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Isotype-specific outcomes in Fc gamma receptor targeting of PspA using fusion proteins as a vaccination strategy against Streptococcus pneumoniae infection

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Isotype-specific outcomes in Fc gamma receptor targeting of PspA using fusion proteins as a vaccination strategy against Streptococcus pneumoniae infection

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AM	Alveolar Macrophage
APC	Antigen presenting cell(s)
BSA	Bovine serum albumin
CBP	Choline-binding proteins
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CTB	Cholera toxin B
DC	Dendritic Cell(s)
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcγR	Fc gamma receptor(s)
HIV	Human immunodeficiency virus
IFNγ	Interferon gamma
iFt	Inactivated *Francisella tularensis*
IL	Interleukin
i.n.	Intranasal
ITAM	Immunoreceptor tyrosine-based activation motif(s)
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KO	Knockout
MFI	Mean fluorescent intensity
Mg	Milligram
MHCII	Major histocompatibility complex class II
Ml	Milliliter
PBS	Phosphate buffered saline
PCV	Pneumococcal conjugate vaccine
PiaA	Pneumococcal iron acquisition
PiuA	Pneumococcal iron uptake
Ply	Pneumolysin
PPV	Pneumococcal polysaccharide vaccine
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
SNP	Single nucleotide polymorphism
Spn	*Streptococcus pneumoniae*
StkP	Serine/threonine-protein kinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
µl	Microliter
WT	Wildtype

Abstract

Streptococcus pneumoniae (Spn) remains a considerable threat to public health despite the availability of antibiotics and polysaccharide conjugate vaccines. The lack of mucosal immunity in addition to capsular polysaccharide diversity, has proved to be problematic in developing a universal vaccine against Spn. Targeting antigen to Fc receptors is an attractive way to augment both innate and adaptive immunity against mucosal pathogens, by promoting interactions with activating Fcγ receptors (FcγR) that mediate diverse immunomodulatory functions. The effect of targeting FcγR is highly influenced by the IgG subclass, which bares differential affinities for activating and inhibitory FcγR. In the current study we demonstrate targeting activating FcγR with fusion proteins consisting of PspA and IgG2a Fc enhance PspA-specific immune responses, and effectively protect against mucosal Spn challenge. Specifically, targeting PspA to FcγR polarized alveolar macrophage to the AM1 phenotype and increased conventional dendritic cell subsets in the lung in addition to augmenting Th1 cytokines and PspA-specific IgG and IgA. In contrast, fusion proteins consisting of PspA fused to the IgG1 Fc provided minimal benefit over administration of PspA alone, as a result of interaction with the inhibitory FcγRIIB. Protective efficacy of the IgG1 fusion protein was significantly enhanced in animals deficient for FcγRIIB accompanied by increased B cell maturation and proliferation levels in these animals. These studies demonstrate FcγR targeting is an effective strategy for inducing potent cellular and humoral responses via mucosal immunization with Fc fusion proteins, however, careful consideration of the Fc region utilized is required since Fc isotype subclass heavily influenced immunization induced effector functions and survival against lethal Spn challenge. Fc-engineering with specific attention to FcγRIIB engagement presents a valuable vaccine strategy for protecting against Spn infection.

I. Introduction

A. Streptococcus Pneumoniae

1. Transmission and Colonization

*Streptococcus Pneumoniae (*Spn) is a gram-positive, extracellular bacterial pathogen responsible for more deaths globally than any other bacterial pathogen (1). Spn is associated with a wide range of diseases, from mild respiratory tract infections such as otitis media and sinusitis, to more severe occurrences such as community-acquired pneumonia, sepsis, and meningitis. Infection is preceded by asymptomatic colonization of the upper respiratory tract with carriage rates of up to 65% in children and 10% in adults (2,3). Once acquired, an individual can carry the colonizing strain for weeks to months before it is effectively cleared from the upper respiratory tract by innate immune mechanisms. Infection is likely spread from person to person by transmission of aerosols, or direct contact with contaminated secretions. Horizontal spread between hosts enables the bacteria to enter the nasal passage and colonize the nasopharyngeal tract. Spn can then penetrate sterile tissues become invasive disease through virulence driven mechanisms or host immune susceptibility.

Colonization establishes a carrier state which can last for weeks to months before clearance. Carriage induces the production of mucosal and systemic antibody but the extent to which carriage is an immunizing event remains unclear. Whole genome sequencing of carriage isolates found genomic variation, such as prophage sequences and SNP heritability, to be more reflective of carriage duration then individual host traits, such as age or previous Spn colonization (4). The influence of genetic variants on colonization may not be surprising, since serotype is known to be strongly associated with carriage duration. Serotype is determined by the capsular-polysaccharide composition on the surface of the microbe, which is important in

determining the bacterial physiology, strain- specific virulence traits, and host immune responses. Over 90 different serotypes have been identified based on the unique characteristics of the polysaccharide capsule, but only a small subset are involved in carriage and invasive disease (5).

2. Virulence Factors

The propensity for varying strains to cause invasive disease is partly attributed to their ability to produce virulence factors. Among the many pneumococcal virulence factors which have been identified the capsule, pneumolysin toxin, and choline-binding proteins have received the considerable attention due to their ability to enhance pathogenesis, and their potential as promising antigen candidates for vaccine development. The capsule is one of the first virulence factors to come into contact with the host and promote effective colonization. The presence of the capsule prevents entrapment and mechanical removal by mucus. The capsule also promotes virulence through effective antiphagocytic activity that prevents opsonophagocytosis. Specifically, the capsule prevents the iC3b from activating the classical complement cascade and can interfere with attached immunoglobulin binding to cognate receptors on the surface of white blood cells (6).

Pneumolysin belongs to a family of pore forming toxins which are produced by virtually all pneumococcal isolates. Pneumolysin is produced as a 52kDa protein that binds to membrane cholesterol to form a ring shaped pore that is composed of up to 50 toxin monomer subunits (7). The membrane inserted oligomer has cytolytic and cell modulatory capabilities such as inhibition of phagocyte respiratory burst, inhibition of ciliary movement on respiratory epithelium and brain ependymal; in addition to activation of a proinflammatory responses associated with

infection (8). Pneumolysin has demonstrated a key role to survival of Spn in the upper and lower respiratory tract in mice, and is likely required for bacterial expansion from the lungs to bloodstream (9).

Choline-binding proteins (CBP) are anchored to the cell surface through a bacterial adhesion molecule phosphorylcholine which is present in the pneumococcal cell wall. Spn expresses 10-15 CBPs which vary by strain (10). Pneumococcal surface protein A (PspA) is one of the most widely studied CBPs as an important virulence factor and potential vaccine antigen. PspA helps the bacterium to evade the immune system by inhibiting C3 complement deposition on the Spn surface, and interfering with innate lactoferrin mediated killing of the invading bacteria (11). PspA knockout mutants have reduced virulence and are vulnerable to bactericidal activity of apolactoferrin demonstrating the importance of this cell surface protein to promoting Spn infection (12).

B. Vaccine Approaches

1. Pneumococcal Polysaccharide and Conjugate Vaccines

Two vaccine approaches are currently available to protect against Spn infection, the pneumococcal polysaccharide vaccine (PPV) and pneumococcal conjugate vaccine (PCV). Both strategies utilize the immunologically and structurally distinct capsular polysaccharide to elicit a protective response, but the effectiveness varies between the two approaches due to the immunogenicity of the polysaccharide antigen. For decades, the 23-valent PPV (PPSV23) consisting of only polysaccharides was the standard of care for invasive disease in all patients. Today PPSV23 is still routinely used in adults, and coadministered with PCV's in children with underlying medical conditions. Although PPSV23 has reduced the rate of invasive disease in

adults, overall carriage rates have remained unaffected (13). Furthermore, PPSV23 is poorly immunogenic in young children and HIV- infected individuals due to the antibody response generated (14).

When polysaccharides are administered alone they induce a type 2 T-cell independent (TI-2) response that fails to induce immunoglobulin class switching, affinity maturation, and memory B-cell responses (15). While T-lymphocyte engagement is not required for antibody production against a TI-2 antigen, it can influence the magnitude of the response in high risk groups such as children, elderly, and immunodeficient populations (16). To combat this weak immunogenicity, PCV's were first introduced in the United States in 2000 with the licensing of a 7-valent pneumococcal polysaccharide-protein conjugate (Prevnar), which was later expanded to cover 10 (Synflorix), and then 13 (Prevnar13) serotypes (Table 1).

By covalently conjugating the polysaccharide antigen to an immunogenic protein carrier, PCV strategies elicit a T-cell dependent response capable of producing more effective immunologic priming and memory, then polysaccharides administered alone. The current PCV has been highly effective at reducing invasive pneumococcal disease and carriage of vaccine covered serotypes in children, which also confers heard immunity to unimmunized populations of all ages (17) . Unfortunately, vaccine coverage is limited, with only a small number of serotypes included in current vaccine formations.

Table 1. Serotype coverage across approved polysaccharide and conjugate vaccines

Vaccine	Regulatory Approval	Serotypes Contained in Vaccine
PPSV23	1983	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F
PCV7	2000	4, 6B, 9V, 14, 18C, 19F, and 23F
PCV10	2009	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F
PCV13	2010	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F

Table 1. Vaccines available for the prevention of *S. pneumoniae* infection and the respective serotypes covered by each formulation (17,18).

2. Limitations to Current Vaccine Strategies

The limited coverage offered by the current polysaccharide-based vaccine strategies is a major concern to public health. Spn serotypes responsible for invasive disease can fluctuate based on the host's age and geographic origin. Furthermore, the serotypes covered in the current vaccines were chosen based on prevalence in the United States, resulting in vaccine coverage that is less relevant to other geographic areas with differing serotype distributions. For example, the serotypes included in PCV7 account for only 39% of disease causing serotypes in Africa, and 48% of the invasive serotypes in Asia (18).

The ability of Spn to naturally take up DNA from other bacteria is also problematic, as this allows for capsular switch events that can replace the serotypes causing invasive disease in a given population, and circumvent the protection conferred from serotype specific vaccination. Additionally, selective pressure from the widespread use of PCV has allowed for increases of non-vaccine type strains in carriage and disease (19). Only 5 years after the introduction of PCV7 a rise in non-vaccine strains 19A and 6A were observed, accompanied by increases of

antibiotic resistance in serotypes not covered by the vaccine (20,21). Similarly, following the introduction of PCV13 a rise in infections from serotype 35B has been observed, suggesting infections will continue to increase from non-vaccine type strains of PCV13 (22). Thus, the long-term usage of PCV strategies may only alter invasive serotype distribution instead of reducing the cumulative burden of disease.

Increasing vaccine valency may help combat emerging invasive strains and geographic variability. However, the possibility of increasing serotype coverage by PCV is limited by financial and technical challenges. Maintaining immunogenicity when including additional serotypes could be complicated, a weakening of immunogenicity has been reported for some common serotypes in children vaccinated with PCV13 compared to those vaccinated with PCV7 (23). Increasing serotype coverage, or introducing regional specific vaccines, could help curb Spn infections in healthy populations, but would fail to address the immunogenicity limitations observed in HIV positive individuals.

People infected with HIV have a 40 fold greater burden of pneumococcal disease compared to healthy individuals (24). HIV- infection disrupts humoral immunity through reduced T-cell engagement, and alterations in quantity and proportion of circulating B-cells, resulting in a diminished response to immunization (25). This likely explains why vaccination with PPSV23 in HIV-infected populations has shown no efficacy in reducing infection by Spn (26). In contrast, while the PCV strategies have been shown to be effective in high risk populations, the immunogenicity levels, duration of protection, and overall efficacy are still lower when compared to healthy controls (27). As a result, current Advisory Committee of Immunization Practice guidelines recommend a vaccination series with PCV13 followed by PPSV23 to help generate higher antibody titers (28).

While the innate immune response is often sufficient to combat most mucosal pathogens the ability of Spn to evade and neutralize these defenses requires the generation of a strong adaptive response, with contributions from both humoral and cellular immunity, to protect against an invasive mucosal pathogen. It is widely believed that intranasal (i.n.) immunization may be the optimal way to effectively induce protection across all recipients, address colonization, and stimulate a long-lasting mucosal and systemic response. Despite the importance of the mucosal niche in the development of invasive disease, current vaccines are administered parenterally via intramuscular injections and are only partially effective at inducing mucosal immunity (29). Intranasal immunization is an attractive approach to prevent colonization and invasive disease in a targeted minimally invasive manner.

3. Common Protein Vaccine

One strategy to combat insufficient coverage, and serotype replacement observed with polysaccharide vaccines, is to find a conserved protein-based antigen that could offer cross-protection against the diverse pool of pneumococcal serotypes. The whole-cell approach is one method of immunizing with a large number of antigens which could provide a broad serotype independent protection, and target both humoral and cellular immunity (30). However, whole cell vaccines can be difficult to deliver and less stable then the counterpart subunit vaccine. An alternative to the whole cell approach is purified surface protein antigens. Several Spn antigens have been studied for their potential to induce protective antibody including PiuA, PiaA, PsaA, RrgA, RrgB, Ply, PcsB, StkP, and PspA (14,18).

One of the best characterized and most promising surface protein vaccine candidates is PspA. PspA is a surface protein virulence factor found on all clinical isolates of Spn. PspA is

non-covalently bound at the C-terminal choline-binding repeat region which anchors the protein to lipoteichoic and teichoic acid in the cell wall. The N-terminal alpha-helical region is antibody accessible and divided into three unique families which are further subdivided into six clades (31). 98% of PspA variants fall within the two major families (family 1 and 2). The major conserved epitopes are located within the first 100 amino acids of the N-terminal region (32). Antibodies directed at the N-terminal region of PspA have been shown to protect against pneumococcal challenge in mice (33). Additionally, immunization with a family 2 PspA provided protection against strains from both major PspA families (26). Adults immunized with PspA produce cross-reactive antibodies that are able to passively protect mice against lethal challenge (34,35). Similarly, the amount of pre-existing PspA specific immunoglobulin in an individual has shown to correlate with susceptibility to colonization and carriage (36). Currently, PspA is being evaluated in the clinic for safety and efficacy as part of a multi-protein vaccine PnuBioVax™ (37).

4. Experimental Mucosal Adjuvants

While PspA appears to be one of the most promising protein vaccine candidates, administration often requires the use of an adjuvant to elicit a protective response. Adjuvants improve immunogenicity and delivery of antigens that would otherwise be unable to mount a significant immune response. A majority of vaccine adjuvants can be separated into two main categories: Immune modulators and delivery systems. The current PCV formulations rely on a nontoxic variant of diphtheria toxin conjugated to the polysaccharide to serve as an adjuvant and enhance immune responses over what is observed in PPSV23, which contain polysaccharides administered alone. One hurdle to developing a mucosal vaccine against Spn is identifying safe,

noninvasive adjuvants that can be used with bacterial antigens to produce protective mucosal and systemic responses. While innate immunity is often enough to protect at the mucosal surface, bacterial adaptations to evade immune protections and infiltrate sterile sites require the generation of innate and adaptive responses to effectively resolve mucosal infections.

One popular mucosal adjuvant, which have been tested with a variety of antigens, is cholera toxin B (CTB). CTB is a subunit of cholera toxin, which has demonstrated effective induction of both humoral and cellular immunity when administered with a bacterial and viral pathogens (38,39). In previously published work we have demonstrated CTB effectively induced systemic humoral immunity against Spn when administered mucosally with PspA (40). While CTB proved to be an effective mucosal adjuvant, concerns have been raised regarding CTB accumulation in olfactory nerves when delivered mucosally (41,42). Additional research is needed to explore creative options for mucosal adjuvants that effectively induce protective immunity while mitigating toxicity by limiting exposure to excess adjuvant and antigenic load.

C. Fc Receptors

1. FcγR Overview and Significance

Fcγ receptors (FcγR) are immunoglobulin specific receptors widely expressed on the surface of cells throughout the immune system. By interacting with the fragment crystallizable (Fc) region of IgG antibodies this class of receptors can both activate and inhibit a variety of effector functions, making this receptor class an attractive target for immune modulation. FcγR's consist of distinct groups of activating and inhibitory receptors with varying expression patterns, cellular distributions, and binding affinities for different IgG subclasses. In humans activating FcγR include FcγRI, FcγRIIA, FcγRIIC, and FcγRIIIA. While in mice the activating receptors are

FcγRI, FcγRIII, and FcγRIV. The receptor FcγRIIB is the only inhibitory receptor in both humans and mice (43). All of the FcγR family, with the exception of FcyRI, exhibit low affinity for monomeric IgG and primarily bind multimeric immune complexes. Binding of multimeric IgG by FcγR triggers receptor clustering and aggregations, thus initiating receptor signaling (44).

Specificity of the IgG Fc domain for different classes of FcγR is largely determined by the primary amino acid backbone sequence of the varying IgG subclasses (IgG1, IgG2, IgG3 and IgG4 in humans) and (IgG1, IgG2a, IgG2b, and IgG3 in mice) (45). As a result, the IgG subclass composition can greatly impact the outcome of Fc engagement *in vivo.* For example in mice the IgG2a immunoglobulin binds to the activating receptors with 100 fold higher affinity then to the inhibitory receptor FcγRIIB. In contrast the subclass IgG1 shows preference for the inhibitory receptor over any of the activating FcγR. Furthermore in mice, IgG1 only binds the activating receptor FcγRIII while all activating mouse FcγR bind IgG2a (Table 2) (46).

In addition to the IgG Fc domain amino acid sequence the quaternary structure of IgG is also designed to facilitate interaction with the Fc domain. The IgG heavy chain is composed of two constant domains CH2 and CH3, with an N-linked glycan structure that spatially separates the two CH2 domains creating a confirmation that is amenable to FcγR binding. The hinge proximal region of the CH2 domain engages in a 1:1 stoichiometric complex with the extracellular IgG binding region of the receptor (47). In addition to the determinants in the IgG Fc domain, differences in the sequence of the FcγR can contribute to the binding outcome. For example, FcγRI is the only high affinity receptor capable of binding monomeric IgG, a property which is attributed to a third extracellular domain that stabilizes the protein-protein interaction with IgG (48).

Table 2. Affinities of Mouse Fcγ Receptors for IgG

	IgG1	IgG2a	IgG2b	IgG3
FcγRI	NB	1×10^8	1×10^5	*
FcγRIIB	3×10^6	4×10^5	2×10^6	NB
FcγRIII	3×10^5	7×10^5	6×10^5	NB
FcγRIV	NB	3×10^7	2×10^7	NB

Table 2. Binding affinity for FcγR by IgG subclasses represented as the association constant depicted in M^{-1} units. (*, binds to the receptor with unknown affinity, NB, indicates no binding) (45,46)

2. Activating Receptors

The majority of FcγR are activating in nature and contain a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) which is phosphorylated in response to receptor clustering and activates cytoplasmic Src and Syk family kinases that initiate a signaling cascade in response to binding of Ab-Ag complexes (49). The capacity of antibodies to interact through their Fc domain with cell surface FcγR is a key facet to initiating critical aspects of innate and adaptive immunity. The diverse immunomodulatory capabilities of the FcγR family can be attributed to the expression patterns across a variety of leukocytes ultimately activating effector functions such as antigen uptake, processing and presentation, regulation of B cell selection and antibody secretion, chemokine and cytokine release, and T-cell activation (50) .

Studies using conditional knockout mice and receptor targeting neutralizing antibodies have provided insight into the action of these receptors *in vivo.* Initially, Fc mediated effector functions were believed to be limited to antibody –dependent cellular cytotoxicity (ADCC) and phagocytosis. Indeed, IgG bound antigen has been shown to increase the rate and amount of

antigen internalized (51). ADCC is another microbial defense mediated by FcγR which allows for the recognition of IgG bound to target cells or pathogen. Cross linking of FcγR on the surface of natural killer cells by opsonized pathogen activates unprimed degranulation and killing of target cells, and secretion of cytokines like interferon gamma (IFN-γ) to recruit adaptive immune cells (52).

While phagocytosis is a common downstream function of the FcγR family, engagement of the Fc receptor initiates a diverse spectrum of pleiotropic immunomodulatory functions. For instance, a number of studies have highlighted the role of FcγR on dendritic cell function and maturation. Dendritic cells are one of the few cell types to express all the mouse activating FcγR and the inhibitory FcγRIIB receptor. Dendritic cell FcγR ligation by antigen-antibody immunocomplexes results in a gene expression profile associated with T-cell activation, enhanced antigen presentation, and induction of CD8+ T cell expansion *in vivo.* These observations were mediated by the activating FcγR and were more pronounced in dendritic cells derived from FcyRIIB knockout mice expressing only the activating receptors (53). Crosslinking FcγR on the surface of DC can also influence cytokine secretion intensity and profile. Studies targeting the activating FcyRI with an anti-hFcyRI-Ag fusion protein induced secretion of both Th1 and Th2 cytokines IL-2, INF-γ, TNFα, and IL-4, IL-5 respectively (54).

In physiological conditions the signaling of the activating FC is balanced by the inhibitory receptor creating a threshold for activation that limits uncontrolled dendritic cell maturation in response to serum immune complexes. To circumvent this co-engagement with other activating receptors, such as toll-like receptors, can act synergistically with activating FcγR and lower the threshold of activation for dendritic cells (55). Additionally, expression of the FcγR are dynamically regulated in response to inflammatory environment which can skew the

receptor density in favor of the activating or inhibitory class.

3. Inhibitory Receptors

As mentioned above, a single inhibitory receptor exists in both humans and mouse which possess an immunoreceptor tyrosine-based inhibition motif (ITIM) associated with the intracellular cytoplasmic tail. The ITIM domain recruits SH2-containing SHIP to transduce inhibitory signals interfering with key activating intermediates such as PIP3 to dampen the ITAM signaling pathway (56). The inhibitory receptor is broadly expressed on tissue macrophage, dendritic cells, and small populations of monocytes, neutrophils, natural killer cells, and memory T cells (57,58). Furthermore, the inhibitory receptor is the only FcγR expressed by mouse B cells, and is the primary FcyR receptor found on human B cells, thereby making it the most widely expressed FcyR (59).

The inhibitory receptor is an important regulator of cellular activation. Crosslinking of FcyRIIB with the B-cell receptor or activating FcγR provides a contrasting cascade that limits the development of excessive inflammation and autoimmunity. Consequently, an FcyRIIB loss of function polymorphism has been identified as the causative agent of systemic lupus erythematosus in some individuals, due in part to the loss of inhibitory function on B-cells (60). Interestingly, the FcyRIIB polymorphism is more prevalent in Asian populations where it has been suggested the loss of inhibitory function provides a protective advantage against malaria, and thus has been subject to positive selection (61). Animal studies have supported the role of FcγRIIB in maintaining tolerance and autoimmunity. For instance, FcγRIIB ligation on the surface of B-cells in the absence of opposing BCR signaling results in apoptosis, eliminating B-cells with low BCR affinity (62). Additionally, FcγRIIB checkpoints in the germinal center

exclude autoreactive B-cells from splenic follicles, thus limiting the production of pathogenic auto-antibodies and demonstrating the importance of FcγRIIB in controlling peripheral tolerance (63).

The mechanisms by which FcγRIIB controls autoimmunity can also play a meaningful role in regulation of humoral and cellular immunity in response to invasive pathogen. For instance, DCs lacking the inhibitory receptor showed enhanced ability to generate antigen-specific T-cells and produce stronger longer lasting immune responses *in vitro* and *in vivo* (64). Additionally, crosslinking FcγRIIB on the surface of DC alters inflammatory gene expression and inhibits TLR4-mediated cytokine secretion (65). Macrophages are also susceptible to FcγRIIB regulation. Macrophage from FcγRIIB knockout mice exhibit increased propensity for phagocytosis and bacterial clearance (66). Additionally, FcγRIIB deficient macrophage produce increased levels of chemoattractant cytokine IL-12p40 accompanied by an enhanced ability to stimulate IFN-y producing CD4 T cells (67). Finally, studies in FcγRIIB knockout mice immunized with immune complexes showed enhanced antibody responses, germinal center development, and immunological memory when compared to WT controls (68).

Evidence supports the critical role of FcγRIIB in maintaining immunological homeostasis and preventing exacerbated inflammatory responses. While this receptor is clearly important to controlling autoimmunity the endogenous inhibitory nature of FcγRIIB simultaneously limits beneficial humoral and cellular responses important for protective immunity.

4. Neonatal FC Receptor

An additional non-classical FcγR exists in both mice and humans which is both structurally and functionally distinct from the activating and inhibitory FcγR previously discussed. The neonatal

Fc receptor (FcRn) plays an important role in recycling and transcytosis of IgG across polarized membranes. FcRn expression on placental syncytiotrophoblasts facilitates transport of immunoglobulin allowing for the passive immunization of offspring with maternal IgG in utero (69). Expression of FcRn is not limited to the placenta and the functional capacity of FcRn to transport IgG persist after infancy allowing for targeted movement of IgG across a broad range of epithelia, endothelia, and hematopoietic cells (70).

In addition to binding IgG the FcRn has a separate distinct binding site for albumin which can be engaged simultaneously with IgG. Together IgG and albumin make up almost 90% of the serum protein content and possess a long serum half-life due in large part to their interaction with FcRn (71). Intracellular expression of FcRn allows for salvage and rescue of both ligands from lysosomal degradation, followed by exocytotic processing in positive recycling endosomes that ultimately leads to release at the plasma membrane (72). The ability of FcRn to effect IgG transcytosis and recycling is dependent on the receptors high affinity for IgG at a mildly acidic pH (5.0- 6.5) but not at a physiological pH (7.4) (73). Our understanding of the role of FcRn in IgG homeostasis has been further supported by conventional and conditional knockout studies. In mice deficient of FcRn serum IgG levels were 20-30% of what is observed in WT animals. Similarly, the half-life was significantly reduced from ~95 hours in WT animals to ~22 hours in FcRn knockouts, further demonstrating the importance of FcRn in maintaining circulating IgG (74,75).

For many years, the emphasis in FcRn studies were focused on half-life and biodistrubution of IgG, thus overlooking the important contribution of FcRn on hematopoietic cells to immune responses involving IgG bound antigen immune complexes. In dendritic cells IgG immune complexes associated with FcRn were diverted into late endosomes or lysosomes

that encouraged retention and were conducive to antigen presentation. Additionally, neutrophils exposed to IgG immune complexes unable to bind FcRn exhibited reduced phagocytosis (76). In line with these observations, mouse and human antigen-presenting cells exposed to WT IgG immune complexes induced greater CD4+ T cell proliferation then groups administered FcRn non-binding variant IgG immune complex (77). While the mechanisms underlying these observations require further investigation, FcRn is likely an important regulator of antigen presentation in addition to IgG transcytosis and homeostasis.

5. FC Targeted Vaccines

Given the important role of the Fc Receptor in mediating a wide spectrum of immunological functions the FcγR family is an attractive target for vaccination strategies to enhance host immune responses. Additionally, by targeting antigen to these immunomodulatory receptors you circumvent the need for adjuvant and may reduce the overall antigen load required to provide protective immunity. Numerous studies have illustrated enhanced cellular and humoral responses when targeting antigen to FcγR receptors (54,78,79). Our lab has demonstrated targeting inactivated *F. tularensis* (iFT) to FcγR using bacterial immune complexes enhanced protection against fatal *F. tularensis* challenge. Using an Fc targeting strategy we demonstrated respiratory and systemic macrophage activation accompanied by a shift in cytokine profile from anti-inflammatory to pro-inflammatory, which helped overcome *F.tularensis* induced immune suppression (80). *In vivo* efficacy conferred by the iFT immune complex is dependent on the FC receptors, and FcγR knockouts are not protected against lethal *F.tularensis* challenge (79). In a separate study by our lab, mucosally administered immune complexes significantly increased the T-helper cell subpopulation TH17 in the lung and spleen post infection. Additionally, TH17

synergistic pro-inflammatory cytokines IL-6 and TGF-β1 were elevated in the lung and spleen suggesting an enhanced TH17 response as a result of FcγR targeting in the context of *F. tularensis* infection (81).

FcγR targeting vaccines are a highly advantageous strategy to generate both humoral and cellular immune responses without the requirement for adjuvant. However, this strategy can be limited by interactions with the inhibitory receptor FcγRIIB. Crosslinking the inhibitory receptor can circumvent Fc protective immunity through inhibition of B-lymphocyte activation and subsequent antibody production. Additionally, FcγRIIB on the surface of macrophage and dendritic cells can have a negative impact on antigen processing and presentation thus limiting T-cell activation. While characterization of the inhibitory receptor in the context of bacterial infection is limited studies have demonstrated FcγRIIB knockout mice to be less susceptible to infection then WT controls (67,82). Interestingly, in some models of infection FcγRIIB knockout mice administered high titer doses had increased mortality rates, likely as a result of increased proinflammatory cytokines and sepsis (83).

An additional benefit to FcγR targeting is the potential for interaction with FcRn. Engaging the FcRn receptor is advantageous for transepithelial vaccine delivery, which helps activate the mucosal immune system. Indeed, Fc targeting vaccines have been proven effective for delivery across pulmonary, oral, and genital mucosal surfaces. Ye and colleagues showed intranasal immunization of herpes simplex virus type-2 glycoprotein gD fused to an Fc fragment protected WT mouse from virulent herpes simplex virus challenge. The construct induced mucosal and systemic immunity which was not observed in FcRn knockout animals (84). It can also not be overlooked the important role FcRn plays in increasing plasma-half-life. As such interactions with FcRn are likely to improve vaccine pharmacokinetics and enhance therapeutic

efficacy.

Many FcγR targeting vaccination studies use immune complexes to target antigen to FcγR on the surface of immune effector cells. This method is necessary in situations where a single immunogenic antigen has not been identified and whole killed or inactivated pathogen is administered complexed with IgG. But therapeutic immune complex studies are complicated by generation of well-defined native immune complex preparations, which require optimal ratios of antibody to antigen. Additionally, immune complex generations are often highly unstable making them impractical for widespread use. One approach to circumvent these hurdles is engineering antigen Fc fusion proteins that could be mass produced at a low cost in a eukaryotic expression system to target immunogenic proteins to FcγR. Fc fusion proteins are bioengineered polypeptides composed of the Fc domain of antibody fused to an effector molecule. An ideal Fc targeting vaccine would bind the activating FcγR while limiting interaction with the inhibitory receptor. One approach to optimize these interactions is to select an IgG subclass whose Fc fragment would maximize interactions with activating FcγR and FcRn while minimizing FcγRIIB involvement. The binding affinities of each IgG subclass for the activating and inhibitory FcγR can be employed to determine the optimal subclass Fc for inclusion in an Fc fusion protein. In mice IgG2a would be isotype of choice with preferential affinity for activating FcγR. In humans, IgG1 would be the equivalent isotype with broad specificity for hFcγRs and lower affinity for FcγRIIB (85).

II. Aims of the Study

The purpose of this thesis is to demonstrate the potential for isotype specific Fc fusion proteins to target PspA to immune effector cells, and provide adjuvant free protection against lethal Spn challenge. To this end we aim to characterize the key contributors of innate and adaptive immunity that are enhanced in response to immunization with Fc fusion proteins. Based on our understanding of isotype and subtype binding hierarchy's we believe a PspA fusion protein consisting of the IgG2a Fc (IgG2a Fc-PspA) would provide superior protection over an IgG1 Fc fusion protein (IgG1 Fc-PspA) in WT mice expressing both activating and inhibitory receptors. In the absence of FcγRIIB we hypothesize a fusion protein containing the IgG1 Fc-PspA will gain efficacy and provide similar protection to IgG2a Fc-PspA.

III. Materials and Methods

A. Mice and Bacteria

C57BL/6 mice were obtained from Jackson Labs (Bar Harbor, Maine). FcγRIIB knockout mice, on the C57BL/6 background, were purchased from Jackson Laboratory stock #002848. All mice were housed in the animal research facility at Seton Hall University and provided ad libitum food and water during the experiment. Animal studies were approved by the Animal Care and Use Committee.

S. pneumoniae strain A66.1 a mouse virulent capsule type 3 serotype expressing family 1, clade 2 PspA was provided by Dr. Gosselin (Albany Medical College, Albany, NY) (86). Bacteria were cultured in Todd-Hewitt broth at 37˚C until mid-log phase. Bacterial suspension was pelleted and washed 3x with Phosphate buffered saline (PBS), then resuspended in fresh broth with 15% glycerol and stored in liquid nitrogen until use.

B. Construction, Purification, and Characterization of IgG1 Fc-PspA and IgG2a Fc-PspA Fusion Proteins

The cDNA encoding the 303 amino acids of the N-terminal region of family 1, clade 2 PspA was amplified from the Rx1 strain plasmid pUAB055 provided by Dr. Edmund Gosselin (Albany Medical College) using the primer pair (5'-CGGAATTCTTCGAGCGAATCTCC-3', 5'ATAGTTTAGCGGCCGCATTTCTGGGGCTGGAG-3) (87). During amplification EcoR1 and Not1 restrictions sites were introduced at the 5' and 3' end of the sense and antisense primers respectively. Amplified PspA fragments were then ligated into a PEF6/V5 expression plasmid containing the hinge, CH2, and CH3 domains of mouse IgG1 Fc (a gift of Dr. Sally Ward, University of Southampton). For construction of the IgG2a Fc-PspA construct PspA was

amplified by PCR, with the same primers described above, with the exception of the antisense primer modified to introduce a Xho1 restriction site. The PspA PCR product containing EcoR1 and Xho1 restriction sites was then ligated into the pFuse-IgG2a-Fc plasmid containing the murine IgG2a heavy chain (InvivoGen, San Diego, CA). Antibiotic-resistant plasmids were screened by PCR and verified by sequencing.

The plasmids containing chimeric IgG1 Fc-PspA or IgG2a Fc-PspA were transfected into Chinese hamster ovary cells (CHO) (ATCC, Manassas, VA) using PolyFect (Qiagen, Hilden, Germany). Cells were maintained in serum-free CHO optimized media (Gibco, Waltham, MA) and Puromycin (IgG1 Fc-PspA) or Zeocin (IgG2a Fc-PspA) resistant clones were selected for secretion of the Fc-PspA fusion proteins by PspA and isotype specific ELISA using anti-PspA (SantaCruz Biotechnology, Dallas, TX) and goat anti-mouse IgG1 or IgG2a antibodies (Thermo Fisher, Waltham, MA). Recombinant proteins were purified from serum-free CHO supernatant by affinity chromatography using protein G packed columns (Thermo Fisher). Further product concentration and buffer exchange was accomplished using size exclusion chromatography columns (Millipore, Burlington, MA). Protein concentrations were measured by Bradford protein assay kit (Thermo Fisher), and endotoxin level of the purified fusion proteins was determined using chromogenic LAL endotoxin assay kit (Pierce, Rockford, IL).

Purified fusion proteins were resolved on a 12% SDS-PAGE gel under reducing or non-reducing conditions and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA and probed with anti-PspA. Bands were detected using IR800 conjugated Donkey anti-goat IgG (Licor, Lincoln, NE) secondary and visualized using the FluorChem Imager (Protein Simple, San Jose, CA). To confirm IgG1 Fc-PspA and IgG2a Fc-PspA retained all structures necessary for FcγR engagement we measured fusion protein binding to murine macrophage FcγR

expressing cell line (RAW 264.7). 1 x 10^6 cells were incubated with 1ug or 5ug of fusion protein for 30 minutes in the presence and absence of Fc receptor block. Cells were washed and stained with FITC anti-mouse IgG1 heavy chain or FITC anti-mouse IgG2a heavy chain secondary antibody (Abcam, Cambridge, MA) and fluorescence was detected by flow cytometry using the MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany).

C. Antigen Presentation Assay

C57BL/6 peritoneal macrophage cell line IC-21 (ATCC® TIB186™) (2 x 10^5 cells/well) were co-cultured with the PspA specific T-cell hybridoma B6D2 (1x10^5 cells/well) with and without the Fc receptor block CD16/32 (Biolegend, San Diego, CA). Co-cultures were treated with titrating amounts of PspA, IgG1 Fc fusion protein, or IgG2a Fc fusion proteins and incubated for 36 hours at 37˚C in 5% CO2. Following incubation, the supernatants were collected and probed for IL-2 by ELISA using the manufacturer's instructions (Biolegend).

D. Immunization and Challenge

Eight to twelve-week-old WT C57BL/6 and FcγRIIB knockout mice were divided into groups consisting of 5 mice. Each mouse was anesthetized with a mixture of 20% ketamine (Vedco, St. Joseph, Missouri) and 5% xylazine diluted in sterile water to provide sedation without respiratory depression. While under light sedation mice were immunized i.n. with 20 ul PBS, 10ug of IgG1 Fc or IgG2a Fc fusion protein, or an amount of PspA (gift of Dr. Snapper, Uniformed Services University of Health Sciences, Bethesda, MD) determined to be equivalent to the PspA present in the fusion proteins. Animals in each immunization group were boosted on days 14 and 28. Two weeks after the last dose animals were challenged with 8 x 10^6 colony-

forming units (CFU) of live A66.1 bacteria and monitored for 21 days. The challenge dose was determined by bacterial titration demonstrating 50% mortality (LD_{50}) in non-immunized mice. Exact CFU administered was verified by culturing and counting inoculum subsequent to challenge on blood agar plates (trypticase soy agar with 5% sheep blood).

E. Bacterial Burden

Following immunization and challenge, lung and spleen were collected in PBS containing protease inhibitor (Roche Diagnostics, Indianapolis, IN) and mechanically homogenized using the Omni tissue homogenizer. Supernatants were then diluted 10-fold in sterile PBS, 10ul of each dilution was spotted on Blood Agar plates in duplicate. Plates were incubated at 37˚C for 24 hours and monitored for growth. The number of colonies were counted and expressed as Log_{10} CFU per milliliter. The remaining tissue homogenate was centrifuged at 12,000 x g for 20 minutes, the clarified supernatant was removed and stored at -20˚C for cytokine analysis.

F. Measurement of cytokine production and *S. Pneumonaie* specific antibody production

S. Pneumoniae specific antibody production in response to immunization was measured in serum 24 hours pre-infection by ELISA. Assay plates were coated in $1x10^7$ CFU/mL of live *S. Pneumoniae* and incubated overnight at 4˚C and then washed four times with PBS containing 0.05% Tween-20. Washed plates were then blocked with superblock (Thermo Scientific, Waltham, MA) as indicated by the manufacture's instructions. Serum was collected one day before infection through submandibular bleed, samples were added in duplicate as a 10-fold serial dilution and incubated for 2 h at 37˚C. Following incubation, plates were washed three

times and incubated with anti-mouse isotype-specific horseradish peroxidase conjugated secondary antibody for 1 h at 37˚C [anti-IgG, anti-IgG2c, anti-IgG1, anti-IgG3, and anti-IgA (Invitrogen, Carlsbad, CA)]. After 1 h incubation with secondary antibody the plates were washed three times and 3,3',5,5' tetramethylbenzidine (TMB) substrate solution was added as per manufacturer instructions, the sample optical density (OD) was read at 650 nm on a microplate reader. Antibody titers are represented as the reciprocal of the highest dilution showing a 2-fold increase in the optical density measured at 650 nm over the background generated from the PBS treated mice.

Cytokines were measured in lung and spleen tissue homogenates collected 24 and 48 hours after infection. Mouse ELISA kits were purchased from Biolegend and performed as recommended by the manufacturer.

G. Flow Cytometry

The lungs of immunized mice were harvested 24 hours post-infection perfused with cold 1x PBS containing a protease inhibitor. The tissue was then shredded into small pieces and placed in digestion buffer containing RPMI (Life technologies), 0.2mg/ml DNaseI (Sigma), 0.4mg/ml Collagenase D (Sigma), and 1M $MgCl_2$. The tissue was incubated in digestion buffer for 30 minutes at 37°C and then forced through a cell strainer to obtain a single cell suspension. The cell suspension was washed and resuspended in RPMI containing 2% FBS, then layered onto 5mL of Lympholyte M (Cedarlane Laboratories, Burlington, NC). The gradient was spun down at 15,000g for 30 minutes at room temperature to obtain the leukocyte rich interface which was removed and added to RPMI with 2% FBS. The single cell lung leukocyte suspension was then preincubated with Fc block (Biolegend) and washed with staining buffer (PBS, 2% bovine serum

albumin, 0.01% sodium azide). Blocked cells were divided into two groups and stained for 30 minutes with fluorescently labeled antibodies for macrophage cell surface markers (F4/80, CD11b, CCR7, MHCII, and CD86) or dendritic cell markers (CD11c, CD11b, CD24, CD103, MHCII) (Table 3).

For analysis of memory B-cell populations splenocytes were processed into a single cell suspension. Cells were counted following isolation, 1 x 10^6 cells per spleen were washed and stained with fluorescently labelled antibodies (CD4, CD19, CD80, and PD-L2) (Table 2), and analyzed using the MACSQuant Analyzer (Miltenyi Biotec). Data analysis was performed using FlowJo and FCS Express.

Table 3. Antibodies used for flow cytometry analysis

Antigen marker	**Fluorophore**	**Clone**	**Panel**
F4/80	PE	BM8	Macrophage
CD11b	FITC	MI/70	
CCR7	Percp/Cy5.5	4BI2	
MHCII	APC	M5/114.15.2	
CD86	APC/Cy7	GL-1	
CD11c	APC	N418	Dendritic Cell
CD11b	FITC	MI/70	
CD24	PB	MI/69	
CD103	PE	REA789	
MHCII	PE/Cy7	M5/114.15.2	
CD4	APC/Cy7	RM4-5	Memory B-Cell
CD19	APC	1D3/CD19	
CD80	FITC	16-10A1	
PD-L2	PE	TY-25	

H. B-Cell Proliferation Assay

Spleens were harvested from PBS, PspA, or fusion protein immunized mice two weeks after the last boost and processed into a single cell suspension as described above. Spleen cells were

incubated with Carboxyfluorescein diacetate succinimidyl (CFSE, Biolegend) at a final concentration of 2uM for 10 minutes at 37˚C. CFSE labeled splenocytes (5 x 10^5 cells) were plated in RPMI 1640 (Gibco) with 10% FBS and cultured in the presence of PspA (5ug/mL) for 4 days at 5% CO2. The cells were then harvested and stained with PE/Cy7 conjugated anti-mouse CD19 (Biolegend) and analyzed by flow cytometry.

I. Statistical analysis

Bacterial burden, cytokine analysis, antibody titers, and cell phenotyping were assessed using a one-way analysis of variances (ANOVA) or the unpaired, two-tailed student t-test. Survival curves were compared using the Log-Rank (Mantel-Cox) test. GraphPad Prism software was used for statistical analysis.

IV. Results

A. *Generation and in vitro characterization of recombinant Fc fusion proteins.*

Fusion protein expression vectors were transfected into Chinese hamster ovary (CHO) cells, a common host for the production of biotherapeutics. Stable clones suitable for protein production in culture were selected using anti-PspA and isotype specific ELISA. Since transfection of CHO expression systems is well characterized, and a selective antibiotic media was used, all tested clones showed evidence of the target protein expression. Stable clones were maintained in culture for supernatant collection. Fusion proteins containing the hinge and heavy chain portion of IgG1 or IgG2a linked to the N-terminal α-helical region of PspA were purified from CHO supernatant using affinity and size exclusion chromatography (Fig. 1A) (88). The expression and antigenic reactivity of the isolated fusion proteins was determined by western blot using antibodies specific to the PspA region expressed in fusion with the isotype specific heavy chain. The molecular weight was detected as predicted, accounting for the posttranslational glycosylation innate to mammalian cell expression systems (Fig.1B). To assess effective binding of the recombinant proteins to endogenous FcγR, fusion proteins were incubated with a murine macrophage FcγR expressing cell line (RAW 264.7) *in vitro* and analyzed by flow cytometry (89). Fusion protein binding to RAW 264.7 cells was detected in a concentration dependent manner and was inhibited in the presence of FcγR blocking antibodies for both IgG2a and IgG1 fusion protein preparations (Fig. 1C, 1D). The functional binding of the recombinant fusion proteins was further assessed through an *in vitro* antigen presentation assay. Fc receptors are expressed on the surface of antigen presenting cells (APC) and are important mediators of core function in both macrophage and dendritic cells, including their capacity to internalize antigen and regulate T-cell responses (90). To test this, C57BL/6 mouse macrophage line IC-21 was

cultured with a PspA-specific T cell hybridoma (B6D2) in the presence of titrating amounts of fusion protein or PspA alone. Antigenic stimulation of the T-cell receptor triggers multiple signaling pathways including the secretion of IL-2 which further commits T-cells to proliferation and differentiation. For this reason, IL-2 is frequently used as a reliable readout of T-cell activation (91). The production of IL-2 was highest in the groups co-cultured with IgG2a Fc-PspA suggesting an enhanced ability for IgG2a fusion protein to target APCs and stimulate T-cells as compared to the IgG1 fusion or PspA alone (Fig. 1E). The production of IL-2 from the PspA-specific T-cell hybridoma was dependent on FcγR engagement and was reduced significantly for both fusion proteins with the addition of FcγR blocking antibodies (Fig. 2).

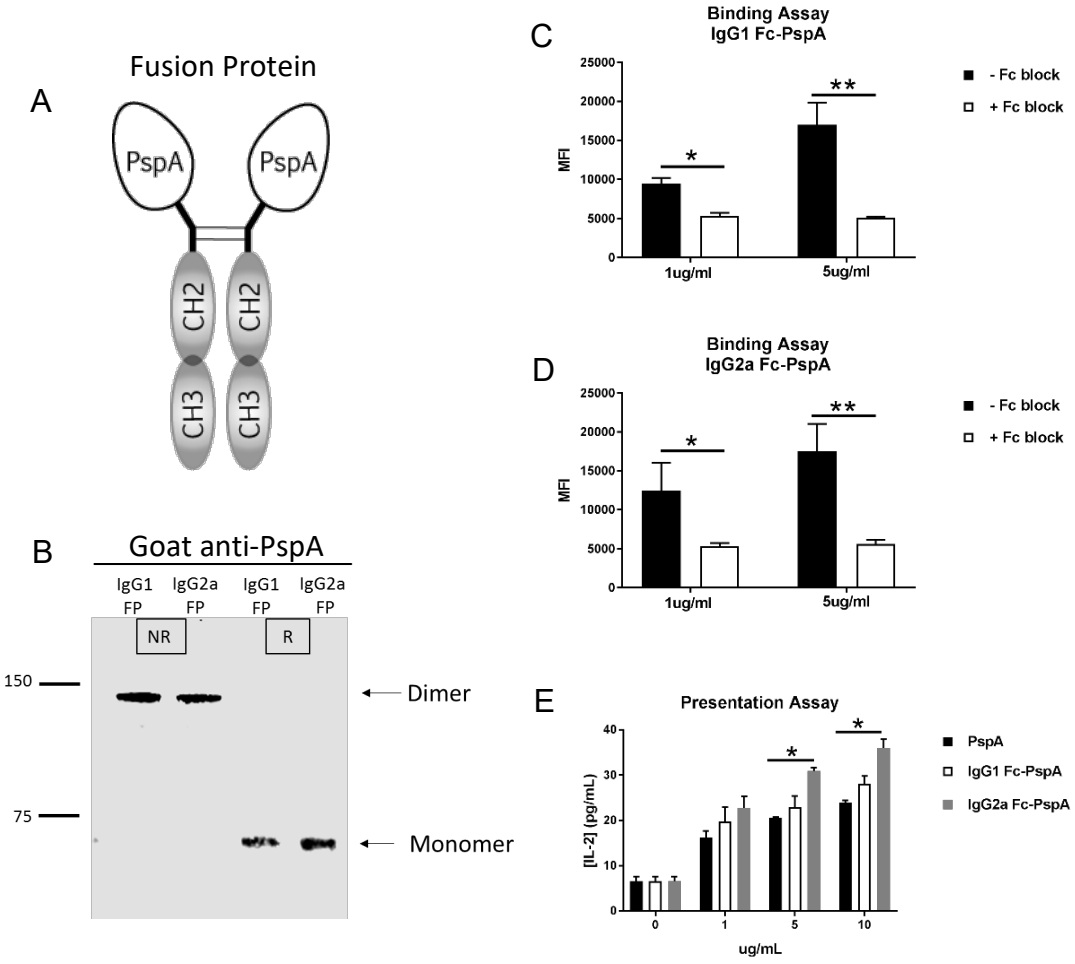


FIGURE 1. Fc Fusion protein construction, purification, and functional characterization. (A) Schematic depiction of the heavy chain Fc fragment fused with the N-terminal α-helical region of PspA to create an IgG Fc-PspA fusion protein. (B) The fusion proteins were secreted by CHO cells and the purified product was detected on western blot when probed with anti-PspA antibody. The fusion protein formed monomers under reducing (R) conditions dimers under nonreducing (NR) conditions. Fusion protein binding to FcγR's on murine macrophage was determined by incubating RAW 264.7 cells with 1 and 5ug/mL of unlabeled IgG1 Fc-PspA (C) and IgG2a Fc-PspA (D) in the presence and absence of FcγR block. Fusion protein treated cells were then washed and stained with APC anti-mouse IgG1 or anti-mouse IgG2a. MFI, mean fluorescent intensity. (E) Functional PspA presentation by macrophage from C57BL/6 mice cultured with PspA-specific mouse (C57BL/6) T cell line (B6D2). IL-2 secretion in the supernatant was measured by ELISA after 30 hours of incubation at 37˚C (*, $P < 0.1$; **, $P < 0.05$) (88).

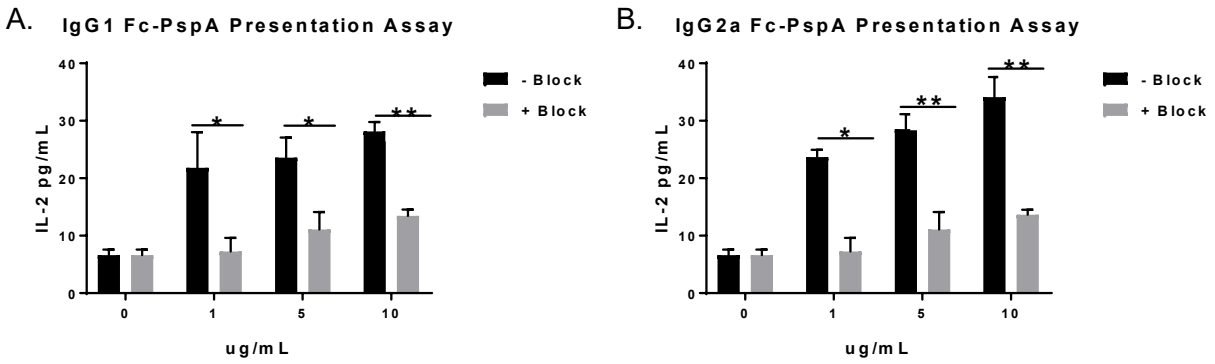


FIGURE 2. IL-2 secretion from PspA specific T-cells is reversed in the presence of FcγR block. Presentation of PspA by fusion protein treated C57BL/6 macrophage to the PspA-specific mouse (C57BL/6) T cell line (B6D2) was measured in a functional antigen presentation assay in the presence and absence of FcγR blocking antibody. IL-2 secretion in the supernatant was measured by ELISA after 30 hours of incubation at 37˚C (*, $P < 0.1$; **, $P < 0.05$).

B. *Targeting activating FcγR with IgG2a Fc-PspA decreases pulmonary bacterial burden and provides superior protection against lethal Spn challenge.*

Naive C57BL/6 mice (WT) were intranasally (i.n.) administered PBS buffered unadjuvanted isotype specific fusion proteins or PspA alone using a double boost strategy consisting of immunization on day 0 with boosters on day 14 and 28, followed by lethal i.n. challenge with Spn strain A66.1 two weeks following the second boost. Immunization with IgG2a Fc-PspA provided superior protection against lethal challenge while IgG1 Fc-PspA provided only limited protection, comparable to the protection observed with PspA alone (Fig 3A). Unimmunized mice succumbed to infection by 48 hours after lethal mucosal challenge. Similarly, 75% of PspA and IgG1 Fc-PspA immunized mice died within 4 days of A66.1 challenge. Protection provided by the IgG2a fusion protein was also consistent with reduced bacterial burden in lung 24 hours post-infection compared to the other treated and non-treated groups (Fig 3B).

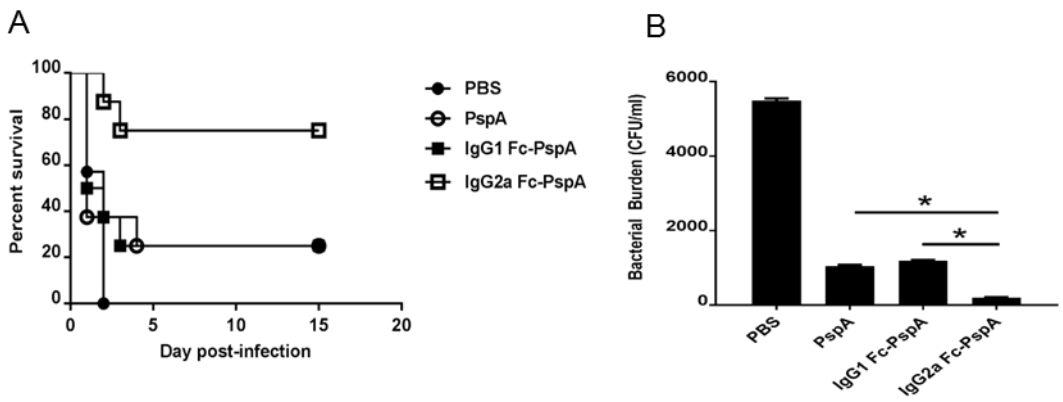


FIGURE 3. Immunization with IgG2a fused to PspA protects against lethal Spn challenge and decreases bacterial burden in the lungs of infected mice. (A) WT mice were immunized i.n with PBS, PspA, IgG1 Fc-PspA, or IgG2a Fc-PspA on day 0 and boosted on days 14 and 28. Two weeks following the last boost mice were challenged i.n. with Spn strain A66.1 (8 x 106 CFU) and monitored for 21 days. (B) Bacterial burden was measured in the lung of immunized animals 24 hours after i.n. challenge with A66.1. Survival studies are representative of three individual experiments consisting of 6 mice per group (*, $P < 0.1$).

C. *Immunization with IgG2a Fc- PspA enhance the Spn specific humoral response and stimulate a TH1 like cytokine profile.*

The importance of antigen specific antibody production has been well established in controlling Spn pathogenesis and providing long-term protection (35,92–94). Specifically, Spn specific antibodies elicit protection by interfering with bacterial adhesion and facilitating complement independent opsonization. For this reason, the levels of PspA-specific antibodies in serum were measured 24 hours pre-infection following i.n. immunization. Mice administered IgG2a Fc-PspA had significantly higher titers of antigen specific antibodies across all tested isotypes, including IgG and IgA (Fig 4A-E). Alternatively, IgG1 Fc-PspA showed no additional induction of PspA specific systemic antibody across all tested isotypes compared to administration of PspA alone. IgA antibodies are particularly relevant to pulmonary infection as IgA is the primary immunoglobulin contributor to humoral mucosal immunity, and has been shown to reduce carriage and prevent colonization of Spn *in vivo* (95).

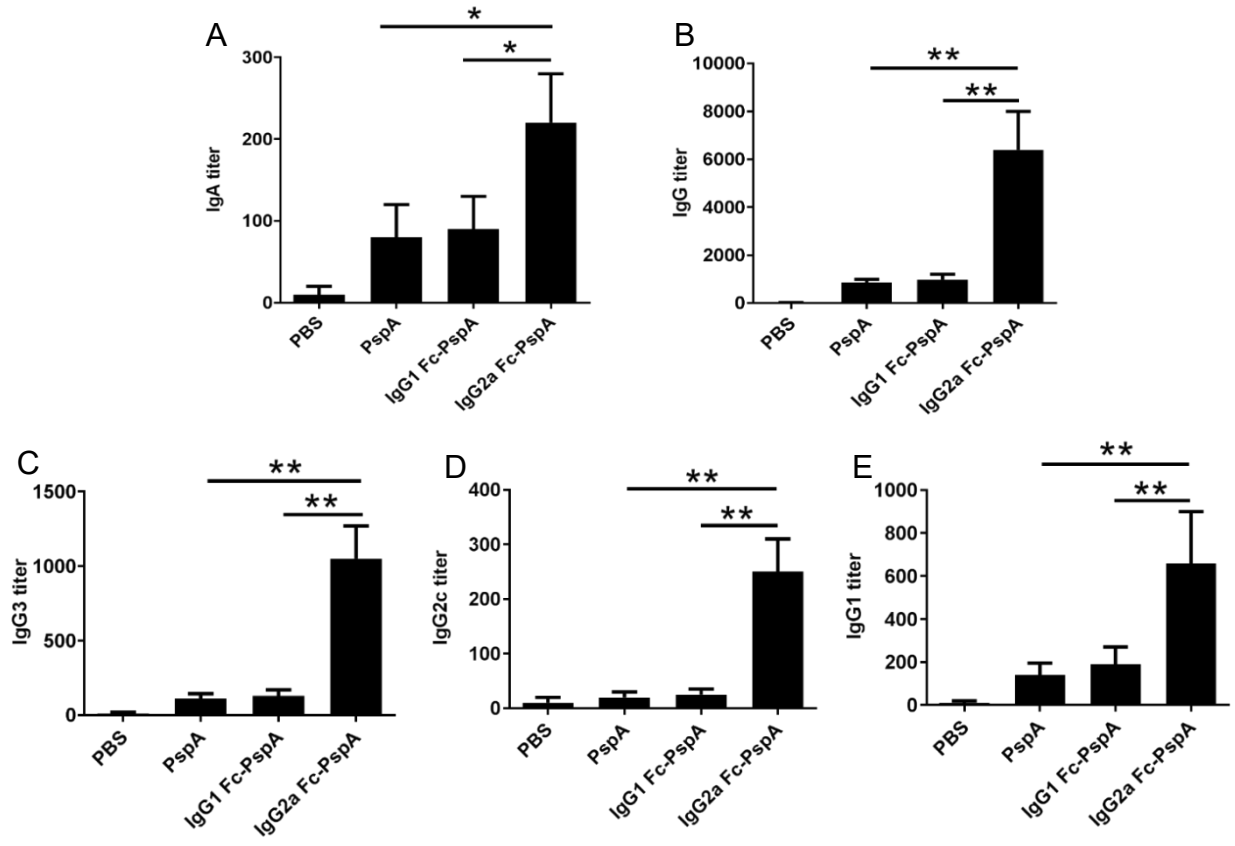


FIGURE 4. PspA specific antibodies are increased in mice immunized with IgG2a fusion proteins. WT animals were immunized using the i.n. double boost strategy as previously described. Two weeks following the final boost serum was collected and PspA-specific antibody titer for IgG (A), IgA (B), IgG3 (C), IgG2c (D), and IgG1 (E) was measured by ELISA. P-values were determined using an unpaired two-tailed t test (*, $P < 0.1$; **, $P < 0.05$).

While both T-helper 1 (Th1) and T-helper 2 (Th2) associated antibody isotypes were elevated in serum, the predominant cytokine profile in lungs of IgG2a Fc-PspA immunized mice was consistent with Th1 differentiation. Type I cytokines, such interleukin (IL)-2, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) were elevated in the lungs of IgG2a Fc-PspA immunized mice (Fig 5).

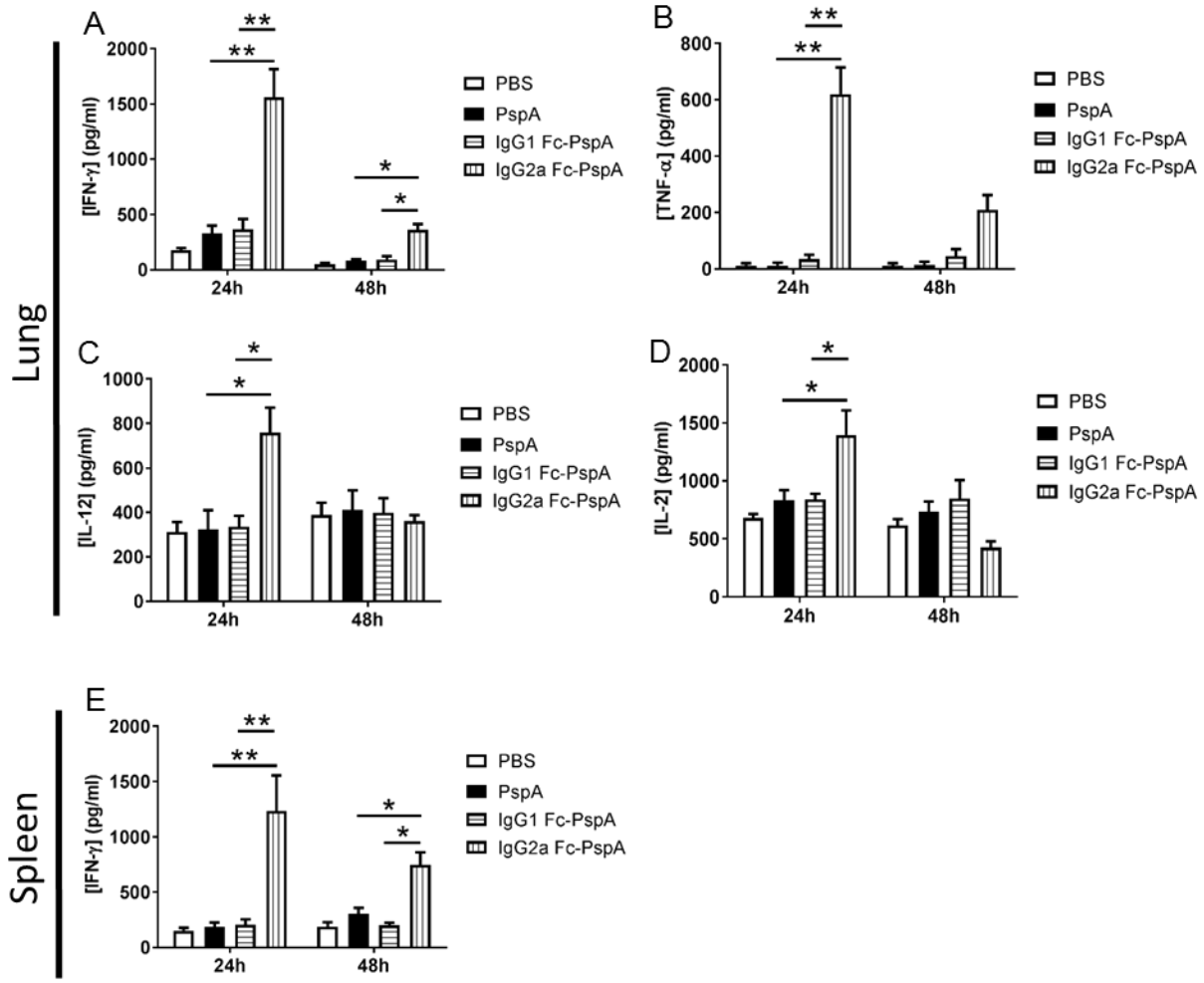


FIGURE 5. IgG2a fusion proteins enhance the early innate immune response. WT mice were immunized with PBS, PspA, or fusion protein (day 0) and then boosted twice (day 14 and 28) before i.n. challenge with A66.1 on day 42. Lung and spleen were harvested 24 and 48 hours following challenge, and cytokine concentrations were measured by ELISA (A-E). Each time point represents independent groups of 6 animals per treatment, immunized and challenged in parallel (*, $P < 0.1$; **, $P < 0.05$).

The amount of IFN-γ and TNF-α reduced significantly by 48 hours, suggesting the early inflammatory response may be key to controlling infection in the lung and quickly resides to mediate pathologic damage (Fig. 5A,5B). IL-12 was also increased in lung, which further supports TH1 polarization, as IL-12 is commonly produced by activated APC's and polarizes T-cell responses to the Th1 phenotype (Fig. 5C) (96). Similarly, levels of IFN-γ were also

significantly increased in spleen of IgG2a Fc-PspA immunized mice (Fig. 5E). No difference was measured between groups in cytokines typically associated with TH2 differentiation including IL-4, IL-5, and IL-10 (data not shown).

D. IgG2a Fc-PspA immunization leads to AM1 macrophage polarization in the lungs of immunized mice 24 hours post lethal challenge

Alveolar macrophages are a critical component to the early stages of innate immunity against mucosal pathogens in the lung. Classically activated alveolar macrophage (AM1) are a pro-inflammatory subtype characterized by increased phagocytic capacity and high levels of IL-12, IL-23, and nitric oxide production (97). In previous studies, depletion of alveolar macrophages in a murine model of Spn pulmonary infection significantly increased bacterial burden, suggesting an important role for alveolar macrophage in bacterial clearance during pneumococcal infection (98). In contrast, alternatively activated alveolar macrophages (AM2) are regarded as the resting phenotype, promoting tissue remodeling and immune tolerance in conjunction with anti-inflammatory cytokine secretion such as IL-10 (99). The AM1 lineage constitute a functionally distinct cell type ideally suited to control bacterial burden and provide the first line of innate cellular defense. For this reason, we used flow cytometry to characterize the macrophage population in isolated pulmonary leukocytes of immunized mice 24 hours after bacterial challenge. AM1 cells were characterized by expression of ($F4/80^{+}$,$CD11b^{int}$,$CCR7^{+}$,MHC class II^{+},$B7.2^{+}$) markers, while the AM2 population were identified as ($F4/80^{+}$,$CD11b^{int}$,$CCR7^{-}$,MHC class II^{-},$B7.2^{-}$) (80). The lungs of mice immunized with IgG2a Fc-PspA had a greater number and frequency of AM1 cells compared to the IgG1 fusion protein and PspA alone (Fig. 6). Increasing the ratio of AM1 to AM2 cells in the lungs by IgG2a Fc-PspA immunization illustrates that targeting the activating FcγR leads to a protective

AM1 alveolar macrophage polarization.

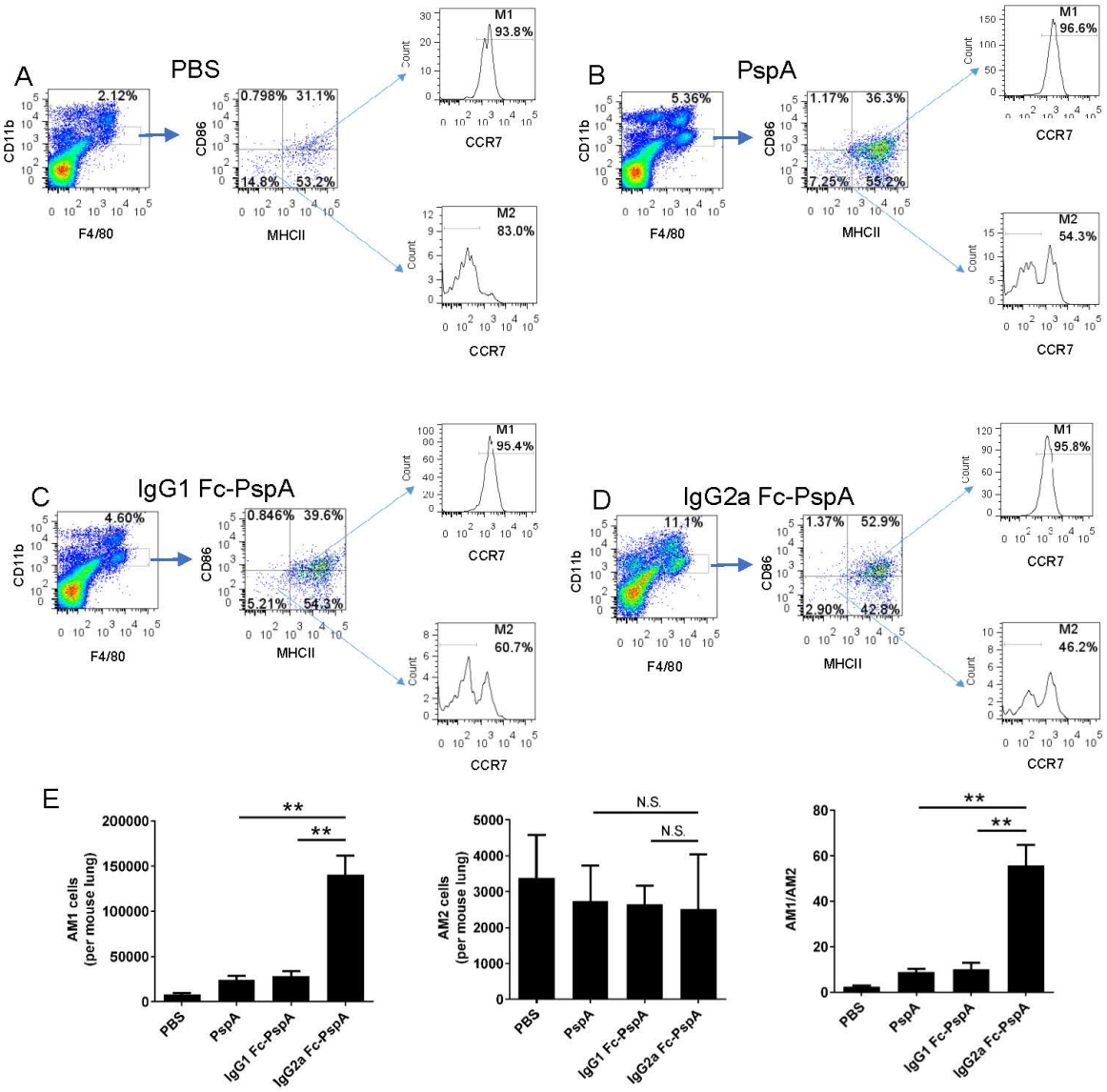


FIGURE 6. Immunization with IgG2a Fc-PspA polarized alveolar macrophage to the AM1 phenotype 24 hours after lethal Spn challenge. Lungs of immunized mice were harvested 24 hours post-infection and cells were separated using density gradient centrifugation. (A-D) Isolated leukocytes were stained with fluorophore conjugated antibodies against CD11b, F4/80, MHC class ll, CD86, CCR7, or the coordinating isotype control and analyzed by flow cytometry. Plots are representative of three independent experiments (E) Average number of AM1 and AM2

cells per mouse lung measured by flow cytometry (*, P < 0.1; **, P < 0.05, N.S., No significant difference).

E. CD11B+ and CD103+ conventional dendritic cells are increased in the lung following PspA targeting to activating FcγR.

Lung resident conventional dendritic cells (cDC) are essential for antigen presentation and subsequent priming of antigen-specific CD4+T-lymphocytes (100). Two main cellular subsets of migratory cDCs have been identified based on the expression of cell surface integrins: CD103 (cDC1) and CD11b (cDC2) (101). Emerging evidence suggests a functional specialization exists between the two subsets, including both a distinct and overlapping propensity to present antigen and initiate specific T-cell responses in a manner dependent on antigenic context and environment stimuli. To our knowledge no studies have explored the role of discrete cDC subsets in promoting specific T-cell polarization after Spn infection.

In the current study we measured cDC1 (CD24+, CD11C+, MHCII+, CD11b-, CD103+) and cDC2 (CD24+, CD11C+, MHCII+, CD11b+, CD103-) in the lungs of immunized mice following lethal Spn challenge. Both subsets were increased in animals immunized with IgG2a fusion protein compared to the IgG1 fusion protein or PspA alone (Fig. 7). While we cannot definitively elucidate the specific contributions of each subset to downstream T-cell activation these findings suggest both groups of cDC are receptive to activating FcγR targeting and contribute to immunity of the lung. Other groups have highlighted a commonality between the two cell types. Shekhar et al. demonstrated both cDC1 and cDC2 capable of inducing Th1 polarization and promoting protective immunity in response to chlamydial lung infection (102). Additionally, depletion of both cDC1 and cDC2 in the lungs abrogated α- galactosyleramide-mediated protection against lethal S. pneumoniae challenge, suggesting a possible role for both cDC subsets in protecting against Spn infection (103).

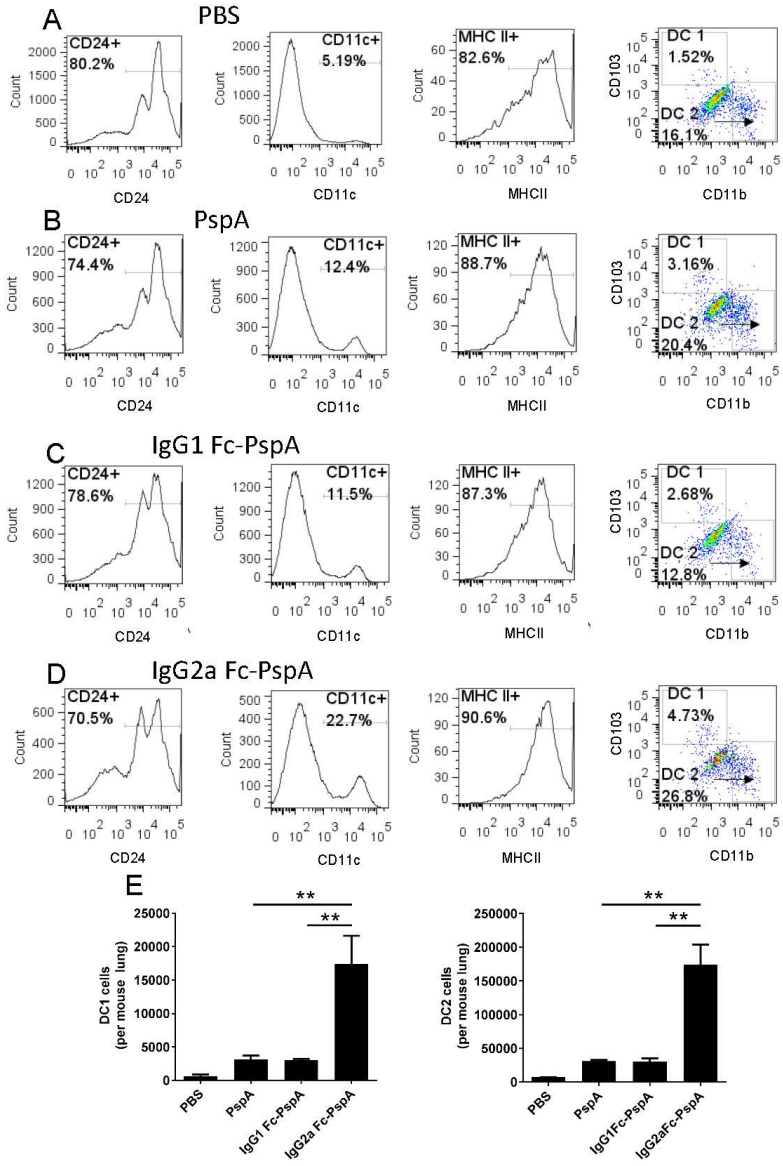


FIGURE 7. IgG2a Fusion protein increases CD103+ (DC1) and CD11b+(DC2) dendritic cells in the lung 24 hours after Spn infection. (A-D) Representative flow cytometry analysis of leukocytes from immunized mice stained with fluorescently labelled antibodies against CD24, CD11c, MHCII, CD11b, CD103, or the coordinating isotype controls. (E) The average number of DC1 and DC2 cells per lung as detected by flow cytometry (*, $P < 0.1$; **, $P < 0.05$).

F. The reduced protection of the IgG1 Fc-PspA fusion protein is reversed in FcγRIIB knockout mice.

Individual receptors of the FcγR family display differential affinity dependent on IgG subclass

which can influence the biological activity of antibodies *In vivo* (Table 2). In the current study, IgG2a Fc fusion proteins provided superior protection over fusion proteins composed of the IgG1 Fc region, which preferentially binds to the inhibitory receptor FcγRIIB. To affirm the role FcγRIIB played on the *in vivo* efficacy of IgG1 Fc-PspA, we investigated the impact of FcγR targeted immunization in FcγRIIB deficient mice (FcγRIIB KO). Consequently, increased protection was observed in IgG1 Fc-PspA immunized FcγRIIB KO mice compared to the WT controls (Fig. 8A).

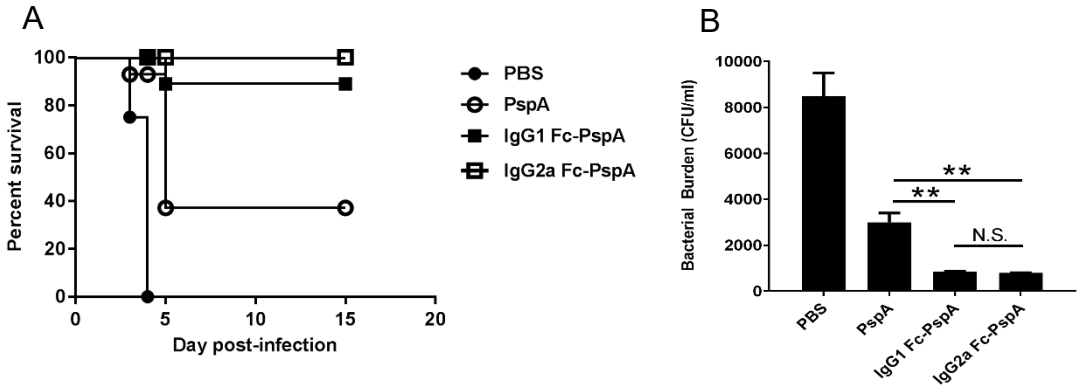


FIGURE 8. The protective efficacy of IgG1 Fc-PspA against Spn is enhanced in FcγRIIB KO mice. (A) FcγRIIB KO mice were immunized as previously described. Two weeks following the second boost animals were challenged with a lethal dose of A66.1 (8 x 106 CFU) and monitored 21 days post-challenge. (B) Bacterial burden is significantly decreased in the lungs of mice immunized with IgG1 and IgG2a fusion proteins 24 hours after lethal challenge. Survival studies are representative of three individual experiments consisting of 6 mice per group (**, $P < 0.05$).

The protection conferred by the IgG1 Fc fusion protein in the KO mice was similar to that of the IgG2a fusion protein observed in the genetically deficient animals as well as the wildtype controls. Additionally, lung bacterial burden was lowest for fusion protein immunized KO mice, and no significant difference was observed between the two fusion protein regiments (Fig. 8B).

G. Immunization with IgG1 fusion protein induces antigen specific antibodies and proinflammatory cytokines in FcγRIIB deficient mice.

Given the improvement in survival observed in the FcγRIIB KO we sought to determine the impact of FcγRIIB on antigen specific antibody production in animals immunized with IgG1 Fc-PspA. The levels of PspA-specific IgG and IgA were both elevated following IgG1 fusion protein immunization in FcγRIIB KO, while PspA alone showed no improvement compared to the titers induced in WT animals (Fig. 9).

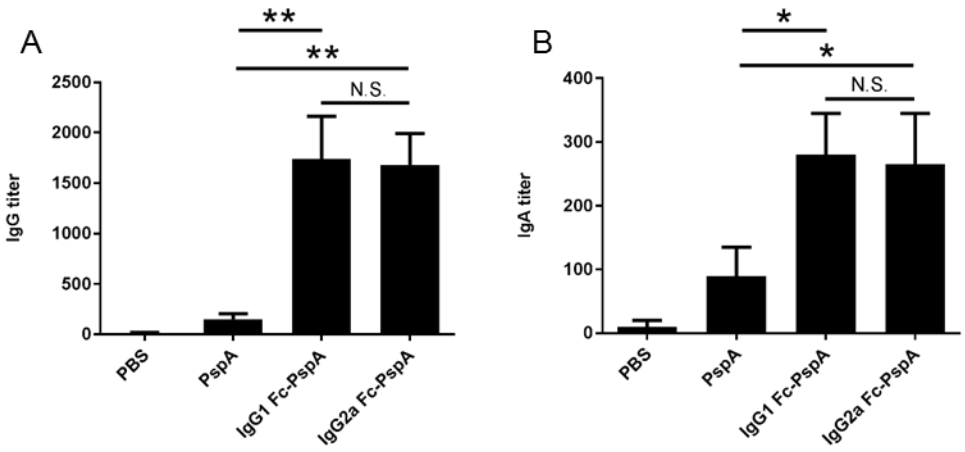


FIGURE 9. The PspA specific humoral response induced by IgG1 Fc-PspA is improved in FcγRIIB KO mice. FcγRIIB KO mice were immunized i.n. on day 0 and 21. Serum was collected on day 35 and levels of PspA-specific IgG (A) and IgA (B) were measure by ELISA. Statistical analysis was performed using an unpaired two-tailed t test (*, $P < 0.1$; **, $P < 0.05$, N.S., No significant difference).

The absence of FcγRIIB improved protective antibody titers to a level similar to what was observed for IgG2a fusion proteins. While previous studies have shown increases in total serum IgG in FcγRIIB KO versus WT mice, we observed no significant differences in the levels of PspA specific antibodies between FcγRIIB KO and WT mice (104). Previous studies have also demonstrated increased proinflammatory cytokine production in the absence of FcγRIIB (67). Consistent with these observations we measured significantly higher amounts of IL-12, IFN-γ, IL-2 and TNF-α in naive FcγRIIB KO mice 24 hours post infection, compared to WT mice at the

same time point. The higher baseline level of proinflammatory cytokine induction made it difficult to ascertain significant differences in cytokine production for IL-2 and IFN-γ in the lung driven by fusion protein immunization and were omitted from the analysis. Even so, levels of TNF-α and IL-12 in the lung were still notably increased over PspA immunization alone (Fig. 10A,10B). Similarly, IFN-γ in the spleen was significantly increased in the animals receiving IgG1 Fc-PspA compared to the PspA immunized group (Fig. 10C).

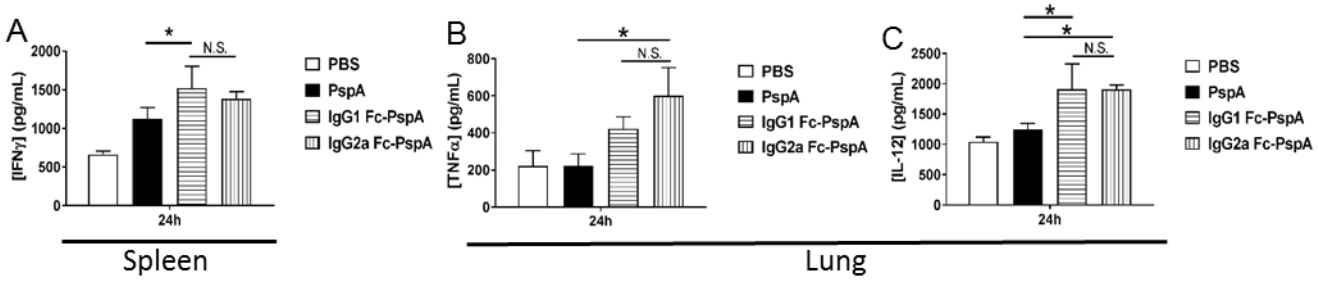
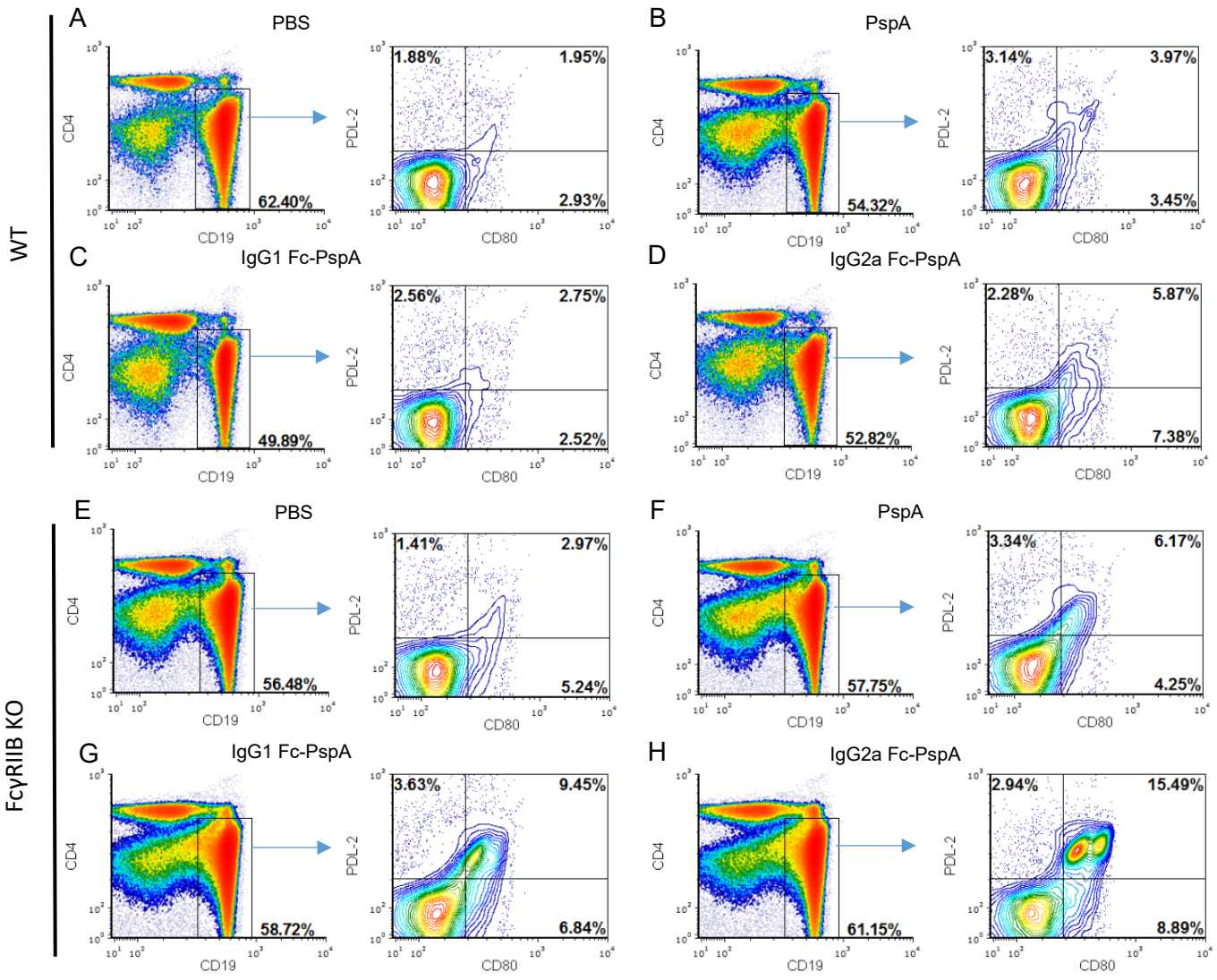


FIGURE 10. Proinflammatory cytokines are increased in the lung and spleen of FcγRIIB knockout mice immunized with IgG1 Fc-PspA. FcγRIIB knockout animals were immunized, boosted and infected on day 0, 14, and 28 respectively. The lung and spleen were harvested 24 hours following infection TNF-α (A), IL-12 (B), and IFN-γ (C) were measured by ELISA. Statistical analysis was performed using an unpaired two-tailed t test (*, $P < 0.1$; **, N.S., No significant difference).

H. B-lymphocytes in the spleen of WT and FcγRIIB knockout mice express memory markers and proliferate in response to PspA ex vivo stimulation following immunization with Fc fusion proteins.

FcγRIIB is the only FcγR receptor expressed on the surface of B-lymphocytes and is a primary modulator of cell responses emanating from the B-cell receptor (BCR). Crosslinking FcγRIIB with the BCR negatively regulates B-cell signaling, antigen uptake, and promotes apoptosis (105). Efficient, long lasting protection against bacterial infection is reliant on humoral immunological memory controlled by memory B-cells which secrete antigen-specific antibodies in response to previously encountered pathogen. Murine memory B-cells can be characterized by

expression of cell surface markers CD80 and PD-L2, independent of BCR isotype. Double positive populations expressing both CD80 and PD-L2 are a functionally distinct memory B-cell subset which differentiate into antibody forming cells upon reoccurring exposure to antigen, thus providing a reservoir for long-lasting humoral immunity (106). B-cells expressing the double positive memory phenotype were highest in WT and FcγRIIB knockout animals immunized with IgG2a Fc-PspA. Similarly, the IgG1 fusion protein immunization elicited a higher frequency of double positive cells than PspA alone, with no significant difference observed between the two fusion proteins (Fig. 11). The response was significantly augmented in the animal's deficient for FcyRIIB. Similarly, CD19+ B-cells from FcyRIIB KO mice immunized with either of the two fusion proteins had a greater propensity to proliferate ex vivo when restimulated with PspA than their WT counterpart (Fig. 12).



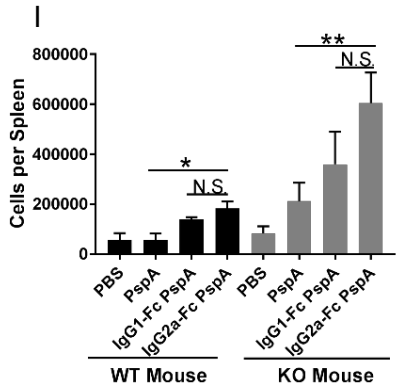


FIGURE 11. Targeting activating FcγR promotes memory-like B-cells in the spleen of immunized mice which is enhanced in the absence of FcγRIIB. Two weeks following the second boost splenocytes harvested from immunized WT (A-D) and FcγRIIB (E-H) knockout mice and stained for functional memory B-cell markers. (I) Average number of CD80, PD-L2 double positive B-cells (DP) in the spleens of immunized WT and FcγRIIB Knockout mice. Statistical significance was determined using an unpaired two-tailed t test (*, $P < 0.1$; **, N.S., No significant difference).

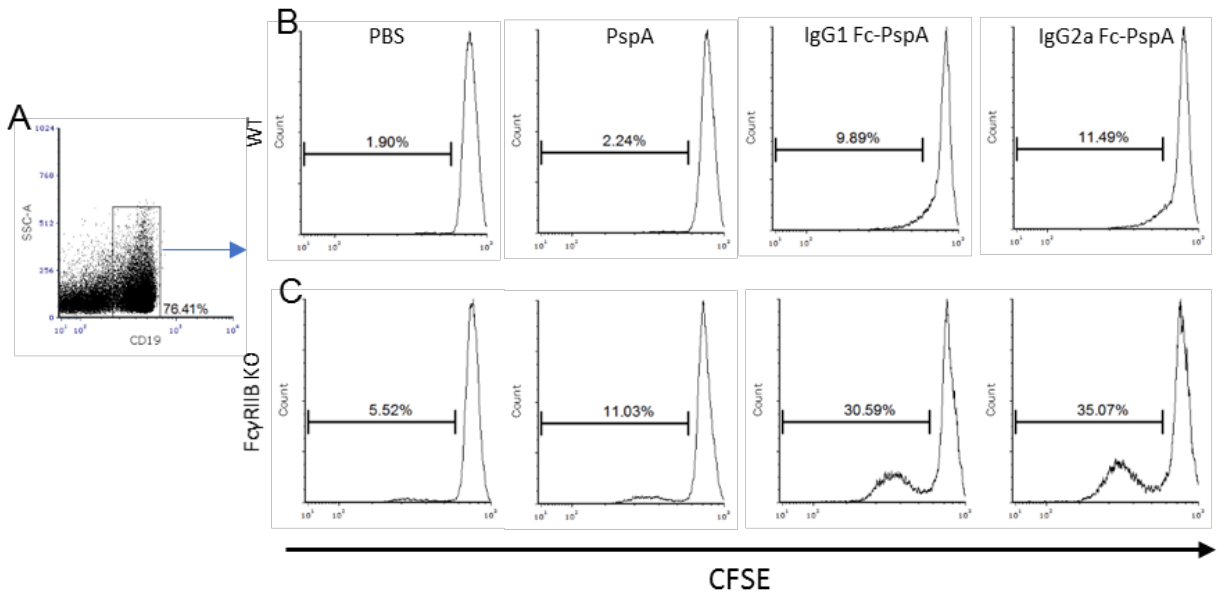


FIGURE 12. CD19+ B-Cells proliferate on secondary exposure to PspA. Single cell suspensions from the spleen of immunized wild-type (WT) and FcγRIIB knockout mice were stained with CFSE and stimulated with 5ug/mL of purified PspA for 4 days. (A) CD19+ B-cells were gated in histograms of florescent intensity versus cell count for both WT (B) and FcγRIIB knockout (C) mice.

V. Discussion

FcγR are primary regulators of IgG effector mechanisms *in vivo* and have been shown to be an effective immunomodulatory target to augment vaccine-induced immunity. Engagement of FcγR with the immunoglobulin heavy chain regulates functions such as antigen presentation, antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, and myeloid cell activation and proliferation. Activating these effector functions would be advantageous to producing mucosal and humoral immunity and protecting against microbial infections. Additionally, an FcγR targeting vaccination would also benefit from interactions with FcRn. While not specifically studied in this thesis FcRn has been shown to facilitate transfer of IgG bound vaccine antigens across the mucosal epithelia (84). Uptake of IgG fusion proteins by FcRn could help shuttle antigen across the mucosal surface and prolong the half-life of circulating IgG, in addition to the benefits conferred by engagement of activating FcγR.

In the current study we harnessed the affinity of IgG2a to target activating FcγR in the form of an adjuvant-free, mucosally administered Fc-PspA fusion protein. We then sought to characterize the protective response in key cell types of innate and adaptive immunity which are endogenously regulated by FcγR such as macrophages, dendritic cells, and B-cells.

Alveolar macrophage are a key facet of innate immunity and protect against early Spn infection in the lung through bacterial clearance and production of microbicidal inflammatory mediators. FcγR signaling in macrophages strongly influences the functional polarization and propensity for pathogen clearance. It was previously shown PspA targeted to human FcγRI enhanced protection against pneumococcal infection through increased bactericidal activity and antibody mediated complement deposition (78). In the current study we utilized the inherent binding affinity of the IgG2a Fc region for activating FcγR, by targeting PspA to FcγR on the

surface of immune effectors leading to enhanced humoral and cellular immune responses. By targeting PspA to activating receptors macrophage in the lung of IgG2a fusion protein immunized mice were polarized to the AM1 phenotype following lethal challenge with Spn. Furthermore IFN-γ, a Th1 cytokine which was abundant in the lung and spleen of IgG2a immunized mice, is a primary cytokine associated with M1 activation. Both AM1 and IFN-γ are important contributors to host defense against Spn. IFN-γ secreted by Th1 cells at the site of infection can stimulate macrophage to release microbiocidal factors such as metalloproteinases and nitric oxide that boost phagocytic killing of invasive bacteria (107). Additionally, IFN-γ has been shown to increase the expression of FcγR on the surface of macrophage and dendritic cells, thus perpetuating the anti-PspA immune response in animals receiving IgG2a fusion proteins (108). To this end, we observed coordinating increases in IFN-γ, AM1 polarization, and cDC recruitment in the lungs of IgG2a Fc-PspA immunized mice who were better protected against lethal Spn challenge.

The proinflammatory cytokine TNF-α was also locally increased in the lung within 24 hours of infection but subsided when measured 48 hours after bacterial challenge. TNF-α is a pleiotropic cytokine produced by multiple effector cells, including stimulated alveolar macrophage, in the first minutes to hours following pathogen recognition and response. Expression of this proinflammatory cytokine serves to regulate neutrophil recruitment and mediate adaptive mechanisms such as T-cell activation and B-cell stimulation (109). In mice administration of a TNF-α blocking antibody increases bacterial infiltration in blood and accelerates mortality of infected mice (110). Even so, prolonged and excess production of TNF-α is deleterious resulting in acute pulmonary damage and sepsis (111). To this end, an increased risk of shock and death has been recorded in patients with excessive TNF-α production (112).

Early resolution of pulmonary inflammation, as was observed in this study, is a beneficial outcome of the fusion protein immunization strategy to address invading Spn while minimizing tissue damage.

Dendritic cells are central regulators of immune responses by providing a bridge between innate and adaptive immunity. Activating and inhibitory FcγR are of central importance in controlling DC activation and thus influencing adaptive immunity. In mice, FcγR crosslinking by immune-complexes on the surface of DCs initiates a gene expression profile consistent with T cell activation which is absent in FcγR knockout mice. In the current study immunization with IgG2a Fc-PspA increased the number of cDC1 and cDC2 subsets in the lungs of mice 24 hours after Spn infection. Both cDC1 and cDC2 subsets in the lung have been attributed to Th1 priming and activation, historically, cDC1 have been associated with type 1 responses due to non-redundant IL-12 production essential for Th1 polarization. Separately, cDC2 appear to regulate IgA class switching both *in vitro* and *in vivo* in mice (113,114). In the current study we saw increases in Th1-like cytokines in the lungs of immunized mice, such as IL-12, as well as antigen specific IgG and IgA. These observations suggest that the increased cDC1 and cDC2 populations in the lungs of immunized mice may have provided the necessary stimulatory signals to T-cells, in the form of increased antigen presentation and IL-12 production, which in turn provided cognate help to B-cells leading to increased antigen specific antibody responses. Effective production of PspA-specific antibodies is especially important in controlling endogenous PspA driven virulence as antibodies directed against PspA can overcome complement inhibition and increase C3 deposition on the surface of infiltrating Spn (115).

While FcγR targeting was broadly beneficial to key facets of cellular and humoral immunity, interaction with the inhibitory FcγRIIB can dampen the response conferred by FcγR

targeting. This was particularly evident in fusion proteins containing IgG1 Fc, which has higher affinity for the inhibitory FcγR. In WT mice, immunization with IgG1 Fc-PspA provided no additional protection over the PspA antigen alone. In the absence of FcγRIIB, immunization with the IgG1 Fc-PspA was comparably efficacious to IgG2a Fc-PspA in terms of protection and generation of cellular and humoral immune responses. For example, targeting FcγR with IgG1 fusion proteins in FcγRIIB deficient mice decreased bacterial burden to a similar level as IgG2a fusion proteins. The reduced bacterial burden was likely driven by increased alveolar macrophage activation as was observed in the WT mice. Consistent with this, the phagocytic capacity of macrophage during C. rodentium infection have demonstrated increased capacity when FcyRIIB is not engaged (116). In the absence of FcγRIIB IgG1 fusion proteins also increased TH1 like cytokines and PspA-specific antibodies demonstrating the importance of FcγRIIB signaling in T and B-cell regulatory functions.

While IgG1 has highest affinity for the FcγRIIB, this isotype can also bind to the activating receptor FcγRIII, which likely drove protective immunity against Spn in the FcγRIIB knockout model. Similarly, improvement in anti-tumor activity of IgG1 has been observed in a murine metastatic melanoma model conducted in FcγRIIB deficient mice (46). Each of the immunizations administered benefited from the absence of FcγRIIB, but to a smaller degree than IgG1 Fc-PspA. Most notably, IgG2a Fc-PspA was 100% effective in the KO model compared to 75% survival achieved in the WT animals. This suggests even the minor association of IgG2a with FcγRIIB can subtly affect the biological activity of this isotype *in vivo.*

The FcγRIIB receptor is the only FcγR expressed on the surface of B-cells, the expression of which is upregulated on activated B-cells. Additionally, IFN-γ which we found to be increased in the spleen of both groups immunized with the two fusion proteins, increases FcγRIIB expression

on the surface of B-cells (117). B-cell maturation and differentiation into the memory compartment is clearly imperative for long lasting protection against Spn, and correlate with protection from acquisition further preventing horizontal transmission. As such, engagement of FcγRIIB on the surface of B-cells could greatly influence vaccine outcomes. For this reason, we investigated the role of FcγRIIB expression on B-cell differentiation and proliferation in our FcγR targeting vaccine model. Subsequently, we found that WT mice immunized with IgG2a Fc-PspA had the greatest number of B-cells expressing memory markers compared to the other treatment groups. Moreover, the total number of these cells were significantly increased in the mice deficient for FcγRIIB. Given the importance of FcγRIIB in B-cell selection, it is possible that BCR crosslinking in the absence of FcγRIIB allowed for survival and activation of low affinity clones that would have otherwise undergone apoptosis in the presence of FcγRIIB. Why this response was more prolific in IgG2a Fc-PspA immunized mice may be dependent on augmented T-cell activation exacerbated by targeting activating FcγR in the absence of FcγRIIB, which can in turn provide the necessary costimulatory signals to promote B-cell proliferation and differentiation. To this point, FcγRIIB KO mice have demonstrated increased activation of antigen-specific T-cell responses following utilization of IgG immune complexes as a targeting vaccine strategy (118). T-cell dependent B-cell memory is developed through two distinct mechanisms. In the first, memory B cells go through affinity maturation in the germinal center by interaction with cognate follicular T-cells. In a second pathway memory T-cells develop independent of the germinal center with the help of systemic T-cells. Newly formed memory B-cells then exit to peripheral circulation and differentiate rapidly to memory plasma cells on re-exposure to antigen (119). Similarly, we observed B-cells isolated from fusion protein immunized mice were more likely to proliferate *ex vivo* when cultured with PspA, and thus

primed to provide a secondary response to PspA exposure. This would suggest the B-cell expansion in the KO animals was largely in response to the immunomodulatory capabilities of the FcγR targeting fusion proteins, instead of maturation of low affinity B-cells, or a lowered threshold of activation for a weak Toll-like receptor ligand, such as PspA. But since antigen specific memory responses were higher in FcγRIIB deficient mice, it appears this inhibitory receptor attenuates many facets of humoral memory following fusion protein vaccination.

VI. Conclusions

Collectively, FcγR targeting by IgG2a Fc-PspA fusion protein proved to be an effective vaccine strategy for inducing both cellular and humoral protection against mucosal Spn challenge. Protection was associated with increased AM1 and cDC subsets in the lung, which decreased bacterial burden and increased Th1 polarization and B-cell activation. Additionally, we show immunization with IgG1 Fc-PspA which selectively binds FcγRIIB caused decreased efficiency in various aspects of protective immunity including bacterial clearance, T-cell activation, antigen presenting cell polarization, and B-cell maturation. Consistent with these observations the potency of IgG1 Fc-PspA was highly influenced by the presence of FcγRIIB, which had only a subtle impact on the efficacy of IgG2a Fc-PspA. These findings emphasize the effectiveness of FcγR targeting, and highlight the role played by FcγRIIB in modulating the efficacy of this vaccination strategy against a respiratory pathogen.

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