Inhibition of the Antibody Response to Heterologous Protein by *Streptococcus pneumoniae,* Mediated by Choline-binding Protein(s)

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled *"**Inhibition of the Antibody Response to Heterologous Protein by Streptococcus pneumoniae, Mediated by Choline-binding Protein(s)**"* submitted by **Saumyaa** ID No **2006PH29001** for the award of Ph.D. degree of the Institute embodies original work done by her under my supervision.

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ABSTRACT

Thesis Title **: Inhibition of the Antibody Response to Heterologous Protein by *Streptococcus pneumoniae*, Mediated by Choline-binding Protein(s)**

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Abstract

We previously demonstrated that intact, inactivated *Streptococcus pneumoniae* (including unencapsulated strain R36A) inhibits IgG responses to a number of co-immunized soluble Ags. In this study, we investigated the mechanism of this inhibition and whether other extracellular bacteria exhibited similar effects. No inhibition was observed if R36A was given 24h before or after immunization with soluble chicken ovalbumin (cOVA), indicating that R36A acts transiently during the initiation of the immune response. Using transgenic cOVA-specific CD4+ T cells, we observed that R36A had no significant effect on T-cell activation (24h) or generation of Foxp3+ (regulatory) T cells (d7), and only a modest effect on T cell proliferation (48-96h) in response to cOVA. However, R36A mediated a significant reduction in the formation of Ag-specific splenic germinal center T follicular helper (GC Tfh) and GC B cells, and antibody-secreting cells in the spleen and bone marrow in response to cOVA or nitrophenyl (NP)-cOVA. Of note, the inhibitory effect of intact R36A on the IgG anti-cOVA response was reproduced using R36A-derived cell walls. Endogenous IL-10 and TGF-β, two cytokines known to play a

role in immunosuppression, did not play any apparent role in R36A-mediated inhibition. In contrast to R36A, neither inactivated, unencapsulated intact *Neisseria meningitidis* nor *Streptococcus agalactiae* inhibited the OVA-specific IgG response. Since expression of phosphorylcholine (PC), previously reported to have immunosuppressive properties, was specific for R36A, we determined whether PC-depleted R36A (R36A^{pc-}) was inhibitory. Indeed, R36A^{pc-} exhibited a markedly reduced level of inhibition of the anti-cOVA response, relative to R36A. However, a soluble covalent conjugate of PC and the protein bovine serum albumin (PC-BSA) failed to induce inhibition of the IgG anti-cOVA response. Further, treatment of R36A with periodate, which selectively destroyed PC residues, had no effect on inhibition. Collectively, these data argued against a direct role of PC in R36A-mediated inhibition. However, R36A^{pc-} is also devoid of a family of choline-binding proteins (CBPs) that are attached to the cell surface by specific binding to PC. Thus, we prepared CBP-depleted R36A (R36A^{cbp-}) by stripping CBPs from the cell surface using a solution of choline chloride, with the bacterial PC expression intact. Of note, R36A^{cbp-} exhibited a marked reduction in the level of inhibition of the IgG anti-cOVA response, strongly suggesting that one or more CBPs mediated R36A-induced inhibition. Indeed, a supernatant of choline chloride-treated R36A, containing CBPs, was inhibitory, whereas intact bacteria treated with the proteolytic enzyme, trypsin, lost its inhibitory activity. These results demonstrate a novel immunosuppressive property of *Streptococcus pneumoniae*, mediated by one or more CBPs.

LIST OF ABBREVIATIONS

Antibody	Ab
Antigen	Ag
Antigen presenting cells	APCs
Antibody-secreting cells	ASC
B cell receptor	BCR
Bone marrow	BM
Bovine serum albumin	BSA
Choline binding domain	CBD
Choline binding protein A	CbpA
Choline binding proteins	CBPs
Chemically defined media	CDM
Carboxyfluorescein diacetate	CFSE
Colony forming units	CFU
Chicken ovalbumin	cOVA
Dendritic cells	DC
Dimethyl sulfoxide	DMSO
Follicular B cells	FB cells
Follicular dendritic cells	FDCs
Fetal bovine serum	FBC
Group B *Streptococcus*	GBS
Germinal center	GC

Gram-negative	GN
Gram-positive	GP
Haemophilus influenza	Hi
Inducible costimulator	ICOS
Interferon	IFN
Immunoglobulin	Ig
Interleukins	IL
Intravenous	i.v.
Keyhole limpet hemocyanin	KLH
Lipoteichoic acid	LTA
Microbe-associated molecular patterns	MAMPs
Meningococcus type C	MenC
Major histocompatibility complex class II	MHC-II
Metallophilic macrophage	MMM
Marginal zone	MZ
Marginal zone macrophage	MZM
4-hydroxy-3-nitrophenylacetyl	NP
Oligodeoxynucleotide	ODN
Platelet activating factor	PAF
Platelet activating factor receptor	PAFR
Periarteriolar lymphoid sheath	PALS
Phosphate buffered saline	PBS
Phosphorylcholine	PC

Phycoerythrin	PE
Prostaglandin	PG
Pneumococcus	Pn
Peanut agglutinin	PNA
Pneumococcal polysaccharide type 14	PPS14
Pattern recognition receptor	PRR
Polysaccharides	PS
Pneumococcal surface adhesin A	PsaA
Pneumococcal surface protein A	PspA
Red blood cells	RBC
Sodium acetate buffer	SAB
Subcutaneous	s.c.
Supernatant	sup
Teichoic acid	TA
T cell receptor	TCR
T cell-dependent	TD
T follicular helper cells	Tfh
Transgenic	Tg
Transforming growth factor	TGF
T helper cells	Th
Todd Hewitt Broth	THB
T cell-independent	TI
Toll-like receptors	TLR

Tumor necrosis factor	TNF
Trinitrophenol	TNP
T regulatory cells	Tregs
Wild-type	WT

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Introduction

1.1 *Streptococcus pneumoniae* (Pn)

Pneumococci are a major cause of pneumonia, empyema, otitis media, septic arthritis, septicemia, and meningitis (1). Pn is one of the most important bacterial causes of respiratory infections in children and adults and third most common cause of meningitides among infants and children (2). It is responsible for nearly half of all the cases of otitis media. Over 2 million cases of pneumonia are reported annually in developing countries (3). Acute respiratory infections, the majority of them caused by Pn, are a major cause of morbidity, and especially mortality, among children in developing countries (4, 5). Their clinical management is becoming challenging due to an alarming rise in the emergence of several antibiotic-resistant pneumococcal strains (6-8). About 25-75% of infants are pneumococcal carriers (symptomatic or asymptomatic) at any given time, with the highest colonization rates among children attending various institutions or child care centers (9).

1.1.1 Colonization and infection

Pneumococcal colonization is a pre-requisite for invasive disease (9). It primarily colonizes mucosal epithelium of the upper respiratory tract (1). Although colonization at this site is typically asymptomatic, Pn may gain access to sterile parts of the airway causing rapid inflammation and clinical disease (10, 11). Occasionally, Pn gains access to the bloodstream via cervical lymphatics resulting in bacteremia and infection of other organ systems (11). Clinical disease occurs in a small percentage of the people who are colonized. Risk factors for developing a pneumococcal disease from a carrier stage depend upon ethnicity, underlying diseases, environmental factors and pneumococcal strain (9). Colonization in childhood may impart protection to various pneumococcal serotypes in adults (10). The ability of pneumococcus to colonize the nasopharynx is dependent upon the colonial morphology of the bacteria, which

varies between two phases, transparent and opaque. Pn undergoes spontaneous reversible phase variations, which are visible in terms of the opacity of the colony on a transparent agar plate. Bacteria with transparent morphology efficiently colonize the nasopharynx, whereas the isogenic opaque variants fail to do so (11, 12). Conversely, the opaque variants are more virulent and are the prevalent phenotype found during systemic infections (13). Pneumococci are enclosed within a polysaccharide capsule that protects it from phagocytosis (1, 14). Opaque variants have a higher expression of polysaccharide capsule and lower expression of cell wall teichoic acids as compared to transparent variants. The thicker capsule in opaque variants could account for their ability to evade the immune response during systemic infections and thus behave in a more virulent fashion (13-15).

1.2 Structure of *S. pneumoniae*

*S. pneumonia*e consists of a plasma membrane, a cell wall and in most cases a polysaccharide capsule (16) (Fig. 1).

1.2.1 Capsule

The 200-400nm thick polysaccharide (PS) capsule forms the outermost layer of *S. pneumoniae* cells (17). Most of these capsules are covalently linked to the outer surface of the peptidoglycan component of the cell wall (18). Pn can express 90 different types of capsular polysaccharides that form the basis of its serotyping (15). Within the same serotype it exhibits variation in the amount of capsule expressed, giving rise to different isogenic variants (13). The capsule is non-toxic in isolated form, but when associated with bacteria, contributes to its virulence, due to its ability to inhibit phagocytosis, the primary mechanism for clearance of

pneumococci (15). Specifically, capsule prevents the interaction of the Fc region of Pn-specific antibody (Ab) or complement bound to the underlying bacterial cell wall, with the cognate receptors on phagocytic cells (i.e. neutrophils and macrophages) (19). A relationship between the serotype of Pn capsule and virulence has also been established (20).

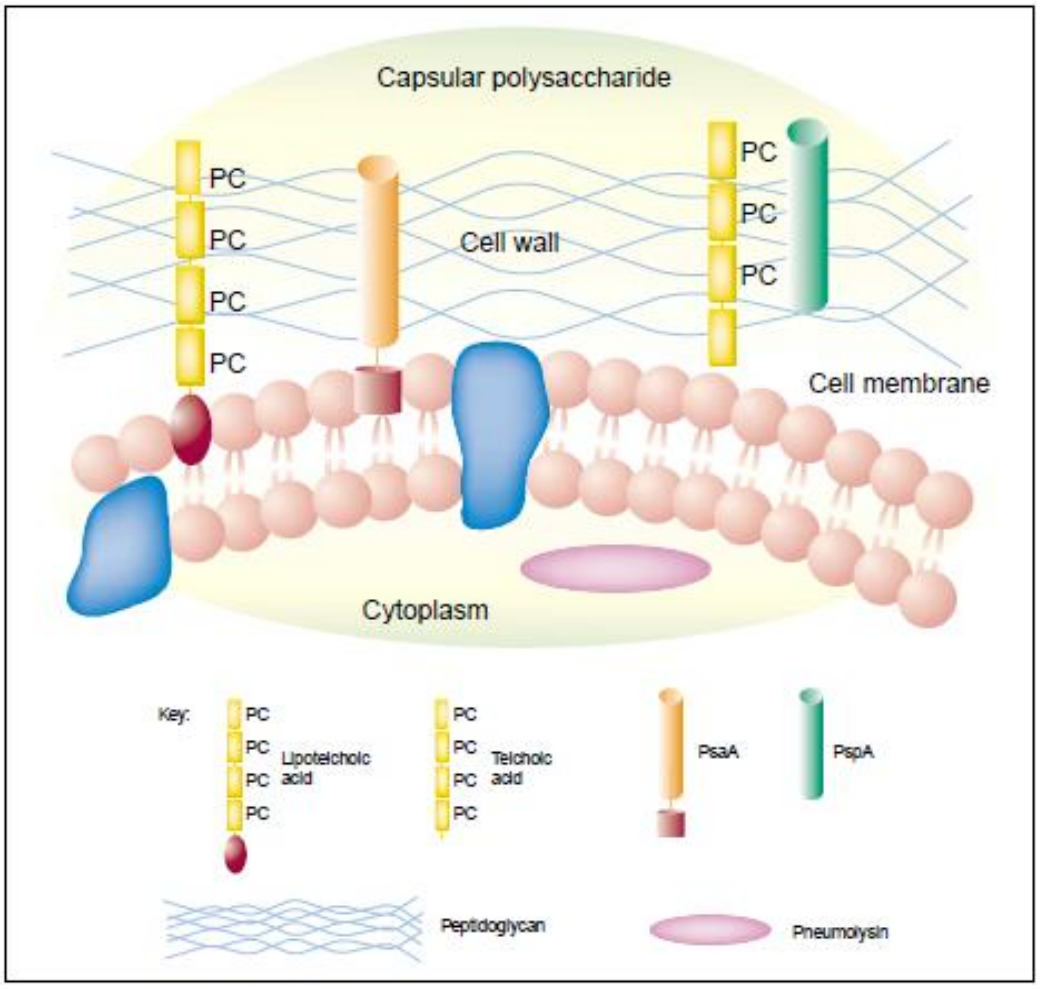

Fig. 1. **Structure of *S. pneumoniae*.** (PsaA: Pneumococcal surface adhesin A, a lipoprotein anchored to the plasma membrane; PspA: Pneumococcal surface protein A, a choline binding protein, PC: phosphorylcholine) (16)

1.2.2 Cell wall and cell membrane

The cell wall is composed of a peptidoglycan backbone. Chains of teichoic acid ([TA], also called C-polysaccharide) expressing phosphorylcholine (PC) are bound to the peptidoglycan layer though phosphodiester bonds. Lipoteichoic acid (LTA) chains are linked to the cytoplasmic membrane through hydrophobic interactions. The TA component of membrane LTA is biochemically identical to the non-lipidated TA of the cell wall. Both TA and LTA chains express PC residues (21). These PC residues anchor a family of surface proteins called choline-binding proteins (CBPs) that are attached to the PC residues through their choline-binding domain. Pn has a nutritional requirement for PC (22, 23) (Fig. 2).

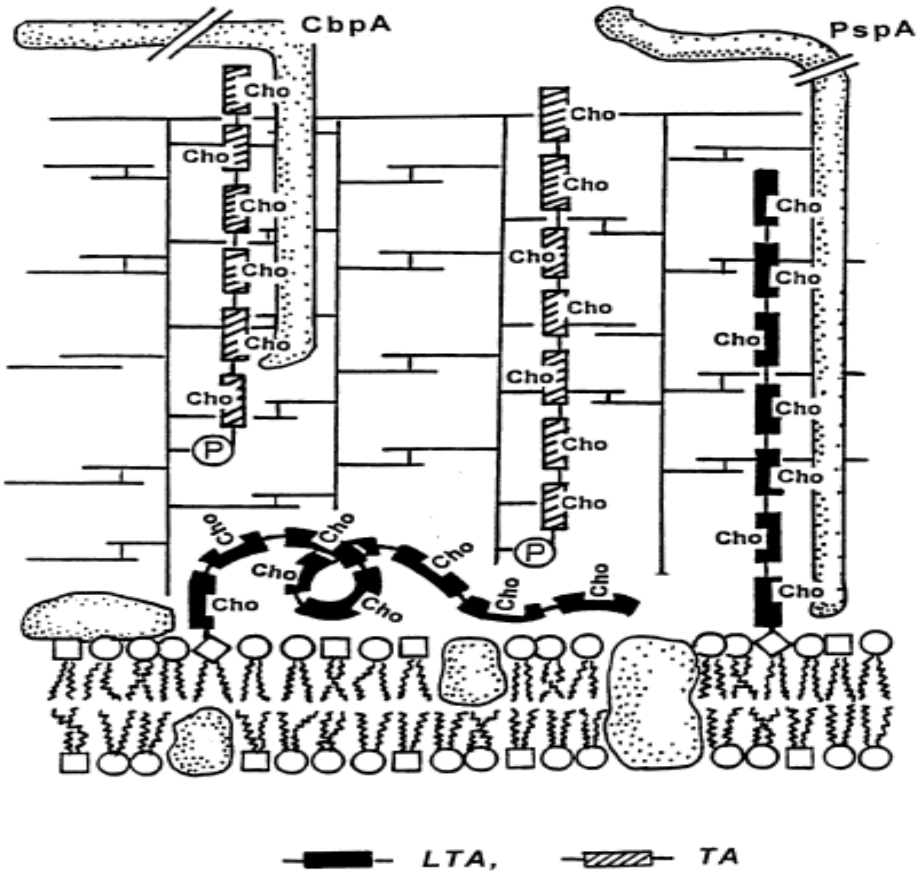

Fig. 2. <u>Cell wall-membrane complex of pneumococci.</u> Cho: phosphorylcholine, CbpA: choline binding protein A (23)

1.2.3 Choline-binding proteins

The CBPs are a diverse class of proteins that associate, non-covalently, with PC residues on TA or LTA. They express repeat sequences of approximately 20 amino acids which form a choline-binding domain (CBD), that enables them to bind to PC (10). Other than their signal peptides and choline-binding domains, there are very few similarities in CBP structures (24, 25). The avidity of CBPs for TA depends on the number of repeats of the choline-binding domain (26). The expression of CBPs correlates with phase variation, with some CBPs such as PspA being expressed in higher amounts in opaque variants, whereas expression of others such as CbpA and LytA are increased in the transparent variants (27). Pn expresses 10-15 distinct CBPs, depending upon the strain (28). They are described in the table below (Table 1).

Name	**Protein**	**Function**	**# CBD repeats**
LytA	Autolysin/amidase	Daughter cell separation, autolysis in stationary phase and penicillin induced lysis, competence for genetic transformation	4-6
LytB	Cell wall hydrolase/ muramidase	Pneumococcal daughter cell separation	7+2
LytC	Cell wall hydrolase/ lysozyme	Lysozyme like activity at 30ºC, competence for genetic transformation	11(N-terminal)
PcpA		Plays role in protein-protein and protein-lipid interactions	6
PspA/ SpsA		Decreases complement deposition, host cell adhesion	10
CbpA/ PspC	Adhesin	Host cell adhesion, binds to IgA and complement protein C3	8
CbpG	Putative serine protease	Host cell adhesion	3

CbpD	Murein hydrolase	Host cell recognition, competence for genetic transformation	4
CbpE		Host cell recognition	8
CbpB		Host cell recognition	
CbpC		Host cell recognition	6+3
CbpI		Not known	6
CbpF		Inhibits autolytic activity of LytC	5+2

Table 1. Choline-binding proteins (22, 23, 28-37)

1.3 Structure of spleen

We have adopted the intravenous (i.v.) route of immunization in our study. Upon i.v. immunization the immune response is elicited within the spleen. The spleen is the body's largest filter of blood. It plays a central role in removing apoptotic cells and senescent red blood cells from the circulation, and is the most important organ for generating immune responses against blood-borne pathogens. As a secondary lymphoid organ it concentrates antigen (Ag), antigen-presenting cells, and antigen-specific lymphocytes for enhanced interactions, critical for initiating the immune response. It is organized as a tree of branching arterial vessels (38). The afferent splenic artery branches into central arterioles, which are sheathed by white-pulp areas, consisting of periarteriolar lymphoid sheaths (PALS) or T cell zones, and adjacent follicles consisting of B cells. The follicles are surrounded by a marginal sinus containing macrophages, dendritic cells, and specialized B cells termed marginal zone B cells (this region is termed the "marginal zone" [MZ]). Blood enters the marginal sinus from the central arteriole. The follicle itself is composed of follicular B (FB) cells (Fig. 3) (38, 39).

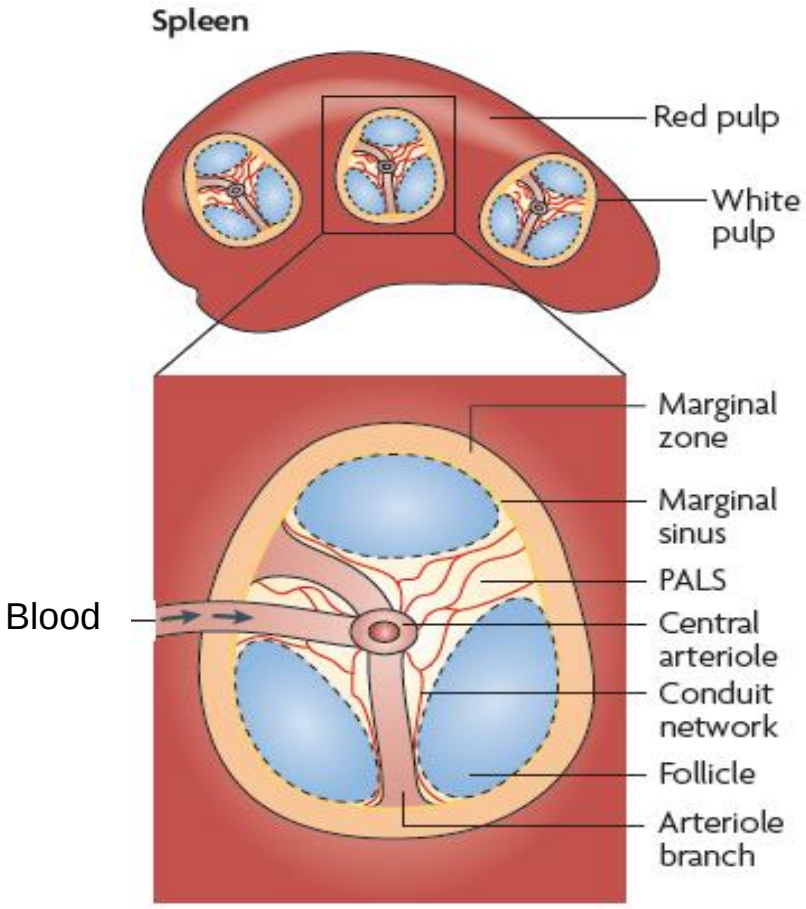

Fig. 3. Structure of spleen (39)

The sluggish blood flow within the MZ sinus that is adjacent to the marginal zone macrophages (MZM) and marginal metallophilic macrophages (MMM), as well as MZ B cells promotes early phagocytosis and destruction of blood-borne pathogens and rapid antibody responses, respectively (40). The MZ is also an important transit area for cells leaving the bloodstream and entering the white pulp. The PALS contains a high density of CD4+ and CD8+ T cells and dendritic cells (DC) and is the site where T cells interact with DCs that have internalized and processed antigen. B cell follicles are the site of clonal expansion of activated B cells that eventually undergo isotype switching and somatic hypermutation, in a process termed

the germinal center (GC) reaction (38, 41). In the spleen both innate and adaptive immune responses are mounted. The innate response is generated within the MZ and the adaptive response is generated both within the MZ and white pulp (38).

1.4 Innate immune response

The MZ is the primary site of screening of blood that enters from the arteries (Fig. 4). The MZ resident macrophages efficiently phagocytose blood-borne pathogens, apoptotic cells, and senescent RBCs. Both MZMs and MMMs express a variety of membrane scavenger receptors for a number of bacterial structures including polysaccharides and are thus are very efficient at taking up bacteria expressing such ligands on their surface (38).

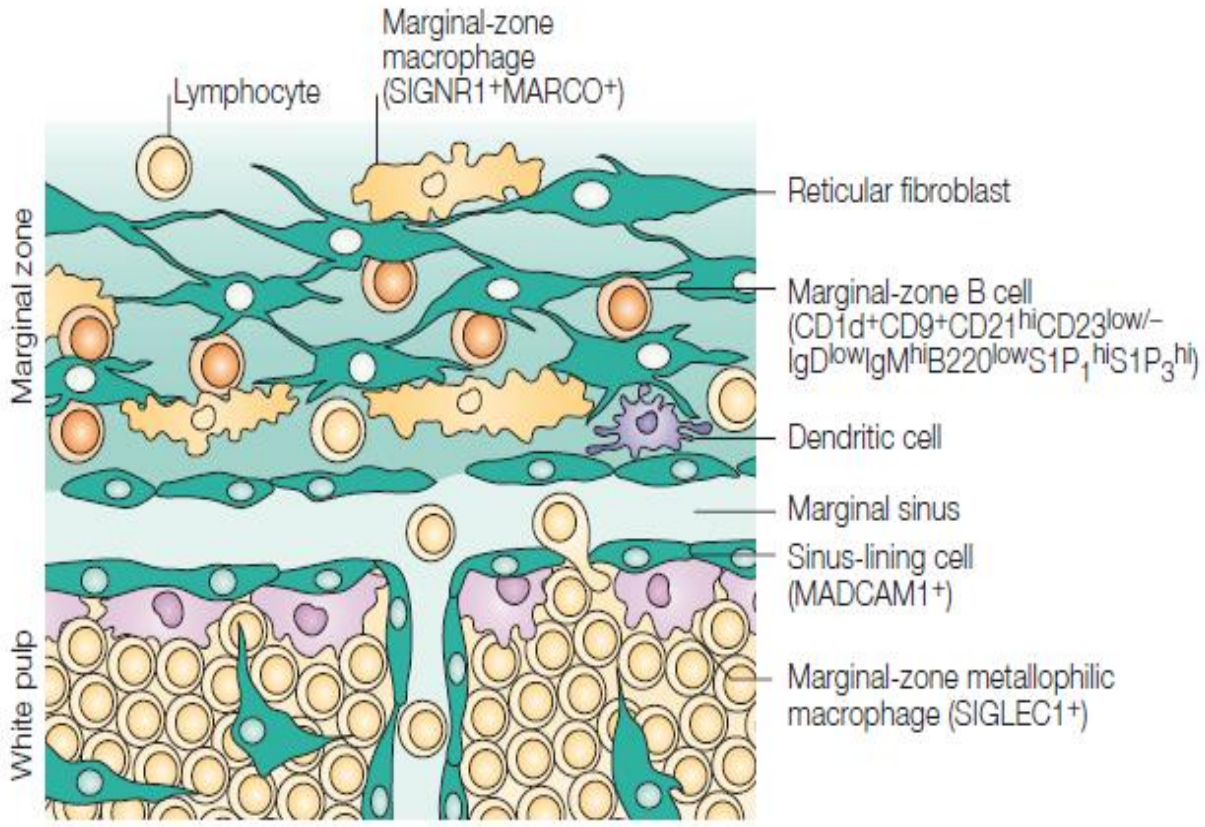

Fig. 4. Marginal zone of spleen (38)

MZB cells, in contrast to FB cells, are programmed to elicit rapid responses to antigen. Depending on the type of Ag they exhibit either a rapid T cell-independent (TI) antibody response, or internalize and process antigen and move to the T cell-B cell border zones to interact with cognate T-cells already primed with Ag by DC. This latter process leads to a T cell-dependent (TD) antibody response. MZB thus typically mediate rapid, short-term (TI and TD) extrafollicular plasma cell responses, but may also participate, along with FB cells, in long-term TD plasma cell and memory B cell responses involving a germinal center reaction (42) (Fig. 5).

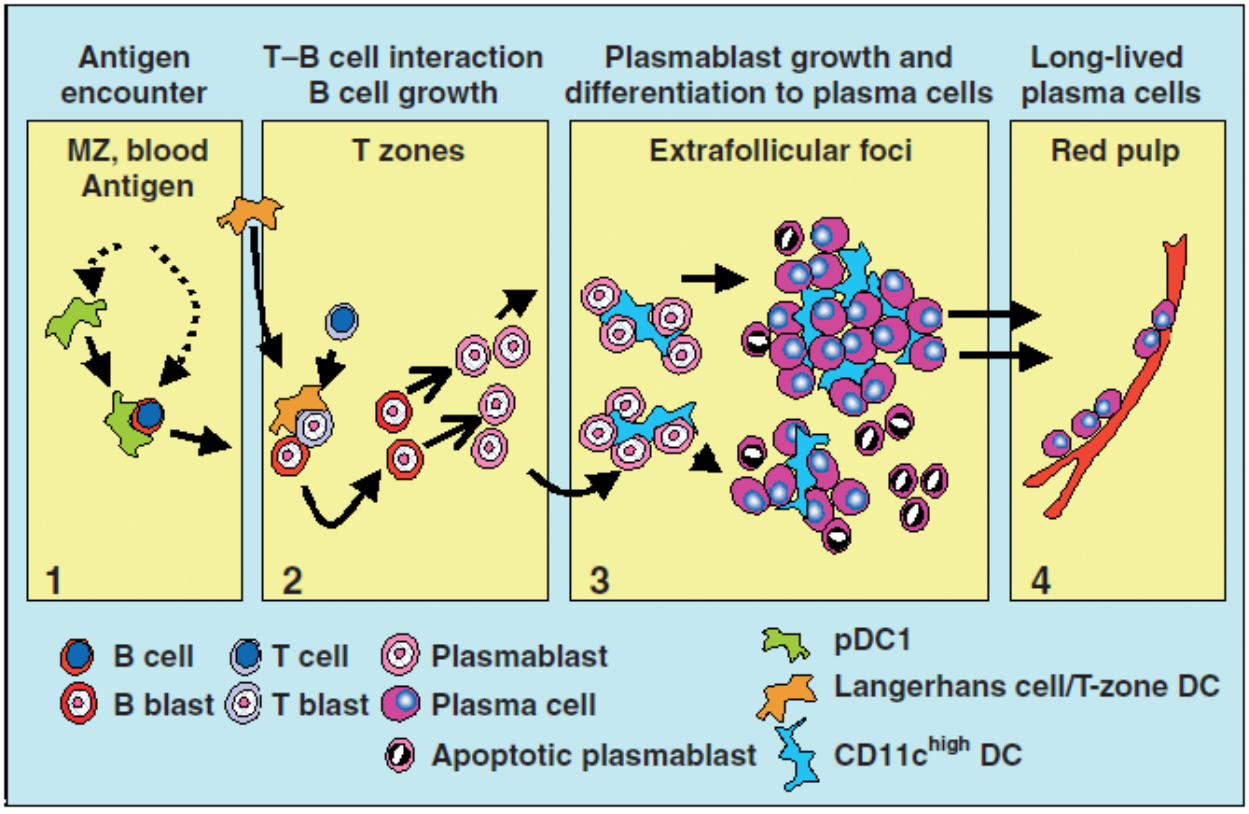

Fig. 5. <u>Splenic T-dependent extrafollicular Ab response</u> (42).

1.5 Adaptive immune response

The first step in adaptive immunity is the entry of antigen presenting cells (APCs) into the T cell zone where they activate T cells. These activated T cells then proliferate and migrate to the T-B border. The B cells also become activated after recognizing Ag through their B cell receptor (BCR) and migrate towards the T-B border to receive T cell help. These B cells then undergo proliferation, immunoglobulin (Ig) class switching, and affinity maturation in the B cell follicles as part of the germinal center reaction (38). The adaptive immune response against a pathogen usually takes days to develop. During this early lag phase, innate immunity is protective. Once antigen-specific B and T cells have differentiated into antibody-secreting and effector cells, respectively, adaptive immunity supersedes in conferring protection (41).

1.6 T cell activation and proliferation

We have evaluated T cell activation and proliferation in response to the protein Ag used in the study (i.e. cOVA), since these are the earliest events involved in the immune response to an Ag. These events are preceded by uptake of Ags by APCs. DCs are the most efficient APCs. They continuously patrol through blood, peripheral tissue and secondary lymphoid organs. Microbe-associated molecular patterns (MAMPs) and inflammatory cytokines interact with specific receptors on DCs to induce DC maturation. DC maturation, including upregulation of class II major histocompatability complex (MHC-II) and costimulatory molecules, results in augmentation of their APC function for T cells. It also upregulates certain chemokine receptors to enable them to migrate to T cell zones for interaction with T cells (43). DCs then internalize Ag, digest it into small peptides and present it on their surface in association with MHC- II molecules, to be recognized by CD4+ T helper (Th) cells.

The interaction between T cells and APCs during the first 24 hours induces T cell activation, cytokine secretion and proliferation. It also plays a role in determining which differentiation path they are going to follow (44). The outcome of the T cell response relies on efficient and productive encounters between APCs and cognate T cells. A productive T cell response requires sustained binding of the T cell receptor with a critical threshold of MHC-II-peptides complexes on the APC (41).

1.7 Extrafollicular response and germinal centers

B cells that have recognized Ag through their Ig receptors move to the T-B border to obtain help from T cells that had earlier been primed by APCs expressing antigen in the T cell zone. Subsequent to this event B cells can follow one of two functional pathways, follicular or extrafollicular. In the extrafollicular pathway, B cells proliferate and differentiate into low affinity, short-lived plasmablasts, which migrate to bridging channels. Bridging channels are where T cell zones meet red pulp. In the follicular pathway, B cells enter the follicles where they seed the GCs. They participate in the GC reaction to form long-lived plasma cells and memory B cells (45) (Fig. 6).

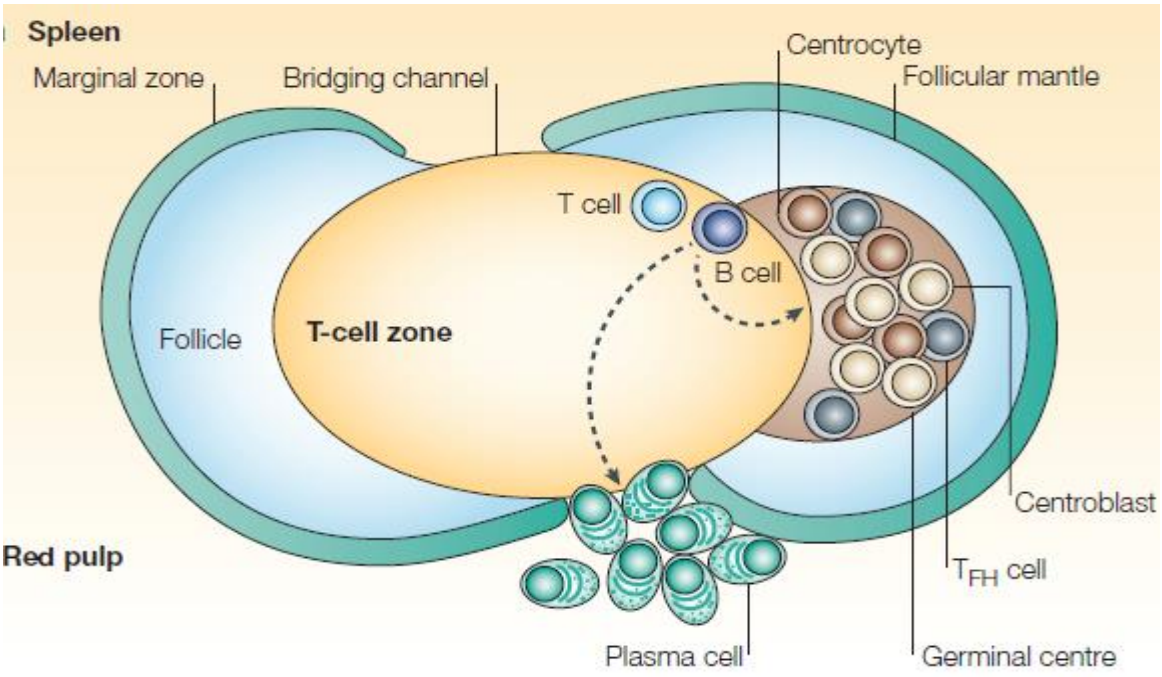

Fig. 6. Follicular and extrafollicular pathways of B cell differentiation (45)

GCs are divided into two zones based on their histological appearance, dark zone and light zone (Fig. 7). The dark zone is towards the T cell zone and is packed with highly mitotic, large B cells called centroblasts that have reduced Ig expression. The light zone is on the far end of the GC and it has a lower density of B cells. Instead it has a high density of follicular dendritic cells (FDCs), which are specialized stromal cells in the follicles that retain Ag in the form of immune complexes through their Fc receptors and present it to GC B cells. The GC B cells in the light zone are smaller, non-mitotic and express surface Ig and are called centrocytes. Light zones also contain T follicular helper (Tfh) cells. Together Tfh cells and FDCs drive the affinity maturation of centrocytes in the light zone and their differentiation to memory B cells or plasma cells, which then exit the GC (46, 47). Cells are retained in their respective compartments by chemokine gradients of CXCL12 and CXCL13, which bind to receptors, CXCR4 and CXCR5, respectively. All GC B cells express high levels of CXCR5, but centrocytes are low in CXCR4

and light zone is high in CXCL13 and low in CXCR12, which keeps the centrocytes in the light zone. Centroblasts by contrast have high CXCR4 and dark zone has more abundance of CXCR12, thereby keeping centroblasts in the dark zones (48)

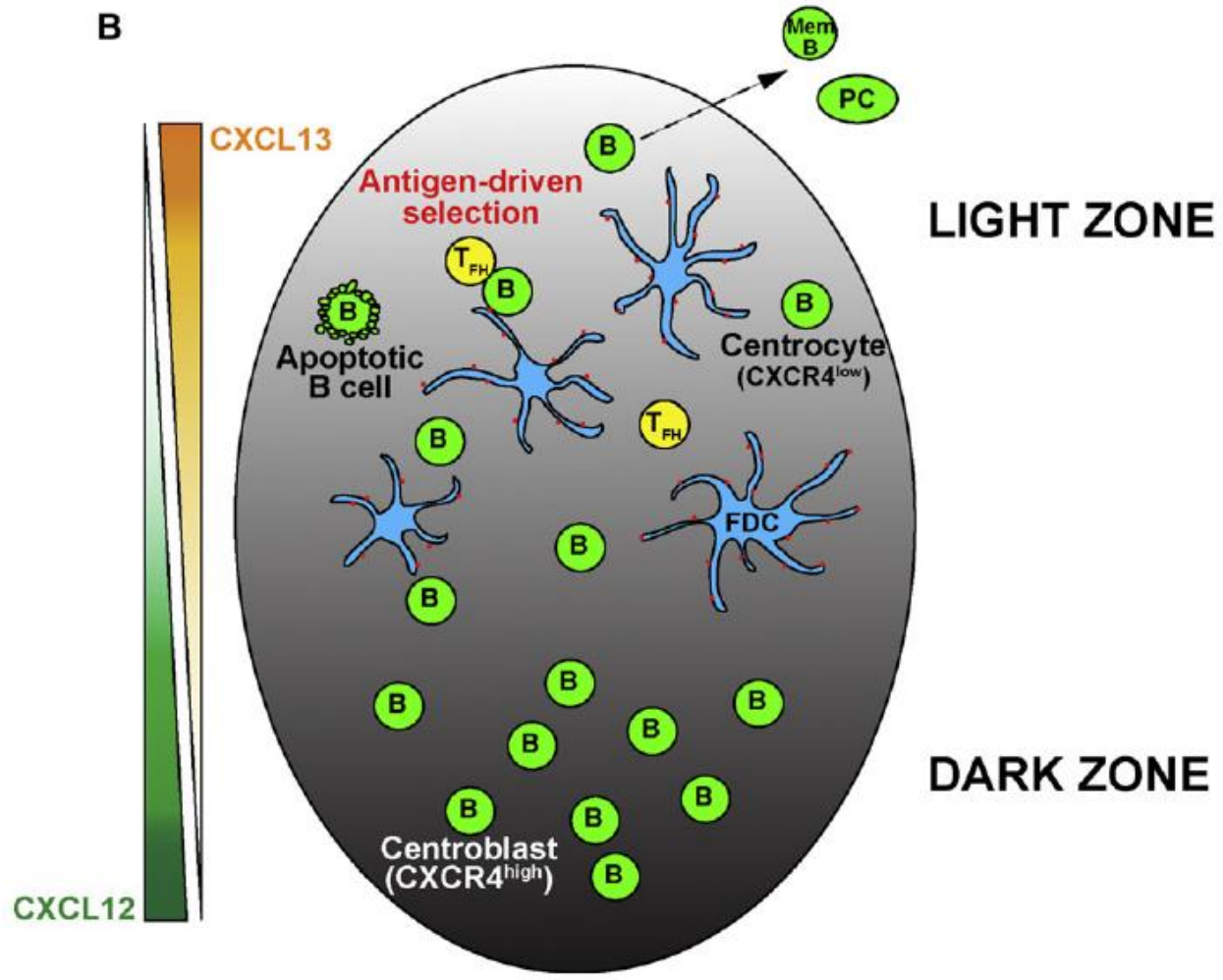

Fig. 7. <u>Organization of GCs</u> (46)

GC responses depend on a series of cellular interactions and movements guided by different chemokine gradients (Fig. 8). Naïve B cells migrate to B cell follicles by expressing high levels of CXCR5, the receptor for chemokine CXCL13 expressed by stromal cells (49).

Naive T cells migrate to the T cell zone by expressing high levels of CCR7, the receptor for T zone chemokines, CCL19 and CCL21 (50). Within a few hours of interaction with Ag, B cells upregulate their expression of CCR7 and EBI2 (a receptor that supports migration in response to 7α,25-dihydroxycholesterol to migrate to the T:B border for cognate T cell help (51, 52). Activated CD4 T cells in the T cell zone downregulate CCR7 and upregulate transcription factor Bcl6 which in turn upregulates CXCR5 (53, 54) to promote T cell movement towards the T:B border to provide help to B cells. This is followed by further upregulation of BCL6 and CXCR5 by T cells and downregulation of CCR7 by B cells to promote their further migration to the interfollicular region (region ~~in~~ between any two follicles inside the B cell zone) (55). The cognate interaction taking place in this location between B and T cells determines the fate of these B cells, which either enter the extrafollicular pathway or form a GC. B cells that enter the GC pathway downregulate EBI2 and maintain high levels of Bcl6 and cluster in the center of follicles along with a few T cells, which also maintain high levels of Bcl6 and remain in close contact with surrounding B cells for long periods of time to help sustain the GC reaction (54, 56, 57).

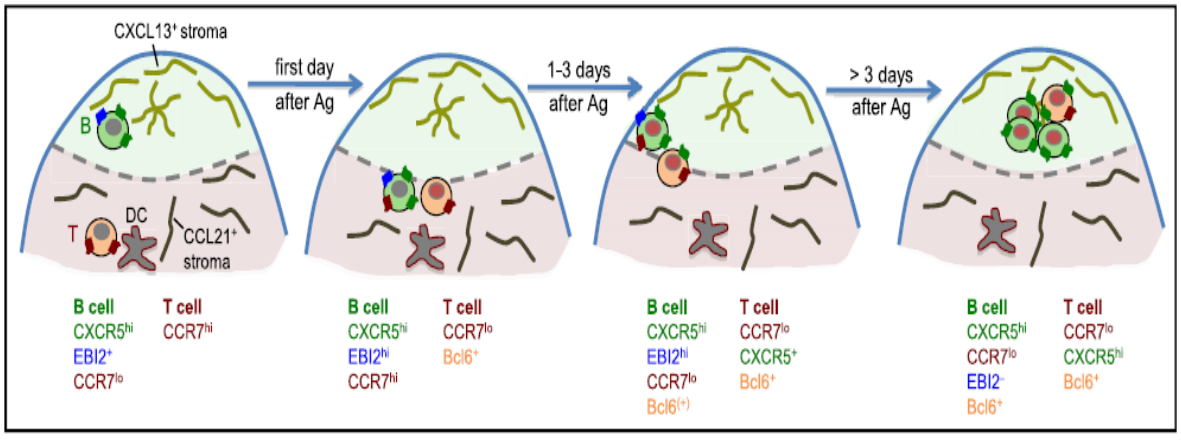

Fig. 8. <u>T and B cell activities during early GC response</u> (57)

1.7.1 T follicular helper cells

In this study we demonstrate that *S. pneumoniae* inhibits the number of T follicular helper cells (Tfh) generated against the co-immunized protein Ag. Tfh cells are defined as T cells providing help to B cells in the follicular region. They are crucial for formation and maintenance of GCs (58, 59). The antigen-specific Tfh cells move to B cell follicles, where they, in association with B cells and FDCs, give rise to the GC reaction (58).

Tfh differentiation begins during the first 24h of the immune response during the period when they are being primed by activated DCs. Inducible costimulator (ICOS), which is induced on activated CD4+ T cells, is critical for providing early differentiation signals and promoting the upregulation of Bcl6 (53, 60). Effector CD4+ T cell differentiation is controlled by specific transcription factors (61, 62). Each lineage of effector CD4+ T cells; Th1, Th2, Th17, and T regulatory, is defined and controlled by a unique master regulator transcription factor; T-bet (63), GATA3 (64), RORγt (65), and Foxp3 (66), respectively. Bcl6 is the master regulator of, Tfh cells (67-69). A clear bifurcation between Tfh and other T helper differentiation pathways can be seen by the second T cell division, based on the expression of the transcriptional repressor Bcl6 and the transcription factor Blimp-1 (67) (Fig. 9).

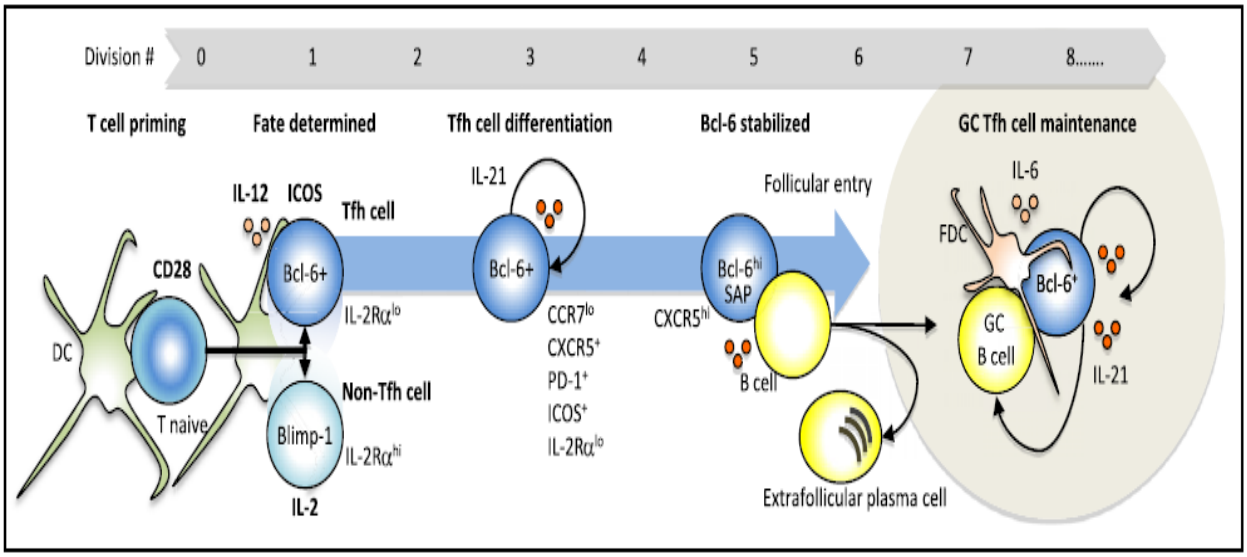

Fig. 9. Tfh and GC Tfh development (57)

Blimp-1 is an antagonist of Bcl6 and Bcl6 can repress Blimp-1 (70-73). The fate of T cells depends on the signal they receive at the time of DC priming. Some CD4+ T cells upregulate Blimp-1 and interleukin (IL)-2 receptor and differentiate towards a non-Tfh phenotype. IL-2 positively regulates IL-2Rα and Blimp-1 expression (74, 75). IL-21, on the other hand, promotes Tfh differentiation and maintenance. It works in an autocrine fashion and maintains high levels of Bcl6 (76, 77). Upregulation of Bcl6 in Tfh cells induces CXCR5, which then drives Tfh to migrate to B cell follicles in a CXCL13-dependent manner. B cells are not essential for early Tfh differentiation, however they are required to maintain it at later time points (53). Tfh interaction with cognate B cells at the T-B border can prime them to enter either the extrafollicular or GC pathway (56) (Fig. 10). Prolonged interaction with B cells favors Bcl6 and further stabilizes Tfh residence in GCs. Inside GCs Tfh provide growth and maintenance signals to GC B cells mediated by cytokines like IL-6 and IL-21 (78). IL-21 is further critical for plasma cell and memory B cell development (79).

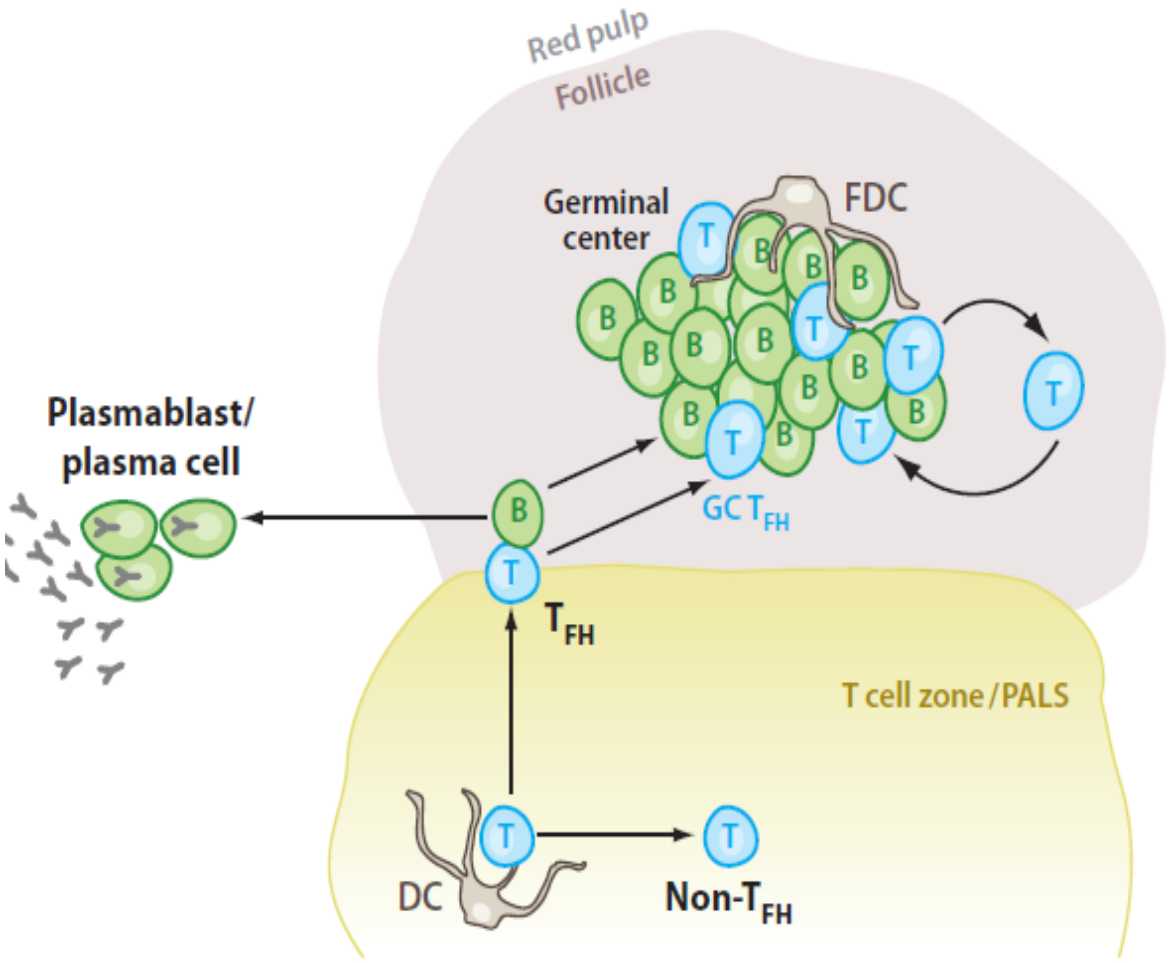

Fig. 10. <u>Tfh differentiation and GC reaction</u> (58)

One of the hallmarks of the GC reaction is the generation of B cell memory. Memory B cells persist for long periods of time after encountering ~~the~~ Ag. They are localized in the MZ and they also recirculate through secondary lymphoid organs. They are capable of responding quickly to a re-encounter with Ag. The GC also promotes migration of activated B cells to the bone marrow where they reside for long periods of time as long-lived plasma cells, critical for maintenance of serum antigen-specific antibody (80).

1.8 Immune response to *S. pneumoniae*

The immune response against *S. pneumoniae* includes a combination of both innate and adaptive responses (81). The initial immunity comes mainly from the rapid innate response involving phagocytosis and clearance of Pn by neutrophils and macrophages. Toll-like-receptors (TLRs) present on host innate immune cells recognize TLR ligands expressed by Pn (82, 83). This TLR-mediated signaling activates innate immune cells and also contributes in the initiation of the adaptive immune response. Cytoplasmic NOD-like receptors (NLRs) that recognize peptidoglycan in the cell wall of Pn may also play a role in innate immunity. The major adaptive immune response against Pn is humoral, involving production of IgG, IgM and IgA Abs against pneumococcal proteins and polysaccharide (84). Ig binding to the bacterial surface leads to Fc and complement mediated opsonophagocytosis of bacteria by macrophages and neutrophils, through binding to Fc and complement receptors present on these cells (81).

1.9 Differential regulation of protein- versus polysaccharide (PS)-specific Ig responses to intact *Streptococcus pneumoniae* and to Pn antigens expressed by intact Pn versus their isolated soluble counterparts

The antibody response to the protein and PS components of Pn differ mechanistically, as well as whether Pn antigens are in particulate (intact Pn) versus soluble form. The nature of the immune response thus depends on the nature of the Ag, the immune cells involved and potential cross-regulatory effects of multiple antigens and their physical form. Studies addressing these differences have been done comparing PS- and protein-specific Ig isotype responses to intact Pn, isolated soluble proteins or PS, soluble protein-PS conjugate vaccines, and on concomitant immunization of intact Pn and a corresponding soluble protein-PS conjugate (84).

1.9.1 Streptococcus pneumoniae

The anti-protein IgG response to intact Pn requires CD4+ T-cell help and CD40- and B7-dependent co-stimulation. It also shows boosting on secondary challenge involving a follicular B cell response and GC reaction and generation of protein-specific memory. The IgG anti-PS response to Pn is also dependent on CD4+ T cells and requires CD40 and B7 co-stimulation, but displays more accelerated kinetics of primary Ig induction than the anti-protein response, with no boosting upon secondary challenge, despite the induction of memory during the primary. This response is extra-follicular and dependent on MZB cells. The IgM anti-PS response to Pn is TI (85-88).

1.9.2 Soluble PS and protein antigen

The Ig anti-PS response to soluble, isolated PS is TI, does not require CD40- or B7-dependent co-stimulation, does not elicit a GC reaction, requires MZB cells, and shows no boosting upon secondary challenge. It also exhibits rapid kinetics of the primary response. The inability of PS to associate with MHC-II molecules and hence their failure to facilitate cognate CD4+ T cell help for Ig induction, may account for this behavior. In contrast, the Ig anti-protein response to soluble protein requires CD4+ T cells, CD40- and B7-dependent co-stimulation, elicits a GC reaction, requires FB cells and exhibits boosting upon secondary immunization (85).

1.9.3 Protein-PS conjugate

The IgG anti-protein and anti-PS responses to a protein-PS conjugate vaccine both require CD4+ T cell help, CD40 and B7 co-stimulation. There is a boosted anti-protein and anti-

PS response on secondary challenge suggesting a follicular response involving FB cells and a GC reaction and generation of protein- and PS-specific memory (87, 89, 90).

1.9.4 Co-immunization of intact Pn and PS-protein conjugate

A previous study from our laboratory demonstrated that Pn significantly inhibits the primary as well as secondary anti-protein and anti-PS IgG response to PS-protein conjugate (i.e. type 14 pneumococcal PS covalently attached to pneumococcal surface protein A [PPS14-PspA]) when Pn and conjugate are co-immunized. The inhibition displayed by Pn was not Pn strain-specific, nor was it dependent solely on its particulate nature or the presence of a PS capsule (91).

1.10 Co-immunization model

Most studies are designed to understand the parameters underlying antibody responses to an isolated, soluble protein or PS antigen. However, during a natural infection with a bacterium, the host encounters multiple bacterial Ags. They could be either associated with the intact bacterium and/or present in soluble form. These different kinds of Ag and their physical forms may determine the way they are processed by and stimulate the immune system, and thus may lead to cross-regulation of distinct antigen-specific antibody responses. Antigen expressed by an intact bacterium is in a particulate form, which by itself may influence an immune response, (92, 93), and is associated with immuno-modulating components of the bacterium, such as TLR ligands, scavenger receptors, NOD-like receptor ligands (82, 94). This highly influences the way Ag is processed by the immune system and the final outcome of the immune response against the Ag. Our studies were designed to simulate conditions closer to natural infections by co-

immunizing protein Ag with intact bacteria and determining how bacteria influence the antibody response against a co-immunized soluble protein Ag.

CHAPTER

2

An immunosuppressive property within the cell wall of *Streptococcus pneumoniae* inhibits the generation of a T follicular helper, germinal center, and plasma cell response to a co-immunized heterologous protein

2.1 **INTRODUCTION**

During the natural course of bacterial infections, the immune system is exposed to both cell-associated as well as soluble microbial components (95-97). Distinct differences exist in the immunologic properties between particulate and soluble antigens (98-102), suggesting the possibility of cross-regulatory processes occurring upon their simultaneous encounter by the immune system. In addition, the expression of innate stimulating moieties (103, 104), scavenger receptor ligands (94), and virulence factors by intact pathogens may further influence the immune response to co-immunizing soluble antigens. A number of studies in mice have demonstrated inhibitory effects of infectious agents on antibody responses, including germinal center reactions, to soluble protein antigens (105-109). Although the underlying mechanisms of these inhibitory effects were not fully clarified, pathogen-mediated inhibition of dendritic cell maturation and induction of regulatory T cells were implicated, depending upon the specific pathogen. An understanding of the interplay between intact microbes and immune responsiveness to soluble antigens may have implications on processes involving natural immunity, autoimmunity, and vaccination.

We previously demonstrated that intact, heat-killed *Streptococcus pneumoniae* inhibited the protein- and polysaccharide-specific IgG responses to a number of soluble conjugate vaccines, as well as soluble chicken ovalbumin (cOVA), upon co-immunization of mice i.p. in the presence of alum + CpG-ODN as adjuvant (110). In contrast, soluble conjugate vaccine had no effect on the IgG response to a pneumococcal protein expressed by the intact bacterium. Of note, co-immunization of a soluble conjugate with 1 μm latex beads failed to inhibit the

subsequent IgG response, indicating that the inhibition did not depend solely on the particulate nature of the bacteria. These data suggested that some structural or biochemical feature of *S. pneumoniae* mediated this suppressive effect. The study, however, left unresolved the mechanism of this inhibition and whether other intact extracellular bacteria exhibited similar suppressive properties on antibody responses to a co-immunized soluble antigen. In this report we determined potential changes in a number of key cellular parameters that could account for the suppression of the cOVA-specific IgG response following i.v. co-immunization of soluble cOVA with intact, heat-killed unencapsulated *S. pneumoniae*. We further evaluated the potential effects of cOVA co-immunization with other extracellular bacteria, i.e. *Neisseria meningitidis* or *Streptococcus agalactiae*. Our results reveal a novel immunosuppresive property of *Streptococcus pneumoniae* expressed in its cell wall that acts transiently during the early stage of the immune response to cOVA. This early event has a marked inhibitory effect on the subsequent cOVA-specific T follicular helper, germinal center, and plasma cell response accounting for the reduction in serum titers of cOVA-specific IgG.

2.2 MATERIALS AND METHODS

Mice. BALB/c and FVB mice were purchased from the National Cancer Institute (Frederick, MD). For IL10 studies IL10-/- mice, strain C.129P2(B6)-Il10^{tm1Cgn}/J mice and BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous DO11.10 mice x RAG-2$^{-/-}$ mice (BALB/c background) [from hereon referred to as "DO11.10 mice"], in which all CD4+ T cells express a transgenic T cell receptor (TCR) that encodes for a cOVA peptide (amino acids 323-339), presented by MHC-II^{d}, were purchased from Taconic Farms (Hudson, NY). For studies using NP-cOVA, BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred and maintained within the Biological Resource Center at National Jewish Health (NJH, Denver, CO). Mice were used between 7-12 weeks of age. These studies were conducted in accordance with the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and were approved by the Uniformed Services University of the Health Sciences and National Jewish Health Institutional Animal Care and Use Committees.

Reagents. cOVA ("Imject OVA") was purchased from Thermo Scientific (Rockford, IL). 4-hydroxy-3-nitrophenylacetyl $(NP)_{19}$-OVA was obtained from Biosearch Technologies (Novato, CA). Alum (Allhydrogel 2%) was obtained from Brenntag Biosector (Denmark). A stimulatory 30 mer CpG-containing oligodeoxynucleotide (CpG-ODN) was synthesized (111) and a truncated (amino acids 1-470) Epstein barr virus (EBV) envelope glycoprotein (gp350) was expressed in SF9 insect cells and purified (110). Anti-TGFβ Ab (clone 1D11.16.8) and

mouse IgG1 isotype control Ab (clone MOPC1), were purchased from BioXcell (West Lebanon, NH).

Bacterial strains. The unencapsulated mutant of D39 (*S. pneumoniae*, capsular type 2), [strain R36A]) was obtained from Dr. David Briles (University of Alabama at Birmingham, Birmingham, AL). The unencapsulated mutant of FAM18 C+ (*Neisseria meningitidis*, type C) [strain FAM18 C-], from hereon referred to as MenC, was obtained from Dr. Mustafa Akkoyunlu (FDA, Bethesda, MD) (112). The unencapsulated mutant of COH1 (*Streptococcus agalactiae*, type III) [strain COH1-13], from hereon referred to as GBS-III, was obtained from Dr. Craig Rubens (Children's Orthopedic Hospital, Seattle, WA) (113). Bacteria from frozen stocks were subcultured on BBL blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Todd Hewitt Broth [THB] (BD Biosciences, San Jose, CA) [for R36A and GBS] or Brain Heart Infusion media (BD Biosciences) [for MenC] to mid-log phase, collected, and heat-killed by incubation at 65°C for 2 h. Sterility was confirmed by subculture on blood agar plates. Bacteria were then aliquoted at 10^{10} colony forming units (CFU)/ml in PBS and frozen at -20°C.

Sonication of R36A. A cell wall preparation of R36A was prepared by sonication of heat killed R36A (10^{9} CFU/ml) at an amplitude of 60 μm for 5 minutes using a MISONIX Ultrasonic Liquid Processor S-4000 (Farmingdale, NY). Sonicated R36A was then centrifuged at 15,000 rpm for 10 minutes to spin down cell wall fragments followed by two washings with PBS before immunization. Complete disruption of bacteria was confirmed by microscopy.

Immunizations. Mice were immunized i.v. with 50 µg of cOVA or NP_{19}-OVA adsorbed on 13µg of alum, with or without CpG-ODN, in the presence or absence of $2x10^{8}$ CFU heat-killed bacteria in phosphate buffered saline (PBS). All secondary immunizations were performed in a similar manner, but in the absence of bacteria. Serum samples for measurement of Ag-specific IgG titers, at different time points, were prepared from blood obtained through the tail vein. For adoptive transfer studies, 2.5×10^{6} spleen cells (containing ~ $5x10^{5}$ Transgenic [Tg] T cells) from DO11.10 mice were injected i.v. into WT BALB/c mice 24 h prior to immunization. For transforming growth factor (TGF) β studies, 100 µg of anti-TGFβ Ab or mouse IgG1 isotype control Ab was injected i.v. into the mice 24 h prior to Ag immunization (114).

Measurement of antigen-specific serum titers by ELISA. Immulon 4 ELISA plates (Dynex Technologies, Chantilly, VA) were coated overnight with 5 µg/ml of cOVA or gp350 in PBS (50 µl/well) at 4ºC. The plates were then blocked with PBS + 1% bovine serum albumin [BSA] (100 µl/well) for 2 h at 37ºC. Three-fold serial dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 1% BSA (50 µl/well) were then added and incubated overnight at 4ºC followed by washing (3x) with PBS + 0.1% Tween-20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG Ab (200 ng/ml, 50 µl/well) in PBS plus 1% BSA was then added and plates were incubated at 37ºC for 1 h. Plates where then washed with PBS + 0.1% Tween-20 and substrate (*p*-nitrophenyl phosphate, disodium; Sigma-Aldrich) was added at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM $MgCl_2$ [pH 9.8]) for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland).

Enumeration of NP-specific antibody-secreting cells (ASC) by ELISPOT. NP-specific ASC were measured in 96-well flat bottom EIA/RIA high-binding plates (Costar, Corning, Sigma-Aldrich) coated overnight at 4°C with 2 μg/ml NIP_{15}–BSA diluted in 0.05 M K_2HPO_4 (pH 8.0). Plates were washed 3x with PBS prior to blocking with warm PBS, 1% gelatin (Sigma-Aldrich) at 37°C for a minimum of 1 h. Plates were washed again 3x with PBS prior to incubation with cells. Single cell suspensions of splenocytes or bone marrow (harvested 2 weeks post-immunization) were seeded in duplicate at 4-6 × 10^6 total viable cells per 100 μl in the first well, and 2-fold serial dilutions were carried out down the plate. Plates were incubated at 37°C in 5% CO_2 for 5-6 h in RPMI Medium (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated Fetal Bovine serum [FBS] (BioSource, Grand Island, NY), 2 mM GlutaMAX-I (Invitrogen), 100 U/ml Penicillin (Invitrogen, Grand Island, NY), 100 μg/ml Streptomycin (Invitrogen), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). Following culture, cells were then lysed with H_20, 0.05% Tween 20 for 10 min at room temperature and subsequently washed 3x with PBS, 0.1% Tween 20. Secreted antibody was detected by incubating plates with an AP-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL) diluted in 1% gelatin in PBS for 1 h at 37°C. After 3 washes with PBS/Tween 20, plates were developed overnight at 4°C with 1 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate *p*-toluidine (Sigma-Aldrich) salt substrate diluted in an alkaline buffer composed of 0.1 M 2-amino-2-methyl-1-propanol, 0.01% NaN_3, 0.5 mM $MgCl_2$, 0.007% Triton X-405, pH 10.25. Plates were washed 3x with deionized H_2O, allowed to dry in the dark at room temperature, and scanned (Epson Perfection 2450 Photo Scanner). Developed spots were counted visually from the scanned images and the frequency of NP-specific ASCs per total number of cells plated was calculated.

Flow cytometric analysis. Individual samples of red blood cell (RBC)-lysed spleen cells from 3-5 mice/group were stained using the following mouse-specific mAbs: Alexa Fluor 405-anti-CD4 (clone RM4-5) and allophycocyanin -anti-DO11.10 TCR (clone KJ1-26) [Invitrogen]; phycoerythrin (PE)-anti-CD69 (clone H1.2F3), PE-anti-CD25 (clone PC61), PE-Texas Red-anti-B220 (clone RA3-6B2), and FITC-anti-T and B cell activation antigen (clone GL7) [BD Biosciences, San Jose, CA]; PE-Cy7-anti-PD1 (clone 29F.1A12) [Biolegend, San Diego, CA]; PE-Cy7-anti-CD25 (clone PC61.5) and PE-anti-Foxp3 (clone FJK-16s) [eBiosciences, San Diego, CA]. Foxp3 staining was performed using the Foxp3 staining kit from eBiosciences as per the manufacturer's protocol. Cells were analyzed using a LSR-II flow cytometer (BD Biosciences) and results were generated using FlowJo (Tree Star, Ashland, OR) and FACSDiva (BD Biosciences) softwares. For detection of PC on bacteria, 1 x 10^5 CFU heat-killed bacteria were incubated overnight at 4°C with 0.25 µg of mouse IgG2aκ anti-PC mAb (clone PCG2a2.A1) was obtained from Dr. J. Kenny (Beth Israel Deaconess Medical Center, Boston, MA), in PBS plus 1% BSA. This was followed by washing with PBS (2x) and incubation with 1.25 µg biotin-goat anti-mouse IgG (Southern Biotech) on ice for 30 min followed by another washing with PBS (2x) and incubation with 0.5 µg PE-streptavidin (BD Biosciences) on ice for 30 min. After 1 more wash with PBS, bacteria were analyzed by flow cytometry using a BD LSR-II flow cytometer. Bacteria incubated with only biotin-goat anti-mouse IgG and PE-streptavidin were used as a negative control. For studies to detect NP^+ cells, the following mAbs were used: PE-Cy7-anti-B220 (clone RA3-6B2) and Allophycocyanin-Cy7-anti-IgD (clone 11.26c.2a) [BioLegend]; biotin-Igκ (clone 187.1, hybridoma), and PE-Cy5.5-CD11c (clone N418) [eBiosciences]. FITC-peanut agglutinin [PNA] (Vector Laboratories, Burlingame, CA) was used for detection of germinal center B cells and 647-OVA (Invitrogen) was used to exclude

non-NP binding B cells. For detection of NP-specific B cells, cells were stained with PE-NP_{40} (Biosearch Technologies). The secondary reagent for detecting biotin-conjugated antibodies was Pacific Blue-streptavidin (Invitrogen). Flow cytometric analyses were performed by acquiring data on a Cyan analyzer (Dako, Denmark) and with FlowJo software (Tree Star).

Measurement of T-cell proliferation by Carboxyfluorescein diacetate (CFSE) dilution. RBC-lysed spleen cells from D011.10 mice (2.5 x 10^7 cells) were incubated in 1 ml of 5 μM CFSE (Vybrant CFDA-SE; Molecular Probes, Grand Island, NY) in PBS for 10 min at 37°C. Cells were then washed 1x, resuspended in prewarmed PBS, and incubated at 37°C for 30 min. Cells were then washed 2x with PBS and transferred i.v. into WT BALB/c mice (2.5 x 10^6 spleen cells/mouse containing ~5 x 10^5 DO11.10 Tg T cells). One day later, mice were immunized with cOVA + alum with or without R36A. 2.5 and 4 d post-immunization, spleen cells were obtained, and gated DO11.10 Tg T cells ($CD4^+$ DO11.10 TCR^+) were analyzed for CFSE dilution using an LSR-II flow cytometer (BD Biosciences) and ModFitLT software (Verity Software House, Topsham, ME).

Statistical analysis. Serum antigen-specific IgG titers were expressed as geometric means $\pm$ SEM of the individual serum titers. Significance was determined by two-tailed Student's *t* test. Values of $p \leq 0.05$ were considered statistically significant. Each experiment was performed at least twice to ascertain reproducibility.

2.3 RESULTS

The degree of R36A-mediated inhibition of the IgG response to cOVA is dependent on the nature of the adjuvant.

We previously demonstrated that an unencapsulated *S. pneumoniae* (strain R36A) inhibited IgG responses to a number of soluble proteins co-injected into mice i.p. in the presence of alum + CpG-ODN adjuvant (110). CpG-ODN, a TLR9 agonist (115), significantly enhances antibody responses to soluble proteins in alum (116), thus making alum + CpG-ODN a more effective adjuvant than alum alone. To extend these findings, we set out to determine whether R36A-mediated inhibition was influenced by the nature of the adjuvant used for the co-injected soluble protein. We immunized BALB/c mice i.v. with cOVA + alum in the presence (Fig. 11A) or absence of CpG-ODN (Fig. 11B), with or without R36A at a dose of 2 x 10^8 CFU/mouse. Mice were similarly boosted on d 14 in the absence of R36A. R36A inhibited the IgG response to cOVA by 3-4-fold when cOVA was injected in alum + CpG-ODN (Fig 11A). In contrast R36A-mediated inhibition was ~10-fold when cOVA was co-injected in alum alone (Fig. 11B). R36A also significantly inhibited the IgG anti-cOVA response to cOVA + alum, at the lower dose of 2 x 10^7, but not 7 x 10^6, CFU/mouse (data not shown). These data suggest an inverse relationship between the effectiveness of the adjuvant used for enhancing an antibody response to protein immunization and the degree of R36A-mediated inhibition of the IgG response. R36A failed to inhibit the cOVA-specific IgG response when mice were first primed with cOVA + alum alone, followed by secondary immunization with cOVA + alum in the presence of R36A (data not shown), indicating that the inhibitory effect of R36A occurred only during the time of primary immunization. Of note, an R36A cell wall preparation produced by sonication followed

by repeated washing and centrifugation, also inhibited the IgG anti-cOVA response to cOVA + alum (Fig. 11C) indicating that the inhibitory moiety was contained within the cell wall, and that inhibition was not dependent on the particulate nature of the bacterium. To see if this inhibitory effect of R36A is specific to BALB/c strain or extends over to other mice strains we co-immunized FVB mice with cOVA with or without R36A. R36A significantly inhibited the primary and secondary anti-cOVA IgG responses suggesting that this suppressive property is not limited to BALB/C mice only (Fig 11D).

Co-immunization via the subcutaneous (s.c.) route with R36A and either cOVA or truncated gp350 (an EBV glycoprotein) in alum + CpG-ODN also resulted in inhibition of the cOVA-specific and gp350-specific IgG responses, respectively (Fig. 12). Inhibition required the injection of R36A and soluble protein at the same site, strongly suggesting that this event occurred within the draining lymph node. Thus, the inhibitory effect of R36A is mediated both within the spleen (i.v. route) or draining lymph node (s.c. route).

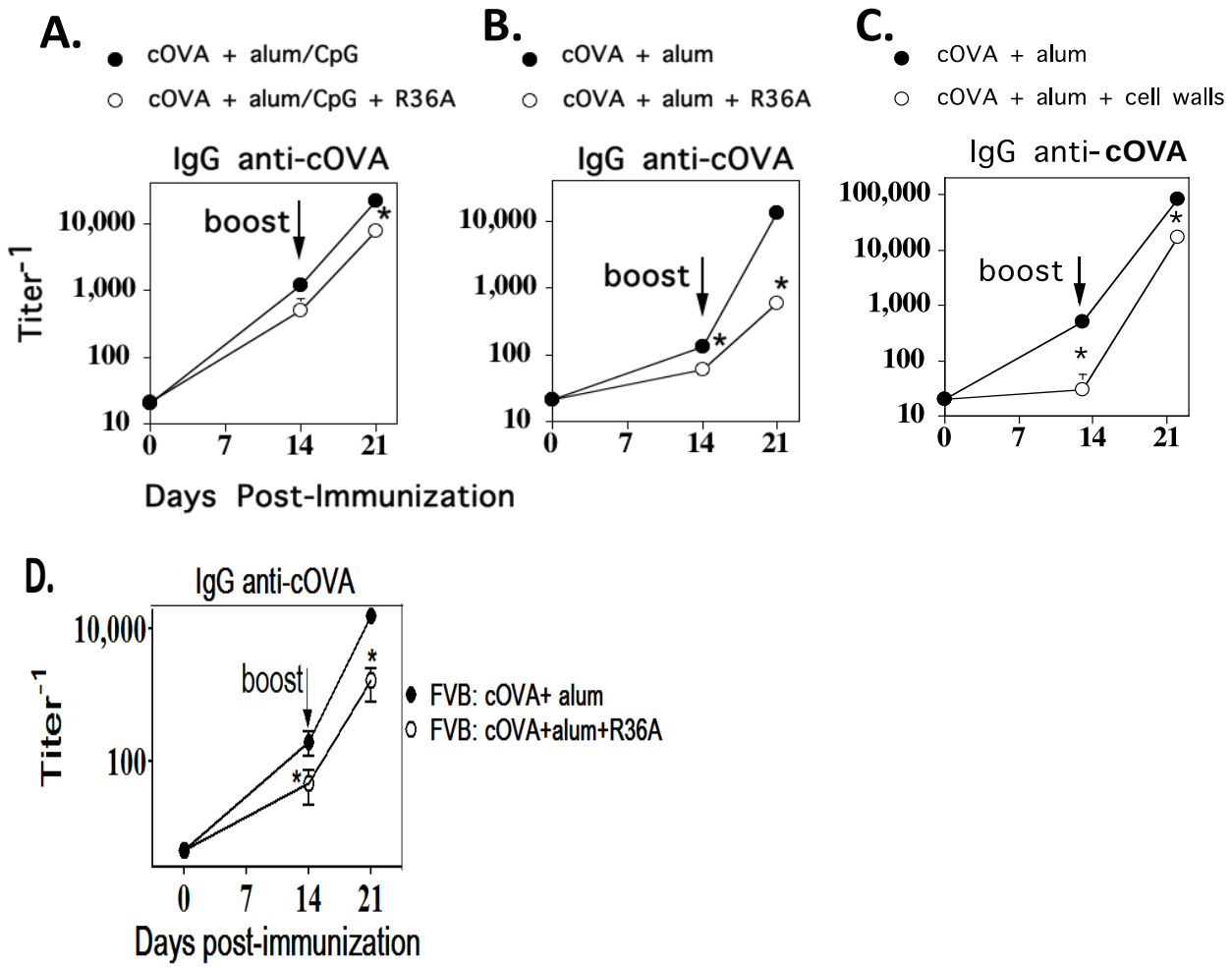

Fig 11. **<u>The degree of R36A-mediated inhibition of the IgG response to cOVA is dependent on the nature of the adjuvant.</u>** BALB/c mice (7 per group) were immunized i.v. with 50 µg cOVA with or without 2 x 10^8 CFU R36A **(A)** in alum + CpG-ODN or **(B)** in alum alone, or **(C)** with cell walls of R36A (2 x 10^8 CFU/mouse equivalents). (**D)** FVB mice (5 mice per group) were immunized i.v. with 50 µg cOVA with or without 2 x 10^8 CFU R36A in alum. All the mice were boosted i.v. with 50 µg cOVA in alum or alum + CpG-ODN on day. Serum titers of cOVA-specific IgG were measured by ELISA. Significance * $p \leq 0.05$.

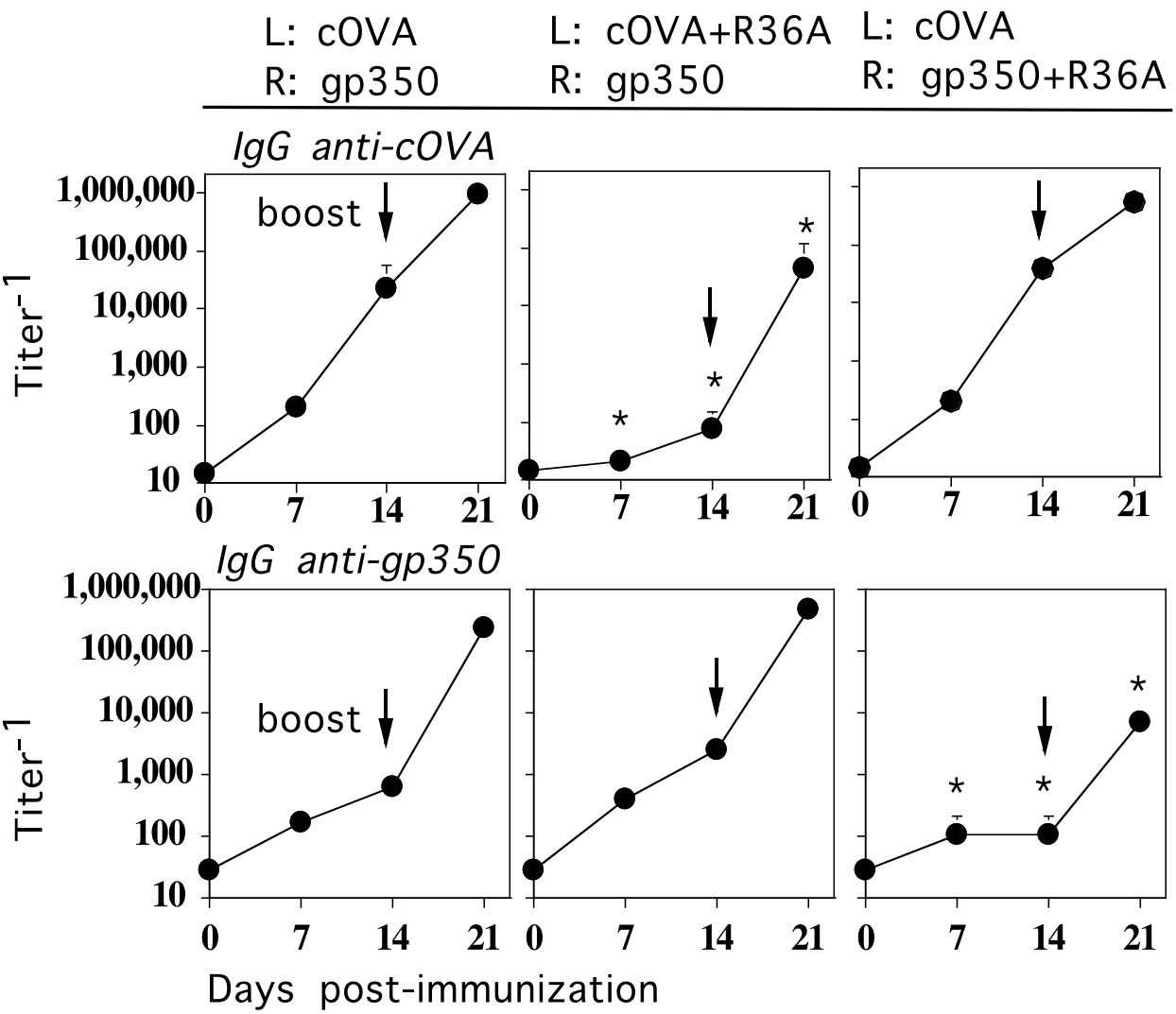

Fig. 12. <u>R36A inhibits the cOVA-specific IgG response via the s.c. route.</u> BALB/c mice (7 per group) were immunized s.c. in the left (L) or right (R) footpad with 50 µg cOVA or 50 µg gp350, respectively, in alum+ CpG-ODN with or without 2 x 10^8 CFU R36A as illustrated. Mice were similarly boosted on day 14 in the absence of R36A. Serum titers of cOVA-specific (upper panels) and gp350-specific (lower panels) IgG measured by ELISA. Significance * $p \leq 0.05$ (middle and right panels) relative to mice immunized in the absence of R36A (left panels).

R36A acts transiently within the first 24 h to cause inhibition of the cOVA-specific IgG response.

Previously, we reported that R36A was effective in mediating inhibition of an IgG anti-polysaccharide response to a soluble pneumococcal conjugate vaccine in alum + CpG-ODN when injected i.p. at the same time, but not when R36A injection was delayed by 24 h (110). This indicated that the primary inhibitory event occurred during the period of initiation of the immune response. In this regard, we further wished to determine whether R36A could mediate this inhibitory effect when injected prior to cOVA immunization. We thus immunized mice with R36A, 0-3 d prior to cOVA immunization (Fig. 13A), or 0-2 d following immunization (Fig. 13B). All mice were boosted with cOVA/alum alone, 14 d following primary cOVA immunization. If R36A was injected either 1-3 d prior or 1-2 d subsequent to immunization with cOVA/alum, no inhibition of cOVA-specific IgG response was observed, in contrast to mice co-immunized with cOVA and R36A. These data demonstrated that R36A acts transiently and early during the immune response to cOVA.

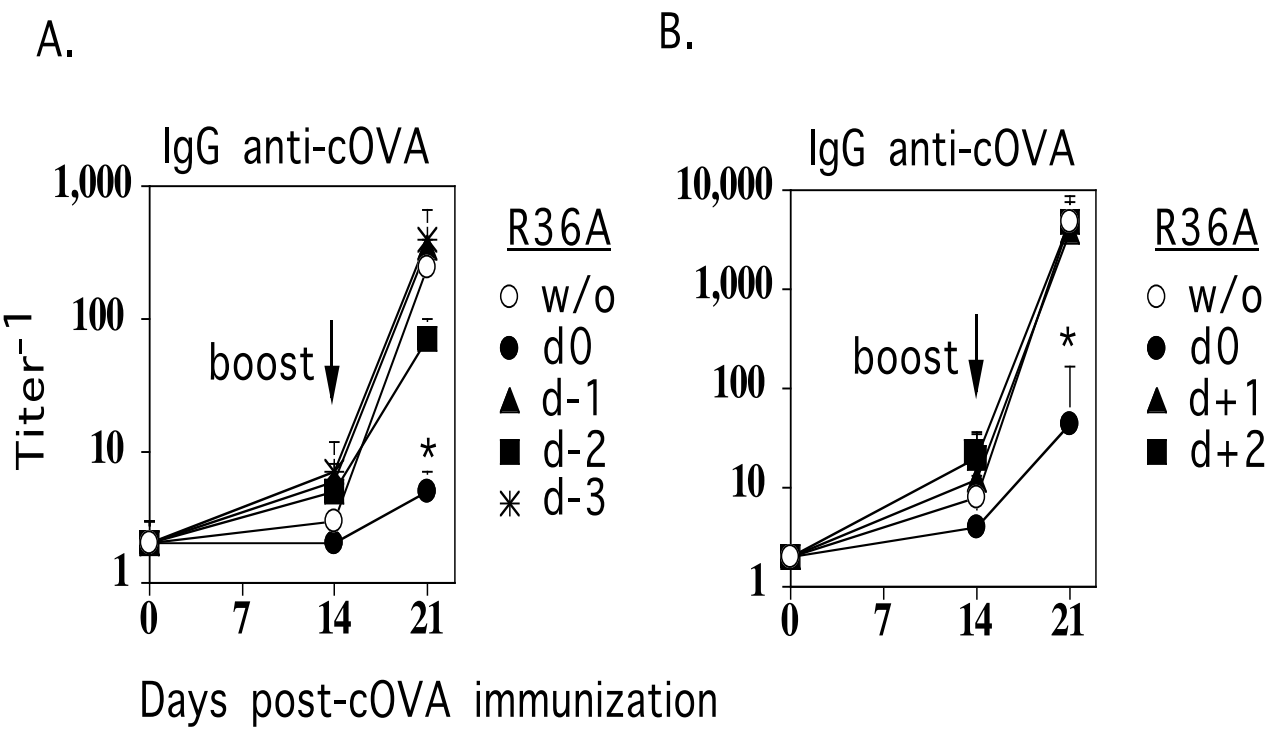

Fig. 13. R36A acts transiently within the first 24 h to cause inhibition of the cOVA-specific IgG response. BALB/c mice (7 per group) were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A given **(A)** 1, 2 or 3 d earlier than cOVA or **(B)** 1 or 2 d after cOVA immunization. All mice were boosted i.v. with 50 µg cOVA in alum alone, 14 d after primary cOVA immunization. Serum titers of cOVA-specific IgG measured by ELISA are shown. Significance * $p \leq 0.05$ in comparison to mice immunized with cOVA + alum in the absence of R36A ("w/o").

R36A has no apparent effect on early cOVA-specific T cell activation, and has only a modest effect on T cell proliferation.

The cOVA-specific IgG respon

se is dependent on CD4+ T cell help. The observation that R36A exerted its inhibitory effect within 24 h following cOVA immunization suggested that it might be mediating an inhibitory effect on initial CD4+ T cell priming. To determine this, we adoptively transferred cOVA-specific CD4+ Tg T cells from DO11.10 mice, i.v. into BALB/c mice, followed by i.v. immunization with cOVA/alum alone or cOVA/alum + R36A, 1 d later. Splenic Tg T cells ($CD4^+$ DO11.10-TCR^+) were evaluated 24 h later by flow cytometry for expression of the T cell activation markers CD69 and CD25 (Fig. 14A). As illustrated, R36A had no effect on the cOVA-mediated upregulation of either CD69 or CD25 on Tg T cells.

To determine the proliferative response of Tg T cells, CFSE-labeled cOVA-specific Tg T cells from DO11.10 mice were adoptively transferred into BALB/c mice 1 d prior to immunization with cOVA/alum alone, or cOVA/alum + R36A. Splenic Tg T cells were analyzed by flow cytometry for CFSE dilution, 2.5 and 4 d post-immunization. At day 2.5, no significant effect on the cOVA-induced Tg T cell proliferative response was observed by co-immunized R36A (data not shown). However, by day 4 we found that R36A mediated a modest, though consistent, reduction in the percentage of Tg T cells that had undergone 9, 10, or 11 proliferative cycles (Fig. 14B). This modest R36A-mediated reduction in proliferation, however, seemed unlikely to account for the more striking inhibition of the subsequent cOVA-specific IgG response.

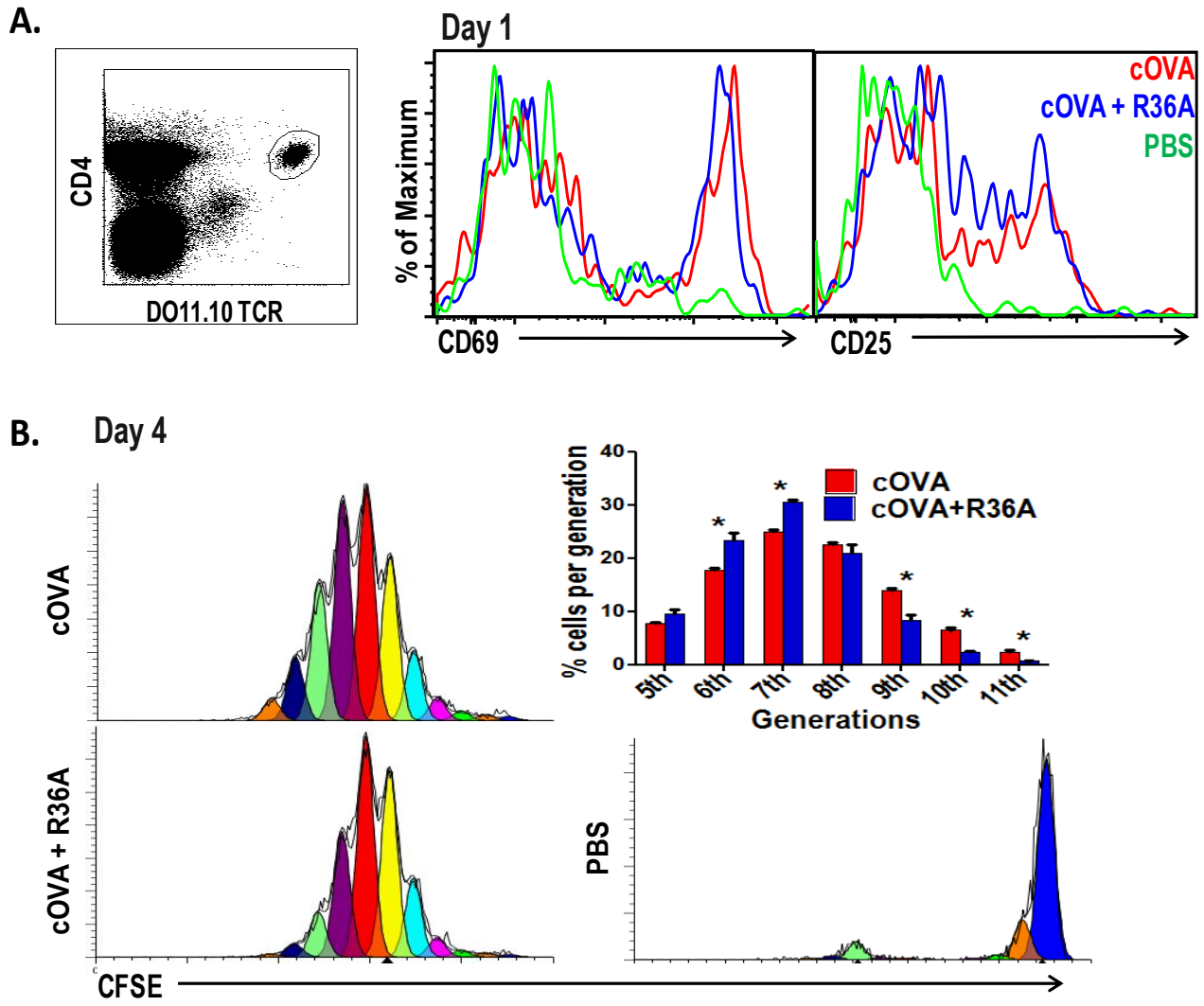

Fig. 14. **<u>R36A has no apparent effect on early cOVA-specific T cell activation, and has only a modest effect on T cell proliferation.</u>** **(A)** cOVA-specific Tg T cells from DO11.10 mice were adoptively transferred into BALB/c mice, and 1 d later immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A, or with PBS alone. Gated $CD4^+$ DO11.10 TCR^+ Tg T cells from spleen cell suspensions were analyzed by flow cytometry, 1 d following immunization, for T cell activation markers CD69 and CD25 (3 mice/group). **(B)** 5 x 10^5 cOVA-specific Tg T cells from DO11.10 mice were labeled with CFSE and adoptively transferred into BALB/c mice. 1 d later mice were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A, or with PBS alone (3 mice/group). On d 4, gated $CD4^+$ DO11.10 TCR^+ Tg T cells from spleen cell suspensions were analyzed for T cell proliferation as reflected by CFSE dilution by flow cytometry. Significance * $p \leq 0.05$

R36A mediates a significant reduction in the generation of GC T follicular helper cells, but has no effect on the numbers of Foxp3+ regulatory T cells (Tregs).

Tregs have been reported to have inhibitory effects on in vivo humoral autoimmune responses (117, 118), although we previously observed no effects of endogenous Tregs on Ig responses to intact *S. pneumoniae* (119). To determine if R36A affected the generation of cOVA-specific Tregs following cOVA immunization, DO11.10 Tg T cells were transferred into BALB/c mice, which were then immunized with cOVA/alum or cOVA/alum + R36A. We then determined the numbers of $Foxp3^{+}CD25^{+}$ Tg T cells 7 d later. As illustrated in Fig. 14A, cOVA immunization resulted in an increase in Tregs at d 7 relative to that observed in unimmunized mice. However, R36A had no significant effect on the numbers of cOVA-induced Tregs present at this time point (Fig. 15A), which is 7 d prior to the time in which mice are typically boosted with cOVA. In addition, co-immunization of cOVA-immunized mice with R36A did not consistently alter the total number of Tg T cells present on either day 7 or 8 (data not shown).

CD4+ GC T follicular helper cells play a key role in promoting TD antibody responses (120). These cells can be reliably identified by flow cytometry as $CD4^{+}GL7^{+}PD\text{-}1^{hi}$ cells, a population that is also $CXCR5^{+}$ (121, 122). In this regard, we set out to determine whether R36A affected the generation of cOVA-induced GC Tfh in the adoptive transfer model using DO11.10 Tg T cells. We analyzed spleen cells 8 d post-immunization, a time point shown previously to exhibit peak numbers of GC Tfh (122). As illustrated in Fig 4B/C, ~10% of Tg T cells from mice immunized with cOVA/alum alone expressed the GC Tfh phenotype at d 8 relative to <0.5% GC Tfh cells in mice injected with PBS. CXCR5 expression was substantially higher on

$CD4^{+}GL7^{+}PD\text{-}1^{hi}$ Tg T cells relative to the endogenous CD4+ T cells from cOVA-immunized mice (data not shown). Of note, we observed a consistent, significant 2-3-fold reduction in the percentage and absolute numbers of splenic Tg T cells expressing the GC Tfh phenotype in mice immunized with cOVA/alum + R36A relative to cOVA/alum alone (Fig. 15B).

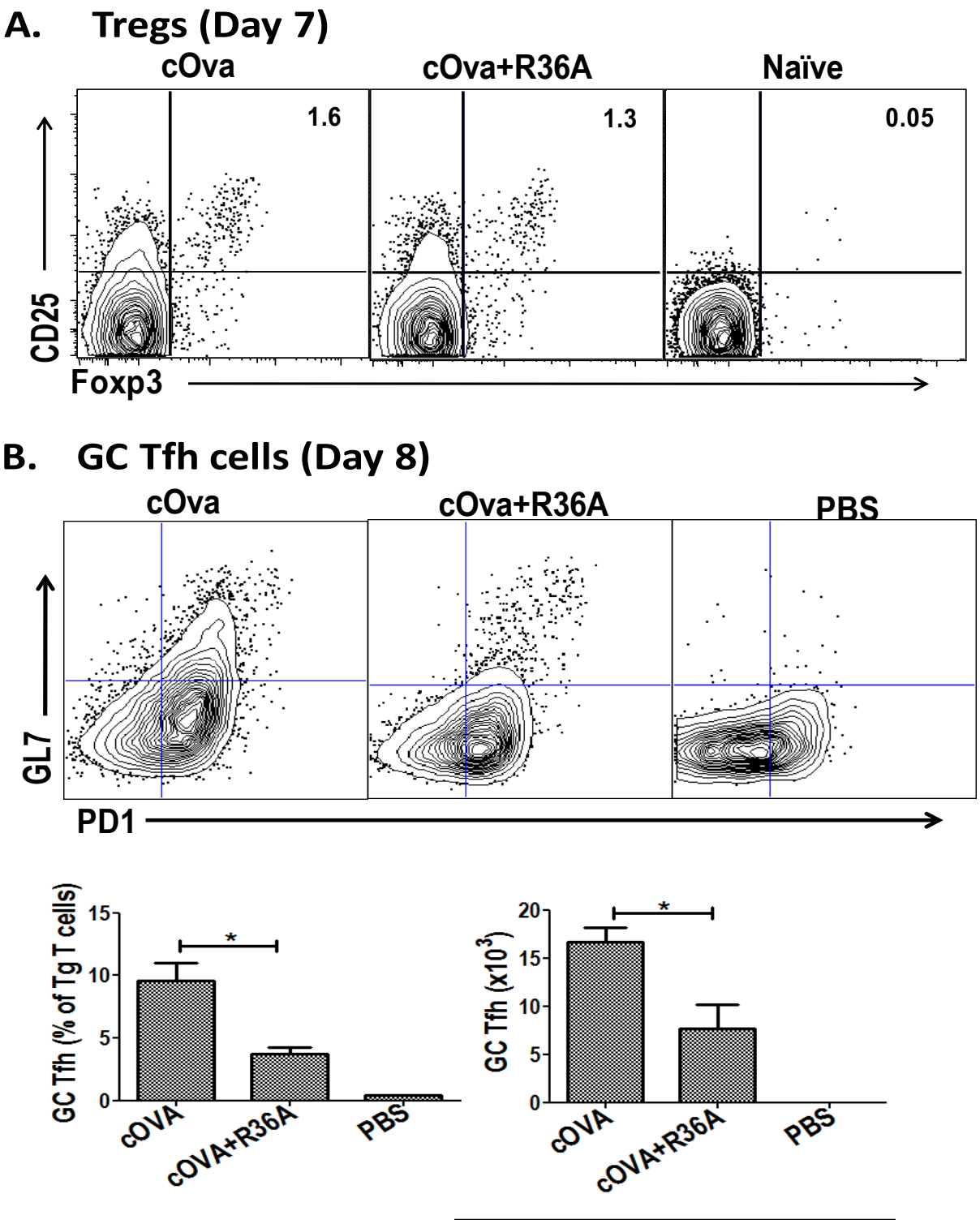

Fig. 15. **R36A mediates a significant reduction in the generation of GC T follicular helper cells, but has no effect on the numbers of Foxp3+ regulatory T cells (Tregs).** **(A)** cOVA-

specific Tg T cells from DO11.10 mice were adoptively transferred into BALB/c mice and 1 d later immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A (3 mice/group). On d 7 following immunization, gated $CD4^+$ DO11.10 TCR^+ Tg T cells from spleen cell suspension were analyzed for Tregs ($CD25^+$ $Foxp3^+$). The numbers represent the mean percentage of total Tg T cells in each population. Flow cytometric dot plots are representative of one sample from each group. **(B)** cOVA-specific Tg T cells from DO11.10 mice were adoptively transferred into BALB/c mice and 1 d later immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A, or with PBS alone (3-5 mice/group). On d 8 following immunization gated $CD4^+$ DO11.10 TCR^+ Tg T cells from spleen cell suspensions were analyzed for GC Tfh cells ($GL7^{hi}$ $PD1^{hi}$) [upper panel]. Quantitation of GC Tfh data [lower panel]. Significance * $p \leq 0.05$.

Unencapsulated variants of intact, heat-killed *Streptococcus agalactiae* (GBS-III) and *Neisseria meningitidis* (MenC) do not mediate inhibition of the cOVA-specific IgG response.

We next set out to determine whether or not the R36A-mediated inhibition of the cOVA-specific IgG response represented a more general suppressive effect of various intact bacteria on T cell-dependent Ig responses to soluble antigens. To evaluate thiswe used a gram positive (GP) bacteria , GBS-III and a gram negative (GN) bacteria, MenC for this pupose. We co-immunized BALB/c mice i.v. with cOVA/alum alone or cOVA/alum + GBS-III or MenC, followed by a boost with cOVA/alum alone on d 14. In contrast to R36A, GBS-III failed to inhibit, and MenC modestly enhanced the cOVA-specific IgG response (Fig. 16A). Of note, whereas R36A induced a significant 2-3 fold reduction in cOVA-induced GC Tfh, 8 d post-immunization, similar to that observed in Fig. 15B, MenC had no significant effect on the generation of cOVA-specific GC Tfh (Fig. 16B).

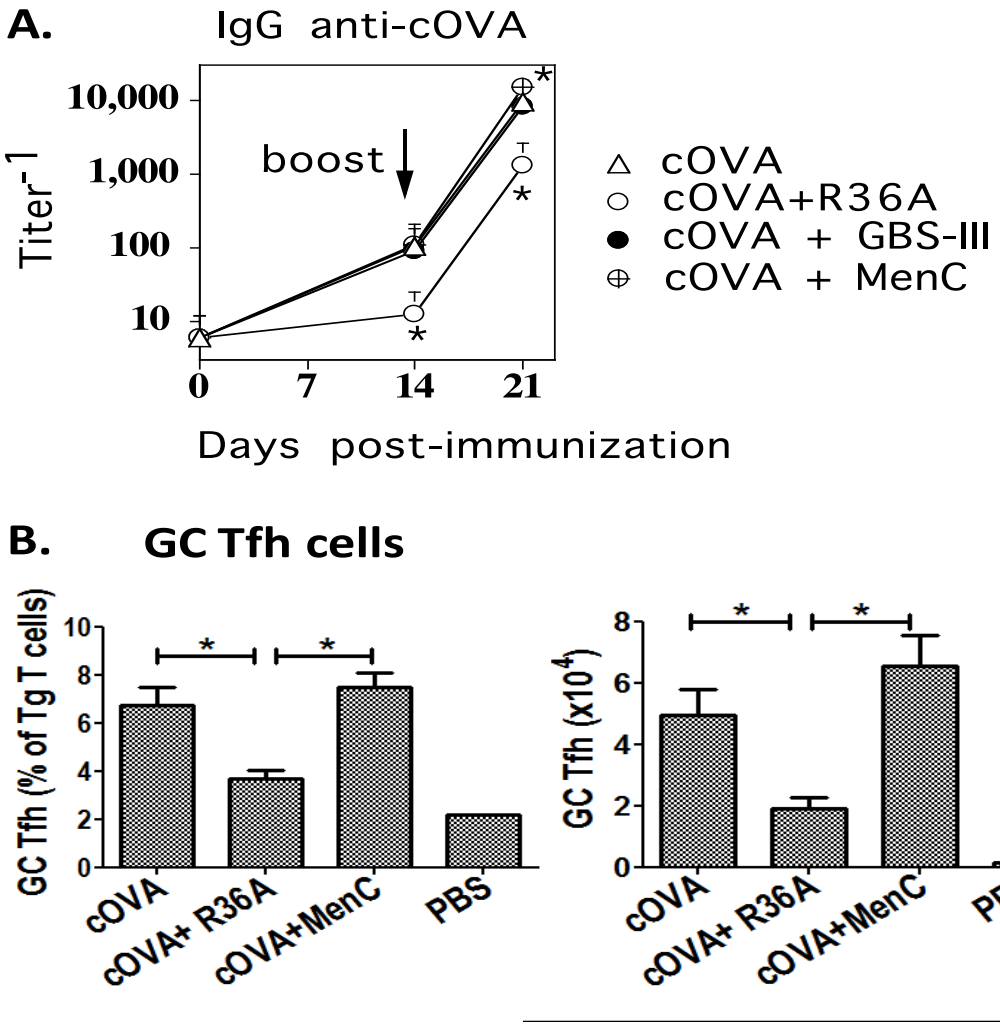

Fig. 16. <u>Unencapsulated variants of intact, heat-killed *Streptococcus agalactiae* (GBS-III) and *Neisseria meningitidis* (MenC) do not mediate inhibition of the cOVA-specific IgG response.</u> (A) BALB/c mice (7 per group) were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU intact heat-killed R36A, GBS-III or MenC (all unencapsulated variants). All mice were boosted i.v. with 50 µg cOVA in alum alone on d 14. Serum titers of cOVA-specific IgG were measured by ELISA. Significance * $p \leq 0.05$ in comparison to mice immunized with cOVA + alum without bacteria ("cOVA"). **(B)** cOVA-specific GC Tfh cells ($GL7^{hi}$ $PD1^{hi}$) were measured [as shown in Fig. 5B] in response to cOVA alone or with R36A or MenC on d 8 post-immunization (5 mice/ group). Significance * $p \leq 0.05$

R36A, but not MenC, inhibits the generation of NP-specific GC B cells, and antibody-secreting cells in the spleen and bone marrow, when co-immunized with NP-cOVA.

The R36A-mediated reduction in the number of cOVA-induced GC Tfh suggested that the generation of cOVA-specific GC B cells and ASC would be correspondingly reduced. To determine this, we immunized BALB/c mice with NP-cOVA/alum alone or NP-cOVA/alum + R36A or MenC, and 14 d later measured the number of NP-specific GC B cells by flow cytometry, defined here as $B220^{+}CD11c^{-}NP^{+}IgD^{-}PNA^{+}$. As illustrated in Fig. 17A/B, R36A, but not MenC, significantly reduced, by 2-3-fold, the percentage and absolute numbers of NP-specific GC B cells, as well as the absolute number of total NP+ B cells generated following NP-cOVA immunization. Consistent with these findings, the generation of NP-specific ASC in the spleen and bone marrow (BM) were reduced by R36A, but not MenC, by ~3-5-fold (Fig. 17C). These data further paralleled the observed generation of cOVA-specific GC Tfh cells in the presence or absence of R36A and MenC (Fig. 16B). Collectively, these data reveal a novel immunosuppresive property of intact *S. pneumoniae* expressed in its cell wall that acts transiently during an early stage of the immune response to co-immunizing cOVA. This early event has a marked inhibitory effect on the subsequent cOVA-specific T follicular helper, germinal center, and plasma cell response.

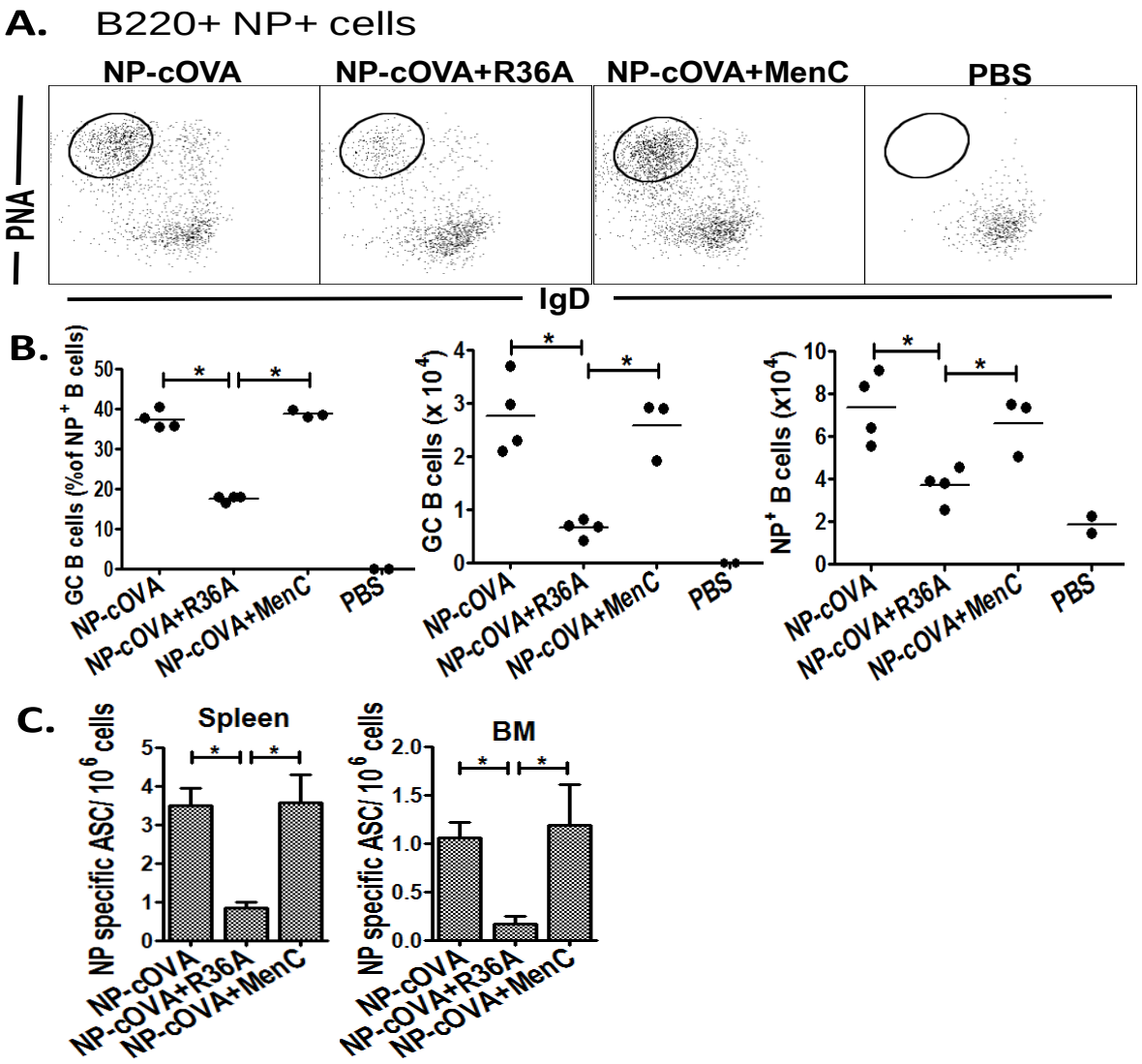

Fig. 17. <u>R36A, but not MenC, inhibits the generation of NP-specific GC B cells, and ASC in the spleen and bone marrow, when co-immunized with NP-cOVA.</u> (A) BALB/c mice (3-4/group) were immunized i.v. with 50 µg NP-cOVA with or without 2 x 10^8 CFU R36A or MenC. On d 14 post-immunization, NP-specific B cells ($B220^+$ $CD11C^-$ NP^+) were analyzed for isotype-switched GC B cells ($IgD^{-/low}$ PNA^+) represented by encircled population. **(B)** Quantitation of NP-specific GC B cell data. **(C)** Quantitation of NP-specific ASC from spleen and BM in response to NP-cOVA alone or with R36A or MenC on d 14 post-immunization (3-4 mice/group). Significance * $p \leq 0.05$.

IL-10 and TGF-β play no apparent role in R36A-mediated inhibition

Many studies have implicated IL-10 and TGF-β in immune suppression (123, 124) including a role in preventing a number of autoimmune diseases (114, 125) and limiting T cell effector responses (126, 127). Mast cells, which are activated early during bacterial infections, secrete IL-10 that suppresses Tfh generation and GC reaction under certain conditions (127). TGF-β can also be released by macrophages early in response to bacteria. Therefore, we examined the role of TGF-β and IL-10 in the immunosuppression exhibited by R36A. To evaluate the role of IL-10, we immunized IL-10-/- mice with cOVA in the presence or absence of R36A. BALB/c mice were immunized similarly as controls. R36A significantly inhibited anti-cOVA IgG responses in both IL-10-/- and control BALB/c mice (Fig. 18A). To determine a potential role of TGF-β, we injected mice with anti-TGF-β Ab or control Ab (mouse IgG1 isotype control), 24h prior to immunization with cOVA in the presence or absence of R36A, and determined IgG responses against cOVA. R36A inhibited the anti-cOVA response similarly, whether they were injected previously with anti-TGFβ Ab or the control Ab (Fig. 18B). In light of the possibility of redundant inhibitory effects of IL-10 and TGF-β, we administered anti-TGFβ Ab to IL10-/- mice or control Ab to BALB/c mice, 24h prior to immunization with cOVA alone or cOVA+ R36A together. R36A significantly inhibited both primary and secondary anti-cOVA IgG responses in both groups of mice (Fig. 18C), strongly suggesting that the mechanism of suppression by R36A, does not involve IL-10 or TGF-β.

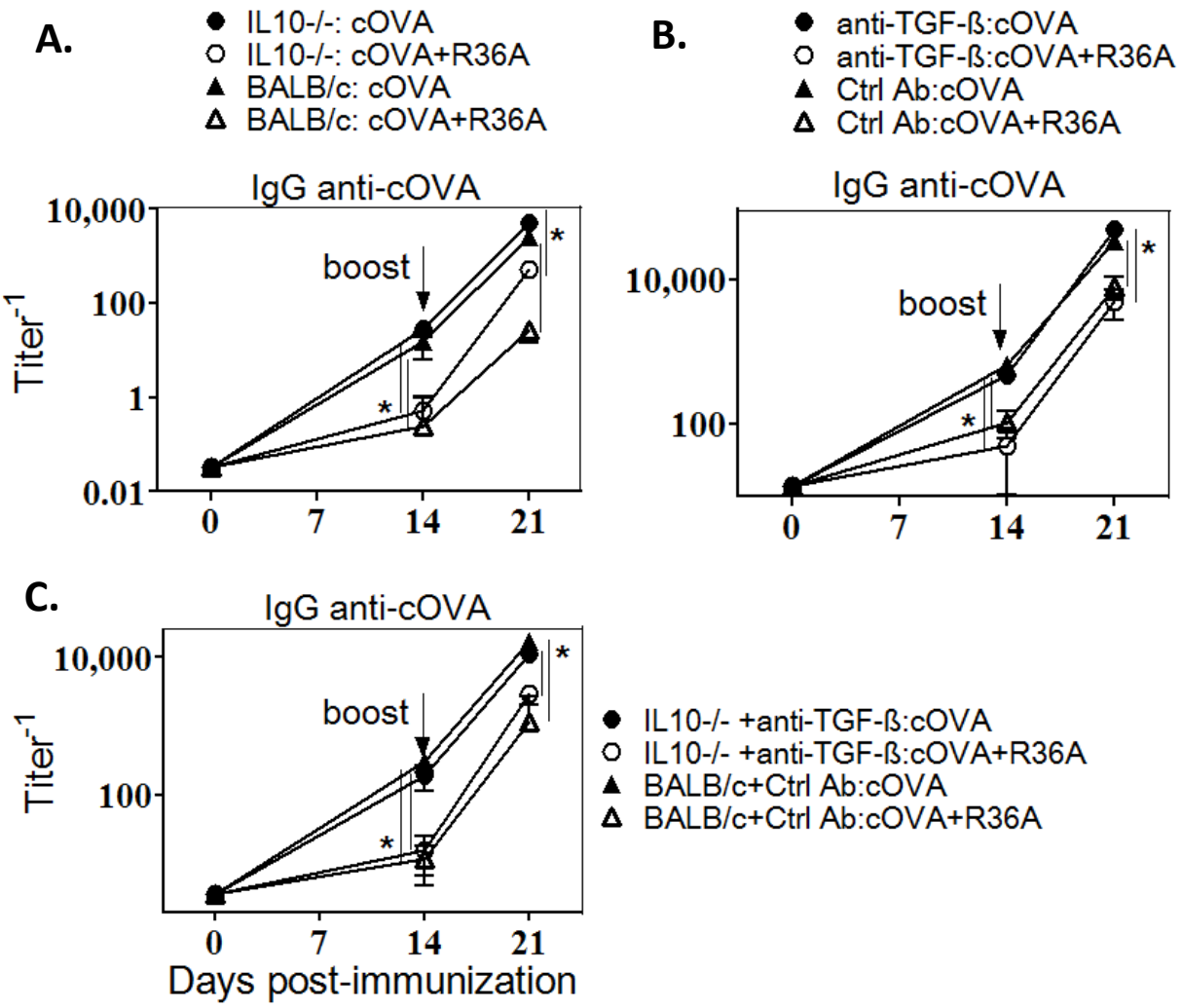

Fig. 18. <u>IL-10 or TGF-β are not involved in the suppressive property of R36A.</u> (A) IL10-/- or BALB/c mice were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A. (**B)** BALB/c mice were immunized i.v. with 100µg anti-TGFβ Ab or Ctrl Ab (mouse IgG1 isotype control). 1d later mice were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A. (C) IL10-/- were immunized i.v. with 100µg anti-TGFβ Ab and BALB/c mice with 100µg Ctrl Ab. 1d later mice were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A (7/group). All mice were boosted i.v. with 50 µg cOVA in alum without bacteria, on d14. Serum titers of cOVA-specific IgG were measured by ELISA. Significance * $p<0.05$.

2.4 DISCUSSION

During the natural course of infections with extracellular bacteria, soluble bacterial proteins are likely to be released (95-97). Little is known however, regarding the immunologic consequences of simultaneous exposure of the intact pathogen and these soluble antigens, to the immune system. We previously demonstrated that co-immunization with intact R36A resulted in a significantly reduced IgG response to a number of soluble TD antigens (110). In the current study we investigated the mechanism of this inhibitory effect using R36A and soluble cOVA as the basis for the experimental system. We demonstrate that, although co-immunization of R36A with cOVA had no obvious effect on early cOVA-specific CD4+ T cell activation or subsequent generation of $Foxp3^+$ Tregs, and only a modest effect on T cell proliferation, it led to a significant downstream reduction in the generation of cOVA-specific GC Tfh (d 8). This likely contributed to the observed decrease in GC B cells, and ASC in the spleen and bone marrow (d 14). This, in turn can account, at least in large part, for the associated reduction in serum titers of cOVA-specific IgG. Of note, we show that this effect is not a general property of all intact extracellular bacteria, in that neither intact unencapsulated GBS-III nor MenC were inhibitory. Although the inhibitory moiety expressed by S. *pneumoniae* remains to be determined, our data indicate that it is present within the cell wall, as a cell wall preparation obtained by sonication of R36A followed by centrifugation and washing was also inhibitory. These data further indicate that inhibition does not require particulation, since sonication resulted in complete bacterial disruption.

Several other pathogens have also been reported to inhibit antibody responses to soluble TD antigens in mice, although by apparently different mechanisms. *Salmonella typhimurium* has

been reported to delay GC formation induced by haptenated proteins (108). Trinitrophenol (TNP)-specific IgG responses to soluble TNP-conjugated proteins are suppressed during infection with *Trypanosoma cruzi*, mediated by nonspecific suppressor T cells (109). Intracellular bacterial infection by *Ehrlichia muris* inhibited splenic, but not lymph node, NP-specific IgG responses to co-administered NP-chicken γ globulin, secondary to impaired generation of GC responses (105). Finally, infection with *Plasmodium chabaudi* (106) or foot-and-mouth disease virus (107) have each been shown to suppress OVA-specific IgG responses to soluble OVA. These suppressive effects were associated with an inhibition of DC maturation and a resultant decrease in T cell stimulatory capacity.

A specialized class of $CD4^+$ helper T cells, T follicular helper, has been described that initiate and maintain the GC reaction and regulate the maintenance, proliferation, and differentiation of GC B cells (120, 128, 129). GC B cells eventually give rise to long-term antibody-secreting plasma cells in the bone marrow and circulating memory B cells. Tfh cell development initiates rapidly during priming by DC and is subsequently maintained by cognate B cells by d 3-4 (130). The GL7 epitope defines a more differentiated subset of Tfh (referred to as GC Tfh) that localize to the GC and express higher levels of IL-4 and IL-21, key cytokines that mediate B cell help (122). ICOS, induced on DC-primed CD4+ T cells, was shown to be a critical early signal for Tfh differentiation via upregulation of Bcl6, which then stimulated the expression of the key Tfh marker, CXCR5. Of note, a bifurcation of Tfh versus effector T cells was observed as early as d 2 following a viral infection, with IL-2Rα^{int} cells expressing Bcl6 and CXCR5 (Tfh) and IL-2Rα^{hi} cells expressing Blimp1, that repressed Bcl6 (effector T helper). Complete polarization was observed by 72 h even in the absence of B cells. These data indicated

that the key signals initiating Tfh differentiation arise within the first 24h during CD4+ T cell-DC interaction (130). Thus, the R36A-mediated reduction in cOVA-induced GC Tfh observed on d 8, may result from a direct or indirect effect of R36A on these early DC-mediated events effecting early Tfh differentiation. This is consistent with our data demonstrating that R36A acts to mediate this inhibition only during the first 24h following cOVA immunization.

In addition to induction of Tfh differentiation, the generation of $Foxp3^+$ Treg is another event that can be controlled by DCs, early during an immune response (131), and thus could be a potential mechanism of R36A-mediated inhibition of IgG secretion in response to soluble antigens. Although Tregs are well-established negative regulators of cell-mediated immunity, their general role in humoral immunity is less clear, with few reports indicating an inhibitory effect on autoantibody production (117, 118). Certain microbial constituents that activate TLR2 on DC can induce Treg (131). In this regard, TLR2 plays a key role in immune responses elicited by *S. pneumoniae* (132). Indeed, *S. pneumoniae* infection has been reported to suppress allergic airway disease by inducing Treg cells (133, 134), although we observed no apparent role of endogenous Treg in regulating antibody production in response to immunization with this bacterium (119). Further, in the current study we observed that although cOVA immunization induced cOVA-specific Tg Treg, the presence of R36A had no additional effect.

IL-10 has emerged as a key inhibitor of immune responses to various pathogens, with a wide range of cellular sources, targets and effects (123). Of note, IL-10 inhibits inflammatory cytokines, such as IL-1, IL-2, tumor necrosis factor (TNF), interferon (IFN)-γ, and granulocyte-monocyte colony-stimulating factor (135). In the absence of IL-10, the pro-inflammatory

cytokines induced in response to R36A are enhanced (136). TLR2-dependent activation of the ERK signaling pathway by teichoic and lipoteichoic acids expressed by certain Lactobacillus strains (also present in R36A), favors the induction of IL-10 (anti-inflammatory) in responding macrophages, as opposed to IL-12 (pro-inflammatory) induction by certain other strains due to lack of potent TLR2 activation (137). Mast cell derived IL-10 has been reported to suppress GC formation by suppressing Tfh cell generation, due to a diminished ratio of BCL-6/ BLIMP-1 expression (127). In contrast, our group has shown previously that appropriate amounts of IL-10 are also required to sustain activated DCs in the circulation and delay the onset of their maturation-associated apoptosis (138). This might be critical in terms of the GC response, as sustained DC-T cell interactions are critical to promote optimal Tfh differentiation (139, 140). TGF-β is another major immunoregulatory cytokine, which has been reported to suppress T cells and macrophages and some chemokines, in order to control inflammation and curb autoimmune responses (126). It plays a major role in apoptosis, by creating an anti-inflammatory environment in association with IL-10 and prostaglandin (PG)E, in order to suppress any inflammation that would otherwise be caused by the dying cell (141). Of note, recognition of phosphatidylserine (which is structurally analogous to phosphorylcholine present on the surface of R36A) on apoptotic cells by receptors present on macrophages, induces the release of these anti-inflammatory cytokines (142). Despite the plausibility that IL-10 and/or TGF- β might account for R36A-mediate immunosuppression, our data using IL-10-/- mice and anti-TGF-β antibodies did not support such a role.

These data further support the notion that R36A-mediated suppression of the cOVA-specific IgG response was secondary to reduced Tfh differentiation and not enhanced generation

of Treg numbers. In summary, our data strongly suggest that *S. pneumoniae* expresses an immunosuppressive cell wall structure that mediates inhibition of T follicular helper and associated GC and plasma cell responses to co-immunized soluble antigens. The identity of this structure, the context in which it mediates suppression and its cellular targets remained to be determined.

CHAPTER

3

Streptococcus pneumoniae-induced immunosuppression is mediated by one or more choline-binding proteins

3.1 INTRODUCTION

Invading pathogens elicit a wide range of immune reactions in the host. The initial host defense is mediated by the innate immune system. This is accomplished mostly through host cell recognition of various molecules expressed on the surface of pathogens or released by them during infection, collectively termed microbe-associated molecular patterns (MAMPs). MAMPs are recognized by a family of pattern recognition receptors (PRRs) present on host cells, including TLRs, NLRs, RLRs, and scavenger receptors (143-145). Upon activation, PRRs stimulate the production of antimicrobial peptides (146), inflammatory mediators such as TNF-α, IL-6, 1 or 8, type I IFN and chemokines, which in turn results in innate host protective measures including recruitment of leukocytes and subsequent activation of an adaptive immune response (147).

In order to propagate and establish infection inside hosts, pathogens have either evolved means to evade the innate and adaptive response against them, or to cause its general or transient suppression. To avoid detrimental effects of PRR activation, they have evolved mechanisms to alter the downstream signaling that takes place after their activation (148), such as inducing production of immunosuppressive IL-10 instead of pro-inflammatory cytokines (149). They promote skewing of Th1 or Th17 responses, which are host-protective, to a more Th2 phenotype, which allows microbial persistence (150). They have molecules that suppress T cell activation and proliferation (151, 152) and superantigens that alter T cell responses (153). At times, pathogens mimic the host's immune modulators to alter the immune response (154).

We hypothesized that a certain moiety expressed on R36A, is adopting one of these mechanisms for its own success as a pathogen and in turn, is leading to the suppression of the immune response generated against a co-immunized non-related soluble protein antigen, and perhaps against soluble proteins secreted by *S. pneumoniae* itself.

As described in Chapter 2, R36A suppressed the Tfh, GC B and plasma cell response against a co-immunized soluble protein Ag (cOVA), leading to the suppression of the anti-cOVA IgG response. We also demonstrated that *S. pneumoniae* expresses an immunosuppressive cell wall structure that mediates this suppression, as a preparation of cell wall fragments of R36A inhibited the anti-cOVA IgG response. However, the identity of this cell wall structure was not determined. Another important finding from our previous study was that other GP and GN bacteria (i.e. GBS-III or MenC respectively) did not exhibit a suppressive effect similar to R36A. We however, could not determine the reason behind this difference in their behavior. In this study we observed that R36A expression of phosphorylcholine (PC), shown to be immunosuppressive when expressed by filarial pathogens, distinguished R36A from the GBS-III or MenC strains used in our earlier study. We demonstrate that although PC in R36A is not directly immunosuppressive, one or more of the CBPs that are anchored to R36A by PC, are responsible for the inhibitory property of R36A.

3.2 MATERIALS AND METHODS

Mice. BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Homozygous DO11.10 mice x RAG-$2^{-/-}$ mice (BALB/c background) [from here on referred to as "DO11.10 mice"], in which all CD4+ T cells express a transgenic T cell receptor that encodes for a chicken cOVA peptide (amino acids 323-339), presented by MHC-II^d, were purchased from Taconic Farms (Hudson, NY). For studies using NP-cOVA, BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred and maintained within the Biological Resource Center at National Jewish Health (NJH, Denver, CO). Mice were used between 7-12 weeks of age. These studies were conducted in accordance with the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and were approved by the Uniformed Services University of the Health Sciences and National Jewish Health Institutional Animal Care and Use Committees.

Reagents. cOVA ("Imject OVA") was purchased from Thermo Scientific (Rockford, IL). $(NP)_{19}$-OVA, $(NP)_{26}$-BSA and PC-BSA were obtained from Biosearch Technologies (Novato, CA). Alum (Allhydrogel 2%) was obtained from Brenntag Biosector (Denmark). Indomethacin was obtained from Sigma (St. Louis, MO). Platelet activating factor (PAF) antagonist, PCA 4282, was obtained from Santa Cruz Biotechnology (Dallas, TX). PC covalently linked to keyhole limpet hemocyanin (KLH), was obtained from Andrew Lees (Fina BioSolutions, Rockville, MD) (155).

Bacterial strains. The unencapsulated mutant of D39 (*S. pneumoniae*, capsular type 2), [strain R36A]) was obtained from Dr. David Briles (University of Alabama at Birmingham, Birmingham, AL). Bacteria from frozen stocks were subcultured on BBL blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Todd Hewitt Broth (BD Biosciences, San Jose, CA) to mid-log phase, collected, and heat-killed by incubation at 65°C for 2 h. Sterility was confirmed by subculture on blood agar plates. Bacteria were then aliquoted at 10^{10} CFU/ml in PBS and frozen at -20°C. The unencapsulated *Neisseria meningitidis* strain C311, was obtained from Dr. Michael Jennings (Griffith University, Southport, Queensland, Australia). *Haemophilus influenzae* type B strain Rd, was obtained from Dr. Jeff Weiser (University of Pennsylvania, PA). *Erysipelothrix rhusiopathiae* strain Fujisawa, was obtained from Yoshihiro Shimoji (National Institute of Animal Health, Tsukuba, Ibaraki, Japan).

Preparation of R36A lacking PC. PC-depleted R36A (R36A^{pc-}) was prepared by growing R36A in a chemically defined medium (CDM) (156) and slowly adapting it to a very low choline chloride concentration (0.000001%), by gradually replacing choline chloride in the medium with ethanolamine (157). As a control wild type (WT) R36A grown in CDM was also used for immunizations.

Depletion of CBPs from R36A and preparation of CBP-containing supernatant. R36A depleted of CBPs [R36A^{cbp-}] was prepared by treating R36A with 2% choline chloride (Sigma-Aldrich, St. Louis, MO) solution (158). Supernatant of choline chloride-treated R36A

(R36A^{cbp-} sup.) was concentrated and dialyzed using an Amicon Ultra-15 filter unit, 10000 MWCO (Millipore Corp., Bedford, MA) and used for immunizations.

Treatment of R36A with trypsin. For trypsin treatment of R36A [R36A (trypsin)], 10^7 CFU heat killed R36A cells were incubated for 15min at 37°C with 100µg of trypsin (Merck Millipore, Billerica, MA) followed by incubation with 100µg of trypsin inhibitor, AEBSF (Merck Millipore, Billerica, MA). Cells were then washed 3 times with PBS and used for immunizations (159).

Destruction of PC on R36A by treatment with periodate. Heat-killed R36A cells were washed with PBS followed by one wash in 100mM sodium acetate buffer, pH 5 (SAB). The pellet was then resuspended in 20mM sodium periodate in 100mM SAB at a concentration of 2ml buffer per $2x10^9$ CFU R36A. Cells were incubated in the dark at 4°C with mixing, for about 1h. A drop of glycerol was added to them and agitated to mix well. Cells were then washed at 4,000 rpm for 20 min. The pellet was resuspended in 15mM glycerol in PBS at a concentration of 2ml buffer per $2x10^9$ CFU R36A and incubated for 30min at 4°C to block the aldehyde groups generated by periodate oxidation. Cells were then washed twice with PBS and used for immunizations (160).

Sonication of R36A. Heat-killed R36A^{pc-} was sonicated in an ultrasonic bath (VWR International), to break the chains (formed while growth in CDM), at a concentration of 10^9 CFU/ml for 15 minutes (operating frequency 35kHz). For a control, WT R36A was also sonicated similarly. Disruption of chains of bacteria was confirmed by microscopy.

SDS PAGE. 2µl of R36A^{cbp-} supernatant (equivalent to 6x10^{7}CFU R36A) or 1x10^7 WT or trypsin treated R36A, was mixed with NuPAGE sample reducing agent (10x) and NuPage LDS sample buffer (4x) (Life Technologies, Carlsbad, CA) to final concentration of 1x in 25ul total sample volume. Samples were run on 4-12% Bis-Tris precast gel in NuPAGE MOPS SDS running buffer (Life technologies), at 195V for 50min. The gel was then subjected to staining with Denville Blue^{TM} protein stain (Denville Scientific Inc.).

Immunizations. Mice were immunized i.v. with 50 µg of cOVA or NP_{19}-OVA adsorbed on 13µg of alum, in the presence or absence of 2x10^8 CFU heat-killed bacteria in PBS, unless otherwise mentioned. All secondary immunizations were performed in a similar manner, but in the absence of bacteria. Serum samples for measurement of Ag-specific IgG titers, at different time points, were prepared from blood obtained through the tail vein. For adoptive transfer studies with DO11.10 mice, 2.5 x 10^6 spleen cells (containing ~ 5x10^5 Tg T cells) from DO11.10 mice were injected i.v. into WT BALB/c mice 24 h prior to immunization. For PAF receptor studies, BALB/c mice (7/ group) were administered 0.1 mg of indomethacin (dissolved in ethanol), i.v. 24 h prior to Ag immunization, followed by 0.1 mg indomethacin and 500nmol PAF antagonist (dissolved in dimethyl sulfoxide [DMSO]), i.v. 2h prior to Ag immunizations. As controls, ethanol at (-24)h followed by ethanol and DMSO at (-2)h was injected i.v. into BALB/c mice. Immunizations were performed with 50µg cOVA with or without 1x10^7 CFU heat-killed R36A.

Measurement of antigen-specific serum titers by ELISA. Immulon 4 ELISA plates (Dynex Technologies, Chantilly, VA) were coated overnight with 5 μg/ml of cOVA, gp350, or PspA, or 10 μg/ml of PC-KLH in PBS (50 μl/well) at 4ºC. The plates were then blocked with PBS + 1% BSA (100 μl/well) for 2 h at 37ºC. Three-fold serial dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 1% BSA (50 μl/well) were then added and incubated overnight at 4ºC followed by washing (3x) with PBS + 0.1% Tween-20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG Ab (200 ng/ml, 50 μl/well) in PBS plus 1% BSA was then added and plates were incubated at 37ºC for 1 h. Plates where then washed with PBS + 0.1% Tween-20 and substrate (*p*-nitrophenyl phosphate, disodium; Sigma-Aldrich) was added at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM $MgCl_2$ [pH 9.8]) for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland).

Enumeration of NP-specific ASC by ELISPOT. NP-specific ASC were measured in 96-well flat bottom EIA/RIA high-binding plates (Costar, Corning, Sigma-Aldrich) coated overnight at 4°C with 2 μg/ml NIP_{15}–BSA diluted in 0.05 M K_2HPO_4 (pH 8.0). Plates were washed 3x with PBS prior to blocking with warm PBS, 1% gelatin (Sigma-Aldrich) at 37°C for a minimum of 1 h. Plates were washed again 3x with PBS prior to incubation with cells. Single cell suspensions of splenocytes or bone marrow (harvested 2 wks post-immunization) were seeded in duplicate at 4-6 $\times$ 10^6 total viable cells per 100 μl in the first well, and 2-fold serial dilutions were carried out down the plate. Plates were incubated at 37°C in 5% CO_2 for 5-6 h in RPMI Medium (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated FBS (BioSource, Grand Island, NY), 2 mM GlutaMAX-I (Invitrogen), 100 U/ml Penicillin

(Invitrogen, Grand Island, NY), 100 μg/ml Streptomycin (Invitrogen), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). Following culture, cells were then lysed with H_20, 0.05% Tween 20 for 10 min at room temperature and subsequently washed 3x with PBS, 0.1% Tween 20. Secreted antibody was detected by incubating plates with an AP-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL) diluted in 1% gelatin in PBS for 1 h at 37°C. After 3 washes with PBS/Tween 20, plates were developed overnight at 4°C with 1 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate *p*-toluidine (BCIP; Sigma-Aldrich) salt substrate diluted in an alkaline buffer composed of 0.1 M 2-amino-2-methyl-1-propanol, 0.01% NaN_3, 0.5 mM $MgCl_2$, 0.007% Triton X-405, pH 10.25. Plates were washed 3x with deionized H_2O, allowed to dry in the dark at room temperature, and scanned (Epson Perfection 2450 Photo Scanner). Developed spots were counted visually from the scanned images and the frequency of NP-specific ASCs per total number of cells plated was calculated.

Flow cytometric analysis. Individual samples of RBC-lysed spleen cells from 3-5 mice/group were stained using the following mouse-specific mAbs: Alexa Fluor 405-anti-CD4 (clone RM4-5) and allophycocyanin-anti-DO11.10 TCR (clone KJ1-26) [Invitrogen]; PE-Texas Red-anti-B220 (clone RA3-6B2), and FITC-anti-T and B cell activation antigen (clone GL7) [BD Biosciences, San Jose, CA]; PE-Cy7-anti-PD1 (clone 29F.1A12) [Biolegend, San Diego, CA]. Cells were analyzed using a LSR-II flow cytometer (BD Biosciences) and results were generated using FlowJo (Tree Star, Ashland, OR) and FACSDiva (BD Biosciences) softwares.

For detection of PC or PspA on bacteria, 1 x 10^5 CFU heat-killed bacteria were incubated overnight at 4°C with 0.25 μg of mouse IgG2aκ anti-PC mAb (clone PCG2a2.A1) or IgG2a anti-PspA mouse IgG2aκ mAb (clone DC10-IA5),obtained from Dr. J. Kenny (Beth Israel Deaconess

Medical Center, Boston, MA) , in PBS plus 1% BSA. This was followed by washing with PBS (2x) and incubation with 0.5µg FITC-anti-mouse IgG2a (BD Biosciences, San Jose, CA) on ice for 30 min followed by 2 more washings with PBS. Bacteria were then analyzed by flow cytometry using a BD LSR-II flow cytometer. Bacteria incubated with only FITC-anti-mouse IgG2a were used as a negative control. For studies to detect NP^+ cells, the following mAbs were used: PE-Cy7-anti-B220 (clone RA3-6B2) and Allophycocyanin-Cy7-anti-IgD (clone 11.26c.2a) [BioLegend]; biotin-Igκ (clone 187.1, hybridoma), and PE-Cy5.5-CD11c (clone N418) [eBiosciences]. FITC-PNA (Vector Laboratories, Burlingame, CA) was used for detection of germinal center B cells and 647-OVA (Invitrogen) was used to exclude non-NP binding B cells. For detection of NP-specific B cells, cells were stained with PE-NP_{40} (Biosearch Technologies). The secondary reagent for detecting biotin-conjugated antibodies was Pacific Blue-streptavidin (Invitrogen). Flow cytometric analyses were performed by acquiring data on a Cyan analyzer (Dako, Denmark) and with FlowJo software (Tree Star).

Statistical analysis. Serum antigen-specific IgG titers were expressed as geometric means ± SEM of the individual serum titers. Significance was determined by two-tailed Student's *t* test. Values of $p \leq 0.05$ were considered statistically significant. Each experiment was performed at least twice to ascertain reproducibility.

3.3 RESULTS

Depletion of phosphorylcholine from R36A results in a significant reversal of its inhibitory effect on the cOVA-specific IgG response.

S. pneumoniae, including strain R36A, expresses high levels of PC on its cell wall teichoic acid and membrane lipoteichoic acid (161, 162). Studies on the PC-containing filarial nematode glycoprotein, ES-62, have demonstrated a role for PC in ES-62-mediated immunosuppression (163). Of note, our strain of GBS-III and MenC, neither of which inhibited the cOVA-specific IgG response (as shown in previous chapter), did not express detectable PC as determined by flow cytometry. Thus, we speculated that PC expression by R36A might account for the immunosuppressive activity of this bacterium. To determine this, we produced R36A that was markedly deficient in PC expression, by growing it in a chemically defined media with gradual replacement of choline chloride with ethanolamine. As illustrated in Fig.19A, this R36A (referred to as R36A$^{\mathbf{pc-}}$) exhibited essentially no detectable PC expression by flow cytometry using an anti-PC mAb, in contrast to high expression of PC by the WT R36A. Of note, co-immunization of BALB/c mice with cOVA/alum + R36A^{pc-} resulted in a cOVA-specific IgG response that was comparable to that observed in mice immunized with cOVA/alum alone, and was in distinct contrast to mice immunized with cOVA/alum + R36A (Fig. 19B, left panel), suggesting the involvement of PC in the R36A-mediated inhibitory effect. As expected, mice immunized with R36A^{pc-}, in contrast to R36A, failed to induce a detectable PC-specific IgG response (Fig. 19B, right panel).

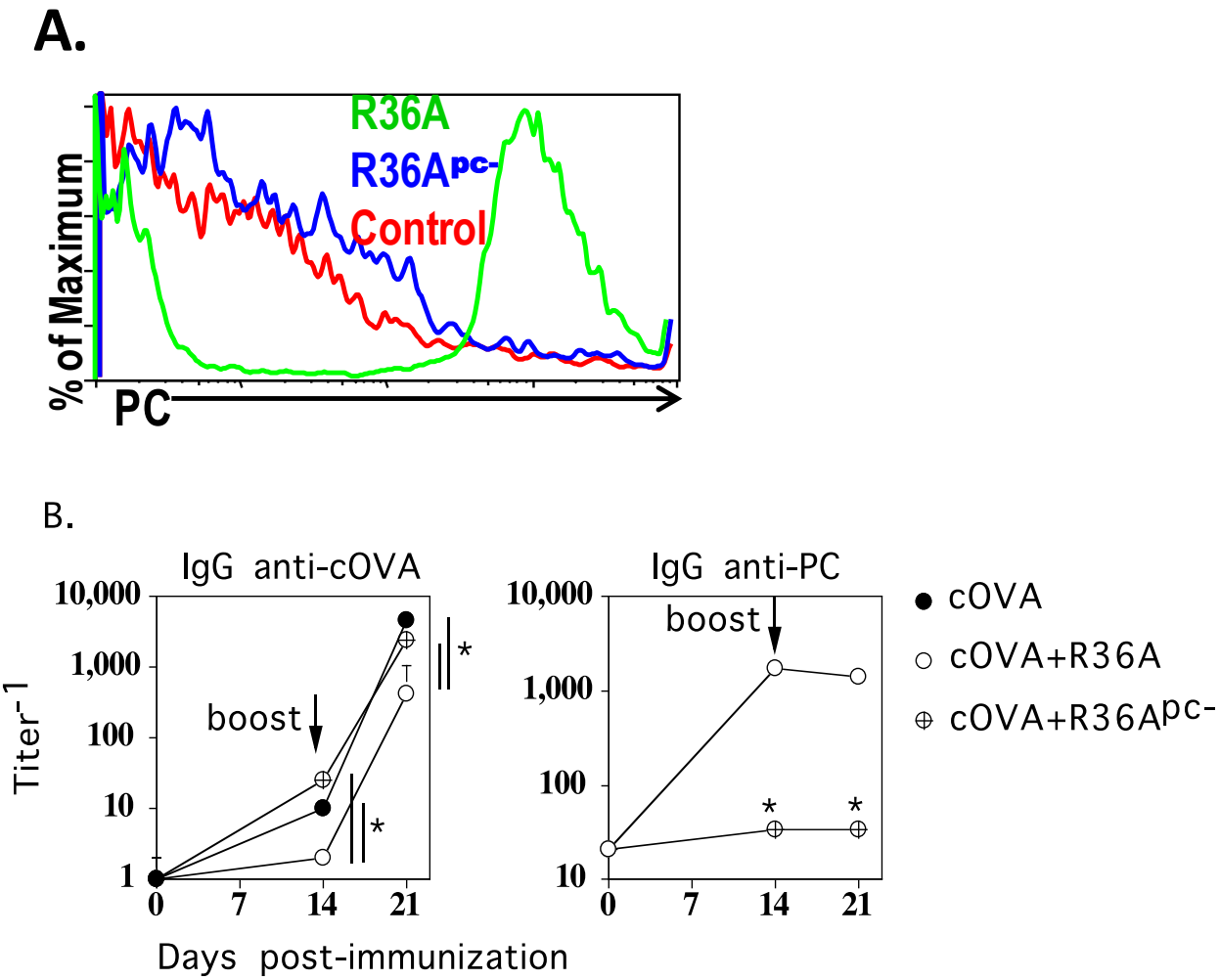

Fig.19. <u>Depletion of PC from R36A results in a significant reversal in its inhibitory effect on the cOVA-specific IgG response.</u> (**A)** PC expression by R36A or R36A^{pc-} by flow cytometry ("control", R36A^{pc-} stained with FITC-anti IgG2a only [no primary anti-PC Ab]). (**B)** BALB/c mice (7 per group) were immunized i.v. with 50 µg cOVA in alum with or without 2x10^8 CFU R36A or R36A^{pc-}. All mice were boosted i.v. with 50 µg cOVA + alum without bacteria, on d14. Serum titers of cOVA-specific and PC-specific IgG were measured by ELISA. Significance * $p \leq 0.05$

Consistent with the data illustrated in Fig. 19B (left panel), R36A^{pc-}, in contrast to R36A, failed to inhibit the generation of GC Tfh cells (Fig. 20A), GC B cells (Fig. 20B), or ASC in the spleen or bone marrow (Fig. 20C) upon co-immunization with cOVA/alum or NP-OVA/alum. Collectively, these data suggested a role for PC in the *S. pneumoniae*-mediated inhibition of IgG responses against cOVA.

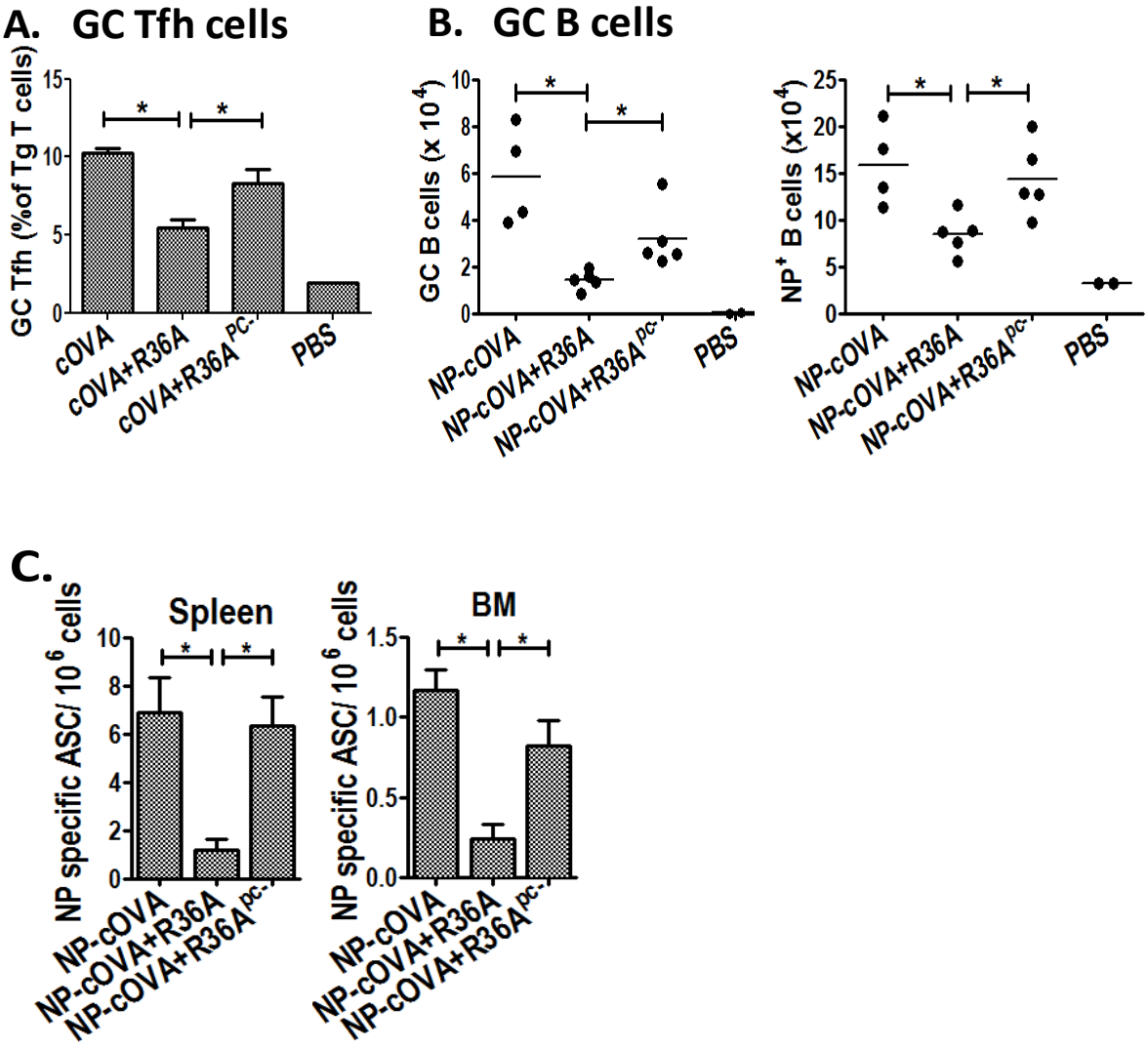

Fig.20. <u>R36A^{pc-} does not cause inhibition of GC response against cOVA.</u> (A) cOVA-specific GC Tfh cells were measured as before, from spleen cell suspensions on d 8 post-immunization with cOVA + alum with or without 2 x 10^8 CFU R36A or R36A^{pc-} (3-5 mice/group). **(B)** NP-specific GC B cells were measured as before, in spleen cell suspensions on d 14 post-immunization with NP-cOVA + alum with or without R36A or R36A^{pc-} (4-5 mice/group). **(C)** Quantitation of NP-specific ASC from spleen and BM in response to NP-cOVA + alum with or without R36A or R36A^{pc-} on d 14 post-immunization (4-5 mice/group). Significance * $p \leq 0.05$

The reversal in inhibition by R36A^{pc-} is not due to chain formation or growth in chemically defined media

Growing bacteria in a different media can alter their cell wall composition and thus their properties (164, 165). We grew R36A^{pc-} in chemically defined media to efficiently control individual component concentration (in this case PC), whereas the WT R36A used throughout the study was grown in THB. Thus, we grew WT R36A in CDM to determine whether it still inhibited the anti-cOVA IgG response. As shown in Fig. 21A, R36A grown in CDM inhibited the anti-cOVA IgG response similar to WT R36A grown in THB.

R36A^{pc-} grows in long chains due to a PC-dependent defect in the enzymatic cleavage of the daughter cells. To determine whether this difference in morphology causes the reversal in cOVA inhibition, we gently sonicated R36A^{pc-}, using an ultrasonic bath, so as to break the chains but not lyse the cells. As a control we also sonicated WT R36A. We verified the disruption of chains of R36A^{pc-} by gram staining the bacteria and viewing it microscopically (data not shown). Upon immunization, sonicated R36A inhibited the anti-cOVA IgG response, similar to WT R36A and sonicated R36A^{pc-} did not (similar to R36A^{pc-} in Fig. 19B), (Fig 21B). This confirms that chain formation by R36A^{pc-} is not the cause of the reversal of inhibition.

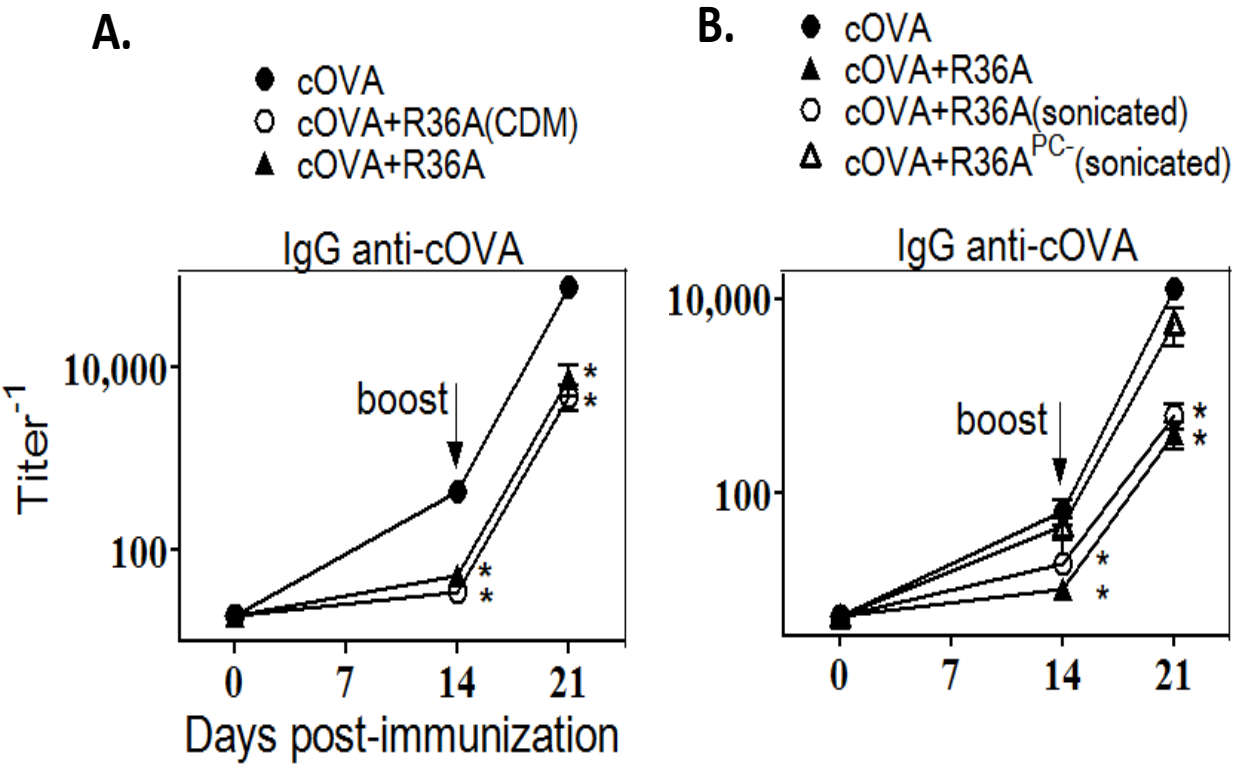

Fig. 21. <u>Different growth medium and chain formation, due to absence of PC, is not a cause of reversal of inhibition by R36A^{pc-}.</u> BALB/c mice (7 per group) were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU (**A)** WT R36A or CDM grown R36A, (**B)** WT R36A, sonicated R36A or sonicated R36A^{pc-}. All mice were boosted i.v. with 50 µg cOVA + alum without bacteria, on d 14. Serum titers of cOVA-specific IgG were measured by ELISA. Significance * $p \leq 0.05$.

Blocking PC interaction with the platelet activating factor receptor (PAFR) does not cause reversal of inhibition.

PC can interact with the PAFR present on various cells, and upregulate COX-2 and IL-10, both of which have immunosuppressive properties. To examine the role of this interaction of PC with PAFR, in R36A-mediated inhibition, we blocked the PAFR by administering the PAF antagonist, PCA4282 (166). We also blocked COX-2 by administering a COX-2 inhibitor, indomethacin (167). DMSO and ethanol, the solvents used to dissolve PCA 4282 antagonist and indomethacin respectively, were administered to the control mice. This was followed by immunization with cOVA with and without R36A. R36A inhibited the anti-cOVA IgG response in the indomethacin+PCA-treated group similar to the control group, suggesting that ligation to PAF receptor may not be the mechanism of R36A mediated immune suppression (Fig. 22).

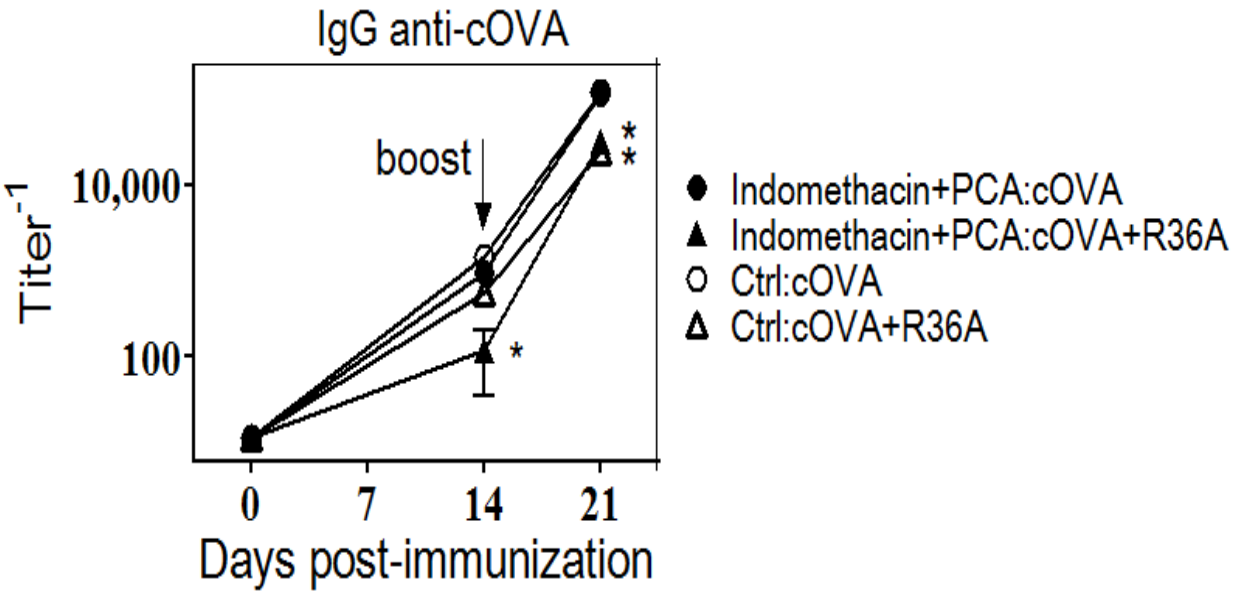

Fig. 22. <u>Binding of R36A with PAF receptor does not play a role in R36A mediated inhibition.</u> 0.1 mg Indomethacin was administered i.v. to BALB/c mice (7/group), at (-24) h followed by 0.1 mg Indomethacin and 500nmol PAF antagonist (PCA 4282) at (-2h). Ethanol at (-24) h followed by ethanol and DMSO at (-2) h was administered to the control groups. Mice were then immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A. All mice were boosted i.v. with 50 µg cOVA + alum without bacteria, on d 14. Serum titers of cOVA-specific IgG were measured by ELISA. Significance * $p \leq 0.05$.

Elimination of PC on R36A by periodate oxidation does not reverse the inhibition of the cOVA IgG response, nor are soluble PC-BSA or other PC-expressing bacteria inhibitory

Periodate oxidation of bacteria is known to destroy the exposed PC on the cell wall, without altering protein epitopes (168). Therefore, to determine whether PC is directly responsible for the R36A-mediated inhibition of the anti-cOVA IgG response, we treated R36A with periodate and confirmed the destruction of PC by flow cytometry (Fig. 23C). In contrast, staining of R36A with an anti-PspA mAb demonstrated that PspA was intact and presumably the other CBPs as well (data not shown). Upon co-immunization of cOVA with periodate-treated R36A, the inhibition of the anti-cOVA IgG response was comparable to that observed with WT R36A (Fig. 23B).

To further explore a potential, direct role for PC in mediating inhibition of the anti-cOVA IgG response, we determined whether a soluble conjugate of PC and bovine serum albumin (PC-BSA) was inhibitory. Thus, mice were immunized with cOVA and soluble PC-BSA (or NP-BSA as a control). As shown in Fig. 23A, PC-BSA did not inhibit the anti-cOVA IgG response.

We further evaluated if PC-expressing bacterial strains other than *S. pneumoniae*, also exhibit a suppressive property similar to R36A. We co-immunized mice with cOVA and *Neisseria meningitidis* (strain C311), *Haemophilus influenzae* (strain Rd), or *Erysipelothrix rhusiopathiae* (strain Fujisawa), which we demonstrated by flow cytometry to express PC, and determined whether the cOVA-specific IgG responses were inhibited (Fig 24A and B). None of these other bacteria exhibited an inhibitory effect on the cOVA-specific IgG response, suggesting that this suppressive property is unique to R36A and not mediated directly by PC.

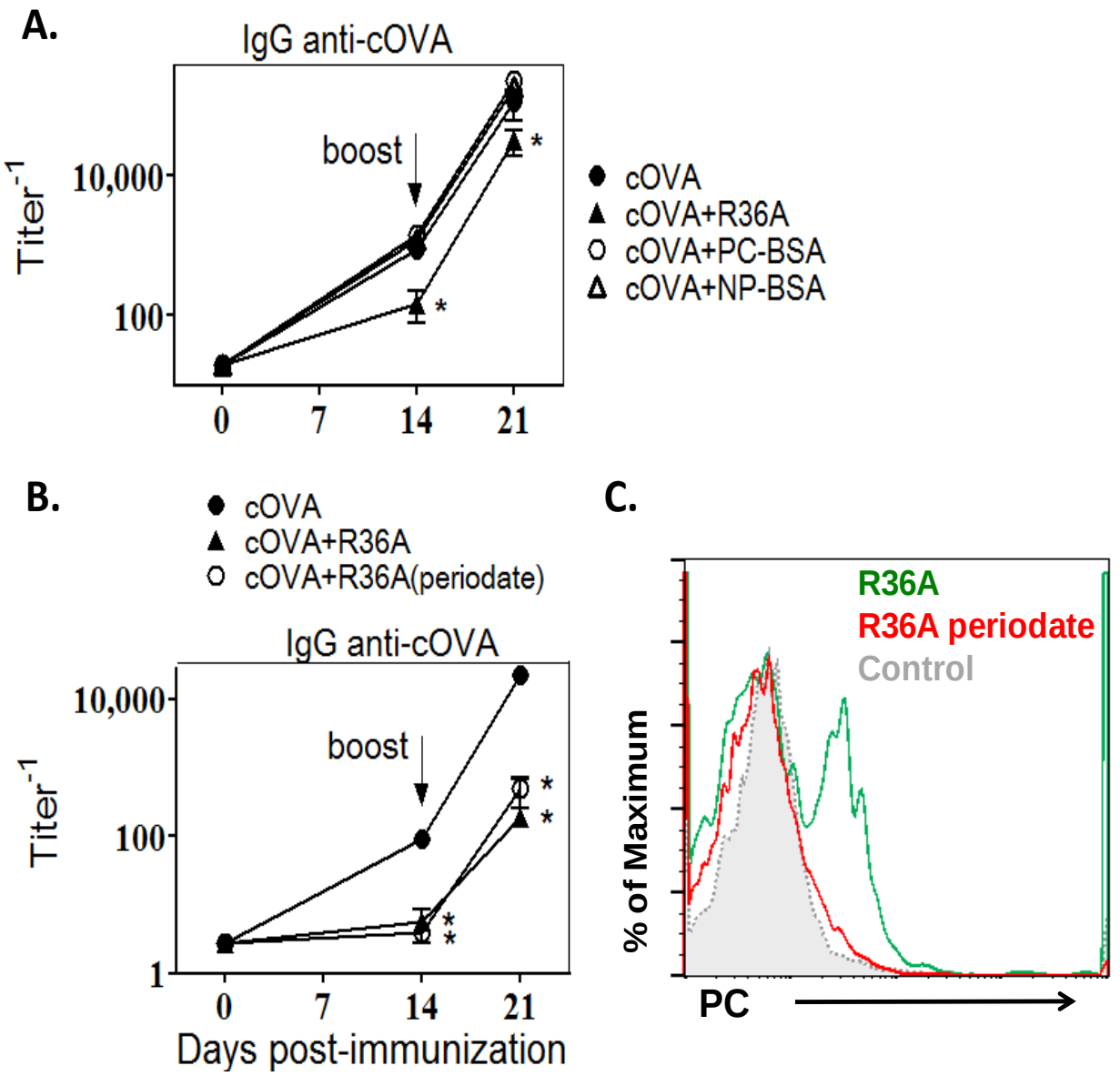

Fig. 23. **Periodate-treated R36A significantly inhibits the anti-cOVA IgG response whereas soluble PC-BSA does not.** BALB/c mice (7/group) were immunized i.v. with 50 µg cOVA in alum with or without **(A)** 50µg PB-BSA or NP-BSA (as control) or 2 x 10^8 CFU R36A, (**B)** 2 x 10^8 CFU WT R36A or periodate treated R36A. All mice were boosted i.v. with 50 µg cOVA + alum without bacteria, on d14. Serum titers of cOVA-specific IgG were measured by ELISA. **(C)** PC expression by R36A or R36A (periodate) by flow cytometry ("control", R36A stained with FITC-anti IgG2a only [no primary anti-PC Ab]). Significance * $p \leq 0.05$.

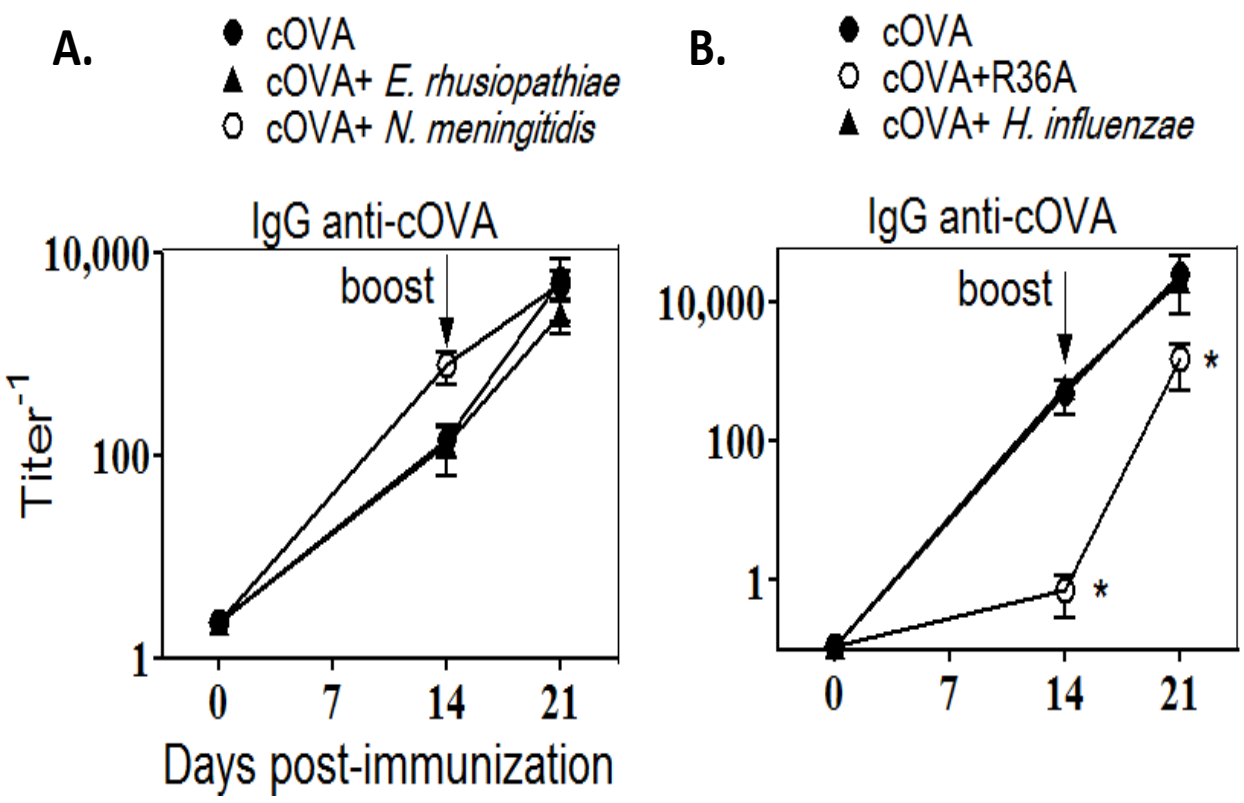

Fig. 24. <u>PC-containing bacteria, *E. rhusiopathiae*, *N. meningitidis* and *H. influenzae*, do not inhibit the anti-cOVA IgG response.</u> BALB/c mice (7 per group) were immunized i.v. with either 50 µg cOVA in alum alone or with **(A)** $4x10^8$ CFU *E. rhusiopathiae* or $2x10^8$ CFU *N. meningitidis* **(B)** $2x10^8$ CFU R36A or $5x10^8$ CFU *H. influenzae*. All mice were boosted i.v. with 50 µg cOVA + alum without bacteria, on d 14. Serum titers of cOVA-specific IgG were measured by ELISA. Significance *$p \leq 0.05$.

PC-binding proteins are responsible for the R36A-mediated inhibition of the IgG anti-cOVA response

PC anchors a number of pneumococcal proteins to the cell wall (referred to as choline-binding proteins) via a non-covalent interaction (169). In this regard, the reversal of the R36A-mediated inhibition of the cOVA-specific IgG response by $R36A^{pc-}$ only, and not by periodate-treated R36A, could be due to the loss of CBPs, and not directly due to the absence of PC. While growing $R36A^{pc-}$, the PC in the media is substituted with ethanolamine leading to absence of PC in the cell wall, which causes CBPs to fall off the cell surface. Whereas in periodate treated R36A, only the exposed PC residues on the cell wall are destroyed by periodate oxidation, with the CBPs remaining bound to the cell wall. To evaluate this, we treated R36A with choline chloride to detach CBPs from the cell wall through competitive binding. R36A depleted of CBPs is referred to as $R36A^{cbp-}$. Co-immunization of mice with cOVA/alum and $R36A^{cbp-}$ resulted in a reversal of inhibition of the cOVA-specific IgG response, comparable to that observed using $R36A^{pc-}$ (Fig. 25A). PC expression by $R36A^{cbp-}$ was confirmed by flow cytometry (Fig. 25B, left panel), and also by demonstrating induction of a PC-specific IgG response following $R36A^{cbp-}$ immunization, which was significantly higher than that observed for R36A, likely due to unmasking of PC, because of the loss of CBPs (Fig. 25C). Removal of CBPs was confirmed by flow cytometry (Fig. 25B, right panel) and by demonstrating a marked loss of the IgG response to one of the CBPs, PspA, in response to $R36A^{cbp-}$ relative to R36A (Fig. 25D). Thus, these data suggest that CBPs expressed on R36A, play a significant role in the R36A-mediated inhibition of the anti-protein immune response.

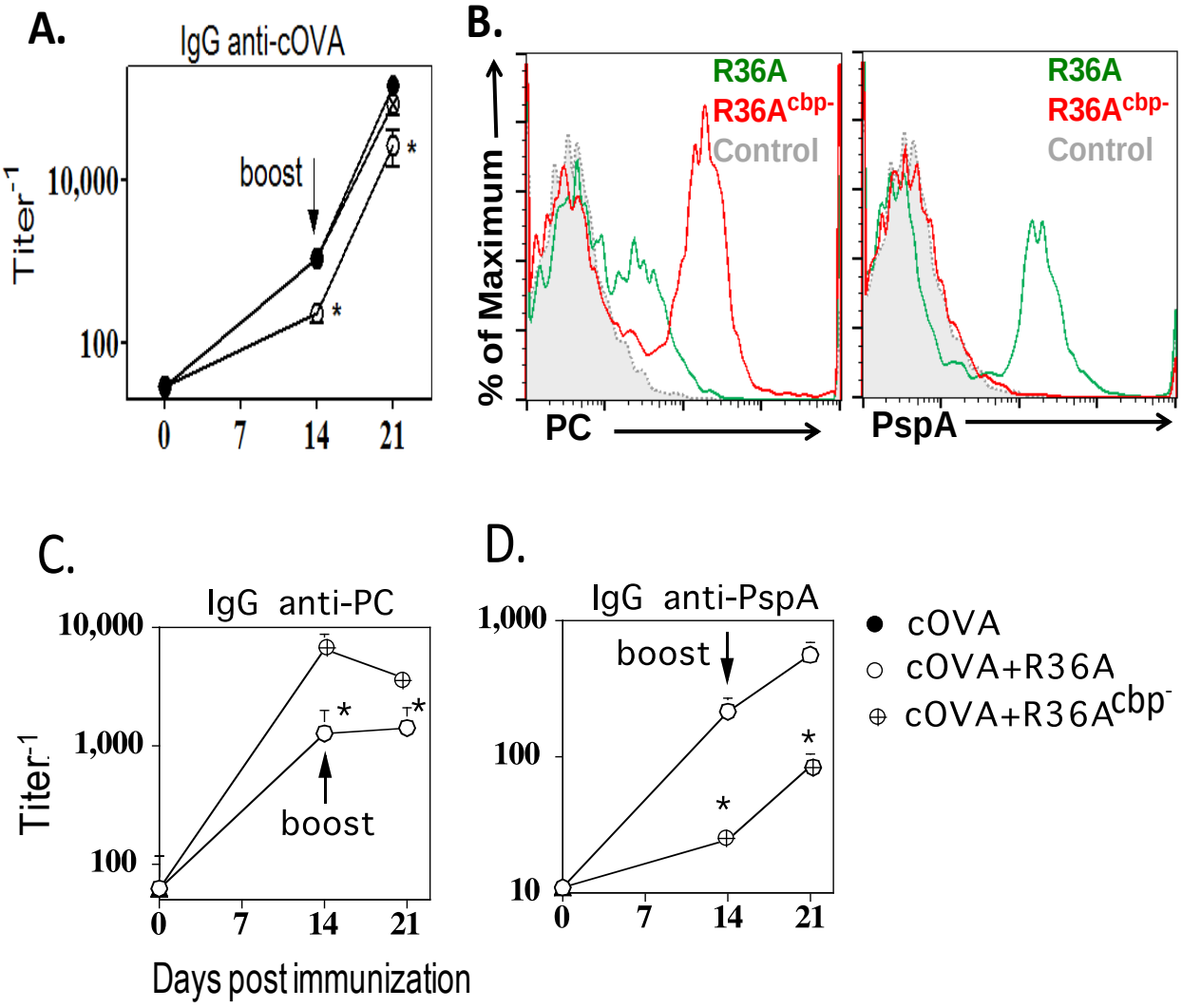

Fig. 25. <u>Depletion of CBPs from R36A results in significant reversal in the inhibitory effect on the cOVA-specific IgG response.</u> BALB/c mice (7 per group) were immunized i.v. with 50 µg cOVA in alum with or without 2 x 10^8 CFU R36A or R36A^{cbp-}. All mice were boosted i.v. with 50 µg cOVA in alum without bacteria, on d14. Serum titers of **(A)** cOVA-specific, **(C)** PC-specific and **(D)** PspA-specific IgG were measured by ELISA. **(B)** PC and PspA expression by R36A or R36A^{cbp-} by flow cytometry ("control", R36A stained with FITC-anti IgG2a only [no primary anti-PspA or anti-PC Ab]). Significance * $p<0.05$.

To confirm if CBPs are responsible for the suppressive property of R36A, we collected supernatant after choline chloride treatment of R36A (R36A^{cbp-}sup.), containing all the CBPs released from the R36A cell surface and evaluated if it can inhibit the anti-cOVA response. Upon co-immunization of mice with cOVA and R36A^{cbp-}sup, R36A^{cbp-}sup was able to significantly inhibit the IgG response against cOVA and it was comparable to inhibition exhibited by intact WT R36A (Fig. 26A, top panel). These data lend further support that the inhibitory property resides in the proteins that were released in the supernatant after choline chloride treatment. To further confirm that proteins are responsible for this inhibitory effect and not any other component released into the supernatant, we treated WT R36A with the protease trypsin and evaluated whether it still exhibits its immunosuppressive property. Trypsin-treated R36A [R36A(trypsin)] failed to inhibit the IgG anti-cOVA response (Fig. 26A, bottom panel). Trypsin treatment successfully digested the pneumococcal proteins, as most of the protein bands visible on SDS-PAGE gel using R36A are not present using R36A(trypsin). Further analysis on SDS-PAGE demonstrated a limited number of bands when using R36A^{cbp-}sup (i.e. CBPs) as compared to the number of bands present when analyzing WT R36A (Fig. 26B). Collectively, these data strongly suggest that CBPs are responsible for the R36A-mediated inhibition of the cOVA-specific IgG response. Whether this inhibitory property is shared among all CBPs, perhaps related to their conserved choline-binding domains, or is a unique property of one or several CBPs, remains to be determined.

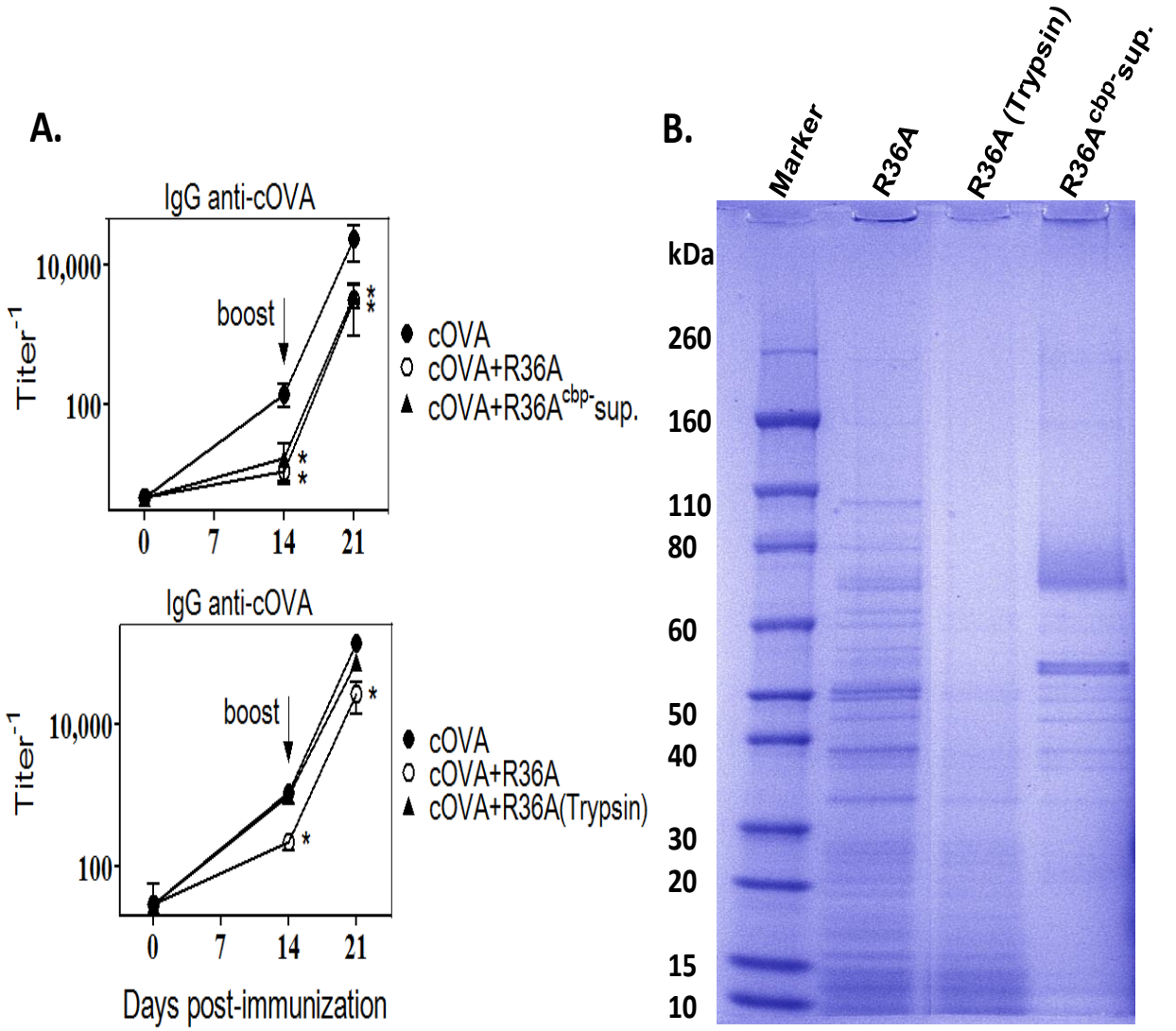

Fig. 26. **<u>Supernatant containing CBPs inhibit cOVA-specific IgG response whereas trypsinized R36A does not.</u>** BALB/c mice (7 per group) were immunized i.v. with 50 µg cOVA in alum with or without **(A, top panel)** $2x10^8$ CFU R36A or supernatant of choline chloride treated R36A ($R36A^{cbp-}$ sup.) equivalent to $2x10^8$ CFU R36A or **(A, bottom panel)** $2x10^8$ CFU R36A or trypsin treated R36A [R36A (trypsin)]. All mice were boosted i.v. with 50 µg cOVA in alum without bacteria, on d 14. Serum titers of cOVA-specific IgG were measured by ELISA. **(B)** Lysates from R36A cells, R36A (trypsin) and $R36A^{cbp-}$ sup., electrophoresed on SDS-PAGE. Significance * $p<0.05$.

3.4 DISCUSSION

In the previous chapter we discussed the ability of R36A, in contrast to our bacterial strains of GBS and MenC, to inhibit the IgG response to co-immunized cOVA, by suppressing the cOVA-specific Tfh, GC B and plasma cell response. In this study we investigated the mechanism by which R36A uniquely exhibited this inhibitory property. One notable difference between our GBS-III or MenC strains and R36A, was the expression of PC by R36A. In light of studies demonstrating a suppressive effect of PC expressed by filarial pathogens (170), we examined the role of PC in R36A-mediated inhibition. We demonstrate that PC-depleted R36A does not inhibit the anti-cOVA IgG response, and that this reversal in its inhibitory property is not due to a different growth medium (CDM) or chain formation. However, we demonstrate that PC is not playing a direct role in this inhibitory property of R36A, as selectively destroying PC residues on R36A, by their periodate-mediated oxidation, did not make R36A any less inhibitory. Also PC-BSA conjugate and other bacteria expressing PC (*E. rhusiopathiae* strain Fujisawa, *Neisseria meningitidis* strain C311 or *Haemophilus influenzae* strain Rd) did not inhibit the anti-cOVA IgG response. Of note, there was a near loss of inhibition when R36A was depleted of choline-binding proteins using choline chloride. This explains why PC-depleted R36A also exhibited a near loss of inhibition, as the CBPs attach to the pneumococcal cell surface via PC, in the absence of which they detach from the cell surface. In addition, the supernatant containing choline chloride-eluted CBPs, significantly inhibited the anti-cOVA IgG response and trypsin treatment of R36A rendered it non-inhibitory. These data strongly suggest that it is the expression of one or more CBPs that imparts R36A its inhibitory property.

Our investigation of a potential role for PC in mediating the R36A-induced inhibition of the cOVA-specific IgG response was based on earlier studies of PC expressed by filarial nematodes. Specifically, PC expressed on a secreted glycoprotein (ES-62), from the filarial nematode *Acanthocheilonmea viteae*, has been widely documented to have immunosuppressive properties. It has been shown to mediate anti-inflammatory effects that downregulate Th1-mediated autoimmunity and Th2-mediated hypersensitivity (163, 171). Various mechanisms have been suggested for such behavior of ES-62, some of which are, desensitization of mast cell degranulation and induction of IL-10 from B-1 B cells, induction of an anti-inflammatory phenotype in macrophages and DC, inhibition of TCR- and BCR-mediated activation of T and B cells and induction of regulatory T cells (163, 171). Of note, we show that neither IL-10, Treg induction, nor TGF−β appears to underlie the mechanism of R36A-mediated inhibition.

PC groups are covalently attached to teichoic acid chains associated with the pneumococcal cell wall peptidoglycan, and on lipoteichoic acid chains present within the cytoplasmic membrane (23, 172). *S. pneumoniae* has a nutritional requirement for PC, in the absence of which bacterial growth ceases (173). However, using a chemically defined media in which PC is substituted by ethanolamine, can allow for growth of R36A. The resulting PC-depleted R36A was not inhibitory. However, this substitution causes several cellular changes, including loss of autolysis in the stationary phase of growth (172), chain formation because daughter cells cannot separate at cell division, loss of competence needed for genetic transformation, and resistance to detergent-induced lysis (22). Some of these effects are indirectly related to loss of PC, since PC is critical for the non-covalent binding and cell wall retention of a number of CBPs (174). Two such proteins, the cell wall lytic enzymes LytA and

LytB are responsible for autolysis and separation of daughter cells respectively (23). In our studies, we utilized R36A, isolated from the exponential phase of growth, before the occurrence of autolysis that is typically observed in the stationary phase. R36A was then subsequently heat-killed, and thus no longer subject to autolysis. The absence of these lytic enzymes did result in chain formation, however, this did not affect the inhibitory properties of R36A^{pc-}, as the disruption of chains had no affect on inhibition. A difference in the media used to grow WT R36A (THB) and R36A^{pc-} (CDM) also appeared to play no role, in that WT R36A grown in CDM exhibited similar inhibition as that of THB-grown R36A. Thus these data, strongly support the notion that the loss of inhibition observed with R36A^{pc-} was not due to structural changes in R36A or changes related to the use of specific growth media.

PC is recognized by a G protein coupled cellular receptor, the platelet activating factor receptor (175). PAFR is expressed by a wide variety of cells and the binding of PAF or PC (a structural analog of PAF) to the receptor, leads to a wide range of effector responses, depending on the cell context (176). In regards to *S. pneumoniae*, PC expression allows bacteria to attach to and invade endothelial and epithelial cells (177). One of the consequences of ligand binding to the PAFR is the induction of prostaglandin E2 and IL-10 (166), both of which can mediate immunosuppression (123, 178). PGE_2 release is induced due to the upregulation of the COX-2 gene, upon activation of the PAFR (166). Thus, these interactions with PAFR might have been playing a role in R36A-mediated inhibition. However, blocking COX-2 and PAF receptor, did not affect the inhibitory property of R36A. It has been reported that PAF receptor shows some heterogeneity in different cell types (179), and perhaps the PAF antagonist used in our experiments blocks one or some of these but not others.

Pneumococcal CBPs bind to the PC residues on TA and LTA through a conserved choline binding motif present on their C-terminal. They have a variable N-terminal region that is responsible for their function (29, 30). Our demonstration of a loss of inhibition using CBP-depleted R36A, could either be due to some property of the choline binding domain of CBPs, in which case the inhibition would be due to all or any of the CBPs, or it could be specific to the specific protein function, in which case one or more specific CBPs would be responsible for the inhibitory effect. Most of the CBPs are not very well-characterized functionally, making speculation on their potential role in R36A-mediated inhibition difficult. There is a possibility that the ability of CbpA to bind to complement proteins may be of importance (180), as complement proteins have also been implicated in enhancing the humoral immune response to Ags (181).

The choline-binding motif was first described for pneumococci, but several other organisms have been found to express proteins with a similar motif, and possibly exhibit functional similarities to some of the pneumococcal CBPs (182). These include toxins A and B of *C. difficile* (183, 184), CspA of *Clostridium acetobutylicum* (185), glucan binding protein of *S. mutans* (186), FibA of *Peptostreptococcus micros* (187) and glycosyltransferases of both *S. mutans* (188-190) and *S. downei* (191). In pneumococcus, expression of these CBPs is phase variable, with CbpA and LytA being more abundant on mucosal surfaces, whereas PspA being expressed strongly in the blood-stream (29). This ability might enable bacteria to regulate the immunosuppression mediated by one or more of these CBPs. CBP expression on other bacteria and the ability to of *S. pneumoniae* to vary their expression in a phase dependent manner, suggest

the possible existence of a novel, more general mechanism for immunoregulation by extracellular bacteria. However, the inhibitory activity of R36A may be related to the function of a specific CBP, and its choline binding properties may be incidental. The mechanism by which these CBPs may be acting to regulate the early events of the immune response, leading to subsequent suppression of GC Tfh and GC B cells, as well as the identity of the specific CBP(s) involved, remains to be determined.

CHAPTER

4

Conclusions

4.1 CONCLUSIONS

Project#1: An immunosuppressive property within the cell wall of *Streptococcus pneumoniae* that inhibits the generation of a T follicular helper, germinal center, and plasma cell response to a co-immunized heterologous protein.

A previous study in our lab demonstrated that co-immunization of *Streptococcus pneumoniae* with a number of different antigens resulted in inhibition of the antigen-specific IgG responses (91). In this project we determined some of the key cellular events underlying this inhibitory effect. We used the unencapsulated *S. pneumoniae* strain R36A, and chicken ovalbumin as the experimental protein Ag. For most of the experiments immunizations were performed with cOVA with or without R36A co-immunized i.v. in alum.

We observed that the R36A-mediated inhibition of the anti-cOVA primary and secondary IgG response, depended on the strength of the adjuvant used, the inhibition being less with a stronger adjuvant like CPG-ODN + alum as compared to alum only. Also inhibition did not depend on the particulate nature of bacteria as the cell wall fraction from a lysed bacterial preparation, was also able to show inhibition. Upon i.v. immunization the inhibition takes place in the spleen. Upon s.c. immunization, inhibition resulted only when the cOVA and R36A were injected at the same site and not when they were injected at separate sites, indicating that it also can occur in the draining lymph node. The time frame when R36A acts to cause the inhibition is within the first 24 hours after co-immunization, as immunizing with R36A at earlier or later time points does not cause inhibition.

We used cOVA peptide-specific transgenic CD4+ T cells to determine whether the activity of these cells were negatively affected by the R36A co-immunization. R36A had no apparent effect on T cell activation in response to cOVA at day 1 or T cell proliferation by day 2.5, but had a modest effect on T cell proliferation by day 4. However this effect seemed unlikely to account for the drastic inhibition in the Ab response that we observed. However, there was a significant reduction in the Ag-specific GC Tfh and GC B cell numbers, as determined by flow cytometry and in Ag-specific antibody secreting cells (ASC) in both bone marrow and spleen, as determined by ELISPOT, suggesting an overall suppression in the GC reaction generated in response to the protein Ag, due at least in part to diminished GC Tfh activity.

In order to evaluate if this inhibitory effect is exhibited by other GP or GN bacteria, we measured the IgG anti-cOVA response upon co-immunization with GP *Streptococcus agalactiae* and GN *Neisseria meningitidis*. Neither of these bacteria were able to inhibit the anti-cOVA primary and secondary IgG responses or the GC reaction. Thus R36A may be relatively unique in exhibiting this immunosuppressive property.

Regulatory T cells have been known to mediate immune suppression or regulation in a wide range of physiological conditions under different kinds of stimuli (117, 118). In case of R36A mediated inhibition, they do not seem to play any role, as is evident by no change in the Ag specific Treg cell numbers in the presence of R36A, at day 10. Other major mediators of immunosuppression, the cytokines IL 10 and TGF-β (123, 124), also played no apparent role in R36A-mediated inhibition.

To conclude, a cell wall component of R36A inhibits an early event during the generation of an anti-protein immune response, leading to subsequent suppression of the GC reaction in

response to the protein, as indicated by the significant reduction in GC Tfh cell, GC B cell and ASC numbers.

Project #2: Pneumococcal choline-binding proteins contain an activity that mediates *Streptococcus pneumoniae*-induced immunosuppression.

One component that was different between the inhibitory R36A and non-inhibitory GBS and MenC strains that we tested was the expression of phosphorylcholine by R36A but not the latter two bacteria. Several published reports have demonstrated immunosuppressive properties of PC (192, 193). Based on these studies we explored the role of PC in R36A-mediated inhibition. Depletion of PC from R36A (R36A^{pc-}), resulted in reversal of R36A-mediated inhibition. Depletion of PC from R36A was confirmed by flow cytometry staining and by measuring the anti-PC IgG response to R36A^{pc-}, by ELISA. R36A^{pc-} also exhibited a reversal of the suppression in GC Tfh cell, GC B cell and ASC numbers, suggesting that PC was implicated, either directly or indirectly, in the suppression exhibited by R36A. However R36A^{pc-} has some additional differences in its composition and properties as compared to WT R36A, because of growth in a chemically defined media (CDM) in the absence of PC (which is a nutritional requirement for R36A), instead of growth in the complex media, THB. Any changes due to growth in CDM are not a cause of R36A^{pc-} being non-inhibitory, as WT R36A grown in CDM shows the same extent of inhibition as WT R36A grown in THB. Due to growth in absence of PC, the R36A^{pc-} daughter cells fail to separate after division, resulting in formation of long chains (23). This chain formation however, was also found not to have any role as sonicated R36A^{pc-} , with chains being disrupted, was still non-inhibitory.

PC is a PAF analog and can interact with PAFR expressed by a number of cells. This interaction can lead to immunosuppression by inducing COX-2 upregulation and secretion of cytokines like IL-10 (166). Mice injected with PAF antagonist and indomethacin (COX-2

inhibitor) to block this pathway, prior to the Ag immunizations, showed no difference in inhibition as compared to the control mice. Therefore this pathway is unlikely to be involved in the pneumococcal inhibition. Soluble PC-BSA was not inhibitory, whereas R36A oxidized with periodate to destroy its exposed PC groups, still inhibited the anti-cOVA IgG response. Other PC containing bacterial strains, *E.rhusiopathae, N.meningiditis* and *Haemophilus influenzae* also did not inhibit the anti-cOVA IgG response. Collectively, these studies argued strongly against a direct role of PC in R36A-mediated inhibition.

PC anchors a family of choline binding proteins on the pneumococcal cell wall (81), which are also absent in R36A$^{\mathbf{pc}-}$ along with PC. We treated bacteria with choline chloride to make R36A depleted only in CBPs (R36A^{cbp-}), to examine the role of CBPs in the inhibition. Depletion of CBPs was confirmed using flow cytometry and by measuring the anti-PspA response to R36A^{cbp-}, by ELISA. R36A^{cbp-} did not inhibit the anti-cOVA IgG response, similar to R36A$^{\mathbf{pc}-}$. The supernatant obtained from choline chloride treatment of R36A, containing all the eluted CBPs inhibited the anti-cOVA IgG response. R36A lost its inhibitory activity upon trypsin treatment, confirming that the inhibitory component is a protein. Collectively, these data strongly suggest a novel immunosuppressive property of pneumococci mediated by one or more CBPs, enabling *S. pneumoniae* to inhibit the humoral immune response against a co-immunized soluble protein Ag. The potential consequences of this inhibitory property on the host during pneumococcal colonization and infection remains to be determined, as well as the identity of the inhibitory CBP(s) and its mechanism of action.

4.2 LIMITATIONS OF WORK

- In this study we have demonstrated that R36A acts early to suppress the immune response against a protein Ag, but the nature of these early events and the target cells underlying this suppressive effect, as well as how this leads to downstream inhibition of the GC response are still unknown.

- Whether R36A suppresses the generation/differentiation of Tfh cells or it suppresses differentiated Tfh cell numbers is not clear.

- This study demonstrates a role for CBPs in R36A-mediated inhibition but the mechanism of action leading to inhibition has not been determined.

- Whether all CBPs exhibit the described immunosuppresive activity, perhaps through their shared choline-binding domains or whether inhibition is due to a single or several specific CBPs has not been determined.

- The potential consequences of this inhibitory activity on the host during pneumococcal colonization and infection, remains to be determined.

4.3 SPECIFIC CONTRIBUTIONS OF THE RESEARCH

- In our work we describe a novel immunosuppressive property that appears potentially unique to *Streptococcus pneumoniae*.

- The common mechanisms of pathogenic immunosuppression, such as suppression of T-cell activation and proliferation or induction of regulatory T cells and IL-10, were not the means of pneumococcal suppression.

- We describe that *S. pneumoniae* suppresses the germinal center reaction generated against co-immunized protein antigens.

- In this study a phosphorylcholine-dependent immunosuppressive property has been described for bacteria.

- In this work we determine that a hitherto unknown property of one or more CBPs can mediate inhibition of a humoral immune response either when expressed by the intact bacterium or in a soluble isolated form.

4.4 FUTURE PERSPECTIVES

Our work demonstrates that *S. pneumoniae* inhibits the humoral immune response to a co-immunized protein antigen by suppression of T follicular helper, germinal center B and antibody secreting cells and that this inhibition is mediated by one or more choline-binding proteins present on the pneumococcal cell wall. This leads to a new set of questions that require further investigation.

- What are the cells involved in R36A mediated inhibition of the anti-cOVA IgG immune response?

- How does R36A suppress T follicular helper cell numbers and germinal center reaction?

- What is the identity of the CBP(s) involved in inhibition, and what is the mechanism of action.

- Are there other bacteria that exhibit immunosuppression using the same mechanism as *S. pneumoniae*?

- Does the expression of this CBP(s) during pneumococcal colonization and/or infection affect the host immune response to pneumococcus itself and/or to other, heterologous antigens.

Some of these questions are currently under investigation. Another interesting aspect would be to verify whether the inhibition in lymph node (upon subcutaneous immunizations) involves the same mechanism as in spleen.

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LIST OF PUBLICATIONS

Journal publications

1) **Saumyaa,** Swadhinya Arjunaraja, Lindsey Pujanauski, Jesus Colino, Raul M. Torres and Clifford M. Snapper. 2013. Immunosuppressive Property within the Streptococcus pneumoniae Cell Wall That Inhibits Generation of T Follicular Helper, Germinal Center, and Plasma Cell Response to a Coimmunized Heterologous Protein. *Infection and Immunity.* 81(9): 3426-3433.

2) **Saumyaa,** Jesus Colino and Clifford M Snapper. Pneumococcal choline-binding proteins contain an activity that mediates *Streptococcus pneumoniae*-induced immunosuppression. *Manuscript in preparation.*

Abstract publications

1) **Saumyaa**, Swadhinya Arjunaraja and Clifford M Snapper**.** The regulation of T cell dependent IgG response to soluble protein antigens in the presence and absence of intact bacteria. *Research Week 2011* at Uniformed Services University of the Health Sciences, Bethesda, MD, USA.

2) **Saumyaa**, Swadhinya Arjunaraja, Raul M. Torres and Clifford M Snapper. Regulation of T cell dependent IgG responses to soluble protein antigens in the presence and absence of intact bacteria. *American Association of Immunologists Meeting 2012* at Boston, MA, USA.

3) **Saumyaa**, Swadhinya Arjunaraja, Raul M Torres and Clifford M Snapper. *Streptococcus pneumoniae* selectively inhibits the humoral immune response to a soluble protein through a phosphorylcholine dependent mechanism. *Research week 2012* at Uniformed Services University of the Health Sciences, Bethesda, MD, USA

4) **Saumyaa**, Jesus Colino and Clifford M Snapper. Pneumococcal choline-binding proteins contain an activity that mediates *Streptococcus pneumoniae*-induced immunosuppression. *American Association of Immunologists Meeting 2014* at Boston, MA, USA

BRIEF BIOGRAPHY OF THE CANDIDATE

Saumyaa obtained her Bachelor of Technology degree in Biotechnology from Ambala College of Engineering and Applied Research, Ambala, Haryana, India. As part of her Bachelor's degree she has done summer internships at Centre for Biotechnology, Jawaharlal Nehru University, Delhi, India; Institute of Genomics and Integrative Biology, New Delhi, India and Department of Zoology, Molecular Biology Division, Delhi University, Delhi, India. She did her Master of Engineering, in Biotechnology from Birla Institute of Technology and Science (BITS), Pilani, Rajasthan, India. As a part of her Master's degree she completed a six month dissertation titled 'Biochemical analysis of oxidative stress in complex diseases', at BITS, Pilani. Subsequently she was selected for a vertical transfer to PhD at BITS, Pilani under a collaborative program at Uniformed Services University of Health Sciences, Bethesda, MD, USA. Since 2008 she has been involved in studying the regulation of immune response to heterologous protein antigens, by *Streptococcus pneumoniae.* She has presented her research work at conferences over the years and has published her findings in a reputed Journal in the field.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Clifford M. Snapper obtained his M.D. from Albany Medical College, Albany, NY, USA, did his residency training in Pathology at Cornell-New York Hospital, NY, NY, USA, and his scientific training in the field of immunology as a Medical Staff Fellow at the National Institutes of Health, Bethesda, MD, USA. He has been doing research in the field of Immunology for the past 25 years. Currently, he serves as a Professor in the Department of Pathology, and Professor, Molecular and Cell Biology and Emerging Infectious Diseases graduate programs at USUHS, Bethesda, USA. He is also Director, Institute of Vaccine Research at USUHS that is tasked to conduct basic and translational immunologic studies that have relevance for the rational design of new or improved vaccines against infectious agents. He has received a number of awards including the Burroughs Wellcome Developing Investigator Award in Immunopharmacology of Allergic Diseases, the Jeffrey Modell Foundation Lifetime Achievement Award, Outstanding Biomedical Graduate Educator Award (USUHS) and the Henry Wu Award (USUHS) for his excellence in basic research. He served as both an Associate and Section Editor for "The Journal of Immunology" and as an Associate Editor for "Infection and Immunity". He has published 94 original research articles in peer-reviewed journals including Science, The Journal of Experimental Medicine, The Journal of Immunology, Infection and Immunity, and The European Journal of Immunology, and has written 23 invited reviews and chapters. He is actively engaged in teaching Pathology to USUHS medical students, teaching Immunology to, and serving as a Ph.D. advisor for graduate students in the BITS-USUHS collaborative program, and USUHS Molecular Cell Biology, and Emerging Infectious Diseases programs.