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GENERATION AND CHARACTERIZATION OF *STREPTOCOCCUS PYOGENES* STREPTOLYSIN O DERIVATIVES AS VACCINE CANDIDATES

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1. Summary

Streptococcus pyogenes or Group A Streptococcus (GAS) is a human pathogen which colonizes the throat and the skin causing non-invasive diseases, as pharyngitis or impetigo, as well as more invasive and life-threatening pathologies, as pneumonia, necrotizing fascitiis or streptococcal toxic shock syndrome (STSS), in addition to non-suppurative sequels, as rheumatic fever or glomerulonephritis. GAS is sensible to penicillins and other antibiotics, but during the last years the increasing appearance of multiple antibiotic resistance strains prompted the research towards the development of an effective vaccine.

One of the most important GAS virulence factors is Streptolysin O and the determination of the antibodies levels against this antigen is routinely used as a marker of recent GAS infections (ASO titre). Streptolysin O belongs to the Cholesterol Depending Cytolysins (CDCs) family, a group of Gram positive secreted toxins which induce cell lysis through pores formation. SLO exerts many additional toxic effects, for instance: i) promoting cytokines and other pro-inflammatory molecules release, ii) interfering with the complement pathway system activation, iii) translocating additional GAS virulence factors (i.e. NAD glycohydrolase) into eukaryotic cells, iv) causing platelets and polymorphonucleated lymphocytes (PMNL) aggregation *in vivo*. Additionally, SLO intravenous injection has been shown to be lethal in several animal models.

We have previously demonstrated that mice immunized with a recombinant form of Streptolysin O (rSLOwt) are protected against intranasal (i.n.) infection with GAS M1 3348 strain (provided by Istituto Superiore di Sanità, Roma). Yet, due to its toxic effects, the possible inclusion of SLO in a vaccine formulation is not recommended. Therefore, we generated genetic mutants showing a lower toxic profile, both *in vitro* and *in vivo,* when compared to the wild type rSLO. In particular, a double mutant derivative (rSLOdm) was chosen for more detailed studies, showing that both, cell binding to eukaryotic cells and oligomerization into the cell membranes, were highly impaired in the mutant with respect to the wild type counterpart. We also demonstrated that rSLOdm could still induce antibodies which neutralized rSLOwt toxicity *in vitro* and that mice immunized

with rSLOdm were protected against i.n. challenge with GAS M1 3348 strain and intravenous (i.v.) injection of the rSLOwt toxin.
On the basis of our results, the inclusion of this detoxified form of SLO in a human anti-GAS vaccine formulation may be considered in order to elicit an effective protective immune response against *Streptococcus pyogenes* infections.

Streptococcus pyogenes o Streptococco di Gruppo A (GAS) è un patogeno prettamente umano che colonizza la gola e la pelle causando patologie non invasive, come faringiti o impetigine, ma anche patologie più severe, come polmoniti, meningiti, fasciti necrotizzanti, e sequelae non suppurative, generalmente associate a fenomeni autoimmuni, come la febbre reumatica o il deposito a livello renale di immunocomplessi con conseguenti glomerulo-nefriti.
Uno dei fattori di virulenza più importanti e conosciuti di GAS è la streptolisina O (SLO) il cui titolo (titolo ASO) nell'uomo viene generalmente utilizzato come marker di recenti infezioni da *Streptococcus pyogenes*. Questa tossina fa parte della famiglia delle *Cholesterol Depending Cytolysins* (CDCs), un gruppo di proteine secrete da batteri Gram positivi che, legando il colesterolo sulle cellule eucariotiche, inducono lisi attraverso la formazione di pori. SLO presenta anche altri effetti tossici, tra cui le capacità di: i) favorire il rilascio di citochine, ii) attivare il complemento, iii) traslocare un altro fattore di virulenza secreto da GAS (la NADasi) dentro cellule target, iv) causare, *in vivo*, la formazione di aggregati piastrine/linfociti polimorfonucleati (PMNL), probabilmente alla base di una sindrome conosciuta come shock tossico streptococcico (STSS) dall'esito così fulmineo che causa ancora oggi la morte nel 30% dei soggetti colpiti. Inoltre è stato dimostrato che animali da laboratorio trattati endovena con pochi microgrammi della tossina muoiono in breve tempo dopo l'inoculo.
All'interno del progetto di ricerca Novartis per un vaccino contro GAS, è stato dimostrato che topi immunizzati con una forma ricombinante di SLO (rSLOwt) sopravvivono (90% di protezione) ad una successiva infezione per via intranasale con una dose letale del ceppo M1 3348 (appartenente alla collezione dell'Istituto Superiore di Sanità – ISS). A causa dei suoi effetti tossici, non è però possibile inserire la proteina nella sua versione *wild type* in un vaccino per utilizzo umano.

Per questo motivo, lo scopo di questo lavoro è stato quello di generare dei mutanti genetici con ridotto profilo tossico che mantenessero, però, le capacità protettive della proteina *wild type*. In particolare, abbiamo generato un doppio mutante (rSLOdm) che presenta una ridotta abilità, rispetto a rSLOwt, nell'indurre emolisi di cellule eucariotiche sia perché la sua capacità di legare le cellule è ridotta sia perché, una volta legato, non è in grado di formare oligomeri attivi. rSLOdm è inoltre meno tossico, *in vivo*, della proteina *wild type*, come mostrato in un modello murino di iniezione endovena. Abbiamo infine dimostrato, *in vitro* ed *in vivo*, che il doppio mutante induce anticorpi in grado di inibire l'attività emolitica di rSLOwt e che topi immunizzati con questa forma detossificata sono protetti sia contro un iniezione endovena di una dose letale di rSLOwt sia contro un'infezione intranasale con il ceppo M1 3348.

Sulla base dei nostri risultati è quindi possibile prendere in considerazione l'inclusione di questa forma detossificata di SLO in un vaccino per GAS ad uso umano per favorire la generazione di una risposta immunitaria protettiva.

2. Group A Streptococcus: general features

Streptococcus pyogenes is a Gram positive, capsulated, facultative anaerobic bacterium. It's a mostly extracellular and exclusive human pathogen. Actually, humans are the only natural bacterial reservoir, with about 10% of healthy people harbouring this bacterium in the respiratory tract without evident clinical manifestations (1). GAS belongs to the beta-haemolytic streptococci family and it was defined in 1933 by Rebecca Lancefield as Group A Streptococcus on the basis of its specific cell wall carbohydrate (2). A serological differentiation based on the most abundant surface exposed antigen, the M protein, is also extensively used in infection diagnosis and, over the years, the number of serotypes has progressively increased up to over 150 (1-3).

GAS colonizes the human throat and skin and GAS-associated infections do not generally cause severe complications. Pharyngitis, impetigo, cellulitis are the most frequent GAS dependent pathologies, but this pathogen is also responsible for more severe and life-threatening diseases, such as necrotizing fascitiis, myositis, pneumonia, meningitis, scarlet fever and a particular form of Toxic Shock Syndrome (TSS) which results in over 30% of death in affected people (4). Many non-suppurative sequelae are also ascribed to GAS. The most common are rheumatic fever, a cardiac autoimmune complication depending on the cross-reactivity between anti-M protein antibodies and human myosin (5), glomerulonephritis, an autoimmune sequelae probably caused by immune complexes deposition in the kidney (6-7), and some Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS), as Tourette's syndrome (8) or Sydenham's chorea (9).

GAS is still sensitive to penicillins and other antibiotics (cephalosporines, erythromycin), but an unexplained resurgence of infections mediated by this pathogen has been observed since the mid-1980s, first in United States (10) and then in some European countries, as United Kingdom and Sweden (3).

This fact, associated to a progressive increase of the number of strains less sensitive to penicillin treatment, the complications following local GAS infections and the economic problems related to cover with antibiotic treatments all the

developing countries, prompted the scientific community towards the development of an effective anti-GAS vaccine.

Most recent vaccine strategies have targeted either the hyper-variable N-term region or the highly conserved C-term region of the M proteins (3). Other GAS proteins such as C5a peptidase (11), pyrogenic exotoxin B (12), or specific glyco-conjugates (13) have been considered, but to date, no vaccine against group A streptococcus infections is commercially available.

2.1 Virulence factors

Virulence factors are defined as bacterial components which contribute either to the infection or to the maintenance of the disease condition once an infection has been established. Virulence factors have been generally grouped into two broad categories: i) those that remain predominantly associated to the bacterial cell; ii) those that are mostly secreted and released from the cell (1). Common virulence factors are adhesins, that allow micro-organism to bind to host cells, proteases, which degrade host proteins, for example of the immune system and toxins that attack host cells. Also those components which can protect the pathogen from the reaction of the host immune-system and antibiotics, such as the bacterial capsule, surface binding-proteins and β-lactamase are considered as virulence factors.

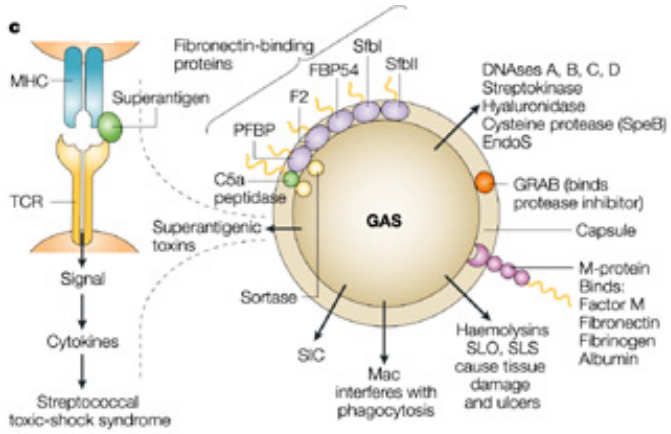

Figure 1: The main GAS virulence factors (14).

Among the most important cell associated GAS virulence factors, the M protein is certainly the most abundant and well characterized and, based on its amino-terminal highly variable protein region, over 150 different M types have been identified so far. This protein has adhesive (15), anti-opsonic (3) and pro-spreading (16-17) properties and, binding to different human proteins, as plasminogen (18), fibrinogen (19) and complement factor H (20), it allows bacteria to escape the immune system. Since the M-protein elicits protective antibodies, it has been considered the best vaccine candidate for a long time. However, since the protection conferred by this antigen is strictly M-type specific and the elicited antibodies have been shown to cross-react with some human tissues, its importance in the last years is progressively decreasing and attempts are being made to identify alternative protein vaccine candidates.

Other important virulence factors are C5a peptidase, a serine protease which degrades the human complement component C5a thus allowing GAS to elude the immune system (21), the hyaluronic-acid capsule, with anti-phagocytic and adhesive properties (22), many fibronectin, fibrinogen, plasminogen, immunoglobulins, etc… binding proteins and Serum Opacity Factor (SOF) that binds serum lipo-proteins (allowing cholesterol release into the serum) and fibrinogen (1).

GAS pathogenicity is also enhanced by its capability to produce and secrete a wide range of virulence factors into the external milieu (1). Some of them are degradative enzymes, like proteases and DNAses, or toxins, like streptolysins and superantigens (Spes) or proteins able to inhibit components of the human complement, therefore interfering with phagocytosis.

3. SLO and other CDCs members features

Among all secreted virulence factors, Streptolysin O is certainly one of the most structurally and functionally characterized. It belongs to a family of Gram positive bacteria secreted toxins, known as Cholesterol Depending Cytolysins (CDCs).
Members of this group (see table 1) are all structurally and functionally related (23), show 30-60% of protein similarity and contain an almost invariant undecapeptide sequence (ECTGLAWEWWR), which is considered the putative cell binding site.

ORGANISM	TOXIN	ABBREVIATION
Arcanobacterium pyogenes	Pyolysin	PLO
Bacillus cereus	Cereolysin	CLY
B. thuringiensis	Thuringiolysin O	
Brevibacillus laterosporus	Laterosporolysin	
Clostridium bifermentans	Bifermentolysin	
C. botulinum	Botulinolysin	
C. chauvoei	Chauveolysin	
C. histolyticum	Histolyticolysin	
C. novyi type A	Oedematolysin	
C. perfringens	Perfringolysin O	PFO
C. septicum	Septicolysin O	
C. tetani	Tetanolysin	
Listeria ivanovi	Ivanolysin O	ILO
L. monocytogenes	Listeriolysin O	LLO
L. seeligeri	Seeligerilysin O	LSO
Paenibacillus alvei	Alveolysin	ALY
Streptococcus canis	Streptolysin O	SLO
S. equisimilis	Streptolysin O	SLO
S. intermedius	Intermedilysin	ILY
S. pneumoniae	Pneumolysin	PLY or PLN
S. pyogenes	**Streptolysin O**	**SLO**
S. suis	Suilysin	SLY

Table 1: List of known members of Cholesterol Depending Cytolysins (23).

Only Perfringolysin O (24) and Intermedilysin (25) have been crystallized so far, but thanks to the high homology of all members of this family, the 3D structure obtained for PFO (figure 2) is generally used to graphically represent the predicted structure of all the other toxins included in the same group.

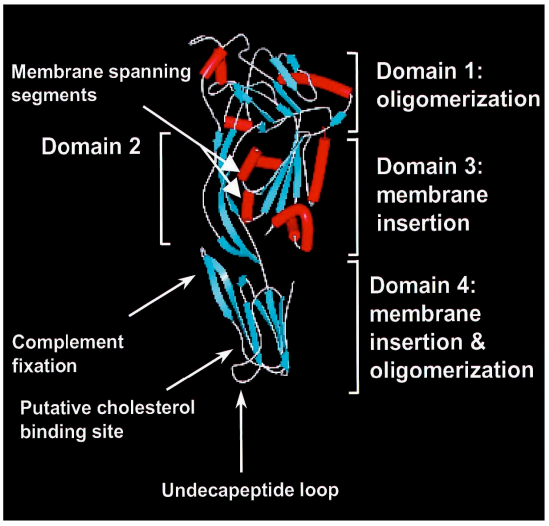

Figure 2: 3D structure of Perfringolysin O of *Cl. perfrigens* (24) generally used to represent all members of CDCs family. Proteins belonging to CDCs family are generally composed by four domains. In the picture, toxic activities directly linked to each single

These proteins are secreted as water soluble monomers and are able to induce eukaryotic cell lysis through a three-step process (26):

1. cholesterol **binding** on cell membranes
2. **self-oligomerization** in 40-80mers
3. **insertion** into the cell lipid bilayer, eventually leading to cell lysis due to pore formation

CDCs were originally described as haemolysins due to their activity on erythrocytes, but their main target may be cells which defend the host from infections, rather than erythrocytes them selves. In fact, even at sub-lytic doses (probably the real amount of toxin released at the infection site) many members of this family show cytotoxicity for human immune system cells. For example, PLY interferes with phagocytic and ciliary clearance of pneumococci (27), PFO and SLO cause vascular dysfunctions (28-29), PLO is cytotoxic for murine macrophages and bovine and ovine polymorphonuclear (PMN) leukocytes (30), and SLO for human PMNs (31). In addition to direct cytotoxicity, CDCs members are also able to interfere with the immune system in a more subtle way, preventing clearance of the pathogen and allowing the infection to proceed. It has been well described that cytokines are not only important to allow the immune system to recognize invading organisms, but they can also be used by pathogens

themselves to create a preferred niche during the infection process (32-33). Many CDCs, as PLY or SLO (23-34-35-36), induce *in vitro* the release of different types of cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-1β, IL-6, IL-8 and these molecules can represent an advantage for the bacterium pathogenicity. Furthermore, PLY and SLO can also activate the complement pathway system, the first one in a direct way (37), the second one in an indirect way (SLO oligomers act as foci for specific antibody dependent complement activation, 38). SLO is also able to translocate into eukaryotic cells another GAS secreted virulence factor, NAD-glycohydrolase (NADase) which, degrading intracellular NAD, deplete cell energy inducing apoptosis (39-40).

Many studies demonstrated that CDCs are important virulence factors for those pathogens which secrete them. For example, mutants deficient for PLY (37), LLO (38), PLO (30) and PFO (39) are either less virulent or completely avirulent in several animal models and, very interestingly, when a revertible *slo* mutant is used to infect mice, the reversion frequency of bacteria isolated from infected animals is more than 100 times higher than the inoculum value, indicating that there is an *in vivo* selective pressure for *slo*+ GAS bacteria (40).

The importance of members of the CDCs family in bacterial pathogenicity can be also deduced by the fact that some of them have been show to confer protection in actively and passively immunized animals. Mice immunized with genetically inactivated PLY toxoids (27-41) are protected against challenge with multiple capsular serotypes of *S. pneumoniae*, while passive immunization with either PLO (42-43) or PFO (44) specific antibodies are able to confer protection to mice against infection with *A. pyogenes* and *Cl. Perfrigens* respectively. Similarly, vaccination with purified SLY, the *S. suis* member of the CDC family, completely protects both, mice and pigs, against challenge with this pathogen (45).

Summarizing, all the available data support the fact that vaccination with CDCs can be suitable to obtain effective vaccines against many Gram positive pathogens, as *S. pneumoniae* or *S. pyogenes*, but genetic or chemical inactivation of this proteins would be required to neutralize their dangerous toxic effects.

4. Aim of the project and experimental approach

In the context of the Novartis vaccine project against group A streptococcus infections, it has been demonstrated that immunization with a recombinant form of wild type SLO (rSLOwt) confers nearly 100% protection to mice intranasally challenged with a lethal dose of GAS M1 3348 strain. However, the use of this protein as vaccine component is hampered by its multiple described toxic effects. In fact SLO is not only able to lyse eukaryotic cells, but also to cause them to release different cytokines (34-35), possibly favouring pathogen establishment in the host tissues. Additionally, SLO can indirectly activate the complement pathway system (38), promote platelets/PMNL aggregation (29) and NADase translocation into eukaryotic cells (39-46-40). SLO is also very toxic *in vivo* since tiny amounts of toxin injected intravenously kill different laboratory animals within few minutes after the treatment (47).

The aim of this study was to generate a genetically modified SLO derivative with a much lower toxic profile, while retaining its immunogenic and protective properties. To achieve this, we generated changes in the protein sequence of two domains either involved in SLO binding to eukaryotic cells or in the formation of oligomers on the eukaryotic cell membranes. The mutants obtained were then assayed both for their capacity to lyse eukaryotic cells and to be toxic *in vivo*, allowing the identification of defined mutations impairing these specific processes. Combination of these changes in a single protein derivative, allowed the obtainment of a highly detoxified SLO mutant, which was still protective in a mouse model of GAS infection, being thus suitable to be proposed as a component in a vaccine formulation for human use.

5. Material and Methods

5.1 Bacterial strains, plasmids and bacterial growth

GAS M1 3348 strain belonging to ISS (istituto superiore di Sanità, Roma) was used for the infection experiments and GAS M1 SF370 strain, purchased from ATCC, was used to obtained the genome by which PCR products were amplified. No differences between *slo* sequence of M1 3348 and M1 SF370 were detected after the sequencing of these genes in these two strains. The two GAS strains were grown in Todd–Hewitt broth (Difco Laboratories) at 37°C without shaking or grown on either trypticase–soy blood agar medium (TSA blood) or THYE agar medium supplemented with 5% (v/v) defibrinated sheep blood (THYE blood). *E. coli* BL21DE3 (Novagen) strain was used to express all the recombinant proteins. It was grown in Luria-Bertani broth (LB - Difco Laboratories) supplemented with ampicillin (Sigma) 100 mg/ml at 37°C, 180rpm shacking or on LB agar medium supplemented with ampicillin at 100 mg/ml. Plasmids used to express the recombinant proteins were pET21b(+) and pET15 for the his-tag mutants and pET24b(+) for the tagless derivatives (all purchased from Novagen).

5.2 DNA techniques

rSLO proteins were cloned using the genome of M1 SF370 strain as template. A stationary phase GAS growth was washed once in PBS and then suspended in TES (Tris 50mM, EDTA 5mM, NaCl 50mM, pH=8,00) buffer + lysozyme (Sigma) 10 mg/ml + mutanolysin (Sigma) 1 KU/ml and incubated in rotation for 1 hour at 37°C. Then, RNAse (Sigma) and proteinase K (Sigma) were added at a final concentration of, respectively, 10 mg/ml and 20 mg/ml and the incubation was allowed for 30 additional minutes. After the addition of Sarcosyl (1% final) EDTA (0,025M final) solution, the incubation was extended for other 30 minutes. Then, a three step extraction procedure was started adding, first SEVAG (1:1 v/v), then phenol-chloroform (1:1 v/v), and, finally, SEVAG (1:1 v/v), again; at each step, the upper watery phase was recovered and extracted again in the next step.

After the last extraction, DNA was precipitated adding Na Acetate (1:10 v/v) and pre-chilled (-80°C) ethanol (2:1 v/v), washed with ethanol 70% in water and then suspended in Tris buffer (pH=8.00) to be quantified spectrophotometrically. Oligonucleotides used to clone the recombinant proteins are listed below.

Oligonucleotides a) and b) were used, as external primers, to clone all the recombinant proteins. As internal primers for the SOE-PCR (Splicing by Overlapping Extension – PCR), oligos c) and d) were used for rSLOP136Ghis mutant, e) and f) for the mutant rSLOP175Ghis, g) and h) for the mutant rSLOP190Ghis, j) and k) for the mutant rSLOP427G, l) and m) for the mutant rSLOP427Lhis and rSLOP427L tagless and, finally, n) and o) for the mutant rSLOW535Fhis. Oligonucleotides a), b), n), o), were used to clone the double mutant derivative rSLOP427L-W535F tagless using the PCR product of rSLOP427L tagless derivative as template.

List of Oligonucleotides used for the cloning of recombinant SLO derivatives

a)WTforGTGCGTGCTAGCGAATCGAACAAACAAAACACTGC
b)WTrevGCATTCGATCCTCGAGCTTATAAGTAATCGAACCATATGGG
c)P136GforATGGTGAAACCATTGAAAATTTTGTTGGTAAAGAAGGCGTTAAGAAAGCTGA
d)P136GrevTCAGCTTTCTTAACGCCTTCTTTACCAACAAAATTTTCAATGGTTTCACCAT
e)P175GforCTCTGTCACTGATAGGACCTATGGAGCAGCCCTTCAGCTGGCTA
f)P175GrevTAGCCAGCTGAAGGGCTGCTCCATAGGTCCTATCAGTGACAGAG
g)P190GforCTAATAAAGGTTTTACCGAAAACAAAGGAGACGCGGTAGTCACCAAGCG
h)P190GrevCGCTTGGTGACTACCGCGTCTCCTTTGTTTTCGGTAAAACCTTTATTAG
j)P427GforATGCTACCTTCAGTAGAAAAAACGGAGCTTATCCTATTTCATACACCA
k)P427GrevTGGTGTATGAAATAGGATAAGCTCCGTTTTTTCTACTGAAGGTAGCAT
l)P427LforATGCTACCTTCAGTAGAAAAAACCCTGCTTATCCTATTTCATACACCA
m)P427LrevTACGATGGAAGTCATCTTTTTTGAGGCGAATAGGATAAAGTATGTGGT
n)W535Ffor=GAGTGCACTGGCTTAGCTTTCGAATGGTGGCGAAAAGTGATC
o)W535Frev=GATCACTTTTCGCCACCATTCGAAAGCTAAGCCAGTGCACTC

Plasmid DNA was isolated from *E. coli* with Qiagen plasmid mini- or maxi-prep kits (Qiagen) according to the manufacturer's protocol. Restriction enzymes and DNA ligase were purchased from BioLabs laboratories and were used according to the manufacturer's protocol. Agarose gel electrophoresis (1% w/v) were performed according to standard techniques (Maniatis).

5.3 Protein expression and purification

rSLO derivatives were expressed in BL21DE3 *E. coli* cells. Bacteria were grown up to OD_{600nm}=0,4-0,6 at 30°C, 180rpm shacking and induced with IPTG 1mM at 25°C for 3 hours, shacking at 180rpm. Cells were then recovered by centrifugation and frozen at -20°C until the purification. *E. coli* pellets were suspended in lysis buffer (10 ml B-PER™ -Pierce-, $MgCl_{2\ at}$ a final concentration of 0.1 mM, DNAsi I - Sigma-, 100 units and lysozyme -Sigma-, at a final concentration of 1 mg/ml) and mixed for 30-40 minutes at room temperature. For his-tag derivatives samples, lysates were centrifuged at 30-40000 x *g* for 20-25 minutes and supernatants were loaded onto wash buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8,0) equilibrated columns (Poly-Prep with 1 ml of Ni-Activated Chelating Sepharose Fast Flow resin). The loaded resin was washed three times with wash buffer A and three times with wash buffer B (70 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Proteins were eluted with elution buffer (250 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) in Eppendorf tubes containing 1mM final of DTT. Total elution proteins are quantified with Bradford reagent and then analyzed by SDS-polyacrylamide 12% Criterion pre-cast gels (Bio-rad).

For tagless proteins, about 80-110 g of bacterial culture pellet were suspended in 200-280 ml B-PER™ reagent (Pierce) supplemented with 6 tablets of COMPLETE® protease inhibitor (Roche), EDTA pH 7.5 at the final concentration of 5 mM, lysozyme (Sigma) 3 mg/ml, DNAse I (Sigma) about 3-500 units and $MgCl_2$ at a final concentration of 0,2 mg/ml. Bacterial lysis was achieved by shaking the bacterial suspension for 60 minutes until a homogeneous suspension was obtained. Following centrifugation for 60 minutes at 25400 x *g*, the supernatant was filtered using a 0.22 µm filter and is diluted with H_2O until a 1.8-1.9 mS conductivity was obtained. The pH was adjusted to 8.0. Protein concentration was determined by the Bradford method. The supernatant was then loaded on an HP 50/10 Q Sepharose column (~200 ml), previously equilibrated with 30 mM TRIS, pH 8.0. The flow-through was collected. Fractions containing the SLO proteins were pooled and dialyzed against 10 mM Na phosphate, pH 6.8. Protein concentration was determined by the Bradford method. Following buffers and experimental conditions were used to elute protein fractions: Buffer A) 30mM

TRIS, pH 8.0, Buffer B) 30mM TRIS, 1M NaCl, pH 8.0, Equilibrium and Loading) 0% B, Gradient) 0-25% B in 5 CV – 25% B 2 CV, Wash) 100%B 2 CV + 3 CV, Flux) 20 ml/min, Fraction volume) 14 ml. A Hydroxyl apatite chromatography followed this last step and the previously obtained pool was loaded on a CHT20 column equilibrated with 10mM Na-phosphate, pH 6.8. The flow through was collected. Following buffers and experimental conditions were used to elute protein fractions: Buffer A) 10mM Na-phosphate, pH 6.8, Buffer B) 500mM Na phosphate, pH 6.8, Wash) 8 CV and 30%B 6 CV, Gradient) 30-100%B (10 CV), Wash) 100%B, Flux) 5 ml/min, Fraction volume) 5 ml. Fraction aliquots were loaded on 12% Criterion pre-cast gels (Bio-rad) under reducing and non-reducing conditions. Fractions containing SLO proteins were pooled and protein concentration was determined by Bradford method. A gel filtration chromatography was the last step in the purification protocol. Collected pool was concentrated using an Amicon filter in order to get a volume < 10 ml. The concentrated material was loaded on a HiLoad Superdex 200 26/60 equilibrated with at least 3-4 column volumes of PBS. Following buffers and experimental conditions were used to elute protein fractions: Buffer) PBS, Elution) Isocratic, Flux) 2.5 ml/min, Fraction volume) 5 ml. Fractions containing SLO proteins were pooled and protein concentration was determined by Bradford. An additional estimation of protein concentration was performed by UV measurement considering Abs 0.1% (=1 g/l) 1.119. Protein purity is analyzed by polyacrylamide gel electrophoresis. No contaminants were found loading up to 10 µg of each sample.

5.4 Haemolytic activity titration and haemolysis inhibition assay

Haemolysis test was used as first screening to asses rSLO derivatives toxicity. Serial dilutions of purified proteins or IPTG induced *E. coli* crude surnatants were prepared in 96-well plates with U-shaped bottoms using PBS + 0.5% BSA or LB + ampicillin 100 mg/ml respectively as dilution buffer. One ml of sheep blood was washed three times in PBS (with centrifugation at 3000 x g), and blood cells were finally suspended in 5 ml of PBS. A volume of 50 µl of this suspension was added to 50 µl or 150 µl of each diluted samples and incubated at 37°C for 30

min. A high rSLOwt dose was used to give 100% haemolysis, and PBS + BSA 0.5% or the *E. coli* IPTG induced crude lysate of strain expressing a non haemolytic protein were used as negative controls. Plates were then centrifuged for 5 min at 1,000 x g, and the supernatant was carefully transferred to 96-well flat-bottomed plates to read the absorbance at 540 nm. Haemolytic activities of purified proteins or *E. coli* lysates were respectively defined as the protein dose (in ng/well) or dilution factor which gave 50% of the maximum haemolysis obtained with the positive control. Analysis was performed using excel software and the function: y=a*ln(x) + b applied only to the linear portion of the curve (no less than 3-4 points for curve). R^2 of the analyzed curves were always higher than 0,98. *E. coli* lysates were also loaded into a polyacrylamide gel (Bio-rad) to compare protein expression following the manufacturer's protocol.

Haemolysis inhibition assay was used to asses the ability of anti rSLO (wild type or double mutant) antibodies to inhibit rSLOwt *in vitro* induced haemolysis. Serial two fold sera dilutions in PBS + BSA 0,5% were incubated with 10 ng (3X 100% haemolysis dose) of rSLOwt for 20 min. at RT. Then, the sheep blood cells suspension was added and the incubation was allowed for additional 30 min. at 37°C. 100% of the haemolysis was reached incubating cells with 10 ng of the toxin with out any serum; as negative control, sera of mice immunized with alum adjuvant alone were used. Plates were then centrifuged for 5 min at 1,000 x g, and the supernatant was carefully transferred to 96-well flat-bottomed plates. The absorbance was read at 540 nm. Haemolysis inhibition titers were defined as the lowest sera dilution factor which completely inhibits rSLOwt induced haemolysis. For the negative control sera, often, the complete inhibition was not reached even at the lowest dilution tested (1:125). In these cases, titres were arbitrarily fixed at 50. Analysis was performed using GraphPad prism5 software and the function: Y= IF(X<X0, Y0,Y0 + (Plateau-Y0)*(1 - exp(-K*(X-X0)))), where X = protein dose in ng and Y = % of haemolysis. R^2 of the analyzed curves were always higher than 0,98. Statistical analysis were performed using Mann-Whitney U-test and the Graph Pad prism5 software. P-values lower than 0,05 were considered statistically significant.

5.5 Circular Dichroism analysis

rSLOwt, rSLOsm and rSLOdm tagless proteins were analyzed in CD to evaluate the secondary protein structure. Proteins were diluted in PBS to a final concentration of 0,2 mg/ml and then analyzed in the far UV spectral region from 195nm to 260nm. Wavelengths lower than 195nm cannot be considered in this analysis due to the interference of PBS buffer in that range. The analysis were performed using the CDNN2.1 algorithm and were expressed as average of 10 scans.

5.6 Binding assay

Human epithelial A-549 cell line was used to evaluate the ability of rSLOwt and the mutant derivatives to bind human cells. A-549 cells were grown in complete DMEM medium (DMEM -Gibco- supplemented with HEPES 250mM, antibiotics (ampicillin and streptomycin 200 U/ml each), scomplemented FBS 10% (v/v)). Confluence cells were washed two times in PBS (Gibco) and then detached with trypsin 0,25% (w/v) + EDTA 1mM (Gibco); incubation was stopped adding complete DMEM. Cells were washed once in PBS and then a suspension of 2.000.000 cells/ml were incubated for 20 min. in agitation at RT in PBS + live and dead reagent (1/1000 v/v according to manufacturer's procedures - Invitrogen) to discriminate dead cells. After two washes in PBS, cells were treated with formaldehyde 2% (v/v) for 20 min. at RT to fix them and then were washed once with PBS and once with pre-chilled (4°C) PBS + BSA 1%. Serial two-fold protein dilutions in PBS + BSA 1% were then added to the formaldehyde fixed cells (200.000 cells/well in a 96 wells plate with U-shaped bottoms) and the incubation was allowed for 45 min. at 4°C. Cells were washed two times in PBS + BSA 1% and then suspended in the same buffer containing mouse polyclonal antibodies (1/1000 v/v) against rSLOdm (which had recognized all the proteins at the same level in ELISA assay). Then cells were washed two times with the same buffer and suspended in a PBS + BSA 1% solution with an anti-mouse phycoerythrin-conjugated secondary antibody (1:100 v/v Jackson immunoresearch laboratories); incubation was prolonged for 30 min. at 4°C. Cells

were finally washed in PBS + BSA 1%, then in PBS and suspended in PBS + EDTA 5mM. Bound proteins were detected in FACS analysis and data were analyzed with FlowJo software. Cells treated only with primary and secondary antibodies and cells treated with proteins, a negative control serum (anti alum alone) and secondary antibodies were both used as blank, giving very similar results. Final delta mean fluorescence intensities of each sample were obtained subtracting a mean of the blank values to Mean Fluorescence intensity (MFI) of each sample.

5.7 Oligomerization assay (SDS-AGE analysis)

SDS-AGE (SDS - agarose gel electrophoresis) analysis was used to evaluate the ability of each single SLO derivative to self-oligomerize into cell membranes. A 1% agarose gel in TGS buffer (Tris 25mM, glicina 192mM, SDS 0,1% w/v, pH=8,3 – Bio-rad) was prepared. A-549 cells were detached as described in the protocol of the binding assay (see above). rSLOwt, rSLOsm and rSLOdm were diluted in PBS and incubated with the cells (100.000 cells/sample) for 30 min. at 37°C. Cells alone were used as negative control, while soluble proteins were used to determine the molecular weight of the monomer after the running into the gel. After this incubation, 200 µl of water were added to each sample to allow the lysis, then washed once in water again and, finally, suspended in protein 4X loading buffer (Tris HCl pH=6,8 200mM, SDS 8% bromophenol blue 0,4% v/v, 40% glycerol in water). Lysed suspended samples were boiled 10' and then loaded into the solidify agarose gel. The run was allowed for about 2 hours at 200V and then the gel was extensively washed in water before transferring the separated proteins onto a nitrocellulose membrane as described elsewhere (55). Samples were transferred in transfer buffer (25mM Tris, 192mM Glycine, 20% v/v ethanol in water pH=8.3) O.N. at RT. Nitrocellulose membrane were then washed three times in water, saturated with TPBS 0,1% (Tween PBS 0,1% v/v) + milk 5% w/v for 1 hour at RT, incubated with a serum anti rSLOdm (1/5000 v/v) in TPBS + milk 5%, 3 hours at RT, washed three times in TPBS 0,1%, incubated with an anti-mouse horseradish peroxidase linked antibody (Amersham - 1/3000

v/v, 1 hour, RT) and finally developed 5 min. at RT with SuperSignal West pico Chemoluminescent reagent (Thermo) according to the manufacture's protocol.

5.8 Mice immunization

Mice were immunized using either rSLOwt or rSLOdm proteins to be, either, intranasally infected with GAS M1 3348 strain or intravenously injected with rSLOwt. Groups of 4-16 mice were immunized intraperitoneally with 20 μg of the recombinant protein at days 0, 21 and 35 using alum 2 mg/ml as adjuvant. Mice sera were collected before the first immunization (pre-immune sera) and 2 weeks after the third immunization (immune sera). Mice of negative control groups were immunized with alum alone.

5.9 GAS intranasal infection model

A few days after the immune bleeding, immunized mice were challenged intranasally with 10^8 cfu (50 μl) of the M1 3348 GAS strains. Mice survival was monitored for a 14 days period. Mice were monitored on a daily basis and euthanized when they exhibited defined humane endpoints that had been pre-established for the study in agreement with Novartis Animal Welfare Policies. All experiments Statistical analysis was performed using the Fisher exact test (GraphPad Prism5 software). P-value lower than 0,05 were considered statistically significant.

5.10 Intravenous injection model

Either wild-type or double mutant SLO were diluted in a solution of PBS + 2 mM DTT, then 100 μl were injected into either, naïve or immunized mice tail vein. Mice survival was observed for at least one week after the inoculum. Injection of rSLOwt typically resulted in death within a few minutes. Mice were monitored on a daily basis and euthanized when they exhibited defined humane endpoints that

had been pre-established for the study in agreement with Novartis Animal Welfare Policies. All experiments Statistical analysis was performed using the Fisher exact test (GraphPad Prism5 software). P-value lower than 0,05 were considered statistically significant.

5.11 In silico analysis

B-factors were downloaded from the Web site Protein Brookhaven data Bank (www.pdb.org) and images were elaborated using the software Swiss PDB Viewer (www.expasy.org).

6. Results

6.1 Mutagenesis of SLO 1st and 4th domains: generation of new protein mutants with impaired haemolytic properties

Several studies demonstrated that many SLO side effects are strongly related to its capability to bind cells and to organize in oligomers which insert into the cell membrane forming active pores (47-48). SLO first domain and N-term region consist of about one half of the whole protein, but 16 out of 21 prolines present in SLO are located here. These aminoacids confer rigidity to the protein, probably giving to the pre-pore complexes the necessary energy to insert into cell membranes. For this reason, the mutagenesis of specific prolines into glycine, the most flexible aminoacid, might inhibit this transition and the following haemolytic properties of the toxin. β-factor (atomic displacement parameter) is an *in silico* parameter that is related to the rigidity conferred by each aminoacid in the native protein structure (49); a high β-factor means low mobility of individuals atoms and side chains. In particular four SLO prolines (P136, P175, P190 and P427) were chosen for their very high (P136 and P427) or very low (P175 and P190) β-factors, and they were mutagenized using SOE-PCR (Splicing by Overlapping Extension – PCR) into glycine.

Moreover, as hypothesized in many models of pore formation (50-24-26-51), P427 is located between 1st and 3rd domain, in a region probably important in monomer-monomer interaction during oligomers formation. In these models, P427 is in the interface between two monomers and mutations in this residue could not only prevent membrane insertion, but also reduce the ability of SLO to form active oligomers. Therefore, mutant P427 into Leucine (P427L) was also generated and i*n silico* analysis of the SLO P427L mutant reveals that the long hydrophobic chain of the mutagenized aminoacid may stretch toward other monomers, creating an hindrance for the formation of an active oligomer.

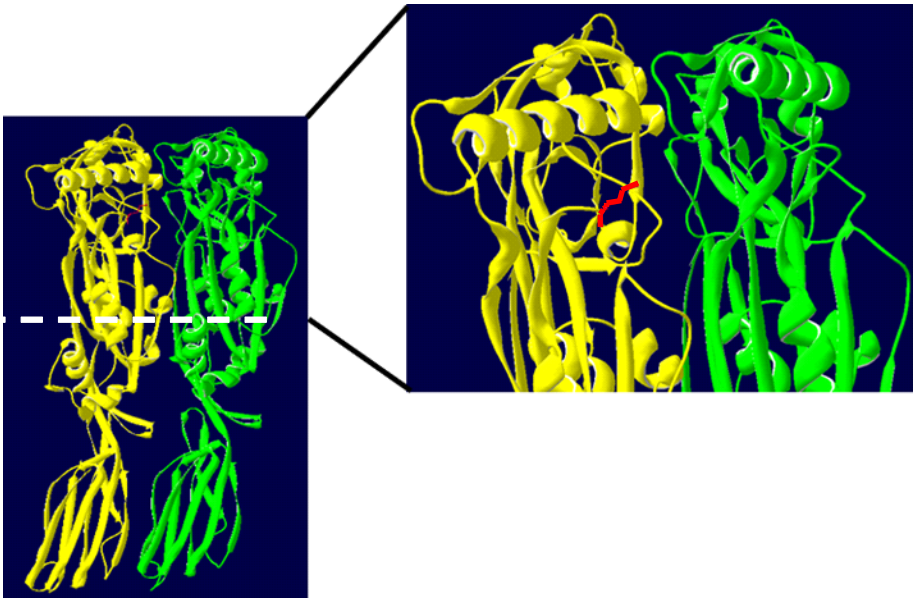

Fig.3: SLO monomer-monomer interaction into eukaryotic cell membranes. After bound, two monomers are supposed interact as shown in the picture on the left; on the right a detail. Leucine 427 (in red) turns its long hydrophobic chain toward another monomer and that may generate an hindrance which interferes with the formation of active oligomers.

So, five prolines mutants were generated as his-tagged proteins and were screened for their ability to induce haemolysis *in vitro* using a crude *E. coli* lysate haemolysis test. After induction with IPTG, *E. coli* strains expressing either, recombinant wild type SLO (rSLOwt), a rSLO proline derivative or a not-correlate protein (neurofilament protein isdB cloned by *Staphylococcus aureus* NCTC8325 strain) as negative control, were lysed and crude lysates were assayed in haemolysis test. The results we obtained (see figure 4 and table 2) were not completely in agreement with the *in silico* predictions, probably due to the high complexity of the transition of a such huge structure (pre-pore complexes) into an active haemolytic form. In particular, mutant P427L was the one with the highest reduction in haemolytic properties among all derivatives, showing a profile nearly 1000 times less toxic than the wild type protein. Also mutants P190G and P175G showed a reduction in haemolytic activity in respect to the wild type counterpart, but less significative than that obtained with P427L. Very interesting, derivative P427G did not present any reduction in haemolytic properties, suggesting that the insertion of a hydrophobic chain in that position rather than the reduction in rigidity itself is responsible for the loss of toxicity.

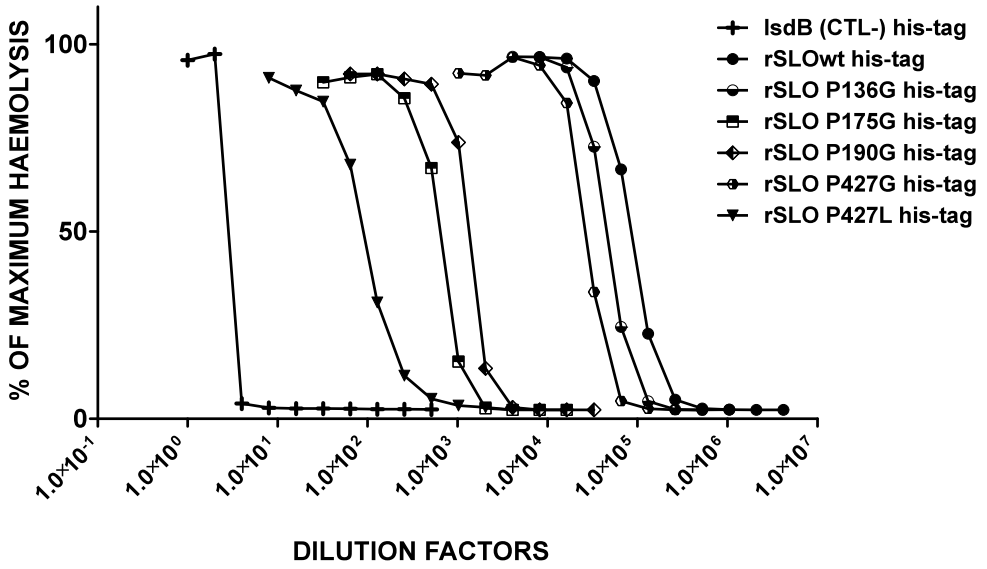

Fig.4: Haemolytic curves of rSLOwt his-tag and rSLO proline his-tag mutants using a crude *E. coli* lysate in the haemolysis test. A crude lysate of a non-haemolytic protein (IsdB of staphylococcus aureus) was used as negative control. The wild type form, as expected, showed the highest haemolytic activity among all proteins tested. Mutants P136G and P175G did not show a significative reduction in haemolytic properties compared to rSLOwt, while a good reduction was observed for the other 2 proline mutants and in particular for rSLO P427L his-tag.

SAMPLE	TITRE (50% max haemolysis)	RATIO WT/SAMPLE
rSLOwt his-tag	84831	1
rSLO P136G his-tag	45278	1,9
rSLO P175G his-tag	611	139
rSLO P190G his-tag	1297	65
rSLO P427G his-tag	25438	3,3
rSLO P427L his-tag	88	968
IsdB his-tag (CTL-)	3	28277

Table.2: Haemolysis titres of rSLO his-tag derivatives in a crude *E. coli* lysate. Titres were measured as 50% of the maximum haemolysis (100% in the graph) and expressed as dilution factor of the tested lysates. The ratios between rSLOwt his-tag and each single mutant haemolytic titres were also reported.

SDS-page analysis of *E. coli* lysates were also performed, showing that all mutant proteins were induced in comparable levels (figure 5).

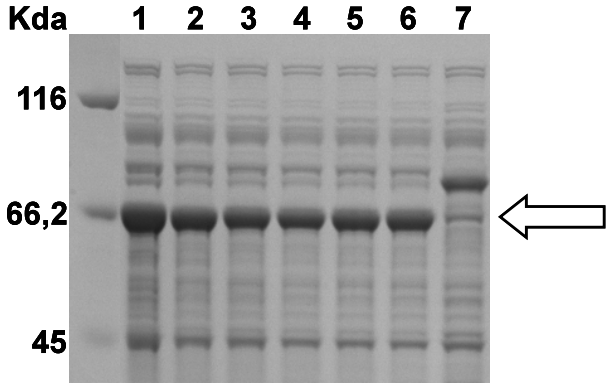

Fig.5: SDS-PAGE analysis of crude *E. coli* lysates. The arrow shows rSLO molecular weight. 1 μl of crude lysates of each sample were loaded into a SDS gel in the following order: 1) rSLOwt his-tag, 2) rSLO P136G his-tag, 3) rSLO P175G his-tag, 4) rSLO P190G his-tag, 5) rSLO P427G his-tag, 6) rSLO P427L his-tag, 7) IsdB. No significative differences in protein expression among the samples were found.

As previously described for Pneumolysin (PLY), mutations in the cholesterol-binding site (4^{th} domain) can also affect the ability of CDCs to lyse eukaryotic cells. In particular, PdB (52-41) is a less toxic derivative of PLY with a mutation into phenylalanine (F) of the first tryptophan (W) in the undecapeptide responsible for cholesterol binding. This mutant is 100-150 times less haemolytic than the wild type counterpart and this reduction is due to an impaired ability in cell binding (23-52). On these basis, a SLO mutant W535F was also generated and, rSLOwt, rSLO P427L and rSLO W535F his-tagged purified proteins were tested for their ability to induce haemolysis. After purification, we obtained similar amount of each protein with very similar levels of purity (data not shown). As expected, both mutations resulted in derivatives with a less toxic profile, and in particular, rSLO P427L his-tag and rSLO W535F his-tag were respectively about 500 times and 200 times less haemolytic than rSLOwt his-tag (figure 6 and table 3).

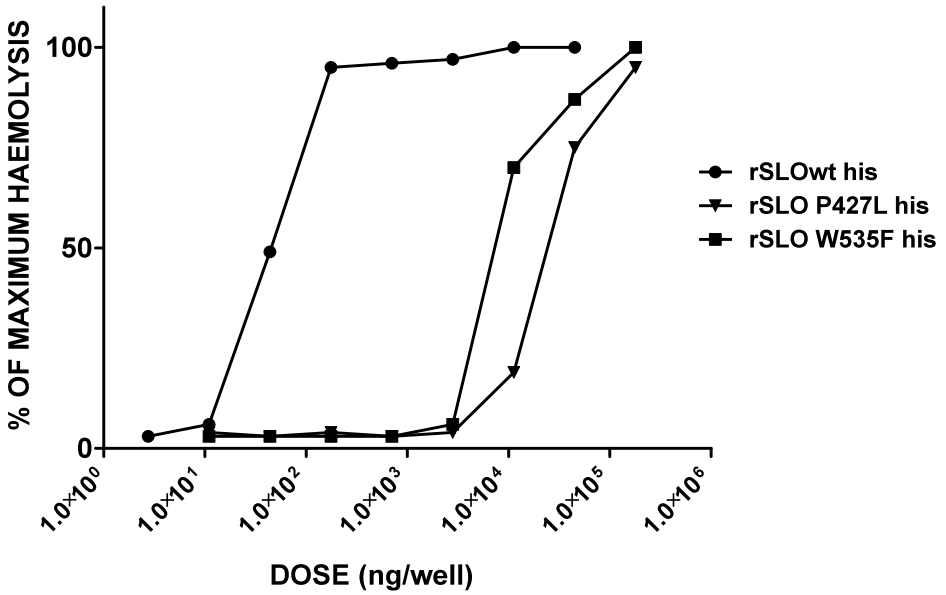

Fig.6: Haemolytic curves of rSLOwt, rSLO P427L and rSLO W535F his-tagged purified proteins. Both mutants showed a reduction in haemolytic properties compared to the activity measured for the wild type form.

SAMPLE	TITRE (50% max haemolysis)	RATIO WT/SAMPLE
rSLOwt his-tag	44,1	1
rSLO P427L his-tag	9730	549
rSLO W535F his-tag	24227	221

Table.3: Haemolysis titres of rSLO his-tagged purified proteins. Titres were measured as 50% of the maximum haemolysis (100% in the graph) and expressed as protein dose, in nanograms, for well. The ratios between rSLOwt his-tag and each single mutant haemolytic titres were also reported in the table.

6.2 SLO genetic detoxification: generation and characterization of a SLO double mutant derivative with reduced toxicity but still able to induce protective immune response

6.2.1 Haemolytic properties of rSLOwt, rSLO P427L and rSLO P427L-W535F tagless proteins

Following the demonstration that single mutations either in the 1st or 4th SLO domains were able to reduce SLO toxicity to a certain extent, we decided to generate a SLO derivative which included both mutations in order to obtain a

highly detoxified genetic mutant. Therefore a double mutant derivative rSLO P427L-W535F (rSLOdm) was obtained as tagless recombinant protein for further and detailed investigations. Similar tagless versions were also generated for the previously described rSLOwt and rSLO P427L (rSLOsm). The three recombinant proteins were tested in haemolysis assay to confirm the data already obtained with the rSLOwt and the rSLOsm his-tagged derivatives and to evaluate the behaviour of the newly generated rSLOdm. As shown in Figure 7 below, tagless rSLOsm is about 2400 times less haemolytic than the wild type counterpart, while we were not able to measure an haemolytic titre for the double mutant derivative due to its very low toxicity. We could however estimate that the haemolytic titre of this mutant would be at least 250.000 times lower than that we obtained for the wild type version (table 4).

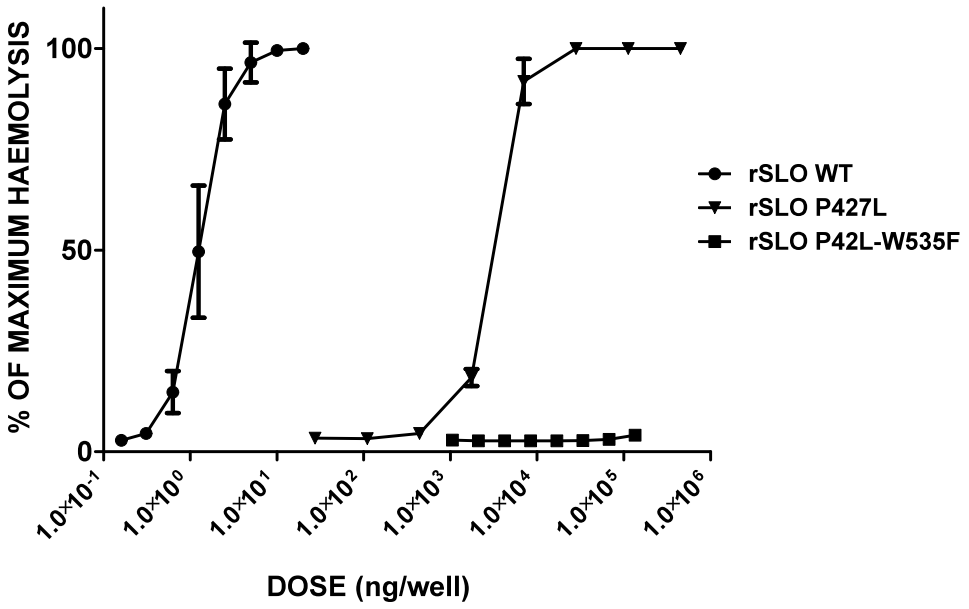

Fig.7: Haemolytic curves of rSLOwt, rSLOsm and rSLOdm tagless proteins. In the graph, the mean and standard deviation of three independent experiments were reported. Single mutant derivative showed a remarkable reduction in haemolytic properties (more than 2000 times) compared to the wild type counterpart. Haemolytic titre of the double mutant derivative was not measurable, but we estimated it would have been at least 250.000 times lower than that obtained for rSLOwt.

SAMPLE	TITRE (50% max haemolysis)	RATIO WT/SAMPLE
rSLOwt	1,25	1
rSLOsm	3049	2439
rSLOdm	356481 (Hyp. Value)	285185

Table.4: Haemolysis titres of rSLO tagless proteins. Titres were measured as 50% of the maximum haemolysis and expressed as protein dose, in nanograms, for well. The ratios between rSLOwt and each single mutant haemolytic titres were also reported in the table.

On the basis of the results we obtained using his-tagged and tagless derivatives, we could conclude that in the double mutant the single mutations synergistically work to highly reduce the haemolysis process.

6.2.2 Secondary structure analysis of rSLO derivatives

The reduction in the toxic activity of the double mutant derivative could be related to the disruption of the normal protein structure rather than to the mutations itself. For this reason, we performed Circular Dichroism (CD) analysis to characterize the secondary structure of the wild-type protein and to compare it to those observed with the genetically mutant derivatives (53). The two SLO derivatives were analyzed in CD and the relative spectra were compared to that of the wild type protein (figure 8). No significative differences were found in the range of analysis between 195-260 nm (far UV), suggesting that the two mutations do not dramatically change the secondary structure of the protein and that the mutations themselves are directly responsible for the reduction in haemolytic properties.

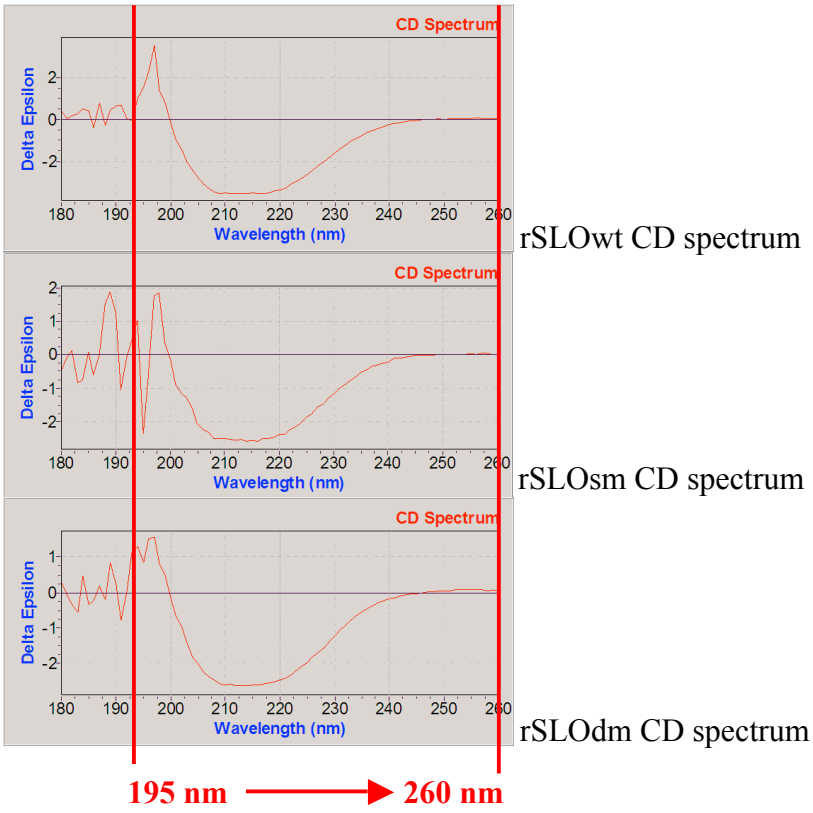

Fig.8: 180-260 nm CD spectra of rSLOwt, rSLOsm and rSLOdm. No significative differences among CD spectra in the far UV from 195nm to 260nm of the three proteins were found, suggesting that protein secondary structures are very similar. Spectra cannot be analyzed in the range between 180-195nm due to the interference with PBS buffer. On the basis of this analysis, we can conclude that the reduction in haemolytic activity we measured were probably linked to the mutations them selves rather than to a change in protein secondary structures.

6.2.3 Functional characterization of rSLO derivatives

As previously described, the formation of an active pore is a process which requires three consecutive steps: binding, oligomerization and insertion into the cell membrane. PLY W433F mutant is impaired in its ability to induce cell lysis due to a reduction in the capability to bind cells and so we hypothesized that the related SLO mutant W535F exhibits a similar impairment of its haemolytic capacities. To verify this hypothesis and to test whether also mutation P427L affected the ability of the toxin to bind eukaryotic cells, we tested the binding properties of the three tagless SLO derivatives to the A549 lung epithelial cell line.

rSLOwt is a very potent toxin and rapidly induces cell death even at very low doses (less than one hundred nanograms). Therefore, testing of SLO binding was carried out on formaldehyde-fixed cells: rSLO bound proteins were detected by FACS analysis using a rSLOwt anti-serum followed by a phycoerythrin-conjugated secondary antibody. This experimental strategy was possible since SLO recognizes cholesterol on the cell membrane (23) and not any other protein structure which could have been damaged by the formaldehyde. The anti rSLOwt serum we used in this experiment was tested in ELISA against all the three coated derivatives giving very similar results and suggesting that it recognizes the two mutants as well as the wild type protein (data not shown). rSLOwt appeared to be a strong cell binder and indeed we were able to detect binding using few nanograms of the protein. The single mutant P427L appeared to bind to the epithelial cells comparably to the wild type counterpart, while the double mutant showed a much lower binding profile (300-500 times less than rSLOwt), but not so significatively lower to account for the observed huge impairment in haemolytic activity (figure 9). Similar results were also obtained using another human cell line (HBMEC, human brain microvascular endothelial cells – data not shown)

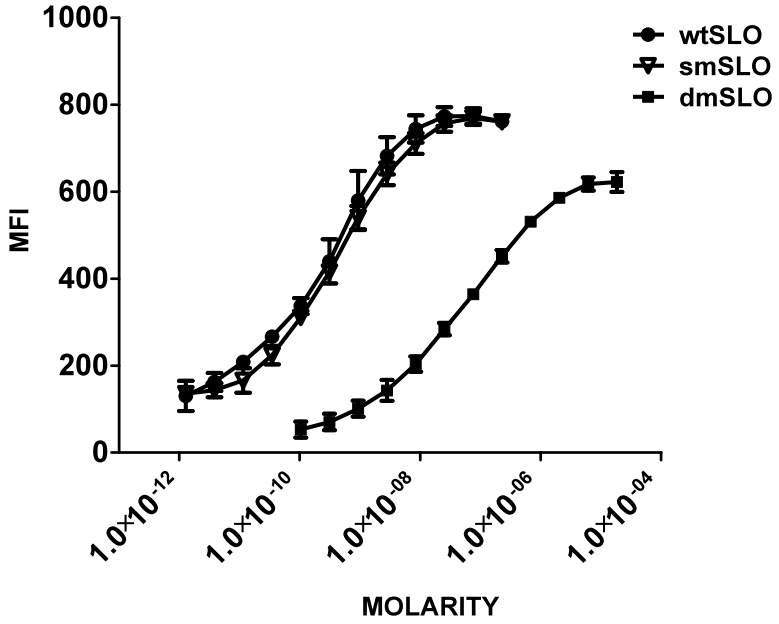

Fig.9: Binding curves of rSLOwt, rSLOsm and rSLOdm tagless proteins on human epithelial cell line (A-549 cells). FACS analysis of stained cells was used to detect rSLOwt and mutants derivatives binding on A-549 cell line. The graph shows Mean Fluorescence Intensity (MFI) versus molarity (rSLOwt MW= 60,8 Kda); the mean and standard deviation of three independent

experiments were reported. rSLOsm bound cells as well as the wild type counterpart, while rSLOdm 300-500 times less. This suggested that the mutation in the 4th domain affected SLO cell binding, while the mutation in the 1st domain did not reduce this SLO ability.

We therefore concluded that, while the mutation in the cholesterol binding site reduces the ability of the protein to bind cells, confirming the data previously obtained with PLY, the additional mutation in proline 427 probably impacts a different step towards the complete pore formation on the eukaryotic cell membrane.

To better understand whether mutation P427L affected the ability of the bound protein to oligomerize into active pre-pore complexes (the second step during pore-formation), we performed a western blot associated to a SDS-Agarose Gel Electrophoresis (SDS-AGE) analysis. SDS-AGE is a technique that allows the separation of protein complexes with a molecular mass up to mega Daltons (Mda) and which was already used to detect the formation of similar pre-pore complexes into cell membranes for SLO as well as for other members of the CDCs family. (54-52). For this analysis, rSLOwt, rSLOsm and rSLOdm were incubated for 30 minutes at 37°C with A-549 cells, cell lysis was then induced by the addition of water to the samples and the resulting membrane bound proteins and free soluble proteins were allowed to migrate in a SDS-agarose gel, using not treated A-549-derived membranes as negative control. On the basis of the binding results, about 0.33 pmol of the wild type and of the single mutant toxin and an equivalent of three hundred more rSLOdm molecules were separated on the gel, which was then transferred to a nitrocellulose membrane to perform a western blot analysis using an anti-rSLOdm antibody. The result obtained, shown below in figure 10, revealed that only rSLOwt was able to oligomerize on the eukaryotic cell membranes forming structures with high molecular weight (called oligomer, in the following image), while rSLOsm and rSLOdm were not able to form similar stable complexes. Similar results were also obtained using sheep blood cells (data not shown). This strongly suggested that the mutation in the proline 427 affected the ability of the toxin derivative to form stable oligomers following binding of the monomers to the eukaryotic cell membrane.

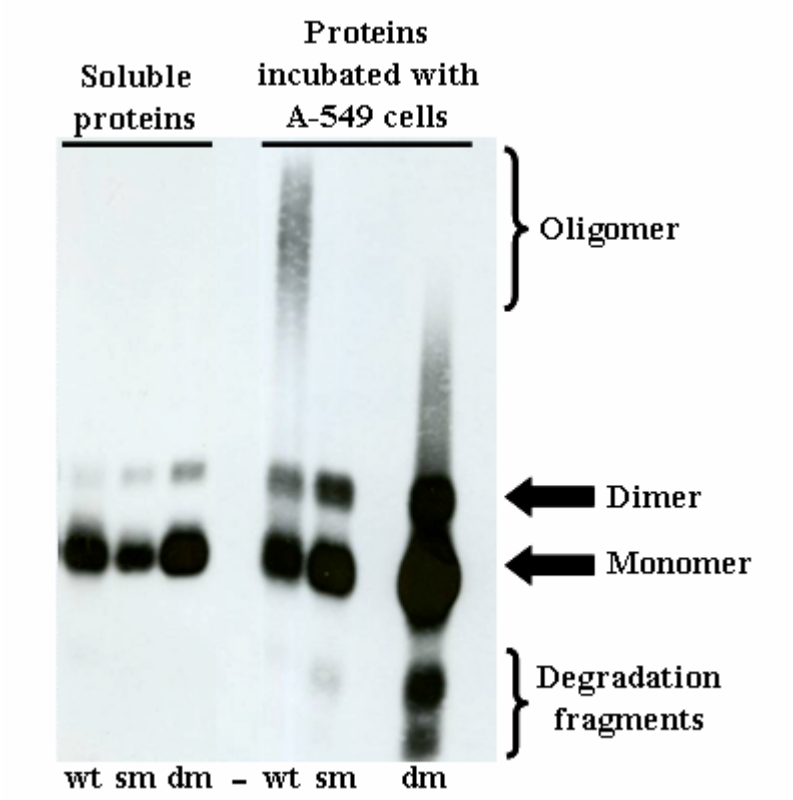

Fig.10: Western blot of a nitrocellulose transferred SDS-AGE analysis of rSLOwt, rSLOsm, rSLOdm bound to human epithelial cells (A-549). In the legend below the picture, wt is rSLOwt, sm is rSLOsm, dm is rSLOdm and – is the negative control (A-549 no treated with any protein). In the experiment, similar amounts of the wild type and single mutant and three hundred more of the double mutant derivative were used. The arrows show protein monomer and dimer forms, while the upper bracket points out an oligomer form. Only rSLOwt was able to oligomerize on cell membrane, while single and double mutants lost the ability to form stable complexes after cell binding. This strongly suggested that mutation in proline 427 to leucine affected the ability of SLO to form active pre-pore complexes.

6.2.4 In vivo analysis of rSLOdm toxicity

A demonstration of SLO high toxic potential is represented by its ability to kill laboratory animals when injected intravenously, even at very low doses (47); therefore rSLOwt and rSLOdm intravenous injection in mice were compared.
Groups of 4-8 mice, approximately 13-15 week old, were injected in the tail vein with different amounts of rSLOwt and rSLOdm and they were observed for at least 1 week after treatment. As reported in Table 5, mice injected with 5 μg of rSLOwt died within few minutes after the injection, while mice treated with 2 μg

showed necrosis at the inoculum site during the days following the injection. On the other hand, mice treated with a dose up to 100 μg of rSLOdm and observed for a week did not show any toxicity, neither locally nor systemically, suggesting that the mutant derivative was at least 50 times less toxic and 20 time less lethal of the wild type form.

DOSE (μg/mouse)	rSLOwt SURVIVED/ TREATED	rSLOdm SURVIVED/ TREATED	Fisher p-val
0,04	4/4		
0,4	4/4		
2	4/4★		
5	0/20		
10	0/20	8/8	<0,0001
50	0/4	4/4	0,0286
100		4/4	

★ **This group of mice showed local toxicity**

Table 5: intravenously injection of rSLOwt and rSLOdm. Serial rSLOwt or rSLOdm dilutions were inoculated in mice tail vein and mortality was followed for 1 week after the treatment. While mice i.v. injected with a dose of 5 μg of rSLOwt died within few minutes after the treatment and mice injected with a dose of 2 μg of the same preparation showed tail necrosis in the following days, no toxicity neither systemic nor local was observed for the mice treated with the double mutant derivative with doses up to 100 μg.

6.2.5 In vitro titration of anti-SLO functional antibodies

The results obtained *in vivo* confirmed the highly reduced toxicity of rSLO double mutant extending the data previously observed *in vitro,* and prompted us to perform additional experiments to verify whether the detoxified mutant had retained the immunogenic and protective properties of the wild type counterpart.
Therefore we first evaluated whether the immunization of mice with rSLOdm could elicit antibodies able to inhibit SLO induced toxicity *in vitro*, measured using the previously described haemolysis assay. Serial two-fold dilutions of collected immune sera were pre-incubated with a 100% lytic dose of rSLOwt, then a sheep blood cells suspension was added and the samples were incubated for 30 additional minutes at 37°C. Sera Haemolysis Inhibition Titres (HITs) were

defined as the lowest dilution factor required to totally inhibit SLO induced lysis. As reported in Figure 11, anti-rSLOwt sera HITs and anti-rSLOdm sera HITs were very similar and significantly higher than HITs obtained using negative control sera (mice immunized with alum alone). This strongly suggested that the double mutant derivative had retained the ability to induce *in vivo* inhibitory antibodies and that these antibodies could be easily measured using a classical *in vitro* assay

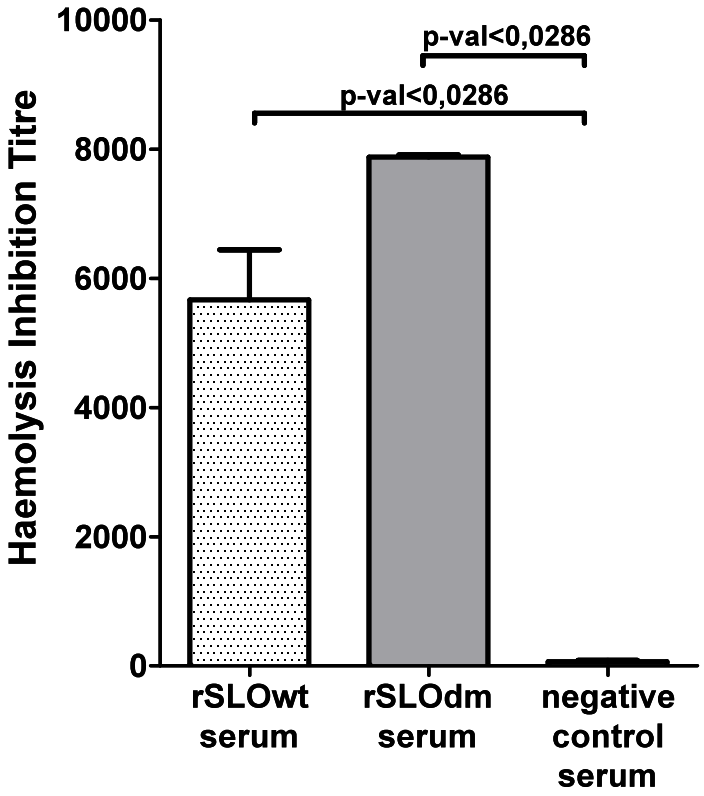

Fig.11: Haemolysis inhibition assay using sera of mice immunized with either alum alone as negative control, rSLOwt or rSLOdm. Haemolysis inhibition titre (HIT) was defined as the lowest serum dilution which completely inhibits rSLOwt induced haemolysis. White dotted bar represents the HIT mean of 4 rSLOwt anti-sera, grey bar represents the HIT mean of 4 rSLOdm anti-sera. No HITs were detected for negative control sera (mice immunized with alum alone). Standard deviations were also reported. Sera against the double mutant derivative inhibited the toxicity induced *in vitro* by the wild type form with titres as high as those obtained immunizing mice with rSLOwt. Statistical analysis was performed with a non parametric Mann-Whitney t-test. P-val < 0,05 is considered statistically significant.

6.2.6 In vivo protection analysis of rSLOdm protein

The ability of anti-rSLOdm antibodies to inhibit toxicity *in vivo* was then tested. Groups of 4-8 animals were immunized either with alum adjuvant alone as negative control or with rSLOwt or rSLOdm and then subjected to injection in the tail vein of different doses of wild-type toxin . While control group animals died if treated with 5 μg of the toxin, mice immunized with rSLOdm survived to this treatment with toxin doses as high as 20 μg. Identical results were obtained for rSLOwt immunized mice, suggesting that immunization with the double mutant derivative could inhibit SLO toxicity *in vivo* comparably to wild-type.

DOSE (μg/mouse)	ALUM ALONE IMMUNIZED MICE SURVIVED/TREATED	rSLOwt IMMUNIZED MICE SURVIVED/TREATED	rSLOdm IMMUNIZED MICE SURVIVED/TREATED
2,5	4/4	ND	ND
5	0/12	8/8 Fisher p-val <0,0001	8/8 Fisher p-val <0,0001
10	ND	4/4	4/4
20	ND	4/4★	4/4★

★ **These groups of mice showed local toxicity**

Table 6: intravenously injection of rSLOwt in mice immunized with either alum alone as negative control, rSLOwt or rSLOdm. Serial two fold dilutions of rSLOwt were inoculated in immunized mice and mortality was followed for a week after the treatment. While mice belonging to the negative control group died within few minutes if injected with 5 μg of the toxin, no mortality was observed for mice immunized with either rSLOwt or rSLOdm treated with a dose as high as 20 μg. Statistical analysis was performed with a Fisher exact test. P-val < 0,05 is considered statistically significant. ND=not done.

A further confirm that rSLOdm could elicit *in vivo* a specific protective immune response came from the demonstration that mice immunized with the double mutant derivative were protected from an intranasal challenge with a lethal dose of GAS 3348 M1 strain. In fact, the cumulative result of two independent experiments showed that 80% of mice immunized with rSLOdm adjuvanted with alum were protected from GAS infection, while less than 5 % of mice immunized with adjuvant only survived.

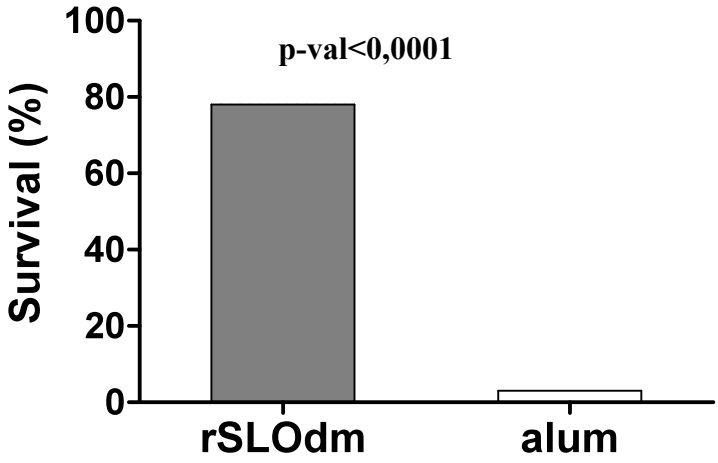

Fig.12: protection results of immunized mice intranasally (i.n.) challenged with a lethal dose of GAS M1 3348 strain. 32 mice/group were immunized with either alum alone as negative control (white bar) or rSLOdm tagless adjuvanted with alum (grey bar). Mice immunized with the double mutant derivative were highly protected against intranasal infection of a GAS M1 strain; the protection rate was perfectly comparable to that obtained immunizing mice with the wild type SLO

7. Discussion

In this study we have demonstrated that a genetically modified derivative of GAS Streptolysin O, belonging to the Cholesterol Depending Cytolysins (CDCs) family of Gram positive secreted toxins, retains the immunogenic and protective properties of the wild type counterpart, while being highly impaired in the typical SLO main toxic characteristics; these two features taken together allow to insert it in a vaccine formulation against Group A Streptococcus.

Using *in silico* analysis and a previously described mutation for another CDC member, Pneumolysin (PLY) of *Streptococcus pneumoniae*, we have generated a double mutant derivative of SLO inserting two point mutations in protein domains directly involved in lytic activity.

In particular, proline 427 is located between first and third protein domains, in a region where the presence of many prolines confers rigidity to the whole structure. This rigidity may become very important during oligomer insertion into cell membranes, the last step of pore formation, conferring to the required high molecular weight and complex structures the necessary energy to lyse the bound cells. Additionally, mutations in this site could also affect the ability of the protein to form active oligomers, due to the fact that it is located in the protein region involved in monomer-monomer interaction during oligomer formation. We mutagenized proline (P) 427 into either glycine (G), the most flexible aminoacid which could reduce the local rigidity, or leucine (L), with a long hydrophobic chain that could interfere with the formation of the pre-pore complex. Both mutants were assessed in haemolysis test using a sheep blood cell suspension and only P427L derivative showed a significant reduction in haemolytic properties, about 1000 times less than wild type SLO. Moreover, using *in vitro* assays, we showed that the single mutant P427L, although able to bind eukaryotic cells as well as the wild type counterpart, is not able to form stable oligomers on the membrane, suggesting that the presence of a long hydrophobic chain in that position do not allow protein monomer oligomerization into an active pre-pore complex.

The second mutation, Tryptophan (W) 535 to Phenylalanine (F), corresponds to the mutation W433F of PLY detoxified derivative PdB. PdB is a very well

characterized mutant of Pneumolysin (41-52), which shows a lytic profile about 100-150 times lower than the wild type counterpart. This reduction in haemolytic activity is essentially due to an impairment of the mutant in cell binding and does not negatively influence the ability of the derivative to generate a protective immune response in a mouse model of Pneumococcus infection (52). Mutation W535F in SLO generated a derivative with reduced haemolytic characteristics comparable to those observed for PdB, and this reduction was strictly correlated with the decrease in cell binding properties.

On these bases, the double mutant derivative rSLO P427L-W535F (rSLOdm) was generated and, assessed in the haemolysis test, appeared to be at least 250.000 times less haemolytic than the wild type rSLO protein. Consequently to this result, rSLOdm was chosen for further investigations using *in vivo* models. As it has been clearly shown (47), intravenous (i.v.) injection of fully active SLO, is highly lethal in different animal models, even when used at very low doses and this harmful feature was related to the high SLO haemolytic activity. We demonstrated that our recombinant form of rSLOwt i.v. injected in mice was able to kill animals within few minutes after a treatment with 5 μg of purified protein, showing local toxicity at a dose of 2 μg. On the contrary, a dose of 100 μg of rSLOdm did not cause any animal toxicity, strongly suggesting that the mutant protein that we have generated is highly impaired in its *in vivo* toxicity, an observation which represents the major result obtained in this work.

SLO is a secreted toxin and, usually, these kinds of proteins confer protection against the pathogens which secrete them through the generation of inhibitory antibodies. We demonstrated that mice immunized with rSLOdm elicited inhibitory antibodies able to block SLO induced toxicity, using both an *in vitro* and an *in vivo* assay. In particular, SLO inhibitory properties of immunized mice sera were checked *in vitro* using haemolysis assay showing that rSLOdm induced a strong immune response with very high inhibition titres, perfectly comparable with those obtained immunizing mice with rSLOwt. Moreover, mice immunized with either the double mutant derivative, the wild type form or alum as negative control were i.v. injected with a lethal dose of rSLOwt and mice immunized with the double mutant derivative were fully protected as well as those immunized with the wild-type protein, a result which is even more relevant since it was obtained in a complex experimental environment represented by an *in vivo* animal model.

Finally, rSLOdm immunized mice were intranasally challenged with a lethal dose of GAS M1 3348 strain and infected animals were followed for 14 days after the treatment demonstrating that immunization with the double mutant rSLO derivative could confer to mice a nearly full protection like that observed in mice immunized with rSLOwt.

A good vaccine candidate should have two main characteristics: i) it should be safe, not showing any detectable toxic effect, ii) it should confer protection against infection from the pathogen.

The results obtained and described in this work strongly suggest that rSLOdm exhibits these two features, being highly impaired in the major SLO toxic properties while retaining the ability to induce a protective response against both i.v. injections of the wild-type toxin and intranasal challenge with a lethal dose of GAS.

On the basis of the above described results and considerations, we believe that the described rSLO double mutant could be proposed as being part of future vaccine formulations against group A streptococcus infections.

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9. Index