Polysaccharide-induced hyporesponsiveness

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Ritgerð til meistaragráðu í Líf og læknavísindum
Háskóli Íslands
Læknadeild
Heilbrigðisvísindasvið

HÁSKÓLI ÍSLANDS

Fjölsykrumiðluð skerðing á mótefnasvari

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Ritgerð til meistaragráðu í Líf og læknavísindum

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Ágrip

Nýburar og ungbörn hafa skerta ónæmissvörun. Sá aldurshópur sem *Streptococcus pneumoniae* sýkir mest eru börn yngri en 2 ára. Fjölsykrur hjúpa *Streptococcus pneumoniae* og hafa verið nýttar í bóluefni. Þar sem fjölsykrur vekja ekki ónæmissvar í börnum yngri en 2 ára hafa pneumókokkafjölsykrur verið tengdar við prótein, og eru slík bóluefni (e. conjugate vaccines) ónæmisvekjandi í ungbörnum og vernda þau gegn alvarlegum pneumókokkasjúkdómum. Þegar bólusett er með hreinni fjölsykru eftir bólusetningu með sömu fjölsykru tengdri við prótein getur ónæmisvarið skerst. Við höfum áður sýnt að endurbólusetning með hreinni pneumókokkafjölsykru (PPS) af gerð 1 skerðir PPS-1-sértækt mótefnasvar og fækkar mótefnaseytandi frumum í milta og beinmerg sem hafa myndast við frumbólusetningu með próteintengdu fjölsykrubóluefni (PCV) af sömu hjúpgerð í nýburamúsum.

Markmið rannsóknarinnar var að athuga hvort 23-gilt fjölsykrubóluefni (PPV23) sem er skráð fyrir notkun í mönnum hafi bælandi áhrif á svör nýburamúsa sem hafa verið bólusettar með tíugildu PCV (PCV10). Jafnramt að kanna hvort frumbólusetning með PPV23 skerði myndun á PPS-sértæku mótefnasvari og fjölda mótefnaseytandi frumna í milta og beinmerg þegar endurbólusett er með PCV10. Einnig hvort áhrif PPV23 á ónæmissvör nýburamúsa gegn PCV10 væru háð magni PPV23 fjölsykra.

Nýburamýs sem voru frumbólusettar með PCV10 og endurbólusettar með PPV23 höfðu lægra sértækt mótefnasvar gegn öllum PPS hjúpgerðum sem mælt var fyrir (PPS1, PPS4, PPS9V, PPS14 og PPS18C) í samanburði við mýs sem voru frumbólusettar með PCV10 og endurbólusettar með saltvatni. Einnig höfðu þær lægri mótefni gegn 4 af 5 PPS (ekki PPS4) en mýs sem voru frum- og endurbólusettar með PCV10.

Nýburamýs sem voru frumbólusettar með PPV23 og endurbólusettar með PCV10 höfðu lægri sértæk mótefni gegn 4 af 5 PPS sem mælt var fyrir (PPS1, PPS4, PPS9V og PPS18C) en mýs sem voru frumbólusettar með saltvatni eða PCV10 og endurbólusettar með PCV10, á einhverjum tímapunkti og við einhvern PPV23 styrk sem var prófaður. PCV10 náði að hluta til að yfirvinna skerðingu mótefnasvars gegn PPS9V og PPS18C með tímanum. PPV23 frumbólusetning lækkaði einnig fjölda PPS-sértækra mótefnaseytandi frumna í milta og beinmerg miðað við fjöldann í músum sen voru frumbólusettar með saltvatni eða PCV10. Áhrif PPV23 frumbólusetningar á svörun við PCV10 voru háð magni fjölsykranna sem frumbólusett var með.

PPV23 hafði þannig hamlandi áhrif á mótefnasvar gegn öllum fjölsykrum sem mælt var fyrir við bólusetningu með PCV10, bæði þegar bólusett var með PPV23 eftir og fyrir bólusetningu með PCV10. Frumbólusetning með PPV23 olli einnig fækkun PPS-sértækra mótefnaseytandi frumna sem mynduðust við endurbólusetningu með PCV10, og reyndust áhrifin háð gerð og magni fjölsykranna í PPV23 bóluefninu.

Niðurstöðurnar benda til að óráðlegt sé að bólusetja ungviði með hreinum pneumókokkafjölsykrum.

Abstract

Neonates and infants elicit poor immune responses. This age group is most susceptible to *Streptococcus pneumoniae* infections. Polysaccharides of the capsules that coat *Streptococcus pneumoniae* have been used in vaccines. Because polysaccharides do not elicit immune response in neonates and infants pneumococcal polysaccharides (PPS) have been conjugated to proteins to make so called pneumococcal conjugate vaccines (PCVs). PCVs are immunogenic in neonates and infants and protect them from invasive pneumococcal diseases. When individuals are boosted with a plain polysaccharide it can decrease the response already generated by the same serotype of conjugate vaccine. We have shown that booster with a plain PPS of serotype 1 decreases PPS1-specific antibody response and reduces the number of PPS1- specific antibody secreting cells (AbSCs) in the bone marrow and spleen that were generated by priming neonatal mice with a PCV of serotype 1. The aim of the study was to assess if a 23-valent PPS vaccine (PPV23) that is licensed for human use compromises the immune response to a ten-valent PCV (PCV10). We assessed if a PPV23 booster decreases the PPS-specific antibody response generated by priming of neonatal mice with PCV10 and PPV23 priming decreases the generation of PPS-specific antibody responses and the number of PPS-specific AbSCs in spleen and bone marrow elicited by a PCV10 booster. Furthermore, we assessed whether the interfering effect of neonatal PPV23 priming was dependent on the dose of PPV23.

Neonatal mice primed with PCV10 and boosted with PPV23 had lower antibody response to all PPS measured (PPS1, PPS4, PPS9V, PPS14 and PPS18C) compared to PCV10-primed mice boosted with saline. Their antibody response to 4 of 5 PPS serotypes measured (not PPS4) was also reduced compared to mice primed and boosted with PCV10.

Neonatal mice primed with PPV23 and boosted with PCV10 had lower immune response to 4 of 5 PPS serotypes measured (PPS1, PPS4, PPS9V and PPS18C) at some time point for one of the PPV doses tested compared to mice primed with saline or PCV10 and boosted with PCV10. The reduced antibody response to PPS9V and PPS18C was partially overcome by PCV10 with time. PPV23 caused a decrease in PPS specific-AbSCs in spleen and bone marrow in mice boosted with PCV10 compared to mice primed with saline or PCV10 and boosted with PCV10. The effect of PPV23 priming on response to PCV booster was dependent on the dose of PPV23.

Thus PPV23 interfered with antibody response to all PPS serotypes measured when mice were immunized with PPV23 before or after PCV10 immunization. The impact of PPV23 priming was dependent on the type and dose of the polysaccharides.

The results suggest that PPV23 vaccination in early life should be avoided.

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Abbreviations

AbSC: Antibody secreting cell

Ab: Antibody

AID: Activation induced deaminase

APC: Antigen presenting cell

APRIL: A proliferation inducing ligand

BAFF: B cell activating factor

BCR: B cell receptor

BCL6: B cell lymphoma 6

BLIMP1: B lymphocyte induced maturation protein 1

CCL, CXCL: Chemokine ligands

CCR, CXCR: Chemokine receptors

CR: Complement receptor

CWPS: Cell wall polysaccharide

BM: Bone marrow

Chop: Phosporylcholine

CpG ODN: CpG oligonucleotides

DC: Dendritic cell

DT: Diphteria Toxoid

ELISA: Enzyme-linked immunosorbent assay

ELISPOT: Enzyme-linked immunosorbent spot assay

EU: ELISA units

FDC: Follicular dendritic cell

FCS: Fetal calf serum

GC: Germinal center

HBSS: Hanks balanced salt solution

IFN: Interferon

IPD: Invasive pneumococcal disease

Ig: Immunoglobulin

IL: Interleukin

LPS: Lipopolysaccharide

LT-K63: Non-toxic mutant of *E. coli* heat labile enterotoxin with a serine to lysine substitution at position 63.

MBL: Mannan binding lectin

MenC: Meningococci of serogroup C

MHC: Major histocompability complex

MZ: Marginal zone

NHEJ: Non homologous end joining

NLR: NOD like receptor

NMRI: Albino outbreed mice with the origin from The Naval Medical Research Institute (NMRI), Silver Spring, MD, USA

OMP: Outer membrane protein

OPA: Opsonophagocytosis assay

PAMP: Pathogen associated molecular patter

PBS: Phosphate saline buffer

PC: Plasma cell

PCV: Pneumococcal conjugate vaccine

PD-1: Program cell death protein 1

PNA: Peanut agglutinin

PPS: Pneumococcal polysaccharide

PPV: Pneumococcal polysaccharide vaccine

PRR: Pattern recognition receptor

PS: Polysaccharide

Psp: Pneumococcal surface protein

RLR: RIG-1 like receptor

Rpm: Revolutions per minute

RT: Room temperature

SBA: Serum bactericidal activity

s.c: Subcutaneously

SCS: Sub capsular sinus

SHM: Somatic hypermutation

STAT-1: Signal transducer and activator of transcription 1

TACI: Transmembrane activator and cyclophilin ligand interactor

TCR: T cell receptor

TD: T cell dependent

T_{FH}: T follicular helper cell

TGF: Transforming growth factor

Th1: T helper cell 1

Th2: T helper cell 2

Th17: T helper cell 17

TI: T cell independent

TLR: Toll-like receptor

Tregs: T regulatory cells

TT: Tetanus toxoid

ZPS: Zwitterionic polysaccharide

1 Introduction

1.1 Vaccination and the immune system

Vaccination is one of the most effective and cost effective ways to prevent disease and mortality (1). When an individual is vaccinated protective immunity is created against pathogens. The host immune system recognizes the vaccine as a foreign particle. The dendritic cells (DCs) of the innate immune system are antigen presenting cells (APCs) that react to the microbial components of the vaccine and activate the adaptive immune system, mainly the B- and T lymphocytes. The lymphocytes that recognize the vaccine components respond and specific immunity is created against the vaccine components leading to protection against forthcoming infection from the pathogen the vaccine is directed against (2, 3).

There are two main types of vaccines, live attenuated vaccines and non-replicating vaccines. Live attenuated vaccines are pathogens that have been weakened and are no longer harmful. Live attenuated vaccines mimic natural infection. Replicating live microorganism rapidly disseminates to the host immune system through the vascular network. DCs or other APCs are activated at multiple sites and migrate to lymphoid organs and present antigens to T and B cells, thus initiating adaptive immune response (4). Live attenuated vaccines create long lasting cellular and humoral immunity. In many natural infections protective immunity is not established and thus does not protect against reinfection, thus live attenuated vaccines must work better than infection (reviewed in (5)). Non replicating vaccines include subunit vaccines like proteins, polysaccharides, glycoconjugates or killed microorganisms. In the absence of replication the vaccines activate local innate response at the site of vaccination, thus only local APCs are activated. Adjuvants are often needed to enhance the response to a non replicating vaccine. Licensed adjuvants are Alum, which is by far most commonly used, MF59, AS04 and AS03 (5, 6).

There are many immune factors that contribute to protection against infections and can potentially be used as correlates of protection. The titer of neutralizing antibodies (Abs) against vaccine antigens, in particular viral vaccines, often correlates with protective efficacy of the vaccine (2, 7). Although Ab titer often correlates with protection, vaccination with antigens may elicit high Ab response that results in low protective efficacy. Vaccination of children with meningococcal C (MenC) polysaccharide is an example where the Ab level does not correlate with protection (8). The vaccination elicits Ab response but protective efficacy is low. In that case the protective efficacy correlates with the bactericidal function of the Abs, which is measured by serum bactericidal activity (SBA). SBA measures the capacity of serum Abs to mediate direct complement mediated killing of the bacteria, in particular Gram negative bacteria. SBA titer is reciprocal dilution of serum needed to kill 50% of

bacteria. Quality of the Ab and its functional capacity matters more in protection against meningococci than Ab levels. Abs can protect against bacterial infection by opsonization and complement activation leading to opsonophagocytosis and killing of the bacteria, in particular Gram positive bacteria, by phagocytic cells, neutrophils and macrophages. Opsonophagocytosis assays (OPA) are used to assess the ability of serum Abs to kill the bacteria through opsonophagocytosis. Opsonization is the coating of bacteria with factors, antibodies and complement that facilitate phagocytosis. OPA is used as a correlate of protection for pneumococcal vaccines. Other assays that measure functional activity of antibodies include hemagglutination assays that measure the ability of antibodies to inhibit hemagglutination of viruses and neutralization assays that measure Abs neutralizing capacity of e.g. toxins or viruses (5, 7). For some viral vaccines, cell mediated immune factors may correlate better with protective efficacy than levels or functional capacity of Abs (9).

Immunological memory is necessary for long term protection against pathogens (10). Ab mediated immunity or humoral immunity involves the generation of memory B cells and long-lived plasma cells (PCs). The measurement of cells that continuously secrete ab is called Ab secreting cells (AbSCs). Abs decline rapidly after vaccination and up to half of vaccinees can be seronegative five years after vaccination (11). Despite the loss of Abs, B cell memory lasts longer and the protective efficacy can still persist although the individual is seronegative (12, 13). Generation of long-lived PCs residing in the bone marrow (BM) that continuously secrete Abs is the other hallmark of immunity based on protection by Abs. For some vaccines long-lived PCs do not persist while for others they survive in the BM lifelong. Persistence of Abs varies between antigens and vaccines, thus the replenishment or survival of long-lived PCs varies between vaccines (13).

Many questions are unresolved regarding the persistence of Abs, but the aim of our study was to understand the impact of a plain polysaccharide on the persistence and magnitude of Abs in serum and of long-lived AbSCs in BM and spleen.

1.2 The immune system – overview

The immune system has been developed to fight different types of pathogens by diverse mechanisms, depending on the nature of the infective organism. The immune system is divided into the innate and the adaptive immune system. After the pathogen has breached anatomic barriers like the skin and mucosal membranes, innate immune mechanisms are initiated. First defenses include soluble molecules present in blood, extracellular fluid and epithelial secretion that kill pathogens or weaken their effects. Antimicrobial enzymes digest bacterial cell wall while antimicrobial peptides lyse bacterial cell membranes and the complement system lyses pathogens and marks them for phagocytosis by neutrophils and macrophages (14, 15).

The innate system recognizes pathogen associated molecular patterns (PAMPs) through germline encoded receptors known as pattern recognition receptors (PRRs). Various PAMPs are widely expressed by pathogens. Different classes of PRRs have been identified, the Toll-like receptors (TLR), the NOD-like receptors (NLR), RIG-I-like receptors (RLRs) and the manifold cytosolic DNA sensors (15, 16). These receptors are activated by conserved microbial molecules, bacterial virulence factors and endogenous molecules released after tissue damage. When activated most PRRs induce secretion of large amounts of inflammatory mediators, such as cytokines and chemokines. The cytokines stimulate immune cells and non immune cells, activate acute phase response, recruit neutrophils and control the adaptive immunity. The innate immune system acts as the early phase of the immune response, while the adaptive immunity plays a key role in destroying the pathogens in the late phase of the infection and in creating an immunological memory (17-19).

The complement system is activated through three different pathways. Activation of each pathway occurs by binding the recognition unit and thus activating the pathway (14, 20, 21). The classical pathway is initiated when C1q binds to antibodies (Ab) that are bound to antigens. The lectin pathway is activated when mannan binding lectin (MBL) or ficolins bind ligands on microbes, which usually are carbohydrates. The alternative pathway is constantly activated by slow and continuous turnover of C3. Independent of the complement activation all three pathways form C3 convertase (C4b2a and C3bBb) which leads to cleavage of C3 into C3b and C3a. The end product of the complement system is the membrane attack complex C5b-C9, also called the lytic complex, which lyses bacteria. C3a and C5a are chemotactic factors that recruit phagocytic cells and promote inflammation and C3b and C4b are opsonins (coat pathogen so they are more actively taken up by macrophages) that are critical for effective opsonophagocytosis (20).

APCs form the bridge between the innate and adaptive immune response. APCs consist of DCs, macrophages and B cells. APCs are the cells that express major histocompatibility complex (MHC) class II and present an antigen on its surface which helper T cells bind to through their T cell receptor (TCR). Macrophages and DCs play critical role in innate immune system as they recognize, phagocytose and degrade the pathogens/antigens but as well activate the adaptive system and form the bridge between the innate response and the adaptive response against pathogens (15, 22). Helper T cells serve many roles and are classified through their different activities. Helper T cells are CD4+ CD8- cells that can be grouped into T helper type 1 (Th1), T helper type 2 (Th2), T helper type 17 (Th17), and T follicular helper (T_{FH}) cells and T regulatory cells (Tregs). Th1 cells secrete interferon (IFN)γ and activate infected macrophages and help B cells to class switch to IgG2. Th2 cells provide help for B cells mainly in switching to IgE and IgG1. Th17 cells enhance neutrophil

response and promote barrier integrity. T_{FH} cells help B cells in follicles and enhance isotype switching and Ab production. Tregs control and inhibit adaptive immune response. The different T helper cell subsets mediate their functions through cytokine production and co-stimulation signaling (23-27).

Immunity against pathogens is developed by clonal expansion of lymphocytes bearing/expressing antigen recognizing/specific receptors that have been generated with gene rearrangement during early developmental stage (reviewed in (28)).

1.2.1 Humoral immunity

Humoral immunity refers to immunity provided by antibodies (Abs). Abs are the secreted version of the surface bound B cell receptor (BCR), also called immunoglobulins (Igs). Any antigen can be a target of an Ab response and response to a single epitope may consist of different Ab molecules, with different specificity and affinity for the epitope. Abs are central mediators of humoral immunity. They provide direct neutralization, opsonization and inhibition of pathogens and pathogen derived products and recruit molecular and cellular immune effectors to eliminate a threat. There are five main Ab classes in mammals, IgA, IgD, IgE, IgG and IgM. They differ in the constant region of the heavy chain resulting in different biological functions, binding to different receptors, ability to neutralize the antigen, activate the complement system and opsonize pathogens (reviewed in (29) and (30)).

B cell responses can be of two types, response to T cell dependent (TD) antigens and response to T cell independent (TI) antigens. Antigens unable to induce Ab response in animals that lack T cells are TD antigens, whereas antigens able to induce Ab response in those animals are TI antigens. B cell responses to TD or protein antigens require antigen-specific T cell help. TI antigens are not enzymatically processed and presented in MHC class II (31).

PCs are the main source of Abs and are divided into two pools. Short-lived PCs secrete low affinity IgM and form extra follicular foci in the spleen or lymph node and die within 3-5 days (32). Long-lived PCs secrete high affinity isotype-switched Abs. They reside in the BM and can persist for years (33). Long-lived PCs mainly arise in response to TD antigens but can arise in response to TI antigens and independently of B cell maturation in germinal centers (GCs) (34). Plasmablasts are activated B cells that still undergo cell cycle and secrete Abs and are a kind of intermediate between B cells and PCs (35). Memory B cells are the other key components of long-lived humoral immunity and are mainly created in response to TD antigens through the GC reaction. They have a long lifespan and are the cause of recall response to a booster immunization (36-38).

Variability in the BCR of naive B cells is generated by rearrangement of gene segments encoding the antigen binding region of the BCR. The genes are arranged with one of each of the multiple variable, diversity and joining gene segments in the heavy chain and one of each of multiple variable and joining gene segments in the light chain. Different combination of these gene segments results in antigen binding diversity (reviewed in (39)).

Naive B cells consist of four classes, follicular B cells, marginal zone (MZ) B cells, B1a and B1b cells that differ by their developmental program, phenotype, location and function. B1a and B1b cells are mostly located in serous cavities (pleural and peritoneal) but are seldom seen in lymphoid tissues (40), follicular B cells recirculate among the B cell follicles in the secondary lymphoid tissues and MZ B cells are located in the splenic marginal zone. B1a cells are the main producers of polyreactive Abs that cross react with commensal antigens (so called natural Abs) and secrete high amounts of Abs after non-specific stimuli, such as by TLR agonists. Follicular B cells, MZ B cells and B1b cells are participants of the adaptive immunity. Follicular B cells respond mostly to TD antigens, while MZ B cells and B1b cells respond mostly to TI antigens (Table 1) (31, 40-42).

Table 1: Comparison on antibody response to T dependent and T independent type 1 and 2 antigens

Antibody response	TD	TI-1	TI-2
Antigen	Proteins	LPS, CpG, lipopeptides, RNA	Capsular polysaccharides
Responding cell	Follicular B cell	MZ B cell, B-1	MZ B cell, B-1
Source of help	T_{FH}, CD40L and IL-21	TLR signaling in B cells	Extensive BCR crosslinking
GC	Yes, productive	Possibly	Can occur, nonproductive
Somatic hypermutations	Yes, leads to affinity maturation	Occurs in certain mouse strains	Limited, no affinity maturation
Memory B cell response	Yes, pre- and post-GC memory	B1 cell-derived memory, GC dependant	B1b cell-derived memory, GC dependant
Long-lived plasma cells	Yes	Not reported	Some bone marrow plasma cells
Extrafollicular response	Yes, delay due to T cell priming	Yes, early and robust	Yes early
Response in neonates	Limited response	?	Little or no response

Adapted from (43) with additional information from (44).

1.2.2 T cell dependent antibody response

When recognizing a TD antigen, a B cell can only be activated if it receives help from a helper T cell that recognizes the same antigen (31).

Follicular B cells can acquire soluble antigens that diffuse into lymphoid follicles (45) or are transported through the lymphoid system of conduits (46). In lymph nodes subcapsular

sinus (SCS) macrophages appear to be the most effective in presenting cell-associated antigens to follicular B cells. Follicular B cells take the antigens presented by SCS macrophages and transport them into the follicles and transfer to follicular dendritic cells (FDCs), a source of antigens for priming naive B cells (47-49). Programmed cell death protein-1 (PD-1) regulates selection and survival of B cells in GC and thus impacts the quantity and quality of long-lived PC cells (50).

T_{FH} cells are a class of T helper cells that regulate follicular B cell immune response. Their key role is to contact and stimulate antigen primed follicular B cells. Expression of CXCR5 and loss of expression of CCR7 positions the T_{FH} cells in the follicular B cell region of lymph nodes, where they express the transcription factor B cell lymphoma (BCL) 6 while other T cells in the lymph node express B lymphocyte induced maturation protein 1 (BLIMP1), which has an opposing function. Development of T_{FH} cells is one option for naive T helper cells after antigen-specific priming by DCs (26).

Initial pre GC contact between B cells and antigen-specific T_{FH} cells promotes a major division in developing B cell response. Some antigen primed B cells differentiate into PCs at this early stage via extra follicular B cell pathway, thus these B cells never enter the GC. B cells that do not get into contact with T_{FH} cells at that time point enter GC reaction (51).

GCs are dynamic structures that are created in follicular regions of lymphoid tissues to support generation of high affinity memory B cells. GCs promote antigen-specific clonal expansion and BCR diversification. Within GC, B cells scan antigens presented by FDCs and following antigen binding make contact with GC T_{FH} cells. In the TD GC, antigen-specific B cells undergo numerous rounds of division, class switch recombination and somatic hypermutation (SHM) in their variable region genes, leading to affinity maturation. SHM is seen through the introduction of single base-pair substitutions. Affinity maturation refers to the rising affinity of antigen-specific antibodies (Abs) that can be measured over time following infection or vaccination (reviewed in (52)).

Ab class switching is an irreversible genetic recombination event. Activation induced deaminase (AID) with an access to single stranded DNA enables AID to deaminate cytosine. This triggers DNA damage machinery that removes the resulting uracil that results in mismatch repair that causes double strand breaks. Nonhomologous end joining (NHEJ) completes the class switching. AID expression is largely restricted to antigen activated B cells (reviewed in (53)).

Cytokines and innate stimuli alone can drive naive IgM positive B cells to switch Ab class. Typical vaccination requires T cell help to generate antigen-specific switched Abs. Early studies demonstrated Ab class switching in both the non GC and GC pathways, suggesting that the earliest events of class switching are controlled at the pre GC phase (54). T cells

produce cytokines at sites of antigen-specific contact with follicular B cells and different cytokines have been reported to drive commitment to different Ab classes. Interleukin(IL)-4 promotes IgG1 and IgE (55), IFNγ induces IgG2a (55) and transforming growth factor beta (TGFβ) directs the switching to IgA (56). IFNγ activates signal transducer and activator of transcription 1 (STAT1) downstream of the IFNγ receptor to induce transcription factor T-bet and promotes IgG2a class switching. Similarly, TGFβ signals run through the TGFβ receptor to activate SMAD and Runx transcription factors that promote IgA class switching (57-59).

FDCs are located in follicles in lymph nodes and spleen (60) and are important for activation and differentiation of B cells into plasmablasts/PCs and memory cells (61). The B cells that are produced in the GCs but do not secrete immunoglobulin are the mature memory B cells. FDCs are activated by binding immune complexes (IC) through surface receptors, such as complement receptor (CR) 1, CR2 and Fc γ receptor (FcγR) (62). FDCs secrete the chemokine CXCL13, which attracts effector B cells that express the chemokine receptor CXCR5 that binds to CXCL13 (63).

After contact with FDCs only few naive GC B cells have contact with T_{FH} cells. Cell death is a common fate of B cells in GC reaction. Positive selection of GC B cells that bind with higher affinity to antigens rescues them from cell death in the GC circle. High affinity GC B cells exit the GC rapidly and produce more PCs than GC B cells with lower antigen binding capacity (52, 64, 65).

Plasma cells negatively regulate BCL6 and IL-21 expression of T_{FH} cells and engage in antigen controlled immune regulation (66). The end product of the GC reaction is either development of plasmablasts or memory B cells. Plasmablasts home to the BM and become long-lived PCs, if able to find a survival niche where they obtain cell to cell contact or are exposed to soluble factors released by stromal cells. A proliferation inducing ligand (APRIL) secreted by stromal cells in the BM binds to B cell maturation protein (BCMA) and is the most important survival factor for long-lived PCs. Meanwhile, memory B cells circulate or stay in the lymphoid tissues. Reintroduction of antigens into the immune system causes memory B cells to undergo clonal proliferation and differentiation into PCs (67-69).

Capsular polysaccharides (PSs) are TI antigens because they induce specific IgM response in wild type and T cell deficient mice without inducing IgM to IgG switching and they fail to induce a booster response. By conjugating a PS to a carrier protein a TD response is generated. The proteins that have been selected as carriers when developing conjugate vaccines are known to be safe and highly immunogenic in infants. Diphteria toxoid (DT), CRM197 (mutated DT), tetanus toxoid (TT), protein D from non-typable *Hemophilus influenza*e and outer membrane protein (OMP) from meningococci are the protein carriers used in licensed polysaccharide conjugate vaccines (70, 71). When multivalent conjugate

vaccines containing several PSs conjugated to the same carrier protein is used, a carrier induced interference can occur. A phenomenon known as carrier induced epitopic suppression leads to a reduced Ab response to one or multiple polysaccharides (or haptens) of conjugate vaccines sharing common protein epitopes that are administered simultaneously (72). Carrier specific enhancement of T cell help may result in an enhanced Ab response to one or several conjugated polysaccharides conjugated to the same carrier protein, when coadministered. Carrier priming may either enhance or reduce the Ab response to a subsequent administration of a PS conjugate containing the same carrier protein (73, 74). These phenomena may influence the immunogenicity of multivalent PS conjugate vaccines.

Conjugating PS to peptides consisting of T cell epitopes can induce cognate recognition and TD response (75, 76). Furthermore, recent studies have shown that conjugating PS to a small peptide is enough to induce a TD response. MHC-II molecules can present PS to T cells if the PS is bound to peptide that the MHC-II recognizes. MHC class II molecules can only bind peptides while the TCR can bind to saccharides (77, 78)

1.2.3 T cell independent antibody response

There are two types of TI antigens. Type 1 or TI-1 antigens are mitogenic stimuli such as lipopolysaccharide (LPS), CpG or poly IC that elicit polyclonal activation through Toll-like receptors, whereas type 2 or TI-2 antigens are for example polysaccharides (PSs) that engage the BCR and crosslink them (79). Class switching of BCRs that bind TI-2 antigens has been shown to be induced by stimulation of TACI or TLRs and the downstream signaling when TACI and TLR are activated by their ligands (80, 81). B cell activating factor (BAFF) and APRIL bind to TACI and promote IgM production and class switching to IgG or IgA. This enables local class switching at mucosal sites and class switching of B cells specific for TI antigens in the marginal zone of spleen (82-84). Response to TI-2 antigens can generate memory B cells and long-lived PCs independent of GC maturation. The phenotype of TI memory B cells and PCs is different from the phenotypes of TD memory B cells and long-lived PCs (34, 85).

Many cell types have been suggested to have a role in establishing help to marginal zone B cells. Neutrophils have recently been implicated in supporting TI responding B cells through a mechanism involving APRIL, BAFF and IL-21 (86). Marginal zone macrophages and metallophilic macrophages (also located in marginal zone) are thought to play a role in acquiring antigens for MZ B cells. Marginal zone macrophages express the receptors SIGN-R1 and MACRO that have been shown to play an important role in protection against pneumococcal disease. SIGN-R1 is also known to bind capsular pneumococcal PSs (87-90). Capsular PSs that encapsulate bacteria and protect them from phagocytosis are examples of

TI-2 antigens (31). The ability to produce specific antibodies (Abs) against capsular PSs is important for protection against encapsulated bacteria (91). PS structure impacts the immune response differently, such as features like variation in polarity and ring forms. Slight structural differences that affect immune response are best shown in the minimal difference between the structure of PS of group B and C of *Neisseria meningitis*. Group C PS is immunogenic in humans while group B PS is not and fails to elicit Ab response due to molecular mimicry, i.e.identical structures of the meningococcal B PS and mammalian tissue (92). Ab responses to PS antigens are influenced by the composition and structure of the bacteria. Immune response to plain PS differs in quality from the response to the same PS in an intact bacteria and the same PS expressed on different subgroups of bacteria may elicit different responses. Immune response to intact pneumococcus expressing PPS14, PPS14 conjugate, intact pneumococcus expressing serotype 14 or Group B streptococcus expressing PS14 recruited distinct B cell clones that expressed different idiotypes (93-96). Bacterial PSs such as MenC-PS (MenC) and streptococcus Group B serotype V PS are able to suppress APRIL and BAFF mediated Ig secretion while NP-Ficoll, a prototype TI-2 antigen, increases BAFF mediated Ig secretion, but both MenC and Group B serotype V PS are weakly immunogenic (97).

The maintenance of PS-specific AbSC in spleen when immunized with plain PS is the result of continuous antigen-induced formation of short-lived plasmablasts in the spleen because of persistence of PS. Persistence of PS (dextran) in spleen can last at least 90 days in mouse, while PS in an intact bacterium or a conjugate is endosomally digested. Persistence of long-lived PCs in BM results in maintenance of Ab level in serum (98). A study conducted to compare response to conjugated pneumococcal PS vaccines (PCV) of serotype 3 and plain pneumococcal PS (PPS) 3 showed that TD PC in the BM had higher secretion capacity and underwent higher contraction phase the first two months after immunization than TI PC (99).

As the capsule of pathogens consists of PS, vaccines have been made that include only bacterial PS. As PSs do not elicit immune response in children younger than 2 years of age, only adults and elderly receive such vaccines. It has been shown that PS immunization may cause lower Ab response than after the first immunization, a phenomenon known as hyporesponsiveness (85, 100, 101).

1.3 Limitations of the neonatal immune system and vaccination of neonates

Neonates and infants are at high risk of infection because their immune system responds poorly to both TD and TI antigens. Infants and young children (<2 years of age) and mice (<3 weeks of age) have a poor ability to respond to TI antigens like polysaccharides (PSs) (102).

The antibody (Ab) response in neonates and infants to vaccines, including PCV, is slow and the half life of Abs is low. Repeated vaccination in the first year of life and a booster in their second year is necessary to uphold the amount of protective Ab levels (103, 104). One reason for low response to vaccines is that maternal Abs may mask epitopes of vaccine antigens, thus inhibiting immune response to the vaccine (105).

Mouse and human neonates have low serum levels of C3, which limits antigen-C3d complex formation, resulting in reduced antigen presentation to B cells and reduced IC in the spleen, thus impairing the GC reaction, resulting in lower TD response. Low serum C3 also causes decreased opsonophagocytosis and complement mediated killing. C3 is the most important molecule in all three complement pathways and low C3 level causes decline in multiple immune mechanisms (106).

Combination of factors in the neonatal immune system cause naive B cells to differentiate into memory B cells rather than PCs. Lower BCR signaling in neonatal GCs promotes differentiation to memory cells rather than PCs, as low affinity naive B cells differentiate into memory B cells while high affinity B cells differentiate into PCs. Thus, in early life memory B cells are preferred rather than Ab persistence in the body.

There are numerous differences between adult and neonatal B cells in spleen. Naive neonatal B cells in mice and human have lower level of the cell surface molecules CD21, CD40, CD80 and CD86, which are co-stimulation factors in T cell help to B cells, reducing the stimulation threshold of B cell response to antigens (reviewed in (44)).

Infant B cells produce lower Ab response to most protein and conjugate antigens. Delay in Ab production against TD antigens in neonatal mice correlates with limited activation of GCs. Delayed development of FDCs in neonates is one of the main causes for limited GC reaction. FDCs retain ICs that are stimulatory for naive B cells and provide signals that push B cells towards SHM and class switch recombination (107, 108).

Short persistence of mouse Abs in early life is because of a failure to establish and maintain long-lived PC pool in the BM. In infant mice, post GC plasmablasts home to the BM but fail to establish themselves there because of lack of APRIL from resident stromal cells (69, 109, 110).

Neonatal CD4+ T cells respond differently from CD4+ T cells in adults, as they exhibit Th2 biased response to antigens. This results in a diminishing response to Th1 antigens and enhanced response to Th2 antigens (107, 111).

Because of limited immune response of neonates and infants to vaccines, numerous studies have attempted to find adjuvants that are able to circumvent limitations of the immune system in early life. It has been shown that certain adjuvants (e.g. CpG, IC31 and LT-K63) are able to circumvent the Th2 bias in neonatal mice and induce protective immunity

when coadministered with pneumococcal and meningococcal conjugates (112-120). LT-K63 has also been shown to circumvent delayed maturity of FDCs in neonatal mice when given with a PCV ((119).

1.4 *Streptococcus pneumoniae*

S. *pneumoniae* is a lancet shaped gram positive bacterium which consists of three main layers: plasma membrane, cell wall and capsule, surrounding the cytoplasm. The capsule is made of polysaccharides (PSs) and protects the bacteria from phagocytosis. The cell wall is made up of cell wall PS (CWPS) that anchors the capsular PS. PSs form the basis for classifying pneumococci by serotypes. Pneumococcal bacteria can be divided into more than 90 serotypes that express antigenically and structurally different capsules (reviewed in (121)).

Pneumococci are a normal component of microflora in the nasopharyngeal mucous membrane. The primary step in pneumococcal pathogenesis is colonization at the mucosal epithelium in the nasopharynx (122). Most colonization causes no symptoms or diseases. However the pneumococci are one of the most common causes of respiratory tract infection and can also cause invasive infections, like meningitis, sepsis and bacteremia. When pneumococcal disease occurs, it is the host's own nasopharyngeal pneumococci that serves as the source of the disease-causing strain (122, 123).

Colonization occurs mainly in early childhood, most infants are colonized with one or more serotype simultaneously or sequentially. Pneumococcal colonization occurs in approx.10% of adult individuals. Some serotypes are more likely to colonize than others (124). In a cross sectional study from around the world, serotypes 1, 4, 5, 7, 14 and 18C were correlated with invasive diseases, while serotypes 3, 6A, 6B, 15, 19 and 23F were correlated with prevalence in colonization. Inverse correlation was found between invasiveness and carriage prevalence. The most invasive strains were the least commonly carried strains (124). The most prevalent serotypes tend to be harder to kill by neutrophils. They are more encapsulated and the capsule PSs of those serotypes tend to be more highly charged and have smaller repeating units. Invasive serotypes tend to be less encapsulated, their PSs tend to have longer repeating units, be less highly charged and less susceptible to C3b deposition and to consequent opsonophagocytosis (125-128).

S. pneumoniae encounters mucous secretion within minutes after entering the nasal cavity. Expression of capsule reduces entrapment in the mucous, thus allowing the bacteria to access the epithelial surfaces (129). When the pneumococcus has reached the epithelium the expression of a thick capsule is disadvantageous because of its inhibitory effect on adherence. Several receptor-ligand interactions have been proposed between pneumococci and the epithelial surface of the nasopharynx. The bacterial adhesion molecules that are

involved include phosphorylcholine (ChoP). ChoP is an unusual bacterial structure present in many microorganisms that colonize the upper respiratory tract. ChoP mediates bacterial adherence in the nasopharynx. Pneumococcal surface protein A (PspA) and C (PspC) are choline binding proteins. Both proteins are important in colonization and for virulence. For example PspC can bind to factor H (a regulator of complement activity) while PspA can inhibit complement binding (121).

The pneumococcal factors that enable host epithelial and tissue barriers to be breached during the progression from colonization to invasive infection are poorly understood. In mice and humans opaque variants that express increased amounts of capsule are more resistant to opsonophagocytic killing and are selected for the transition from mucosal surface to the bloodstream. The capsule protects the pneumococcus from direct phagocytosis by macrophages and neutrophils, complement factor binding and entrapment by neutrophil extracellular traps (130-134). The effect of the capsule on complement factor binding varies and effectiveness of protection against phagocytosis varies between serotypes (126). To kill pneumococci, it is necessary to opsonize them by antibodies (Abs) and complements so that macrophages and neutrophils can engulf them. Fc receptors on macrophages and neutrophils bind to the Fc part of Abs bound to the pneumococcal capsule, which initiates engulfment of the bacteria. Capsule binding Abs can also activate the complement system and the binding of C3b on bacterial surface enhances phagocytosis (121).

Although opsonization with complements and/or Abs followed by phagocytosis is required to kill *S. pneumoniae* in invasive disease*,* the time length of colonization is unaffected in mice that fail to generate specific Abs or lack neutrophils or complement (135). Mice that lack TLR2 (136) and mice that do not express MHC-II show prolonged carriage (137). It has been shown that pneumococcal colonization can protect against subsequent colonization; this protection is dependent on $CD4^+$ T cells but independent of Abs. Abs specific for PPS antigens do correlate to protection against colonization (135, 137, 138). Passively administered human PS Abs protect against colonization in infant rat (139).

A factor that limits the effectiveness of the Ab response in the mucosal system is the bacterial expression of a secreted zinc metalloprotease that targets human IgA1, which constitutes to more than 90% of total IgA in the human airway. Cleavage of bound IgA1 produces bacterial surface antigens bound to Fab which masks the epitopes from being recognized by other Abs, thus protecting the bacteria from other epitope recognizing molecules. Ab-mediated clearance might occur with sufficient amounts of Abs of other classes and subclasses (140, 141).

Pneumococcal colonization induces a variety of immune responses in humans including production of Abs specific for surface proteins and capsule PSs. Ab production against

pneumococcal proteins begins in infancy, whereas production of IgG specific for capsular PS does not start until around the second or third year of life, although salivary IgA specific for some serotypes is present in young children (142-149).

Highest rate of infection by *S. pneumoniae* is in infants and the elderly. About one million children younger than 5 years of age die from diseases caused by *S. pneumoniae* every year worldwide (150-152). Shortly after birth placental transfer of maternal Abs protects neonates against infection (153). The maternal Abs decline with time which results in increasing infections because of limitation in the immune system of neonates and infants (103, 154).

Several types of pneumococcal vaccines are under development but only two types are licensed (plain PS vaccines and PPS-protein conjugate vaccines).

There is one licensed plain PPS vaccine (PPV) (PPV23, MSD (Merck and Sanofi Pasteur)) that incorporates 23 serotypes (1, 2, 3, 4, 5, 6B, 7F 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 20, 22F, 23F and 33). Those 23 serotypes cover 85-90% of invasive infections in the USA. PPV23 is ineffective in children under the age of 2 and does not induce memory, thus repeated vaccinations are needed in adults as well (155, 156). The reason for its limitations is mainly because PSs are TI-2 antigens (31).

There are three licensed multivalent pneumococcal conjugate vaccines. Seven (PCV7) and 13 (PCV13) valent (Prevenar®, produced by Pfizer Inc.) and 10 valent (PCV10) (Synflorix™, produced by GSK). In PCV7 there are 7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) individually conjugated to a non-toxic variant of diphtheria toxin (CRM197). This heptavalent vaccine was licensed in the USA for infants and children in the year 2000. The selected serotypes represented 80-90% of invasive pneumococcal disease (IPD) cases (157) and 76% of pneumococcal serotypes causing otitis media (158) in young children at that time point (159).

Vaccination of children with PCV7 has dramatically decreased pneumococcal disease and provided herd immunity protecting unvaccinated children and adults. The PCV7 vaccination decreased all IPD and IPD caused by the serotypes in PCV 45% and 94%, respectively, in USA from 1997 to 2007. The largest reduction occurred in children under the age of 5. Two thirds of the reduction of IPD can be attributed to herd immunity (160). PCV13 is the replacement vaccine for PCV7 and has six additional serotypes (1, 3, 5, 6A, 7F and 19A). All the serotypes in PCV13 are conjugated to CRM197. To be licensed, PCV13 had to fulfill three criteria: 1) immune responses to serotypes that were in PCV7 had to be noninferior to PCV7, 2) Ab titer to new serotypes had to be >0.35 µg/ml (measured by ELISA) and the responders had to have OPA titer of at least 8 one month after primary vaccinations for the new serotypes, and 3) there had to be evidence of memory after boosting. All three criteria were fulfilled (161).

PCV10 is the competing vaccine to the PCV13. PCV10 has three additional serotypes (1, 5 and 7F) that PCV7 has not. Eight of the 10 serotypes are conjugated to protein D from non-typeable strain of *H. influenza*; serotype 18C is conjugated to tetanus toxoid and 19F to diphtheria toxoid. Aluminum phosphate acts as an adjuvant for all the licensed pneumococcal conjugate vaccines. PCV10 has been shown to be noninferior to PCV7 for 8 out of 10 serotypes (5 of 7 serotypes common to both vaccines) (162). It has been shown that PCV10 is protective against invasive pneumococcal disease in a 2+1 schedule in infants (163).

PCVs are expensive and increased incidence of non-PCV serotypes has been observed after vaccination. New non-PS based vaccines are being developed, which are cheaper and provide broad serotype and geographical coverage. These include mixtures of conserved pneumococcal proteins that are widely expressed by pneumococci. Another approach is whole cell vaccine, which in theory could provide protection against most serotypes (reviewed by (164)).

Conjugate vaccines are expensive, thus the use of PPV23 to boost the response to PCV has been proposed as a possible solution to decrease cost and increase serotype coverage as PPV23 includes more serotypes than PCVs that have been licensed. The main hindrance is that PS have been shown to cause hyporesponsiveness to PCV vaccination (165).

Hyporesponsiveness is defined as immune response that is lower after booster vaccination than it was after primary vaccination. Hyporesponsiveness can be established by measuring the quantity of specific Ab response with enzyme linked immunosorbent assay (ELISA) (166) or the functional capacity of the Abs with assays like SBA or OPA (167, 168).

Hyporesponsiveness was first seen for PS vaccines when children, who had been primed with meningococcal C conjugate vaccine in infancy, were boosted with meningococcal C PS 5 years of age. They had lower specific Ab response if they had received a PS booster in the second year of life than children who had not received a PS booster earlier (169). Hyporesponsiveness has been seen in clinical studies in response to meningococcal and pneumococcal polysaccharide booster and priming, and results from mouse studies using those PS from other pathogens show similar effects. Children and elderly primed with PCV as infants have shown reduced PPS-specific IgG response when boosted with PPV (165, 170-174). Children, adolescents and elderly primed with PPV23 and boosted with PCV had lower PPS-specific IgG levels and/or lower OPA than those that only received PCV (100, 175-177). Priming with meningococcal PS has been shown to cause hyporesponsiveness to subsequent meningococcal conjugate vaccination in children and adolescents (178, 179).

Although Ab response to PSs in intact pneumococci has been shown to differ slightly from response to plain PPS, their suppressive effects on immune response seem to be similar

(94). Colonization with pneumococci may cause reduced serotype-specific Ab response to PCV in infants. Colonized infants had lower Ab response specific to the serotype that they were colonized with compared with a control group that was not colonized before vaccination or colonized with a different serotype (166, 180). Invasive pneumococcal infection has also been shown to cause serotype-specific reduction of Ab levels (181).

PS-induced hyporesponsiveness has been demonstrated in our neonatal mouse model. Neonatal NMRI mice primed with monovalent PCV of serotype 1 and boosted with PPS1 as infants had lower PPS1-specific IgG Ab levels in serum and fewer IgG+ AbSCs in BM and spleen than mice that received saline booster (118, 182). The immunological mechanism behind hyporesponsiveness is unknown; however, it has been shown in a neonatal mouse model that in mice primed with MenC-PS conjugate as neonates, MenC-PS increased apoptosis of MenC-PS-specific B cells in spleen and BM, in particular within the memory B cell and PC pool (183).

2 Aims

The overall aim of the study was to investigate the effects of immunizing with a licensed multivalent plain pneumococcal polysaccharides vaccine, PPV23, on immune response to a ten-valent pneumococcal conjugate vaccines, PCV10, i.e. whether the polysaccharide affects the PPS-specific humoral immunity elicited by PCV immunization.

The study consisted of two parts addressing two specific aims:

1. To investigate whether a PPV23 booster decreases the antibody response in mice primed as neonates with PCV10 and whether hyporesponsiveness is induced by multiple PPS serotypes.
2. To investigate whether priming with a multivalent PPV23 delays or reduces the antibody response and whether PPV23 reduces the number of PPS-specific AbSCs in spleen and BM created by a subsequent PCV10 immunization. Furthermore, to assess whether such interference is caused by multiple PS serotypes and whether it is dose dependent.

3 Materials and Methods

3.1 Mice

Adult NMRI mice were purchased from Taconic (Ry, Denmark). They were kept in micro-isolator cages with free access to commercial food pellets and water, and housed under standardized conditions first at the institute of Experimental Pathology at Keldur (Reykjavik, Iceland) and then later at Arctic LAS (Reykjavik, Iceland) with regulated daylight humidity and temperature. The mice adapted for at least one week after arrival and then one male and one female were put in the same cage for two weeks. Two weeks later each female mouse was put in a breeding cage that was checked daily for new births and the pups were kept with their mother until weaning at the age of 4 weeks. The animal experiments are authorized by the Experimental Animal Committee of Iceland and complied with animal welfare act 15/94.

3.2 Immunization and blood sampling

Neonatal (7 days old) mice (7 to 9 per group) were immunized subcutaneously (s.c.) in the scapular girdle region with either PCV10 (Synflorix™ produced by GSK), PPV23 (Pneumovax II, Sanofi Pasteur MSD) or saline. PCV10 was used at ¼ dose for human, which results in 0.25 µg of PPS1, PPS5, PPS6B, PPS7F, PPS9V, PPS14 and PPS23F conjugated to protein D, 0.75 µg of PPS4 conjugated to protein D, 0.75 µg of PPS18C conjugated to tetanus toxoid and 0.75 µg of PPS19F conjugated to diphtheria toxoid. The doses for PPV23 was 1/10 of human dose which is 2.5 µg for each plain polysaccharide (PS) in the vaccine, 1/5 of human dose which is 5 µg for each plain PS or 2/5 of human dose which is 10 µg for each plain PS. Mice were reimmunized 16 days later with PCV, PPV23 or saline. Blood was taken from the tail vein at week 2, 3, 4, 5, 6 and 12 after first immunization.

To assess the effect of PPV23 booster on immune response, two independent experiments were performed that included 3 immunized groups (PCV10-PCV10, PCV10-saline, PCV10-PPV23) and a control group (saline-saline). The results from both experiments were comparable, all the results shown are from one experiment. The results for both experiments are showed in appendix.

To assess the effect of neonatal PPV23 priming on the immune response four experiments were performed, two identical experiments included three immunized groups and one control group (PCV10-PCV10, Saline-PCV10, PPV23-PCV10 and saline-saline) and two identical experiments included two additional PPV23 priming groups to compare 3 different doses of PPV23 (PCV10-PCV10, saline-PCV10 PPV23 (2.5, 5 or 10 µg/serotype)-PCV10 and saline-saline). The results from both sets of experiments were comparable, all the results shown are from one experiment including 5 immunized groups and a control group. Results for all experiments are showed in appendix.

3.3 Antibody measurement

Antibodies (Abs) specific for PPS were measured in serum by ELISA, a protocol designed according to the standardized ELISA protocol recommended by the ELISA workshop at the Centers for Disease Control with a few modifications as described (184, 185). Microplates (Maxisorp; Nunc AS, Roskilde, Denmark) were coated with 5 µg/ml PPS1 (American Tissue Culture Collection (ATCC), Rockville, MD), 10 µg/ml PPS4 (ATCC), 10 µg/ml PPS6B (ATCC), 10 µg/ml PPS9V (ATCC), 2.5 µg/ml PPS14 (ATCC), 2.5 µg/ml PPS18C (ATCC), or 5 µg/ml of PPS19F (ATCC), and incubated for 5 h at 37°C. After incubation the plates were stored at 4°C until they were used. When the plates were used they were washed with PBST (PBS containing 0.05% Tween 20 (Sigma, St. Louis, MO)). Plates were washed using Elx405 ELISA washer (Biotek Instruments Inc, Winooski, VT) with an ELISA stacker (Biotek Instruments Inc). Then the plates were blocked with 0.1% BSA (Millipore, Kanakee, IL) in PBS. The sera was adsorbed with 500 µg/ml CWPS (Statens Serum Institute, Copenhagen Denmark) for 30 min at room temperature (RT), and serially diluted in the plates and incubated in duplicates for 2 h at RT. Plates were washed in PBST and then horse radish peroxidase (HRP)-labeled monoclonal Abs against mouse IgG (Southern Biotech Associates Inc., Birmingham, AL) was incubated for 2 h at RT. Plates were then developed with tetramethylbenzidine (Kem-En-Tec Diagnostics, Taastrup, Denmark) for 10 minutes and the reaction was stopped using 0.18 M H_2SO_4. The absorbance was measured at 450 nm in an ELISA spectrophotometer (Thermo Electron Corporation, Vantaa, Finland). Reference serum was included to calculate titers, which are expressed in ELISA Units (EU) per ml.

3.4 ELISPOT for measurement of the number of AbSC

PPS-specific AbSCs numbers from spleen and BM were assessed by enzyme-linked immunosorbent spot (ELISPOT) assay four weeks after secondary immunization, i.e. 6 weeks after the first immunization.

Spleen was removed, cut into several pieces and then pressed through a sterile nylon cell strainer (BD Biosciences, San Diego, CA). BM in tibia and femur was flushed with complete medium, i.e. RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK) containing 25 mM Hepes buffer (Gibco), 100 µg/ml penicillin/100 µg/ml streptomycin (Gibco), 2mM L-glutamine (Gibco) and 10% fetal calf serum (FCS) (Gibco).

Cells from BM and spleen were dissolved in Hanks balanced salt solution (HBSS; Gibco BRL) containing 2% (FCS and centrifuged at 1000 rpm for 10 minutes. Single cell suspensions (10^8 cells/ml) from spleen and BM were treated for 5 minutes with ACK lysing buffer (0.15 M NH_4CL, 10 mM $KHCO_3$ and 0.1 mM of Na_2EDTA) to eliminate red blood cells, whereafter HBSS was added to diminish the ACK lysing. Cells were then centrifuged at 1000 rpm for 10 minutes and the cells resuspended in 10 ml of HBSS. The cells were allowed to

sediment for 10 minutes, whereafter 9 ml were apprehended and put into a new tube. The cells were centrifuged for 10 minutes at 1000 rpm for 10 minutes. After washing cells were resuspended in complete medium and counted using Countess automated counting (Invitrogen). Thereafter the cells were centrifuged at 1000 rpm and resuspended in complete medium at a concentration of 10^8 cells/ml. Multi-screen nitrocellulose bottomed plates (Millipore Corporation, Bedford, MA) were coated with 20 µg/ml of PPS1, 20 µg/ml of PPS4, 20 µg/ml of PPS9V or 5 µg/ml of PPS18C overnight at 37°C, washed with PBST and blocked with RPMI 1640 containing 10% FCS. Serial dilutions of cells were then incubated for 5 h at 37°C in the plates. After washing the plates, the plates were incubated with alkaline phosphates (ALP)-conjugated goat Abs against mouse IgG (Southern Biotechnology Associates Inc.) overnight at 4°C. After washing, the plates were developed by substrate solution (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium in AP color development buffer) (BioRad Labs, Hercules, Ca, US). Tap water was used to stop the reaction. Spots were counted and analyzed with an ELISPOT reader (KS ELISPOT, Zeiss, Germany).

3.5 Statistical analysis

Mann-Whitney test was used to compare Ab titers or AbSC between groups and time-points. A P value of <0.05 is considered statistically significant. Prism (Graphpad software inc, CA, US) was used for statistical analysis and to make figures.

4 Results

4.1 Effect of plain pneumococcal polysaccharide booster on antibody specific response from immunization with pneumococcal polysaccharide conjugate vaccine

Neonatal mice (7 days old) were immunized s.c with ¼ of human PCV dose to study the impact of PPV23 booster on antibodies (Abs) response induced by PCV10 immunization. Sixteen days after the first immunization, mice were boosted s.c. with 1/5 of human dose of PPV23 which includes 5 µg of each polysaccharide (PS), 1/4 dose PCV or saline. Control group received saline at both injections. Mice were bled 2, 3, 4, 5, 6 and 12 weeks after first immunization. Two independent experiments were performed and comparable results were obtained (Figure A1). IgG Abs specific for PPS1, 4, 9V, 14 and 18C were measured by ELISA and the results from one of the two experiments are shown in figures 1 and 2.

Mice from all immunized groups had higher PPS1-specific IgG Abs than unimmunized control mice (figure 1a). PCV10 primed neonatal mice boosted with PPV23 had a diminished PPS1-specific IgG Ab response compared with mice boosted with PCV10 or saline (figure 1a). PPS1-specific IgG Ab levels were significantly lower in PCV10 primed mice that were boosted with PPV23 than in PCV10 primed mice that were boosted with saline or PCV10 6 and 12 weeks after first immunization (figure 1b). There was no difference regarding PPS1-specific IgG Ab response between PCV10 primed mice that were boosted with saline or PCV10 (figure 1b).

Mice from all immunized groups had higher PPS4-specific IgG Abs than unimmunized control mice (figure 1c). PPV23 booster diminished the PPS4-specific IgG Ab response in PCV10 primed neonatal mice compared with PCV10 or saline booster (figure 1c). At week 3 and 4 there was a clear difference between PCV10 primed mice boosted with PCV10 or PPV23, but the difference diminished with time (figure 1c). PPS4-specific IgG Ab levels in mice primed with PCV10 was significantly lower after PPV23 booster than after saline booster at 6 and 12 weeks after first immunization (figure 1d). No difference was in PPS4-specific IgG Ab response between PCV10 primed mice that were boosted with PCV10 compared with mice boosted with PPV23 or saline at week 6 and 12 after first immunization (figure 1d).

Mice from all immunized groups had higher PPS9V-specific IgG Abs than unimmunized control mice (figure 1e). PPV23 booster decreased PPS9V-specific IgG Ab response compared with PCV10 or saline booster in PCV10 primed mice (figure 1e). PPS9V-specific IgG Ab response in PCV10 primed mice were significantly lower after booster with PPV23 than after booster with saline or PCV10 6 and 12 weeks after first immunization (figure 1f).

No difference was found between PCV10 primed mice that were boosted with saline or PCV10 (figure 1f).

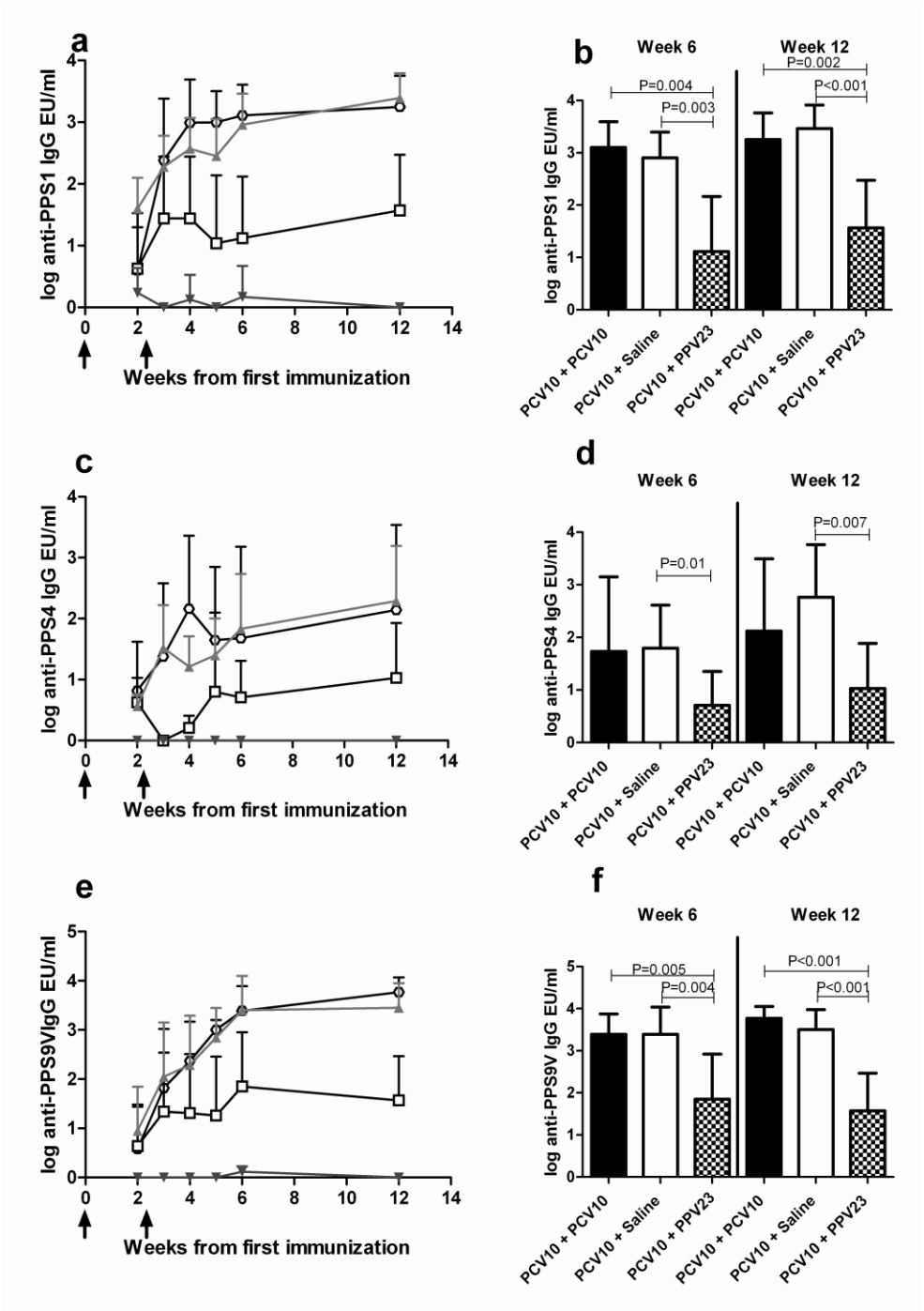

Figure 1 PPS1-, PPS4- and PPS9V-specific IgG Abs in PCV10-primed mice boosted with saline, PCV10 and PPV23.

a) Kinetics of PPS1-specific IgG response from 2 to 6 weeks after first immunization. PCV10-PCV10 (●), PCV10-saline (▲), PCV10-PPV23 (■) and saline+saline (▼). b) PPS1-specific IgG levels 6 weeks after first immunization. c) Kinetic of PPS4-specific IgG response from 2 weeks after first immunization through to 6 weeks after first immunization. PCV10-PCV10 (●), PCV10-saline (▲), PCV10-PPV23 (■) and saline-saline (▼). Arrows indicate time of immunization. d) PPS4-specific IgG levels 6 weeks after first immunization. e) Kinetics of PPS9V-specific IgG response from 2 weeks after first immunization through to 6 weeks after first immunization. PCV10-PCV10 (●), PCV10-saline (▲), PCV10-PPV23 (■) and saline-saline (▼). Arrows indicate time of immunization. f) PPS9V-specific IgG Ab levels 6 weeks after first immunization. P-values are shown if the difference between the groups is significant.

Mice from all immunized groups had higher PPS14-specific IgG Abs than unimmunized mice (figure 2a, figure A 2). PPV23 booster diminished PPS14-specific IgG Ab response in PCV10 primed neonatal mice compared with saline or PCV10 booster (figure 2a). The difference was evident from 2 to 10 weeks after booster (figure 2a). PPS14-specific IgG Abs in PCV10 primed mice was significantly lower in mice that received PPV23 booster than in PCV10 primed mice that received saline booster or PCV10 booster 6 and 12 weeks after first immunization (figure 2b). No difference was observed in total PPS14-specific IgG Ab response between PCV10 primed mice that were boosted with saline or PCV10 (figure 2b).

Mice from all immunized groups had higher PPS18C-specific IgG Abs than unimmunized control mice (figure 2c). PCV10 primed neonatal mice boosted with PPV23 had diminished PPS18C-specific IgG Abs after the PPV23 booster than mice boosted with PCV10 or saline (figure 2c). PPS18C-specific IgG Abs in PCV10 primed mice was significantly lower in mice boosted with PPV23 than in mice boosted with saline or PCV10 6 and 12 weeks after first immunization (figure 2d). No difference was in PPS18C-specific IgG Ab response between PCV10 primed mice that were boosted with saline or PCV10 (figure 2d).

There was no difference between the PCV10 primed groups before the booster administration, except for certain abnormalities that were not found for the same serotype in both experiments performed and can be explained by coincidental difference.

In summary, significantly lower IgG Ab response specific for PPS1, 9V, 14 and 18C was observed in PCV10 primed mice that received a booster with PPV23 compared with PCV10 primed mice boosted with saline or PCV10, 6 and 12 weeks after immunization. IgG Abs specific for PPS4 in PCV10 primed mice was significantly lower after booster with PPV23 than in PCV10 primed mice boosted with saline 6 and 12 weeks after immunization.

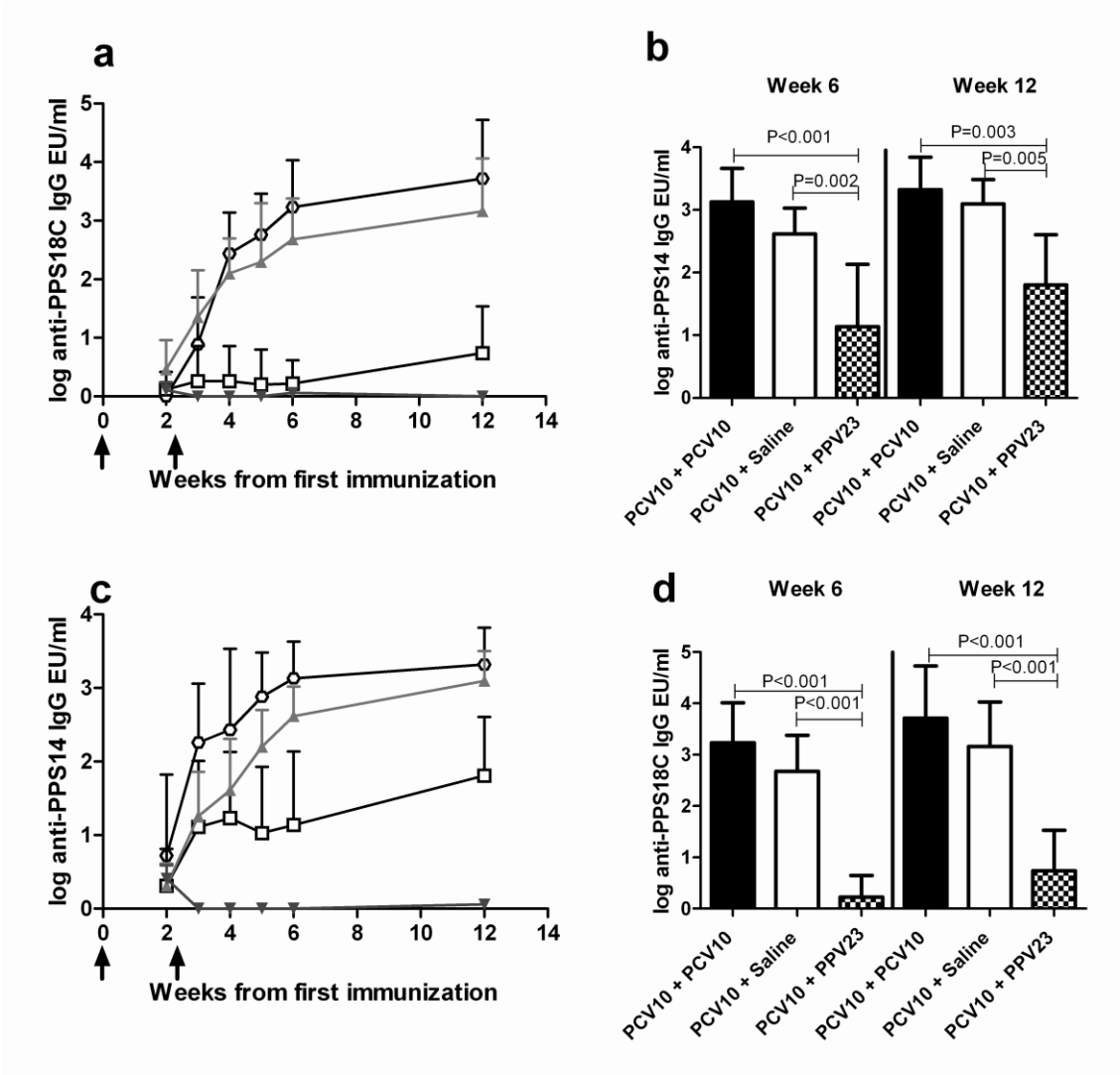

Figure 2 : PPS14- and PPS18C-specific IgG Abs in PCV10-primed mice boosted with saline, PCV10 or PPV23.

a) Kinetics of PPS14-specific IgG Ab response from 2 weeks after first immunization through to 6 weeks after first immunization. PCV10+PCV10 (●), PCV10+saline (▲), PCV10+PPV23 (■) and saline+saline (▼). Arrows indicate time of immunization. b) PPS14-specific IgG Ab response 6 weeks after first immunization. c) Kinetics of PPS18C-specific IgG Ab response from 2 weeks after first immunization through to 6 weeks after first immunization. PCV10-PCV10 (●), PCV10-saline (▲), PCV10-PPV23 (■) and saline+saline (▼). Arrows indicate time of immunization. d) PPS18C-specific IgG Ab response 6 weeks after first immunization. P-values are shown if the difference between the groups is significant.

4.2 Effect of priming with native/plain pneumococcal polysaccharide on immune response when boosting with pneumococcal polysaccharide conjugate vaccine

Neonatal mice (7 days old) were immunized s.c. with 5 µg of each polysaccharide (PS) in PPV23 (1/5 of human dose). For comparison neonatal mice were immunized s.c. with saline or PCV10 (¼ of a human infant dose). Sixteen days later all groups were immunized with PCV10 (¼ of human dose). Control mice received saline at both injections. Two days before booster, mice were bled and they were also bled 3, 4, 5, 6 and 12 weeks after the first

immunization. As the initial results showed differences between PS serotypes, it was decided to assess if the effect was dose dependent. Therefore, neonatal mice were immunized s.c. with 2.5, 5 and 10 µg of each PS in PPV23 and then boosted 16 days later with PCV10. IgG specific antibodies (Abs) for PPS1, 4, 9V, 14 and 18C were measured by ELISA to establish the effect of PPV23 priming on PPS-specific IgG Ab response compared with priming with saline or PCV10 with all groups receiving PCV10 booster. Four independent experiments were performed to investigate the effect of PPV23 priming on immune response to PCV10 booster, in two experiments the dose effect of PPV23 was also investigated. Comparable results were obtained (figure A 2). Results representative of one of these experiments is shown in figure 3-6.

In neonatal mice primed with 5 µg/serotype in PPV23, a booster with PCV10 elicited lower PPS1-specific IgG Ab response than in mice primed with PCV10 or saline (figure 3a). All three PPV23 doses tested caused lower PPS1-specific IgG Ab response to PCV10 than in mice primed with saline. There was a clear difference between PPV23 doses, as mice that received 10 µg/serotype PPV23 had a lower PPS1-specific IgG Abs than mice that received 2.5 and 5 µg dose. The mice that were primed with a 5 µg dose had a lower PPS1-specific IgG Ab response than mice primed with 2.5 µg dose of PPV23 (figure 3a).

PPS1-specific IgG Ab response after PCV10 booster in mice primed with 5 µg/serotype in PPV23 was significantly lower than in mice primed with saline or PCV10 6 weeks after priming (figure 3b, table A 2). The other doses of PPV23 used for priming, 2.5 and 10 µg/serotype, resulted in significantly lower PPS1-specific IgG Ab response after PCV10 booster than in saline primed and PCV10 primed mice . Mice primed with 2.5 µg/serotype in PPV23 had significantly higher PPS1-specific IgG Ab response 4 weeks after PCV10 booster than mice that received 5 and 10 µg/serotype in PPV23. Mice that received 5 µg/serotype in PPV23 had significantly higher PPS1-specific IgG Ab response 4 weeks after PCV10 booster than mice that were primed with 10 µg/serotype in PPV23. There was no difference between mice primed with saline or PCV10 and all groups had significantly higher PPS1-specific IgG Abs than unimmunized control mice, 6 weeks after first immunization (figure 3b, table A 2 in appendix).

PPV23 priming resulted in lower PPS1-specific IgG Ab response to PCV10 booster and the reduction increased with increasing dose of PPV23 administered. Because of PPV23 blunting the PPS-specific serum IgG Ab response we investigated whether PPV23 affected AbSC number in spleen and BM (4 weeks after booster).

Four weeks after the PCV10 booster significantly higher number of PPS1-specific IgG secreting AbSCs than in unimmunized control mice was found in mice primed with saline, PCV10 or 2.5 µg/serotype in PPV23 in spleen and mice primed with saline or PCV10 had

significantly higher PPS1-specific IgG secreting AbSC BM than unimmunized control (Figures 3c and 3d). The number of PPS1-specific IgG secreting AbSCs in spleen (figure 3c) and BM (figure 3d, table A 3 and 4) was significantly lower in mice that were primed with 5 and 10 µg/serotype in PPV23 than in mice primed with saline or PCV10. A dose effect of PPV23 was seen, as mice primed with 2.5 µg/serotype in PPV23 had significantly higher number of PPS1-specific IgG secreting AbSCs in spleen than PCV10 boosted mice primed with 5 and 10 µg/serotype in PPV23. The dose dependent difference was not significant for PPS1-specific IgG secreting AbSCs in BM, although the trend was the same. The detrimental effect of PS priming on PPS1-specific IgG secreting AbSCs in spleen and BM was dose dependent.

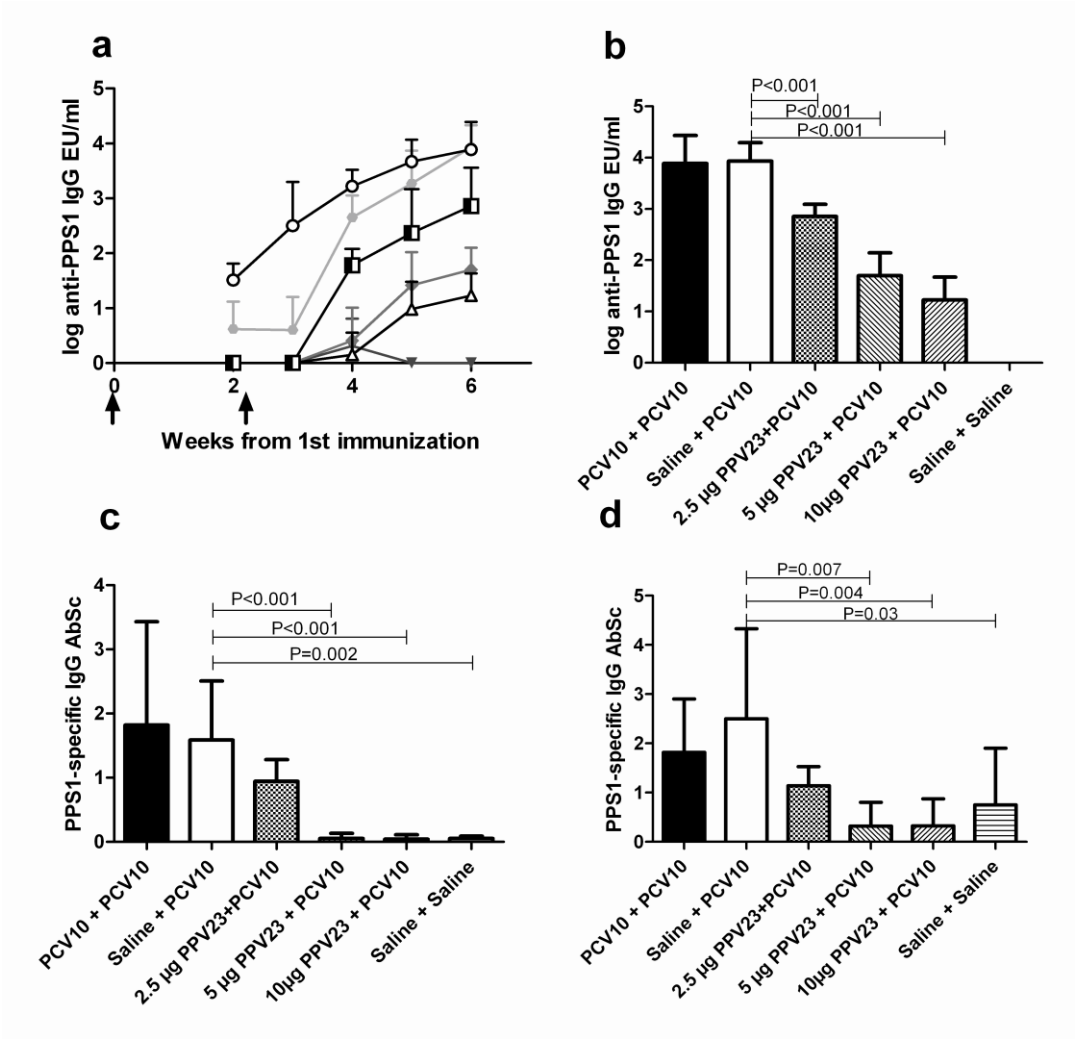

Figure 3: PPS1-specific IgG Abs and number of PPS1-specific IgG AbSCs in PCV10-boosted mice primed with saline, PCV10 and different doses of PPV23.

a) Kinetics of PPS1-specific IgG Ab response from 2 weeks after first immunization to 6 weeks after first immunization. PCV10-PCV10 (●), saline-PCV10 (○), 2.5 µg PPV23-PCV10 (◧), 5 µg PPV23-PCV10 (◆), 10 µg PPV23-PCV10 (△) and saline+saline (▼). Arrows indicate time of immunization. b) PPS1-specific IgG Abs levels 6 weeks after first immunization. c) PPS1-specific IgG+ AbSCs of 10^6 cells in spleen 6 weeks after first immunization. d) PPS1-specific IgG+ AbSCs of 10^6 cells in BM 6 weeks after first immunization. P-values are shown if the difference between the test group and the saline-PCV10 group is significant.

For serotype 4, the PCV10 boosted neonatal mice primed with 5 µg/serotype in PPV23 mounted a lower PPS4-specific IgG Ab response than PCV10 or saline primed mice (figure 4a). Regarding the dose effect of PPV23, two of three doses tested caused reduction of PPS4-specific IgG Ab response compared with mice primed with saline. The effect was dose dependent, as mice that received 10 µg/serotype in PPV23 had a lower PPS4-specific IgG Ab response than mice that received 2.5 or 5 µg/serotype in PPV23 and mice that received 5 or 10 µg dose had a lower PPS4-specific IgG Ab response than mice that received 2.5 µg dose. PCV10 boosted mice that received 2.5 µg/serotype in PPV23 had lower PPS4-specific IgG Ab response than PCV10 boosted mice that received saline at week 6 but not lower than PCV10 (Figure 4a).

PPS4-specific IgG Ab response in mice primed with 5 and 10 µg/serotype in PPV23 was significantly lower than in mice primed with saline or PCV10 4 weeks after the PCV10 booster (figure 4b). Mice that received 2.5 µg/serotype in PPV23 had significantly lower PPS4-specific IgG Ab response than mice primed with saline but not than mice primed with PCV six weeks after priming. Mice that received 2.5 µg/serotype in PPV23 had significantly higher PPS4-specific IgG Ab response 4 weeks after PCV10 booster than mice that received 5 or 10 µg/serotype in PPV23, respectively (figure 4b). Mice that received 5 µg/serotype in PPV23 had significantly higher PPS4-specific IgG Ab response four weeks after PCV10 booster than mice that were primed with 10µg/serotype in PPV23 (figure 4b). Mice primed with saline had significantly higher PPS4-specific IgG Ab response than mice primed with PCV10 (figure 4b). All groups had significantly higher PPS4-specific IgG Abs than unimmunized control 6 weeks after first immunization (figure 4b, table A 2).

Significantly higher number of PPS4-specific IgG secreting AbSCs was found in spleen in PCV10 boosted mice that were primed with saline, PCV10 or 2.5 µg/serotype in PPV23 than unimmunized control mice 6 weeks after first immunization and in BM for mice primed with PCV10 or saline (figures 4c and 4d and table A 3 and 4).

The number of PPS4-specific IgG secreting AbSCs in spleen (figure 4c) and BM (figure 4d) was significantly lower in mice that were primed with 5 or 10 µg/serotype in PPV23 than in mice primed with saline, PCV10. There was a clear dose effect as significantly higher number of PPS4-specific IgG secreting AbSCs in spleen was found in mice primed with 2.5 µg/serotype in PPV23 than in mice primed with the other two PPV23 doses (Table A 3 and 4). The detrimental effect of PPV23 on AbSCs was only seen for the two higher PPV23 doses with regards to number of PPS4-specific IgG secreting AbSCs cells in spleen.

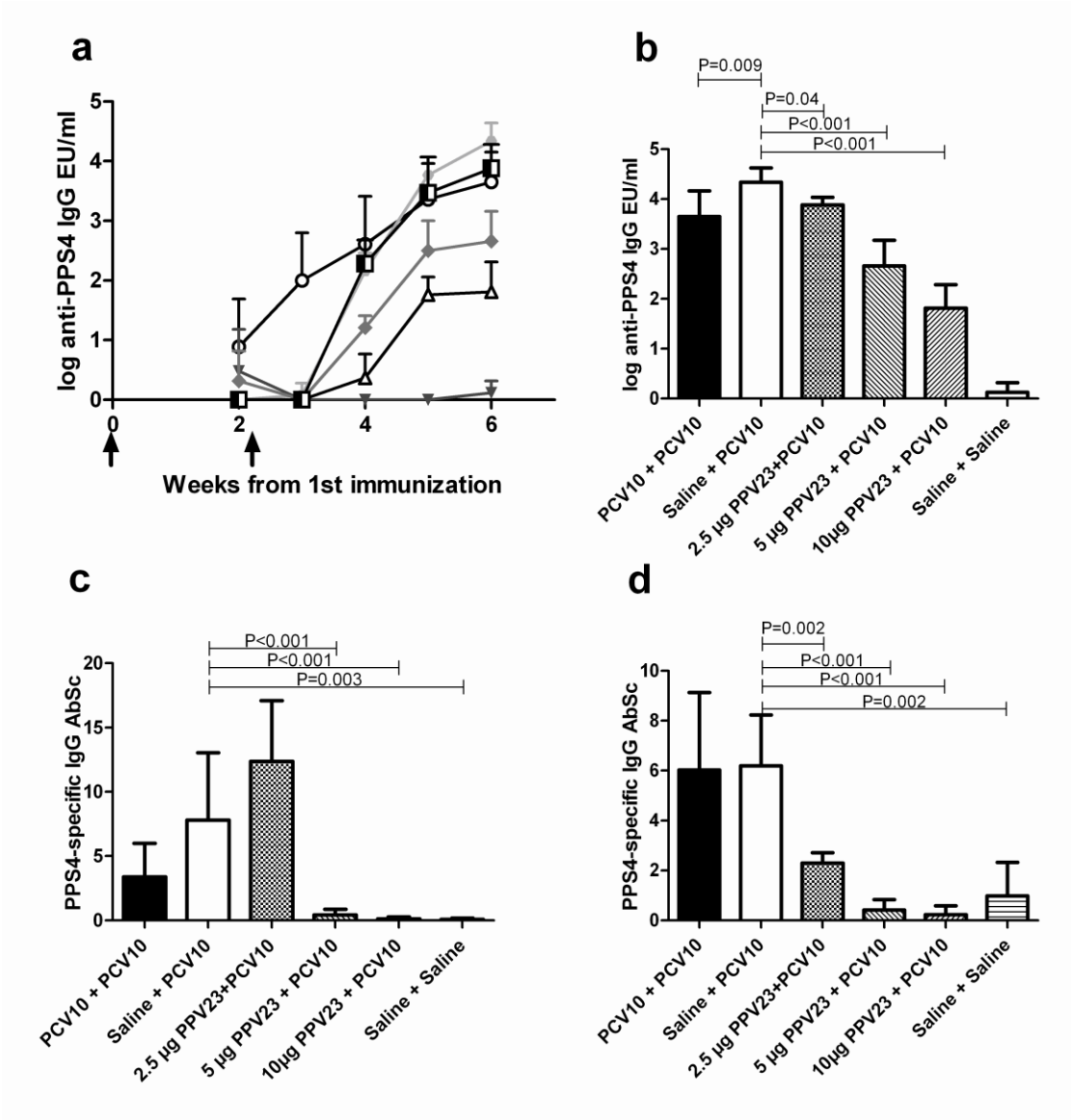

Figure 4: PPS4-specific IgG Abs and number of PPS4-specific IgG+ AbSC in PCV10-boosted mice primed with saline, PCV10 and different doses of PPV23.

(a) Kinetics of response of PPS4-specific IgG Abs from two weeks to six weeks after first immunization for each group. PCV10+PCV10 (●), saline+PCV10 (●), 2.5 µg PPV23+PCV10 (■), 5 µg PPV23+PCV10 (♦), 10 µg PPV23+PCV10 (△) and saline+saline (▼). Arrows indicate time of immunization. b) PPS4-specific IgG Ab levels six weeks after first immunization. c) Number of PPS4-specific IgG+ AbSCs of 10^6 cells in spleen. d) Number of PPS4-specific IgG+ AbSCs of 10^6 cells in BM. P-values are shown if the difference between the test groups and the saline-PCV10 group is significant.

When the response to PPS9V was studied, PCV10 boosted neonatal mice primed with 5 µg/serotype in PPV23 mounted an initially reduced PPS9V-specific IgG Ab response compared with PCV10 or saline primed mice, but with time the difference decreased (figure 5a, table A 2). Priming with 5 or 10 µg/serotype in PPV23 initially reduced the PPS9V-specific IgG Ab response more than priming with 2.5 µg/serotype in PPV23, but with time the diminished Ab response was partially overcome. There was a dose effect, as mice that received 2.5 µg/serotype in PPV23 did not show hyporesponsiveness and had similar PPS9V-specific IgG Ab levels over time as mice primed with saline (Figure 5a). PPS9V-

specific IgG Ab response was significantly lower in mice primed with 5 and 10 µg/serotype in PPV23 than in mice primed with saline 4 weeks after PCV10 booster. The effect was dose related as mice primed with 2.5 µg/serotype in PPV23 had significantly lower PPS9V-specific IgG Abs compared with mice primed with 5 µg and 10 µg dose. PCV10 boosted mice primed with saline, 5 or 10 µg/serotype in PPV23 did not have lower PPS9V-specific IgG Ab response than PCV10 primed mice 4 weeks after PCV booster. Mice that received 2.5 µg/serotype in PPV23 did not have lower PPS9V-specific IgG Ab response than mice primed with saline or PCV. All immunized groups had significantly higher PPS9V-specific IgG Ab response than unimmunized control mice 6 weeks after first immunization (figure 5b, table A 2).

Four weeks after PCV10 booster there was a significantly higher number of PPS9V-specific IgG secreting AbSCs in spleen of neonatal mice primed with saline, PCV10, 2.5/serotype in PPV23, 5/serotype in PPV23 and 10 µg/serotype in PPV23 than in spleen of unimmunized control mice (figure 5c). Mice primed with saline had a significantly higher number of PPS9V-specific IgG secreting AbSCs in spleen than mice primed with PCV10 or 10 µg/serotype in PPV23. Mice primed with 2.5 µg/serotype in PPV23 had significantly higher number of PPS9V-specific IgG secreting AbSCs than those primed with 10 µg/serotype in PPV23 (figure 5c, table A 3).

Priming with PPV23 had no clear effect on PPS14-specific IgG Ab response after PCV10 booster. PCV10 primed mice showed higher PPS14-specific IgG Ab response than saline or PPV23 primed mice (all doses) (figure 5d). PCV10 primed mice had significantly higher PPS14-specific IgG Ab response than mice primed with saline, 2.5 µg/serotype in PPV23, 5 µg/serotype in PPV23 or 10 µg/serotype in PPV23, 4 weeks after a PCV10 booster. No difference was seen between different PPV23 doses or saline priming (figure 5e). PCV10 elicited a booster response to PPS14 but there was no evidence of PPV23 diminishing the PPS14-specific IgG Ab response to PCV10 booster. All immunized groups had significantly higher PPS14-specific IgG Ab response than unimmunized control mice (figure 5e, table A 2).

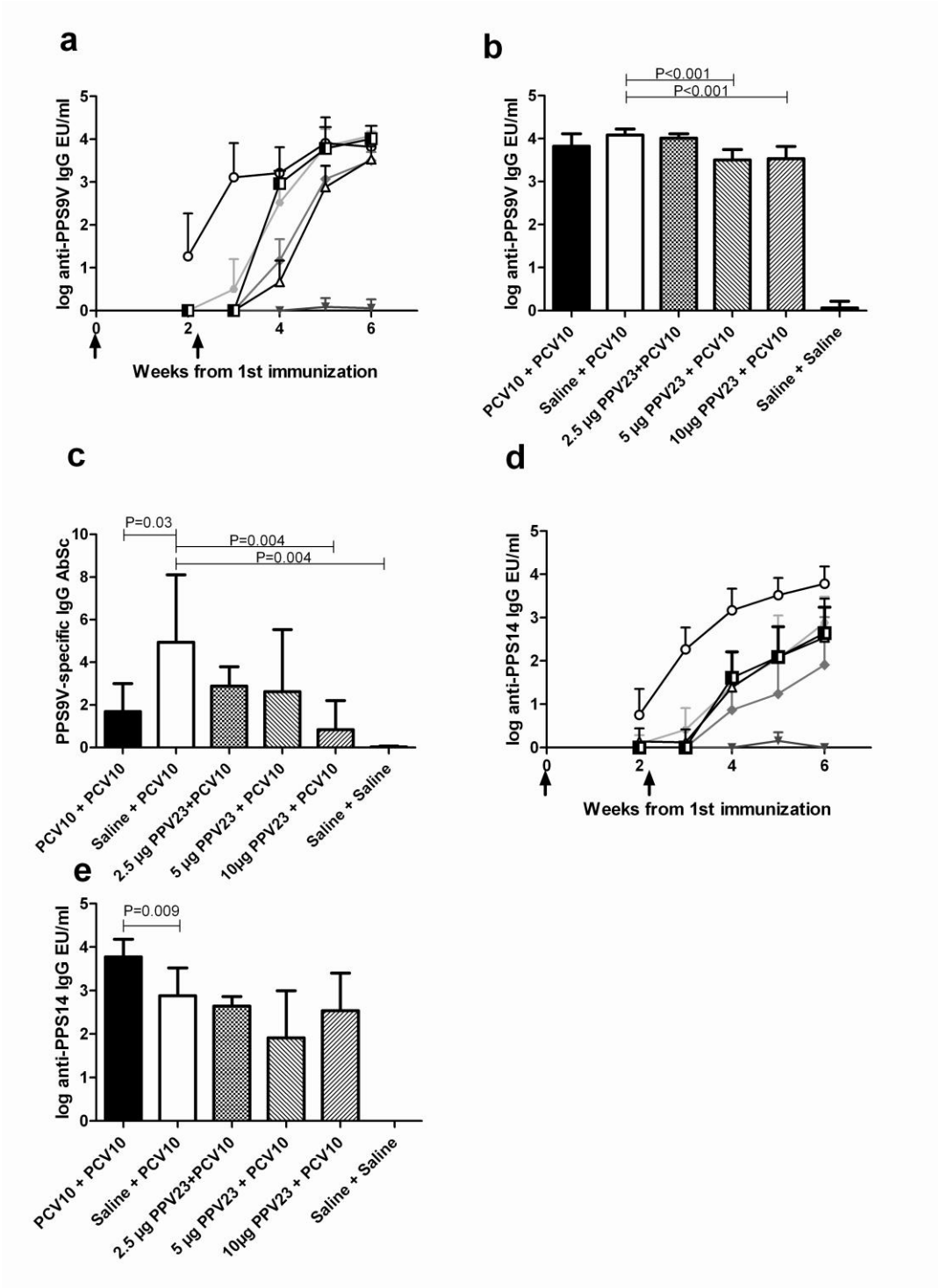

Figure 5: PPS9V- and PPS14-specific IgG Abs and number of PPS9V-specific IgG+ AbSCs in spleen in PCV10-boosted mice primed with saline, PCV10 and different doses of PPV23.

a) Kinetics of PPS9V-specific IgG Ab response from two weeks to six weeks after first immunization for each group. PCV10-PCV10 (●), saline-PCV10 (●), 2.5 µg PPV23-PCV10 (◧), 5 µg PPV10-PCV10 (♦), 10 µg PPV23-PCV10 (△) and saline-saline (▼). Arrows indicate time of immunization b) PPS9V-specific IgG Ab levels six weeks after first immunization c) Number of PPS9V-specific IgG+ AbSCs of 10^6 cells in spleen. d) Kinetic of PPS14-specific IgG Ab response two weeks to six weeks after first immunization for each group. PCV10-PCV10 (●), saline-PCV10 (●), 2.5 µg PPV23-PCV10 (◧), 5 µg PPV23-PCV10 (♦), 10 µg PPV23-PCV10 (△) and saline-saline (▼). Arrows indicate time of immunization. e) PPS14-specific IgG Ab levels six weeks after first immunization. P-values are shown if the difference between test groups and the saline-PCV10 group is significant.

PPV23 priming had a detrimental effect on PPS18C-specific IgG Ab response after PCV10 booster (figure 6a, table A 2). The effect was dose dependent, as PCV10 boosted mice primed with 5/serotype in PPV23 or 10 µg/serotype in PPV23 had lower PPS18C-specific IgG Ab response than mice that received saline as priming, while mice primed with 2.5 µg/serotype PPV23 did not show compromised response after PCV10 boosting. PCV10 booster after PCV10 priming elicited a clear booster response (Figure 6a). Priming with 5 or 10 µg/serotype in PPV23 significantly reduced the PPS18C-specific IgG Ab response compared with saline, 4 weeks after a PCV10 booster. There was an evident dose effect of PPV23, as mice that received 2.5 µg/serotype in PPV23 had significantly higher PPS18C-specific IgG Abs than mice primed with 5 µg/serotype in PPV23 or 10 µg/serotype in PPV23 doses. PCV10 boosted mice primed with PCV10 had significantly higher PPS18C-specific IgG Abs than those that received saline, 2.5 µg/serotype in PPV23, 5 µg/serotype in PPV23 or 10 µg/serotype in PPV23. All immunized groups had significantly higher PPS18C-specific IgG Abs than unimmunized control mice (figure 6b, table A 2).

Priming with PPV23 had a clear effect on the generation of PPS18C-specific IgG secreting AbSCs in spleen 4 weeks after booster (figure 6c). PCV10 boosted mice primed with PCV10, saline or 2.5 µg of PPS18C in PPV23 had significantly higher number of PPS18C-specific IgG secreting AbSCs in spleen than the unimmunized control mice. The effect of the PPV priming was significant, with more AbSCs in saline and PCV10 primed mice than mice primed with 5 µg PPS18C in PPV23 and 10 µg PPS18C in PPV23. The effect was dose dependent, mice that received 2.5 µg/serotype in PPV23 had higher number of PPS18C-specific IgG secreting AbSCs than mice primed with 5 µg PPS18C in PPV23 or 10 µg µg PPS18C in PPV23 (P=0.002). In mice primed with 2.5 µg/serotype in PPV23 there was no difference between the numbers of PP18C-specific IgG secreting AbSCs in spleen compared with mice primed with PCV10 or saline (figure 6c, table A 3).

PPV23 tended to reduce the number of PPS18C-specific IgG secreting AbSCs in BM, but no immunized group had significantly higher number of PPS18C-specific IgG secreting AbSCs than the unimmunized mice in BM (figure 6d, table A 4). No conclusions can be drawn regarding effects of PPV23 on the PPS18C-specific IgG secreting AbSCs in the BM.

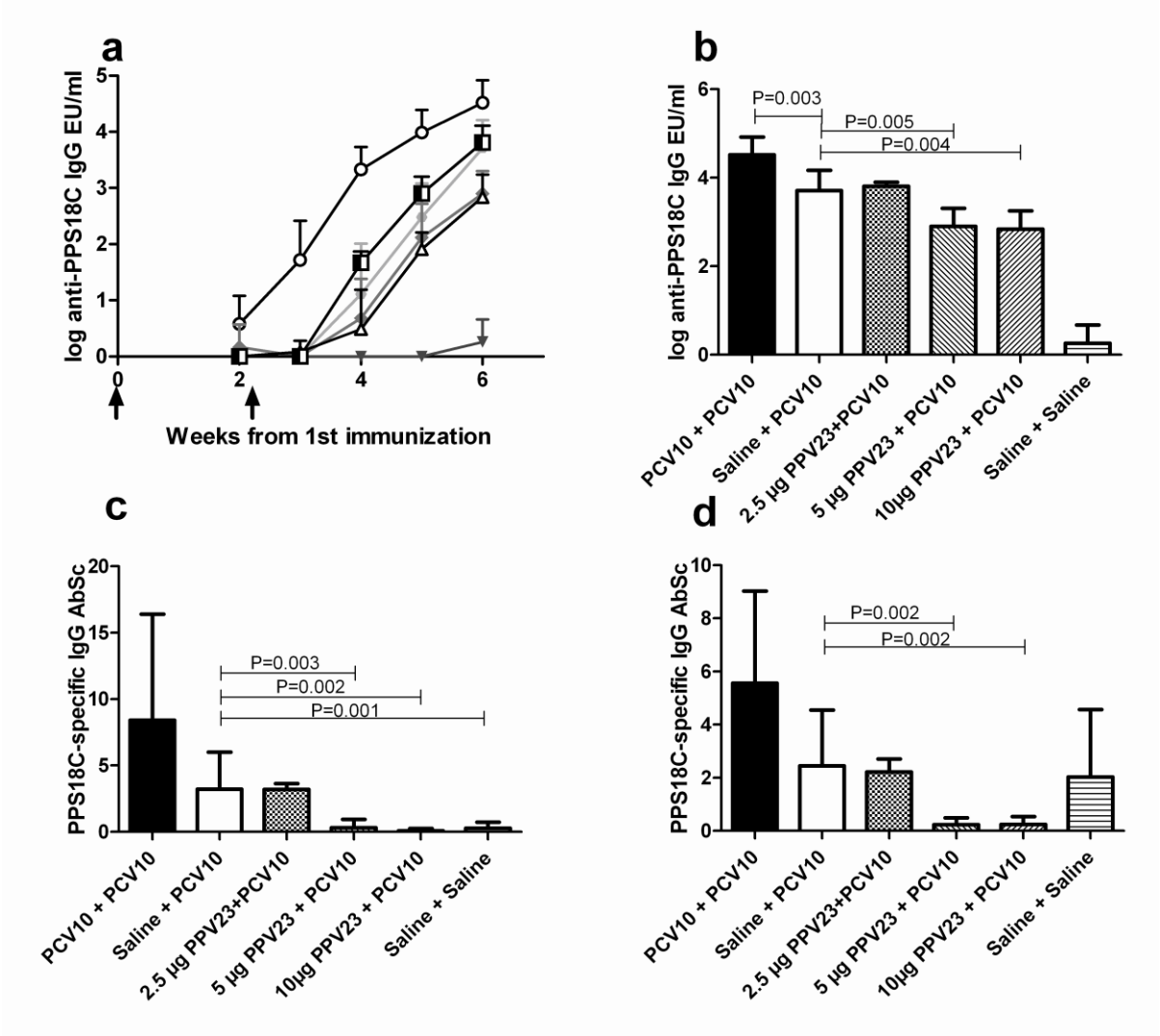

Figure 6: PPS18C-specific IgG Abs and number of PPS18C-specific IgG+ AbSCs in PCV10-boosted mice primed with saline, PCV10 and different doses of PPV23.

a)Kinetics of PPS18C-specific IgG Ab response from two weeks to six weeks after first immunization for each group. PCV10-PCV10 (●), saline-PCV10 (●), 2.5 µg PPV23-PCV10 (◧), 5 µg PPV23-PCV10 (◆), 10 µg PPV23-PCV10 (△) and saline-saline (▼). Arrows indicate time of immunization. b) PPS18C-specific IgG Abs six weeks after first immunization. c) Number of PPS18C-specific IgG+ AbSCs of 10^6 cells in spleen. d) Number of PPS18C-specific IgG+ AbSCs of 10^6 cells in BM. P-values are shown if the difference between test groups and the saline-PCV10 group is significant.

In summary, hyporesponsiveness to a booster of PCV10 was induced by priming mice with PPV23 for all PPSs measured except PPS14. The PS-induced reduction of IgG Ab response could be overcome for certain PSs, as results for PPS9V and PPS18C implied. The PPV23-induced hyporesposiveness was dose dependent and higher doses had greater effect than lower doses on IgG Abs and AbSCs specific to all serotypes, except IgG Ab response specific for PPS14.

5 Discussions

5.1 Effect of native/plain pneumococcal polysaccharide booster on antibody specific response from immunization with pneumococcal polysaccharide conjugate vaccine.

In order to obtain a better immune response against pneumococci for decreasing pneumococcal disease globally, the use of a polysaccharide (PS) booster has been considered a possible solution, mainly in the third world as it is less expensive than PCVs and would theoretically provide protection against more serotypes (165), as PPV23 contains 23 serotypes but the PCVs contain 7 – 13 serotypes. The effect of bacterial capsular PS boosters on already established antibody (Ab) response to PCVs has been the major drawback, since many studies have shown significant decrease in quality and/or quantity of PS specific Abs (174, 186). PS-induced hyporesponsiveness caused by PS boosters has been seen in clinical studies in infants (174), and middle aged adults and elderly (172, 187). These are the most studied age groups regarding response to pneumococcal vaccines, since they are most susceptible to pneumococcal disease (151, 152).

The effects of PS on immune response to conjugate vaccines have been studied in neonatal mouse models. Our group has previously shown that boosters with monovalent MenC-PS, PPS1 or PPS19F decrease the levels and quality of PS-specific IgG Abs in mice primed as neonates with conjugate vaccines. Similar effects have been observed in adult mice when model PSs like NP-ficoll were used (118, 170, 183).

Our aim was to establish whether PS-induced hyporesponsiveness was induced similarly to many PPS serotypes in a multivalent licensed pneumococcal vaccines in our neonatal mouse model. Here we demonstrate that a booster with a multivalent licensed PPV23 causes a reduction in PPS-specific IgG Abs in neonatal mice primed with a licensed multivalent PCV10. In this study, results for Ab measurement are shown for 5 of 10 PPS in PCV10. IgG specific to 6B and 19F have been measured 6 weeks after priming. IgG levels were undetectable for PPS19F and low for PPS6B with no significant difference between the immunized groups, although there was a trend for hyporesponsiveness. Compared with saline a PPV23 booster caused a reduced IgG Ab response specific to all PS serotypes measured and compared with PCV10 booster the IgG Ab levels to all PSs measured except PPS4 were reduced.

In mice primed with PCV10 both PCV10 and saline boosters induced lower PPS4-specific IgG Ab response to PPS4 than expected. Although not compared directly in this study both groups had lower PPS4-specific IgG Ab response than neonatal mice primed with saline and boosted with PCV10 (in a different experiment). This may indicate that the dose of PPS4-conjugate was too high for neonatal mice. Animal studies have shown that when more than

seven serotypes were included in a PCV reduced serotype-specific Ab responses were observed at higher conjugate doses (reviewed in (188)). The concentration of PPS4 conjugated to protein D in PCV10 is higher than any of the other PPS conjugated to protein D and higher than the concentration of 7 out of 9 serotypes in the vaccine. The PPS4-conjugate is composed of 0.75 µg of PPS4 conjugated to protein D, while PPS1, PPS5, PPS7F, PPS9V, PPS14 and PPS23F are composed of 0.25 µg conjugated to protein D, and PPS18C and PPS19F conjugates are composed of 0.75 µg conjugated to TT and DT, respectively.

When comparing mice primed 7 days old with PCV and mice primed 7 days old with PCV and boosted with PCV 16 days later there was no clear PCV booster response to PPS1, PPS9V, PPS14 and PPS18C, further suggesting that the PCV dose was too high. We chose 1/4 of human dose that for most serotypes was similar to the doses of monovalent PCVs in previous studies that were likely to elicit measurable Ab response to all PS serotypes in the vaccine. However, PPS19F-specific IgG Abs was undetectable, indicating that the PPS19F-conjugate dose was too low (data not shown).

The PPS-induced reduction of serum Ab response could possibly be due to lack of creation of new plasmablasts/short-lived PCs in spleen/lymph nodes or due to decreased survival of long-lived PCs in BM. Earlier study has shown that in mice primed with a monovalent conjugate of serotype 1 that received PPS1 booster the number of PPS1-specific IgG secreting AbSCs in BM and spleen was decreased compared with mice that received saline booster (182). The results showed that AbSCs that had already reached the BM when PPS booster was given did not decrease. The likeliest explanation is that the PPS booster decreased the number of PPS1-specific AbSCs in the spleen thus decreasing homing of PPS1-specific IgG secreting AbSCs to the BM. A further support for the effect occurring in the spleen rather than BM was the observation that a PPS1 booster decreased GC reaction (PNA+ GCs) in spleen compared with a saline booster (182). It was as well shown in previous studies that a MenC-PS booster in MenC-CRM primed mice caused a decrease in newly activated MenC-PS-specific PCs and naive B cells in spleen. Shortly after the MenC-PS-booster increased apoptosis of newly activated MenC-PS-specific B cells was observed in the spleen and fewer newly activated PCs travelled to the BM (183). This was most likely because PS caused increased apoptosis in the GC, leading to reduced number of PS-specific memory and naive B cells in the spleen. Thus the contact with the PS causes fewer PCs to home to the BM which results in lower Ab level (182). A similar effect on memory and naive B cells was observed in adult mice after administration of NP-Ficoll as a model PS compared to NP-CGG as the conjugate. B cells with the highest affinity were most inclined to be killed after a NP-Ficoll booster in NP-CGG primed mice (170).

Most clinical studies addressing PS-induced hyporesponsiveness have been done in infants and a few in middle-aged adults and elderly. Our group showed that in terms of Ab levels and protective efficacy the PS-induced hyporesponsiveness was most prominent in mice primed with PCV as neonates or infants while it was less evident in adult mice. Therefore, we used a neonatal mouse model in this study and previous studies to investigate hyporesponsiveness induced by multivalent PPV, although we would anticipate that PPV23 booster might compromise the Ab response serotypes in PCV10 primed adult mice in either the quantity and/or quality of Abs (118, 170).

Whether the PS-induced hyporesponsiveness we observed could have clinical effects is hard to know without doing a protection experiment or *in vitro* protection assay like OPA. Our group has shown that for serotype 1 the protective serum Ab levels (anti-PPS1 IgG) was approx. 1.3 log EU/ml and 2.5 log EU/ml for lung infection after intranasal pneumococcal challenge with aspiration (112). In the current study on multivalent vaccines and a previous study on monovalent vaccines the PPS1-specific IgG Ab levels in mice boosted with PPV23 or PPS1, respectively, were below the protective levels for both bacteremia and lung infection (118). Those low Ab levels are similar to clinical studies that show no difference in quantity of Abs but affected protective efficacy of the Abs measured by SBA or OPA (112, 182). Increased hospitalization because of illness in the lower respiratory tract among Australian aboriginals was observed in individuals that were boosted with a PS dose compared with those who were not, indicating that hyporesponsiveness can compromise the protective efficacy induced by PCV immunization (189). It is difficult to extrapolate mouse data into clinical relevance in humans. However, our results show that hyporesponsiveness to the majority of pneumococcal serotypes tested is induced by PPV23 booster reflected in reduced Ab levels. These results suggest that PPV23 booster is likely to reduce the humoral effector and memory responses and compromise the protective immunity elicited by PCV immunization.

5.2 Effect of priming with plain pneumococcal polysaccharide on immune response to a booster with pneumococcal polysaccharide conjugate vaccine.

Priming children younger than 2 years old with a plain capsular polysaccharides (PSs) has only been suggested as a possible solution in an epidemic in recent years. Clinical studies on both meningococcal and pneumococcal polysaccharides have shown that priming of young children with PS decreases serotype specific antibody (Ab) response against a subsequent conjugate or PS booster (175). The negative effect of PS priming on generation of immune memory and response to subsequent conjugate vaccination has been shown in other age groups like adolescents (178), young adults (190) and middle aged adults to

elderly (176, 177). Similarly, pneumococcal colonization and infection can also cause a serotype specific reduction of Ab response to subsequent PCV immunization (166, 180, 181), emphasizing the need to understand how PSs affect cells of the immune system and cause hyporesponsiveness. Studies have shown that immune responses to PSs in intact bacteria are different from immune responses to plain capsular PSs. In intact bacteria there are numerous immune enhancing and suppressing molecules, that may modulate the responses. Understanding how and why plain capsular PSs affect subsequent responses would help to understand the effects of exposure to intact encapsulated bacteria on responses to subsequent vaccination with conjugate vaccines (93, 94, 96) and hopefully to find ways to circumvent PS-induced hyporesponsiveness.

Our aim was to investigate whether priming with PPV23 would affect immune response to a subsequent PCV booster.

Our results indicate that some PSs are more effective than others in suppressing the response to subsequent PCV10 immunization. PPV23 priming induced hyporesponsiveness to PPS1, shown in significantly decreased PPS1-specific IgG response to PCV10. PPV23 priming significantly reduced PPS4-, PPS9V- and PPS18C-specific IgG Ab response. Reduced response to PPS9V in PPV23 primed mice was overcome to some degree with time. PPV23 priming caused no reduction in PPS14-specific IgG response to PCV10 booster. The number of PPS-specific AbSCs in spleen was in agreement with the IgG response in serum.

We asked ourselves whether the dose of PPV23 could affect hyporesponsiveness because the PSs have different effects on PPS-specific IgG and AbSCs response. In a previous study it was shown that PPS1 booster caused hyporesponsiveness in conjugate primed mice and all doses tested caused a similar reduction in PPS1-specific Ab levels (118). In our study priming with three doses of PPV23 was compared; 2.5, 5 and 10 µg of each serotype before a PCV10 booster was given. A clear dose effect was seen for three of five serotypes, an increasing dose of PPV23 caused more reduction of PPS-specific IgG responses. For PPS4, PPS9V and PPS18C the lowest dose (2.5 µg of each PPS serotype) did not induce hyporesponsiveness. For PPS14 no hyporesponsiveness was seen with any dose of PPV23. All the doses tested decreased the PPS1-specific IgG response. Some dose effect was seen on the number of PPS-specific AbSCs. Mice primed with the lowest PPV23 dose (2.5 µg of each PPS) had similar numbers of AbSCs in spleen as saline-primed mice, whereas AbSC numbers were reduced in mice primed with higher doses of PPV23 (5 or 10 µg of each PPS).

The reduction observed in AbSCs in spleen is evident in the BM as well, demonstrating that reduction of AbSCs in spleen was not due to their homing to the BM. For serotype 9V

AbSCs were only measured in spleen. PCV primed mice had significantly lower PPS9V-specific AbSCs cells in spleen than saline primed mice. This may be because PPS9V-specific AbSCs cells have already moved to the BM, since PPS9V-specific Ab levels were not reduced, but the GC reaction in spleen has started to wane and fewer new AbSCs being generated. Our results cannot prove this, since the limited number of BM cells only allowed us to measure AbSCs for three serotypes, and PPS9V-specific AbSCs were not measured in the BM.

Why some PSs more effectively suppress serotype specific Ab response than others is quite a mystery. The variation in general inability of PSs to induce Ab response in 1 week old mice could be one of the reasons that some PSs induce hyporesponsiveness to subsequent PCV booster while other PSs do not. Earlier studies have shown that PS specific Abs regulate recall response to the same antigen and diminish the response (85).

PPS are poorly immunogenic in children younger than 2 years of age, but it is known that some PPS induce better Ab responses in children than others. (191)

A metaregression was used to compare responses to plain PPS in healthy children under the age of two, reported in 29 publications. The results showed that PPS of serotypes 6B, 14 and 19F were poorly immunogenic, PPS of serotypes 1, 4 and 18C were showed to be more immunogenic while PPS9V was most immunogenic. The response to PS in children was influenced by the age of the children and possible interference by maternal Abs and possible hyporesponsiveness caused by pneumococcal colonization and infection. In many studies Abs are only measured against fractions of the PSs (180, 191, 192). PPS14 is a worse inducer of Ab response in infants than many of the other serotypes. No natural Abs recognized PPS14 in mice (PPS14 was the only neutral PPS measured), while natural Abs to the other PPS measured did exist (193). This may explain poor response to PPS14 in mice as natural Abs contributes to induction of adaptive immune response to vaccines. In rats plain PPS14 has been shown not to locate in the follicles of spleen, while other plain PPS were shown to locate in the follicles (194-196). A study in adult mice has also shown that priming with PPS14 and colonization with pneumococci of serotype 14 did not reduce antibody response to a subsequent immunization with conjugate of serotype 14 (197).

The main reason for different effects of different PS on the immune response is probably related to their structure. Structure of PSs impacts their ability to bind certain immune-enhancing–receptors, such as TLR or MHC-II. PPS1 is the only pneumococcal zwitterionic PS (ZPS). ZPSs are both positively and negatively charged and have been shown be able to stimulate CD4+ T cells *in vivo* and *in vitro.* ZPSs are depolymerized in the endosomes of APCs into molecules of 10-15 kDA, which can bind to MHCII molecules. Anionic PS that are chemically changed to ZPS gain the ability to stimulate CD4+ T cells and ZPS that are

changed to anionic/neutral PS lose this ability. The induction of Ab response by ZPS is different from that of other PS, which could possibly be a reason for PPS1 being a strong inducer of hyporesponsiveness (198-201). Of the PSs in PCV10 serotypes 7F and 14 are neutral while the other are anionic. Some PSs like MenC-PS and Group B streptococci serotype V PS can negatively influence the BAFF/APRIL system which plays an important role in Ab response against TI-2 antigens, while the neutral model PS NP-Ficoll enhances BAFF/APRIL signaling. As BAFF/APRIL is important for creation and persistence of Abs, impacting that system would affect specific Ab response (97).

One of the questions we would like to answer is why PPS-induced hyporesponsiveness is overcome for some polysaccharides. Clinical studies have indicated that some conjugates are able to overcome PS-induced hyporesposiveness (202). However, two PCV boosters did not overcome reduction caused by PPV23 priming of elderly individuals (176). Immunogenicity of pneumococcal conjugates varies greatly even though they have the same carrier. Many factors influence the immunogenicity of conjugates. The immunogenicity of the polysaccharide, polysaccharide/protein ratio and the length of the polysaccharide are examples of aspects that influence the immunogenicity of conjugates (203, 204). Differences in immunogenicity of conjugates could explain why some PCVs overcome the reduction of PPS-specific IgG response in PPS primed mice. Response to PPS9V was partially overcome by PCV10, at initial time points the Ab response significantly reduced in PPV23 primed mice compared with saline primed mice, but at the sixth week those differences has diminished. Some carriers increase the immunogenicity of a PS more than others and having serotypes conjugated to the same carrier can either enhance or suppress Ab responses to the serotypes (72-74, 203, 205-207)

There are indications that carriers could affect hyporesponsiveness. We did a pilot study using PCV7 instead of PCV10. In PCV7 all the serotypes (4, 6B, 9V, 14. 18C, 19F and 23F) are conjugated to the CRM197 carrier. We immunized neonatal mice with 0.5 µg of each pneumococcal conjugate except PPS6B where the dose was 1 µg. PPS19F-specific IgG response was measurable after PCV7 booster and reduction was evident in PPV23 primed mice (208). Whereas there was no response to PPS19F detected after PCV10 immunization (data not shown). It has been reported that different responses to PPS19F conjugates in PCV7 and PCV10 are due to different conjugation methods used in PCV7 and PCV10 that affect their immunogenicity. Reductive amination which is used to conjugate PPS19F to CRM197 in PCV7 appeared to create an additional epitope while opsonophagocytic activity of the Abs did not increase. Cyanylation was used to conjugate PPS19F to the DT carrier protein in PCV10, inducing less PPS19F-specific Abs without affecting Ab mediated opsonophagocytosis of pneumococci of serotype 19F compared with PCV7. Thus there are

many factors that may have an impact on immunogenicity of conjugates which may also influence their ability to overcome hyporesponsiveness induced by the corresponding PPS serotypes (209).

Our results show that PPV23 priming reduces PPS-specific AbSCs and IgG Abs, although the cellular mechanism has not been fully explained. There have been indications from other studies, for example a decreased number of memory and B1b cells responding to PCV7 in PPV23 primed middle aged adults to elderly individuals (210). Our project also raises questions that are related to reasons behind different responses to different serotypes and why the effect is dependent on the PPS dose. To investigate the reasons the different response PPV priming has on Ab response to each PPS certain variants must be taken out of the equation. For example the effect of different carriers, conjugation methods, multivalent effect and dose of the conjugate. If these factors do not affect the differences regarding the PSs some structural studies could be done.

To further expand the results of this study it is important to investigate whether PPV affects the quality of the Abs where it does not affect the quantity.

It is difficult to extrapolate clinical relevance in humans from mouse data. Our results show that hyporesponsiveness to the majority of pneumococcal serotypes tested is induced by priming with PPV23 which is not only reflected in reduced Ab levels but also rapid depletion of AbSCs in the spleen and reduced number of AbSCs in the BM. The results suggest that priming with PPV23 is likely to reduce the humoral effector and memory responses and compromise the protective immunity elicited by PCV immunization.

6 Conclusion

In this study we evaluated the effect PPSs on response to subsequent and already established response to PCV. The main results are summarized in tables 2 to 4.

We have demonstrated that PPV23 decreases response to a PCV10 immunization. We have shown that a PPV23 booster decreases PPS-specific IgG antibodies (Abs) that were created by PCV10 immunization. IgG response specific for all measured polysaccharides were lower in mice boosted with PPV23 than in mice boosted with saline and for 4 of 5 measured polysaccharides compared with mice boosted with PCV10. We conclude that PPV23 booster clearly decreases the response induced by PCV10 (summarized in table 2).

We show that priming with PPV23 causes hyporesponsiveness, since a decrease in PPS-specific IgG and number of PPS-specific AbSCs in spleen and bone marrow (BM) to a subsequent PCV10 immunization was observed. IgG response specific to 4/5 PPS measured were lower in PPV23 primed mice compared with mice primed with saline or PCV. We show that the effect is dependent on the type of PS as the effect is significant for 4 of 5 PPSs (not for PPS14) and that the response to PPS9V is partially overcome (summarized in table 3). We saw that priming with PPV23 reduced the number of PPS-specific AbSCs in spleen and BM for all measured PPSs (PPS1, 4, 9V and 18C) (summarized in table 4). Because the effect of PPS-specific IgG response and number of PPS-specific AbSCs was different between serotypes we speculated that the dose of the PPS would affect the response. We compared the effect of three different PPV23 doses (2.5, 5 and 10 µg of each serotype) on PPS-specific IgG and number of AbSCs in spleen and BM before a PCV booster. We demonstrated that the effect of priming with PPV23 was dose and serotype dependent, because there was significant difference in hyporesponsiveness induced by different doses, which varied between serotypes (summarized in table 3 and 4).

Table 2: Summary of results on the effects of PPV23 booster on IgG response in mice primed with PCV10 6 and 12 weeks after first immunization

Weeks after first immunization	Priming	Boosting	IgG anti-PPS1	IgG anti PPS4	IgG anti-PPS9V	IgG anti-PPS14	IgG anti-PPS18C
Week 6	PCV10	PCV10	+++	++	+++	+++	++
	PCV10	Saline	+++	++(+)*	+++	+++	++
	PCV10	PPV23	++	++	++	++	+
	Saline	Saline	+	+	+	+	+
Week 12	PCV10	PCV10	+++	++	+++	+++	+++
	PCV10	Saline	+++	++(+)*	+++	+++	+++
	PCV10	PPV23	++	++	++	++	++
	Saline	Saline	+	+	+	+	+

Mice were primed as neonates (1 week of age) with PCV10 and boosted 16 days later with PCV10, saline or PPV23. An unimmunized control group received saline-saline. An additional + means that there is significant difference between groups P<0.05 (++ is significantly higher than +, etc).

* PCV10 primed mice boosted with saline had significantly higher PPS4-specific IgG than PCV10 primed mice boosted with PPV23.

Table 3: Summary of results on the effects of priming with PPV23 on PPS-specific IgG response in mice boosted with PCV10 six weeks after first immunization

Priming	Booster	IgG anti-PPS1	IgG anti PPS4	IgG anti-PPS9V	IgG anti-PPS14	IgG anti-PPS18C
PCV10	PCV10	++++	+++	++	+++	++++
Saline	PCV10	++++	++++	++(+)*	++	+++
2.5 µg/serotype in PPV23	PCV10	+++	+++	++(+)*	++	+++
5 µg/serotype in PPV23	PCV10	++	++	++	++	++
10 µg/serotype in PPV23	PCV10	++	++	++	++	++
Saline	Saline	+	+	+	+	+

Mice were primed as neonates (1 week of age) with PCV10, saline or 2.5, 5 or 10 µg/serotype PPV23 and boosted 16 days later with PCV10. An unimmunized control group received saline-saline. An additional + means that there is significant difference between groups P<0.05 (++ is significantly higher than + etc,)

*****Saline and mice primed with 2.5 µg/serotype in PPV23 had significantly higher PPS9V specific IgG response than mice primed with 5 or 10 µg/serotype PPV23.

Table 4: Summary of results on the effect of priming with PPV23 on PPS specific AbSC in spleen (SP) and bone marrow (BM) six weeks after first immunization in mice boosted with PCV10.

Priming	Booster	ABSC in SP specific for PPS1	ABSC in SP specific for PPS4	ABSC in SP specific for PPS9V	ABSC in SP specific for PPS18C	AbSC in BM specific for PPS1	AbSC in BM specific for PPS4	AbSC in BM specific for PPS18C
PCV10	PCV10	++	++	++	++	++	++	+
Saline	PCV10	++	++	++(+)*	++	++	++	+
2.5 µg/serotype in PPV23	PCV10	++	++	++(+)**	++	+(+)***	+	+
5 µg/serotype in PPV23	PCV10	+	+	++	+	+	+	+
10 µg/serotype in PPV23	PCV10	+	+	++	+	+	+	+
Saline	Saline	+	+	+	+	+	+	+

Mice were primed as neonates (1 week of age) with PCV10, saline or 2.5, 5 or 10 µg/serotype in PPV23 and boosted 16 days later with PCV10. Saline-saline is an unimmunized control group. An additional + means that there is significant difference between groups $P<0.05$ (++ is significantly higher than +, etc).

* PCV10 boosted mice primed with saline had significantly higher PPS9V-specific AbSC response than PCV10 boosted mice primed with PCV10 and 10 µg/serotype. ** PCV10 boosted mice primed with 2.5 µg/serotype in PPV23 had higher PPS9V specific AbSC in spleen than mice primed with 10 µg/serotype in PPV23. *** PCV10 boosted mice primed with 2.5 µg/serotype did not have lower PPS1 specific AbSCs in BM than PCV10 boosted mice primed with saline or PCV10 but not significantly higher than unimmunized control mice.

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Appendix

Table A1: Statistical comparison of PPS1-, PPS4-. PPS9V-, PPS14- and PPS18C-specific IgG Ab levels in mice primed with PCV10 and boosted with PCV10, PPV23 or saline.

IgG anti-PPS1	**Week 6**			**Week 12**		
Groups	**PCV10-Saline**	**PCV10-PPV23**	**Saline-saline**	**PCV10-saline**	**PCV10-PPV23**	**Saline-saline**
PCV10-PCV10	P=0.67	**P=0.004**	**P<0.001**	P=0.43	**P=0.002**	**P<0.001**
PCV10-Saline		**P=0.003**	**P<0.001**		**P<0.001**	**P<0.001**
PCV10-PPV23			**P=0.049**			**P=0.002**
IgG anti-PPS4	**Week 6**			**Week 12**		
Groups	**PCV10-Saline**	**PCV10-PPV23**	**Saline-saline**	**PCV10-saline**	**PCV10-PPV23**	**Saline-saline**
PCV10-PCV10	P=0.7	P=0.14	**P=0.002**	P=0.28	P=0.19	**P=0.007**
PCV10-Saline		**P=0.01**	**P=0.002**		**P=0.007**	**P<0.001**
PCV10-PPV23			**P=0.04**			P=0.16
IgG anti-PPS9V	**Week 6**			**Week 12**		
Groups	**PCV10-Saline**	**PCV10-PPV23**	**Saline-saline**	**PCV10-saline**	**PCV10-PPV23**	**Saline-saline**
PCV10-PCV10	P=0.63	**P=0.005**	**P<0.001**	P=0.36	**P<0.001**	**P<0.001**
PCV10-Saline		**P=0.004**	**P<0.001**		**P<0.001**	**P<0.001**
PCV10-PPV23			**P=0.002**			**P=0.002**
IgG Anti-PPS14	**Week 6**			**Week 12**		
Groups	**PCV10-Saline**	**PCV10-PPV23**	**Saline-saline**	**PCV10-saline**	**PCV10-PPV23**	**Saline-saline**
PCV10-PCV10	P=0.11	**P<0.001**	**P<0.001**	P=0.36	**P=0.003**	**P<0.001**
PCV10-Saline		**P=0.002**	**P<0.001**		**P=0.005**	**P<0.001**
PCV10-PPV23			**P=0.04**			**P=0.002**
IgG Anti-PPS18C	**Week 6**			**Week 12**		
Groups	**PCV10-Saline**	**PCV10-PPV23**	**Saline-saline**	**PCV10-saline**	**PCV10-PPV23**	**Saline-saline**
PCV10-PCV10	P=0.17	**P<0.001**	**P<0.001**	P=0.3	**P<0.001**	**P<0.001**
PCV10-Saline		**P<0.001**	**P<0.001**		**P<0.001**	**P<0.001**
PCV10-PPV23			P=0.645			**P=0.04**

Bold P value is significant.

Table A2: Statistical comparison of PPS1-, PPS4-, PPS9V-, PPS14- and PPS18C-specific IgG Ab levels between mice primed with PCV10, different doses of PPV23 or saline and boosted with PCV10 six weeks after first immunization.

IgG anti-PPS1	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=0.96	**P=0.006**	**P<0.001**	**P<0.001**	**P=0.002**
Saline+PCV10		**P<0.001**	**P<0.001**	**P<0.001**	**P=0.002**
2.5 µg PPV23+PCV10			**P=0.007**	**P<0.001**	**P=0.002**
5 µg PPV23+ PCV10				**P=0.04**	**P=0.002**
10 µg PPV23+PCV10					**P=0.002**
IgG anti-PPS4	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	**P=0.009**	P=0.48	**P=0.001**	**P<0.001**	**P=0.003**
Saline+PCV10		**P=0.04**	**P<0.001**	**P<0.001**	**P=0.002**
2.5 µg PPV23+PCV10			**P=0.001**	**P<0.001**	**P=0.002**
5 µg PPV23+ PCV10				**P=0.01**	**P=0.002**
10 µg PPV23+PCV10					**P=0.002**
IgG Anti-PPS9V	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=0.054	P=0.4	P=0.09	P=0.07	**P=0.003**
Saline+PCV10		P=0.3	**P<0.001**	**P<0.001**	**P=0.002**
2.5 µg PPV23+PCV10			**P=0.003**	**P=0.006**	**P=0.002**
5 µg PPV23+ PCV10				P=1	**P=0.002**
10 µg PPV23+PCV10					**P=0.002**
IgG Anti-PPS14	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	**P=0.009**	**P=0.007**	**P=0.009**	**P=0.002**	**P=0.002**
Saline+PCV10		P=0.5	P=0.065	P=0.43	**P=0.002**
2.5 µg PPV23+PCV10			P=0.07	P=0.8	**P=0.002**
5 µg PPV23+ PCV10				P=0.15	**P=0.002**
10 µg PPV23+PCV10					**P=0.002**
IgG Anti-PPS18C	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	**P=0.004**	**P=0.002**	**P<0.001**	**P=0.001**	**P=0.003**
Saline+PCV10		P=0.72	**P=0.005**	**P=0.004**	**P=0.002**
2.5 µg PPV23+PCV10			**P=0.002**	**P<0.001**	**P=0.002**
5 µg PPV23+ PCV10				P=0.8	**P=0.002**
10 µg PPV23+PCV10					**P=0.002**

Bold P value is significant.

Table A3: Statistical comparison of numbers of PPS1-, PPS4-, PPS9V- and PPS18C-specific IgG+ AbSC in spleen between mice primed with PCV10, different doses of PPV23 or saline and boosted with PCV10.

PPS1-AbSCs	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=1	P=0.27	**P=0.002**	**P=0.002**	**P=0.003**
Saline+PCV10		P=0.13	**P<0.001**	**P<0.001**	**P=0.002**
2.5 µg PPV23+PCV10			**P=0.003**	**P=0.003**	**P=0.001**
5 µg PPV23+ PCV10				P=0.74	P=0.4
10 µg PPV23+PCV10					P=0.54
PPS4-AbSC	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=0.07	P=0.12	**P=0.004**	**P<0.001**	**P=0.003**
Saline+PCV10		P=1	**P<0.001**	**P<0.001**	**P=0.002**
2.5 µg PPV23+PCV10			**P<0.001**	**P<0.001**	**P=0.002**
5 µg PPV23+ PCV10				P=0.16	P=0.08
10 µg PPV23+PCV10					P=0.44
PPS18C-AbSC	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=0.054	P=0.65	**P=0.001**	**P=0.001**	**P=0.003**
Saline+PCV10		P=0.09	**P=0.002**	**P=0.001**	**P=0.004**
2.5 µg PPV23+PCV10			**P=0.002**	**P<0.001**	**P=0.002**
5 µg PPV23+ PCV10				P=0.87	P=0.84
10 µg PPV23+PCV10					P=0.56
PPS9V-AbSC	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	**P=0.03**	P=0.47	P=0.75	P=0.12	**P=0.006**
Saline+PCV10		P=0.23	P=0.12	**P=0.004**	**P=0.004**
2.5 µg PPV23+PCV10			P=0.53	**P=0.045**	**P=0.008**
5 µg PPV23+ PCV10				P=0.093	**P=0.006**
10 µg PPV23+PCV10					**P=0.003**

Bold P value is significant.

Table A4: Statistical comparison of numbers of PPS1-, PPS4- and PPS18C-specific IgG+ AbSC in spleen between mice primed with PCV10, different doses of PPV23 or saline and boosted with PCV10.

PPS1-AbSCs	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=0.86	P=0.12	**P=0.004**	**P=0.006**	**P=0.04**
Saline+PCV10		P=0.16	**P=0.007**	**P=0.004**	**P=0.03**
2.5 µg PPV23+PCV10			**P=0.045**	**P=0.04**	P=0.27
5 µg PPV23+ PCV10				P=0.87	P=0.55
10 µg PPV23+PCV10					P=0.54
PPS4-AbSCs	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=0.86	**P=0.01**	**P=0.002**	**P=0.001**	**P=0.008**
Saline+PCV10		**P=0.002**	**P<0.001**	**P<0.001**	**P=0.002**
2.5 µg PPV23+PCV10			**P=0.002**	**P=0.002**	P=0.07
5 µg PPV23+ PCV10				P=0.17	P=1
10 µg PPV23+PCV10					P=0.6
PPS18C-AbSCs	**Saline+PCV10**	**2.5 µg PPV23+PCV10**	**5 µg PPV23+ PCV10**	**10 µg PPV23+PCV10**	**Saline +Saline**
PCV10+PCV10	P=0.07	P=0.09	**P=0.003**	**P=0.003**	P=0.054
Saline+PCV10		P=0.75	**P=0.002**	**P=0.002**	P=0.47
2.5 µg PPV23+PCV10			**P=0.002**	**P=0.002**	P=0.56
5 µg PPV23+ PCV10				P=0.96	P=0.5
10 µg PPV23+PCV10					P=0.43

Bold P value is significant.

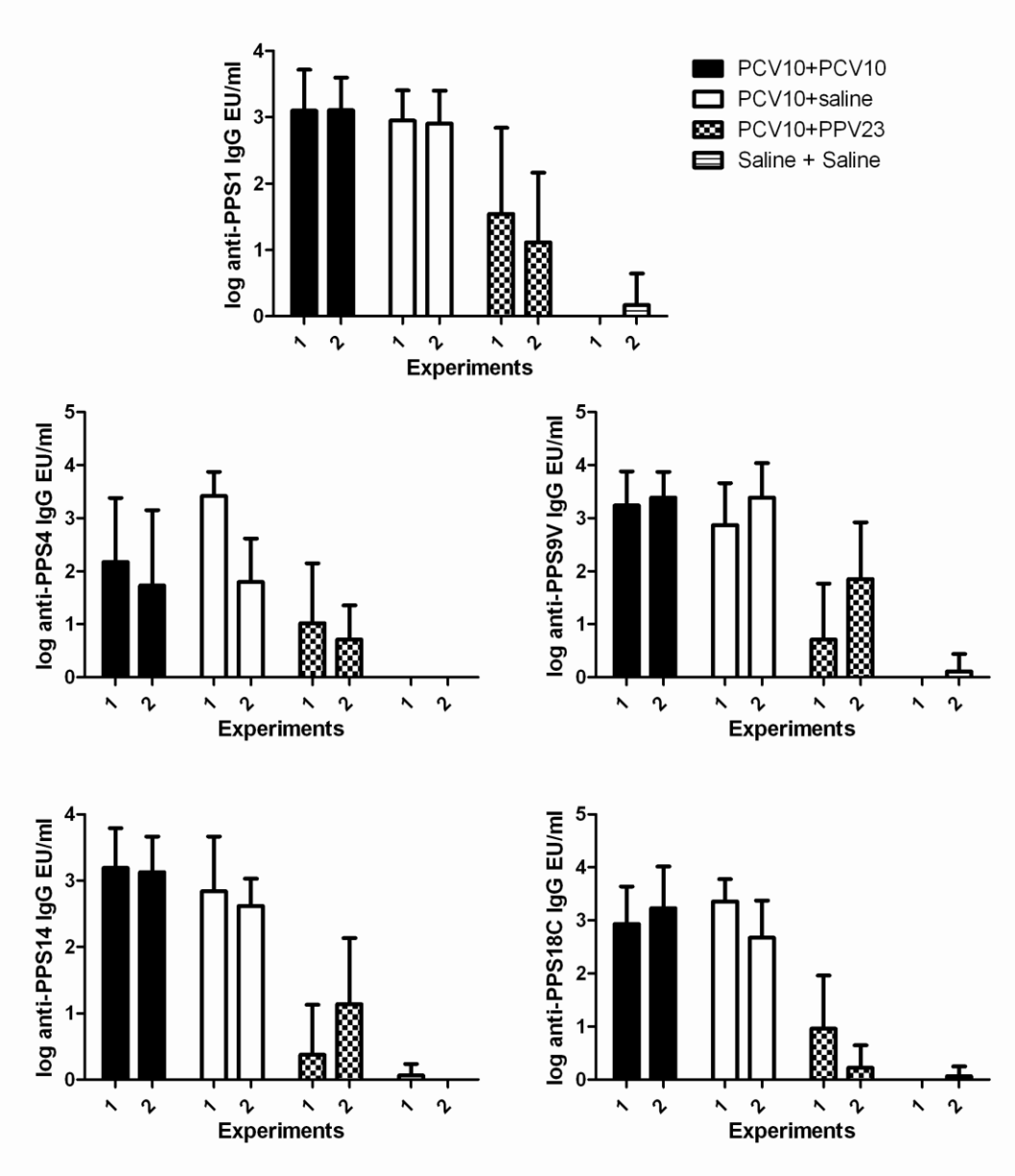

Figure A 1: Comparison of results from 2 experiments regarding the effects of boosting with PPV23 on PPS-specific IgG response.

a)Comparison between different experiments on PPS1-specific IgG for PCV10 primed mice that were boosted with saline, PCV10 or PPV23, b) Comparison between different experiments on PPS4-specific IgG for PCV10 boosted mice that were boosted with saline, PCV10 or PPV23 c) Comparison between different experiments on PPS9V-specific IgG for PCV primed mice that were boosted with saline, PCV10 or different doses of PPV23, d) Comparison between different experiments on PPS14-specific IgG for PCV primed mice that were boosted with saline, PCV10 or different doses of PPV, e) Comparison between different experiments on PPS18C-specific IgG for PCV primed mice that were boosted with saline, PCV10 or different doses of PPV23.

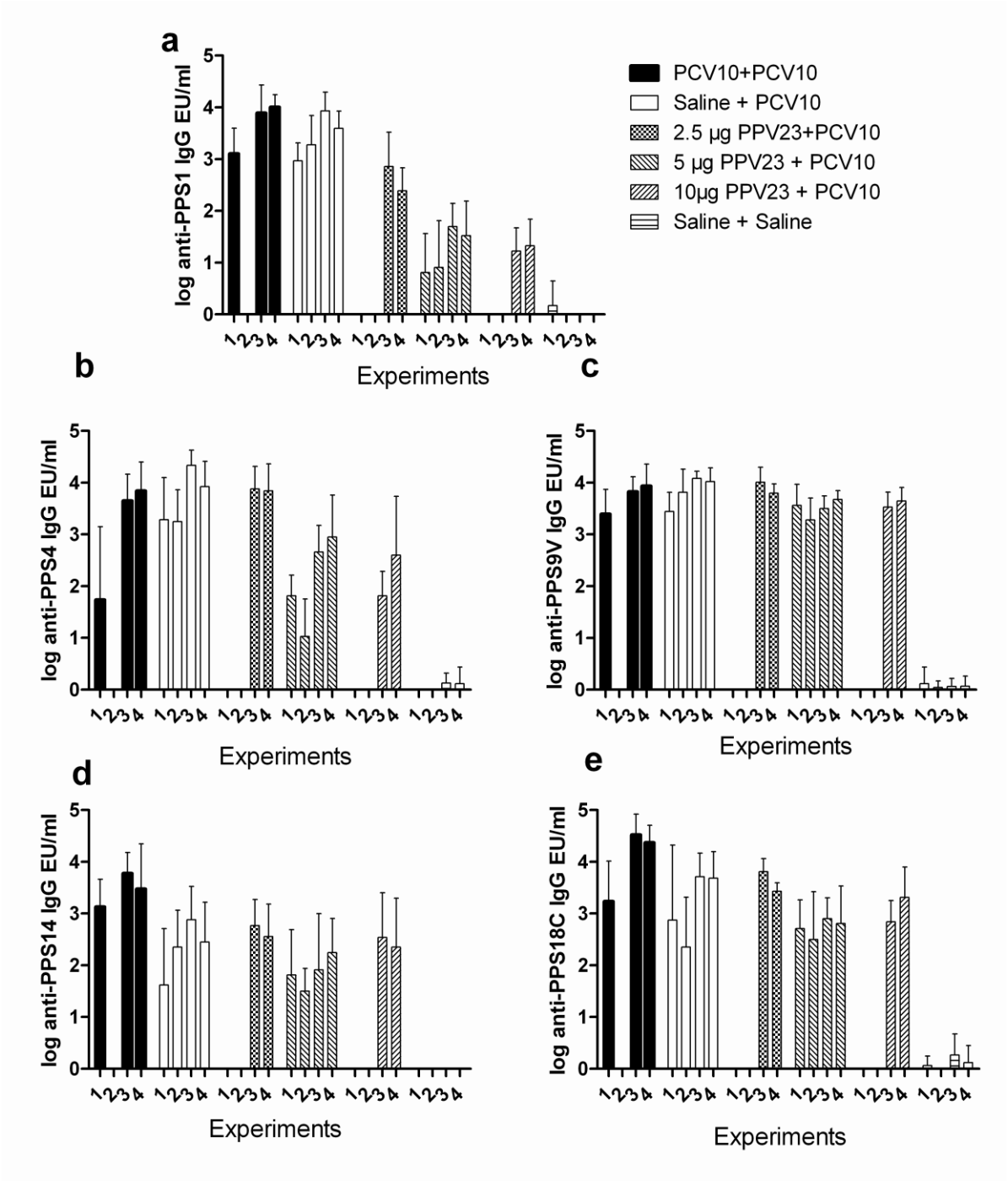

Figure A 2: Comparison of results from 4 experiments regarding the effects of priming with PPV23 on PPS-specific IgG response.

a)Comparison between different experiments on PPS1-specific IgG for PCV boosted mice that were primed with saline, PCV10 or different doses of PPV23, b) Comparison between different experiments on PPS4-specific IgG for PCV10 boosted mice that were primed with saline, PCV10 or different doses of PPV c) Comparison between different experiments on PPS9V-specific IgG for PCV10 boosted mice that were primed with saline, PCV10 or different doses of PPV23, d) Comparison between different experiments on PPS14-specific IgG for PCV10 boosted mice that were primed with saline, PCV10 or different doses of PPV23, e) Comparison between different experiments on PPS18C-specific IgG for PCV10 boosted mice that were primed with saline, PCV10 or different doses of PPV23.