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TITOLO TESI

Analysis of the memory B cell response against glycoconjugate vaccines

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ABSTRACT

The development of vaccines directed against polysaccharide capsules of *S. pneumoniae*, *H. influenzae* and *N. meningitidis* have been of great importance in preventing potentially fatal infections. Bacterial capsular polysaccharides are T-cell-independent antigens that induce specific antibody response characterized by IgM immunoglobulins, with a very low IgG class switched response and lack of capability of inducing a booster response. The inability of pure polysaccharides to induce sustained immune responses has required the development of vaccines containing polysaccharides conjugated to a carrier protein, with the aim to generate T cell help. It is clear that the immunogenicity of glycoconjugate vaccines can vary depending on different factors, e.g. chemical nature of the linked polysaccharide, carrier protein, age of the target population, adjuvant used. The present study analyzes the memory B cell (MBC) response to the polysaccharide and to the carrier protein following vaccination with a glycoconjugate vaccine for the prevention of Group B streptococcus (GBS) infection. Not much is known about the role of adjuvants in the development of immunological memory raised against GBS polysaccharides, as well as about the influence of having a pre-existing immunity against the carrier protein on the B cell response raised against the polysaccharide component of the vaccine. We demonstrate in the mouse model that adjuvants can increase the antibody and memory B cell response to the carrier protein and to the conjugated polysaccharide. We also demonstrate that a pre-existing immunity to the carrier protein favors the development of the antibody and memory B cell response to subsequent vaccinations with a glycoconjugate, even in absence of adjuvants. These data provide a useful insight for a better understanding of the mechanism of action of this class of vaccines and for designing the best vaccine that could result in a productive and long lasting memory response.

INTRODUCTION

1.1 Characteristics of glycoconjugate vaccines

Pathogenic extracellular bacteria often express in their surface large molecular-weight polysaccharides, usually in the form of a capsule. The development of vaccines directed against polysaccharide capsules of *Streptococcus pneumoniae*, *Haemophilus Influenzae type b* (Hib) and *Neisseria meningitidis* have been of great importance in preventing potentially fatal infections.

Bacterial capsular polysaccharides are T-cell-independent antigens. They generally stimulate only short-lived B-cell responses by cross-linking the B-cell receptor, which drives the differentiation of B cells to plasma cells producing specific antibodies characterized by IgM immunoglobulins, with a very low IgG class switched response (Fig 1a). Memory B cells are not produced in response to most polysaccharide vaccines, lacking therefore the ability of inducing a booster response (Kelly, DF. 2006). Instead, the terminal differentiation of memory B cells to plasma cells depletes the memory B-cell pool, resulting in hyporesponsiveness to future vaccine doses (MacLennan, J. 2001; Granoff, DM. 2007). Furthermore, plain polysaccharide vaccines are not generally immunogenic in infants (Smith, DH. 1973), not allowing their use as vaccines to prevent disease in children caused by polysaccharide-encapsulated bacteria, that have their highest incidence in the first year of life.

The inability of pure polysaccharides to induce sustained immune responses has required the development of vaccines containing these important components conjugated to a carrier protein. Chemical conjugation of the polysaccharides to highly immunogenic modified bacterial proteins used as carriers, such as tetanus toxoid (TT), diphtheria toxoid (DT), crossreactive

material 197 (CRM197) has been applied to develop several conjugate vaccines already in the market or in ongoing clinical studies.

The first explanation for the mechanism of induction of polysaccharide specific antibodies by glycoconjugate vaccines is that they are able to generate T cell help through different steps. Polysaccharide-protein conjugates bind to the B cell receptor (BCR) of polysaccharide-specific B cells and are internalized into the endosome. Once inside the cell, the protein carrier portion is digested by proteases and the resulting peptides are presented to carrier-specific T cells in association with MHC class II molecules. Carrier peptide/MHCII activated T cells release cytokines that play a role in stimulating B cell maturation and induction of immunoglobulin class switching from IgM to polysaccharide specific IgG (Fig.1b). Some aspects of the precise molecular mechanisms underlying glycoconjugate processing and presentation in the MHCII pathway have not been yet fully dissected and understood. For example, it is not well known what happens to the carbohydrate once inside the endosome and if the covalent linking to the carrier protein is broken during the enzymatic digestion. Recent studies suggests that the carbohydrate portion of the glycoconjugate remains linked to the carrier peptide and is presented on the surface of APCs in the context of MHCII molecules (Avci, FY. 2011). It is clear that the immunogenicity of glycoconjugate vaccines can vary depending on different factors, such as the chemical nature of the linked polysaccharide, the carrier protein used, the age of the target population and the adjuvant used for the formulation. Therefore, the definition of the immunogenicity of a glycoconjugate vaccine is extremely important for a better understanding of the mechanism of action of this class of vaccines and for designing the best glycoconjugate-adjuvant formulation that could result in a productive and long lasting memory response.

In this PhD project I focalize my attention on the study of the immunogenicity of a glycoconjugate vaccine against *Streptococcus agalatiae*, also named Group B Streptococcus (GBS). GBS is a leading cause of morbidity and mortality in neonates in US and globally; its infection gives bacteremia and sepsis in the first week of life as well as meningitis beyond the first week of life (between 8-90 days of life). Until now, no vaccine exists to prevent this disease; since 1996, Centers for Diseases Control (CDC) recommended intrapartum antimicrobials for women identified with GBS colonization before delivery, but the development of a preventive vaccine would be highly recommended.

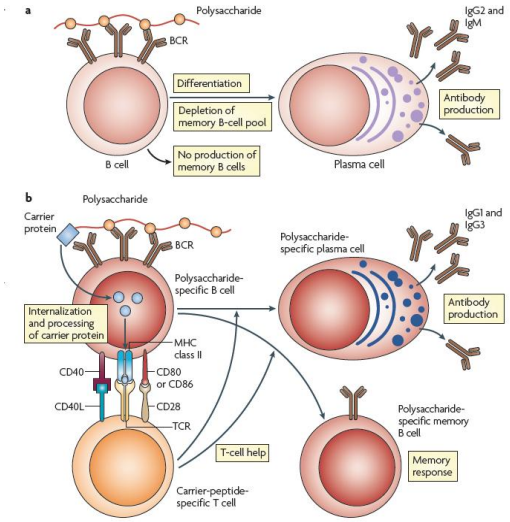

Figure 1: The immune response to polysaccharide and protein–polysaccharide conjugate vaccines. a) Polysaccharides from the encapsulated bacteria that cause disease in early childhood stimulate B cells by cross-linking the B-cell receptor (BCR) and drive the production of immunoglobulins. This process results in a lack of production of new memory B cells and a depletion of the memory B-cell pool, such that subsequent immune responses are decreased. **b)** The carrier protein from protein–polysaccharide conjugate vaccines is processed by the polysaccharide-specific B cell, and peptides are presented to carrier-peptide-specific T cells, resulting in T-cell help for the production of both plasma cells and memory B cells.

1.2 The importance of the glycoconjugate vaccines

The polysaccharide-encapsulated bacteria *Streptococcus pneumoniae*, *Haemophilus influenzae type b* (Hib) and *Neisseria meningitidis* (meningococcus) are the leading causes of serious bacterial infections in young children, accounting for most of the cases of bacterial pneumonia and meningitis worldwide. Between 800.000 and 1 million children under 5 years of age die from pneumococcal disease annually (Scott, JA. 2007), and Hib and meningococcus are thought to account for approximately 400.000 and 50.000 deaths, respectively, each year (WHO 2006; Tikhomirov, E. 1997). These bacteria have a polysaccharide capsule that surrounds the organism and is thought to be important in reducing desiccation and phagocytosis. They are common commensals of the human nasopharynx that rarely invade through the mucosa to cause invasive disease. Despite the availability of effective antibiotics and intensive care management, case–fatality rates and morbidity among survivors remain high (Watt, JP. 2009; O'Brien, KL. 2009). The huge global burden of disease and death caused by these bacteria comes despite the availability of highly effective vaccines. In the United Kingdom, the Hib vaccine was introduced into the infant immunization schedule in 1992, the serogroup C meningococcal (MenC) vaccine in 1999 and the pneumococcal vaccine in 2006. However, only 26% of children worldwide received a course of Hib vaccine in 2006 and less than 10% received other conjugate vaccines. An all-party parliamentary group report on pneumococcal disease was launched in the House of Lords in the UK Parliament on 15 October 2008, highlighting the importance that the UK Government has placed on the global disease burden that is caused by *S. pneumoniae*. In addition, the World Health Organization (WHO) has recommended the widespread introduction of Hib and pneumococcal vaccines, as well as the use of serogroup A meningococcal (MenA) vaccines in the meningitis belt of Africa (a vast

area across sub-saharan Africa that suffers cycles of epidemic meningococcal disease) (LaForce, FM. 2007). In the next decade, these initiatives could change the picture of global child health.

The polysaccharide capsules of *S. pneumoniae, H. influenzae* and *N. meningitidis* are virulence determinants that are composed of repeating saccharide units, the chemical nature of which defines the capsular type of the organism. For example, there are 91 different polysaccharides (serotypes) associated with pneumococci and 13 polysaccharides associated with meningococci (although only five serogroups of meningococcal polysaccharide — A, B, C, Y and W135 — commonly cause disease). Four pneumococcal polysaccharides were first used for the development of a vaccine in 1945 (MacLeod, C. 1945), and a vaccine containing 23 pneumococcal polysaccharides was developed in 1983 and is now in widespread use for the elderly population in many developed countries, including the United Kingdom. Natural immunity against encapsulated bacteria is principally mediated through the binding of antibody to specific bacterial antigens, including the polysaccharide capsule, followed by complement deposition and complement-mediated lysis or opsonophagocytosis (Pollard, AJ. 2001). The highest incidence of invasive bacterial disease is in young children, reflecting the low levels of specific antibody in early childhood (Goldschneider, I. 1969). Through childhood exposure to non virulent strains of these bacteria and other organisms bearing cross-reacting surface structures, the level of antibodies directed against these organisms increases to eventually provide adult levels of protection (Troncoso, G. 2000). The aim of immunization with protein–polysaccharide conjugate vaccines in early infancy is to provide protection during the period of susceptibility in early childhood.

The limitations of the B-cell response in infants is true for most of the polysaccharides encapsulating the bacteria that cause severe infections in humans, including Hib, MenC and most pneumococcal polysaccharides, but there are some exceptions. For example, unlike MenC polysaccharides, and for unknown reasons, MenA polysaccharides are immunogenic from early infancy. In addition, in some studies (Jokhdar, H. 2004), but not in others (Borrow, R. 2000), MenA polysaccharides did not induce antibody hyporesponsiveness. Another exception are some zwitterionic polysaccharides (that is, having both a positive and negative charge), such as the *Bacteroides fragilis* capsule (Kalka-Moll, WM. 2002) and serotype 1 pneumococcal polysaccharide (Velez, CD. 2008), which can be presented in an MHC class II-dependent manner. Based on mouse studies (Vinuesa, CG. 2003), it has been suggested that marginal-zone B cells are involved in polysaccharide-induced immune responses (Weller, S. 2005). The maturation of the splenic marginal zone and its ability to respond to polysaccharides in humans both occur at about 18 months to 2 years of age (Weller, S. 2004). However, direct evidence of this has not been obtained from human studies. Chemical conjugation of the polysaccharide to a protein carrier — such as tetanus toxoid, diphtheria toxoid or CRM197— directs processing of the protein carrier by polysaccharide-specific B cells and presentation of the resulting peptides to carrier-peptide-specific T cells in association with MHC class II molecules. So, a conjugate polysaccharide vaccine induces a T-cell-dependent response from early infancy and induces an anamnestic (memory) response to a booster dose of the vaccine (Kelly, DF. 2006). The main B-cell subset that is involved in the immune response to conjugate vaccines in humans is unknown; however, the characteristics of the immune response that is induced by conjugate vaccines (such as the induction of immunological memory and avidity maturation) strongly indicate that follicular B cells are

probably activated and form germinal centers. Unlike the response to plain polysaccharide vaccines, these responses to conjugate vaccines might provide long-term immunity through the production of new memory B cells. The immunogenicity of different conjugate vaccines varies as a result of differences in the chemical nature of the polysaccharide (such as the length of the saccharide chain) (Pichichero, ME. 1998), the amount of unconjugated polysaccharide in the vaccine and the nature of the carrier protein (Decker, MD. 1992). For example, the Hib–outer membrane protein (Hib–OMP) conjugate vaccine is markedly more immunogenic than Hib–CRM197 (Bulkow, LR. 1993).

1.3 The use of CRM197 as carrier protein

CRM197 is a mutated form of diphteria toxoid that differs in one amino acid residue in the ‘fragment A’ region (Giannini, G. 1984). Alteration of fragment A removes its enzymatic activity, making CRM197 non-toxic. It is thought that the conformation of CRM197 differs from Diphteria Toxoid (DT), leading to lower B cell responses. Since CRM197 is not treated with formaldehyde as it is DT, the T-helper epitopes appear to be better preserved, explaining the better carrier effect of CRM197 versus DT.

In the last years CRM197 has been used in some glycoconjugate vaccines that are in the market such as: the multivalent pneumococcal vaccine (13-valent 4, 6B, 9V, 14, 18C, 19F, 23F,1,3,5,6A, 7F, & 19A) (Kieninger, DM. 2008; Klinger, CL. 2008; Grimprel, E. 2008), the monovalent meningococcal serogroup C vaccine (Richmond, P. 2001) and the multivalent meningococcal vaccine including ACWY serogroups (Snape, M.D. 2008).

1.4 The immune response to the polysaccharide capsule

The polysaccharide capsule of the encapsulated bacteria is a T-cell-independent antigen; it cannot be processed and bound to the MHC class II molecules for presentation to T-helper cells. The polysaccharide capsule is composed of multiple identical antigenic epitopes in close proximity to each other that cross-link multiple membrane immunoglobulins on a B cell to allow activation without the help of T cells (Lesinski, GB. 2001). Because of the lack of T-cell help, there is no germinal center reaction or the associated isotype switching and avidity maturation of the B-cell receptors, or the production of memory B cells. Although naive follicular B cells can recognize T-cell-independent antigens, the most important subset of B cells recognizing T-cell-independent antigens are B1 cells and marginal zone (MZ) B cells (Fig 2).

B1 cells produce low-affinity polyreactive IgM (IgA, IgG3), also called natural antibodies because they are a component of innate immunity and their production does not require exposure to an antigen (Martin, F. 2001). These antibodies recognize autoantigens and many bacterial antigens, including polysaccharide and lipopolysaccharide (Boes, M. 2000). B1 cells constitute a major proportion of the B cells in children but only a minor proportion in adults (Baumgarth, N. 2005) and develop earlier than the other B cells so that they are already present during fetal and neonatal life. They are responsible for producing short-term low-affinity antibody responses that provide a first line of defense against pathogen invasion.

Marginal zone B cells respond mainly to T-cell-independent antigens, such as the polysaccharide capsule of the encapsulated bacteria, and produce IgM. These cells have a mutated immunoglobulin receptor and high expression of CD21, which facilitates attachment to

complement-coated polysaccharides. They are principally localized in the MZ of the spleen, which is a peripheral region of splenic lymphoid follicles that contains macrophages that are particularly efficient at trapping polysaccharide antigens. The location of the MZ B cells presumably permits them to respond rapidly to blood-borne pathogens filtered through the spleen (Pillai, S. 2005).

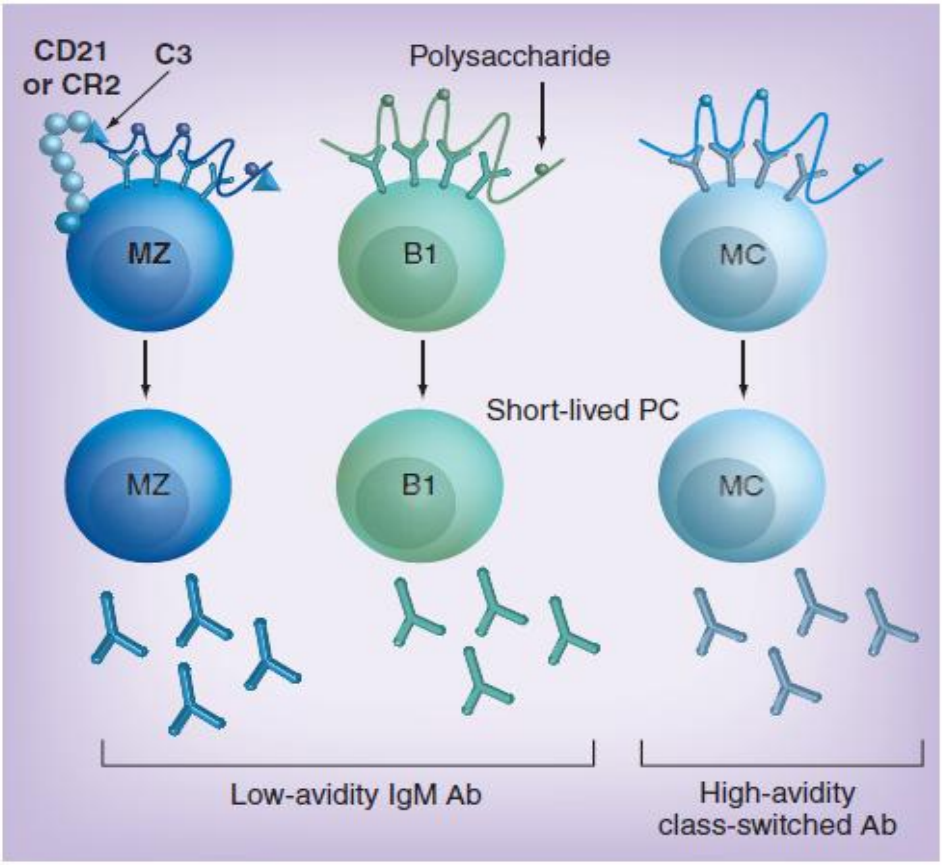

Fig 2: **The B-cell responses to a plain polysaccharide capsule of an encapsulated bacteria.** This vaccine induces MZ B cells and B1 B cells to differentiate into short-lived PCs, which secrete low-avidity IgM Abs. Additionally, polysaccharide-specific memory B cells are activated to differentiate into PCs, which can secrete high-avidity, class-switched Ab.

1.5 The role of B cells in the persistence of anticapsular antibody

Immunization with a protein–polysaccharide conjugate vaccine induces naive B cells, B1 cells and MZ B cells to differentiate within extrafollicular foci into plasma cells that produce polysaccharide-specific antibody of low avidity and of IgM isotype (Jacob, J. 1991). These cells seem to be short-lived with a half-life of between 1 and 10 days (Nossal, GJ. 1962). However, the naive B cells that recognize the polysaccharide antigen of the protein–polysaccharide conjugate vaccine take up and process both the polysaccharide and protein carrier and present the derived peptides alone or together with the polysaccharide in MHC class II molecules on their surface, allowing activation of T-helper cells. The MHC class II-restricted cognate interaction between B cells and T cells provides the necessary costimulatory signals to the B cells to begin the process of germinal center reaction with the generation of somatically mutated class-switched B cells that will secrete high-avidity IgG antibody against the polysaccharide antigen (Lai, Z. 2009; Klein, U. 1998; Smith, KG. 1997).

It is not clear how antibody production is sustained in humans after priming with protein–polysaccharide conjugate vaccines. Antibody might simply depend on the half-life of antibody, 3 weeks, (Vieira, P. 1988) and, therefore, would mostly be determined by the level of antibody reached after primary immunization with protein–polysaccharide conjugate vaccines. However, it is likely that long-lived plasma cells and turnover of memory B cells generated in the germinal center during primary immunization with protein–polysaccharide conjugate vaccine also contribute to antibody persistence.

Plasma cells are terminally differentiated non dividing cells that are conventionally considered to be short-lived and continuously generated de novo from the memory B cells

produced in germinal center reactions following immunization with protein–polysaccharide conjugate vaccines (Crotty, S. 2004). Studies in mice have demonstrated, however, that a fraction of plasma cells generated in the germinal center reaction are long-lived and can secrete antibody for extended periods of time in the absence of memory B cells with a lifespan from 3 months to more than a year (Manz, RA. 2005; Slifka, MK. 1998). Their survival appears to depend on signals provided by stromal cells in a limited number of niches situated in the bone marrow, and it is believed that these signals protect plasma cells from apoptosis (DiLillo, DJ. 2008; Fairfax, KA. 2008). The existence of long-lived plasma cells in humans is supported by a study of patients with rheumatoid arthritis and treated with rituximab, which causes a selective depletion of the circulating plasma cells. In these patients the concentration of immunoglobulins remained in the normal range during treatment (Edwards, JC. 2004).

Memory B cells can persist for more than 50 years following immunization with smallpox vaccine (Crotty, S. 2003). They may continuously recirculate between secondary lymphoid organs through the blood and may also persist in secondary lymphoid organs, such as the lymph nodes and spleen (Maruyama, M. 2000) where the antigen may be kept in follicular dendritic cells. They are thought to continuously differentiate into plasma cells in response to antigen-dependent stimuli (cross-reactive antigen or persisting antigen) (Ochsenbein, AF. 2000) or antigen-independent stimuli (polyclonal stimulation of B cells by microbial products which stimulate B cells via TLRs or through bystander T-cell help) (Bernasconi, NL. 2002).

The continuous activation of memory B cells, the survival of long-lived plasma cells in bone marrow and the antibody half-life are likely to contribute to the long-term maintenance of specific antibody after priming.

1.6 The importance of memory in the glycoconjugate vaccines

Following an immune response two types of differentiated B cells persist in the memory pool: plasma cells, which confer immediate protection by the secretion of specific antibodies; and memory B cells, which confer rapid and enhanced response to secondary challenge. Recent advances in understanding the heterogeneity, dynamics, and persistence of human memory B cells and plasma cells as well as new methods to isolate human monoclonal antibodies have offered new insights into the human B cell response, which are relevant for vaccination and therapeutic intervention.

It is well established that in the course of a T cell dependent B cell response, naive B cells proliferate and differentiate to memory B cells and long-lived plasma cells (Rajewsky, K. 1996; Radbruch, A. 2006; McHeyzer-Williams, LJ. 2005). Using highly purified human naive B cells it was shown that optimal expansion, differentiation, and class switch requires, in addition to BCR triggering and T cell help, a third signal that can be delivered by TLR agonists or by cytokines produced by activated dendritic cells (Ruprecht, CR. 2006). These findings are consistent with mouse experiments that addressed the requirements for TLR expression on B cells in certain types of T-dependent responses (Pasare, C. 2005; Gavin, AL. 2006). Polysaccharides behave as T cell-independent antigens and activate B cells by crosslinking the BCR. Polysaccharide vaccines have been available for decades, but have shown to provide only short-term protection and to be unable to generate B cell memory. By contrast protein–polysaccharide conjugate vaccines elicit T-dependent responses and long lasting memory, at least in adults (Pollard, AJ. 2009). Longevity remains the key aspect of immunological memory and applies to both plasma cells and memory B cells. In humans all

memory B cells recirculate through the blood, but their main reservoir is represented by lymphoid tissues, such as the bone marrow and the spleen (Paramithiotis, E. 1997; Mamani-Matsuda, M. 2008).

Subsets of memory B cells and plasma cells can be defined on the basis of the expression of surface markers. Although CD27 has been widely used as a marker for memory B cells (Klein, U. 1998) there is a substantial fraction of bona fide memory B cells that lack CD27 expression (Wirths, S. 2005). These CD27- memory B cells have been mistakenly taken as naive cells, a fact that has generated some confusion in the field.

The immunological memory is generally defined as an anamnestic response to a booster dose of a vaccine. B-cell memory responses have been observed even among those who did not make a detectable primary response to the vaccine (McVernon, J. 2003). In addition, B-cell memory theoretically could provide long-term protection in those individuals for whom antibody levels have waned below the protective threshold. Unfortunately, in susceptible individuals the encapsulated bacteria are known to invade rapidly after acquisition, often within a few days. In this case, the memory B-cell response, which takes 4 or more days to become established after re-encounter with antigen, is too slow (Kelly, D F. 2005; Snape, MD. 2006), except in those cases where there is a prolonged incubation period. For example, in children who suffer from Hib disease despite prior vaccination (vaccine failures), the immune response to Hib infection is greater than the response in an unvaccinated individual who suffers from the disease. These children mount a memory immune response to infection but still suffer from Hib disease, which supports the concept view that the presence of immunological memory does not guarantee protection (McVernon, J. 2003). These observations strongly suggest that B-cell

memory might not be as important as long lasting antibodies for long-term protection against a rapidly invasive pathogen.

1.7 Glycoconjugate vaccines: immune interference and immune enhancement

The majoriry of the glycoconjugate vaccines that are in the market, contain three most used carrier proteins: TT, DT and CRM197. Several studies have reported controversial hypothesis on the effect that the response to the carrier protein might have in interferring with the response to the polysaccharide. Two are the common interference mechanisms that we can observe following vaccination with conjugate vaccines: carrier-induced epitopic suppression (CIES), whereby pre-existing immunity to a carrier (conjugate protein) suppresses subsequent responses to a hapten/ saccharide linked to the same carrier; and bystander interference, whereby coadministration and/or combinations of vaccines containing a given conjugate protein induce interference that extends to unrelated antigens that are part of the combinations in use (Dagan, R. 2010).

CIES may arise from one or more immune mechanisms (Dagan, R. 2010): pre-existing antibodies to the carrier protein may interfere with B-cell responses to polysaccharide either by preventing binding of polysaccharide-specific B cells, or by promoting anti-carrier B-cell responses over anti-polysaccharide B-cell responses. Carrier-specific B cells may consume local sources of immune help (T cells and associated cytokines) to the detriment of

polysaccharide B cells. Finally, responses to polysaccharide may be impaired by the presence of carrier regulatory T cells.

Bystander interference may occur when there is local competition between antigens for immune help, or when there are changes in the T-cell milieu including induction of T-cell regulatory mechanisms. Conjugate vaccines using diphtheria toxoid variant (CRM197) as a carrier, although less likely to reduce responses to polysaccharides through CIES, are more likely to induce bystander interference, as observed in the UK via diphtheria toxoid (DT) present in diphtheria-tetanus-acellular pertussis and Haemophilus influenzae type b vaccine (DTPa/Hib) (Dagan, R. 2010). Hib responses appear particularly vulnerable to bystander effects (Dagan, R. 2008, 64). Hepatitis B responses also seem to be susceptible to bystander interference, although more data are needed to substantiate the observation (Dagan, R. 2010). Bystander effects are probably mediated via T-cell mechanisms shared between DT and CRM197 and spreading to other coadministered antigens.

Immune enhancement occurs when specific T-helper cells to one vaccine antigen increase the response to the same antigen in another vaccine. The best recognized form of immune enhancement occurs when vaccines using tetanus toxoid (TT) as the conjugate protein are coadministered with Hib-TT. Anti-PRP (polyribosyl-ribitol-phosphate) antibody concentrations increase when Hib-TT is coadministered with meningococcal serogroup C-TT conjugate (MenC-TT), or 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV) that includes 18C-TT (Kitchin, NRE. 2007; Southern, J. 2006; Tejedor, JC. 2006; Schmitt, H. 2007; Knuf, M. 2009). On the other hand, immune responses to TT-conjugated pneumococcal polysaccharides and MenC-TT decrease in the

presence of large amounts of coadministered TT through CIES (Dagan, R. 1998; Dagan, R. 2004; Peeters, CC. 1991).

Immune enhancement mechanisms also contribute to optimal responses to vaccines that use DT and CRM197 as conjugate proteins. Unlike TT, DT/CRM197 conjugates need T-cell enhancement via DT/CRM197 priming or co administration to maximize immune responses (Granoff, DM. 1994; Shelly, MA. 2001; Granoff, DM. 1993). In general, CRM197 and TT when engaged as carrier lead to higher anti-polysaccharide responses than DT, as exemplified by Hib-TT/CRM197 versus Hib-DT (Bulkow, LR. 1993), and meningococcal ACWY-TT/CRM197 versus ACWY-DT (Rennels, M. 2004; Snape, MD. 2008; Ostergaard, L. 2009). Whole cell pertussis (Pw) antigen has an adjuvant effect on TT, but not DT, resulting in enhanced responses to conjugate vaccines using TT as carrier. The effect of DTPw vaccines on the immunogenicity of coadministered protein conjugate vaccines in clinical trials was recently reviewed by Dagan et al (Dagan, R. 2010).

1.8 Group B Streptococcus (GBS)

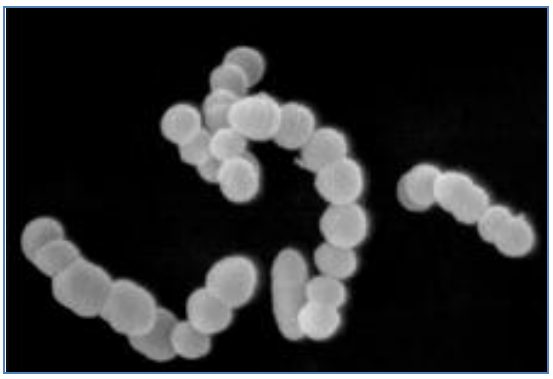

Fig 3: Image of *Streptococcus agalactiae*

Streptococcus agalactiae, also referred to as Group B Streptococcus (GBS), (Fig.3) is one of the most common causes of life-threatening bacterial infections in infants. GBS is a Gram-positive pathogen that colonizes the urogenital and the gastrointestinal tracts of more than 30% of the healthy population and, in particular, it colonizes the vagina of 25–40% of healthy women (Dillon, HC. 1982; Schurcat, A. 1998; Hansen, SM. 2004). GBS, first recognized as a pathogen in bovine mastitis, is distinguished from other pathogenic streptococci by the cell wall-associated group B carbohydrate. The microorganism also expresses a capsular polysaccharide (CPS) that allows GBS isolates to be classified in 9 different serotypes based on the distinct structure and antigenicity of the capsule (Kong, F. 2002). There are currently nine GBS serotypes identified by reactivity of specific antibodies with the surface capsular polysaccharides (CPSs). Serotypes Ia, Ib, II, III and V are responsible for most GBS disease in North America and Europe. Serotypes VI and VIII have thus far been prevalent mainly in Japan; and type IV, although rarely reported worldwide, was the predominant serotype among colonised pregnant women in United Arab Emirates. To date,

only a few cases of GBS type VII have been reported. Neonatal GBS infections can result in pneumonia, sepsis, meningitis and in some cases, death (McCracken, GH. 1973; Schrag, SJ. 2000). Moreover, GBS infections are increasing also among adults, especially in the elderly, immunocompromised and diabetic adults (Schrag, SJ. 2000; Blancas, D. 2004; Skoff, TH. 2009).

However, around 8–14% of the clinical isolates in Europe and in the USA are non-typeable strains because they cannot be distinguished on the basis of CPS antigenicity (Bisharat, N. 2005; Skoff, TH. 2009). Despite this low rate of progression to disease, GBS remains the leading cause of bacterial infections in the newborn, with an incidence rate in 1995/1996 of 1.4 cases per 1000 births, compared with 0.6 cases per 1000 births for Escherichia coli. Neonatal GBS disease was the original impetus for the GBS vaccine effort, and despite declining incidence rates (0.6 cases per 1000 births in 1998) due to the implementation of intrapartum antibiotic prophylaxis, it remains a strong motivating force behind the GBS vaccine effort. GBS disease in newborns has been divided in early-onset disease (EOD) and late-onset disease (LOD) depending on the infants' age and disease manifestation. Early-onset disease manifests in the first week of life and the neonate is usually infected by exposure to GBS during birth. The transmission from mothers to newborns usually occurs when the neonate aspirates contaminated amniotic and vaginal fluids. Early-onset disease can progress as pneumonia and the bacteria can spread into the bloodstream resulting in septicaemia, meningitis and osteomyelitis (Rubens, CE. 1991; Puopolo, KM. 2005). Infants who present with late-onset disease do not show signs of infection in the first 6 days of life. LOD (7–90 days) is less frequent than EOD and the mortality rate is lower but morbidity is high, as around 50% of neonates that survive to GBS infection suffer complications, including

mental retardation, hearing loss and speech and language delay (Schurcat, A. 1998; Schrag, SJ. 2000; Edwards, MS. 2005). The introduction in the US of national guidelines for GBS disease prevention, first issued in 1996 and updated in 2002, recommending universal screening of pregnant women for rectovaginal GBS colonization at 35–37 weeks' gestation and administering intrapartum antimicrobial prophylaxis to carriers, was associated with a decline in the incidence of EOD in the United States (Boyer, KM. 1983; Baker, CJ. 1997; CDC 2002). But EOD still occurred with an incidence of 0.34 per 1000 live births in 2003–2005 in the USA. Not surprisingly, late-onset GBS infections did not decline despite the implementation of prophylactic measures and occurred in 1999–2005 with an incidence averaging 0.34 per 1000 live births in USA (Phares, CR. 2008).

1.8.1 Polysaccharide based vaccines against GBS

GBS is still a public health concern for human infants and adults and the introduction of additional prevention and therapeutic strategies against GBS infection is highly desirable. Vaccination represents the most attractive strategy for GBS disease prevention. An April 1999 NIAID commissioned study from the Institute of Medicine cited GBS as one of the four most favourable infectious disease vaccine targets. Effective vaccines would stimulate the production of functionally active antibodies that could cross the placenta and provide protection against neonatal GBS infection.

During the last two decades, polysaccharide based vaccines against GBS have been extensively studied but also several promising protein antigens have been identified leading to the development of universal protein-based vaccines (Martin, D. 2002; Maione, D. 2005; Margarit, I. 2009, 96,97,98).

Glycoconjugate vaccines against all nine GBS serotypes have been made and all have been shown to be immunogenic and efficacious in a mouse maternal immunization-neonatal challenge model of GBS disease. Without exception, all GBS conjugate vaccines prepared with TT are significantly more immunogenic than uncoupled CPS in mice and rabbits (McDonald, HM. 2000; Wessels, MR. 1995; Paoletti, LC. 1992). The conjugate vaccines elicited type-specific antibodies, primarily IgG, that were active in opsonising homologous GBS for killing "*in vitro"* by human peripheral blood monocytes in the presence of complement. Baboons mounted a strong immune response to GBS conjugate vaccines in presence of alum as adjuvant compared with that measured with uncoupled CPS (Paoletti, LC. 1996), and they, like mice, readily transferred antibody transplacentally to their offspring (Paoletti, LC. 2000). Vaccination of female mice with a mixture of four monovalent GBS conjugate vaccines (Ia, Ib, II and III) provided simultaneous protection against neonatal pup challenge with the serotypes of GBS covered by vaccination. The first proof that vaccination with uncoupled GBS CPSs was well tolerated came from early clinical trials that clearly indicated its safety (Fisher, G. 1983; Baker, CJ. 1985). Because early efforts were focused on developing a maternal vaccine to prevent neonatal GBS disease, healthy non pregnant women (18-45 years) were recruited to participate in the first clinical trials with GBS CPS conjugate vaccines (Baker, CJ. 2000; Kasper, DL. 1996). GBS type Ia linked to tetanus toxoid (Ia-TT), Ib-TT, II-TT and III-TT were the first four conjugate vaccines individually prepared for evaluation in four separate Phase I clinical trials. The main purpose of a Phase I clinical trial, is to asses the safety of a vaccine in a small number of healthy adults and these vaccines have shown to be well-tolerated with minimal reactogenicity (Mattheis, MJ. 1999). A secondary goal of Phase I clinical trials is the evaluation of an immune response, which for protection against neonatal GBS disease is the

amount, specificity and functional activity of CPS –specific IgG. Induction of IgG is important in the development of a maternal vaccine, as it is the only antibody class that crosses the mammalian placenta. Consistent with the preclinical studies in mice, rabbits and baboons, significant increases in the serum levels of CPS-specific IgG were elicited in adult women who received the conjugated CPS, as opposed to those that received the uncoupled CPS. All conjugates elicited significantly higher levels of CPS-specific IgG than uncoupled CPS administered at equivalent dose (Baker, CJ. 2000; Kasper, DL. 1996). The improved antibody response elicited by the conjugate vaccines clearly reflected the transformation of the CPSs from T cell-independent to T-cell dependent antigens.

Phase II clinical trials evaluated safety and immunogenicity of GBS conjugate vaccines administered at different doses. GBS conjugate vaccines Ia-TT, Ib-TT, II-TT and III-TT (initially administered at doses of 57-63 µg as CPS in Phase I trials) were administered at two fourfold dilutions to determine optimal responses in Phase II clinical trials. A dose-dependent, CPS –specific IgG response was measured for all GBS conjugate vaccines. Except for the II-TT, which was still highly immunogenic when administered at the 3,6 µg CPS dose (Baker, CJ. 2000), the intermediate dose 14-15 µg of CPS was the lowest dose administered that elicited type-specific IgG at levels significantly exceeding those obtained with uncoupled CPS.

Other advanced clinical trials were undertaken to prove the observation made in animal models that administration of the GBS conjugate CPS with adjuvant would improve the immunogenicity of the vaccine, but this was not the case. Infact, in healthy humans GBS III-TT adsorbed to aluminium hydroxide was not more immunogenic than the same vaccine administered without alum (Paoletti, LC. 2001). Another aspect of the immunogenicity of the GBS vaccine that has been investigated was the need for a second dose to achieve optimal

and lasting immunity, as it is needed for many vaccines currently in use. In the case of subjects vaccinated with two doses of GBS III-TT it has been shown that some recipients, naive with respect to GBS type III CPS have a response that represented a classical “prime and boost” IgG response. In another group of subjects, already primed by previous exposure to type III GBS, a second dose of vaccine does not induce an increase in the specific IgG levels.

1.8.2 Protein-based vaccine

Efforts to develop a GBS vaccine capable of protecting against all serotypes have focused on identification of universally recognized protein antigens. Proteins should induce protective T-cell-dependent antibody responses and long-lasting immunity. Conserved surface proteins are considered promising candidates for vaccines against GBS because antibodies directed against surface antigens can interfere with bacterial virulence factors and can promote complement dependent opsonophagocytosis.

1.8.3 Reverse vaccinology

For more than a century, vaccines were developed by isolating, inactivating and injecting the cause of the infection. This traditional approach is time-consuming and expensive. Moreover, it usually identifies only abundant antigens that are expressed under in vitro culture conditions (Andre, F. E. 2003). When the first complete microbial genome sequences became available in 1995, a new era, ‘the genomic era’, began and it changed completely the approach to vaccine development (Rappuoli, R. 2001; De Groot, AS. 2004). This new approach, named

reverse vaccinology (Pizza, M. 2000; Mora, M. 2003) has provided a new impulse to the vaccinology field because the vaccine research starts from the genome and not from the pathogen itself (Capecchi, B. 2004). In reverse vaccinology, antigen discovery is achieved by using the integration of several techniques such as genomics, bioinformatics, and molecular biology. Reverse vaccinology shows several advantages respect to the conventional approach. In fact, it permits the identification also of less common, low expressed and/or not expressed in vitro antigens. Moreover, the reverse vaccinology approach can also be applied to non-cultivable microorganisms. One of the major disadvantages is that the reverse vaccinology can be applied only for the discovery of proteins antigens and not for other antigens like lipopolysaccharides and glycolipids (Serruto, D. 2006). The reverse vaccinology approach was applied to the development of a vaccine against GBS starting from the sequencing of the complete genome of a virulent GBS strain (2603v/r, serotype V). But a comparative genome hybridization (CGH) analysis showed that the genetic variability within the GBS isolates was too high, suggesting that more genome sequences were necessary for the identification of vaccine candidates (Tettelin, H. 2005). From this analysis it was clear the need to include genome sequences of more serotypes for the selection of protein antigens. In order to study the genome variability in GBS, Tettelin and coworkers sequenced the genome of six GBS strains that represent the most frequent disease-causing serotypes (serotype Ia strains A909 and 515, type Ib strain H36B, type II strain 18RS21, type III strain COH1 and type V strain CJB111). By a comparative analysis of all available genomes, it was possible to identify two subgenomes: the ‘core genome’ and the ‘variable genome’, together defined as ‘pan-genome’ (Maione, D. 2005; Medini, D. 2005). The ‘core genome’ includes genes present in all the strains and constitutes around 80% of each genome. It contains all genes necessary for the

basic biology of the bacteria. The ‘variable genome’ is responsible for strain diversity and comprises genes that are dispensable and unique to each strain. The introduction of the concept of the ‘pan-genome’ represented a huge potential for the application of reverse vaccinology to the identification of novel vaccine candidates. The availability of the antigens on the bacterial surface for antibody recognition is a prerequisite for a protective immune response. Maione and coworkers, by using modern computer algorithms and bioinformatic software, selected within the GBS pan-genome the genes coding for putative surface-associated and secreted proteins. Different bioinformatic tools were used in order to identify the presence of signal peptides (Signal IP, PSORT), transmembrane domains (TMPRED), lipoproteins and cell-wall anchored proteins (motifs), and homology to other bacterial surface proteins (FastA). Around 589 putative surface proteins were selected, 396 belonged to the core genome and 193 were variable genes (Maione, D. 2005). The proteins predicted to contain more than three transmembrane domains were excluded from the selection because they are difficult to produce in E. coli. By using an high-throughput cloning and expression approach, 312 of the selected GBS genes were successfully produced in E. coli. Each of the genes was cloned with either an N-terminal 6XHis Tag or a C-terminal GST tag, and the expressed proteins were purified by affinity chromatography (Maione, D. 2005; Medini, D. 2005; Telford, JL. 2008). All the 312 purified recombinant GBS antigens were tested by an active maternal immunization/neonatal pup challenge mouse model of GBS infection. Briefly, the antigens were used to immunize intraperitoneally groups of 6–8 CD-1 female mice (6–8 weeks of age) with a three-dose immunization schedule. After the last immunization, mice were mated and their pups were challenged, within 48 h after birth, with a lethal dose of GBS. The survival of the neonates was monitored for 3 days. Immune sera were also collected from

immunized mice for in vitro analysis. Immunoblot assays were used for the identification of the protein in GBS total protein extracts, while flow cytometry assays were carried out to confirm the surface exposure of the antigens.

From this first systematic screening, four antigens were identified as capable of significantly increasing the survival rate of challenged infant mice. When the four antigens were mixed and administered simultaneously, an almost universal protection was achieved against challenge model using a panel of strains comprehensive of the most pathogenic GBS serotypes. In particular, the levels of protection reached were similar to those achieved using the polysaccharides-based vaccines. Only one (SAG0032) of these four antigens was part of the ‘core genome’, and this protein was the already described Sip protein. The other three antigens – GBS67 (SAG1408), GBS80 (SAG0645), and GBS104 (SAG0649) – were present in the variable portion of the subgenome.

AIM OF THE THESIS

Aim of my PhD project is the assessment of the memory B cell (MBC) and antibody responses to the polysaccharide and to the carrier protein of a glycoconjugate vaccine against *Streptococcus agalactiae*, also referred as, Group B streptococcus (GBS). GBS is a leading cause of morbidity and mortality in the neonate in US and globally; its infection gives bacteremia and sepsis in the first week of life as well as meningitis beyond the first week of life (between 8-90 days of life). Until now, no vaccine exists to prevent this disease; since 1996, Centers of Diseases Control (CDC) recommended intrapartum antimicrobials for women identified with GBS colonization, but the development of a preventive vaccine would be highly recommended. Immunological memory characterized by MBC and the persistence of antibodies are important characteristics required to a vaccine. In particular memory B cells are the key players to provide a faster antibody response upon antigen-re-exposure; they are useful to induce the generation of class-switched plasma cells secreting high-avidity antibodies and to maintain protective antibodies levels over time. The first part of the work has been dedicated to the setting up of the most appropriate assays to measure MBC frequencies against the carrier and the polysaccharide component of the vaccine. I have then focused my attention to the understanding of how immunological memory can be better induced by vaccination with a glycoconjugate and maintained. We can act on two fields; the presence of adjuvants and the possible influence of a pre-existing immunity to the carrier protein on the B cell response to the GBS –Ps.

RESULTS

2.1 ELISA-based Serial Limiting Dilution Assay (sLDA)

The most widely used assays to measure the frequency of antigen specific memory B cells are ELISpot (Slifka, MK. 1996) and more recently serial Limiting Dilution Assay (sLDA) (Bernasconi, NL. 2002).

In order to evaluate the performance of the two assays for assessing frequencies of polysaccharide specific memory B cells induced by vaccination, we have immunized mice and applied the two assays in parallel to evaluate the B cell response 20-30 days after the last vaccine dose.

To perform sLDA, splenocytes collected from mice vaccinated with CRM-GBS PsIII were plated starting from 8-4x10^5 cells/well in serial two-fold dilutions, 6 replicates per dilution, with or w/o polyclonal B cell activators (CpG 5µg/ml and IL2 1000U/ml). After 10 days of culture, supernatants from each single well were collected and analyzed by ELISA for the presence of total and antigen-specific (CRM and PsIII) IgG. Using the Optical Density (OD) values obtained from the ELISA results, the number of wells that were positive and negative for the presence of Ag-specific antibodies was determined for each cell dilution of the sLDA plate. The definition of positive and negative culture wells for Ag-specific antibodies has been based on the OD cut-off value of the assay obtained from the non-stimulated culture wells . In particular, the cut-off value for selecting positive culture wells was calculated as the mean OD value of all supernatants collected from non-stimulated culture wells plus three standard deviations. On the basis of the non-stimulated control OD values obtained in three different

experiments, the cut-off values for positivity have been fixed at OD=0.20 for the measurement of total IgG, OD=0.47 for CRM specific IgG and OD=0.32 for GBS- PsIII specific IgG.

Fig.4 shows an example of layout of an ELISA plate coming from a typical sLDA assay, where positive and negative wells are represented with a different color code. Frequencies of circulating Ps and CRM specific B cells are calculated as percentage of antigen-specific MBC on total IgG MBC. One of the advantages of the sLDA assay is that it allows the simultaneous analysis in the B cell culture supernatants of carrier and Ps specific antibodies produced by MBC.

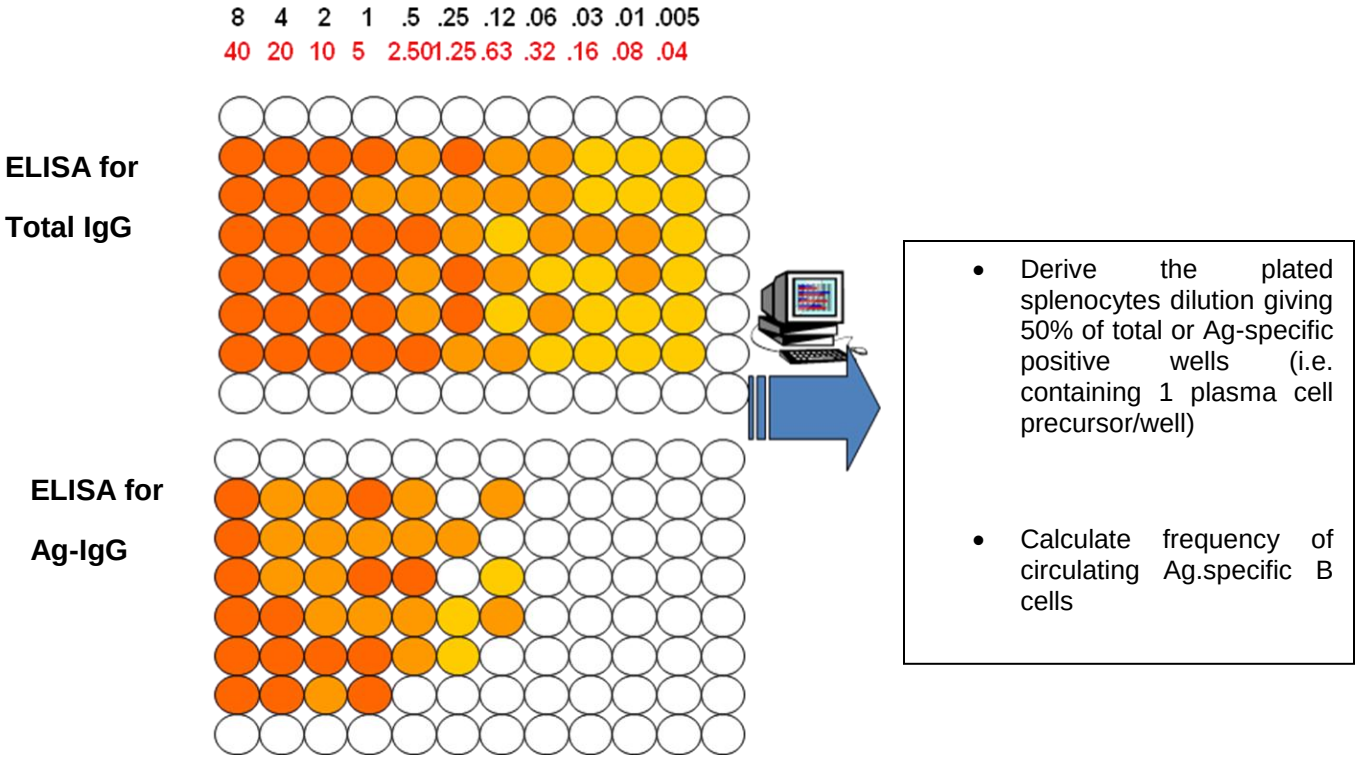

Fig 4: Layout of an ELISA plate coming from a typical sLDA assay. Splenocytes from immunized mice were plated in complete medium with 5% FBS in serial 2-fold dilutions starting from $8x10^5$ cells/well, 6 replicates per dilution in 96-well U-bottom plates containing 5 µg/ml of CpG and 1000 units/mL of IL2. After 10 days of culture, supernatants from each single well were collected and analyzed by ELISA for the presence of total and antigen-specific (CRM and PsIII) IgG.

2.2 ELISpot (Direct and Reverse) for the MBC detection

ELISpot assay is an alternative method to sLDA that has the advantage of allowing not only the assessment of the frequency of antigen specific memory B cells, but also of plasmablasts. Two different ELISpot methods have been set up and compared, the first one named “Direct” and the second one named “Reverse”.

The “Direct” ELISpot allows to capture the antibodies secreted by each antigen-specific B cell through their direct binding to the antigen coated on the membrane of the plates. To perform this assay, spleen cells collected from vaccinated mice 20-30 days after the last vaccine dose were stimulated in bulk with 5 µg/ml CpG and 1000U/ml IL2 for 5 days. After stimulation, viable cells were plated on ELISpot plates coated with the antigens GBS-Ps, CRM and HSA as negative control or with anti-mouse Ig. After incubation O.N. at +37°C, 5% CO_2, cells were washed away and antibodies secreted by single B cells and bound to the plate were revealed with a biotin-conjugated anti-mouse Ig antibody and Streptavidin-HRP. AEC substrate was used to stain the spots. By performing several experiments we observed that the “Direct” ELISpot often shows a high background on HSA, used as negative control (Fig.5, Panel A). Furthermore the shape of the spots detected in the wells coated with CRM is not very well defined, making them often uncountable (Fig.5, Panel B). Instead, the spots detected in the wells coated with the Ps have a well defined shape and are countable easily and precisely (Fig.5, Panel C).

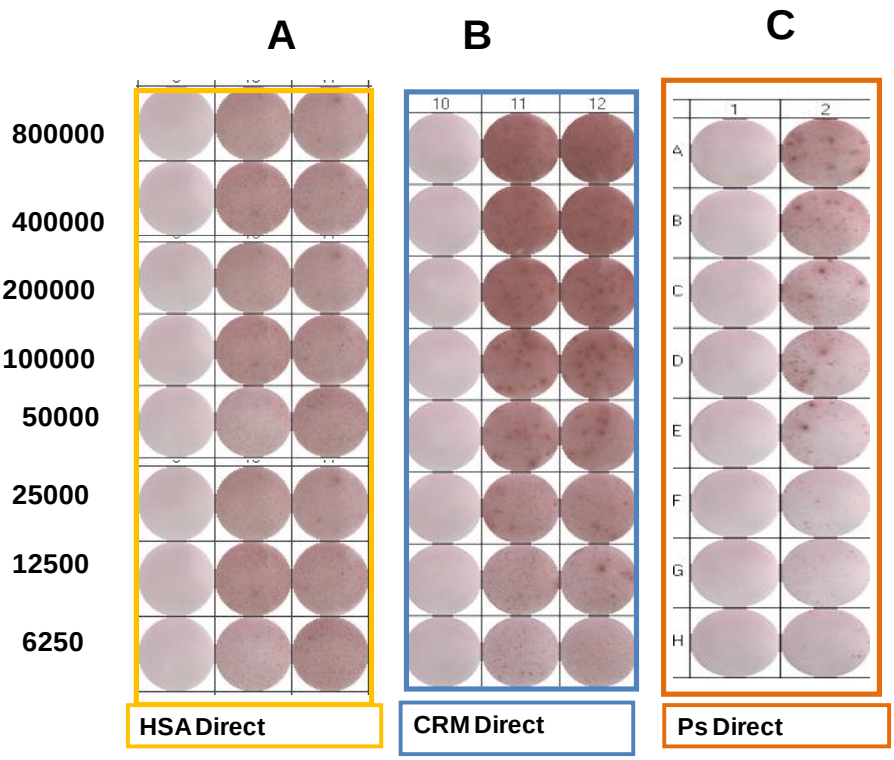

Fig 5: Picture of wells from a "Direct" ELISpot plate to detect IgG specific for HSA, CRM and GBS Ps. Splenocytes collected from vaccinated mice 20-30 days after the last vaccine dose were stimulated in bulk with 5 µg/ml CpG and 1000U/ml IL2 for 5 days. After stimulation, viable cells were plated on ELISpot plates coated with the antigens, HSA (Panel A), CRM (Panel B) and GBS-Ps (Panel C). After incubation O.N. at +37°C, cells were washed away and antibodies secreted by single B cells and bound to the plate were detected with a biotin-conjugated anti-mouse Ig antibody. Spots were then revealed by Streptavidin-HRP followed by AEC substrate.

On the other hand, the "Reverse" ELISpot allows to immobilize on the membrane of the wells all the immunoglobulins secreted by the B cells, as the wells are coated with anti-mouse Ig polyclonal antibodies. Specific antibodies produced by B cells and interacting with the antigen are then detected by using a biotin-labeled antigen. Spots are revealed with Streptavidin-HRP and AEC substrate. To set up this method, CRM and plain GBS polysaccharide III have been biotinylated and used to detect antibodies specifically reacting

with them. As shown in Fig.6 by Reverse ELISpot: (Panel A) biotinylated-HSA does not show any background signal on negative controls; (Panel B) CRM-specific B cells are visualized with cleaner results than with the Direct method when detected with CRM-biotinylated antigen, as this technique allows to visualize spots clearly defined in their size and shape; (Panel C) clear spots are also obtained when biotinylated GBS-polysaccharide is used to detect antigen specific antibodies produced by B cells.

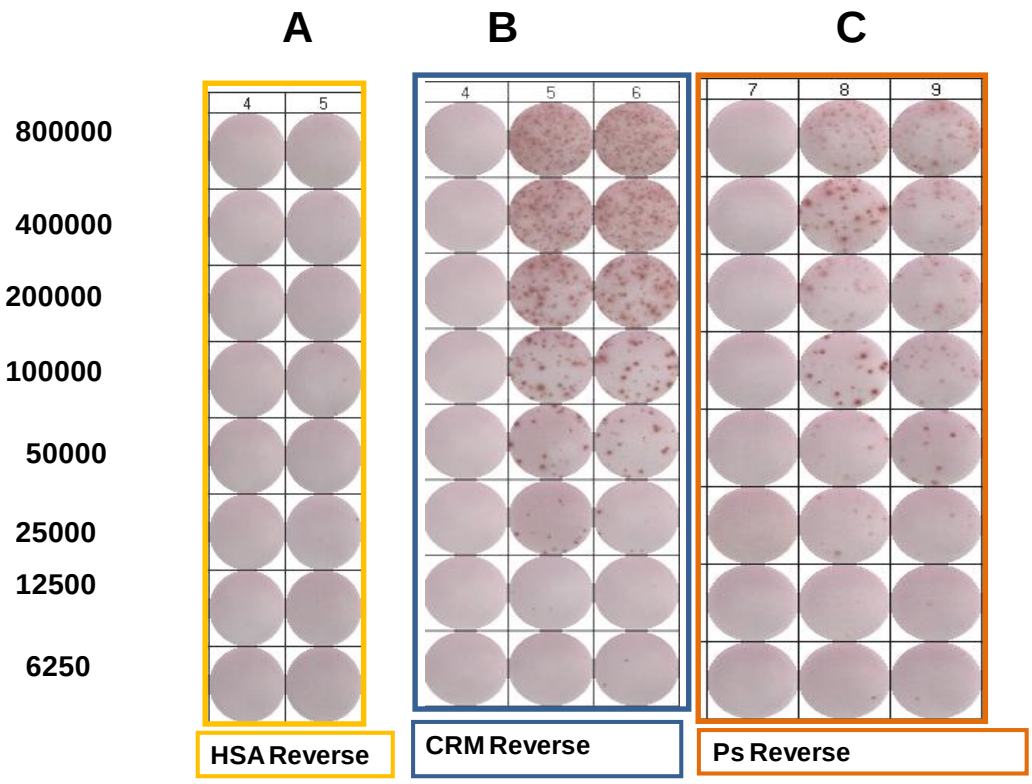

Fig 6: Picture of wells from a "Reverse" ELISpot plate to detect IgG specific for HSA, CRM and GBS Ps. Splenocytes collected from vaccinated mice 20-30 days after the last vaccine dose were stimulated in bulk with 5 µg/ml CpG and 1000U/ml IL2 for 5 days. The "Reverse" ELISpot allows to immobilize on the membrane of the wells all the immunoglobulins secreted by the B cells, as the wells are coated with anti-mouse Ig polyclonal antibodies. Specific antibodies produced by B cells and interacting with the antigen are then detected by using a biotin-labeled antigen, HSA (Panel A), CRM (Panel B) and GBS-Ps (Panel C). Spots are revealed with Streptavidin-HRP and AEC substrate.

The comparison between the Direct and the Reverse ELISpot to assess CRM and GBS-Ps specific MBC shows that (Fig.7) CRM-specific MBC can be clearly measured by using the Reverse method, while there is no difference between the two methods in terms of measured frequency of polysaccharide specific MBC, but also in this case the spots are better defined when using the Reverse method. We have therefore demonstrated that, by performing the "Reverse" ELISpot, it is possible to increase the quality of the assay since the background on negative antigen (HSA) is lower, spots are more clearly defined in size and shape for all the antigens used and in some cases it allows the detection of higher frequencies of antigen-specific B cells (as for CRM).

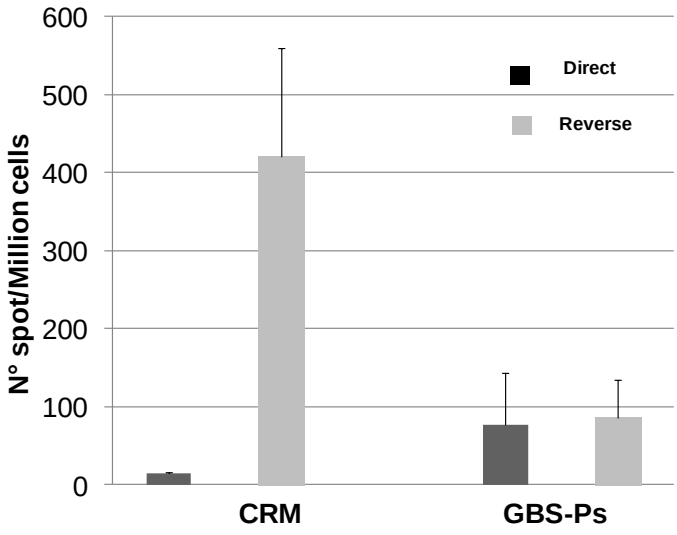

Fig 7: Comparison between Direct and Reverse ELISpot assays to assess CRM and GBS-Ps specific MBC. Frequencies of antigen-specific MBC (N°IgG MBC x10^6 plated cells) have been assessed by using Direct ELISpot (dark gray bars) and Reverse ELISpot (light gray bars). Bars represent the mean value and error bars represent the Standard Deviation (SD). CRM-specific MBC can be clearly measured by using the Reverse method, while there is no difference between the two methods in terms of measured frequency of polysaccharide specific MBC, but also in this case the spots are better defined when using the Reverse method.

Evaluating the strenghts and the limitations of the tested assays it is clear that although Reverse ELISpot can provide clear results, it does not allow a simultaneous analysis in the same well of anti-Ps, anti-CRM and total IgG antibodies, therefore requiring a labor intensive work to detect all these parameters simultaneously. On the other hand, B cell culture supernatants coming from sLDA plates, although requiring 10 days of cell culture and two days of ELISA assay, allow the simultaneous detection of both Ps and CRM specific antibodies. These considerations brought us to choose sLDA for the analysis of MBC response in mice vaccinated with CRM-GBS Ps glycoconjugate.

2.3 MBC responses to the carrier (CRM) and the polysaccharide (PsIII) increase in presence of adjuvant

In order to evaluate the effect of different adjuvants in increasing the memory B cell response to the conjugated polysaccharide, we have performed experiments in mice vaccinated with CRM-conjugated GBS polysaccharide III (CRM-GBS PsIII) with or without adjuvant. BALB/c mice have been immunized with three doses of CRM-GBS PsIII vaccine with or w/o adjuvants (Alum/MF59). Twenty-one days after the last dose, spleens have been taken and splenocytes isolated and put in culture with polyclonal stimuli (CpG and IL2) for 10 days. At day 10, supernatants have been recovered and the quantity of CRM and GBS PsIII antigen specific memory B cells has been evaluated by ELISA as percentage of antigen specific MBC on total IgG.

On the basis of the results (Fig 8), we can demonstrate that both adjuvants (Alum/MF59) significantly increase the IgG MBC response to CRM (P=0.0005 and P=0.0008

respectively) in respect to the group of mice receiving the vaccine without adjuvant. The MBC response to PsIII is significantly increased by the presence of MF59 as adjuvant in respect to mice receiving the vaccine without adjuvant (P=0.0011). Also mice receiving the vaccine with Alum show an increased frequency of PsIII specific MBC, although not statistically significant. The P value is calculated with T Test in respect to the group immunized with the plain vaccine.

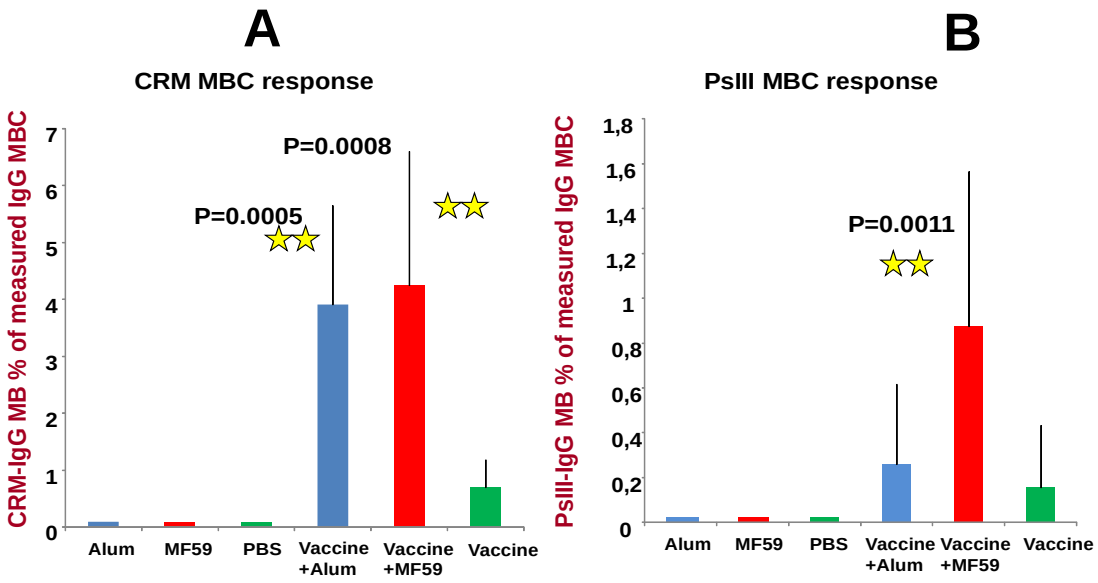

Fig 8: Adjuvants significantly increase the IgG MBC response to CRM and to PsIII. Alum and MF59 significantly increase the IgG MBC response to CRM in respect to the group of mice receiving the vaccine without adjuvant (P=0.0005 and P=0.0008 respectively, Panel A). The MBC response to PsIII is significantly increased only in mice immunized with the MF59-adjuvanted vaccine (P=0.0011, Panel B). The P value is calculated with T Test in respect to the group immunized with the plain vaccine.

2.4 Serum Antibody responses to the carrier (CRM) and the polysaccharide (PsIII) significantly increase in presence of adjuvant

To confirm the observation that usually adjuvants have a positive effect on antibody responses, we investigated whether they improve the immunological responses also in the case of mice vaccinated with CRM-conjugated GBS polysaccharide III (CRM-GBS PsIII) with or without adjuvant. BALB/c mice have been immunized with three doses of CRM-GBS PsIII vaccine with or w/o adjuvants (Alum/MF59). 21 days after the third dose sera have been collected to determine IgG and IgM antibody titers by ELISA. As shown in fig 9 the presence of both adjuvants, Alum and MF59, significantly increases titers of circulating IgG specific for CRM ($P=0.0008$ and 0.00009 respectively, panel A) and also for PsIII ($P=0.0011$ and 0.0006 respectively, panel B).

The comparison of the PsIII specific IgG antibodies induced by the two adjuvants shows that mice vaccinated with MF59 develop significantly higher titer as compare to Alum vaccinated mice ($P= 0.0026$).

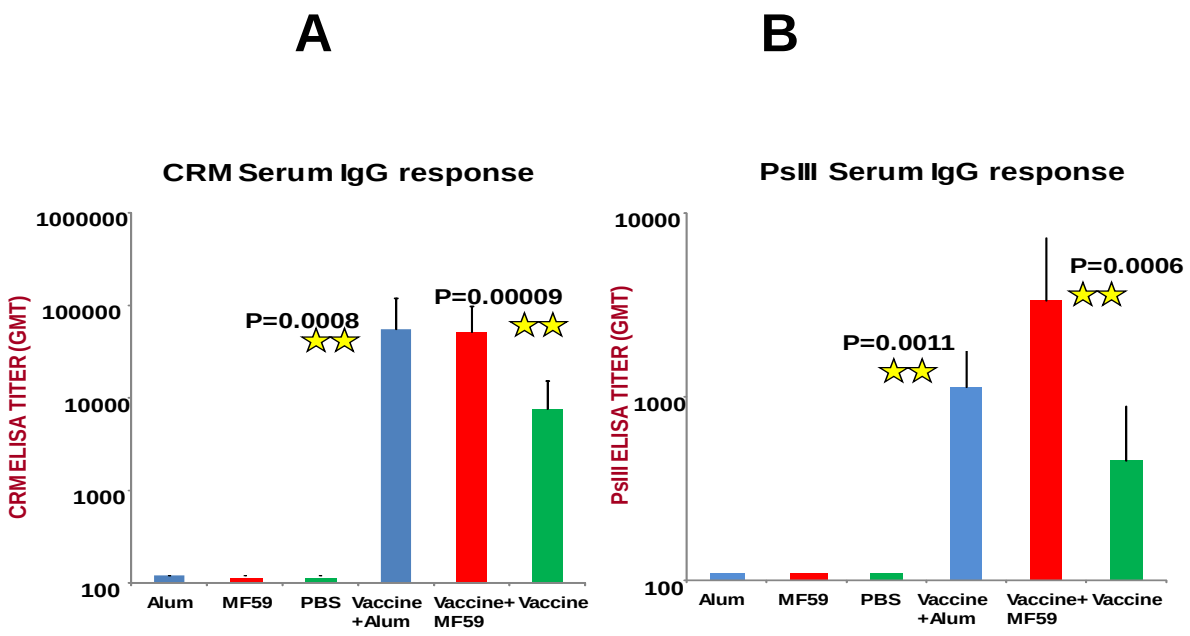

Fig 9: Adjuvants significantly increase titers of circulating IgG specific for CRM and GBS-PsIII. Alum and MF59 significantly increase the IgG serum titer to CRM (P=0.0008 and 0.00009 respectively, Panel A) and the IgG serum titer to PsIII (P=0.0011 and 0.0006 respectively, Panel B). The P value is calculated with T Test in respect to the group immunized with the plain vaccine. The anti-PsIII IgG titers induced by vaccine + MF59 are significantly higher than that induced by vaccine + Alum (P=0.0026, Panel B). In this case the P value is calculated with T Test comparing the two groups receiving vaccine + adjuvants.

We also assessed the presence of circulating IgM, but only anti-GBS-PsIII antibodies were detectable with a statistically significant increase in mice receiving the vaccine with adjuvants as compared to mice receiving the plain vaccine (P=0.01 and 0.003 for Alum and MF59 respectively) (Fig 10). No detectable anti-CRM IgM circulating antibodies were induced when mice were immunized both in presence or absence of adjuvants (data not shown).

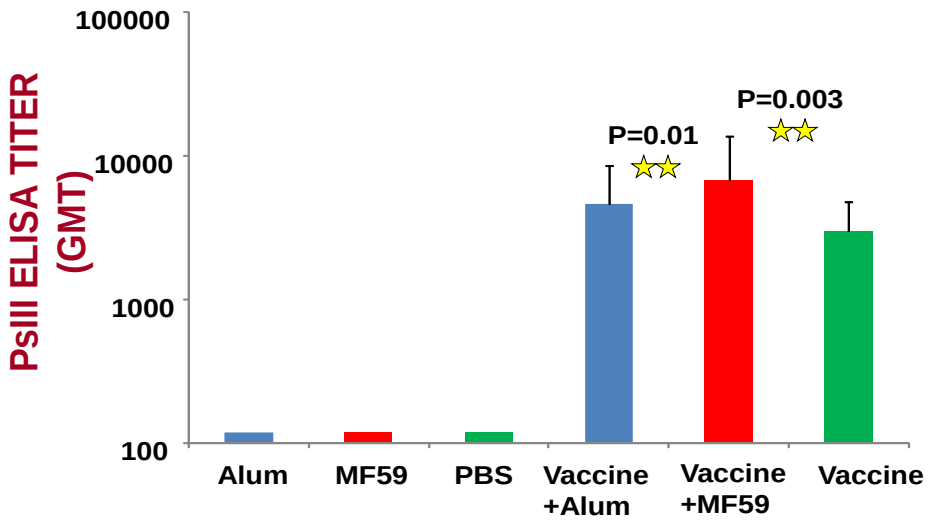

Fig 10: Adjuvants significantly increase titers of circulating anti-PsIII specific IgM. Administration of the glycoconjugate vaccine with Alum and MF59 significantly increase the IgM serum titer against PsIII (P=0.01 and 0.003 respectively). The P value is calculated with the T Test in respect to the group immunized with the plain vaccine.

2.5 Priming with CRM promotes the generation of a pool of MBC specific for the PsIII either in presence or absence of adjuvant

Beyond the presence of an adjuvant, also a pre-existing immunity to the carrier protein can influence the MBC response to the polysaccharide component. To evaluate the effect of a priming with the carrier CRM on the GBS-PsIII response in terms of MBC, BALB/c mice of 6 weeks have been primed with CRM+Alum/MF59 or Alum/MF59 and than immunized i.p. 2 times with CRM-GBS PsIII conjugated vaccine with or without adjuvants (Alum or MF59). As previously described, to measure antigen specific MBC, spleens have been taken 21 days after the last dose and splenocytes isolated and put in culture with polyclonal stimuli (CpG and IL2) for 10 days. At day 10, supernatants have been recovered and the quantity of CRM and GBS PsIII antigen specific memory B cells have been evaluated by ELISA as % of antigen specific MBC on total IgG. By comparing naïve to primed mice, on the basis of the glycoconjugate vaccine they received, primed mice show a MBC response to the polysaccharide significantly increased when the glycoconjugate vaccine is administered w/o adjuvant (P=0.02) or with Alum (P=0.07) (Fig.11, Panel A and B). When the glycoconjugate vaccine is administered with MF59, it induces comparable MBC response in naïve and primed mice. Since we confirm the previous observation that immunization of naïve mice with MF59 induces already a significant increase in the frequency of anti-Ps specific MBC (Fig.8, Panel B) and (Fig.11, Panel A) priming with the carrier does not induce any further increase of MBC frequency in primed mice (Fig.11, Panel B).

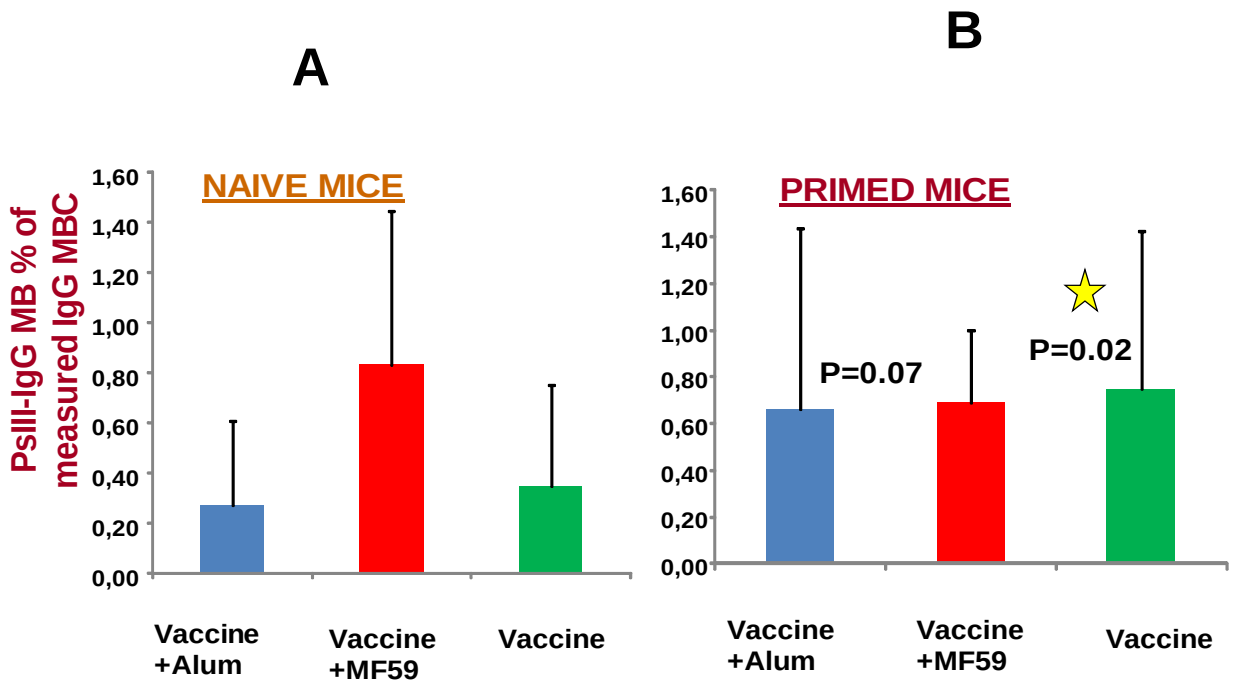

Fig 11: Priming with the carrier CRM has a positive effect on the subsequent MBC response to a glycoconjugate vaccine administered in presence or absence of adjuvants. (Panel B) Primed mice receiving a glycoconjugate vaccine administered with Alum or w/o adjuvant show a statistically significant increase in the frequency of polysaccharide specific MBC as compared to naïve mice (Panel A). Since MF59 significantly increases the IgG MBC response to PsIII already in naïve mice (Panel A), no further increase is observed in terms of MBC frequency in primed mice (Panel B). The P value is calculated with T Test in respect to the group of naïve mice immunized with the corresponding glycoconjugate vaccine.

The frequency of carrier specific (CRM) MBC has been also evaluated in all groups of mice (Fig.12) after the glycoconjugate vaccination. On the basis of the results, primed mice, as compared to naïve mice, show an increase of the CRM specific MBC response independently from the received vaccine (P=0.05 for Alum, 0.01 for MF59 and 0.0004 for plain vaccine).

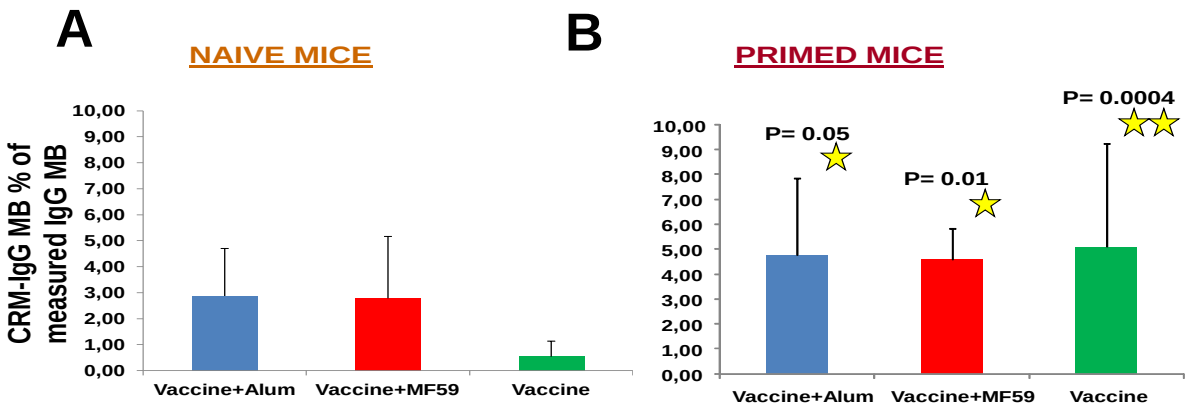

Fig 12: Priming with the carrier affects the subsequent MBC response to CRM after immunization with the glycoconjugate-vaccine administered in presence or absence of adjuvants. (Panel B) Primed mice receiving the glycoconjugate vaccine with or w/o adjuvants show an increase of the CRM specific MBC frequencies (P=0.05 for Alum, 0.01 for MF59 and 0.0004 for plain vaccine) as compared to naïve mice receiving the corresponding vaccine (Panel A). The P value is calculated with T Test in respect to the group of naïve mice immunized with the same glycoconjugate vaccine.

2.6 Priming with CRM promotes enhanced antibody response to PsIII also in absence of adjuvant

To evaluate the effect of a priming with the carrier CRM on the GBS-PsIII response in terms of circulating serum IgG titers, BALB/c mice of 6 weeks have been primed with one injection of CRM+Alum/MF59 or Alum/MF59 as control and than vaccinated i.p. 2 times with CRM-GBS PsIII conjugated vaccine with or without adjuvants (Alum or MF59). Sera have been collected at different time points after immunization: 21 days after priming and 21 days after the first and the second dose of glycoconjugate vaccine and an ELISA assay on CRM and PsIII has been performed to determine circulating antibody titers. In order to analyze if priming with one dose of CRM with adjuvant is able to induce detectable anti-CRM antibody titers and how they increase after glycoconjugate-vaccination, we have assessed the serum IgG antibody titers to the carrier CRM developed after priming and after vaccination (Fig.13). We have observed that priming with CRM allows to reach a mean antibody titer of 367 GMT. After two doses of glycoconjugate vaccination, primed mice (Panel B) develop a higher IgG antibody response to the carrier as compared to the naïve mice (Panel A) in presence of Alum ($P=0.0002$), or MF59 ($P=0.002$) or in absence of adjuvant ($P=0.0001$). It is remarkable to note that, in primed mice the anti-CRM IgG antibody titers reached already after one dose of glycoconjugate vaccine are comparable both in presence or absence of adjuvant. No further significant increase in the CRM antibody titers is observed after a second dose of vaccine with or without adjuvant.

In naïve mice, it is necessary to administer two doses of vaccine with adjuvant in order to reach an anti-CRM antibody response comparable to that obtained in primed mice already after one single dose. Furthermore, if in naïve mice the vaccine is administered without adjuvant, the antibody response reached after the second dose is significantly lower (about 1 Log) than in mice receiving the vaccine with both adjuvants.

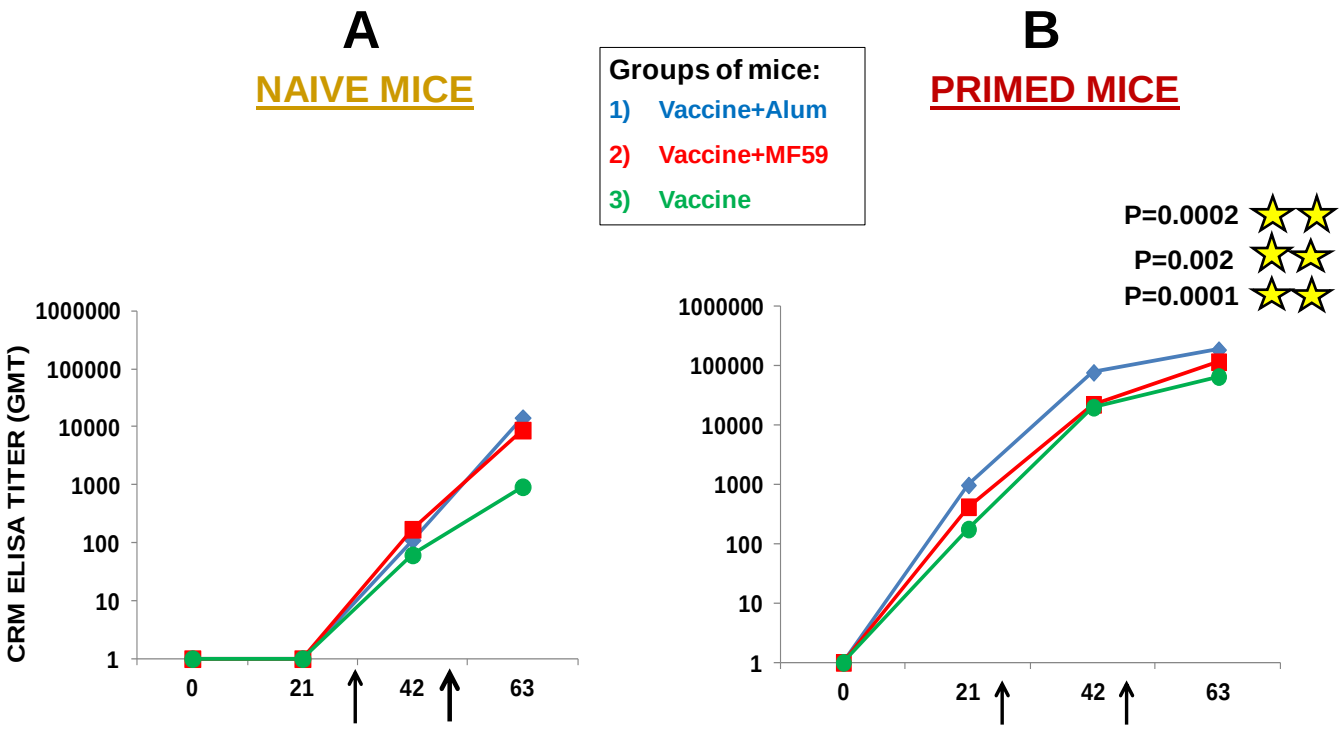

Fig 13: Priming with one dose of CRM with adjuvant is able to induce detectable anti-CRM antibody titers. Detectable anti-CRM IgG titers are measurable 21 days after priming with the carrier CRM administered with adjuvant (GMT=367). After two doses of glycoconjugate vaccination primed mice (Panel B) develop a higher IgG antibody response to the carrier as compared to the naïve mice (Panel A) in presence of Alum (P=0.0002) or MF59 (P=0.002) or in absence of adjuvants (P=0.0001). The P value is calculated with T Test in respect to the group of naive mice immunized with the same glycoconjugate vaccine.

In figure 14 the anti-polysaccharide circulating IgG measured in naïve (Panel A) and primed mice (Panel B) are represented. By comparing primed to naïve mice, on the basis of the glycoconjugate vaccine they received, the IgG antibody response to the polysaccharide is significantly increased in primed mice when the glycoconjugate vaccine is administered w/o (P=0.002) or with Alum (P=0.0005), as compared to naive mice receiving the same vaccination (Fig.14, Panel A and B). When the glycoconjugate vaccine is administered with MF59, since it already increases the IgG serum response to PsIII in naïve mice as compared to the other groups (Panel A), no further significant increase is observed in terms of serum IgG titers in primed mice (Panel B). It is interesting to observe that primed mice, that have a mean anti-CRM IgG titer of 367 GMT at the day of the glycoconjugate vaccination (21 days post priming), respond equally well to the polysaccharide showing comparable anti-Ps IgG antibody titers when receiving the vaccine with both adjuvants or w/o (Fig 14, Panel B).

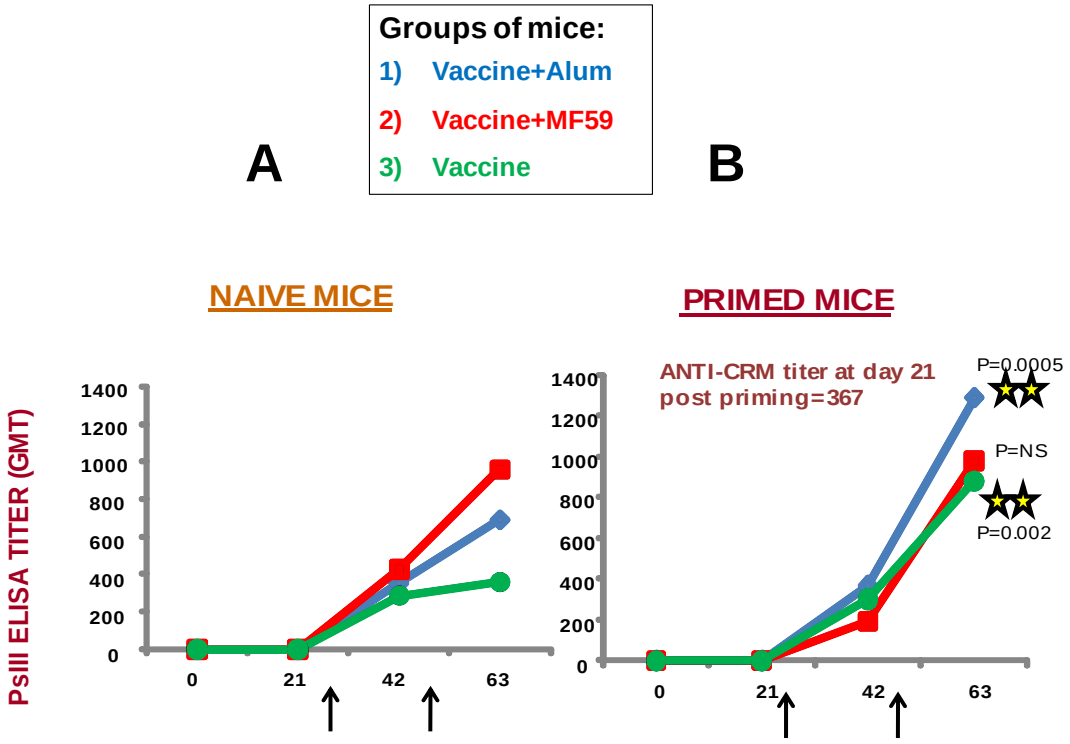

Fig 14: Priming with the carrier CRM has a positive effect on the subsequent anti-polysaccharide IgG response to a glycoconjugate-vaccine. Primed mice show a statistically significant increase in the frequency of anti-Ps IgG titers (Panel B) as compared to naïve mice (Panel A) when receiving the glycoconjugate vaccine with Alum or w/o adjuvant. Since MF59 already increases the IgG serum response to PsIII in naïve mice (Panel A), no further significant increase is observed in terms of serum IgG titers in primed mice (Panel B). The P value is calculated with T Test in respect to the group of naive mice immunized with the same glycoconjugate vaccine.

DISCUSSION

Glycoconjugate vaccines have dramatically reduced the rate of invasive infections due to *S. pneumoniae, H. influenzae* and *N. meningitidis* in children. The inability of pure polysaccharides to induce sustained immune responses has required the development of vaccines containing these potent vaccine candidates conjugated to a carrier protein. Chemical conjugation of the polysaccharides to highly immunogenic modified bacterial proteins used as carriers (such as tetanus toxoid, diphtheria toxoid, crossreactive material 197, CRM197) has been applied to several conjugate vaccines already in the market or in ongoing clinical studies; some examples are reported in the Box 1:

Box1: **Polysaccharide and protein–polysaccharide conjugate vaccines**

***Haemophilus influenzae* type b vaccines**

Polysaccharide vaccines for *Haemophilus influenzae* type b (Hib) were first used in 1985 but were rapidly replaced in 1989 by protein–polysaccharide conjugate vaccines containing the Hib polysaccharide polyribosyl ribitol phosphate chemically conjugated to a protein carrier, such as diphtheria toxoid, tetanus toxoid or meningococcal outer membrane protein. These vaccines continue to be widely used either alone or in combination with other vaccine antigens for the prevention of Hib-mediated disease, mainly among preschool children.

Pneumococcal vaccines

A vaccine containing 14 different polysaccharides from *Streptococcus pneumoniae* was licensed in the United States in 1977 and was replaced by a 23-valent vaccine in 1983 for the prevention of pneumococcal disease in the elderly. This vaccine was first introduced in the United Kingdom in 2003 for universal immunization of adults aged over 65 years. A protein–polysaccharide conjugate pneumococcal vaccine containing seven serotypes was first used in the United States in 2000 (and in the United Kingdom in 2006). In this vaccine, the carrier

protein is crossreacting material 197 (CRM197; which contains a glycine to glutamic acid point mutation at position 52 in the A subunit of diphtheria toxoid). Two new conjugate vaccines are in development, one containing 10 polysaccharides (conjugated to protein D from *H. influenzae)* and another with 13 serotypes (conjugated to CRM197).

Meningococcal vaccines

A quadrivalent meningococcal vaccine containing serogroup A, C, Y and W135 polysaccharides of *Neisseria meningitidis* was first licensed in the United States in 1981, and a bivalent A plus C vaccine is also available in some countries. The polysaccharide of serogroup A is *N*-acetyl mannosamine-1-phosphate, that of serogroup C is α2-9 *N*-acetyl neuraminic acid (NANA), that of serogroup Y is a co-polymer of NANA with glucose and that of serogroup W135 is a co-polymer of NANA with galactose. Serogroup C (MenC) conjugate vaccines were first used in the United Kingdom in 1999 (conjugated to either tetanus toxoid or CRM197), and a quadrivalent A, C, Y and W135–diphtheria toxoid conjugate vaccine has been available in North America since 2005. Several new combination conjugate vaccines are in development, including A, C, Y and W135–CRM197 and A, C, Y and W135–tetanus toxoid.

This thesis focuses on the development of a glycoconjugate vaccine against Group B streptococcus (GBS). GBS is a leading cause of morbidity and mortality in the neonate in US and globally; its infection gives bacteremia and sepsis in the first week of life as well as meningitis beyond the first week of life. Until now, no vaccine exists to prevent this disease; since 1996, CDC recommended intrapartum antimicrobials for women identified with GBS colonization, but the development of a preventive vaccine would be highly recommended.

The most cost-effective and potentially lasting method of preventing invasive group B streptococcal infections in all age groups is active immunization (Mohle-Boetani, JC. 1993). The decline in the incidence of early-onset GBS disease in neonates that has been associated

with the widespread use of maternal intrapartum antibiotic prophylaxis could also be associated with the emergence of antibiotic-resistant organisms (Pearlman, MD. 1998; Schrag, SJ. 2000).

Phase 1 and phase 2 trials in healthy adults have tested conjugate vaccines for the five serotypes of GBS that account for an estimated 98% of invasive disease cases in the United States. Further, successful preclinical studies of GBS types VI and VIII conjugate vaccines (serotypes prevalent thus far only in Japan) suggest the ability, if necessary, to extend vaccine coverage (Paoletti, LC. 1999)

Vaccines against invasive GBS disease must be safe and sufficiently immunogenic to evoke protective and durable concentrations of GBS-specific antibodies.

A good vaccine has to be characterized by immunological memory and persistence of antibodies. In this thesis we have focused our attention mainly on the characterization of immunological memory to a glycoconjugate vaccine, since only few studies have analyzed it so far. Memory B cells (MBC) are key players to provide a faster antibody response upon antigen-re-exposure, to induce generation of class-switched plasma cells secreting high-avidity antibodies and to maintain protective antibody levels over time.

In this work two assays, serial limiting dilution (sLDA) and ELISpot, have been compared to measure frequencies of MBC against the carrier and the polysaccharide component of the vaccine, in order to define the best tool to measure immunological memory. Our results suggest that both methods can be used to assess low frequencies of memory B cells, such as the polysaccharide specific-memory B cells.

sLDA is more laborious, but offers the advantage of measuring simultaneously in the B cell culture supernatants the frequency of carrier (CRM) and polysaccharide (PsIII) memory B cells.

On the other hand, ELISpot is the most widely used method to assess frequencies of MBC or plasmacells. The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual antibody secreting B-cells (Slifka, MK. 1996). When the ELISpot method is used, the created spots on the membrane show an imprint of those cells originating them, therefore giving the advantage of providing an image of the single antibody secreting cells producing antibodies specifically binding to the antigen coated on the membrane of the well. The other advantage of this method is the fact that the spots are long lasting, and they can be evaluated visually as well as by means of image analysis. Unfortunately, the procedure might present low sensitivity and reproducibility due to variable background intensity problems and difficulties in separating true from false spots. We have therefore attempt to improve the standard method, named in this thesis as “Direct”, by reverting the principle of the assay and setting up the so called “Reverse ELISpot”.

We have set up and compared the two different of ELISpot methods: the “Direct” and the “Reverse”. The principle at the basis of the “Direct” ELISpot is that it allows to capture the antibodies secreted by each antigen-specific B cell through their direct binding to the antigen coated on the membrane of the plates. Otherwise, the principle at the basis of the “Reverse” ELISpot is that all the immunoglobulins secreted by the B cells are captured on the membrane of the well on the ELISpot plate by interacting with anti-mouse Ig polyclonal antibodies coating the wells of the plate. Antigen specific antibodies are then revealed by binding to a tagged antigen. As shown in the results, for the detection of the carrier protein CRM specific MBC, the

“Reverse” assay is the only one that allows to see spots clearly defined in size and shape; while when measuring GBS-Ps MBC comparable results are obtained in both ELISpot assays. Unfortunately ELISpot has some limitations, since, differently by sLDA, it does not allow simultaneous analysis in the same well of carrier and Ps specific antibodies produced by MBC. We have therefore choosen sLDA for the analysis of MBC response in mice vaccinated with CRM-GBS Ps glycoconjugate.

The development of immunological memory can be positively influenced by the presence of adjuvants during the vaccination protocol. Adjuvants represent an important component of many modern vaccines, as they increase the immunogenicity of co-administered antigens such as purified, soluble recombinant proteins, which are “per se” less immunogenic than whole or split, killed or attenuated pathogens used in the past. There is still a debate ongoing on the role of adjuvants in increasing the response to Ps. Although a number of glycoconjugate vaccines induce high antibody titers without adjuvants, in many cases adjuvants are used to induce a protective immune response.

As far as GBS is concerned, the majority of published studies on human clinical trials have described the use of conjugate vaccines without an adjuvant. In one study however, subjects received a TT-GBS Ps III glycoconjugate vaccine in which the vaccine was adsorbed with aluminum hydroxide. This did not enhance the immune response to either the polysaccharide or the tetanus component of the vaccine (Paoletti, LC, 2001).

We have here demonstrated that in naive mice MF59 induces a significant increase of the IgG memory B cell response to both components of the glycoconjugate vaccine CRM and GBS-PsIII as compared to mice receiving the vaccine with Alum or plain. The response to the vaccination has been analyzed also in terms of circulating IgG and IgM specific antibodies

present in sera. On the basis of the antibody titers measured, is relevant that MF59 and Alum significantly increase the IgG serum antibody response to CRM and PsIII as compared to mice receiving the plain vaccine. Titers of circulating antigen-specific IgM are also influenced by the presence of adjuvant in the vaccine, but IgM are induced only in response to the polysaccharide component of the glycoconjugate vaccine. No detectable IgM antibodies have been measured against the carrier protein CRM.

Since, as reported by Paoletti et al., there is no clear-cut effect of Alum in enhancing the immune response to the polysaccharide in adults, based on the results we obtained in animals we can speculate two possible explanations. On one hand, MF59 instead of Alum plays a major role in increasing the memory B cell response and the circulating antibody levels to the polysaccharide component of the vaccine. On the other hand, the results obtained in clinical trials reflect the non-immunological naivety of human adults, in particular to the protein component of the glycoconjugate vaccine. This, as a consequence, may favor in humans the response to the vaccine, also in absence of adjuvant.

In case of neonatal immunization, the perspective is completely different. It is well known that vaccination in early infancy is not ideal, as the immune response at this age is generally of low magnitude and memory is more difficult to establish, leading to low persistence of protective antibody levels. One clear example comes from studies performed to analyse the memory and antibody response to MenC vaccination in different age groups. Antibody titers induced by infant immunization, even after three doses of MenC glycoconjugate vaccine, do not persist well; antibody levels fall below the protective threshold in 50% of infants by 1 year of age, and only as few as 12% of vaccinated infants have persistent seroprotection by 4 years of age (Snape, MD. 2005). Vaccination induces sustained levels of protective

antibody in a high proportion of vaccinees only much later in childhood or in adolescence (Snape, MD. 2008).

These observations can bring up adjuvants as a tool to increase the immunogenicity of glycoconjugate vaccines in infants, leading also to a better persistence of protective antibody levels and therefore to a better vaccine effectiveness.

Since, as we mentioned above, the non-immunological naivety of the adult human population might have an effect in the response to the glycoconjugate vaccine, we next evaluated the influence of a pre-exisiting immunity to the carrier protein CRM, in the development of the memory B cell and the IgG antibodies response to subsequent vaccinations with the glycoconjugate vaccine.

By our results, priming with CRM has such an effect on the subsequent vaccination with the glycoconjugate vaccine, that the presence or absence of the adjuvant in the vaccine formulation becomes less relevant in affecting the immune response to both components of the vaccine. Therefore, in primed mice MBC and antibody IgG responses to the GBS polysaccharide III are similar when the glycoconjugate vaccine is administered with or w/o adjuvants. Overall, the frequencies of MBC and the level of circulating polysaccharide specific antibodies are significantly increased as compared to naive mice receiving the same formulation of vaccine. This remarkable advantage of being primed to the carrier protein is less evident when the vaccine is administered with MF59 as adjuvant. In this case naive and primed mice develop an immune response to the glycoconjugate vaccine of similar magnitude. This result can be explained with the strong adjuvant effect that MF59 has already in naive mice, in significantly increasing their capacity to respond to both Ps and CRM components of

the vaccine. Therefore, MF59 does not provide any further advantage to mice receiving priming with CRM as compared to naive mice receiving the same vaccine.

Priming with CRM brings also other advantages in response to the polysaccharide. We have observed that good anti-polysaccharide antibody titers are reached already after two doses of vaccine, either in presence or absence of adjuvant. Naive mice, instead, require adjuvant and three doses of the vaccine in order to develop anti-Ps antibody titers comparable to those observed in primed mice. To our knowledge this study is the first evidence of the positive effect of being primed to the carrier protein in the development of a MBC response to the polysaccharide after GBS-glycoconjugate vaccination, also in absence of adjuvants. It also highlights that being primed to the carrier protein requires less vaccine doses to develop higher frequencies of MBC and good levels of anti-polysaccharide circulating antibodies.

Other studies have described the impact of coadministration of glycoconjugate-vaccines with different carriers on the enhancement of the antibody response to the polysaccharide. The literature reports that the best recognized form of immune enhancement occurs when vaccines using tetanus toxoid (TT) as the conjugate protein are coadministered with Hib-TT. Anti-PRP (polyribosyl-ribitol-phosphate) antibody concentrations increase when Hib-TT is coadministered with meningococcal serogroup C-TT conjugate (MenC-TT), or 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV) that includes 18C-TT (Kitchin, NRE. 2007; Southern, J. 2006; Tejedor, JC. 2006; Schmitt, H. 2007; Knuf, M. 2009). Immune enhancement mechanisms also contribute to optimal responses to vaccines that use DT and CRM197 as conjugate proteins. In general, CRM197 and TT when engaged as carrier lead to higher anti-polysaccharide responses than DT, as exemplified by Hib-TT/CRM197 versus Hib-DT (Bulkow, LR. 1993), and meningococcal

ACWY-TT/CRM197 versus ACWY-DT (Rennels, M. 2004; Snape, MD. 2008; Ostergaard, L. 2009). It has also been demonstrated that DT/CRM197 glycoconjugates maximize their antibody immune response in presence of a DT/CRM197 priming, that probably contributes to a more efficient T-cell help (Granoff, DM. 1994; Shelly, MA. 2001; Granoff, DM. 1993).

Unlike TT, whole cell pertussis (Pw) antigen has an adjuvant effect on TT, but not DT, resulting in enhanced responses to conjugate vaccines using TT as carrier. The effect of DTPw vaccines on the immunogenicity of coadministered protein conjugate vaccines in clinical trials was recently reviewed by Dagan et al (Dagan, R. 2010).

Clearly, more studies are needed to address the effect of adjuvants, in particular in immunologically naive backgrounds, at increasing the development of a persistent immune response to polysaccharides. Adjuvants could be useful in the development of a GBS vaccine targeting infants or children in their first years of life, where is important to increase the persisting antibodies to the polysaccharide that are generally low due to the immaturity of their immune system.

Furthermore, since new and different carriers are now under evaluation for the development of novel glycoconjugate vaccines, a better understanding of the effect of being primed to the carrier protein becomes fundamental in the analysis of the immune response to the vaccine. This might direct the choice of the best carrier protein to be used for the glycoconjugate vaccine. Beyond Tetanus toxoid and CRM 197, other candidate proteins have been used already in animal studies but until now, none of them have yet progressed to human trials. Therefore, the effect of being primed to the target carrier protein should be extensively evaluated, in particular considering that until now the ideal timing of administration

of a GBS vaccine is considered to be early in the third trimester of pregnancy or during adolescence.

In the last five years, many human trials with GBS conjugate vaccines have been completed. These include trials in pregnant women, trials with trivalent conjugate vaccines and trials of persistence of antibody to 2 years post vaccination. This activity is grounds for considerable optimism and suggests that a suitable vaccine might be developed and trialed over the next 5 years. Coupled with this, recent recommendations for routine immunization during pregnancy with H1N1 and other influenza vaccines in many countries suggest that this approach might be now more acceptable. In the next 5 years is likely to see further Phase II human studies with capsular conjugate vaccines, monovalent and polyvalent, with carrier proteins other than tetanus, studies of their immunogenicity and reactogenicity in pregnant women and increased efforts to educate the general public (including pregnant women) regarding GBS and the potential for its prevention through vaccination. There is a real possibility that such efforts might now lead to a Phase III clinical trial to assess the safety and efficacy of a GBS conjugate vaccine in pregnant women.

MATERIALS AND METHODS

4.1 Mice

Female BALB/c mice of 6 weeks were purchased from Charles River Laboratories and maintained in the Novartis Animal Facility (Siena, Italy). Animal studies were conducted in accordance with Italian laws regarding animal protection and with European Community Council Directive 86/609/EEC for the protection of animals used for experimental purposes. All experiments were approved by the local Institutional Animal Care and Research Advisory Committee and authorized by the local government.

4.2 BALB/c mouse immunizations

In order to evaluate the effect of different adjuvants in increasing the memory B cell response in a glycoconjugate vaccine against GBS; BALB/c mice were immunized intraperitoneally (i.p.) with three doses of CRM-GBS –PsIII conjugated vaccine (Vaccine) with or without adjuvants (Alum/MF59); negative control mice received three doses of Alum or MF59 or PBS alone. Sera was obtained 14 days after the second dose and 21 days following the final immunization to evaluated the IgG and IgM antibodies response versus the carrier CRM e the GBS- PsIII by ELISA assay. Then to evaluate the MBC response, spleens have been taken from 20 to 30 days after the last dose (Fig 15).

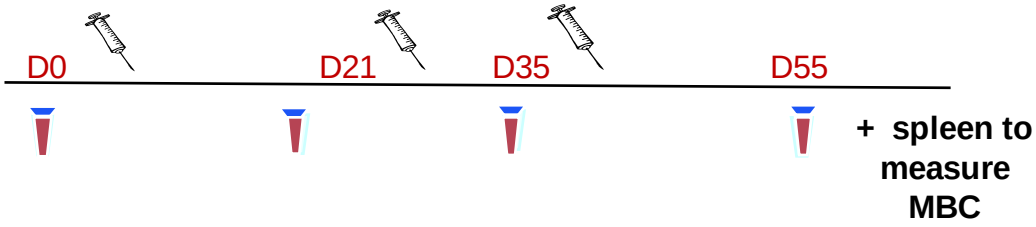

Fig 15: Example of a schedule of vaccination where BALB/c mice have been immunized three times with CRM-GBS PsIII conjugated vaccine with or without adjuvants (Alum or MF59).

To investigate the influence of a pre-existing immunity to the carrier protein CRM on the GBS-PsIII response in terms of MBC; BALB/c mice were primed with CRM+Alum/MF59 or Alum/MF59 and then immunized 2 times with the CRM-GBS PsIII conjugated vaccine with or without adjuvant (Alum or MF59). Sera were obtained 21 days after priming and 21 days after the first and the second dose to measured the antibody response against the carrier CRM and the GBS PsIII by ELISA assay. To evaluate the MBC response, spleens have been taken from 20 to 30 days after the last dose (Fig 16). All animals were treated in accordance with institutional guidelines.

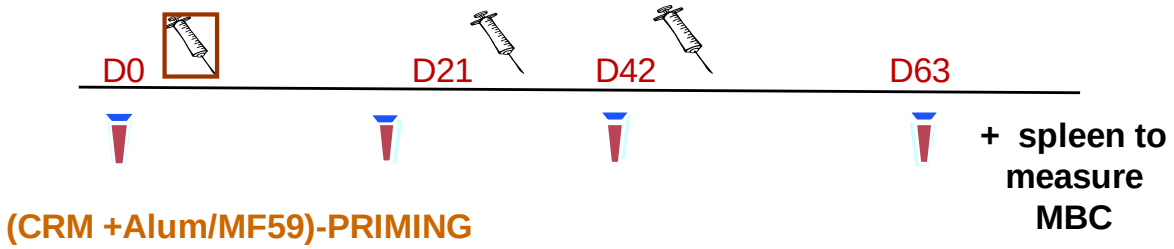

Fig 16: Example of a schedule of vaccination where BALB/c mice have been primed with CRM + Alum/MF59 or PBS as control and then immunized two times with CRM-GBS PsIII conjugated vaccine with or without adjuvant (Alum or MF59).

4.3 Splenocytes preparation

Spleen are taken from donor BALB/c mice from 20 to 30 days after the last immunization and splenocytes were prepared by homogenization than washed and diluted in complete medium (RPMI with 100 units/mL of penicillin,100 µg/mL of streptomycin, 2 mM of L-glutamine, nonessential amino acids, sodium pyruvate and 0.5 mM of β-mercaptethanol) with 5% Fetal Bovine Serum (FBS) (Hyclone).

4.4 Enumeration of CRM and GBS-PsIII-Specific Memory B Cells (MBC) by Serial limiting dilution assay (sLDA)

Frequencies of MBC were determined by the ELISA-coupled serial-limiting dilution assay. Splenocytes were plated in 0.2 ml of RPMI with 5% FBS in serial 2-fold dilutions, 6 replicates per dilution, starting from $8X10^5$ cells/well, in 96-well U-bottom plates containing 5 µg/ml of a phosphorothioate CpG oligonucleotide (ODN 1826) and 1000 units/mL of rhIL-2 (Proleukin, Novartis). Parallel control cultures of splenocytes were run in medium alone. On day 10, individual supernatants were collected and kept at -20 °C until tested by ELISA assay for their content in CRM and GBS PsIII-specific and total IgG. ELISA assays were run on Maxisorp plates (Nunc) pre-coated with either CRM 197 and GBS- PsIII (1µg/ml in PBS, pH 7.5), or a polyclonal anti-mouse IgG (Sigma) (5 µg/mL in PBS, pH 7.5) ON at +4°C. Then plates are saturated with 1% BSA for 1 h at +37°C. Supernatants are added at 1:2 dilution and incubated for 2 hours at +37°C. Antibodies are revealed with an alkaline-phosphatase-conjugated anti-mouse IgG (1:4000 in PBS, 0.05% Tween, 3% BSA), followed by incubation

with the substrate p-nitrophenylphosphate (pNPP) (Sigma). Using the OD values obtained from ELISA results, the number of wells that were positive and negative for the presence of antigen-specific antibodies was determined for each cell dilution of the sLDA plate. The OD obtained from the non-stimulated cultures served as a cut-off in order to define the positive and negative culture wells for antigen-specific antibodies. Without polyclonal stimuli, memory B cells are not expected to differentiate into plasma cells and, therefore, the antibodies detected in non-stimulated culture wells correspond to pre-existing antigen-specific plasma cells. To eliminate the “background” antibodies from pre-existing antigen-specific plasma cells, the cut-off for selecting positive culture wells was calculated as the mean OD value of all supernatants collected from non-stimulated culture wells plus three standard deviations. On the basis of the non-stimulated control OD values obtained in three different experiments, the cut-off values for positivity have been fixed at OD=0.20 for the measurement of total IgG, OD=0.47 for CRM specific IgG and OD=0.32 for GBS- PsIII specific IgG.

The splenocytes dilution containing one antibody-secreting cell precursor was derived by applying the Reed and Muench algorithm (Reed, LJ. 1938, 123) to the distribution of antibody positive and -negative wells among replicates. Frequencies of CRM and GBS PsIII-IgG secreting cell precursors (CRM-IgG MBC and PsIII-IgG MBC) were expressed as percentage of the total IgG MBC precursors measured.

4.5 “Direct” ELISpot assay to detect memory B cell responses

In the “Direct” ELISpot, 96 wells ELISpot plates (Millipore MultiScreen HTS-HA Billerica, MA, US) were coated with 100 µl/well of PBS containing human serum albumin (HSA), CRM (at 5 µg/ml), GBS PsIII-HSA (at 10 µg/ml) and 5 µg/ml of goat Anti-mouse Ig (SBA cat 1010-01), for 16-20 hrs at +4°C. Unspecific binding sites were blocked with 200 µl of PBS containing 10% FBS for 2 hrs at room temperature.

To enumerate antigen specific memory B cells, splenocytes were cultured for 5.5 days at +37°C, 5% CO_2 in complete medium containing 5 µg/ml of phosphorothioate CpG oligonucleotide (ODN 1826) and 1000 units/mL rhIL-2 (Proleukin, Novartis).

Suspensions of 4-8 x 10^6 splenocytes in complete medium were seeded in triplicate wells and serially diluted 2-fold up to the 7^{th} dilution, in a final volume of 100 µL/well.

Plates were incubated at +37°C, 5% CO_2 for 20 hours before stopping the assay by extensive washing with PBS 0.05% Tween 20 (Sigma). Spots of antibody secreting cells were revealed by adding 100 µl/well of PBS containing Biotinylated Anti-mouse Ig antibody (BD Pharmingen cat.553999) and 1% of bovine serum albumin (Sigma Aldrich). After 2 hours, plates were washed and further incubated for 30 min with 100 µL/well of PBS containing 1.25 µg/ml of horse radish peroxidase (HRP)-conjugated streptavidin (Endogen, Cambridge, MA, US).

Plates were then stained in the dark with the HRP substrate AEC kit (Sigma) for 40 minutes and then extensive washings with de-ionized water. Antigen-specific and total Ig antibody-secreting cells were enumerated using the CTL Immunospot S5 UV Analyzer (CTL Europe, Bonn, Germany).

4.6 “Reverse” ELISpot assay to detect memory B cell responses

In the “Reverse” ELISpot, 96 wells ELISpot plates (Millipore MultiScreen HTS-HA Billerica, MA, US) were coated with 100 µl/well of PBS containing 5 µg/ml of goat Anti-mouse Ig (SBA cat 1010-01) for 16-20 hrs at 4°C. Unspecific binding sites were blocked with 200 µl of PBS containing 10% FBS for 2 hrs at room temperature.

To enumerate antigen specific memory B cells, splenocytes were cultured for 5.5 days at +37°C, 5% CO_2 in complete medium containing 5 µg/ml of phosphorothioate CpG oligonucleotide (ODN 1826) and 1000 units/mL rhIL-2 (Proleukin, Novartis).

Suspensions of 4-8 x 10^6 splenocytes in complete medium were seeded in triplicate wells and serially diluted 2-fold up to the 7^{th} dilution, in a final volume of 100 µL/well.

Plates were incubated at 37°C, 5% CO_2 for 20 hours before stopping the assay by extensive washing with PBS 0.05% Tween 20. Spots of antibody secreting cells were revealed by adding 100 µl/well of PBS containing biotynilated antigens like GBS PsIII (5µg/ml), CRM and HSA (0.5µg/ml both) and 1% of bovine serum albumin. After 2 hours plates were washed and further incubated for 30 min with 100 µL/well of PBS containing 1.25 µg/ml of horse radish peroxidase (HRP)-conjugated streptavidin.

Plates were then stained in the dark with the HRP substrate AEC kit for 40 minutes and then extensive washings with de-ionized water. Antigen-specific and total Ig antibody-secreting cells were enumerated using the CTL Immunospot S5 UV Analyzer (CTL Europe, Bonn, Germany).

4.7 ELISA assay for the detection of CRM and PsIII specific mouse IgG and IgM antibodies

ELISA assay was performed using sera from single mice in each group. Flat bottom, Maxisorp 96 well plates (Nunc, Thermo Scientific) were coated with CRM and GBS-PsIII-HSA (1 µg/ml both) and incubated at 4°C overnight (ON). Plates were washed with PBS containing 0.05% Tween-20, and blocked with 1% bovine serum albumin (BSA) (Sigma). Following washing as described, plates were incubated for 2 hr at +37 °C, with 8-fold serially diluted sera in 0.1% BSA in PBS with 0.05% Tween-20. Plates were washed and incubated for 2 hr at 37 °C with anti-mouse IgG or anti-mouse IgM, both alkaline phosphatase conjugate (SBA) followed by development with pNPP liquid substrate (Sigma) for 40 min. OD values were determined at 405/540 nm by spectrophotometry.

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