Bivalent mucosal peptide vaccines administered using the LCP carrier system stimulate protective immune responses against Streptococcus pyogenes infection

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Running Title: Lipid core peptide vaccination against *S. pyogenes*

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ABSTRACT

Despite the broad knowledge about the pathogenicity of *Streptococcus pyogenes* there is still a controversy about the correlate of protection in GAS infections. We aimed in further improving the immune responses stimulated against GAS comparing different vaccine formulations including bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP) and BPPCysMPEG, a derivative of the macrophage-activating lipopeptide (MALP-2), as adjuvants, respectively, to be administered with and without the universal T helper cell epitope P25 along with the optimized B cell epitope J14 of the M protein and B and T cell epitopes of SfbI. Lipopeptide based nano carrier systems (LCP) were used for efficient antigen delivery across the mucosal barrier. The stimulated immune responses were efficient in protecting mice against a respiratory challenge with a lethal dose of a heterologous *S. pyogenes* strain. Moreover, combination of the LCP based peptide vaccine with c-di-AMP allowed reduction of antigen dose at the same time maintaining vaccine efficacy.

Keywords: Streptococcus pyogenes, lipid core peptide system, lipopeptides, peptide-based vaccines, mucosal adjuvants

Word count abstract = 150

Word count complete manuscript = 4997

Number of Figures = 8 (7 plus 1 suppl. Figure)

Number of tables = 0

Number of references = 60

Funding:

Conflict of interest:

Carlos A. Guzman and Thomas Ebensen are named as inventors in a patent application covering the use of c-di-AMP as adjuvant (PCT/EP 2006010693). Other authors declare no competing financial interest.

Prior presentations:

There is no conflict of copy rights.

Introduction

Infection with *Streptococcus pyogenes* (GAS) is able to produce a wide range of mild to severe diseases including pharyngitis, scarlet fever, sepsis, toxic shock syndrome and necrotizing fasciitis, as well as autoimmune diseases like rheumatic fever and rheumatic heart disease. Despite significant advances in widening the knowledge about the pathogenesis of GAS as well as in developing safe and efficient vaccines against it, which even entered human clinical trials, GAS is still one of the most dreaded pathogens with a global estimate of 18.1 million cases of severe GAS disease, and 517,000 deaths per year. Leading vaccine candidates are applied using parenteral immunization strategies stimulating systemic protection.[3-5] Nonetheless, although systemic immunity seems to be efficient in preventing GAS dissemination and associated disease, parenteral immunization is unable to stimulate mucosal immunity against GAS. Certainly, this would be especially preferable as the mucosal surfaces of the upper respiratory tract (URT) constitute a primary portal of entry of GAS, leading to colonisation of the URT and a subsequent disseminated infection (reviewed in [6]). Thus, mucosal immunity would not only prevent diseases caused by GAS but also colonisation, thereby affecting transmission from man to man.

One of the most studied and promising vaccine candidates against GAS is the surface M protein. The M protein is a major virulence factor playing a key role in adherence to host cells, intracellular invasion and in preventing bacterial clearance by complement-mediated phagocytosis. Based on the highly variable amino-terminal region of the M protein, more than 200 GAS serotypes are defined.[6, 9] In contrast, the carboxy-terminal C-repeat region of the M protein is highly conserved. Vaccination approaches based on the M protein resulted in protective immune responses in human volunteers. However, due to molecular mimicry between the M protein and host proteins autoimmune responses have been induced.[10-12]

Therefore, efforts have been made to develop a C-repeat region based GAS vaccine with a broad strain coverage, but excluding the cross-reactive B cell and T cell epitopes located within this region.[13] In this context, a C-repeat-region restricted conformational peptide, J8, has been identified as minimal B cell epitope able to stimulate protective antibodies in animal-based trials but lacking a human heart cross-reactive T cell epitope.[14-16] However, the type-specificity of the M protein seems to require the implementation of multivalent vaccines. In this context, fibronectin-binding proteins, which play a key role in bacterial attachment to host cells and colonisation of the pharyngeal mucosa, as well as in cellular invasion, have also been shown to function as potent vaccine candidates.[17-19] For example, intranasal (i.n.) immunization of mice with the fibronectin-binding protein I of *Streptococcus pyogenes* (SfbI) or even with its fibronectin-binding repeats region (FNBR) provided efficient protection against mucosal GAS infection.[20-22] Therefore, in order to develop a more universal GAS vaccine, we settled a dual antigen component vaccine based on the M protein derived J8 peptide (QAEDKVKQ-SREAKKQVEKAL-KQLEDKVQ) encompassing a truncated B cell epitope of the 20-mer epitope p145 and two SfbI derived peptides encompassing linear B cell and T cell epitopes of the FNBR region.[23-25] The peptides were delivered using the lipid core peptide (LCP) system, which has been described as a vaccine delivery system allowing the conjugation of lipoamino acids to a polylysine core thereby forming nanoparticle (NP). In brief, incorporation of lipoamino acid into the vaccine peptides in aqueous media resulted in formation of particles 5nm to 15nm in size with a polydispersity (PDI) of 0.3-0.5. Whereby self-assemble into small nanoparticles seems to depend on an optimal balance between the hydrophilic (J14) and hydrophobic (FNBR) segments.[28] NP formulation was monitored by analytical HPLC and dynamic light scattering.[26] Mice vaccinated with these formulations showed up to 100% protection against a lethal challenge with a heterologous *S. pyogenes* strain sustaining their potential as mucosal vaccine against a

broader spectrum of *S. pyogenes* strains.[24] However, compared to the immune responses observed using the 20-mer epitope p145 (LRRDLDASREAKKQVEKALE) immunogenicity of the minimal B-cell epitope J14i (ASREAKKQVEKALE) seems to be reduced.[16] The reason for this loss of potency might be related to the loss of the helical conformation of the native epitope.[16] Thus, to overcome this hurdle, Hayman and co-workers embedded the minimal epitope into a helix-promoting sequence of the yeast protein GCN4, resulting in the J14 peptide (KQAEDKVK-ASREAKKQVEKALE-QLEDKVK), which shows the conformational structure of the native protein. Immunization with J14 not only stimulated antibodies which show an increased opsonic activity compared to those stimulated by J8, but also J14-specific T cells possessing a higher proliferative capacity with respect to J8-specific T cells.[16] Hayman and co-workers hypothesize that the T cell epitope of peptides J8 and J14 is formed by the sequence spanning the B cell epitope and the flanking regions. Furthermore, J14 was also shown to be protective against GAS infection in a mouse model.[30-33] In the present work we demonstrated that vaccination of BALB/c mice by i.n. route using the LCP nano carrier system loaded with peptides J14 and FNBR-B (VETEDTKEPGVLMGGQSESVEFTKDTQTGM), which are derived from the conserved C-repeat region of the M protein and the FNBR region of SfbI, respectively (Figure 1), in combination with the mucosal adjuvant BPPCysMPEG stimulated protective humoral and cellular immune responses similar to those observed following immunization with the LCP-J8-FNBR-B system.[24] Furthermore, we demonstrate that incorporation of the universal T helper cell epitope P25 (KLIPNASLIENCTKAEL) together with the mucosal adjuvant c-di-AMP resulted in increased cellular immune responses, and allows to reduce the antigen dose needed to stimulate protective immunity.

Methods

Mice

Studies were performed using female BALB/c mice (4 weeks old) obtained from Harlan Winkelmann GmbH (Borchen, Germany). Animals were maintained under specific pathogen-free conditions at the animal facility of the Helmholtz Centre for Infection Research (Germany). All animal experiments in this study have been performed with ethical agreement by the local government of Lower Saxony (Germany) with the No. 33.42502/07-05.05.

Bacterial strains and media

Infection studies were performed using GAS strain NS192, a blood isolate of a patient from the Australian Northern Territories, characterised as M-type 106 and V-type 3.2 (more details in supplementary materials).

Construction and purification of lipopeptide and LCP vaccine formulations

Polypeptides encompassing B cell epitopes of the M protein (J14) and the SfbI protein (FNBR-B) were synthesized on the LCP system as described previously (more details in supplementary materials).

Immunization protocol

Groups of 15 BALB/c mice were immunised i.n. (10 µl/nostril) with LCP-J14-FNBR-B (30 µg/dose) co-administered with 0.5 µg ($6x10^{13}$ units) of BPPCysMPEG, a pegylated derivative of the TLR2/6 agonist MALP-2 as mucosal adjuvant, on days 0, 7, 14 and 28. In the second approach mice were immunised following the same protocol with a combination of lipopeptides loaded with P25 and J14 (20 µg/dose) or P25 and FNBR-BT (20 µg/dose), respectively. The lipopeptide assortment was co-administered with 5 µg ($4.5x10^{15}$ units) of the

STING agonist c-di-AMP as mucosal adjuvant. Control animals received phosphate buffered saline (PBS) alone.

Sample collection

Blood samples from immunized mice were taken from the retro-orbital complex on days 0, 6, 13, 27 and 38, and stored at -20 °C prior to the determination of J14-specific and FNBR-specific antibodies. On day 38, mice (n = 5) were sacrificed and saliva and cells from cervical lymph nodes and spleen were collected and monitored for the presence of antigen-specific secretory IgA and T-cells, respectively (more details in supplementary materials).

Detection of antigen-specific antibodies in sera and saliva

Stimulation of antigen-specific antibodies following i.n. vaccination with the different formulations was determined by ELISA as described previously (more details in supplementary materials).

T-cell proliferation

Antigen-specific T-cell responses were determined by a T-cell proliferation assay using splenocytes and cervical lymph nodes derived cells from vaccinated animals (more details in supplementary materials).

ELISpot-assay

To determine the amount of IFN-γ and IL-4 secreting cells, murine ELISpot kits (BD Pharmingen) were used according to the manufacturer's instructions (more details in supplementary materials).

Challenge studies

Mice were challenged with 10^8 CFU of the heterologous virulent GAS strain NS192. The mortality was recorded daily up to 21 days after the challenge (more details in supplementary materials).

Statistical analysis

The significance of the differences observed in the immunogenicity studies was analysed using non-parametric Mann-Whitney test, and two-way ANOVA test, respectively. Differences were considered significant at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***), respectively.

Results

Intranasal immunization with LCP conjugated with M protein and SfbI derived epitopes stimulate efficient antibody responses in serum and mucosal secretions when co-administered with BPPCysMPEG

In a first step we evaluated the strength of J14- and FNBR-B-specific antibody production following immunization of mice using the LCP system (LCP-J14-FNBR-B) in combination with the MALP-2 derivative BPPCysMPEG. Vaccination of mice by i.n. route stimulated similar levels of both J14- and FNBR-B-specific IgG in sera, which were significantly increased as compared to values of animals from the untreated control group (Figure 2A). As expected, the IgG titre observed when ELISA plates were coated with both antigens were about the sum of both J14- and FNBR-B-specific IgG titres (Suppl. Figure 1A). Moreover, the obtained titres were also strongly increased compared to those reported for the physical mixture of LCP-J8 and LCP-FNBR-B.[24] The same is true when analysing the peptide-specific IgA titres stimulated in saliva of vaccinated animals. Thus, high titres of FNBR-B-

specific IgA antibodies have been detected in saliva 10 days after the last boost followed by those of J14-specific IgA (Figure 2B). This is in contrast to what we reported before when LCP-J8 and LCP-FNBR-B were admixed, which stimulated stronger J8-specific IgA responses with respect to those observed for FNBR-B.[24] However, i.n. immunization of mice with the LCP-J14-FNBR formulation co-administered with BPPCysMPEG also stimulated J14- and FNBR-B-specific Th2 dominated immune responses, as indicated by the 7.3 and 9.6 times increased IgG1 titres compared to the corresponding IgG2a titres, respectively (Figure 2C). These observations are in line with the results previously obtained after co-administration of LCP-J8 and LCP-FNBR-B.[24]

Intranasal immunization with LCP conjugated M protein and SfbI derived epitopes stimulate increased cellular responses at local and systemic level when co-administered with BPPCysMPEG

In order to evaluate the J14 and FNBR-B peptide-specific cellular immune responses stimulated after i.n. immunization with the LCP-J14-FNBR-B co-administered with BPPCysMPEG, the proliferative capacity of splenocytes and lymph node derived cells was measured after restimulation with the corresponding antigenic peptide. In addition, we evaluated the cytokine profiles stimulated by the different peptides for further elucidating the pattern of the stimulated antigen-specific responses. While J14-specific immune cells showed increased proliferative capacities both at local and systemic level, enhanced proliferation of FNBR-B-specific cells was observed only in draining cervical lymph nodes (Figure 3A, B). Similar observations have been done following i.n. immunization of mice with the LCP-J8-FNBR system.[24]

When evaluating the cytokine profiles produced by spleen-derived lymphocytes after restimulation with the corresponding peptide, the number of both IFNγ and IL-4 producing

J14-specific cells was increased as compared to the FNBR-B-specific immune cells (Figure 3C, D). Interestingly, while the number of IFNγ producing J14-specific cells was increased compared to those obtained when J8 peptide was conjugated to LCPs, the number of IFNγ producing FNBR-B-specific cells was significantly reduced.[24] Con A stimulated control lymphocytes from each group efficiently produced IFNγ (103.5-166.8 SFU/10^6 cells) and IL-4 (20.5-33.6 SFU/10^6 cells) while only 0.3 to 2.3 SFU/10^6 cells have been observed for the background values.

Immunization by i.n. route with LCP conjugated M protein and SfbI derived epitopes stimulate protective immune responses when co-administered with BPPCysMPEG

Mice immunised with LCP-J14-FNBR-B were challenged intranasally 10 days after the last immunization with the mouse adapted heterologous GAS strain NS192, which expresses both the SfbI and the M106 proteins. The percentage of survival in each group was determined up to 15 days post-challenge. Ninety percent of the infected animals receiving LCP-J14-FNBR-B co-administered with BPPCysMPEG survived, whereas the mean survival time of the mice receiving only PBS prior infection was 4.25 days (Figure 4).

Lipopeptides conjugated M protein and SfbI derived B and T cell epitopes stimulate efficient antibody responses in serum and mucosal secretions when co-administered with c-di-AMP

Although vaccination of mice by i.n. route with LCP-J14-FNBR-B co-administered with BPPCysMPEG stimulated already increased antibody production with respect to the foregoing J8 based formulation, we wondered if incorporation of another mucosal adjuvant facilitating a more balanced Th1/Th2 response as well as the universal T helper cell epitope P25 would further enhance the vaccination efficacy. Thus, J14, FNBR-BT (in addition encompassing a T

cell epitope) and P25 peptides were formulated with LCPs to give a lipopeptide based vaccine formulation. Lipoamino acids included were longer compared to the formulations without P25 as longer lipoamino acids are expected to further enhance the stimulated immune responses (Figure 1).[34] As for LCP-J14-FNBR-B also LCPs encompassing P25 have been shown earlier to form nanoparticles which were smaller 10nm in size (determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM)). In brief, lipoamino acids included are unnatural α-amino acids that bear an alkyl side chain that can be readily synthesized and coupled using standard solid-phase peptide synthesis (SPPS) methods. Lipoamino acid synthesis was performed using a microwave-assisted tert-butoxycarbonyl (Boc) strategy.[35] The generated products were analysed by ESI-MS and analytical RP-HPLC with each product obtained at greater than 99% purity.[35] Furthermore, LCPs showed a PDI of 0.57 ± 0.08 and a Zeta potential of about 19.0 ± 1.5 mV.[36] When mice were immunized by i.n. route with about 4 $x10^{14}$ molecules less compared to the antigen dose used in the LCP-J14-FNBR-B system, co-administration of lipopeptides LCP-P25-FNBR-BT + LCP-P25-J14 including the B and T cell epitopes with c-di-AMP as mucosal adjuvant stimulated similar J14-specific IgG titres in mice as compared to the LCP-J14-FNBR-B system (Figure 5A). In contrast, the obtained FNBR-BT-specific titres were remarkably reduced. However, FNBR-BT-specific IgG titres were still increased respect to those previously obtained using a mixture of LCP-J8 and LCP-FNBR as vaccine.[24] In addition, as observed for the LCP-J14-FNBR-B system, the IgG titre obtained for both antigens were about the sum of the indiviual J14- and FNBR-BT-specific IgG titres (Suppl. Figure 1B). When analysing peptide-specific IgA responses in the broncho-alveolar mucus of immunized mice the highest IgA titres were obtained against J14, whereas the FNBR-BT-specific IgA titres were reduced as compared to those observed using the LCP-J14-FNBR-B system (Figure 5B). Therefore, while co-administration of LCP-J14-FNBR-B with BPPCysMPEG seems to favour FNBR-specific

antibody responses at the inductive site, co-administration of the lipopeptides LCP-P25-FNBR-BT + LCP-P25-J14 encompassing in addition the universal T cell epitope P25 with c-di-AMP seems to favour the stimulation of J14-specific antibody production (Figure 5B). Furthermore, incorporation of T cell epitopes together with the use of c-di-AMP also modified the balance between the peptide-specific Th1 and Th2 responses, as indicated by the IgG1/IgG2a ratios observed following vaccination (Figure 5C). Thus, J14- as well as FNBR-BT-specific Th1 and Th2 responses have been modified in favour of Th1 responses, thereby resulting in a more balanced Th1/Th2 response (Figure 5C).

Lipopeptides conjugated M protein and SfbI derived B and T cell epitopes stimulate strong J14-specific cellular immune responses when co-administered with c-di-AMP

Similar to what was observed in terms of peptide-specific humoral immune responses i.n. immunization of mice using the lipopetides LCP-P25-FNBR-BT + LCP-P25-J14 co-administered with c-di-AMP stimulated also predominantly J14-specific cellular responses (Figure 6). Hence, J14-specific splenocytes and lymph nodes derived cells show strong proliferative capacities after restimulation with the corresponding peptide (Figure 6A). In addition, high numbers of IFNγ- and IL-4-producing J14-specific cells have been detected, further attesting a more balanced Th1/Th2 response as compared to that stimulated by the LCP-J14-FNBR-B system (Figure 6B, C). Both proliferative capacity and cytokine production was remarkably increased with respect to those obtained using about 20% more antigen in combination with LCPs and BPPCysMPEG as adjuvant. Nevertheless, no significantly increased FNBR-BT-specific cellular responses could be observed (Figure 6).

Lipopeptides conjugated M protein and SfbI derived B and T cell epitopes stimulate protective immune responses when co-administered with c-di-AMP

In order to evaluate the efficacy of the peptide-specific immune responses modified by the incorporation of c-di-AMP as mucosal adjuvant as well as the impact of the universal T helper cell epitope P25 and a SfbI derived CD4+ T cell epitope, respectively, mice were challenged by i.n. route 10 days after the last immunization with 3.5 $x10^9$ CFU of the mouse adapted heterologous GAS strain NS192. Although the antigen dose used for immunization was about 20% less of what was used for the LCP-J14-FNBR-B system and the infection dose at the same time was 35 times increased, 30% of the animals survived, whereas the mice from the PBS group died within 10 days with a mean survival time of 3.7 days (Figure 7).

Discussion

Several years ago we argued that developing an effective vaccine able to protect against the broad range of GAS serotypes would need to include multiple antigens and should ideally be applied by mucosal route, since most infections caused by the more than 200 different serotypes known today are restricted to the mucosa.[37-39] Currently, although lots of effort has been taken to develop efficient vaccines, there is still no GAS vaccine available and also antibiotic treatment did not solve the problem.[40] Thus, in most of the cases treatment of GAS infection with penicillin remains effective. Nevertheless, several studies reported on penicillin failure by now. Consequently, GAS still constitutes a highly evolved pathogen able to circumvent host defence mechanisms by the use of a multiplicity of virulence factors. Moreover, due to horizontal gene transfer, new dominant variants emerged resulting in a substantial global disease burden with high rates of morbidity and mortality worldwide. Even though there is extensive knowledge available on (i) the molecular pathogenesis of GAS infection, (ii) the multiple virulence factors, and (iii) the immune responses needed for

protecting humans, there is still an urgent need of new vaccination strategies in order to efficiently eradicate GAS infection and disease.[40]

The M protein as major virulence factor was extensively studied and shown to stimulate protective immune responses after both natural infection and vaccination. For example, different multivalent vaccine formulations based on the hypervariable N-terminal region of the M protein showed already promise in clinical trials.[6] However, protection usually is only serotype-specific, which is why recent approaches focus on the conserved C-terminal region of the M protein as vaccine candidate. Albeit, in this case it is essential to exclude those sequences including B cell and T cell epitopes cross-reactive with host tissue, which can lead to autoimmune reactions. Alternatively, in order to circumvent the possibility of inducing such tissue cross-reactive antibodies, different protective antigens have been described (e.g. C5a peptidase, SOF, SfbI, exotoxins B and C) that might provide broader serotype independent coverage against GAS.[40] Nevertheless, although most of the vaccine antigen candidates tested in experimental animal models have proved reasonably effective in inducing protective immunity against GAS infections, their use for human vaccines is limited as they fail either to fulfil all the requirements in terms of broad coverage and safety, or show different performance efficacy with respect to the route of vaccination. We have previously demonstrated that the SfbI protein is a promising vaccine candidate conferring protection in mice when administered by i.n. route.[20] Moreover, we identified the highly conserved functional domain responsible for bacterial attachment, colonisation and invasion, the fibronectin-binding repeats, as minimal fragment needed to stimulate protective immunity. This conserved region is found in a large number of clinical isolates from patients from different geographic areas affected with different forms of disease, making it an ideal candidate for a multi-component vaccine formulation. To this end, we developed earlier a nanoparticulated epitope-based formulation containing conserved determinants from the M

(J8) and the SfbI (FNBR) proteins which are non-cross reactive with host tissues. We demonstrated that immunization of mice with a combination of J8 and FNBR peptides delivered by the LCP nano carrier system and co-administered with the mucosal adjuvant MALP-2 induced strong mucosal and systemic antigen-specific immune responses, which were able to protect mice against a heterologous challenge with GAS.[24] Whereby the efficacy of the combination vaccine was improved compared to the single epitope formulations. We postulated that the reason for the superior efficacy of the bivalent formulation could be due to a synergistic effect of the humoral responses stimulated against the two different antigens and the T cell help enhanced by the included T cell epitope of the FNBR region from SfbI. In addition, co-administration of the TLR-2/6 agonist MALP-2 increased the survival rate of immunized mice as compared to those receiving the LCP formulation alone (data not shown). Indicating that TLR-2 signalling via both heterodimers (TLR2/6 by the diacylated lipopeptide MALP-2 and TLR2/1 by the triacylated lipopeptide LCP) further enhances the stimulated antigen-specific immune responses. However, there is still a controversy about the correlate of protection in GAS infections. Thus, opsonizing antibodies have been correlated previously with the ability to protect against infection.[49] Though, depending on the animal model used protection seems not exclusively depend on the presence of opsonizing antibodies.[50] For example, immunization of mice by s.c. route using either J8 or J14 peptide containing vaccine formulations stimulates opsonic antibodies resulting in protection against GAS infection. The same peptides applied by i.n. route stimulated production of antibodies with only low opsonic activity, but animals were also protected (reviewed in). Accordingly, for further improving the immune responses stimulated against GAS, different adjuvants were included here administered with and without a universal T helper cell epitope along with the optimized B cell epitope J14 of the M protein and the B and T cell epitopes of SfbI. Furthermore, by the combination of the diacylated lipopeptide of this approach and c-di-AMP we aimed in

enhancing the stimulated antigen-specific immune responses due to an additive or synergistic effect of the TLR2/6 and STING signalling induced by these two moieties.

In contrast to the previously used J8-based formulation in which LCP-J8 and LCP-FNBR have been co-administered, the LCP-J14-FNBR-B system used in the present work encompasses both peptides in one molecule. Indeed, the protective humoral and cellular immune responses stimulated following vaccination of mice with the bivalent LCP-J14-FNBR-B system are clearly superior to those obtained with the J8-based system.[24] The same is true using the combination of lipopeptides LCP-P25-FNBR-BT and LCP-P25-J14 co-administered with c-di-AMP. Interestingly, the J14-specific antibody titres obtained for both formulations were similar to those observed using complex LCP-based polymeric nanoparticles. Thus, Marasini and co-workers have demonstrated recently that incorporation of LCP-P25-J14 in polymeric nanoparticles further enhances vaccine efficacy following i.n. immunization of mice.[52] Therefore, incorporation of either BPPCysMPEG or c-di-AMP seems to allow avoiding the need of complex nanoparticles to overcome the mucosal barrier even though an additional booster immunization is needed. However, when the universal T helper epitope P25 was incorporated J14-specific cellular immune responses were further increased compared to the LCP-J14-FNBR-B system even without fusion of J14 and FNBR in one molecule. Thus, immunization with a combination of the lipopeptides LCP-P25-FNBR-BT and LCP-P25-J14 co-administered with c-di-AMP as mucosal adjuvant not only increased the proliferative capacity of J14-specific immune cells, but also modified the immune responses in direction of a more balanced Th1/Th2 response, as compared to those obtained using the TLR2/6 agonist BPPCysMPEG. This is indicated by the observed IgG1/IgG2a ratios, as well as by the numbers of IFNγ and IL-4 producing splenocytes. Interestingly, while the J14-specific cellular immune responses have been strongly enhanced by the inclusion of P25 in the formulation, none or only marginal FNBR-specific responses could be detected. This is in contrast to what

was observed here using the bivalent LCP-J14-FNBR-B system co-administered with BPPCysMPEG and the previously used combination of LCP-J8 and LCP-FNBR co-administered with MALP-2.[24] The use of these two formulations promoted strong FNBR-specific local cellular responses, as well as J8- and J14-specific responses at systemic level. The poor cellular responses stimulated by the FNBR peptides can be explained, at least in part, by the intrinsic immune modulatory properties of the SfbI protein. Thus, the FNBR peptides included in the formulations encompass the minimal domain required for adjuvanticity of SfbI and might have further supported J14-specific responses. Similar effects have been also described by other groups demonstrating that using peptides derived from a fibronectin binding protein or pro-inflammatory cytokine stimulated DC maturation and activation. Nevertheless, an alternative explanation could be the nature of the used formulations. While in the LCP-based system J14 was conjugated together with FNBR in one lipopeptide molecule, LCP-P25-FNBR-BT and LCP-P25-J14 were admixed. This is in line with observations showing that co-administration of vaccine antigens can result in immune interference, thereby preventing immune reactions against a certain antigen of the formulation. Still, despite the reduced FNBR-specific cellular response stimulated by the combination of lipopeptides LCP-P25-FNBR-BT and LCP-P25-J14, mice receiving the P25-based lipopeptides showed a statistically significant increased mean survival time of 6.7 days as compared to 3.7 days in the control group with $P<0.05$. This is particularly important considering that there is a mismatch of 4 out of the 14 amino acids in the M1-derived J14 peptide respect to the corresponding sequence of the M106 protein of the challenge strain.[59]

Although the sequence encompassed by the J14 peptide is highly conserved, there is still a certain degree of variability among different M serotypes. Hence, mice immunised with J14-dT co-administered with CTB as adjuvant showed 70% survival when challenged with a homologous GAS M1 strain, whereas the protection level of J14-specific antibodies against

heterologous strains was slightly reduced compared to J14 sequence variants.[15] In contrast, the sequences encompassed by the FNBR peptides seem to be almost 100% conserved. This strongly argues for the bivalent vaccine formulation. It is important to highlight, that incorporation of either BPPCysMPEG or c-di-AMP as mucosal adjuvant allowed reduction of the antigen dose needed to protect mice against a heterologous challenge, as compared to what was published before. However, minimum effective dose studies need to be performed in order to validate this antigen dose sparing effect. Thus, similar J14-specific antibody titres in sera and saliva of vaccinated animals have been observed even after three immunizations. Furthermore, co-administration of the mucosal adjuvants BPPCysMPEG or c-di-AMP resulted in strong humoral J14-specific responses

In conclusion, the results presented here demonstrate the superior immunogenicity of the LCP based vaccine formulations encompassing the conformational optimized antigenic peptide epitope J14 as well as the mucosal adjuvants BPPCysMPEG and c-di-AMP with respect to those described previously encompassing the J8 peptide. Efficient responses were stimulated at both mucosal and systemic levels after i.n. vaccination with both LCP based bivalent vaccine formulations. Moreover, incorporation of the adjuvant c-di-AMP allowed the reduction of antigen needed in order to stimulate protective immune responses in vaccinated animals. These data further support the feasibility of incorporating multiple antigenic peptide epitopes in a nanoparticulated multivalent vaccine formulation for the development of a mucosal GAS vaccine.

Figure caption

Figure 1. Schematic structure of the LCP based vaccine formulations encompassing peptides J14 and FNBR co-formulated in one LCP moiety (A) or conjugated to different LCP moieties incorporating the universal T helper cell peptide P25 (B).

Figure 2. Peptide-specific antibody responses stimulated after intranasal immunization of mice with the LCP-J14-FNBR system. Peptide-specific IgG titres in sera (A), and IgA titres in saliva (B) of immunized mice 38 days after the first immunization. Results are expressed as the last dilution (end point dilution, e.p.d.) giving the double value (OD450 nm) of the background value (negative control). (C) Peptide-specific IgG subclasses in sera of immunized mice. Results are expressed as the ratio between the IgG1 and IgG2a titres. Standard error of mean (SEM) is indicated by vertical lines. Differences between groups were considered significant at $p<0.05$ (*) and $p<0.01$ (**).

Figure 3. Peptide-specific cellular immune responses stimulated after intranasal immunization of mice with the LCP-J14-FNBR system. Spleen and lymph nodes cells were pooled (n=5) and re-stimulated *in vitro* during 4 days with 30 µg/ml of J14-peptide (A) and FNBR-peptide (B), respectively. Results are expressed as the ratio between values (average of triplicates) from stimulated and non-stimulated samples (stimulation index). For the characterization of cytokine producing cells spleen-derived lymphocytes were cultured for 16 h with 20 µg/well of the corresponding peptide. (A) INF-γ and (B) IL-4 production was determined in culture supernatants by ELISPOT. The SEM is indicated by vertical lines. The observed differences were statistically significant in comparison with the control PBS group at $p<0.05$ (*) and $p<0.001$ (***).

Figure 4. Survival of mice intranasally challenged with the heterologous GAS strain NS192 38 days after intranasal immunization with the LCP-J14-FNBR system. Mortality of vaccinated animals (n = 10) was recorded daily.

Figure 5. Peptide-specific antibody responses stimulated after intranasal immunization of mice with the lipopeptides P25-FNBR-BT and P25-J14. Peptide-specific IgG titres in sera (A), and IgA titres in saliva (B) of immunized mice 38 days after the first immunization. Results are expressed as the last dilution (end point

dilution, e.p.d.) giving the double value (OD450 nm) of the background value (negative control). (C) Peptide-specific IgG subclasses in sera of immunized mice. Results are expressed as the ratio between the IgG1 and IgG2a titres. Standard error of mean (SEM) is indicated by vertical lines. Differences between groups were considered significant at $p<0.05$ (*) and $p<0.01$ (**).

Figure 6. Peptide-specific cellular immune responses stimulated after intranasal immunization of mice with the lipopeptides P25-FNBR-BT and P25-J14. Spleen and lymph nodes cells were pooled (n=5) and re-stimulated *in vitro* during 4 days with 30 µg/ml of J14-peptide (A) and FNBR-peptide (B), respectively. Results are expressed as the ratio between values (average of triplicates) from stimulated and non-stimulated samples (stimulation index). For the characterization of cytokine producing cells spleen-derived lymphocytes were cultured for 16 h with 20 µg/well of the corresponding peptide. (A) INF-γ and (B) IL-4 production was determined in culture supernatants by ELISPOT. The SEM is indicated by vertical lines. The observed differences were statistically significant in comparison with the control PBS group at $p<0.05$ (*) and $p<0.001$ (***).

Figure 7. Survival of mice intranasally challenged with the heterologous GAS strain NS192 38 days after intranasal immunization with the LCP-J14-FNBR system. Mortality of vaccinated animals (n = 10) was recorded daily. The differences between control group and animals vaccinated with the lipopeptides P25-FNBR-BT and P25-J14 were significant at $p< 0.05$(*).

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