas têm seu uso limitado devido aos severos eventos adversos associados a esse tipo de vacina, como dor, náusea e febre. Além disso, têm proteção restrita aos sorovares presentes na vacina e ainda conferem imunidade por um período pequeno de tempo, o que gera a necessidade de re-vacinações periódicas (FAINE et al., 1999). No intuito de suprir as deficiências da bacterina, estudos moleculares e celulares têm focado na motilidade bacteriana, lipopolissacarídeos, lipoproteínas, proteínas da membrana externa e fatores de virulência em potencial (WANG et al., 2007). Preparações vacinais contendo antígenos proteicos têm sido avaliadas em modelos experimentais, como o hamster *(Mesocricetus auratus),* que é considerado o modelo mais adequado para mimetizar a doença que ocorre em humanos sendo, portanto, bastante requerido em ensaios de imunoproteção e utilizado por órgãos reguladores, como o FDA, na avaliação de vacinas comerciais (US GOVERNMENT PRINTING OFFICE, 2010).

Vários antígenos recombinantes têm sido avaliados em vacinas contra essa zoonose (DELLAGOSTIN et al., 2011). Entre outros, o nosso grupo de pesquisa vem trabalhando com o peptídeo recombinante LigA (Leptospiral immunoglobulin-like protein A), uma adesina pertencente à família Big (Bacterial immunoglobulin-like) que é constituína de proteínas com repetidos domínios e que estão expostas na superfície da bactéria. Uma característica das proteínas Ligs, que faz com que elas sejam interessantes alvos vacinais, é o fato de estarem presentes nas leptospiras patogênicas, mas não nas saprófitas (KOIZUMI; WATANABE, 2004; SILVA et al., 2007). Originalmente relacionadas com os fatores de virulência intiminas de *Escherichia coli* (LUO et al., 2000) e invasinas de *Yersinia pseudotuberculosis* (HAMBURGER et al., 1999), atualmente as proteínas Lig sabidamente medeiam interações com proteínas que compõem a matrix extracelular das células do hospedeiro, como fibronectina, fibrinogênio, colágeno, laminina, elastina e tropoelastina (CHOY et al., 2007; LIN et al., 2009).

A proteína LigANI, que corresponde a região carbóxi terminal não idêntica de LigA, tem sido expressa e avaliada em modelos experimentais de leptospirose (COUTINHO et al., 2011; HARTWIG et al., 2010; KOIZUMI; WATANABE, 2004; PALANIAPPAN et al., 2006; SILVA et al., 2007). Resultados promissores foram obtidos utilizando esse alvo vacinal, sendo que o melhor efeito protetivo foi atingido utilizando o adjuvante completo de Freud (CFA) (COUTINHO et al., 2011; KOIZUMI; WATANABE, 2004; SILVA et al., 2007). CFA é um adjuvante frequentemente avaliado e bem caracterizado sendo que seu mecanismo de ação envolve a lenta liberação do antígeno e a ligação dos PAMPs de micobactérias aos seus PRRs, levando a ativação e proliferação de células T (FREUND, 1956). O componente micobactéria presente no CFA especialmente ativa uma resposta imune do tipo Th1, resultando na reação de hipersensibilidade tardia no sítio de aplicação da vacina. Além da reação local, sérios efeitos sistêmicos também foram observados, caracterizados pela proliferação de células Mac-1^{+} da linhagem mielóide (MATTHYS et al., 2001), e pela presença de parafina que se mostrou ser não degradável e tóxica. O antígeno LigA também foi avaliado utilizando-se como adjuvante o hidróxido de alumínio (PALANIAPPAN et al., 2006), o ácido poli-lactico-co-glicólico (PLGA) e lipossomas (FAISAL et al., 2009).

O desenvolvimento de vacinas eficazes e seguras, especialmente as compostas por antígenos proteicos produzidos pela tecnologia do DNA recombinante, que são caracteristicamente pouco imunogênicas, está cada vez mais, dependente da seleção de um adjuvante apropriado. Poucos adjuvantes são, atualmente, aprovados para uso humano e, esses induzem principalmente uma resposta imune humoral, existindo, portanto, uma necessidade, ainda não contemplada nessa área, de desenvolvimento de adjuvantes efetivos e seguros que possam estimular a imunidade celular, de mucosa e/ou humoral dependendo do requerimento de proteção específico de cada doença infecciosa.

3 ARTIGO 1

Xanthan gum as an adjuvant in a subunit vaccine preparation against leptospirosis

(artigo submetido ao periódico International Journal of Macromolecules)

Xanthan gum as an adjuvant in a subunit vaccine preparation against leptospirosis

Short title: Xanthan adjuvant in a leptospirosis vaccine

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Abstract

Leptospiral immunoglobulin-like (Lig) proteins are of great interest due to their ability to act as mediators of pathogenesis, serodiagnostic antigens and immunogens. Purified recombinant LigA protein is the most promising subunit vaccine candidate against leptospirosis reported to date however, as purified proteins are weak immunogens the use of a potent adjuvant is essential for the success of LigA as a subunit vaccine. In the present study, we compared xanthan polysaccharides produced by *Xanthomonas arboricola* pv pruni (strain 106), aluminium hydroxide (alhydrogel) and CpG ODN as adjuvants in a LigA subunit vaccine preparation. Preparations containing xanthan induced a strong antibody response comparable that observed when alhydrogel was used. Upon challenge with a virulent strain of *L. interrogans* serovar Copenhageni, significant protection (Fisher test $P < 0.05$) was observed in 100%, 100%, and 67% of hamsters immunized with rLigANI-xanthan, LigA-CpG-xanthan, and rLigANI-alhydrogel, respectively. Furthermore, xanthan did not cause cytotoxicity in Chinese hamster ovary (CHO) cells *in vitro*. The use of xanthan as an adjuvant is a novel alternative for enhancing the immunogenicity of vaccines against leptospirosis and possibly against other pathogens.

Keywords: Adjuvant; xanthan; *Leptospira*; leptospirosis.

1. Introduction

Leptospirosis is a zoonotic disease that occurs worldwide and is caused by a pathogenic species of *Leptospira*. With 500,000 cases reported each year, this disease remains a significant public health concern [1]. Parenteral immunizations with whole-cell, heat-killed vaccine preparations are very protective, but their use is limited due to severe side effects (pain, nausea, fever), serovar-restricted protection and short-term immunity [2]. Recombinant vaccines have the potential to overcome these limitations. Subunit vaccines consist of purified antigens that are specifically recognized by cells of the adaptive immune system. However, they lack intrinsic pathogen-associated molecular patterns (PAMPs) and the ability to activate the immune system in an appropriate way is impaired so, the use of adjuvants is required [3]. Several leptospiral antigens have been evaluated, as established in a recent review by Dellagostin et al., 2011 [4], and recombinant LigA peptides, including the non-identical carboxy-terminus named LigANI, have been expressed and evaluated in experimental models of leptospirosis using different adjuvants [4,5].

The main adjuvant currently approved for human use by the US Food and Drug Administration (FDA) are aluminium based mineral salts, the most commonly used of which is aluminium hydroxide (generically known as alhydrogel). For decades, alhydrogel has been used successfully in vaccine preparations and has a good safety record [6]. However, alhydrogel is a weak adjuvant for antibody induction against protein subunits and is a poor adjuvant for cell-mediated immunity. In addition, alhydrogel can cause severe local reactions such as erythema, subcutaneous nodules, and contact hypersensitivity [7]. Therefore, there is a need for the development of new adjuvant strategies.

Xanthan is a polysaccharide derived from *Xanthomonas* spp., a plant-pathogenic bacterium genus, which has viscous properties and is widely used as a thickener or viscosifier

and a stabilizer in the food industry, as well as other industries [8-10]. Chemically, it is considered an anionic polyelectrolyte, with a cellulosic backbone chain linked to a trisaccharide side chain consisting of two D-mannose units with alternating D-glucuronic acid residues that can be acetylated or pyruvated at different levels, which influences both the chemical and physical properties of xanthan [11]. The intrinsic adjuvant properties of xanthan gum as a murine lymphocyte activator was originally described in the 1980s but has remained largely unexplored in the following decades [12]. More recently, xanthan has been identified in antitumor effects of [11], and it has been successfully used in bio-adhesive formulations for intranasal influenza virus immunizations [10,13].

In the present study, we demonstrated that the rLigANI protein used in combination with xanthan conferred protection against lethal challenge in the standard Golden Syrian hamster model for leptospirosis. Together, LigANI and xanthan induced a strong IgG response and the xanthan polysaccharides did not demonstrate cytotoxicity in Chinese hamster ovary (CHO) cells, *in vitro*.

2. Materials and methods

2.1 *Bacterial strains and growth conditions*

L. interrogans serovar Copenhageni strain FIOCRUZ L1-130 was cultivated at 30 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium, supplemented with Leptospira Enrichment EMJH (Difco, USA). Bacterial growth was monitored using dark-field microscopy. *Escherichia coli* strain BL21 (DE3) pLysS (Invitrogen) was grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) at 37 °C with the addition of 50 $\mu g.mL^{-1}$ chloramphenicol and 100 $\mu g.mL^{-1}$ ampicillin.

2.2 *Ethics Statement*

Animal experiments described in this study were carried out in strict accordance with the guidelines of the National Council for Control of Animal Experimentation, Brazil (CONCEA, nº 11,794) and approved by the Ethics Committee in Animal Experimentation, Federal University of Pelotas, Brazil (Permit Number: 7777). Hamsters were monitored daily and were euthanized upon the appearance of clinical symptoms of leptospirosis. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.3 Xanthan production

Xanthan gum used in this study was produced by *X. arboricola* pv pruni strain 106 in a 10 L bioreactor (BioStat B Braun Biotech International®) with 7 L of fermentation medium, as previously described [14]. The fermented broth was heated to 121 °C for 15 min, and the polysaccharides were recovered by precipitation with 96% ethanol, dried at 56 °C until maintaining a constant weight and then powdered to particle size using 60-150 mesh. The xanthan pruni used in these experiments was pooled from four fermentations and characterized by viscosity, moisture, ash nitrogen, acetyl and pyruvate content, as previously described by Burdock [15] and the Food and Agriculture Organization of the United Nations (FAO) [16].The quantification of the monosaccharides and derivative acids were determined as previously described [17].

2.4 *rLigANI subunit vaccine preparation*

The cloning, expression and purification of the rLigANI polypeptide was performed as previously described [18]. The purified rLigANI was used in a subunit vaccine preparation with one of three adjuvants: xanthan, alhydrogel, or CpG ODN. Xanthan was diluted with purified water (1.25%, w/v) and stirred until uniformly distributed. The xanthan solution was added to a final concentration of 0.5% (w/v) [13]. When alhydrogel (Invivogen) was used in the vaccine preparation it was added to a final concentration of 15% [19]. The vaccine preparation that contained CpG ODN was comprised of 10 µg phosphotioated CpG ODN (5'-TCG TCG TCG TTC GAA CGA CGT TGA T) also known as ODN-M362 (Alpha DNA, Canada) as described previously [20].

The antigenicity of vaccine preparations containing rLigANI were evaluated by Western Blotting (WB). The rLigANI vaccines were electro-transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare) and, after incubation in blocking buffer (0.05 M PBS pH 7.4, 0.05% (v/v) Tween 20, 5% (PBS-T) and (w/v) non-fat dried milk) overnight at 4 °C, the membranes were subjected to three washes (5 min per wash) in PBS-T and incubated for 1 h with mouse polyclonal anti-LigAni antibody (1:300 in PBS) followed by 3 washes (5 min per wash) in PBS-T. Rabbit anti-mouse IgG peroxidase conjugate (Sigma Aldrich), diluted 1:6,000 in PBS, was added and incubated for 1 h at 37 °C. The membranes were washed (5 x in PBS-T), and the reaction was developed using 3,3-diamino-benzide-tetra-hydrochloride (DAB) (Sigma Aldrich).

2.5 *Xanthan in vitro cytotoxicity*

The viability of CHO cells was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] compared with water insoluble formazan [10]. Briefly, cells were seeded at a density of 2×10^4 cells per well in a volume of 100 µL in 96-well plates and grown at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h prior to the cell viability assay. The CHO cells were incubated with different concentrations of aqueous xanthan solution (0.25, 0.5 and 1.0% w/v) for 24 h. The media was removed and 180 µL of medium and 20 µL of MTT (5 mg MTT/mL solution) were added to each well. The plates were incubated for an additional 3 h, and the medium was discarded. Two hundred microliters of DMSO was added to each well, and the formazan was solubilized by shaking for 5 min at 100 × g. The absorbance of each well was read on a microplate reader (MR-96A, Mindray Shenzhen, China) at a wavelength of 492 nm. The cell inhibitory growth rate (%) was determined as follows: inhibitory rate = $(1- Abs_{492\text{treated cells}}/Abs_{492\text{control cells}}) \times 100$. All observations were validated by at least three independent experiments performed in triplicate.

The rate of apoptosis was determined using the Guava Nexin assay (Guava Technologies), according to the manufacturer's instructions. Cells were treated with aqueous xanthan at 0.25, 0.5 and 1.0% for 48 h. Briefly, 2.0×10^4 to 1.0×10^5 cells (100 µL) were added to 100 µL of Guava Nexin Reagent. The cells were incubated in the dark at room temperature for 20 min and samples (2,000 cells per well) were analysed using the flow cytometer Guava EasyCyte System. In this assay, an annexin V-negative and 7-AAD-positive result indicated nuclear debris, annexin V-positive and 7-AAD-positive indicated late apoptotic cells, annexin V-negative and 7-AAD-negative indicated live healthy cells, and annexin V-positive and 7-AAD-negative indicated early apoptotic cells.

2.6 *Hamster Immunization*

Female golden Syrian hamsters 5-6 weeks old, weighing 82.13 g ± 5.39, were divided into nine groups consisting of 6 animals each: group 1: 15% alhydrogel in PBS (alhydrogel-PBS); group 2: rLigANI in PBS (rLigANI); group 3: rLigANI in 15% alhydrogel (rLigANI-alhydrogel); group 4: CpG in PBS (CpG-PBS); group 5: rLigANI and CpG (rLigANI-CpG); group 6: xanthan in PBS (xanthan-PBS); group 7: rLigANI and xanthan (rLigANI-xanthan); group 8: rLigANI, CpG, and xanthan (rLigANI-CpG-xanthan); group 9: bacterin vaccine consisting of 1×10^9 heat-killed whole-leptospires (bacterin), produced as previously described [21]. Two independent experiments were carried out using 50 µg of recombinant protein, with a standard volume of 500 µL applied at a single injection site.The animals were immunized subcutaneously on day 0 and boosted on day 14. Blood was collected by retro-orbital bleeding from the venous plexus before each immunization and challenge (days 0, 14 and 28); the sera were collected and stored at -20 °C.

2.7 *Hamster challenge study*

On day 28 after the first immunization, the hamsters were challenged with an intraperitoneal inoculum of 1.3×10^3 leptospiras, equivalent to equivalent to 36 × the 50% lethal dose (LD_{50}) of *L. interrogans* serovar Copenhageni (strain Fiocruz L1-130). Hamsters were monitored daily and euthanized when clinical signs of terminal disease were observed. Surviving hamsters were euthanized on day 36 post-challenge and blood samples were collected by cardiac puncture. Kidney and lung tissues were harvested for culture isolation and histopathology studies as described previously [21]. Two independent challenge experiments were carried out.

2.8 *Evaluation of the antibody response in immunized hamsters*

Serum samples collected on day 0, 14 and 28 were evaluated for the presence of specific immunoglobulin G (IgG) by an ELISA using rLigANI as the antigen. A checkerboard analysis was performed to identify the optimal antigen concentration and dilutions of the hamster sera and the antibody conjugate. The ELISA plates (Polysorp Surface, Nunc) were coated with 200 ng of rLigANI protein per well, diluted in carbonate-bicarbonate buffer pH 9.6 and incubated overnight. The plates were washed three times with PBS-T and incubated with 200 µl of 5% blocking buffer at 37 °C for 1 h. After 3 washes with PBS-T, hamster serum (diluted 1:50) was added and the plates were incubated for 1 h at 37 °C. After three washes with PBS-T, the goat anti-hamster IgG peroxidase conjugate (Serotec, USA) diluted 1:6,000 was added and the plates were incubated at 37 °C for 1 h. After 5 PBS-T washes, the reactions were developed using o-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide. The reaction was stopped by adding 0.1 M sulphuric acid and the absorbance was determined at 492 nm with a microplate reader (MultiskanMCC/340, Titertek Instruments). The mean values were calculated from serum samples that were assayed in triplicate.

2.9 *Statistical analysis*

The results are expressed as the mean ± SEM and the significant differences between groups were determined using an analysis of variance (ANOVA), P values < 0.05 were considered statistically significant. Protection against mortality was evaluated using the Fisher exact test using Epi Info 6.04d software (Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA) and the survival curves were compared using a Log-rank analysis (Mantel Cox test) using Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1 Antigenicity of rLigAni vaccine preparations

The antigenicity of rLigANI associated to xanthan polymer and CpG was evaluated by WB using mouse polyclonal anti-LigAni that recognized a 63 kDa protein in the rLigANI vaccine formulations. The antibody was used to show protein associations and indicated that no significant changes occurred.

3.2 Xanthan characterization

The xanthan gum used in this experiment had good viscosity, in accordance with the recommendations by the FAO [16] and Burdock [15] for xanthans used as food additives. The moisture, ash, nitrogen acetyl and pyruvate content (Table 1) were in accordance with the recommendations of the FAO [16]. In addition, none of the aqueous xanthan solutions (0.25, 0.5 and 1.0% w/v) demonstrated significant *in vitro* cytotoxicity (Figure 1) and no statistically significant differences in the growth rate were observed at the different xanthan concentrations ($P > 0.05$). Furthermore, the annexin-PE results indicated that the aqueous xanthan solutions did not induce apoptosis at the concentrations tested. The percentage of apoptosis was 1.98, 2.48 and 2.30% when 0.25, 0.5 and 1.0% of aqueous xanthan solutions were used, respectively, which was similar to that observed for the negative control (3.52%).

3.3 *Antibody responses induced by rLigANI vaccines*

Blood samples from hamsters immunized with either alhydrogel-PBS, rLigANI, rLigANI-alhydrogel, CpG-PBS, rLigANI-CpG, xanthan-PBS, rLigANI-xanthan, rLigANI-CpG-xanthan or the bacterin were collected on days 0, 14 and 28, and the corresponding

antibody response was determined by ELISA. After the first immunization, sera from the hamsters immunized with rLigANI-alhydrogel, rLigANI-xanthan or rLigANI-CpG-xanthan demonstrated significant antibody titres (two-way ANOVA, $P < 0.05$), Fig. 2. However, after the boost immunization, all of the hamsters immunized with rLigANI (with or without adjuvant) presented with detectable antibody titres (two-way ANOVA, $P < 0.05$). Neither bacterin or negative control groups induced dectable levels of IgG antibodies against rLigANI. Although there were localized reactions at the sites of injection that were associated with erythema and alopecia, the hamsters showed no signs of pain or discomfort. No lesions were observed 28 days post-immunization. Of note, the *in vitro* tests found that the xanthan was not cytotoxic.

3.4 *Protective effect of the vaccine preparations following lethal challenge with* L. interrogans *serovar Copenhageni*

In the first experiment, hamsters at day 28 post-immunization, with an average weight of 128.72 g ± 1.69, were challenged and observed daily for signs of disease. The groups of hamsters immunized with either rLigANI-xanthan, rLigANI-CpG-xanthan or the bacterin preparation survived (100%), see Table 2 and Fig. 3. Furthermore, of the hamsters immunized with rLigANI-alhydrogel, a significant number survived, (66.7%, Fisher, $P < 0.05$). However, hamsters immunized with rLigANI-CpG were not significantly protected against lethal challenge (16.7% survival). None of the hamsters in the control groups, alhydrogel-PBS or CpG-PBS, survived and there was only one survivor in the xanthan-PBS group. In addition, rLigANI when administered without an adjuvant failed to protect the hamsters against challenge. In the follow-up challenge experiment to further evaluate the efficacy of the rLigANI-xanthan vaccine preparation, 100% of the hamsters survived challenge and there

were no survivors in the control group. The bacterin control group in both experiments conferred 100% protection, Table 2.

Of note, we observed a correlation between the antibody titre and survival in hamsters immunized with the rLigANI preparations (Spearman correlation coefficient 0.6845, $P < 0.05$). Furthermore, the surviving hamster in the rLigANI-CpG group showed significant seroconversion after the first immunization. However, there were no differences between the hamsters that survived and those that did not survive in the rLigANI-alhydrogel group (t-test $P > 0.05$).

3.5 *Histopathology and culture isolation*

The culture assay showed that 100% (Exp.#1) and 66.7% (Exp.2) of the rLigANI-xanthan and 100% of rLigANI-alhydrogel surviving immunized hamsters harboured leptospires in their kidneys indicating that none of the subunit vaccine preparations afforded sterilizing immunity. None of the groups vaccinated with the bacterin vaccine, showed positive culture (Table 2). The histopathological analyses revealed that the surviving hamsters developed an acute leptospirosis with lesions dominated by moderate pulmonary injury as evidenced by oedema and alveolar haemorrhage. The lesions in the kidneys were characterized by cell degeneration, necrosis, and hyaline deposition (Figure 4). In addition, there were histopathological changes in 33.3% (Exp.#1) and 16.7% (Exp.#2) of the kidneys of hamsters in the rLigANI-xanthan groups, and 75% of the rLigANI-alhydrogel group also showed changes in the kidneys. Of note, no kidney changes were observed in the bacterin control group (Table 2).

4. Discussion

Using the recombinant protein LigANI as a target for a leptospirosis vaccine yielded promising results, and the best protective effect was obtained using Freund's complete adjuvant [5,18], an efficient Th1 inducer. However, this caused a high and generally unacceptable level of adverse local effects. In this study, we compared the efficacy of three different adjuvants used in conjunction with LigANI: xanthan polysaccharide, CpG ODN and alhydrogel. We believe that this is the first study of the application of xanthan as an adjuvant in a recombinant subunit vaccine preparation. In combination with rLigANI, xanthan protected 100% of immunized hamsters (Figure 3). Xanthan has a backbone chain consisting of (1,4) β-D-glucan cellulose and is a negatively charged polymer with intrinsic adjuvanticity [12]. It has been used as an FDA-approved food additive and rheology modifier since 1969 and is commonly used as a food thickening agent and stabilizer, demonstrating its biosafety [8,9]. However, the biological properties and the mechanism of xanthan adjuvanticity are not clear and have remained unexplored until recently.

A previous study found that the oral administration of xanthan gum as a biological response modifier enhances antitumor activity in mice through toll-like receptor (TLR)-4 recognition [11]. This innate immune response is characterized by the production of pro-inflammatory cytokines, via transcription factor NF-κB. The induction of adaptive immunity through the activation of innate immunity is vital for vaccine development. This pathway leads to the expression of co-stimulatory molecules that are essential for the induction of an effective adaptive immune response. Studies in mice have demonstrated that signalling through TLRs is sufficient to initiate an adaptive immune response, which is characterized by Th1 induction and antibody production [22,23].

The rLigANI-xanthan vaccine preparation induced a robust humoral response comparable to the response of the rLigANI-alhydrogel preparation. The antibody-specific titre induced by the rLigANI-CpG ODN preparation was significantly lower than that induced by the xanthan or alhydrogel adjuvants together with rLigANI (Figure 2). Note that CpG ODN M362 and xanthan did not promote a significant decline in antibody titre, which demonstrated the efficiency of xanthan in triggering a humoral response. The demonstration that bacterial DNA and not vertebrate DNA, has a direct immune-stimulatory effect on immune cells led to the identification of the CpG class of adjuvants [24]. Preclinical studies indicate that CpG ODN improve the activity of vaccines targeting cancer and infectious diseases caused by viruses, bacteria and protozoa [25,26]. CpG ODN M362 is a type C human/murine TLR-9 ligand and was used in the present study due to the lack of a specific ligand for a hamster model. Therefore, it is possible that this ligand is not appropriate for the model studied and studies with other CpG ODNs will be necessary before we can dismiss the use of this adjuvant in subunit vaccines preparations against leptospirosis.

Studies carried out using recombinant LigA polypeptides and alhydrogel as an adjuvant report between 50 [27] and 100% [28] survival, however, in some studies the unvaccinated control groups the survival rates were over 50%. In the present study, we found a significant protective effect in 4/6 hamsters when alhydrogel was used as the adjuvant. Although the rLigANI-xanthan vaccine preparation protected a greater number of hamsters, 6/6 in two separate challenge experiments, the increased efficacy was not significant compared to rLigANI-alhydrogel. There was a survivor in the control group immunized with xanthan-PBS in the first experiment and while 100% of the hamsters survived, this reduced vaccine efficacy to 83.3%. Xanthan did not show *in vitro* toxicity in CHO cells at the levels tested in the present study. The *in vitro* cytotoxicity of xanthan in chicken splenic macrophages [10] and in L929 mouse fibroblast cells [29] was previously evaluated, and no

change in cell viability was observed. Chellat and co-workers [30] investigated the *in vitro* and *in vivo* biocompatibility of a chitosan-xanthan polyionic complex, and they did not observe any cytotoxic effects in male Wistar rats.

5. Conclusion

In summary, this study demonstrated the adjuvant effect of xanthan when used with rLigANI, a poorly immunogenic antigen, in a subunit vaccine preparation against leptospirosis. Furthermore, the xanthan polysaccharide enhanced the immune response and the immune protection induced by rLigANI.

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Figure Legends

Figure 1. Cytotoxicity of the aqueous xanthan solution. (A) The effect of different concentrations of aqueous xanthan solutions on the inhibition of CHO cells was determined using an MTT assay. The data are expressed as the means ± SEM. (B) After 48 h the results from the apoptosis assay are shown after flow cytometry and annexin V-PE/7-AAD staining of CHO cells treated with 0.25, 0.5 and 1.0% xanthan solutions and the control groups. The viable cells are in the lower left quadrant, the early apoptotic cells are in the lower right quadrant, the late apoptotic cells are in the upper right quadrant and the nuclear debris is shown in the upper left quadrant. The numbers indicate the percentage of cells in each quadrant.

Figure 2. Inducing the humoral immune response in hamsters immunized with different immunogens. Fifty micrograms of the recombinant protein were used. The groups of hamsters were immunized subcutaneously on day 0 and boosted after 2 weeks (day 14). Blood was collected on days 0, 14 and 28 post-immunization. The specific IgG responses stimulated by the different immunogens were determined by an ELISA of the hamster serum diluted 1:50. The values presented are the means ± SEM for two independent experiments.

Figure 3. The protective effect of immunization against lethal challenge in a hamster model. Nine- to ten-week-old hamsters were challenged with an intraperitoneal inoculum of 1.3×10^3 leptospires 14 days after the second immunization (day 28). (A-C) Experiment # 1 (D) Experiment # 2. The survival conferred by rLigANI-alhydrogel against the lethal challenge was statistically significant ($P < 0.05$). The same phenomenon occurred using

rLigANI-xanthan and rLigANI-CpG-xanthan as immunogens. The protective effect of immunization using rLigANI-CpG was not significantly different from the negative control group (P >0.05). Fifty micrograms of rLigANI, 0.5% xanthan (w/v) and 10 µg CpG were used. Survival curves were compared using log rank analysis (Mantel Cox test). Bacterin: heat-killed whole-leptospires

Figure 4. Histopathological changes in organs of hamsters after leptospiral challenge. Panel showing representative HE-stained (400x) kidney (A-D) and pulmonary (E e F) sections. (A) Normal tubular kidney epithelium (B) Discrete cell degeneration and necrosis, (C) hyaline deposition and (D) severe leukocyte infiltration. (E) Normal lung epithelium (F) Moderate oedema and alveolar haemorrhage.

Figure 1

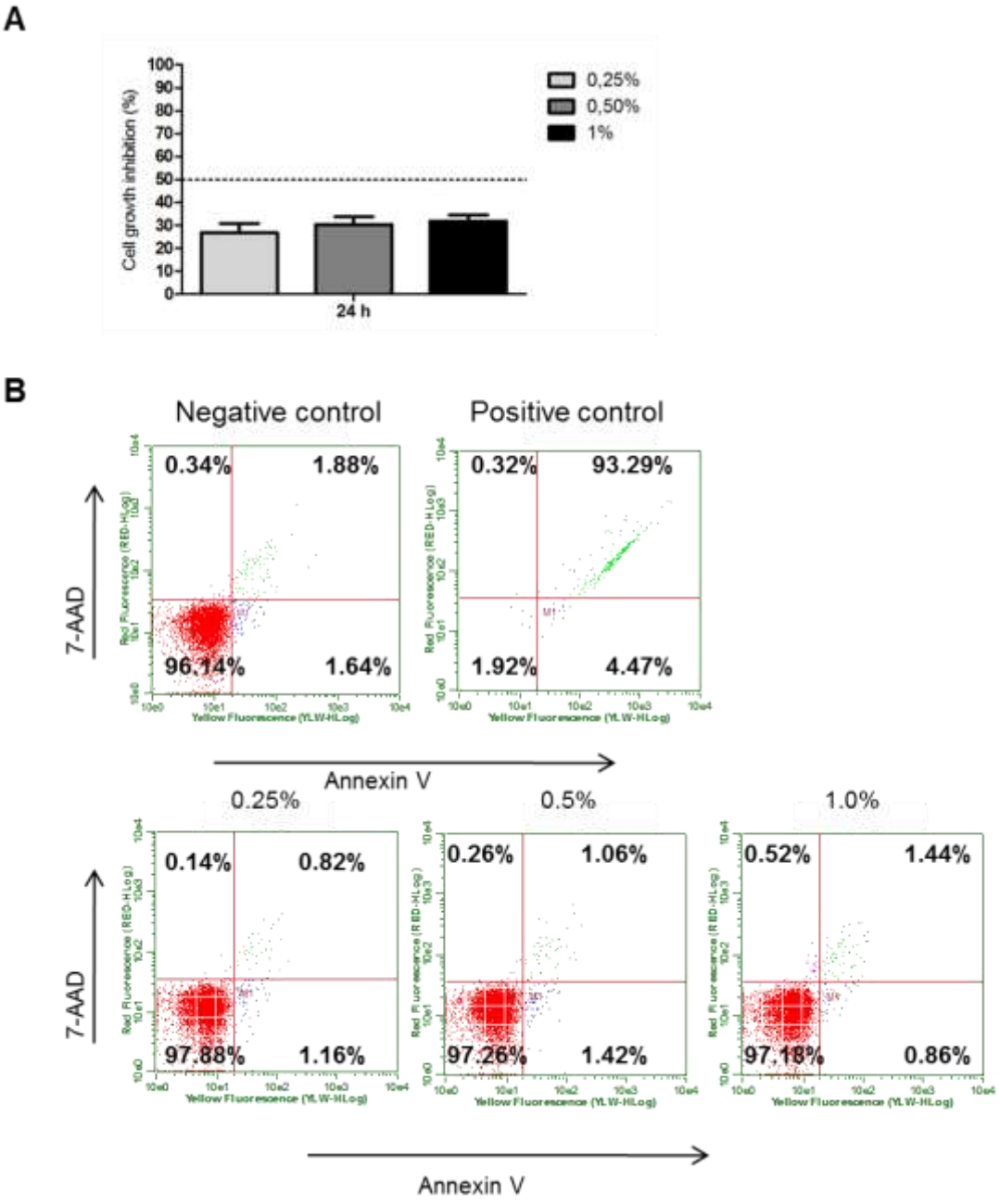
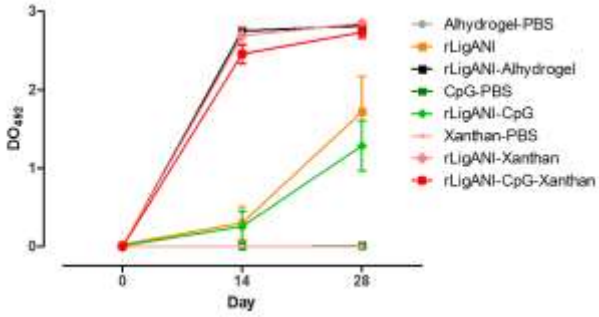

A
Cell growth inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
24 h
0,25%
0,50%
1%
B
Negative control
Positive control
0.34%
1.88%
96.14%
1.64%
0.32%
93.29%
1.92%
4.47%
7-AAD
Annexin V
0.25%
0.5%
1.0%
0.14%
0.82%
97.88%
1.16%
0.26%
1.06%
97.26%
1.42%
0.52%
1.44%
97.18%
0.86%
Red Fluorescence (RED-HLog)
Yellow Fluorescence (YLW-HLog)

Figure 2


3
2
1
0
DO_{450}
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14
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Day
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rLigANI
rLigANI-Alhydrogel
CpG-PBS
rLigANI-CpG
Xanthan-PBS
rLigANI-Xanthan
rLigANI-CpG-Xanthan

Figure 3

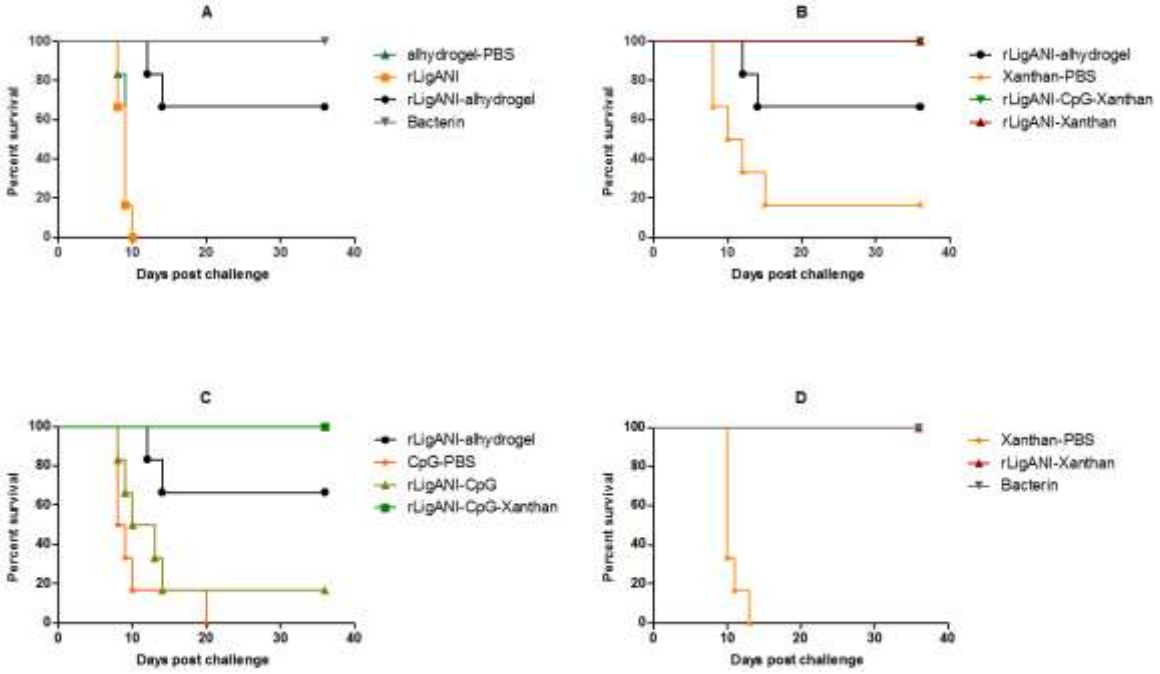
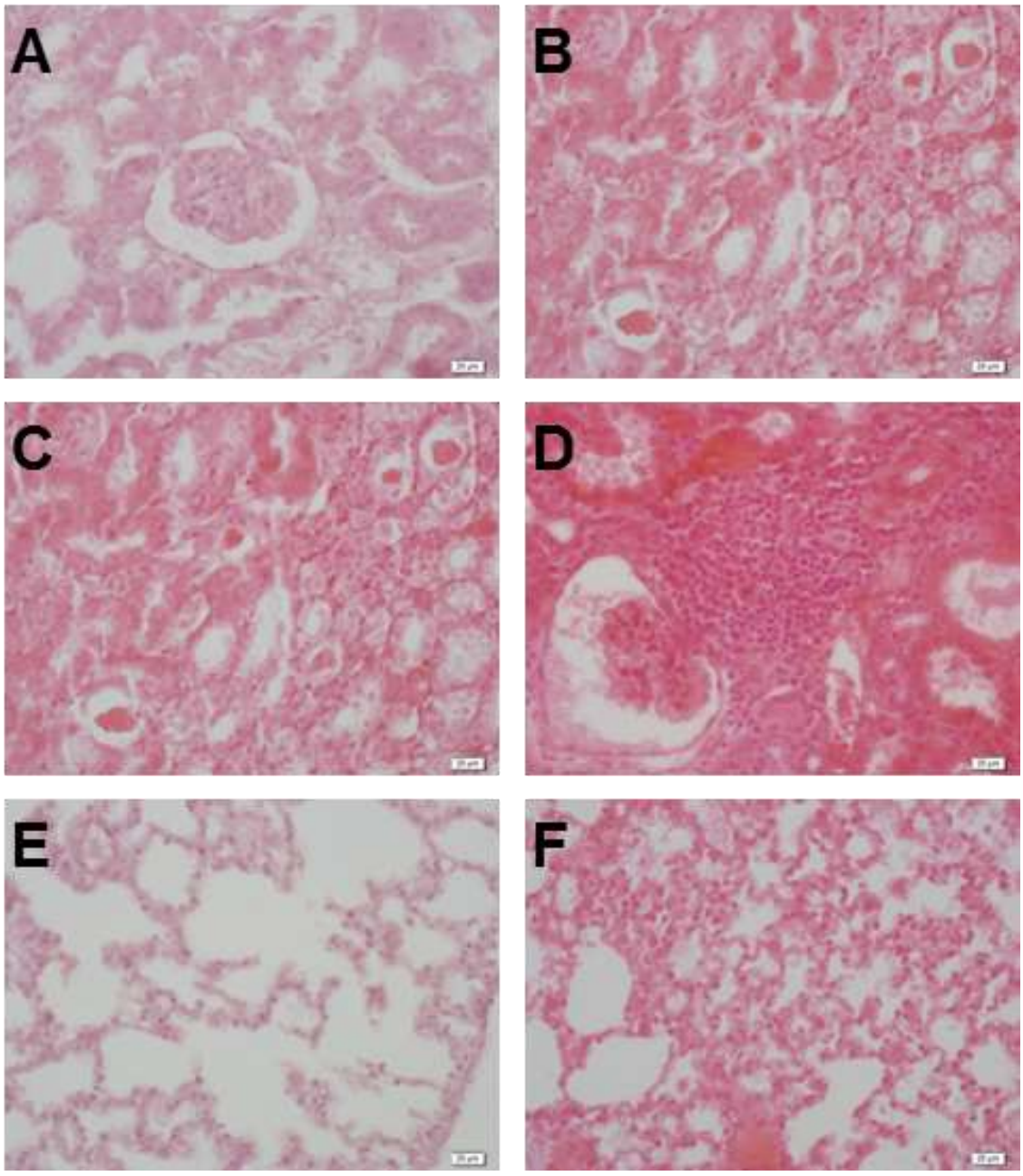

A
Percent survival
Days post challenge
alhydrogel-PBS
rLigANI
rLigANI-alhydrogel
Bacterin
B
Percent survival
Days post challenge
rLigANI-alhydrogel
Xanthan-PBS
rLigANI-CpG-Xanthan
rLigANI-Xanthan
C
Percent survival
Days post challenge
rLigANI-alhydrogel
CpG-PBS
rLigANI-CpG
rLigANI-CpG-Xanthan
D
Percent survival
Days post challenge
Xanthan-PBS
rLigANI-Xanthan
Bacterin

Figure 4



Tables

Table 1. Moisture, ash, nitrogen, acetyl, and pyruvate content (%w/v) of xanthan produced by *X. arboricola* strain 106.

Analysis	Content	Limits (%)*
Moisture	5.0 ± 0.03	≤ 15
Ash	14.37 ± 0.04	≤ 16
Nitrogen	1.06 ± 0.01	≤ 1.5
Acetyl	2.45 ± 0.10	-
Pyruvate	1.93 ± 0.06	-

Values are the means ± SD. * Limits established by FAO, 1999.

Table 2. Protection conferred by immunization and culture isolation and histology among survivors.

Vaccine preparation	Exp. #	% Protection (No./total)	% Culture positive	Evidence of lesions (%)		
				Alveolar haemorrhage	Cell degeneration	Leukocyte infiltration
rLigANI	1	0 (0/6)	NA	NA	NA	NA
rLigANI-Al	1	66.7 (4/6)*	100 (4/4)	100 (4/4)	75.0 (3/4)	100 (4/4)
rLigANI-CpG	1	16.7 (1/6)	100 (1/1)	100 (1/1)	100 (1/1)	100 (1/1)
rLigANI-Xa	1	100 (6/6)*	100 (6/6)	100 (6/6)	33.3 (2/6)	100 (6/6)
	2	100 (6/6)*	66.7 (4/6)	100 (6/6)	16.7 (1/6)	33.3 (2/6)
rLigANI-CpG-Xa	1	100 (6/6)*	100 (6/6)	100 (6/6)	33.3 (2/6)	100 (6/6)
Bacterin	1	100 (6/6)*	0 (0/6)	100 (6/6)	0 (0/6)	0 (0/6)
	2	100 (6/6)*	0 (0/6)	100(6/6)	0 (0/6)	0 (0/6)

Xanthan-PBS	1	16.7 (1/6)	100 (1/1)	100 (1/1)	0 (0/1)	100 (1/1)
	2	0 (0/6)	NA	NA	NA	NA
Al-PBS	1	0 (0/6)	NA	NA	NA	NA
CpG-PBS	1	0 (0/6)	NA	NA	NA	NA

ND: not determined; NA: not applicable; Al: Alhydrogel; Xa: Xanthan.

*Statistically significant compared to the relevant control, Fishers exact test, $P < 0.05$.

[c]The percentage of animals positive with evidence of a particular lesion.

4 ARTIGO 2

Carbon nanotubes: adjuvant effect in a leptospiral immunoglobulin-like A protein (LigA) recombinant subunit vaccine

(artigo formatado de acordo com as normas do periódico International Journal of Pharmaceutics)

NOTE

Carbon nanotubes: adjuvant effect in a leptospiral immunoglobulin-like A protein (LigA) recombinant subunit vaccine

Running title: Carbon nanotubes as subunit vaccine adjuvant

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ABSTRACT

Immunizations with leptospiral immunoglobulin-like A protein (LigA) as a recombinant subunit vaccine delivered through carbon nanotubes as an adjuvant generated anti-leptospira antibodies, but was not protective. The use of carbon nanotubes as an adjuvant in subunit vaccines against leptospirosis is a novel approach and shows efficacy for improving specific IgG production.

Subunit recombinant vaccines consist of purified antigens that are specifically recognized by cells of the adaptive immune system. However, these vaccines lack intrinsic pathogen-associated molecular patterns (PAMPs) and are therefore often weakly immunogenic, and require the addition of adjuvants to appropriately activate the immune system (Burnette, 1991; Gupta et al., 1993).

In the past decade, inorganic nanomaterials such as nanocrystals, nanowires, and nanotubes have been received increasing attention for their potential biomedical applications, including drug design (Prato et al., 2008), drug delivery (Bhirde et al., 2009; Lodhi et al., 2013), tumor therapy (Thakare et al., 2010), tissue engineering (Zanello et al., 2006), vaccine vehicle (Yandar et al., 2008), and DNA recognition (Tu et al., 2009). Carbon nanotubes (CNTs) can be single-walled (SWCNT), double-walled (DWCNT) or multiwalled (MWCNT) depending on the production process. Generally, CNTs are not easy to process due to their lack of solubility in many solvents. However, the sidewall of CNTs presents an excellent platform for chemical functionalization, particularly at some reactive sites. Noncovalent and covalent CNT functionalization has been utilized to overcome the problem of processability (Niyogi et al., 2002; Pantarotto et al., 2003). For instance, MWCNT were functionalized with

carboxyl groups to improve the dispersion of CNT in water (Zhao et al., 2010). Generally, CNT binding to proteins is thought to be driven by various weak interactions such as π–π stacking, hydrophobic, and electrostatic interactions (Zuo et al., 2012).

Here, we evaluated the IgG antibody response and protective effect of the non-identical carboxy-terminus recombinant leptospiral immunoglobulin-like A protein (named rLigANI). We tested this protein associated with carboxyl (COOH) - MWCNT, in an experimental model of leptospirosis. This subunit vaccine had been previously evaluated with Freund's complete adjuvant (Coutinho et al., 2011; Silva et al., 2007), aluminum hydroxide (Alhydrogel, Invivogen, San Diego, CA, USA) (Palaniappan et al., 2006), poly-lactide-co-glycolic acid (PLGA), and liposomes (Faisal et al., 2009) as adjuvants, but not with inorganic nanomaterials.

To evaluate the prophylactic effect of rLigANI, its effect in association with three adjuvants was examined: COOH-MWCNTs, Alhydrogel, and ODN CpG. MWCNT obtained from Sigma® was carboxylated according to methodology described by Stefanie *et al*. (Stefani et al., 2011). The oxidation and characterization of COOH-MWCNTs was carried out using X-ray photoelectron spectroscopy (XPS) and Raman spectroscopy (Gong et al., 2013). For vaccine formulation, an aqueous solution of COOH-MWCNT (0.25 mg mL^{-1}) was added to a final concentration of 15 µg mL^{-1} (Zeinali et al., 2009). To evaluate the adjuvant activity of CpG ODN, 10 µg of fully phosphotioated CpG ODN (25 bp in length) (5′-TCG TCG TCG TTC GAA CGA CGT TGA T-3′) (Alpha DNA, Montreal, Quebec, Canada), was added to the vaccine formulation. The cytotoxic effect of carbon nanotubes (2.5, 5, 10, 15, 25, and 50 µg mL^{-1}) on Chinese hamster ovary cells was determined by measuring the reduction of soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Chiou et al., 2009). Three independent experiments were performed.

Female 5–6 week-old Golden Syrian hamsters were used in this study. All experiments were approved by the Committee on the Ethics of Animal Experiments of the Federal University of Pelotas (Permit Number: 7777). Animals were allocated into 9 groups of 6 animals each, and were administered the following: 1) Alhydrogel 15% in phosphate-buffered saline (PBS) (Alhydrogel-PBS), 2) recombinant LigANI in PBS (rLigANI), 3) rLigANI in Alhydrogel 15% (rLigANI-Alhydrogel), 4) CpG in PBS (CpG-PBS), 5) rLigANI and CpG (rLigANI-CpG), 6) COOH-MWCNT in PBS (COOH-MWCNTs-PBS), 7) rLigANI and COOH-MWCNTs in PBS (rLigANI-COOH-MWCNTs), 8) rLigANI, CpG, and COOH-MWCNT in PBS (rLigANI-CpG-COOH-MWCNTs), and 9) a bacterin vaccine consisting of 1×10^9 heat-killed whole-leptospires (KWL), produced as previously described (Seixas et al., 2007). Two independent experiments were performed. The recombinant protein dose used for immunizations was 50 µg. The hamsters were immunized subcutaneously on d 0 and boosted on d 14. Blood was collected on d 0, 14, and 28. Sera was stored at -20ºC and serum IgG levels were subsequently evaluated through an enzyme-linked immunosorbent assay (ELISA), using 200 ng of rLigANI as the capture antigen (Seixas et al., 2007). Twenty-eight days after the first immunization, the hamsters were challenged with an intraperitoneal inoculum of 1.3×10^3 leptospires of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. Hamsters were monitored daily and euthanized when clinical signs of terminal disease appeared (moribund) and counted as dead. Surviving hamsters were euthanized on d 36 post-challenge and blood was collected by cardiac puncture. Kidney and lung tissues were harvested for culture and histopathology studies.

Aqueous COOH-MWCNT demonstrated no significant *in vitro* cytotoxic activity at any of the concentrations tested, in the MTT assay. This finding is consistent with previous results that showed that functionalized CNT does not exert mitogenic or toxic effects on activated or nonactivated lymphocytes (Bianco et al., 2005). Hamsters immunized with rLigANI-COOH-

MWCNTs and rLigANI-CpG-COOH-MWCNTs produced a significant ($P < 0.05$) anti-leptospiral IgG response to rLigANI after the first immunization, although the antibody titer was lower than that in the rLigANI-Alhydrogel group. However, after administration of the booster dose, the humoral response in hamsters immunized with rLigANI-COOH-MWCNTs did not differ from that in rLigANI-Alhydrogel-immunized animals, demonstrating the ability of COOH-MWCNTs to augment the humoral immune response produced by the recombinant rLigANI protein (Fig. 1). KWL, rLigANI, rLigANI-CpG and negative control groups failed to mount an antibody response to the leptospiral antigen.

The survival rate in groups immunized with rLigANI-CpG and rLigANI-CpG-COOH-MWCNTs was 17% *(*$P > 0.05$*)*. In contrast, 67 and 100% of animals immunized with rLigANI-Alhydrogel and KWL (bacterin) survived, respectively ($P < 0.05$) (Fig. 2). None of the animals in the rLigANI, rLigANI-COOH-MWCNT, and negative control groups survived.

Functionalized and solubilized CNTs possess a remarkable ability to function as peptide and protein carriers across cell membranes (Kam et al., 2006; Pantarotto et al., 2004). A delivery system such as CNT can be used in combination with immunostimulatory adjuvants; accordingly, we supplemented the vaccine formulation with ODN CpG to determine whether the immune response could be improved. While recombinant LigANI administered in combination with CpG induced detectable antibody levels after a booster dose, the increase was lower than that induced by rLigANI-COOH-MWCNTs or rLigANI-Alhydrogel. Although the increase in antibody levels induced by the recombinant protein associated with COOH-MWCNTs was comparable to that induced by the administration of rLigANI-Alhydrogel, the vaccine failed to induce a protective immune response.

In conclusion, our findings suggest that COOH-MWCNTs are an effective delivery vehicle to introduce the recombinant proteins into target cells, and can be used in

immunization approaches when a humoral response is necessary and sufficient to induce protection. However, for leptospirosis, further studies are required to evaluate an appropriate adjuvant that associated to this delivery system can generate a protective immune response.

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Figure Legends

Fig.1. Induction of IgG antibodies response in hamsters immunized with different rLigANI vaccine preparations evaluated by ELISA. Values are means ± SEM of two independent experiments.

Fig.2. Protective effect of immunizations against lethal challenge in the hamster model. Percent survival conferred by rLigANI-alhydrogel and KWL (bacterin) against lethal challenge was significant ($P< 0.05$) in comparison to negative control group. Survival curves were compared using the log rank (Mantel Cox test) analysis.

Figure 1

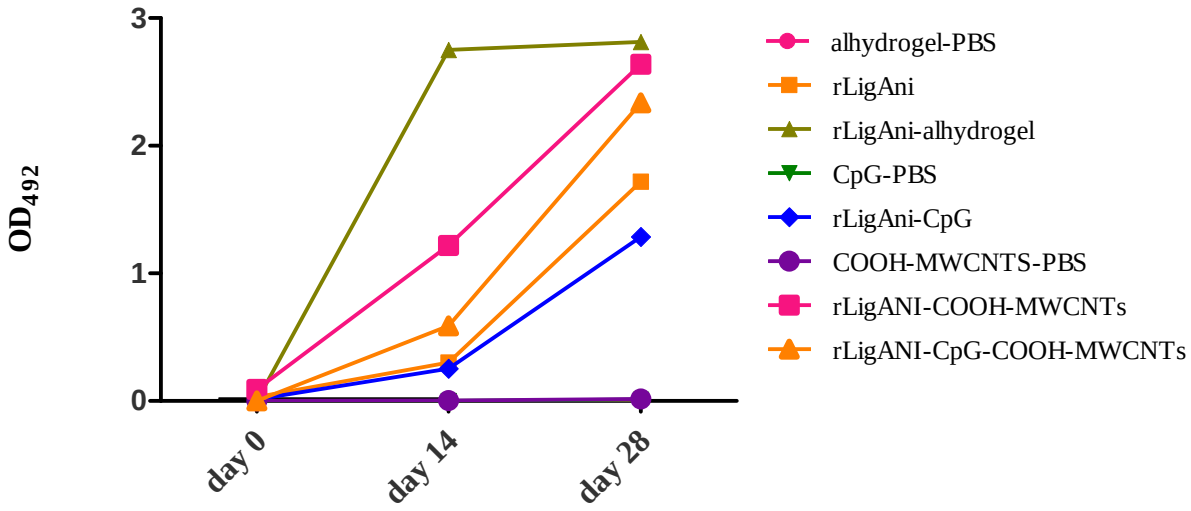
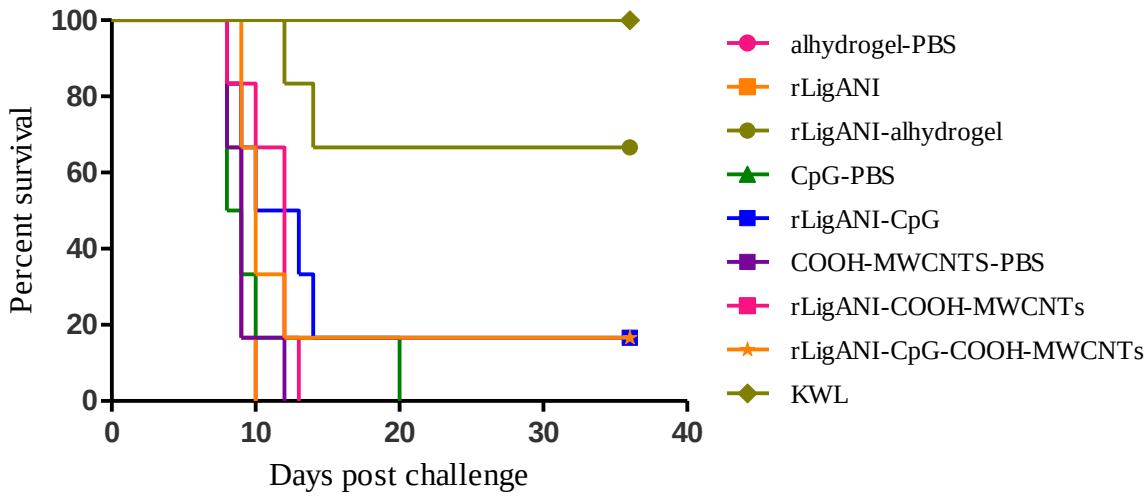

OD492
3
2
1
0
day 0
day 14
day 28
alhydrogel-PBS
rLigAni
rLigAni-alhydrogel
CpG-PBS
rLigAni-CpG
COOH-MWCNTS-PBS
rLigANI-COOH-MWCNTs
rLigANI-CpG-COOH-MWCNTs

Figure 2


Percent survival
100
80
60
40
20
0
0
10
20
30
40
Days post challenge
alhydrogel-PBS
rLigANI
rLigANI-alhydrogel
CpG-PBS
rLigANI-CpG
COOH-MWCNTS-PBS
rLigANI-COOH-MWCNTs
rLigANI-CpG-COOH-MWCNTs
KWL

5 ARTIGO 3

Xanthan as a novel adjuvant for subunit vaccines

(artigo formatado de acordo com as normas do periódico Carbohydrate Polymers)

Xanthan as a novel adjuvant for subunit vaccines

Short title: Xanthan as vaccine adjuvant

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Abstract

Recombinant subunit vaccines require potent adjuvants in order to elicit a strong immune response. Xanthan gum possesses adjuvant properties when administered with protein antigens. Here we report the evaluation of three xanthan gum preparations as an adjuvant to a recombinant vaccine against leptospirosis. Xanthan pruni 106 (X1), xathan pruni 101(X2) and a commercial xanthan (XC) were evaluated. Hamsters were immunized subcutaneously on day 0 and boosted on day 14. Upon challenge with a virulent strain of *Leptospira interrogans* serovar Copenhageni, significant protection was observed in 100 and 50% of hamsters immunized with groups rLigANI-X1 and rLigANI-X2, respectively. The protective rate in these groups was higher than that in rLigANI-XC group (Fisher test $P < 0.05$). Interestingly, protective effect was also obtained when a single dose was administered. Xanthan constitutes a natural carbohydrate-based immune adjuvant with favourable properties such as biocompatibility, safety and its use may preclude the use of booster doses.

Keywords: Adjuvants; xanthan; subunit vaccines; polysaccharide.

1. Introduction

Xanthan gum is a high molecular weight extracellular polysaccharide produced by fermentation of *Xanthomonas* spp., a plant-pathogenic bacterium genus, which has viscous properties and is widely used as a thickener or viscosifier and a stabilizer in the food and pharmaceutical industry, as well as other (Becker, Katzen, Puhler, & Ielpi, 1998; Chiou et al., 2009; Sutherland, 1998). It is a generally recognised as safe (GRAS) product and its use was licensed by the FDA (Food and agriculture organization of the Unites Nations) in 1969 (FAO/WHO, 1999).

Chemically, xanthan is considered an anionic polyelectrolyte, with a cellulosic backbone chain linked to a trisaccharide side chain consisting of two D-mannose units with alternating D-glucuronic acid residues that can be acetylated or pyruvated at different levels, which influences both the chemical and physical properties of xanthan (Becker et al., 1998). This is the classic chemical composition for the commercials xanthans, which since its discovery, in the 50s year, have been produced using *Xanthomonas campestris* strains (Kool et al, 2013). However, another species or mutant strains can produce xanthans with different chemical compositions. *Xanthomonas arboricola* pv pruni can produce a polymer that has rhamnose in its composition. This xanthan gum was named xanthan pruni (Vendrusculo, Moreira, Souza, Zambiazi, & Scamparini, 2000).

The use of polymers as adjuvant could be a useful substitute for conventional adjuvants. In immunology, an adjuvant is defined as a substance that increase or modulate the immunogenicity of a weak antigen via activation of innate and adaptive immune responses (Shakya & Nandakumar, 2013). Natural products are source of many pharmaceuticals used today and remain a rich resource from which new adjuvants can be discovered (Newman & Cragg, 2007; Rey-Ladino, Ross, Cripps, McManus, & Quinn, 2011). The intrinsic adjuvant

properties of xanthan gum as a murine lymphocyte activator was originally described in the 1980s (Ishizaka, Sugawara, Hasuma, Morisawa, & Moller, 1983). Recently, its antitumor effects was described (Takeuchi et al., 2009), and it has been successfully used in bio-adhesive formulations for intranasal influenza virus immunizations (Bertram, Bernard, Haensler, Maicent, & Bodmeier, 2010; Chiou et al., 2009).

Subunit vaccines have the potential to overcome the limitations of traditional vaccines, especially with respect to safety (Moyle & Toth, 2013). This approach, consist on the use of highly purified protein antigens that are recognized by cells of the adaptive immune system. However compared with whole-cell or virus based vaccines, they are poorly immunogenic and trigger insufficient immune responses for protective immunity, mainly due to the lack of intrinsic pathogen-associated molecular patterns (PAMPs) that activate the immune system in an appropriate way (Burnette, 1991; Foged, 2011; Gupta et al., 1993). Adjuvants are therefore required in these vaccine formulations.

For leptospirosis, an infectious zoonotic disease, recombinant LigA peptides, among others, has been expressed and evaluated in experimental models of leptospirosis as a promising target for a vaccine with several promising results (Coutinho et al., 2011; Faisal, Yan, McDonough, & Chang, 2009; Koizumi & Watanabe, 2004; Palaniappan et al., 2006; Silva et al., 2007). In previously studies, we demonstrated the adjuvant power of xanthan in a recombinant LigANI vaccine to leptospirosis. We have also shown that it has no toxic effect in Chinese hamster ovary (CHO) cells, *in vitro*. In the present study, we evaluated the adjuvanticity of different xanthan polysaccharides in a leptospirosis vaccine, administered as single or multiple doses and in two different concentrations.

2. Experimental Section

2.1 Production and characterization of Xanthans

For this study, three different xanthans were evaluated. Two of them, were produced by *X. arboricola* pv pruni strain 106 (X1) and 101 (X2) in bioreactor (BioStat B Braun Biotech International®) at a 10 L vessel with 7 L of fermentation medium under pH 7.0 (Universidade Federal de Pelotas, 2005). The fermented broths were thermally treated at 121 ºC during 15 min and the polysaccharides were recovered by precipitation with ethanol 96%, dried at 56 ºC until constant weight and then were powdered to particle size using 60-150 mesh. The pruni xanthans used in the experiments resulted of a mix of four fermentations; the product resulting from each fermentation was analyzed regarding production and viscosity and no significant difference were observed among these. Commercial xanthan (XC) was purchase from Sigma Aldrich$^{(TM)}$ lot 100M0218V. The polymers were diluted with purified water (1.25%, w/v) and stirred until uniformly distributed, sterilized and then stored at 4 ºC.

The pruni and commercial xanthans were characterized regarding viscosity, moisture, ash, nitrogen, acetyl and pyruvate content, according to Burdock (1997) and FAO (1999). The monosaccharides and derivative acid were qualitatively determined by Thin Layer Chromatography (Moreira, Vendruscolo, Gil-Tunes, & Vendruscolo , 2001).

2.2 *Subunit vaccine preparation*

For evaluation of xanthan as adjuvant, the *L. interrogans* non-identical carboxy-terminus of LigA protein, named LigANI, was expressed in *Escherichia coli* and formulated with different adjuvants for evaluation as vaccines in experimental model of leptospirosis. The cloning, expression and purification of the rLigANI polypeptide was performed as

previously described (Silva et al., 2007). The xanthans solutions were added to a final concentration of 0.5% or 0.3% (w/v) (Bertram et al., 2010).

2.3 *Bacterial strains and growth conditions*

L. interrogans serovar Copenhageni strain FIOCRUZ L1-130 (Ko, Galvão, Ribeiro Dourado, Johnson, & Riley, 1999), was cultivated at 30 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium, supplemented with Leptospira Enrichment EMJH (Difco, USA). Bacterial growth was monitored using dark-field microscopy. *E. coli* strain BL21 (DE3) pLysS (Invitrogen) was grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) at 37 °C with the addition of 50 $\mu g.mL^{-1}$ chloramphenicol and 100 $\mu g.mL^{-1}$ ampicillin.

2.4 *Ethics Statement*

Animal experiments described in this study were carried out in strict accordance with the guidelines of the National Council for Control of Animal Experimentation, Brazil (CONCEA, nº 11,794) and approved by the Ethics Committee in Animal Experimentation, Federal University of Pelotas, Brazil (Permit Number: 4515). Hamsters were monitored daily and were euthanized upon the appearance of clinical symptoms of leptospirosis. All surgical procedures were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.5 *Hamster Immunization*

Different xanthan compositions and concentrations and vaccination schemes were evaluated. Female golden Syrian hamsters 5-6 weeks old were allocated into seven groups consisting of 6 animals each – group 1: 0,5% xanthan strain 106 in PBS (Xanthan-PBS); group 2: rLigANI and 0.5% xanthan strain 106 (rLigANI-X1 0.5%); group 3: rLigANI and 0.5% xanthan strain 106 1 dose (rLigANI-X1 0.5% - 1 dose); group 4: rLigANI and 0.3% xanthan strain 106 (rLigANI-X1 0.3%); group 5: rLigANI and 0.5% xanthan strain 101 (rLigANI-X2 0.5%); group 6: rLigANI and 0.5% commercial xanthan (rLigANI-XC 0.5%); group 7: bacterin vaccine consisting of 1×10^9 heat-killed whole-leptospires (bacterin). The experimental vaccines were prepared using 50 μg of recombinant protein, with a standard volume of 500 μL and application at a single injection site. The animals were immunized subcutaneously on day 0 and boosted on day 14 (except for group 3 that was not boosted). Blood samples were collected by retro-orbital bleeding from the venous plexus before each immunization and challenge (days 0, 14 and 28), and sera were stored at -20 °C.

2.6 *Hamster challenge study and serum antibody levels detection*

On day 28 after the first immunization, hamsters were challenged with an intraperitoneal inoculum of 1.3×10^3 leptospiras, equivalent to 36 × the 50% lethal dose (LD_{50}) of *L. interrogans* serovar Copenhageni (strain Fiocruz L1-130). Hamsters were monitored daily and euthanized when clinical signs of terminal disease were observed. Surviving hamsters were euthanized on day 36 post-challenge and blood samples were

collected by cardiac puncture. Kidney and lung tissues were harvested for culture isolation and histopathology studies (Seixas et al., 2007).

Serum samples collected on day 0, 14 and 28 were evaluated for the presence of specific immunoglobulin G (IgG) by an ELISA using rLigANI as the antigen as previously described (Silva et al., 2007).

2.7 *Statistical analysis*

The results are expressed as the mean ± SEM and the significant differences between groups were determined using an analysis of variance (ANOVA), P values < 0.05 were considered statistically significant. Protection against mortality was evaluated by the Fisher exact test using Epi Info 6.04d software (Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA) and the survival curves were compared using a Log-rank analysis (Mantel Cox test) using Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results and Discussion

3.1 Xanthans characterization

The polysaccharides used in this experiment had levels of moisture, ash, nitrogen, acetyl and pyruvate in accordance with the recommendations by the FAO (FAO/WHO, 1999) and Burdock (Burdock, 1997) for xanthans used as food additives. Commercial (XC) and pruni 106 (X1) xanthans had good viscosity. The results are shown in the Table 1.

The *in vitro* tests previously performed (unpublished data) showed that the xanthan pruni was not cytotoxic by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Guava Nexin assays. Similarly, in vitro assays with *Drosophilla melanogaster* revealed that the xanthan pruni did not cause mutation or recombination in somatic cells by direct or indirect action (Rodrigues, 2010). Carbohydrate-based immune adjuvants have the advantage that they are well tolerated by the body, have high biocompatibility and low toxicity. Furthermore, carbohydrates are easily metabolized or excreted, with little risk of generating toxic metabolites or long-term tissue deposits, as may occur with aluminum salts in the condition known as macrophagic myofascitis (Petrovsky & Cooper, 2011).

3.2 Changes in antibody levels

The antibody response to rLigANI associated to xanthan vaccine formulations in golden Syrian hamsters was analysed at various time points by ELISA. The results shown in Fig.1 clearly reveal a significant antibody levels after immunization. On day 14 after first immunization, the antibody titers in immunized groups (2-5) increased and were significantly higher than those in the negative control group (1) (two-way ANOVA, $P < 0.05$). After the boost immunization, antibody levels were maintained. The bacterin and negative control groups failed to induce detectable levels of antibodies against rLigANI. No difference was observed between groups vaccinated with the different xanthans and are illustrated in Fig. 1A. Similarly, when the concentration of xanthan was decreased to 0.3% or when a booster dose was not administered, the antibody titers showed no difference compared to rLigANI-X1 0.5% group (Fig. 1B). The antibody levels of hamsters that survived the challenge are illustrated in Fig. 1, and showed no difference between xanthan strain 106, 101 or commercial xanthan. The specific immunoglobulin G levels of groups rLigANI 0.3% and rLigANI 0.5%

(1 dose) are the same as the control group rLigANI-X1 0.5% at euthanasia day (two-way ANOVA, $P > 0.05$).

3.3 Protective effect of the subunit leptospiral vaccines

The protective effects of the subunit vaccines with xanthan as adjuvant are illustrated in Fig. 2. Percentage of survival of rLigANI-X1 0.5%, rLigANI-X2 0.5% and rLigANI-XC 0.5% groups were 100, 50 and 33%, respectively. Protection of 100% in rLigANI-X1 0.5% group was achieved in two independent experiments. None of the hamsters in the control xanthan-PBS group survived. In addition, the bacterin positive control group also showed 100% survival. The protection rate of rLigANI-X1 0.5% and rLigANI-X2 0.5% groups did not show difference and was higher than that of rLigANI-XC 0.5% group ($P < 0.05$) (Fig. 2A). The survival of rLigANI 0.3% group was 83% while of rLigANI 0.5% (1 dose) group it was 50%. Even though the survival rate of rLigANI 0.5% group was 100%, it was not statistically different than the survival rates of rLigANI 0.3% and rLigANI 0.5% (1 dose) groups ($P > 0.05$) (Fig. 2B).

Previous study (unpublished data) showed that rLigANI administered without an adjuvant failed to protect hamsters against lethal challenge Alum-based mineral salts are the main adjuvant currently approved for human use by the US Food and Drug Administration (FDA). The reason for this is not so much its potency but its long record of usage and safety (Petrovsky & Cooper, 2011). Carbohydrate-based immune adjuvants, like xanthan pruni, can be an alternative to conventional adjuvants because of its safety and tolerability.

Xanthan is a negatively charged polymer consisting of a backbone chain of (1,4) β-D-glucan cellulose (Ishizaka et al., 1983). Many carbohydrate-containing compound adjuvants

has been tested, including dextran (Bachelder et al., 2010), chitin (Arca,Gunbeyaz, & Senel, 2009), β-1,3-glucan (Huang, Ostroff, Lee, Specht, & Levitz, 2010), deltin (Silva, Cooper, & Petrovsky, 2004) and mannan (Stambas, Pietersz, McKenzie, & Cheers, 2002),, some of them in human phase I and II clinical trials. These adjuvants have the ability to enhance the immunogenicity of antigens in vaccination through binding to specific innate immune receptors designed to recognize pathogen-associated microbial patterns. These receptors include the TLRs, NOD2 and C-type lectins (e.g., mannan-binding lectin), the binding of which results in activation of NF-κB and production of inflammatory cytokines including TNF-α and IL-1β (Petrovsky & Cooper, 2011; Shakya & Nandakumar, 2013). These cytokines then act as co-stimulatory molecules to enhance antigen-specific T- and B-cell responses. An animal lectin, named rhamnose binding lectin (RBL) has been characterized first from eggs of various species of teleost fishes (Tateno, Ogawa, Muramoto, Kamiya, & Saneyoshi, 2002). The preferred binding monosaccharide of RBL is rhamnose. Xanthan pruni 106 and 101, chemically differ from commercial xanthan by the presence of rhamnose in its structure (Vendruscolo et al., 2000). Further studies are required but recognition of rhamnose by a RBL could be the key for the development of an effective and protective immune response to leptospirosis.

Polymers in general work on the principle of depot generation for slow release of the antigen for a longer period of time and act as an immune-modulator via strong antigen presentation (Shakya & Nandakumar, 2013). The xanthan produced in our laboratory by *X. arboricola* pv pruni strain 106 (X1) and 101 (X2) have different viscosity levels. The xanthan pruni X1 is more viscous than X2. This difference may have contributed to the higher percentage of survival of the animals immunized with rLigANI X1 (100%) compared to the animals immunized with rLigANI X2 (50%). Although this difference was not statistically significant, it is likely to be clinically important.

Multiple doses of conventional vaccines for the activation of the desired immune responses are very difficult and challenging, especially in developing countries. Polymers in vaccine formulations could improve the delivery of antigens and thus reduce the booster doses required for an appropriate immune response (Shakya & Nandakumar, 2013). Here we demonstrate that a single priming dose of a rLigANI vaccine using xanthan pruni 106 as adjuvant can protect hamsters from lethal challenge. Protection induced by a single dose vaccine would increase the acceptability of immunization strategies and increase vaccination coverage with decreasing vaccination costs.

3.4 Histopathology and culture isolation

The culture assay is illustrated in Table 2. None of the groups vaccinated with the bacterin vaccine showed positive culture. In contrast, surviving immunized hamsters harboured leptospires in their kidneys at different levels (Table 2). Although none of the subunit vaccine preparations afforded 100% sterilizing immunity, the culture assay of rLigANI 0.5% (P = 0.066) and rLigANI 0.5% - 1 dose (P = 0.44) groups did not differ from the relevant control bacterin group.

The histopathological analyses revealed that most surviving hamsters developed an acute leptospirosis with lesions dominated by moderate pulmonary injury as evidenced by oedema and alveolar haemorrhage. The lesions in the kidneys were characterized by hyaline deposition and severe leukocyte infiltration (Table 2).

4. Conclusion

Our results indicate that xanthan pruni exerts an adjuvant effect in a rLigANI vaccine capable of conferring protection against leptospirosis. This effect is dependent of xanthan

characteristics and seems that the presence of the monosaccharide rhamnose plays an important role in this effect. A single vaccine dose was protective against a lethal challenge.

Highlights

- Xanthan pruni is a carbohydrate polymer with adjuvant properties.
- The adjuvant effect seems to be dependent on xanthan characteristics.
- A single vaccine dose of a rLigANI-xanthan can protect hamsters from lethal challenge.

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Author Contributions

KLB, DDH and OAD conceived and designed the vaccination experiments. The vaccination experiments were performed by KLB, DDH, TLO, FKS, MA. CTV, ASM and AAR produced the xanthans pruni and analysed all xanthans used. KLB, DDH, AAR, ASM and OAD analysed the data and wrote the manuscript.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of manuscript.

Competing Interests

OAD is inventor on a patent submission entitled: LigA and LigB proteins (Leptospiral Ig-like (Lig) domains) for vaccination and diagnosis (Patent nos. BRPI0505529 and WO 2007070996). CTV and ASM are inventors on a patent submission entitled: Process for preparing a xanthan biopolymer. (Patent no. WO 2006047845). The other authors declare no competing interests.

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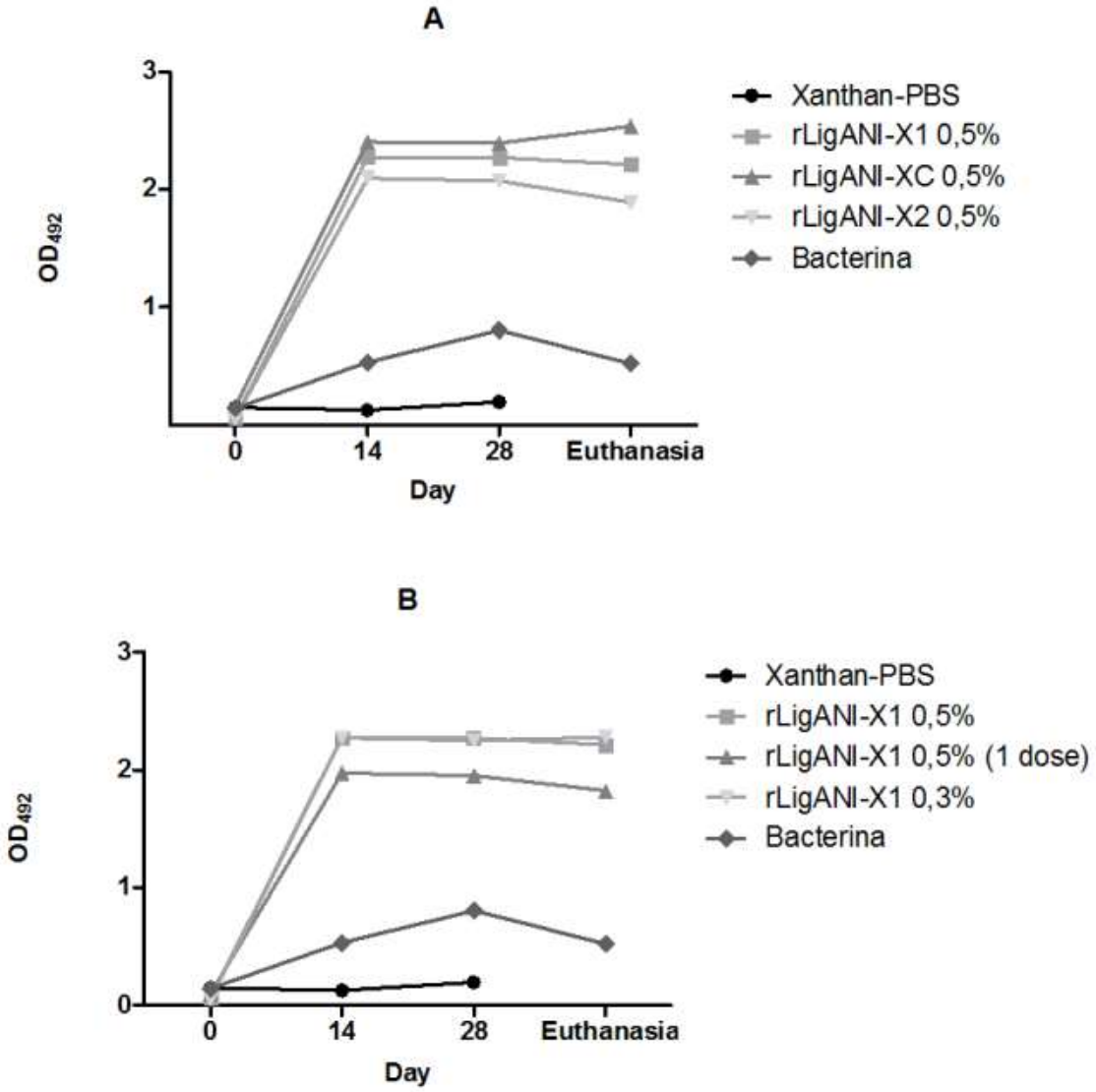
Figure Legends

Figure 1. Specific IgG response in hamsters immunized with different vaccine formulations, determined by ELISA. Fifty micrograms of the recombinant protein were used. The groups were immunized subcutaneously on day 0 and boosted after 2 weeks (day 14). Blood was collected on days 0, 14 and 28 post-immunization. (A) rLigANI-X1 0.5%, rLigANI-X2 0.5% and rLigANI-XC 0.5%. No difference was observed between groups vaccinated with the different xanthans (two-way ANOVA, $P > 0.05$). (B) rLigANI 0.3%, rLigANI 0.5% (1 dose) and rLigANI-X1 0.5%. No difference was observed between groups vaccinated with the different xanthans (two-way ANOVA, $P > 0.05$).

Figure 2. Protection against lethal challenge in a hamster model. Nine- to ten-week-old hamsters were challenged with an intraperitoneal inoculum of 1.3 x 10^3 leptospires 14 days after the second immunization (day 28). (A) The protective rate in groups rLigANI-X1 0.5% and rLigANI-X2 0.5% are the same ($P > 0.05$). Survival was lower in rLigANI-XC 0.5% group ($P < 0.05$). (B) No difference was observed in rLigANI 0.3%, rLigANI 0.5% (1 dose) and rLigANI-X1 0.5% groups ($P > 0.05$). Survival curves were compared using log rank analysis (Mantel Cox test). Bacterin: heat-killed whole-leptospires

Figure 1


A
3
2
1
OD492
0
14
28
Euthanasia
Day
Xanthan-PBS
rLigANI-X1 0,5%
rLigANI-XC 0,5%
rLigANI-X2 0,5%
Bacterina
B
3
2
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OD492
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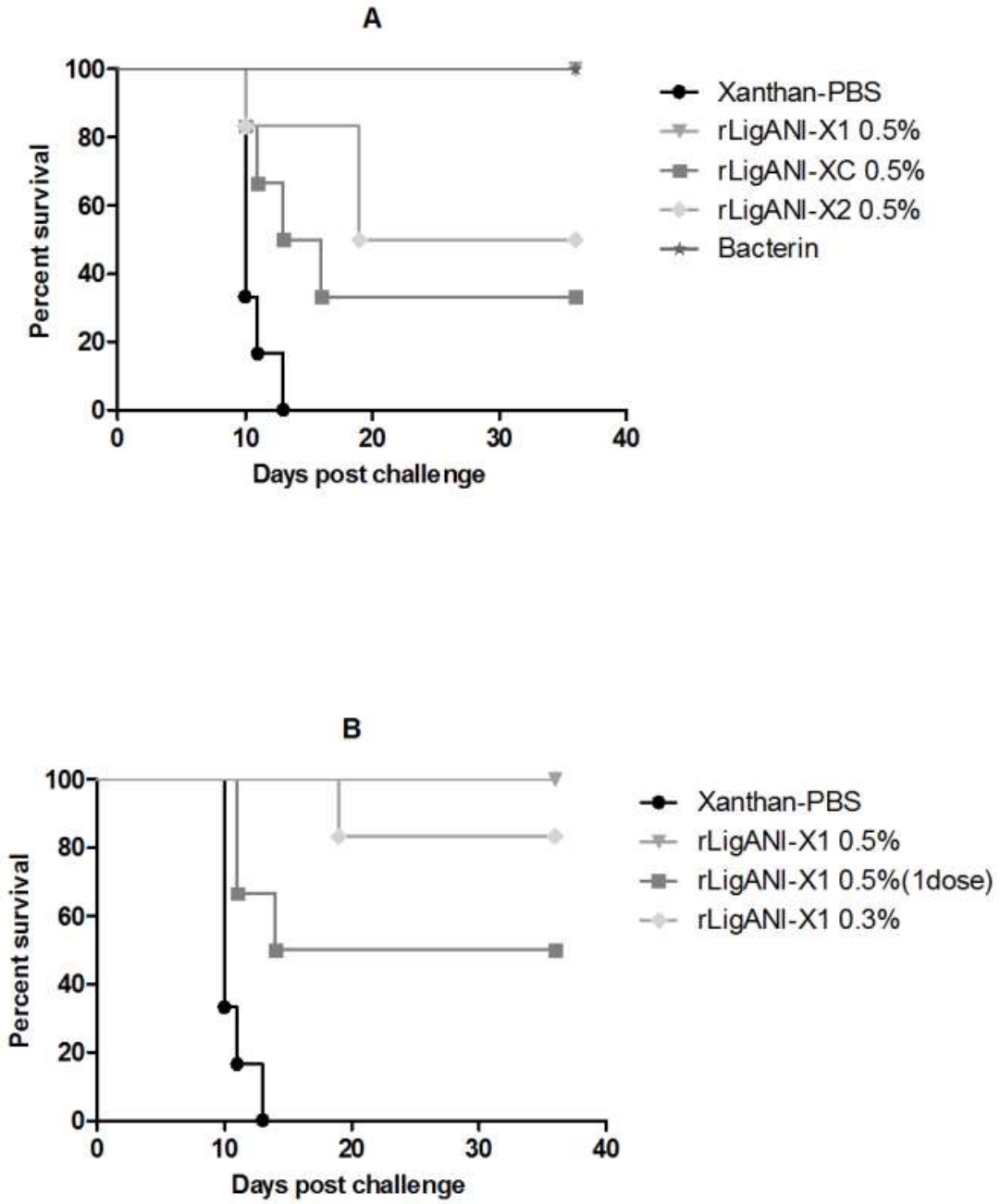
14
28
Euthanasia
Day
Xanthan-PBS
rLigANI-X1 0,5%
rLigANI-X1 0,5% (1 dose)
rLigANI-X1 0,3%
Bacterina

Figure 2


A
Percent survival
Days post challenge
Xanthan-PBS
rLigANI-X1 0.5%
rLigANI-XC 0.5%
rLigANI-X2 0.5%
Bacterin
B
Percent survival
Days post challenge
Xanthan-PBS
rLigANI-X1 0.5%
rLigANI-X1 0.5%(1dose)
rLigANI-X1 0.3%

Tables

Table 1. Moisture, ash, nitrogen, acetyl and pyruvate content (%, w/v), and viscosity (mPa.s) of pruni 106, pruni 101 and commercial (Sigma®) xanthans.

Analysis	Strain 106 (X1)	Strain 101 (X2)	Sigma® (XC)	Limits*
Moisture	5.0 ± 0.03	7.0 ± 0.04	6.0%± 0.04	≤ 15
Ash	14.37 ± 0.04	11.42 ± 0.08	12.63 ± 0.05	≤ 16
Nitrogen	1.06 ± 0.01	1.38 ± 0.03	0.96 ± 0.02	≤ 1.5
Acetyl	2.45 ± 0.10	2.52 ± 0.13	4.14 ± 0.16	-
Pyruvate	1.93 ± 0.06	0.62 ± 0.03	4.43 ± 0.02	-
Viscosity**	1203	129	1128	600

Values are the means ± SD. * Limits established by Burdock, 1997 and FAO, 1999. **at 60 rpm.

Table 2. Vaccine evaluation on basis of histopathological analysis.

		Groups					
	Score	rLigANI-X1 0.5% % (nº/total)	rLigANI-X1 0.5% - 1dose % (nº/total)	rLigANI-X1 0.3% % (nº/total)	rLigANI-X2 0.5% % (nº/total)	rLigANI-XC 0.5% % (nº/total)	Bacterin % (nº/total)
% Culture positive		67 (4/6)	33 (1/3)	100 (5/5)*	100 (3/3)*	50 (1/2)	0 (0/6)
Alveolar haemorrhage	0	0 (0/6)	0 (0/3)	0 (0/5)	0 (0/3)	0 (0/2)	0 (0/6)
	1	100(6/6)	0 (0/3)	0 (0/5)	66.7 (2/3)	0 (0/2)	33.3 (2/6)
	2	0 (0/6)	100 (3/3)	80 (4/5)	33.3 (1/3)	50 (1/2)	66.7 (4/6)
	3	0 (0/6)	0 (0/3)	20 (1/5)	0 (0/3)	50 (1/2)	0 (0/6)
Kidney Cell degeneration	0	83.3 (5/6)	100 (3/3)	100 (5/5)	66.7 (2/3)	100 (2/2)	100 (6/6)

		1		16.7 (1/6)	0 (0/3)	0 (0/5)	33.3 (1/3)	0 (0/2)	0 (0/6)
		2		0 (0/6)	0 (0/3)	0 (0/5)	0 (0/3)	0 (0/2)	0 (0/6)
		3		0 (0/6)	0 (0/3)	0 (0/5)	0 (0/3)	0 (0/2)	0 (0/6)
Kidney infiltration	leukocyte		0	66.6 (4/6)	0 (0/3)	0 (0/5)	0 (0/3)	0 (0/2)	33.3 (2/6)
			1	16.7 (1/6)	33.3 (1/3)	40 (2/5)	33.3 (1/3)	0 (0/2)	50 (3/6)
			2	16.7 (1/6)	0 (0/3)	60 (3/5)	66.7 (2/3)	0 (0/2)	16.7 (1/6)
			3	0 (0/6)	66.7 (2/3)	0 (0/5)	0 (0/3)	100 (2/2)	0 (0/6)

X1: Xanthan strain 106; X2: Xanthan strain 101; XC: Xanthan Sigma®.

The lesions in lung and kidney were graded on a scale of severity with 0 as normal, 1 as mild, 2 as moderate and 3 as severe. *Statistically significant compared to the relevant control group bacterin, Yates'corrected Chi-squared test, $P < 0.05$.

6 PATENTE – DEPÓSITO DE PEDIDO

Depósito de pedido de patente no Instituto Nacional da Propriedade Industrial - INPI

Denominação da invenção:**“XANTANA COMO ADJUVANTE EM VACINA DE SUBUNIDADE RECOMBINANTE”**

Número do registro: PI1020120218100 (Anexo A).

7 CONCLUSÕES

- A proteína recombinante de *Leptospira* LigANI, expressa em *Escherichia coli* cepa BL21 (DE3) pLysS, produziu formulações estáveis e antigênicas quando associada aos adjuvantes hidróxido de alumínio, xantana, CpG e nanotubos de carbono;

- As vacinas contendo rLigAni associada à xantana e aos nanotubos de carbono são capazes de induzir uma resposta imune específica em hamster, comparável à produzida pela proteína recombinante associada ao hidróxido de alumínio;

- A resposta IgG específica induzida pelas vacinas contendo a proteína administrada sem adjuvante ou em associação com o CpG, é inferior à induzida pela vacina rLigANI-hidróxido de alumínio;

- Os adjuvantes xantana e nanotubos de carbono, não são citotóxicos para células CHO, nas concentrações analisadas neste estudo;

- rLigANI associada à xantana pruni cepas 106 e 101, comparável ao que ocorre com ohidróxido de alumínio, protegem hamsters contra desafio letal com cepa virulenta de *L. interrogans* sorovar Copenhageni, contudo a vacina não se mostrou esterilizante.

- As formulações contendo rLigANI associada ao CpG e aos nanotubos de carbono, não produzem uma resposta imunoprotetora no referido modelo animal;

- rLigANI, formulada com xantana pruni cepa 106, é capaz de induzir uma resposta protetora contra leptospirose, mesmo quando administrada em dose única.

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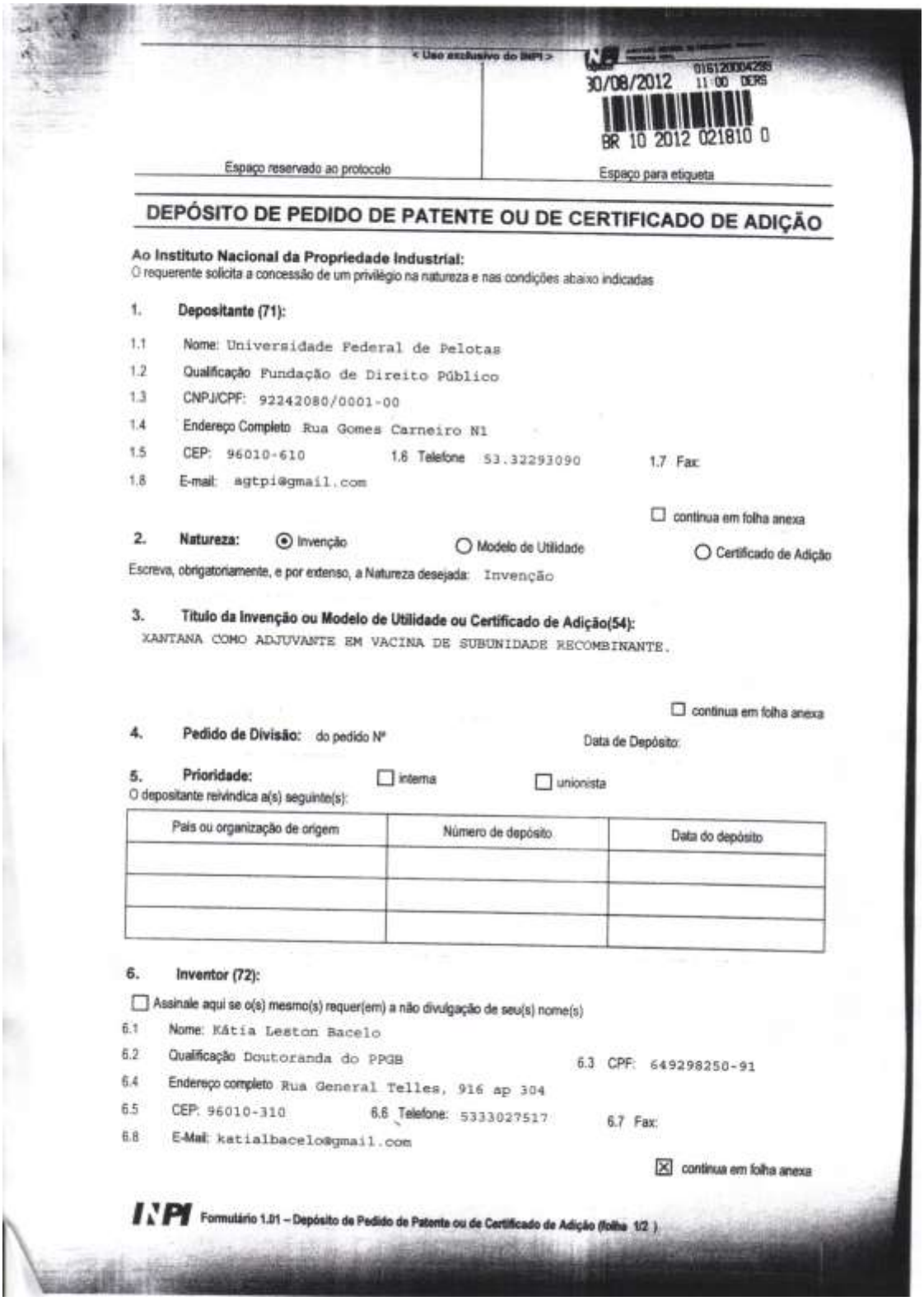
ANEXO A - Depósito de pedido de patente no Instituto Nacional da Propriedade Industrial - INPI

Denominação da invenção:“XANTANA COMO ADJUVANTE EM VACINA DE SUBUNIDADE RECOMBINANTE”



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DEPÓSITO DE PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas

1. Depositante (71):

1.1 Nome: Universidade Federal de Pelotas
1.2 Qualificação Fundação de Direito Público
1.3 CNPJ/CPF: 92242080/0001-00
1.4 Endereço Completo Rua Gomes Carneiro N1
1.5 CEP: 96010-610 1.6 Telefone 53.32293090 1.7 Fax:
1.8 E-mail: agtpi@gmail.com

☐ continua em folha anexa

2. Natureza: ◉ Invenção ○ Modelo de Utilidade ○ Certificado de Adição

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: Invenção

3. Título da Invenção ou Modelo de Utilidade ou Certificado de Adição(54):
XANTANA COMO ADJUVANTE EM VACINA DE SUBUNIDADE RECOMBINANTE.

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº Data de Depósito:

5. Prioridade: ☐ interna ☐ unionista
O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

6.1 Nome: Kátia Leston Bacelo
6.2 Qualificação Doutoranda do PPGB 6.3 CPF: 649298250-91
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