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A PROMISING NOVEL FORMULATION OF *STAPHYLOCOCCUS AUREUS* VACCINE PROTECTS MICE VIA ANTIBODIES AND $CD4^+$ EFFECTOR T CELLS

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Summary

Life-threatening *Staphylococcus aureus* infections and the emergence of antibiotic-resistant strains make vaccination a major medical need. Novartis Vaccines developed a vaccine candidate (Combo) that consists of Hla_{H35L}, a non-toxic mutant of the α-toxin, EsxAB, a fusion of the secreted factors EsxA and EsxB, and of two surface proteins, FhuD2 and Sur2. Combo±alum has shown efficacy in pre-clinical mouse models and has recently been tested in a human phase I clinical trial.

To improve this vaccine further, Combo antigens were formulated with alum/SMIP-7.10 (alum-S-7), a novel adjuvant formulation composed of a Toll-like receptor 7 agonist small molecule adsorbed to alum. We characterized the immune responses elicited by this novel formulation, and compared its efficacy to that induced by Combo±alum in a mouse model of *S. aureus*-induced peritonitis.

Combo+alum-S-7 showed increased efficacy than Combo+alum, with 75%, respectively 40%, of mice surviving a lethal challenge. Compared to Combo+alum, Combo+alum-S-7 induced higher vaccine-specific antibody titers, and polarized $CD4^+$ T-cell responses towards Th1 and Th17 effectors (IFN-γ or IL-17 production, respectively). *In vivo* depletion of $CD4^+$ effector T cells in mice vaccinated with Combo+alum-S-7 reduced the efficacy of this vaccine by 20%. The residual protection was likely due to Combo-specific

antibodies since passive transfer of sera from mice immunized with Combo+alum-S-7 to naïve mice resulted in 30% of survival. The requirement of B cells/antibodies to confer protection against *S. aureus* in the peritonitis model by Combo vaccination was confirmed by the death of 96% of B-cell ko J_H mice vaccinated with Combo+alum/S-7 or Combo+alum. *In vivo* neutralization of IL-17A, either alone or together with IFN-γ, but not of IFN-γ alone, increased bacterial loads in kidneys of mice immunized with Combo+alum-S-7 as did the depletion of $CD4^+$ effector T cells.

Overall these data show that adding SMIP-7.10 to Combo+alum increases the efficacy of this candidate vaccine through combined antibody and Th17 responses in an animal model of *S. aureus* infection.

Riassunto

L'emergenza di ceppi di *Staphylococcus aureus* resistenti agli antibiotici rende il trattamento dei pazienti più difficile e, al tempo stesso, più urgente la necessità di un vaccino. Novartis Vaccines ha sviluppato un candidato vaccino (Combo) che consiste di: Hla_{H35L}, un mutante atossico della tossina-α; EsxAB, fusione delle due proteine secrete EsxA e EsxB; FhuD2 e Sur2, due proteine di superficie. È stato dimostrato che Combo±alum è efficace in modelli animali preclinici ed il vaccino è stato recentemente testato nella fase I di un clinical trial.

Al fine di migliorare ulteriormente il vaccino, gli antigeni di Combo sono stati formulati con alum/SMIP-7.10 (alum-S-7), un nuovo adiuvante composto da una small molecule, agonista del Toll-like receptor 7, adsorbita all'alum. Abbiamo caratterizzato le risposte immunitarie indotte da questa nuova formulazione e abbiamo confrontato la sua efficacia con quella di Combo±alum in un modello murino di peritonite indotta da *S. aureus*.

Combo+alum-S-7 ha dimostrato un'efficacia superiore a quella di Combo+alum, con il 75% dei topi, rispetto al 40%, che sopravvivevano all'infezione letale. In confronto a Combo+alum, Combo+alum-S-7 ha indotto titoli anticorpali specifici per il vaccino più elevati e ha polarizzato le riposte T $CD4^+$ verso risposte di tipo Th1 e Th17 effettrici (produzione di

IFN-γ o IL-17, rispettivamente). La deplezione *in vivo* delle cellule T $CD4^+$ effettrici nei topi vaccinati con Combo+alum-S-7 ha ridotto l'efficacia del vaccino del 20%. La protezione residua era verosimilmente dovuta agli anticorpi specifici per il vaccino: un passive transfer di sieri di topi immunizzati con Combo+alum-S-7 in topi naïve ha infatti conferito una protezione del 30%. La necessità della presenza di cellule B/anticorpi per proteggere i topi vaccinati con Combo dall'infezione con *S. aureus* nel modello di peritonite è stata confermata dalla morte del 96% dei topi J_H (deficienti delle cellule B) vaccinati con Combo+alum/S-7 o Combo+alum. La neutralizzazione *in vivo* di IL-17A, sia da sola che in combinazione con IFN-γ, ma non del solo IFN-γ, ha aumentato la carica batterica nei reni dei topo immunizzati con Combo+alum-S-7, come successo in seguito a deplezione delle cellule T $CD4^+$ effettrici.

Nell'insieme, questi dati dimostrano che l'aggiunta dello SMIP-7.10 al vaccino Combo+alum ne aumenta l'efficacia in un modello animale di infezione con *S. aureus* attraverso l'induzione di anticorpi e risposte Th17.

Contents

Chapter 1

Introduction

1.1 *Staphylococcus aureus*

Staphylococci are gram-positive bacteria belonging to the family of Micrococcaceae that form grape-like clusters on Gram's stain (Figure 1.1). These organisms are catalase-positive, non-motile, aerobic, and facultative anaerobic. They are capable of prolonged survival on environmental surfaces in varying conditions. *S. aureus* is distinguished from other staphylococcal species on the basis of the gold pigmentation of colonies and positive results of coagulase, mannitol fermentation, and deoxyribonuclease tests [1].

1.1.A *S. aureus* structure and components

S. aureus has a nuclear body surrounded by a plasma membrane composed primarily of proteins and phospholipids (about 3:1). The cell wall is 50% peptidoglycan (PGN), confers the characteristic cell shape and provides the cell with mechanical protection. Most staphylococci present a microcapsule [2].

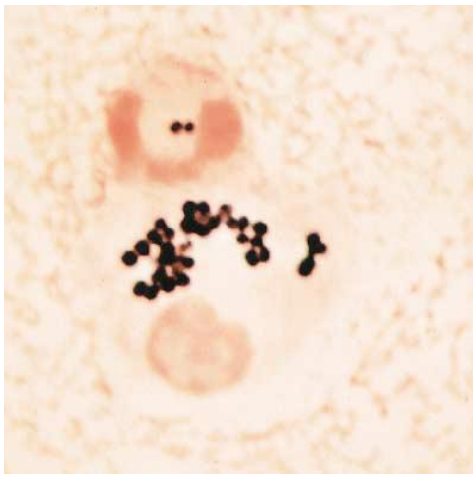


Figure 1.1 Gram's stain of *S. aureus*. Leukocytes in a sputum sample (Gram's Stain, 1,000x) [3].

1.1.A.1 Genome

The staphylococcal genome consists of a ≈2,800 bp circular DNA, with prophages, plasmids, and transposons. Virulence and resistance to antibiotics are regulated by genes found on the chromosome, as well as on extra-chromosomal elements [3]. Comparative analysis revealed that most regions of the staphylococcal genome are well conserved, whereas several large sequence blocks display high variability. The overall combination of variable sequence elements and the encoded spectrum of virulence properties varies from strain to strain and appears to be reflective of the overall large spectrum of clinical disease manifestations in humans [4].

1.1.A.2 Cell wall

The staphylococcal cell wall is made of a thick layer of PGN, which consists of alternating polysaccharide subunits of N-acetylglucosamine and N-acetylmuramic acid with 1,4-β linkages. The PGN chains are cross-linked by tetrapeptide chains bound to N-acetylmuramic acid and by a pentaglycine bridge specific for *S. aureus*. In this layer, there are PGN-anchored (teichoic acid) and membrane-anchored (e.g., lipoteichoic acid) glycopolymers as well as lipoproteins and covalently and non-covalently attached proteins [5]. The best-characterized pathogen-associated molecular patterns (PAMPs) of *S. aureus* are components of its cell wall or their fragments (reviewed in [6]).

1.1.A.3 Capsule

The capsule is composed by polysaccharides: 11 types of microcapsular polysaccharide serotypes have been identified. Serotypes 5 and 8 account for 75% of human infections; most methicillin-resistant *S. aureus* isolates are type 5. The chemical composition of only 4 of these antiphagocytic polysaccharides, including types 5 and 8, has been determined [7].

1.1.A.4 Surface proteins

Many staphylococcal surface proteins are characterized by common features which are implicated in the proper secretion and attachment of these proteins to the cell surface and in the interaction with the host. These common features are a secretory signal sequence at the

N-terminal, positively charged amino acids that extend into the cytoplasm, a hydrophobic membrane-spanning domain, and a cell-wall–anchoring region, all at the C-terminal [8]. A ligand-binding domain at the N-terminal that is exposed on the surface of the bacterial cell enables some of these proteins to function as adhesins. Protein A is the prototype of these proteins and has anti-phagocytic properties due to its ability to bind the Fc portion of immunoglobulins. Several of these related proteins bind extracellular matrix molecules and play an important part in the ability of *S. aureus* to colonize host tissues [3].

1.1.A.5 Toxins

Depending on the strain, *S. aureus* is capable of secreting several exotoxins, which can be categorized into 3 groups on the basis of their mechanism of action. Many of these toxins are associated with specific diseases. Cytotoxins, such as the α-toxin, also known as α-hemolysin (Hla), cause pore formation in mammalian cells and induce pro-inflammatory cytokine release. The consequent cellular damage may contribute to manifestations of the sepsis syndrome [9] [10]. The pyrogenic-toxin superantigens bind to major histocompatibility complex class II proteins, causing non-specific activation of T cells resulting in polyclonal T-cell activation and massive cytokine release [11] leading to the toxic shock syndrome and food poisoning [12]. The exfoliative toxins cause skin erythema and separation, as seen in the staphylococcal scalded skin syndrome. Their mechanism of action remains controversial. Panton Valentine leukocidin is a leukocytolytic toxin that has been epidemiologically associated with severe cutaneous infections [13].

1.1.B *S. aureus* epidemiology and pathogenesis

S. aureus is frequently part of the commensal flora found in the nose and on skin. Approximately 20% of individuals almost always carry one type of strain and are called persistent carriers. A large proportion of the population (≈60%) harbors *S. aureus* intermittently, and the strains change with varying frequency. Such persons are called intermittent carriers. Finally, a minority of people (≈20%) almost never carry *S. aureus* and are called non-carriers [14].

S. aureus is both a commensal organism and a pathogen. It is responsible for nosocomial and community-acquired infections that range from minor skin infections, such as pimples, impetigo, and scalded skin syndrome, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, and toxic shock syndrome. Infections of the blood can carry the bacteria to various deep tissues, including bones, joints, organs, and the respiratory system. *S. aureus* is a pluripotent pathogen, causing diseases through both toxin-mediated and non–toxin-mediated mechanisms [3].

More than 30 staphylococcal species are pathogenic: among them *S. aureus* is the most virulent one. The “other” staphylococci are collectively designated coagulase-negative staphylococci (CoNS), as only *S. aureus* produces coagulase, a surface enzyme that converts fibrinogen to fibrin. CoNS are considerably less virulent than *S. aureus* but remain important pathogens in infections associated with prosthetic devices [15].

1.2 The immune system

The word *immunity* means protection against foreign substances that are not normally present in the organism or are not part of it. The cells and molecules responsible for immunity constitute the *immune system*, and their collective and coordinated response to the introduction of foreign substances is called the *immune response*. The key to a healthy immune system is its remarkable ability to distinguish between the body's own cells, recognized as "self," and foreign cells, or "non-self." The body's immune defenses normally coexist peacefully with cells that carry distinctive "self" marker molecules. But when immune defenders encounter foreign cells or organisms carrying non-self markers they quickly launch an attack. Anything that can trigger this immune response is called an antigen (Ag). Ag (ANTIbody GENerator) was originally defined as a structural molecule that binds specifically to the antibody (Ab), but the term now also refers to any molecule or molecular fragment that can be recognized by highly variable Ag receptors on B cells (BCR) or T cells (TCR) of the adaptive immune system. An Ag can be a microbe, such as a bacterium or a virus, or a part of a microbe such as a bacterial protein.

In abnormal situations, the immune system can mistake self for non-self and launch an attack against the body's own cells or tissues. The result is an auto-immune disease. Some forms of arthritis and diabetes are auto-immune diseases. In other cases, the immune system responds to a seemingly harmless foreign substance such as ragweed pollen. The result is allergy, and this kind of Ag is called an allergen.

In order to escape host's immunity, microbes tend to evolve introducing a greater variability in their phenotype. Therefore different and complex mechanisms are necessary to face microbial invasion, and indeed the immune system is composed of different cell types and an enormous

number of molecular mediators. Defense against microbes is mediated by different types of immune responses: innate and adaptive.

Innate immunity (also called natural or native immunity) provides the early line of defense against microbes [16]. This type of response is very fast, highly efficient and addresses broad classes of pathogens in an Ag–non-specific manner.

The principal components of innate immunity are physical and chemical barriers, phagocytic cells (neutrophils, Nϕ, and macrophages, Mϕ), natural killer (NK) cells, blood proteins (including members of the complement system and other mediators of inflammation), and proteins called chemokines and cytokines that regulate and coordinate many of the activities of the cells of the immune system. When a pathogen enters in the body for the first time, it is recognized and is phagocytosed by different immune cells like Mϕ, Nϕ, monocytes (Mo), and dendritic cells (DC), which produce inflammatory cytokines and chemokines that attract more specialized immune cells to the site of infection. The mechanisms of innate immunity are specific for broad classes of microbes and may not distinguish fine differences between foreign substances.

Adaptive immunity is induced by exposure to infectious agents and increases in magnitude and defensive capabilities with each successive exposure to the same microbe. This form of immunity develops as a response to a particular infection and adapts to it, and therefore is called adaptive immunity. The defining characteristics of adaptive immunity are specificity, and ability to “remember” and respond more vigorously to repeated exposures to the same microbe. Because the adaptive immune system has an extraordinary capacity to distinguish between different, even closely related, microbes and molecules, it is also called specific immunity.

The main components of adaptive immunity are B and T lymphocytes and their secreted products, such as antibodies (Abs). B and T lymphocytes go through a long maturation and selection process during the first period of their life. At the end of this period, only functional B and T lymphocytes with a low affinity for self-Ag are selected. When these cells encounter the Ag for which they are specific, they undergo clonal expansion leading to a very specific immune response. The invading pathogen is eliminated through release of specific Abs and the action of effector T cells. Moreover, some of the Ag-specific B and T cells can differentiate into memory cells that can recognize immediately the same pathogen upon re-encounter. As a result the memory immune response is faster and stronger [16].

1.3 Innate immunity

The innate immunity constitutes the first line of defense against infection and in many cases is sufficient to eliminate the invading agent. In other cases it cannot cope with the infection, but it initiates the development of an adaptive immune response.

Innate immune recognition of infectious agents is based on two main principles: the detection of PAMPs and aberrant localization of specific classes of molecules.

PAMPs are microbial molecular structures that are evolutionarily conserved, and hence shared between different microbial species [17]. Most PAMPs are essential for microbial growth and survival of the microorganisms to which they belong, and therefore these molecular patterns are rarely modified by the microorganism as a mean to avoid innate

recognition [16] [17]. PAMPs include lipids, lipopeptides, polysaccharides and nucleic-acid structures that are broadly expressed by different groups of microorganisms. The cells of the innate immune system are able to recognize these molecular patterns by pattern recognition receptors (PRRs), which are innate immune receptors extremely conserved throughout evolution.

The second principle in innate recognition is the presence of specific molecular structures in unusual intracellular compartments. Viruses, intracellular bacteria, and some parasites replicate inside the cells they infect. The innate immune system senses the presence of viruses through detection of viral glycoproteins at the cell surface, or viral nucleic acids in endosomes or in the cytoplasm. Some bacteria replicate inside eukaryotic host cells either in endosomes or in the cytoplasm. These bacteria activate the innate system through lipid components, cell wall products, and bacterial nucleic acids. These microbial molecules are recognized by specialized PRRs as non-self molecules due to their unusual intracellular localization, thus stimulating innate response.

All the principal components of the innate immunity cooperate at the elimination or the attenuation of the infection.

Epithelial barriers not only constitute a physical obstacle to the infection, but they also produce chemical substances that are microbicidal or that inhibit microbial growth, such as defensins that are peptides with a high bactericidal activity.

Phagocytic cells, such as Nϕ, Mo, and Mϕ capture the microbe and kill it in the phagolysosomes through different mechanisms. Mϕ and Nϕ recognize pathogens by means of cell-surface receptors that can discriminate between the surface molecules displayed by pathogens and

those of the host. In many cases, binding of a pathogen to these cell-surface receptors leads to phagocytosis, followed by the death of the pathogen inside the phagocyte. Phagocytosis is an active process in which the pathogen is first surrounded by the phagocyte membrane and then internalized in a membrane-enclosed vesicle, known as a phagosome or endocytic vacuole. The phagosome then becomes acidified upon fusion with one or more lysosomes. Lysosomes are membrane-enclosed granules that contain proteolitic enzymes and peptides that can attack the microbe. The phagosome fuses with one or more lysosomes to generate a phagolysosome in which the lysosomal contents are released to destroy the pathogen. Upon phagocytosis, Mϕ and Nϕ produce a variety of other toxic products that help kill the engulfed microorganism. The most important of these toxic products are the antimicrobial peptides and nitric oxide (NO), the superoxide anion, and hydrogen peroxide which are directly toxic to bacteria.

NK cells are the effector cells of the innate immune response and are able to kill microbes by producing perforins or by enhancing the phagocytic activity of Mϕ. NK cells can recognize microbes and cells infected by viruses or intracellular bacteria in an Ag–non-specific manner.

Finally, important components of innate immunity are soluble molecules, such as cytokines, chemokines, and plasma proteins that constitute the complement system. These molecules are produced by cells of the innate system and are active on cells of both the innate and adaptive immunity. Chemokines release favors the leukocyte mobility from the lymph nodes to the site of infection through the endothelial barriers. Cytokines promote the phagocytic ability of Mϕ and Nϕ, the cytotoxic activity of NK cells, and the differentiation and activation of T and B cells [16] [17] [18]. The complement proteins react with one another to opsonize pathogens and induce a series of inflammatory responses that help to fight infection.

The innate immunity, although characterized by non-specific and short-term responses, plays an important role in the initiation of the adaptive immunity.

1.4 Adaptive immunity

The adaptive response is divided into *humoral* response and *cell-mediated* immune response. The main components of adaptive immunity are cells called *B and T lymphocytes* and their secreted products, such as Abs. These cells are responsible for exerting effector functions upon receiving signals from innate cells. B and T lymphocytes go through a long maturation and selection process during the first period of their life. B lymphocytes mature in the bone marrow and mount the humoral immune response, while T lymphocytes mature in the thymus and are responsible for the cell-mediated immune response. Mature B and T cells leave the bone marrow or the thymus, enter the bloodstream and migrate to the peripheral lymphoid organs. Circulating mature B and T cells that have not encountered their specific Ags are called naïve B and T cells, respectively.

Mature B cells express a unique membrane-bound BCR, an Ab molecule specific for a given Ag. When a B cell recognizes its cognate Ag through the BCR, the B cell proliferates and differentiates into a terminally differentiated B cell, called plasma cell, able to secrete *Abs* (also called *Immunoglobulins*, Igs) of the same Ag specificity of its BCR [19]. Secreted Igs have two separate functions: one is to bind specifically molecules from the pathogens, leading to their neutralization; the other is to mediate the

phagocytosis of the pathogen bound to Igs through a process known as opsonophagocytosis.

There are different types of Abs, known as IgM, IgA, IgG, IgD and IgE. Each Ig *isotype* differs in its biological properties and in the capability to bind different Ags. All B cells initially express and synthesize IgM, but switch to other isotypes upon gene rearrangement, a mechanism known as isotype-switch. Abs of switched isotypes maintain their specific Ag-recognition capacity but differ in other domains of the molecule that determine their functionality. IgA are usually present in different mucosal body fluids and are important in the control of the local infections. IgG are the most abundant Ig isotype present in the serum, and can be divided in 4 different classes in humans (IgG1, IgG2, IgG3, and IgG4) and in mouse (IgG1, IgG2a, IgG2b, and IgG3). IgD are present on the surface of mature but not immature B lymphocytes; they bind the Ag and stimulate the cells to grow and differentiate in plasma cells. IgE are important against parasite infections, and in the allergic reactions.

Intracellular microbes, such as viruses and some bacteria, can survive and proliferate inside phagocytes and other cells, where they are inaccessible to circulating Abs. Defense against such infections is a function of cell-mediated immunity, which promotes the destruction of microbes residing in phagocytes or the killing of infected cells to eliminate reservoirs of infection.

T lymphocytes are the cells responsible for the cell-mediated immune response; to participate in an adaptive immune response, a naïve T cell must first encounter an Ag presenting cell (APC) that presents specific antigenic peptide to the TCR. After priming by APCs, naïve T cells can proliferate and differentiate into effector or memory T cells [20]. T cells belong to two main classes characterized by the cell-surface proteins *CD4*

and *CD8*. The expression of CD4 and CD8 determines the interactions the T cell makes with other cells.

T cells detect peptides derived from foreign Ags: after Ags are degraded within APCs, their peptide fragments are complexed with MHC molecules and this complex is displayed on the cell surface. The *MHC molecules* are glycoproteins encoded in the large cluster of genes known as the Major Histocompatibility Complex. There are two class of MHC molecules, called MHC class I and MHC class II, which differ in both their structure and expression pattern on the tissues of the body.

MHC class I molecules are expressed on virtually all nucleated cells and consist of two polypeptide chains. The α chain is encoded in the MHC (on chromosome 6 in humans) and is non-covalently associated with a smaller chain, β2-microglobulin, which is not polymorphic and is encoded on a different chromosome (chromosome 15 in humans). The α chain folds into three domains: α1, α2, and α3. The α3 domain and β2-microglobulin closely resemble Ig-like domains in their folded structure. The folded α1 and α2 domains create a long cleft or groove, which is the site at which antigenic peptides bind to the MHC molecules. The MHC molecules are highly polymorphic, and the major differences between the different forms are located in the peptide-binding cleft, influencing which peptides will bind and, thus, the specificity of the dual Ag presented to T cells.

The MHC class II molecules are present only on professional APCs, such as DC, B lymphocytes, and Mϕ. They consist of a non-covalent complex of two transmembrane glycoprotein chains, α and β. The MHC class II α and β chains are both encoded within the MHC. Each chain has two domains and the two chains together form a compact four-domain structure similar to that of the MHC class I molecule. The crystallographic structure of the MHC class II molecule shows that it is folded very much like the MHC class I

molecule, but in MHC class II molecules the peptide-binding cleft is formed by two domains from different chains, the α_1 and β_1 domains. The major differences lie at the ends of the peptide-binding cleft, which are more open in MHC class II molecules than in MHC class I molecules. Consequently, the ends of a peptide bound to MHC class I molecules are substantially buried within the molecule, whereas the ends of peptides bound to MHC class II molecules are not.

Each MHC molecule binds a wide variety of different peptides, but the different variants preferentially recognize sets of peptides with particular sequence and physical features. Peptides derived from intracellular pathogens that multiply in the cytoplasm are carried to the cell surface by MHC class I molecules and presented to CD8 T cells. These cells differentiate into *cytotoxic T cells* that kill the infected cells. Antigenic peptides derived from pathogens multiplying in intracellular vesicles and those derived from phagocytosed extracellular bacteria and toxins are carried to the cell surface by MHC class II molecules and presented to $CD4^+$ T cells [20].

1.5 $CD4^+$ T cells

Upon encountering microbial pathogens, Ag-inexperienced, “naïve” $CD4^+$ T cells can differentiate into 4 (and possibly more) effector *T helper* (Th) cell subsets with different cytokine profiles and distinct effector functions. In this manner, they tailor their responses to the character of the threat encountered, providing help to B lymphocytes and $CD8^+$ T cells, and activating the cells of the innate immune system.

The differentiation of naïve $CD4^+$ T cells into effector Th cells is initiated by engagement of their TCR (signal 1) and costimulatory molecules (signal 2) by the specific antigenic peptide complexed to MHC class II molecules, in the presence of a complex network of specific cytokines (signal 3) produced by the innate immune system upon encounter of pathogens [21].

Naïve $CD4^+$ T cells differentiate into T helper type 1 (*Th1*), *Th2*, *Th17*, or T regulatory (*Treg*) lineages depending on the instructional cues delivered during initial activation (Figure 1.2) [22].

Interleukin (IL)-12, produced by APCs, initiates the differentiation of Th1 cells, which express the signature transcription factor T-bet and secrete the signature cytokine interferon (IFN)-γ that in turn drives Th1 differentiation. Th1 cells protect the host against infections with intracellular pathogens [23]. In contrast, IL-4 triggers the differentiation of Th2 cells that express GATA-3 as signature transcription factor and secrete IL-4 as signature cytokine. Th2 cells eradicate extracellular pathogens and help B cells to produce Abs [24] [25].

Th17 cells are a more recently described effector cell lineage that develops via cytokine signals distinct from, and antagonized by, products of the Th1 and Th2 lineages [26]. Th17 cells express RORγt as signature transcription factor and secrete IL-17A (IL-17) as signature cytokine. Th17 cells contribute to clearance of extracellular bacterial and fungal infections [27] [28].

Treg cells express the signature transcription factor Foxp3 [29]. They suppress immune reactions and ensure that our intrinsically self-recognizing repertoire of lymphocytes does not mount a response against our own tissues or innocuous environmental Ags [30]. Studies have shown that these cells function by an as yet unclear mechanism of cell-to-cell contact

and/or the production of suppressor cytokines such as IL-10 and transforming growth factor-β (TGF-β) [31] [32].

A number of variables influence $CD4^+$ Th cell differentiation, including TCR affinity for peptide/MHC complex, Ag dose, costimulatory signals, and the local concentration of inflammatory cytokines [22].

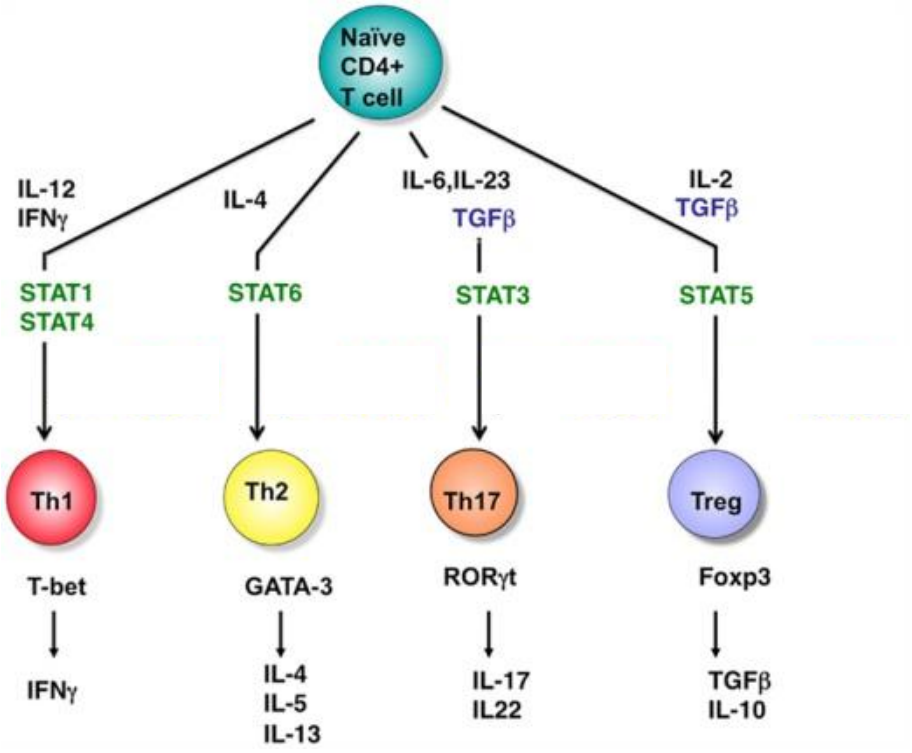


Figure 1.2 Helper T-cell differentiation. Cell differentiation from naïve $CD4^+$ cells into the various functional subtypes (e.g., Th1, Th2, Th17, and Treg cells) primarily depends on the action of cytokines as indicated (modified from [33]).

1.5.A Th1 cells

Th1 cells mediate the elimination of intracellular pathogens through IFN-γ. IFN-γ can promote Mϕ to express inducible nitric oxide synthase (iNOS) and other factors to eliminate intracellular pathogens. Another function of IFN-γ is to deliver the isotype switch signal for B cells to produce IgG2a Abs [24].

There are several transcription factors that are required for Th1 cell differentiation: signal transducers and activators of transcription (STAT)1, STAT4, and T-bet are the best characterized. IL-12 is a cytokine produced by DC that activates STAT4, which positively regulates many aspects of the Th1 genetic program. STAT1 is activated in response to IFN-γ signaling and reinforces the Th1 phenotype in a positive feedback loop. STAT1 and STAT4 also contribute to the regulation of Tbx21, the gene that encodes T-bet. T-bet is a T-box transcription factor that is required for the induction of many Th1 signature genes. T-bet is also needed for the repression of genes specific to alternative T helper cell developmental programs [34].

1.5.B Th2 cells

Th2 cells produce IL-4, IL-5, IL-13, and IL-6 and mediate the clearance of large extracellular pathogens such as helminthes. Th2 cells are also involved in the isotype switching of B cells to produce IgE and thus in allergic reactions [24].

IL-4 is the critical cytokine that drives Th2 development [35]. Upon binding to its receptor, IL-4R, IL-4 can initiate the phosphorylation and activation of Janus activated kinase (JAK)1 and JAK3, which in turn phosphorylate IL-4R subunits [36]. Phosphorylated IL-4 receptors can

further recruit and activate STAT6 through phosphorylation. Phosphorylated STAT6 forms a dimer, which can enter the nucleus and initiate the transcription of Th2-specific genes. GATA3 activates expression of the Th2 cytokine locus, binding to multiple sites proximal and distal to IL-4, IL-5, and IL-13 genes [37]. GATA-3 is assumed to be the central factor whose level/activity decides the fate of Th cell differentiation, not only through its capacity to induce Th2 cytokine production, but also through its capacity to block Th1 cytokine production via down-regulation of STAT4 [38].

1.5.C Th17 cells

Th17 cells are a more recently described lineage of $CD4^+$ effector T cells characterized by production of IL-17 [39] [40] [26].

Th17 differentiation has been linked to TGF-β and IL-6 in mice. IL-6 suppresses TGF-β–induced Foxp3 expression and Treg generation, promoting the development of Th17 cells, suggesting that Treg cells and Th17 effectors differentiate from the same precursor in a mutually exclusive fashion, depending on whether they are activated in the presence of TGF-β or TGF-β plus IL-6 [41]. Moreover, TGF-β acts in Th17 development to confer IL-23 responsiveness [42]. IL-23 is dispensable for the differentiation of Th17 cells, but it is indispensable for a fully effective, protective Th17 response [43]. In addition, IL-6 and TGFβ greatly promote chromatin remodeling of the Il17-Il17f locus [44], which is further reinforced by IL-23 [45] [46]. At the transcriptional level, the differentiation of Th17 lineage induced by cytokines requires the retinoic orphan nuclear receptors γt or α, RORγt or RORα, which are selectively expressed in Th17 cells. RORγt and RORα are lineage-specifying master regulators in Th17 development and

induce the transcription of the genes encoding IL-17A and the related cytokine IL-17F. RORγt and RORα are induced by TGF-β and IL-6 in a STAT3-dependent manner [27] [47]. Indeed, overexpression of a hyperactive STAT3 enhancs Th17 differentiation, whereas STAT3 deficiency impairs Th17 differentiation *in vitro* [45] and *in vivo* [46].
Th17 differentiation is negatively regulated by IFN-γ, IL-4, IL-27, and IL-2 [48] [49] [50]. STAT3 upregulates the transcription factor Aiolos in T lymphocytes and silences the gene encoding IL-2 thus limiting Th17 expansion [51].
Recently, IL-21 was reported as an autocrine factor induced by IL-6 to regulate Th17 differentiation in combination with TGF-β [52] [46] although not as efficiently as IL-6+TGF-β. TGF-β and IL-6 together initiate the differentiation of Th17 cells, which in turn can produce IL-21 and thus further amplify the Th17 differentiation process [53]. STAT3 is necessary for IL-6 induction of IL-21 expression and is required for IL-21–mediated Th17 differentiation [52].
Th17 cells produce IL-17A, IL-17F, which share 55% sequence identity [54], and IL-22, all of which induce a massive tissue reaction [55] [56] [57] [58].

Th17 cells have considerable plasticity and readily acquire the ability to produce IFN-γ in addition to IL-17 production or completely shut off IL-17 production *in vitro* [59] [14] [60] [61] [62]. Hirota et al. suggested that the fate of Th17 cells is shaped by different inflammatory conditions *in vivo*, which allow distinct patterns of plasticity. Whereas IFN-γ producing “ex-Th17” cells that are linked to pathogenicity develop under chronic inflammatory conditions, clearance of an infection that results in resolution creates an anti-inflammatory environment that precludes Th17 cell plasticity and the adoption of the production of alternative cytokines (*bona fide* Th17 cells) [63].

1.6 IFN family and IFN receptor

The IFNs were originally discovered as agents that interfere with viral replication [64]. Initially, they were classified by the secreting cell type but are now classified into type I and type II according to receptor specificity and sequence homology.

The type I IFNs are comprised of multiple IFN-α subtypes (14-20, depending on species), IFN-β, IFN-ω, and IFN-τ, all of which are structurally related and bind to a common heterodimeric receptor. Although type I IFNs are secreted at low levels by almost all cell types, hematopoietic cells are the major producers of IFN-α and IFN-ω, whereas fibroblasts are a major cellular source of IFN-β [65]. IFN-β is also produced by Mϕ under appropriate stimulus. Viral infection is the classic stimulus for IFN-α and IFN-β expression [65] [66]. Secretion of IFN-τ has only been reported in ruminants [67].

IFN-γ is the sole type II IFN. It is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus. Initially, it was believed that $CD4^+$ Th1 lymphocytes, $CD8^+$ cytotoxic lymphocytes, and NK cells exclusively produced IFN-γ [65] [68]. However, there is now evidence that other cells, such as B cells, natural killer T (NKT) cells, and APCs secrete IFN-γ [69]. IFN-γ production by professional APCs acting locally may be important in cell self-activation and activation of nearby cells [70] [71]. IFN-γ secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFN-γ in the adaptive immune response [70] [72].

IFN-γ production is controlled by cytokines secreted by APCs, most notably IL-12 and IL-18. These cytokines serve as a bridge to link infection

with IFN-γ production in the innate immune response. Mϕ recognition of many pathogens induces secretion of IL-12 and chemokines [e.g., macrophage-inflammatory protein-1α (MIP-1α)]. These chemokines attract NK cells to the site of inflammation, and IL-12 promotes IFN-γ synthesis in these cells. In Mϕ, NK and T cells, the combination of IL-12 and IL-18 stimulation further increases IFN-γ production.
Negative regulators of IFN-γ production include IL-4, IL-10, TGF-β, and glucocorticoids [73].

Given the complexity of IFN-γ regulation, it is not surprising that inbred mouse strains vary in their ability to secrete this cytokine; for example, T lymphocytes of C57BL/6 mice secrete significantly higher amounts of IFN-γ compared with the T lymphocytes of BALB/c mice. Increased IFN-γ production in these strains is associated with greater resistance to bacteria and viruses [74] [75] [76].

Both the type I IFN receptor and the type II IFN receptor have multichain structures, which are composed of at least two distinct subunits: IFNAR1 and IFNAR2 for the type I IFN receptor, and IFNGR1 and IFNGR2 for the type II IFN receptor. Each of these receptor subunits interacts with a member of the JAK family [77] [78]. In the case of the type I IFN receptor, the IFNAR1 subunit is constitutively associated with tyrosine kinase 2 (TYK2), whereas IFNAR2 is associated with JAK1 [79] [77] [48] [80]. In the case of the type II IFN receptor, the IFNGR1 subunit associates with JAK1, whereas IFNGR2 is constitutively associated with JAK2 [79] [10]. The initial step in both type-I and type-II-IFN-mediated signaling is the activation of these receptor-associated JAKs, which occurs in response to a ligand-dependent rearrangement and dimerization of the receptor subunits, followed by autophosphorylation and activation of the associated JAKs.

Activation of IFN-receptor–associated JAKs seems to regulate, either directly or indirectly, several other downstream cascades. Such diversity of signaling is consistent with the pleiotropic biological effects of IFNs on target cells and tissues.

IFNs are defined by their ability to block virus replication. In addition to this property, these potent cytokines have many other effects on cellular physiology, especially in cells of the immune system.

Types I and II IFN up-regulate multiple functions within the class I Ag presentation pathway to increase the quantity and diversity of peptides presented on the cell surface in the context of class I MHC. Up-regulation of cell-surface class I MHC by IFN-γ is important for host response to intracellular pathogens, as it increases the potential for cytotoxic T-cell recognition of foreign peptides and thus promotes the induction of cell-mediated immunity.

IFN-γ alone can efficiently up-regulate the class II Ag presenting pathway and thus promote peptide-specific activation of $CD4^+$ T cells.

IFN-γ is a major product of Th1 cells and further skews the immune response toward a Th1 phenotype.

One of the most important effects of IFN-γ on Mϕ is the activation of microbicidal effector functions. Mϕ activated by IFN-γ display increased pinocytosis and receptor-mediated phagocytosis as well as enhanced microbial killing ability. IFN-γ–activated microbicidal ability includes induction of the NADPH-dependent phagocyte oxidase (NADPH oxidase) system (“respiratory burst”), priming for NO production, tryptophan depletion, and up-regulation of lysosomal enzymes promoting microbe destruction. Similar microbicidal mechanisms are activated by IFN-γ in Nϕ [69].

Most, if not all, of these effects are mediated by the products of cellular genes, whose expression is highly induced by IFN treatment of cells. These IFN-stimulated genes (ISGs) number in the hundreds, but the biochemical and cellular functions of only a handful of them have been delineated as yet [81]. Some of these genes are regulated by both type I and type II IFNs, whereas others are selectively regulated by distinct IFNs [82].

1.7 IL-17 cytokine family and IL-17 receptors

The best characterized member of IL-17 family is *IL-17A* (IL-17). The five additional IL-17 family members are IL-17B to IL-17F. Among the IL-17 family members, the *IL-17F* isoforms 1 and 2 have the highest degree of homology with IL-17A (55 and 40% respectively), followed by IL-17B (29%), IL-17D (25%), IL-17C (23%), and IL-17E (also named IL-25) being most distant (17%). Th17 cells produce only IL-17A and IL-17F [83].

Although produced by T cells, IL-17 acts primarily on epithelial, endothelial and stromal cells. Genes induced by IL-17 encode antimicrobial proteins (β-defensins, cathelicidins, RegIII, lipocalin 2, salivary histatins), Nϕ-activating factors (granulocyte colony-stimulating factor, G-CSF, and CXC chemokines), and inducers of the acute phase response (IL-6) [84].

IL-17A and IL-17F have distinct biological effects. Studies comparing *Il17a*$^{-/-}$ mice with *Il17f*$^{-/-}$ mice indicate that IL-17A has a more important role in driving auto-immunity than IL-17F, probably owing to its more potent signaling strength [85]. Instead, both IL-17A and IL-17F are important for host defense against opportunistic infections [86]. IL-17F-

induced responses are 10-30 fold weaker in terms of downstream gene activation than those of IL-17A, with IL-17A–IL-17F heterodimers having an intermediate activity [87].

A striking feature of IL-17A, IL-17F, and IL-17A/F is their modest signaling activity, even at high concentrations. However, they exhibit potent synergy, not only with tumor necrosis factor (TNF) but also with lymphotoxin (LT)α, IFN-γ, and IL-1β. The mechanisms underlying synergy are not fully defined, but a major component is cooperative enhancement of mRNA stability of certain IL-17 target genes [84] [88].

The receptor for IL-17A, IL-17F and the IL-17A/F heterodimer is composed of IL-17RA paired with IL-17RC [84]. There are notable differences between the human and mouse IL-17R systems. Human IL-17RA has much higher affinity for IL-17A than for IL-17F, and human IL-17RC binds both ligands with similar affinity. By contrast, murine IL-17RC alone binds primarily to IL-17F [89] [54].

After either IL-17RA or IL-17RC bind their respective ligand, affinity for the reciprocal subunit is increased [89]. IL-17 receptor signaling requires the adaptor Act1 and results in the activation of the transcription factor nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinases [88].

IL-17RC expression is limited to non-hematopoietic cells whereas IL-17RA is expressed ubiquitously, at particularly high levels in lymphoid tissues such as thymus, spleen, and lymph nodes [86]. This expression pattern is interesting, as the main responses to IL-17A occur in epithelial cells, endothelial cells, and fibroblasts, although Mϕ and DC are also responsive (reviewed in [90]). Only a limited number of IL-17A–induced genes has been documented in lymphocytes, and these genes are distinct from those induced by IL-17A in other cell types [86]. Although its

expression is widespread, IL-17RA can be dynamically regulated; for example, phosphoinositide 3-kinase (PI3K) limits the expression of IL-17RA by T cells [84]. This could be biologically important, as IL-17A–induced signaling strength correlates with cell surface expression levels of IL-17RA; in contrast to most cytokine receptors, high levels of IL-17RA seem to be required for effective responses to IL-17A [91] [92]. Another function of IL-17RA might be to limit signaling by receptor-mediated internalization of the ligand. Indeed, surface expression of IL-17RA rapidly decreases after IL-17A binding, theoretically internalizing IL-17A and clearing it from the inflammatory milieu [93].

A number of genetic disorders have begun to reveal that IL-17 is surprisingly determinant to control just a few infections, namely those caused by *Candida albicans* and *Staphylococcus aureus*. Humans with hyper-IgE syndrome (HIES or Job's Syndrome) have mutations in STAT-3. Presumably because of defects in signal transduction of Th17-inducing cytokines such as IL-6, IL-21 and IL-23, which activate STAT-3, these patients exhibit a selective paucity of Th17 cells [94] [95] [96]. HIES patients exhibit a wide spectrum of clinical manifestations and are highly susceptible to mucosal candidiasis and staphylococcal abscesses, implicating Th17 cells in immunity to these organisms. Moreover, *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ mice develop spontaneous *S. aureus* skin abscesses [86].

1.8 Immune response to natural *S. aureus* infection

Polymorphnuclear (PMNs) cell recruitment is the primary host response to *S. aureus* infection [15]. Indeed, patients with Nϕ disorders, such as chronic granulomatous disease and Job's syndrome, suffer from an increased incidence of *S. aureus* infections [97] [98].

Both Abs and complement enhance the bactericidal effect of Nϕ and indeed the presence of opsonizing Abs directed against *S. aureus* capsule or PGN facilitates phagocytosis *in vitro* [3] [99]. Nevertheless, the role of Abs in clearing the staphylococcal infection *in vivo* is less certain since the titer of anti-staphylococcal Abs is not correlated with protection from infection. Moreover patients with defects in humoral immunity are not particularly prone to *S. aureus* infection. For example, patients with hereditary (X-linked) agammaglobulinemia rarely have clinically important *S. aureus* infections, although *Streptococcus pneumoniae* and *Pseudomonas* species are a serious problem for these patients [100] [101], often leading to death. In these subjects, the lack of Abs against *S. aureus* must be compensated for by other immune mechanisms.

Recent studies showed the importance of Th17 cells and IL-17 for protection against *S. aureus* infections [102] [103]. IL-17, produced by Th17 cells, is crucial for Nϕ recruitment and activation [103]. Of interest, *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ mice develop spontaneous *S. aureus* skin abscesses [86] and humans with IL-17 defects are also prone to *S. aureus* skin infections [104]. Further support for the involvement of Th17 cells in *S. aureus* immunity comes from studies of bacterial pneumonia in mice [105]. It has been shown that influenza A inhibits Th17-mediated host defense in mice via induction of type I IFNs. This caused impaired bacterial clearance of *S. aureus* and resulted in increased mortality. Treating mice with exogenous IL-23

stimulated production of IL-17 and IL-22, which markedly improved clearance of *S. aureus* and reduced mortality. Of note is that the highly virulent community-associated methicillin-resistant *S. aureus* strains can induce type I IFNs within the lungs of mice [106], thereby inhibiting the Th17 cell pathway. All these evidences tie together cell-mediated immunity and Nϕ effector activities, with Abs playing a supportive role for opsonization and/or neutralization of virulence factors.

The innate immune system contributes to protection from *S. aureus* infection through activation of Toll-like receptor (TLR)2/6 and myeloid differentiation primary response protein (MyD)88 pathways by staphylococcal surface components [6] [107]. Moreover, *S. aureus* α-hemolysin activates the immune system via a TLR2-independent mechanism whereby NOD2 signaling results in protection against *S. aureus* murine skin infections [108] [109].

However, the immune system is thwarted by multiple staphylococcal virulence factors: toxins that lyse phagocytes, capsule that prevents efficient opsonization, protein A that inhibits the opsonic activity of Abs and induces apoptosis in B cells, adhesins to host tissues that allow uptake by non-professional phagocytes thereby providing a site hidden from the immune system, and anti-immune system factors that target Abs, complement, selectins, intercellular adhesion molecule 1 (ICAM-1), and innate immune system receptors [110] [111] [112]. These entire virulence factors enable *S. aureus* to evade the host immune response.

1.9 Vaccines

Vaccines are among the greatest successes in the history of public health. Vaccines have now led to the eradication of smallpox, near eradication of polio, and prevention of untold millions of deaths from infectious diseases each year, and are one of the most effective public health measures available [113].

Vaccines are biological products that mimic a disease-causing agent thus stimulating the immune system to mount a response against it. An ideal vaccine will induce the expansion and maturation of naïve pathogen-specific lymphocyte clones that exist at low frequency in uninfected or unimmunized individuals [114].

Immunization can be derived from either passive or active means. Passive immunization occurs with the transfer of preformed Abs to an unimmunized individual, who would then develop a temporary immunity to a particular organism or toxin due to the presence of these Abs. Once Abs have been eliminated, the individual would no longer have immunity to the microorganism or toxin. Active immunization occurs with the exposure of an unimmunized individual to an Ag against which the immune system would then develop immunity. In contrast to passive immunization, active immunization typically produces long-term immunity due to the stimulation of the immune system.

Long-term protection requires the persistence of vaccine-specific Abs and/or the generation of immune memory cells capable of rapid and effective reactivation upon subsequent microbial exposure. The determinants of immune memory induction, as well as the relative contribution of persisting Abs and of immune memory to protection against specific diseases, are thus essential parameters of long-term vaccine

efficacy. The predominant role of B cells in the efficacy of current vaccines should not shadow the importance of T-cell responses: T cells are essential to the induction of high-affinity Abs and immune memory, and novel vaccine targets have been identified against which T cells are likely to be the prime effectors [115].

1.10 Vaccines against *S. aureus*

S. aureus has demonstrated its versatility by remaining a major cause of morbidity and mortality despite the introduction of several new antibiotics to treat methicillin-resistant *S. aureus* infections. Therefore, effective vaccination against *S. aureus* would be a major health achievement.

Despite the continued efforts by a combination of university, small biotech, and large pharmaceutical company researchers to develop a *S. aureus* vaccine, multiple clinical trials have failed.

The first vaccine against *S. aureus* tested in humans was StaphVax. It included *S. aureus* capsular polysaccharides type 5 and 8 (CP5 and 8) conjugated with recombinant *Pseudomonas aeruginosa* exoprotein A. The vaccine failed in phase III clinical trial. It has recently been shown that *S. aureus* USA300, one of the most important clones worldwide, has no detectable capsule due to a point mutation in the promoter region of *cap-5*, necessary for CP5 biosynthesis [116]. Moreover, the role of CPs in *S. aureus* virulence is not clear and their expression is limited to the stationary phase [7] [117]. These reasons may have contributed to vaccine failure.

Another vaccine against *S. aureus* was V710, based on a single surface staphylococcal protein, IsdB [99] [118]. IsdB is a protein conserved and expressed throughout different *S. aureus* strains, which mediates iron uptake from hemoglobin [99] [119]. Immunization with IsdB formulated on amorphous aluminum hydroxyphosphate sulfate adjuvant increased murine Ab titers by up to 20-fold. Increased Ab titers correlated with enhanced survival in a murine disseminated challenge model. Also, monoclonal Ab to IsdB had *in vitro* opsonophagocytic activity and efficacy in rodent challenge models [99]. The phase II/III vaccine clinical trial was interrupted; the reason is not yet completely understood [118].

A number of passive approaches have also been undertaken: Veronate, based on polyclonal Ab against the S*. aureus* surface protein ClfA; Altastaph, containing CP5 and CP8 Abs purified from subjects vaccinated with StaphVax; Tefibazumab, monoclonal Ab against ClfA; and Aurograb, single chain Ab against an ATP binding cassette transporter of the pathogen. They all failed to show efficacy against *S. aureus* infection in humans [117] [120] [121].

Other vaccines are still being evaluated. An investigational vaccine, NDV-3, contains the N-terminal portion of the *Candida albicans* agglutinin-like sequence 3 protein (Als3p) formulated with an aluminum hydroxide adjuvant in phosphate-buffered saline. Preclinical studies demonstrated that the Als3p vaccine Ag protects mice from both intravenous challenge and skin and soft tissue infection with *S. aureus*. The vaccine was safe and well-tolerated in a phase I clinical trial [122]. Protection was found to be mediated by T cells, specifically Th17 and Th1 cells, and was lost in mice that lacked Nϕ producing reactive oxygen species [123].

In other studies, mice were immunized with clumping factor A (ClfA). ClfA is a fibrinogen-binding surface protein involved in staphylococcal

adhesion to host tissues. It was included in a tetra-Ag vaccine that underwent a first-in-human (phase I/II) study in the United States [124]. Wild type mice were protected from *S. aureus* infection after immunization with ClfA, whereas IL-17A–deficient mice were not [125].

All the vaccines against *S. aureus* tested so far in humans failed to show protection. As suggested by Bagnoli et al., they all targeted a single component of the pathogen. Since *S. aureus* expresses a plethora of toxins and immune evasion factors, efficacy of monovalent vaccines is likely to be insufficient in humans. Moreover, animal studies as well as clinical observations indicated that both humoral as well as cell-mediated immunity are involved. On the basis of that, the authors proposed a model in which vaccine efficacy is gained through three major immune responses: (i) Abs to directly inhibit bacterial viability and/or toxicity; (ii) Abs to mediate opsonophagocytosis; and (iii) cell-mediated immunity to stimulate recruitment of phagocytes at the site of infection. It is very likely that only a combination of staphylococcal Ags with different properties and functions formulated with adjuvants able to elicit a potent Ab production, but also the proper cellular response, will satisfy the three criteria [126].

1.11 Novartis Vaccines vaccine against *S. aureus*

Novartis Vaccines has engaged in the research and development of an effective vaccine against *S. aureus* infections. Using different approaches, comprising reverse vaccinology, proteomic and comparative genomics analyses, a combination of four highly conserved Ags (*Combo*) was selected and proposed as potential vaccine. The vaccine comprises

two surface proteins (FhuD2 and Sur2) and two secreted proteins (Hla_{H35L} and EsxAB).

Hla_{H35L} is the non-toxic mutant of Hla, a secreted toxin that assembles into a membrane-perforating homoheptamer upon binding to its eukaryotic cellular receptor, the membrane-associated protease ADAM10, which facilitates Hla-mediated disruption of epithelial integrity [8] [125] [126]. Hla expression is down-regulated when bacteria reach a high concentration through the *agr* locus that encodes a quorum-sensing system [127]. The substitution of histidine 35 with leucine produced a mutant toxin (H35L) without hemolytic or lethal activity, as pore formation on the eukaryotic cell membrane is impaired [71].

EsxAB derives from the fusion of the proteins EsxA and EsxB. *S. aureus* secretes EsxA and EsxB across the bacterial envelope. Staphylococcal esxA and esxB are clustered with six other genes and some of these are required for synthesis or secretion of EsxA and EsxB. Mutants that fail to secrete EsxA and EsxB display defects in the pathogenesis of *S. aureus* murine abscesses, suggesting that this specialized secretion system may be a general strategy of human bacterial pathogenesis [128].

FhuD2 (ferric hydroxamate uptake) was first discovered as a ferric hydroxamate siderophore binding protein, which mediates iron uptake through a dedicated ATP binding cassette transporter [129]. The protein was demonstrated to have a discrete, punctate localization on the bacterial surface, to be important in invasive infection, and function as an efficacious vaccine target [130].

Sur2 is a highly conserved protein, present in multiple copies (paralogs) in all the *S. aureus* strains sequenced by Novartis Vaccines. The protein function is still unknown.

All 4 vaccine Ags are consistently expressed by clinically relevant strains.

NVD Combo vaccine adjuvanted with alum has shown efficacy in pre-clinical mouse models (pneumonia, abscess, and peritonitis model) and has recently been tested in a human phase I clinical trial.

To improve this vaccine further, Combo Ags were formulated with *alum/SMIP-7.10* (*alum-S-7*), a novel adjuvant formulation composed of a TLR7 agonist (TLR7a) small molecule (SMIP-7.10) adsorbed to alum (Figure 1.3).

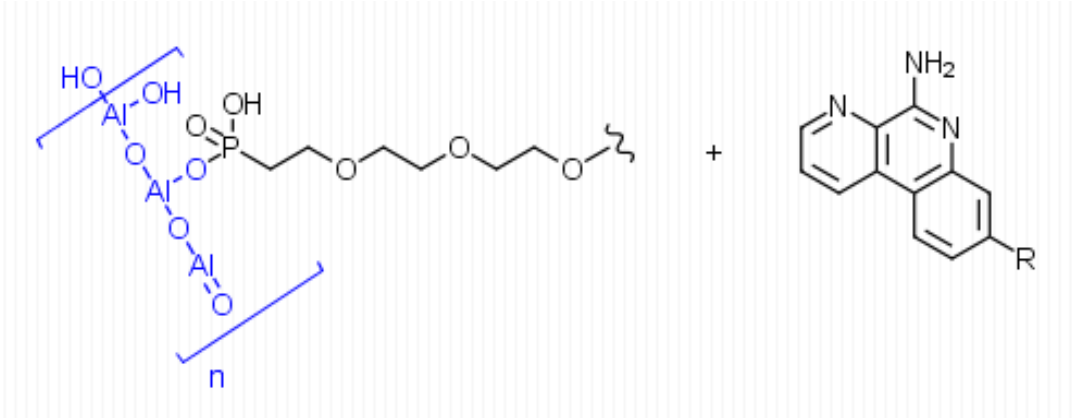


Figure 1.3 alum/S-7 structure. The TLR7a small molecule is a soluble benzonaphtyridine (right) to which a phosphonate group was added via a linker (left) to allow adsorption to alum (in blue).

Adjuvants that incorporate TLR7a are able to induce more polarized Th1 cell responses [117] that have been shown to be important for protection against *S. aureus* [97].

1.12 Vaccine adjuvants

Vaccines typically contain two main components: one or more Ags to which the immune response is desired and an *adjuvant* that enhances the immunogenicity of Ags and that acts accelerating, extending, or improving the quality of specific immune responses to vaccine Ags.

Vaccine adjuvants are represented by different classes of compounds that display adjuvant activity in pre-clinical models. These include microbial products, mineral salts, emulsions, microparticles, nucleic acids, small molecules, saponin, and liposomes, which exert their function by diverse and often poorly characterized mechanisms of action [131] [132] [133].

Currently, there are very few vaccine adjuvants that are approved for human use. Among them there are alum (aluminum hydroxide) and the oil-in-water emulsions MF59 and AS03. Alum has been widely used in human vaccines for more than 70 years, while MF59 has been licensed for human use a decade ago, and AS03 only few years ago. The molecular mechanism of action and the target cells of alum, MF59, and AS03 have only been under investigation.

Alum adjuvant consists of precipitates of aluminum hydroxide to which Ags are adsorbed. Alum absorption increases Ag uptake [134] and stability at the site of injection. In addition, alum induces a local pro-inflammatory reaction that can increase immunogenicity [6] [108]. Alum is used primarily to enhance Ab production and does not utilize TLRs for its function *in vivo* [109]. In humans, responses to proteins with alum tend to be a mix of Th2 and Th1 cells [110]; however, in mice (especially BALB/c mice) alum induces a profoundly polarized Th2 cell response, with Th2 cell-dependent Ab isotypes, to nearly all protein Ags.

1.13 Toll-like receptors and their agonists

TLRs are an evolutionary conserved family of membrane-bound PRRs [135] [136] that were named after Drosophila Toll. Toll was first identified as the product of a gene that was shown to play a critical role on the dorso-ventral axis formation of drosophila embryos [137]. The high homology between the intracellular regions of Drosophila Toll and mammalian IL-1R, as well as the similarity in the signaling pathways triggered by these two receptors, both of which result in NF-κB activation, led to the hypothesis that Toll could also have a function in the immune response [62] [138]. Indeed, Toll is required for Drosophila response to fungi being part of a PGN sensing cascade that ends in the production of antifungal peptides by cells of the fat body [139] [140].

All mammals TLRs play a primary role in innate immune response, but are not involved in mammalian development. Humans have ten TLRs, each of which plays a distinct role in innate immunity, since each of them recognizes a specific microbial pattern [135] [136]. They recognize a broad spectrum of pathogen components, such as bacterial and fungal cell wall components, bacterial lipoproteins, and bacterial and viral nucleic acids. Usually more than one TLR is expressed by the same APC, which during infection can be simultaneously stimulated by many of TLRs. Some TLRs are expressed at the cell surface, whereas others are expressed on the membrane of endocytic vesicles or other intracellular compartments. TLRs are composed of a transmembrane ectodomain of leucine-rich repeats (LRRs), which are involved directly or through accessory molecules in ligand binding, and a cytoplasmatic Toll/interleukin-1 receptor (TIR) domain that interacts with TIR-domain-containing adaptor molecules.

TLR2 can heterodimerize with TLR1 or TLR6 to recognize respectively triacyl or diacyl lipopetides, which are present on the surface of bacteria. TLR4 is specialized in responding to lipopolysaccharide (LPS) expressed by Gram-negative bacteria, while flagellin, a protein of bacterial flagella, is recognized by TLR5. All these TLRs are expressed on the cell surface and allow the immediate detection and response to microorganisms. A second class of TLRs is expressed in the endolysosomal compartment and preferentially recognizes microbial nucleic acids that are able to get through the cytoplasmic membrane barrier. TLR3 binds double-stranded RNA synthesized by viruses. The natural ligand of *TLR7* and TLR8 is single stranded viral RNA, yet several TLR7a and/or TLR8a small molecule immune potentiators (SMIPs) have been described. Among them, imidazoquinolines, like Resiquimod (R848) that activates TLR7 and TLR8, and Imiquimod (R837) that activates TLR7 only, and purine-like molecules, like SM360320 (9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine) that activates TLR7 only. TLR9 recognizes double-stranded DNA, preferentially characterized by unmethylated cytosine guanine oligodeoxynucleotide (CpG-ODN) motifs, common to both bacterial and viral DNA. The intracellular localization of these TLRs is very important to avoid their activation by endogenous nucleic acids, which can lead to autoimmunity (Figure 1.4).

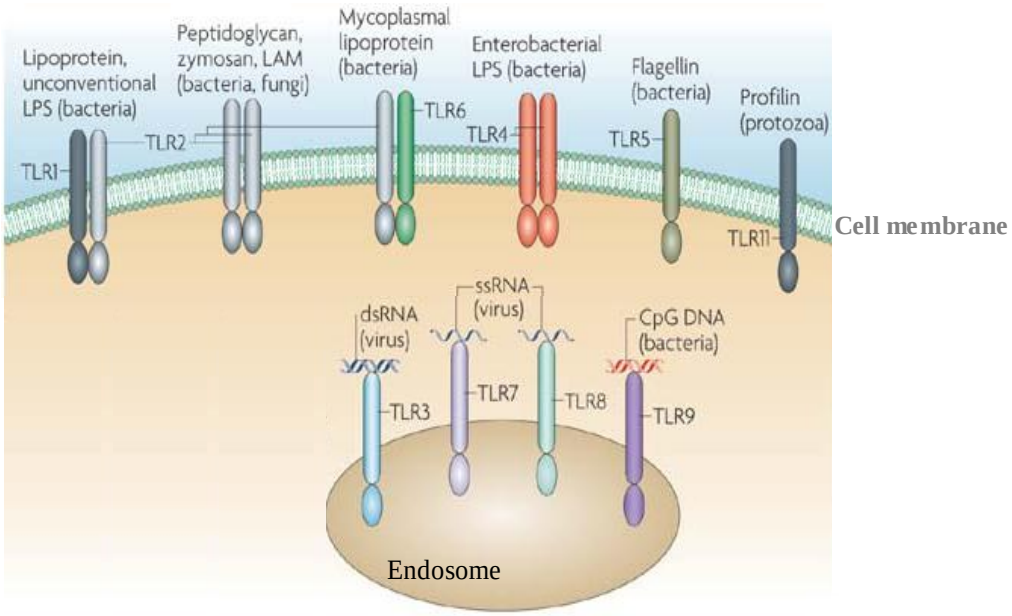


Figure 1.4 Toll-like receptor localization. TLRs, expressed either on the cell surface or on the membrane of endocytic vesicles or intracellular compartments, recognize a diverse range of microbial and viral ligands (modified from [141]).

TLRs are expressed on many immune and non-immune cells. Their expression pattern is different in different DC subsets. Myeloid dendritic cells (mDC) express all surface TLRs plus TLR3, TLR7, and TLR8, thus mounting very strong anti-bacterial responses, while plasmacytoid dendritic cells (pDC) express only TLR7 and TLR9, thus being specialized to face viral infections. TLR7 and TLR9 reside in the endoplasmatic reticulum before stimulation and are rapidly recruited to endosomal compartment upon activation. It has been shown that UNC93B1, a poorly characterized multi-transmembrane-domain–containing protein, specifically binds and delivers TLR7/9 from the endoplasmatic reticulum to endosomal compartment for ligand recognition [142].

Important differences exist between mice and humans with regard to tissue expression and function of TLR7 and TLR8. In both species, TLR7 is expressed in B cells, Nϕ, and pDC; however, in mice TLR7 is expressed by Mϕ and $CD8^-$, but not $CD8^+$, DC subsets [116]. TLR8, in contrast, is expressed by Mo lineage cells and mDC in man, whereas it may not be a functional receptor in mice [143].

Recently, several synthetic agonists of TLR4, TLR7, TLR8, and TLR9 have been identified as a major class of molecules that have potential utility as vaccine adjuvants.

One such adjuvant is the TLR4a monophosphoryl lipid A (MPL), a derivative of the bacterial component LPS. MPL adsorbed to alum (known as AS04) is currently approved for use against human papilloma virus and hepatitis B [144]. Recent studies to elucidate the mechanism of action of AS04 revealed that AS04 binds TLR4 and promotes cytokine production, Ag presentation, and migration of APCs to T-cell area of draining lymph nodes, allowing for an efficient priming of naïve T cells [144].

Another adjuvant is represented by non-methylated CpG oligonucleotide used in both preclinical and clinical studies as adjuvant in cancer vaccines [145]. CpG acts through TLR9 expressed by human pDC and B cells inducing humoral and cellular responses.

The TLR7/8 pathway, specialized in recognition of single stranded viral RNA pathways, has demonstrated promising pre-clinical results as a target for potential vaccine adjuvant. Small molecules TLR7a, such as Imiquimod, or TLR7/8a, such as Resiquimod, were shown to improve the immunogenicity of a variety of vaccine adjuvants if adequately formulated or directly conjugated to protein Ags [145] [146]. Imiquimod has demonstrated immunomodulating effects as a topic vaccine adjuvant. Injection of

immature DC into Imiquimod-pretreated skin led to DC activation and migration to draining lymph nodes in cancer patients, as well as to enhanced DC immunostimulatory capacity [145]. Importantly, Imiquimod is licensed as a topical therapy for malignant and non-malignant skin cell disorders, demonstrating a safety and efficacy track record for this class of TLRa as therapeutics.

1.14 Rationale and aims

The aim of the PhD project described in this thesis is to characterize the immune responses elicited by the novel Combo vaccine formulation with the alum/S-7 adjuvant, and to compare its efficacy to that induced by Combo±alum.

For this purpose, a mouse model of *S. aureus*-induced peritonitis was chosen. Peritonitis remains a frequent complication of continuous ambulatory peritoneal dialysis (CAPO), a major form of renal replacement therapy worldwide. This is despite the considerable improvements made in the delivery systems, which have significantly reduced peritonitis incidence [147] [148] [149]. Moreover, intraperitoneal challenge has been utilized in studies examining the efficacy of vaccine Ags targeting *S. aureus*, owing to the ability to easily deliver large inocula and generate highly reproducible data in lethal dose challenge experiments [150] [151] [152] [153].

S. aureus Newman strain, used in this study, was isolated in 1952 from a human infection [154]. It has been used extensively in animal models of staphylococcal disease due to its robust virulence phenotypes.

BALB/c mice were immunized once intramuscularly (i.m.) with Combo+alum or Combo+alum/S-7 and challenged intraperitoneally (i.p.)

with a lethal dose of *S. aureus* Newman strain 12 days after the immunization. Combo+alum/S-7 efficacy was compared to that of Combo±alum and vaccine-specific humoral and T-cell responses were characterized. Experiments with B-cell ko J_H mice and serum transfer studies, $CD4^+$ effector T-cell depletion and IL-17A/IFN-γ *in vivo* neutralization experiments were performed to determine the arm of the immune response essential for protective immunity against *S. aureus* peritonitis conferred by Combo+alum/S-7 immunization.

Chapter 2

Materials and methods

2.1 Cloning and purification of vaccine antigens

Vaccine Ags were amplified by PCR from *S. aureus* NCTC8325 strain and cloned as tagless constructs. Proteins were purified and suspended in 10 mM potassium phosphate, pH 7.2 (5 mM EDTA was added for EsxAB buffer). Endotoxin content was controlled with limulus amoebocyte lysis (LAL) test.

2.2 Vaccine antigen formulation with alum or alum/S-7

Ags were adsorbed to alum by incubating 10 µg of each Ag with 2 mg/ml aluminum hydroxide±0.5 mg/ml SMIP-7.10, at pH 6.5-7 and osmolality 0.308±0.060 Osm/Kg, with slow stirring for few hours at room temperature (RT).

The formulation pH and osmolality were controlled with the use of a pH meter (Eutech Instruments) and an osmometer (Gonotech), respectively.

To control Ag adsorption to alum or alum/S-7 and protein integrity, formulations were analyzed by SDS-PAGE. Samples were centrifuged for 10 min at 13,000 rpm. Pellets were suspended in 100 µl of 4X desorption buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol, 0.5 M KH_2PO_4) and denatured for 30 min at 100°C. 200 µl of supernatant from each sample were treated with 10 µl of 0.5% deoxycholate (DOC) for 10 min and then with 8.5 µl of 60% trichloroacetic acid (TCA) and finally centrifuged for 10 min at 13,000 rpm. Supernatant was then removed and pellet was suspended in 10 µl of 4X loading buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol) plus 10 µl Tris-HCl pH 8.5 and denatured 30 min at 100°C. 10 µl of denatured pellet and 20 µl of denatured supernatant were loaded on a 4-12% Bis-Tris gel (Invitrogen).

Finally, to control SMIP-7.10 adsorption to alum, samples were analyzed by HPLC. An aliquot of each sample was centrifuged 10 min at 13,000 rpm and the supernatant was diluted 1:2 in 10 mM NaOH. A second aliquot was treated with an equal volume of 1 M KH_2PO_4 pH 9 for 30 min at 100°C, shaking, and then centrifuged for 10 min at 13,000 rpm. Finally, the supernatant was diluted 1:5 in 10 mM NaOH. The standard curve was prepared with 1:2 serial dilutions of SMIP-7.10 in 10 mM NaOH pH 9. SMIP-7.10 content in the two aliquots of each sample was determined by HPLC-UV (high performance liquid chromatography-ultraviolet) (Waters). The analytical column was C18, UV wavelength was 268 nm, and the mobile phase was a gradient of 1% trifluoroacetic acid (TFA) in water and 0.1% TFA in water at flow rate of 1.2 ml/min.

2.3 Animal care and compliance statement

Mice were monitored daily and euthanized at the appearance of humane endpoints, in accordance with Novartis Animal Welfare Policies and Italian laws. Experimental protocols were reviewed and approved by the Italian National Institute of Health, ISS (Istituto Superiore di Sanità). Five-week old female BALB/c mice, purchased from Charles River Laboratories, and five-week old J_H mice and matching BALB/c mice, purchased from Taconic, were used for the experiments.

2.4 Immunization studies

For active immunization, BALB/c mice were immunized once i.m. with:

- Combo: 10 µg of each purified Ag (Hla_{H35L}, EsxAB, FhuD2, and Sur2) in solution.
- Combo+alum: 10 µg of each purified Ag adsorbed to aluminum hydroxide (alum, 2 mg/ml).
- Combo+alum/S-7: 10 µg of each purified Ag adsorbed to alum (2 mg/ml) together with 50 µg SMIP-7.10.

50 µl of vaccine were injected in each hind leg quadricep (100 µl/mouse). Control mice received equal amounts of saline, alum, or alum/S-7.

For passive immunization studies, pools of sera were prepared from BALB/c mice vaccinated either with Combo+alum/S-7 or alum/S-7 alone 32 days before bleeding. One hundred and fifty microliters of these pooled sera

were injected in the tail vein of naive BALB/c mice the day before infection with *S. aureus*.

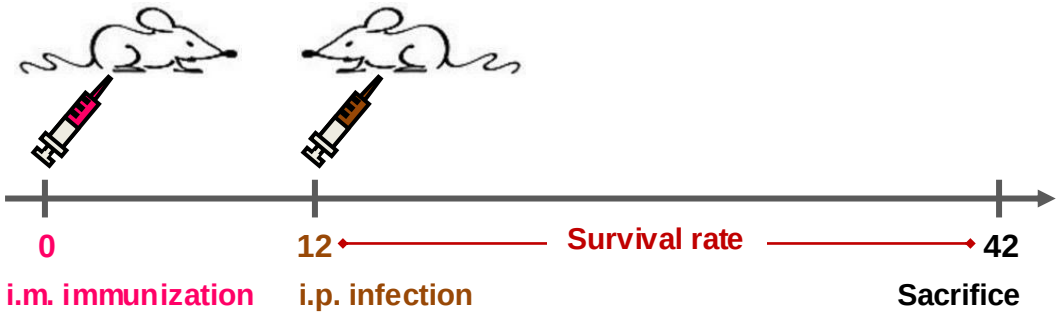


Figure 2.1 Experimental design of active immunization studies. BALB/c mice were immunized i.m. on day 0 and challenged i.p. with *S. aureus* on day 12. Mice survival was monitored for 15-30 days.

2.5 Peritonitis model

Frozen cultures of *S. aureus* were inoculated 1:25 into fresh tryptic soy broth and grown for 2 h and 30 min with shaking (250 rpm) at 37°C until the optical density at 600 nm (OD_{600}) of the bacterial culture diluted 1:10 in PBS was between 0.20 and 0.22. Staphylococci were sedimented, washed, and suspended in sterile PBS (Gibco) to obtain an inoculum of 5 x 10^9 colony forming units (CFU)/ml. Mice were challenged injecting 100 µl of bacterial inoculum (5 x 10^8 CFU) i.p.. Actual CFUs in the inoculum were determined plating it at serial dilutions on tryptic soy agar and enumerating colonies after overnight (o/n) incubation at 37°C. Infected animals were monitored for survival over a period of 15 to 30 days.

2.6 Abscess and CFU enumeration

Fifteen or 30 days after infection, mice were killed by CO_2 inhalation and subjected to necropsy. Abscesses located at the peritoneal membrane were enumerated by visual inspection. Kidneys were excised and homogenized in PBS (Gibco) using an automated lab homogenizer (Miltenyi Biotec). Homogenates were serially diluted in PBS, plated on tryptic soy agar, and incubated o/n at 37°C for CFU enumeration 24 h later.

2.7 Histopathology

Mouse kidneys were cut in two equal halves along the sagittal plane, fixed in 4% buffered formaldehyde, and processed for paraffin embedding. Four µm-sections were cut from each kidney and stained with hematoxylin and eosin (HE). Images of each HE-stained kidney were acquired by Mirax Scan 150 slide scanner (Zeiss) equipped with 40x lens. Two sections for each kidney were examined.

2.8 Combo-specific antibody quantification

For serum collection, mice were bled 12 or 32 days after the immunization. Blood was kept at RT for 1 h in the untreated collection tubes, and then centrifuged 15 min at 1,500 × g, 4°C. Serum was collected and stored at -20°C until the day of analysis.

Ag-specific Abs in serum were quantified using Luminex Tecnology (Luminex® Corporation) as described below.

2.8.A Multi-analyte COOH microspheres

Ags were covalently conjugated to the free carboxyl groups on xMAP multi-analyte COOH microspheres (Luminex Corporation). Carboxylated microspheres were brought to RT and sonicated, followed by vortexing for approximately 1 min. An aliquot of 2.25 x 10^7 microspheres from the stock vial was added to a clear 1.5 ml copolymer microcentrifuge tube and pelleted by centrifugation. Microspheres were then washed with 500 µl of 0.1 M NaH_2PO_4 pH 6.2, suspended by sonication and then pelleted. To activate the carboxyl sites on microspheres, 10 µl of a 50 mg/ml solution of N-hydroxysulfosuccinimide (sulfo-NHS; Pierce) and 10 µl of a 50 mg/ml solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Pierce) solution were added to microspheres suspended in 480 µl of 0.1 M NaH_2PO_4. Tubes were incubated for 30 min at RT in the dark. Following activation, microspheres were washed with 500 µl of either 50 mM 2-(N-morpholino) ethan sulfonic acid buffer pH 5.0 (MES; Sigma) to remove residues of EDC and sulfo-NHS.

2.8.B Coupling

0.02 mg of each Ag were incubated for 2 h at RT in a 1.5 ml eppendorf with activated beads. At the end of this time, beads were washed with solution PBS 1x + 0.05% Tween-20 and stored in a solution PBS 1x + 0.02% Tween-20 + 0.1% BSA (BSA blocks activated sites remaining on the beads).

2.8.C Test coupling

Pre-mixing of beads is necessary to test the beads coupling and to ensure that there is no cross-reactivity between beads coupled to the different Ags. 50 µL of internal control sera were loaded in a plate with 5 µL of pre-mixed beads per well, incubated for 30 min at RT and then washed. 50 µL of phycoeritrin (PE) secondary Ab was added and incubated for 15 min at RT. The plate was washed two times and 200 µL of PBS 1x were added for the instrument reading.

2.8.D Assay

Sera were diluted 1:10,000 in PBS buffer 1x. 50 µL of sera were loaded in a 96-well microtiter plate. The plate was incubated 30 min at RT and then washed with PBS 1x. 50 µl of a solution of PE-anti-mouse IgG 1:200 in dilution buffer were dispensed in each well of the plate with 50 µL of PBS buffer 1x. The plate was incubated 15 min at RT and then washed. 200 µl of PBS buffer 1x were dispensed in each well of the plate and then Luminex reading started. The reader detected individual beads by flow cytometry. The fluidics system of the reader aligned the beads into single file as they enter a stream of sheath fluid and then a flow cell. Once the beads were in single file within the flow cell, each bead was individually interrogated for bead color (which corresponded to a particular *S. aureus* Ag) and assay signal strength (PE fluorescence intensity) after excitation at 532 nm. The median fluorescence intensity (MFI) emitted by PE at 575 nm was directly proportional to the amount of Ab bound to microspheres.

2.8.E Calculation

All results were expressed in MFI at fixed dilution (1:10,000). The lower limit of quantitation for Hla_{H35L}, EsxAB, FhuD2, and Sur2 were respectively 32, 4.56, 5.92, and 6.59 MFI. MFI was then converted in relative light units per ml of serum (RLU/ml) using a mouse serum as standard.

2.9 Hla neutralization assay

Ability of Abs elicited by Combo vaccine to inhibit Hla-induced hemolysis was evaluated in an *in vitro* assay. Seven serial two-fold dilutions of mouse sera were incubated with 12.5 nM Hla for 30 min at 37°C in agitation (350 rpm). Then, erythrocytes derived from de-fibrinated rabbit blood (rabbit red blood cells, RRBC) were added and incubation prolonged for further 30 min at 37°C. Plates were then centrifuged for 5 min at 1,000 x g and the OD_{540} of supernatants was determined with a SpectraMax® 340PC384 Absorbance Microplate Reader (Molecular Devices). OD_{540} of supernatants derived from samples incubated with Hla without serum was considered as 100% hemolysis. Incubation with 1% Triton X-100 in water was used as positive control (maximal hemolysis), while incubation without Hla was the negative control. Percent hemolysis was calculated using as denominator the OD_{540} obtained following lysis of RRBC in absence of mouse serum.

2.10 Spleen processing

Spleens were harvested from mice 12 days after the immunization and placed in 5 ml of cold RPMI 1640 (Gibco) supplemented with 25 mM HEPES (Gibco), 2% heat inactivated fetal bovine serum (FBS; low endotoxin, HyClone), and 1x penicillin/streptomycin (Pen/Strep; 100x, Gibco) in a 15 ml tube. Spleens were dissociated using the pestle of a 5 ml syringe through a 70 µM cell strainer placed on top of a 50 ml Falcon tube. Red blood cells (RBC) were lysed by incubating the cell pellet with 1 ml of lysis buffer (Biolegend) for 3 min on ice. The reaction was stopped by adding 20 ml of PBS (Gibco) and splenocytes were then suspended in 10 ml of complete medium: cold RPMI 1640 (Gibco) supplemented with 25 mM HEPES (Gibco), 10% heat inactivated FBS (low endotoxin, HyClone), 1x Pen/Strep (100x, Gibco), and 50 µM β-mercaptoetanol (Sigma). Cell suspension was filtered on a 70 µm strainer and cell counts determined using Vi-CELL XR 2.03 (Beckman Coulter, Inc.). Splenocytes were finally suspended at 2 x 10^7 cells/ml in complete medium and kept on ice until plating.

2.11 Intracellular cytokine staining

Splenocytes were plated at 2 x 10^6 cells/well in 96-well plates in complete medium and stimulated with anti-CD28 and anti-CD49d (2 µg/ml each; BD Biosciences) plus the combination of Hla_{H35L}, EsxAB, FhuD2, and Sur2 (each 10 µg/ml) (Combo) or with 2 µg/ml *Staphylococcus* enterotoxin B (SEB; Sigma) at 37°C, 5% CO_2 for 20-22 h, in the presence of Brefeldin A (5 µg/ml) for the last 4 h.

Cells were stained with Live/Dead yellow (Invitrogen) diluted 1:1,000 in PBS for 20 min at RT, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 min on ice, washed in Perm/Wash buffer 1x (BD Biosciences), incubated with anti-CD16/CD32 Fc block (BD Biosciences) for 20 min at RT, stained with fluorochrome-conjugated monoclonal Abs (mAbs) diluted in Perm/Wash buffer 1x (see Table 2.1 for Abs) for 20 min at RT, washed twice in Perm/Wash buffer 1x, and finally suspended in PBS. Samples were acquired on a LRSII SOS1 flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Fluorochrome-conjugated mAbs
CD3 PerCP-Cy5.5 (BD Pharmingen)
CD4 V500 (BD Pharmingen)
CD8 PE-Texas Red (Invitrogen)
CD44 V450 (BD Pharmingen)
TCR γ/δ PE-Cy5 (eBioscience)
IL-2 APC (BD Pharmingen)
TNF Alexa 700 (BD Pharmingen)
IFN-γ PE (BD Pharmingen)
IFN-γ FITC (BD Pharmingen)
IL-4 A488 (eBioscience)
IL-13 A488 (eBioscience)
IL-17A PE-Cy7 (eBioscience)
IL-17A A-488 (eBioscience)
IL-17F A-647 (BD Pharmingen)
RoRγt PE (BD Pharmingen)

Table 2.1 (previous page) Fluorochrome-conjugated mAbs used for flow cytometry analysis. The appropriate dilution of each fluorochrome-conjugated mAb was determined with Ab titration prior to staining. The Abs listed were used in 3 different staining panels:
Panel 1: CD3 PerCP-Cy5.5, CD4 V500, CD8 PE-Texas Red, CD44 V450, IL-2 APC, TNF Alexa 700, IFN-γ PE, IL-4 A488, IL-13 A488, IL-17A PE-Cy7.
Panel 2: CD3 PerCP-Cy5.5, CD4 V500, CD8 PE-Texas Red, CD44 V450, TCR γ/δ PE-Cy5, IFN-γ PE, IL-17A A-488.
Panel 3: CD3 PerCP-Cy5.5, CD4 V500, CD8 PE-Texas Red, CD44 V450, TCR γ/δ PE-Cy5, IFN-γ FITC, IL-17A PE-Cy7, IL-17F A-647, RoRγt PE.

2.12 Cytokine quantification in splenocyte culture supernatants

Splenocytes were plated at 2 x 10^5 cells/well in 96-well plates in complete medium and stimulated with 30 µg/ml ovalbumin (OVA; Hyglos), or Combo (10 µg/ml each Ag), or with 2 µg/ml SEB (Sigma) at 37°C, 5% CO_2 for 48 h. Plates were then centrifuged at 300 x g for 5 min and supernatants were harvested, divided in aliquots, and stored at -20°C until analysis.

Supernatants were analyzed for TNF, IL-2, IFN-γ, and IL-4 content using the MSD 96-Well MULTI-SPOT Mouse Th1/Th2 9-plex Tissue Culture kit (Meso Scale Discovery). IL-17A and IL-13 were detected using the MSD 96-Well MULTI-SPOT Mouse 4-plex custom kit (Meso Scale Discovery). Cytokine detection was conducted according to the manufacturer's instructions. In brief, undiluted samples (25 µl) were incubated on 96-well plates pre-spotted with capture Abs against the cytokines for 2 h. The SULFO-TAG detection Ab solution (25 µl) was added and co-incubated with samples on the plate for other 2 h. After washing, MSD Read Buffer T was

added and the plate was read immediately on a Sector Imager 6000 (Meso Scale Discovery). Data analysis used MSD Discovery Workbench software 2.0 (Meso Scale Discovery).

Supernatants were analyzed for IL-17F content using the Bio-Plex Pro™ Mouse Cytokine Th17 Panel B 8-Plex (Bio-Rad). Cytokine detection was conducted according to the manufacturer's instructions. In brief, the capture Ab-coupled beads were first incubated with Ag standards or undiluted supernatants followed by incubation with biotinylated detection Abs. After washing away the unbound biotinylated Abs, the beads were incubated with a reporter streptavidin-phycoerythrin conjugate (SA-PE). Following removal of excess SA-PE, the beads were passed through the Bio-Plex 200 array reader (Bio-Rad). Incubations were performed at room temperature. All washes were performed using a Bio-Plex® Handheld Magnetic Washer (Bio-Rad). Data acquisition was performed using Bio-Plex Manager™ software (Bio-Rad) at low PMT setting.

2.13 Cytokine quantification in mouse sera

Mice were bled 3 h post immunization. Sera were stored at -20°C until the day of analysis.

Samples were analyzed for IL-6 content using the MSD 96-Well MULTI-SPOT Mouse Pro-inflammatory 7-plex Tissue Culture kit (Meso Scale Discovery). IL-23 was detected in mouse sera using the Bio-Plex Pro™ Mouse Cytokine Th17 Panel B 8-Plex (Bio-Rad). TGF-β was quantified with the Bio-Plex Pro™ TGF-β 3-plex assay (Bio-Rad). Cytokine detection was conducted according to the manufacturer's instructions.

Samples were tested undiluted on the MSD kit, diluted 1:4 in sample diluent for IL-13 detection, and diluted 1:16 in sample diluent for TGF-β quantification.

2.14 Proliferation assay

Splenocytes were plated at 1 x 10^6 cells/well in 96-well plates in complete medium and stimulated with 30 µg/ml OVA (Hyglos), or Combo (10 µg/ml each Ag), or with 1 µg/ml plate-bound anti-CD3 (BD Biosciences) at 37°C, 5% CO_2 for 90 h, in the presence of 10 µM 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) in complete medium for the last 18 h.

The cells were then stained with Live/Dead yellow (Invitrogen) for 20 min at RT, incubated with anti-CD16/CD32 Fc block (BD Biosciences) for 20 min at RT, and stained with fluorochrome-conjugated mAbs: anti-CD44-APC (BD Pharmingen), anti-CD4-PB and anti-CD8-PE-Texas Red (Invitrogen) diluted in PBS for 20 min at RT. Samples were then fixed with Cytofix (BD Biosciences) for 15 min on ice, washed twice with 0.1% BSA in PBS, and suspended in 100 µl/well of Click-iT™ EdU reaction cocktail (Invitrogen). Cells were incubated 30 min at RT in the dark, washed twice with 1x saponin-based permeabilization/wash buffer (Invitrogen), and finally suspended in 0.1% BSA in PBS. Samples were acquired on a LRSII SOS1 flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

2.15 $CD4^+$ T-cell depletion

BALB/c mice were treated i.p. with 100 µg of a rat anti-mouse CD4 mAb (IgG2b; clone GK1.5; BioXCell) or isotype control (rat IgG2b; BioXCell) 2 and 4 days before *S. aureus* infection. The day before infection, mice were bled to control the efficacy of the $CD4^+$ T-cell–depleting treatment. For this purpose, blood was diluted 1:4 in PBS (Gibco) and incubated for 10 min on ice with RBC lysis buffer (Biolegend). Blood cells were then washed with PBS and stained with Live/Dead yellow (Invitrogen) diluted 1:1,000 in PBS for 20 min at RT. Anti-CD16/CD32 Fc block (BD Biosciences) was added for 20 min at RT. $CD4^+$ and $CD8^+$ T cells were identified by staining cells with anti-CD3-PerCP-Cy5.5 (BD Pharmingen), anti-CD4-PB, and anti-CD8-PE-Texas Red (Invitrogen) mAbs for 20 min at RT. The appropriate dilution of each fluorochrome-conjugated mAb was determined with Ab titration prior to staining. Cells were washed twice with 0.1% BSA in PBS, fixed with Cytofix (BD Biosciences) for 20 min on ice, and washed with 0.1% BSA in PBS. Samples were acquired on a LRSII SOS1 flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

2.16 *In vivo* cytokine neutralization

To neutralize IL-17A and/or IFN-γ *in vivo*, mice immunized with alum/S-7±Combo were treated with 100 µg/mouse of a neutralizing mouse anti-mouse IL-17A mAb (IgG1; clone 17F3; BioXCell) and/or a neutralizing rat anti-mouse IFN-γ mAb (IgG1; clone XMG 1.2; BioXCell) i.p. every other day starting from 3 h prior to *S. aureus* challenge until sacrifice. Control

mice received the same amount of isotype-matched control mAbs (mouse IgG1 and rat IgG1; BioXCell) or the same volume of PBS (Gibco).

2.17 Statistical analysis

Mouse survival was analyzed for significance using the log-rank (Mantel-Cox) test. The bacterial load following *S. aureus* infection, represented as the $\log_{10}$ CFU/kidneys, was analyzed for statistical significance with the unpaired Student's *t* test, two-tailed. Quantification of mouse peritoneal abscess formation and of Ag-specific Abs was analyzed for statistical significance using the unpaired Mann Whitney U-test, two-tailed. Effective dose 50 (ED_{50}) of Combo-specific Abs able to inhibit Hla-induced hemolysis was calculated using a "log(agonist) *vs*. normalized response" nonlinear regression curve. T-cell responses to vaccination were analyzed using ANOVA and Tukey's or Bonferroni's post-test correction. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc.). All experiments were examined for reproducibility.

Chapter 3

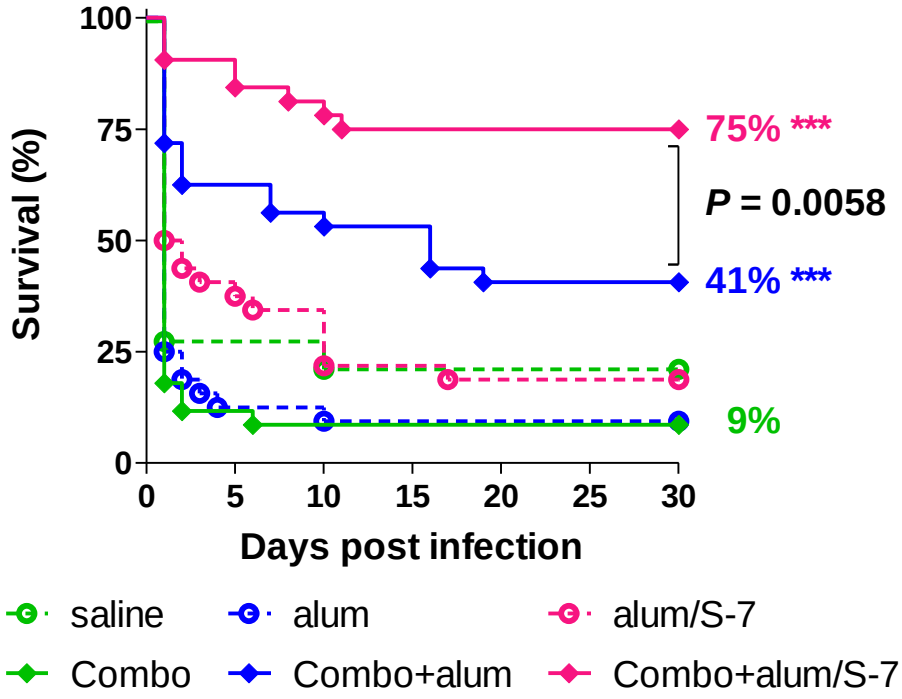
Results

3.1 Combo+alum/S-7 protects mice from *S. aureus*-induced peritonitis more efficiently than Combo or Combo+alum

To assess whether Combo vaccine adjuvanted with alum/S-7 afforded greater protection against staphylococcal infection than the vaccine either non-adjuvanted or adjuvanted with alum, a head to head comparison of the vaccines was performed in the peritonitis model of *S. aureus* infection. Combo Ags, alone or formulated with alum or alum/S-7, were used to immunize BALB/c mice i.m., while negative controls received identical courses of buffer alone (saline) or adjuvant alone (alum or alum/S-7). On day 12 after immunization mice were injected i.p. with a lethal dose of *S. aureus* Newman strain (used throughout all experiments) containing 5×10^8 CFU. Preliminary titration showed that this dose caused the death of approximately 80% of untreated mice (i.e. lethal dose 80, LD_{80}). Survival and symptoms of infected animals were monitored for 30 days after infection (Figure 3.1 A).

As expected, injection of 5 x 10^8 CFU of *S. aureus* Newman strain caused approximately 78% of infected mice to succumb to infection (22% survival in the saline group). Of note, the majority of these animals died within 24 h. Combo vaccine without adjuvant conferred no protection in this model (9% survival) while Combo adjuvanted with alum or alum/S-7 protected more than adjuvant alone, 41% (*P*=0.0002 Combo+alum *vs.* alum) and 75% (*P*<0.0001 Combo+alum/S-7 *vs*. alum/S-7), respectively. Most importantly, the survival of mice vaccinated with Combo+alum/S-7 was significantly higher than that of mice vaccinated with Combo+alum (*P*=0.0058).

A



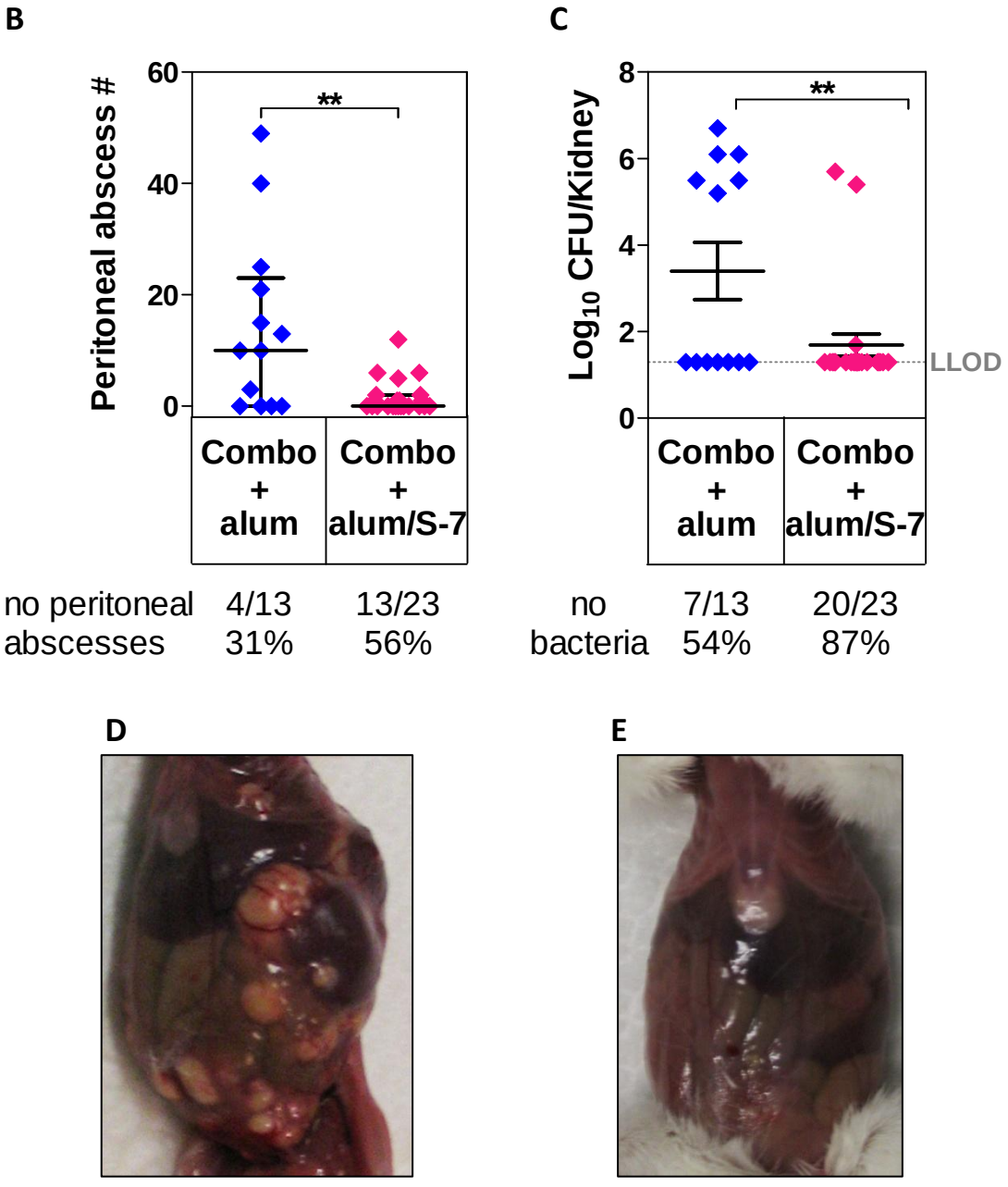


Figure 3.1 (pages 58-59) Survival, peritoneal abscesses and bacterial load in kidneys of BALB/c mice immunized with Combo Ags alone or formulated with alum or alum/S-7 and infected intraperitoneally with *S. aureus*. On day 0, 5-week-old BALB/c mice (n=32) were immunized i.m. with 10 µg of each Ag alone or adsorbed to 2 mg/ml alum±50 µg SMIP-7.10. On day 12, mice were challenged

with *S. aureus* Newman (5 x 10^8 CFU) i.p. (**A**) Mice were monitored for survival for 30 days post challenge. Log-rank (Mantel-Cox) test: survival of Combo+alum/S-7 immunized mice *vs*. alum/S-7 immunized mice and of Combo+alum immunized mice *vs*. alum immunized mice, *** $P<0.001$. Thirty days after *S. aureus* inoculation, mice vaccinated with Combo+alum or Combo+alum/S-7 that survived to infection (Combo+alum: n=13; Combo+alum/S-7: n=23) were euthanized, abscesses in the peritoneal cavity were enumerated, kidneys were excised and their staphylococcal load was determined by plating homogenized tissue on agar plates and enumerating the CFU. (**B**) Number of abscesses in the peritoneal cavity; bars represent the median±interquartile range of each group. Mann Whitney U-test, two-tailed: ** $P<0.01$. (**C**) Log_{10} CFU/kidney; lines and error bars represent the mean±SEM. Unpaired t test, two-tailed: ** $P<0.01$. LLOD=Lower Limit Of Detection. (**D**) Peritoneal cavity of mice belonging to Combo+alum or (**E**) to Combo+alum/S-7 groups, respectively.

To investigate whether *S. aureus* was cleared more efficiently upon vaccination with Combo+alum/S-7 than with Combo+alum, survivors were sacrificed at day 30 after challenge, peritoneal abscesses were enumerated by visual inspection and the staphylococcal load in renal tissue was determined by counting the CFU in kidney homogenates.

Peritoneal abscesses were found in mice belonging to both vaccination groups (Figure 3.1 B, D, E), however their number was significantly lower in the Combo+alum/S-7 group than in the Combo+alum group ($P<0.01$). In addition, not only the size of abscesses was considerably smaller (not shown) but also the percentage of mice free of peritoneal abscesses was remarkably higher in mice vaccinated with Combo+alum/S-7 than in mice vaccinated with Combo+alum (50% *vs*. 31%, Figure 3.1 B).

In addition, one month after the injection of *S. aureus* no staphylococci were detected in kidneys of 87% of mice immunized with Combo+alum/S-7 *vs*. 54% of mice immunized with Combo+alum (Figure 3.1 C).

Together, these data showed that alum/S-7 is a more efficacious adjuvant than alum for Combo vaccine since it conferred not only better protection from mortality (75% of survivors *vs*. 41%) but also promoted a more efficient clearance of bacteria not only at the infection site (peritoneum) but also at distant organs (e.g., kidney). Remarkably, no signs of infection were found at either location in half of the mice vaccinated with Combo+alum/S-7.

3.2 Combo+alum/S-7 outperforms Combo or Combo+alum at inducing Combo-specific IgG

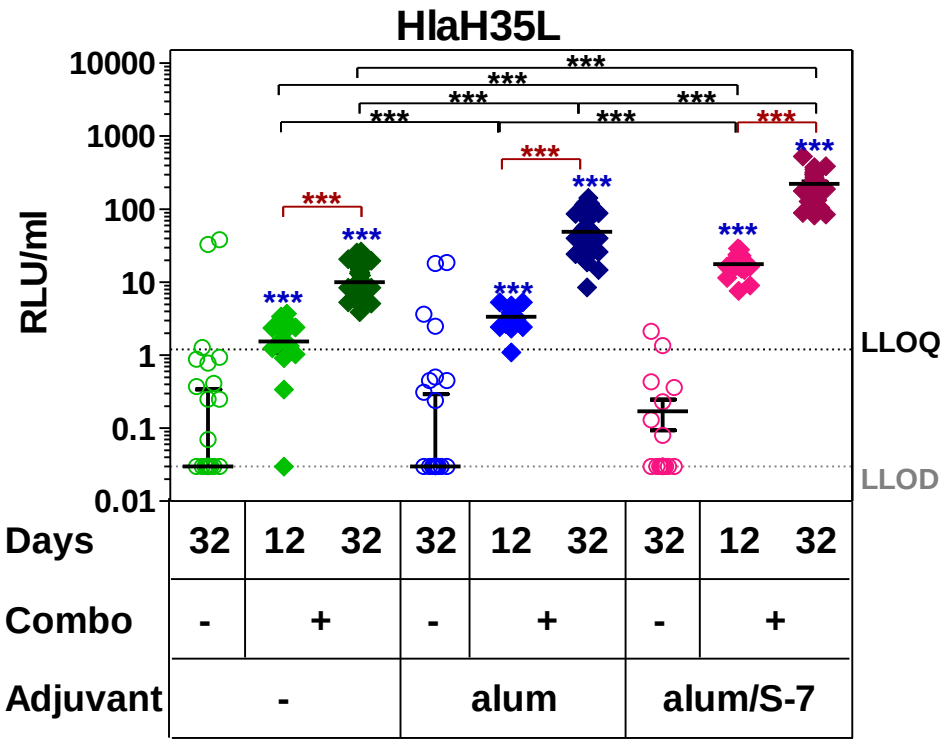
The capacity of Combo-based vaccines to induce production of Abs specific for each of the vaccine Ags was evaluated by collecting sera 12 and 32 days after vaccination.

Twelve days after vaccination, low Hla_{H35L}-specfic IgM titers were measured in sera from mice vaccinated with Combo adjuvanted with alum or alum/S-7, while titers were below the detection limit for non-adjuvanted Combo. Ag-specific IgM titers were below detection limit for EsxAB, FhuD2, and Sur2 (data not shown).

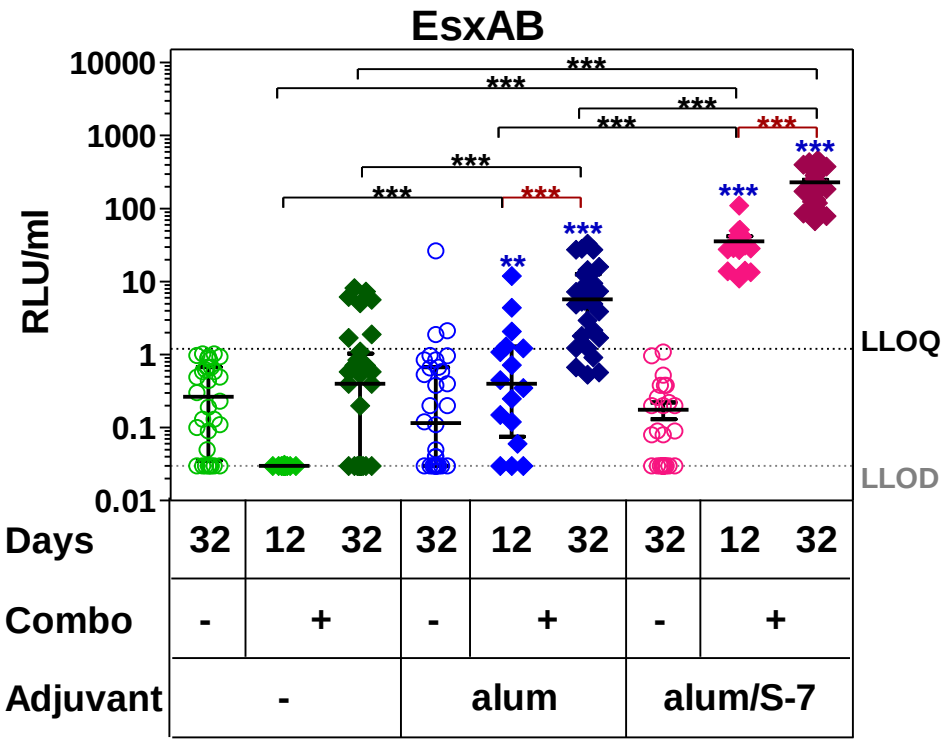
In sera from mice vaccinated with Combo, IgG specific for Hla_{H35L} (but not for the other Combo Ags) were detected by day 12 (Figure 3.2). At day 32 after vaccination, anti-Hla_{H35L} IgG were significantly increased and anti-EsxAB IgG were detected in some mice.

In sera from mice vaccinated with Combo+alum, IgG specific for Hla_{H35L}, EsxAB, and FhuD2 (but not Sur2) were detected by day 12 after vaccination.

A


HlaH35L
RLU/ml
10000
1000
100
10
1
0.1
0.01
LLOQ
LLOD
Days
32 12 32 32 12 32 32 12 32
Combo
- + - + - +
Adjuvant
- alum alum/S-7

B


EsxAB
RLU/ml
10000
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10
1
0.1
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LLOQ
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Days
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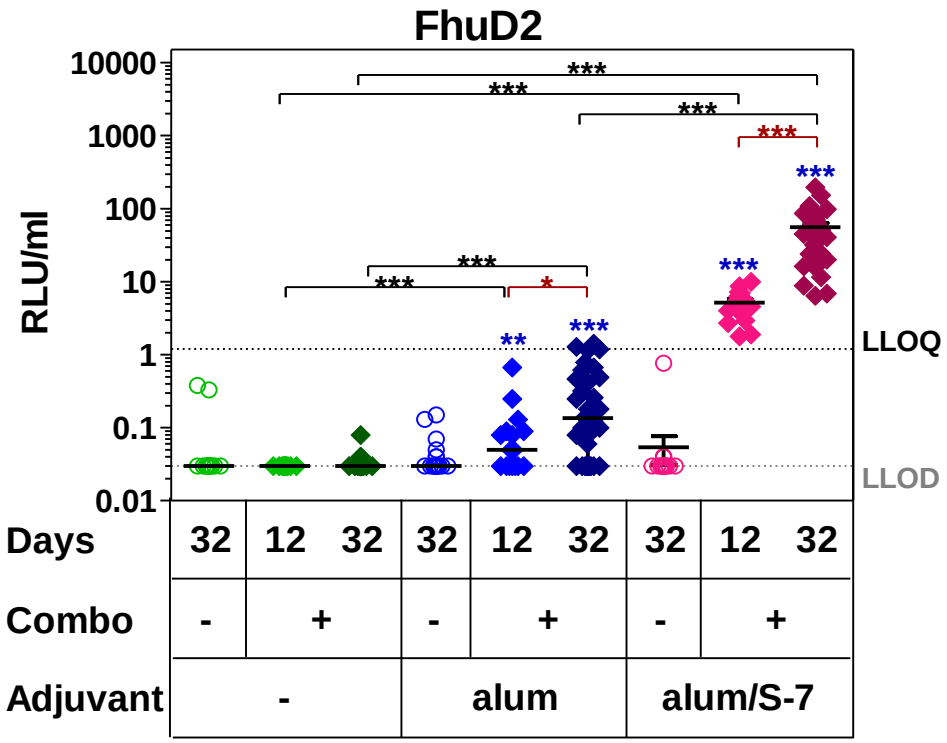
Combo
- + - + - +
Adjuvant
- alum alum/S-7

C


FhuD2
RLU/ml
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Combo
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Adjuvant
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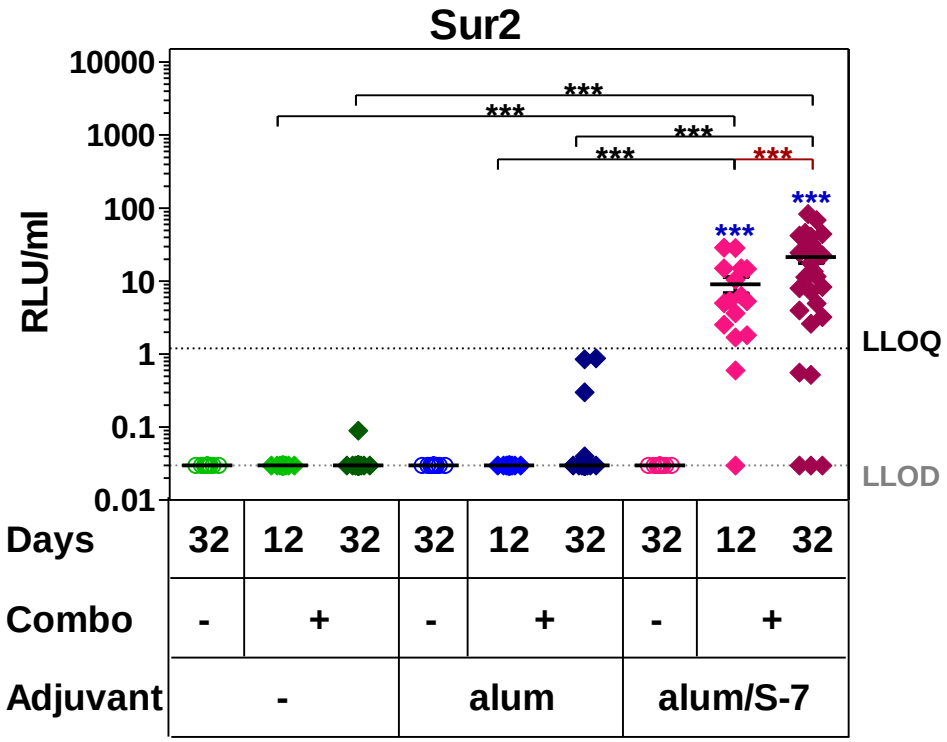
D


Sur2
RLU/ml
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0.01
LLOQ
LLOD
Days
32 12 32 32 12 32 32 12 32
Combo
- + - + - +
Adjuvant
- alum alum/S-7

Figure 3.2 (pages 62-63) Combo-specific IgG in mouse serum 12 or 32 days after immunization with Combo alone or formulated with alum or alum/S-7. BALB/c mice (32/group from 3 independent experiments) were bled 12 or 32 days after vaccination. Levels of total IgG specific for each Combo Ag were measured in sera by Luminex technology and expressed as RLU/ml. Lines and error bars represent median±interquartile range. Mann Whitney U-test, two-tailed: *** $P<0.001$ and ** $P<0.01$. Blue stars indicate differences between Combo±saline/adjuvant *vs*. saline/adjuvant alone. Brown stars indicate differences between day 12 and day 32 within the same vaccination group. Black stars indicate differences between different vaccination groups at the same time. LLOD=Lower Limit Of Detection; LLOQ=Lower Limit Of Quantitation.

IgG levels specific for these Ags increased significantly at day 32 after vaccination.

In sera from mice vaccinated with Combo+alum/S-7, IgG specific for each of the Combo Ags were detected already by day 12 after vaccination and increased significantly at day 32 after vaccination. Remarkably, IgG levels specific for each Combo Ag of mice vaccinated with Combo+alum/S-7 were always significantly higher than those of mice vaccinated with Combo+alum bled at the same time after vaccination.

Overall, alum/S-7 is a more efficacious adjuvant than alum for Combo vaccine since it elicited faster and stronger Combo-specific Ab production.

3.3 Combo+alum/S-7 induces Hla-neutralizing antibodies

It has recently been shown that Hla plays a central role in causing the death of mice infected i.p. with *S. aureus*. In addition, Abs against Hla are protective in the peritonitis model [155]. Therefore, we decided to test whether anti-Hla Abs induced by vaccination with Combo formulated with alum/S-7 were not only more abundant but also more effective at neutralizing Hla toxic activity than those induced by vaccination with Combo+alum.

For this purpose we performed a Hla hemolytic activity neutralization assay [156] in which wild type Hla (12.5 nM), pre-incubated or not with serial dilutions of pooled serum from vaccinated mice, was incubated with rabbit erythrocytes. Erythrocyte lysis was measured reading the optical density at 540 nm (OD_{540}), where the emission of hemoglobin can be detected in sample supernatants.

No hemolysis inhibition was detected with pre-immune sera or with sera from mice immunized with saline, alum, or alum/S-7 alone (data not shown). At day 12 after vaccination, dose-dependent inhibition of hemolysis was observed only in sera from mice vaccinated with Combo+alum/S-7 while at day 32, Hla neutralizing activity was measured in sera from mice vaccinated with Combo, Combo+alum, and Combo+alum/S-7 (Figure 3.3).

In conclusion, Combo+alum/S-7 vaccine induced stronger and faster Hla neutralizing activity than Combo+alum vaccine.

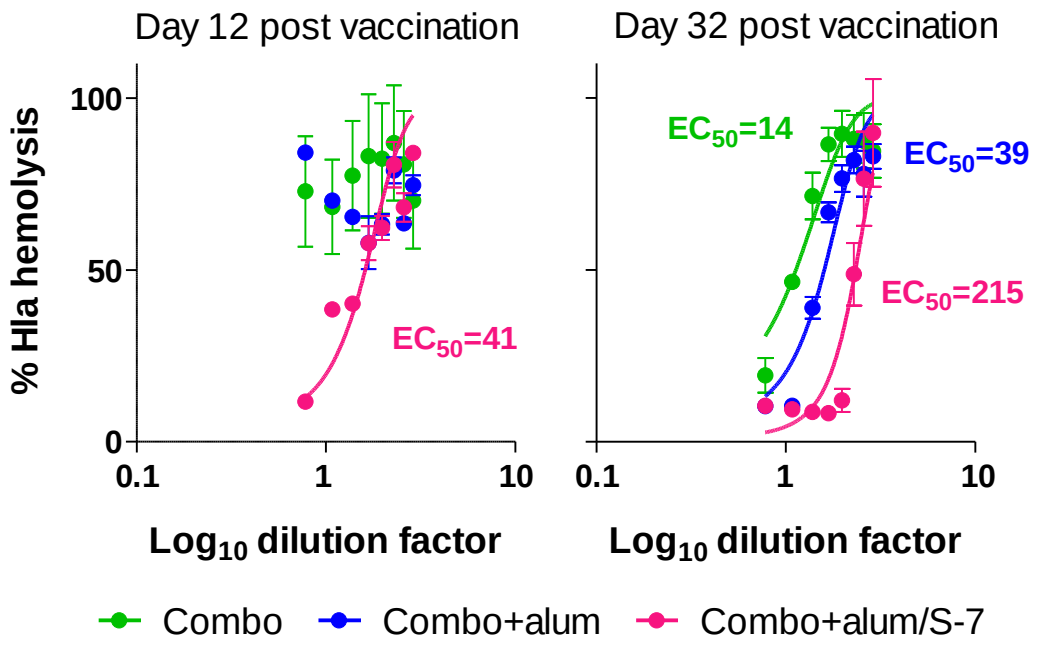


Figure 3.3 Hla neutralizing activity of sera from vaccinated mice. Sera from mice vaccinated with Combo (green), Combo+alum (blue) or Combo+alum/S-7 (pink) were collected 12 and 32 days after vaccination. *S. aureus* α-hemolysin (12.5 nM) was pre-incubated for 30 min with serial two-fold dilutions of pooled sera (triplicates). Then, RRBC were added and incubation continued for another 30 min. Percent hemolysis was calculated reading the OD_{540} of supernatants and using as denominator the OD_{540} of RRBC incubated with Hla in the absence of mouse serum. Dots correspond to the mean of triplicates and bars represent the SEM. Data were interpolated and the effective dose that neutralized 50% of Hla activity (ED_{50}) was calculated using a "log(agonist) *vs*. normalized response" nonlinear regression curve in GraphPad Prism. Each sample was obtained pooling sera from 16 mice from one experiment. Data are representative of 2 independent experiments.

3.4 Antibodies/B cells are needed to achieve protection with Combo+alum and Combo+alum/S-7 vaccination

To establish if Abs induced by vaccination with Combo+alum or Combo+alum/S-7 are required to protect mice against peritoneal challenge with *S. aureus,* we used J_H mice that are genetically unable to produce Abs. J_H mice carry a deletion in the JH gene that does not allow B cells to produce a recombined version of the variable region of the heavy chain of Igs. Therefore, B cell differentiation is blocked at the large, $CD43^+$ precursor stage ($CD220^+CD43^+sIg^-$). No IgM or IgG can be detected in the serum, while these mice have apparently normal T-cell development, surface phenotype, and numbers in spleen.

J_H mice immunized with Combo, Combo+alum, Combo+alum/S-7, or with adjuvants only were infected i.p. 12 days later with 5×10^8 CFU of *S. aureus,* and their survival was monitored for 10 days after infection. J_H mice were maintained in a Murine Pathogen Free (MPF) isolated barrier unit.

As shown in Figure 3.4, 96% of J_H mice died within 24 h following infection. Only 2 J_H mice survived until day 10 after infection: 1 in the group immunized with Combo+alum (4%) and 1 in the group immunized with Combo+alum/S-7 (4%). (These 2 mice had neither abscesses in the peritoneum nor CFU in kidneys suggesting that they might have been not infected).

In parallel experiments, immunization with Combo or Combo+alum protected BALB/c mice housed in a MPF isolated barrier unit very poorly (8% and 12% survival, respectively) while Combo+alum/S-7 still protected 88% of BALB/c mice. The increased sensitivity to i.p. infection with *S. aureus* of BALB/c mice housed in MPF conditions (Figure 3.4) *vs*. BALB/c mice housed in non-isolated conditions (Figure 3.1 A) suggested

that mice housed in non-MPF conditions may be exposed to and develop protective Abs against *S. aureus*.

Altogether, these data showed that Abs/B cells are absolutely required to mediate protection against an i.p. infection with *S. aureus* in response to vaccination with Combo adjuvanted with alum or alum/S-7.

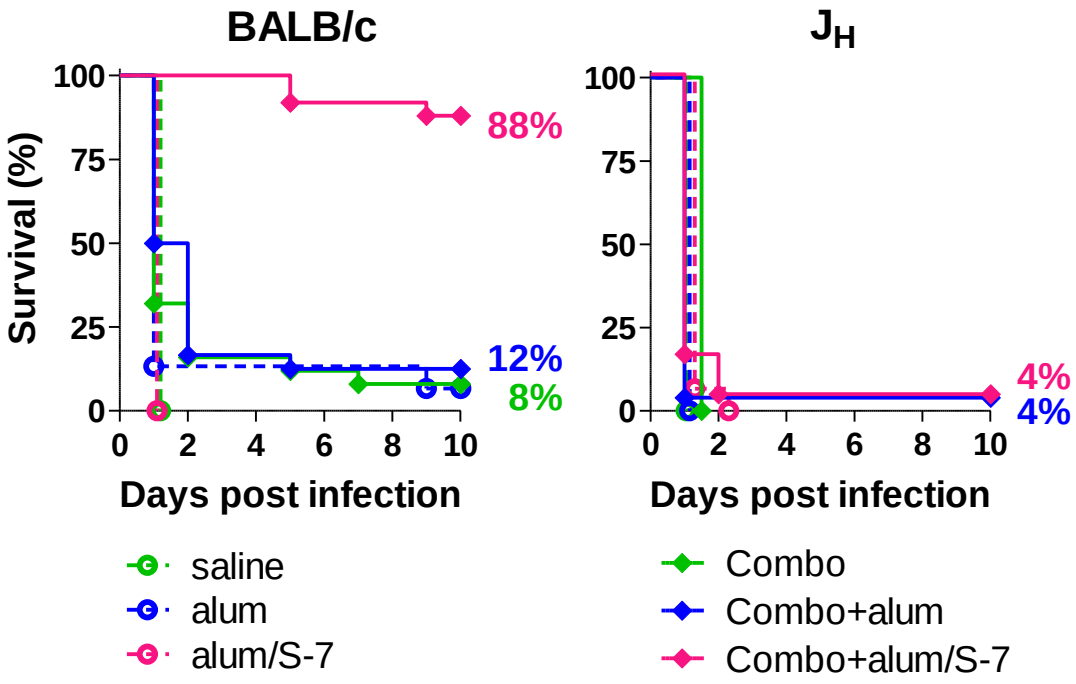


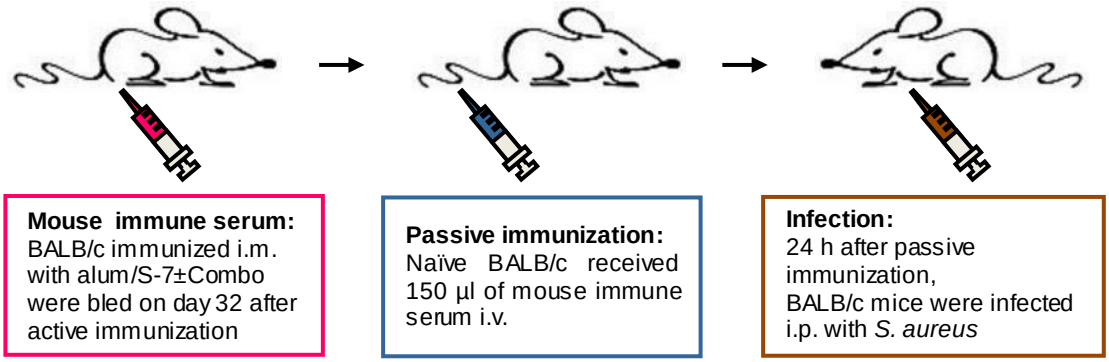
Figure 3.4 Survival of immunized BALB/c and J_H following lethal challenge with *S. aureus*. BALB/c or J_H mice immunized with Combo, Combo+alum, or Combo+alum/S-7, or with adjuvant alone were challenged i.p. with *S. aureus* 12 days after immunization, and their survival was followed for 10 days. Data were pooled from 4 independent experiments (Combo±adjuvant groups: n=25; adjuvant groups: n=15). Log-rank (Mantel-Cox) test: survival of Combo+alum/S-7 immunized BALB/c mice *vs*. alum/S-7 immunized BALB/c mice, *** $P<0.001$.

3.5 Antibodies induced by Combo+alum/S-7 immunization have a protective role against *S. aureus*-induced peritonitis

We have shown that Combo+alum/S-7 protected mice from *S. aureus* infection more efficiently than Combo or Combo+alum and induced higher levels of Combo-specific IgG and Hla-neutralizing Abs. In addition, experiments performed in J_H mice have shown that Abs and/or B cells are required for Combo vaccine-induced protection. Thus, to establish more directly the role played by Abs in the protection conferred by the Combo+alum/S-7 vaccine, we performed passive transfer experiments in which serum from mice vaccinated with Combo+alum/S-7 was injected intravenously (i.v.) in naive mice 1 day before infection. As negative control, we used serum from mice vaccinated with alum/S-7 alone. Mouse survival after infection was followed for 15 days. For comparison, a group of mice was vaccinated actively with Combo+alum/S-7. Serum for passive immunization experiments was prepared bleeding mice 32 days after active immunization since, theoretically, anti-Combo IgG titers achieved by passive transfer of 150 µl/mouse should result in IgG titers comparable to those induced by active immunization at the time of challenge (12 days post vaccination). To control that this was indeed the case, 2 mice/group were bled just before infection and anti-Combo IgG measured.

As shown in Figure 3.5 B, levels of anti-Combo IgG were comparable in mice vaccinated either actively or passively. When mice were injected with serum derived from Combo+alum/S-7 immunized mice, 28% of the animals survived to the lethal challenge for 15 days as compared to 0% in the group that received serum from mice immunized with alum/S-7 alone (Figure 3.5 C). Of note, all mice were protected up to 4 days post infection by passive transfer of serum from Combo+alum/S-7 immunized mice.

A



B

Combo+alum/S-7 immunization	Total IgG (RLU/ml)				ED_{50}
	EsxAB	FhuD2	Sur2	Hla_{H35L}	Hla
Active	70.4	7.3	15.6	56.9	11.7
Passive	32.0	5.8	3.0	52.2	6.4

C

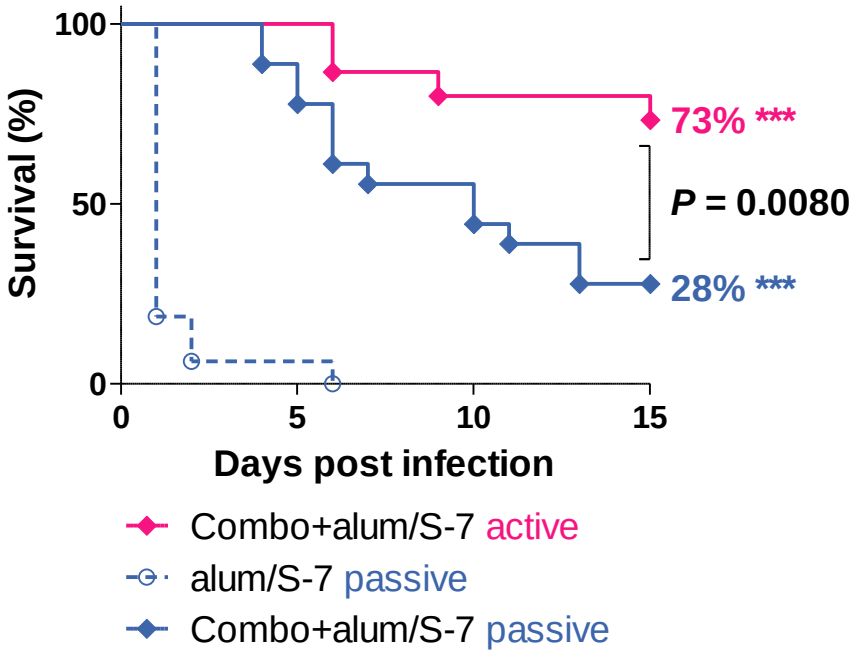


Figure 3.5 (previous page) Passive transfer of mouse serum from Combo+alum/S-7 immunized mice protected mice from lethal challenge with *S. aureus*. (**A**) Serum from mice immunized with alum/S-7 or Combo+alum/S-7 was collected 32 days post-vaccination, pooled (36 mice/group), and injected into the tail vein of BALB/c mice (150 µl/mouse); 24 h later, animals were infected i.p. with *S. aureus* Newman. (**B**) Mice from one experiment (n=2) were bled 1 h before infection and sera were analyzed for Combo-specific IgG content by Luminex technology. (**C**) Mice were monitored for survival for 15 days post challenge. Data were pooled from 2 independent experiments (n=16). Log-rank (Mantel-Cox) Test: survival of Combo+alum/S-7 *vs*. alum/S-7 passively or actively immunized mice, *** $P<0.00$; survival of Combo+alum/S-7 passively *vs*. actively immunized mice, *** $P<0.001$.

These data showed that Combo-specific Abs induced by a single immunization with Combo+alum/S-7 could protect mice from a lethal i.p. challenge with *S. aureus*.

However, passive immunization was not as effective as active immunization with Combo+alum/S-7 at protecting mice either from lethal challenge 15 days post infection (28% *vs*. 73% survival, respectively; $P=0.008$) or from bacterial growth in the peritoneum and in kidneys (Figure 3.6).

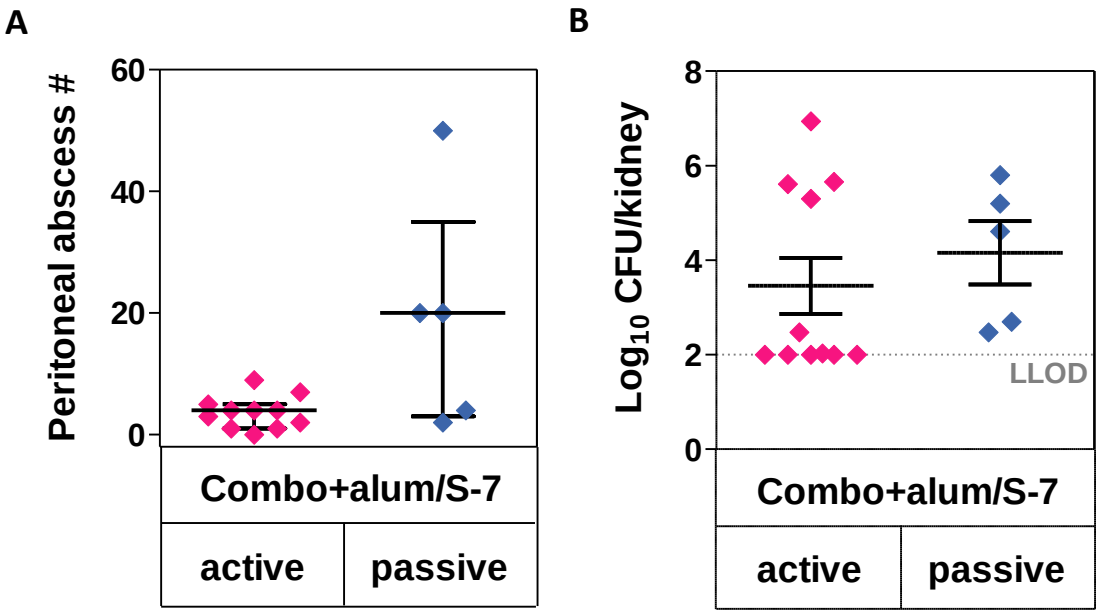


Figure 3.6 Effect of passive transfer of mouse serum from Combo+alum/S-7 immunized mice on the number of peritoneal abscesses and bacterial load in kidneys of mice following lethal challenge with *S. aureus*. 150 µl of serum from alum/S-7 or Combo+alum/S-7 immunized mice were injected into the tail vein of naïve BALB/c mice 1 day prior to i.p. infection with *S. aureus* Newman. (**A**) Fifteen days after inoculation, animals were euthanized and abscesses in the peritoneal cavity were enumerated. Data were pooled from 2 independent experiments (Combo+alum/S-7 active immunization: n=11; Combo+alum/S-7 passive immunization: n=5). Lines and error bars represent median±interquartile range. Mann Whitney U-test, two-tailed. (**B**) Staphylococcal load in kidneys was determined by plating homogenized tissue on agar plates and enumerating CFU. Lines and error bars represent mean±SEM. Unpaired Student's *t* test, two-tailed.

Altogether, these data showed that Combo-specific Abs elicited by one immunization with Combo+alum/S-7 play a central role in protection against *S. aureus* in the peritonitis model, and suggested that other immune mechanisms could contribute to the very good protection achieved by active vaccination with Combo+alum/S-7.

3.6 Combo-specific $CD4^+$ T cells induced by Combo+alum/S-7 immunization do not proliferate *in vitro*

The relevance of $CD4^+$ T cells in protection against *S. aureus* infections has been suggested by clinical observations as well as by mouse studies [157] [158] [159] [160] [161] [162]. Therefore, we decided to evaluate the contribution of the T-cell response to the efficacy of Combo vaccine adjuvanted with alum/S-7.

First, we assessed if vaccination induced detectable Combo-specific $CD4^+$ T-cell responses by looking at $CD4^+$ T-cell proliferation. For this purpose, splenocytes from BALB/c mice vaccinated with Combo, Combo+alum, or Combo+alum/S-7 were collected 12 days after immunization and stimulated *in vitro* with or without Combo (10 µg/ml each Ag) for 90 h. Click-IT EdU incorporation was measured by flow cytometry, as described in paragraph 2.14.

Proliferation assay showed that Combo-specific $CD4^+$ T cells induced by immunization with Combo or Combo+alum were able to proliferate after in vitro stimulation, while those induced by immunization with Combo+alum/S-7 were not (Figure 3.7).

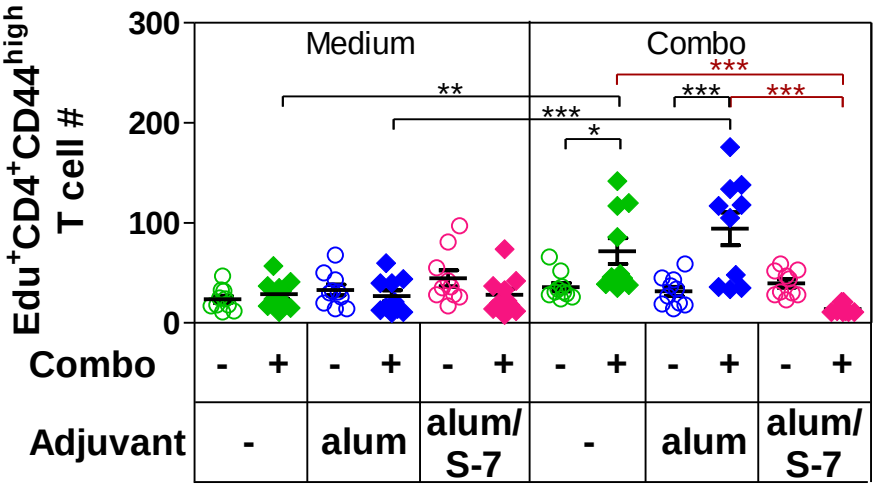


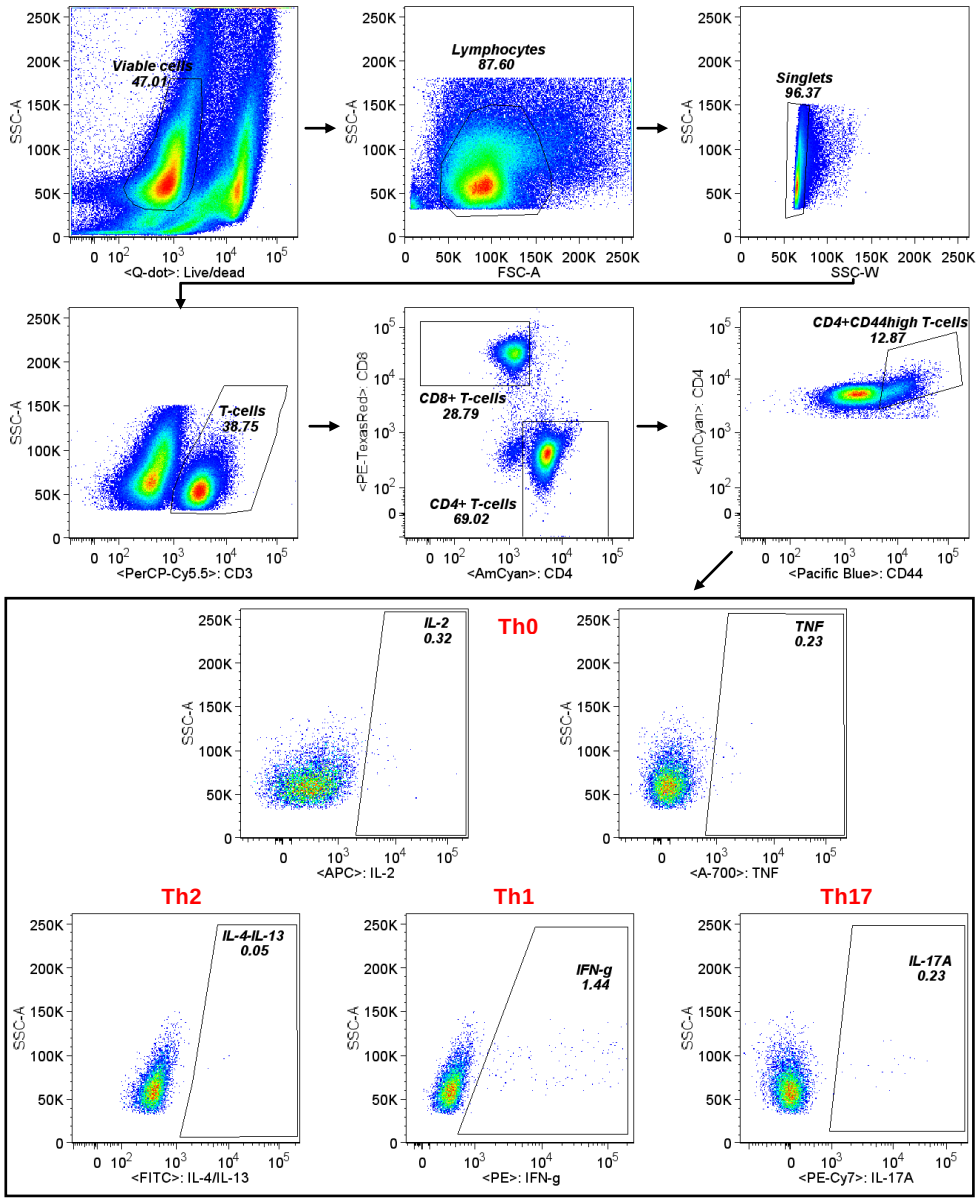
Figure 3.7 Combo-specific CD4$^+$ T-cell proliferation. BALB/c mice were immunized with Combo (close green symbol), Combo+alum (close blue symbol), or Combo+alum/S-7 (close pink symbol) or, as negative controls, with saline (green open symbol), alum (blue open symbol), or alum/S-7 (pink open symbol) only. Twelve days after vaccination, mice were sacrificed and splenocytes from each mouse were stimulated *in vitro* with complete medium or Combo for 96 h. The graph shows the numbers of Edu$^+$CD4$^+$CD44^{high} T cells. Lines and error bars represent mean±SEM. Data were pooled from 2 independent experiments (n=10). ANOVA and Tukey's post-test correction: * P<0.05 and *** P<0.001.

3.7 Combo+alum/S-7 polarized Combo-specific CD4$^+$ T-cell responses to Th1/Th17

We have shown that Combo and Combo+alum immunization are able to elicit proliferating Combo-specific CD4$^+$ T cells. Therefore, we assessed if vaccination induced a detectable Combo-specific CD4$^+$ T-cell response by flow cytometry analysis. For this purpose, splenocytes from BALB/c mice vaccinated with Combo, Combo+alum, Combo+alum/S-7, or adjuvant alone were collected 12 days after immunization and stimulated *in vitro* with or without Combo for 18 h. Brefeldin A was added to the

cultures during the last 4 h to block cytokine secretion. Cells were then loaded with Live/Dead stain to allow exclusion of dead cells from the analysis, fixed, permeabilized to allow staining of intracellular cytokines, and stained with fluorescently-labeled mAbs against T-cell markers: CD3, CD4, and CD8, the activation marker CD44, and against cytokines: TNF, IL-2, IFN-γ (Th1 signature cytokine), IL-17 (Th17 signature cytokine), IL-4/IL-13 (Th2 signature cytokines). mAbs anti-IL-4 and anti-IL-13 were labeled with the same fluorophore (see panel 1). Cells were analyzed by multiparametric flow cytometry according to the gating strategy reported in Figure 3.8.

Figure 3.8 (next page) Gating strategy for intracellular cytokine staining analysis of $CD4^{+}CD44^{high}$ T cells. Spleen cells from a mouse vaccinated with Combo+alum/S-7 stimulated with Combo for 18 h (Brefeldin A was added for the last 4 h). Cells were stained with Live/Dead yellow, fixed, permeabilized and stained with monoclonal mAbs labeled with different fluorochromes directed against CD3, CD4, CD8, CD44, TNF, IL-2, IFN-γ, IL-17A, IL-4, and IL-13 (same fluorochrome anti-IL-4 and anti-IL-13, see panel 1), and analyzed by multiparametric flow cytometry. Live cells were identified based on Live/Dead staining. Lymphocytes were identified first based on a morphological gate (forward scatter area, FSC-A *vs*. side scatter area, SSC-A). Single cells were identified based on forward scatter properties (side scatter width, SSC-W *vs*. SSC-A). $CD4^{+}CD44^{high}$ T cells were then identified based on expression of CD3, CD4, and CD44 markers. Inside the $CD4^{+}CD44^{high}$ T-cell population, cells producing TNF, IL-2, IFN-γ, IL-17A, or IL-4/IL-13 were identified setting gates on non-stimulated cells (not shown). Boolean gates were used to identify the total number of cells that produced any cytokine in each cell sample.



The response of non-stimulated cells was subtracted from that of cells stimulated with Combo. Stimulation with an irrelevant Ag, OVA, did not increase the percentages of cytokine-producing $CD4^{+}CD44^{high}$ T cells (data not shown). Figure 3.8 shows representative flow cytometry dot plots of

spleen cells from a Combo+alum/S-7 vaccinated mouse stimulated with Combo.

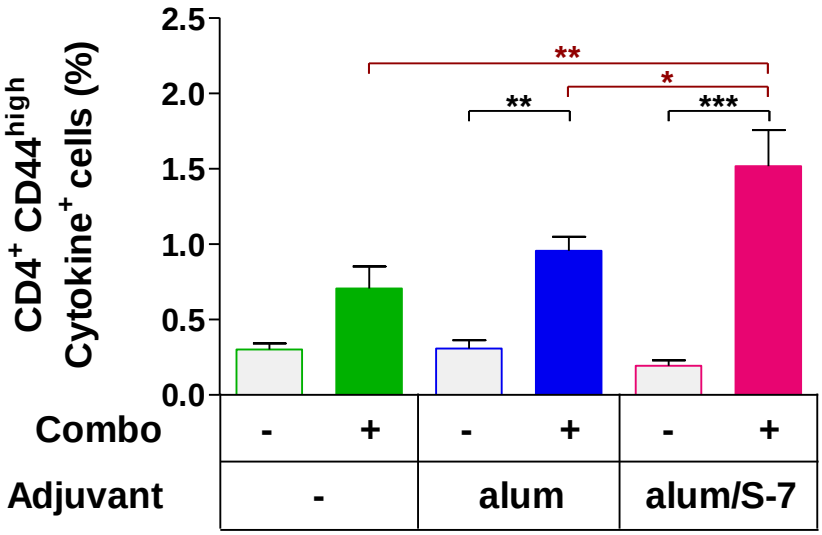


Figure 3.9 Magnitude of Combo-specific CD4$^+$ T-cell responses in mice vaccinated with Combo, Combo+alum, or Combo+alum/S-7. BALB/c mice were immunized with Combo (close green bar), Combo+alum (close blue bar), or Combo+alum/S-7 (close pink bar) or, as negative controls, with saline (green open bar), alum (blue open bar), or alum/S-7 (pink open bar) only. Twelve days after vaccination, mice were sacrificed and splenocytes from each mouse were stimulated or not *in vitro* with Combo Ags for 18 h. The response of non-stimulated cells was subtracted from that of stimulated cells. The graph shows the percentages of CD4$^+$CD44^{high} T cells producing any combination of TNF, IL-2, IFN-γ, IL-17A, IL-4, IL-13 (panel 1) in response to Combo stimulation. Bars represent mean±SEM (n=16 from 3 independent experiments). ANOVA and Tukey's post-test correction: ** P<0.01 and *** P<0.001.

Vaccination with Combo alone induced a low percentage of cytokine-producing CD4$^+$CD44^{high} T cells not statistically significantly different from vaccination with saline alone. On the other hand, vaccination

with either Combo+alum or Combo+alum/S-7 induced statistically significantly higher percentages of cytokine-producing $CD4^{+}CD44^{high}$ T cells as compared to vaccination with alum or alum/S-7 only. The magnitude of the $CD4^{+}$ T-cell response of mice immunized with Combo+alum/S-7 was higher compared to that of mice vaccinated with Combo or Combo+alum (Figure 3.9).

Then, we proceeded to analyze the quality of cytokines produced by Combo-specific $CD4^{+}$ T cells from mice vaccinated with Combo+alum or Combo+alum-S-7. We found comparable percentages of Combo-specific IL-2–producing $CD4^{+}CD44^{high}$ T cells in mice vaccinated with Combo+alum or Combo+alum/S-7, while higher percentages of TNF-producing cells were found in the Combo+alum/S-7 group. On the contrary, higher percentages of IL-4/IL-13–producing cells were found in the Combo+alum group. In addition, much higher percentages of IFN-γ–producing cells were found in the Combo+alum/S-7 *vs*. Combo+alum group, while low percentages of IL-17A–producing cells were found in the Combo+alum/S-7 group only (Figure 3.10).

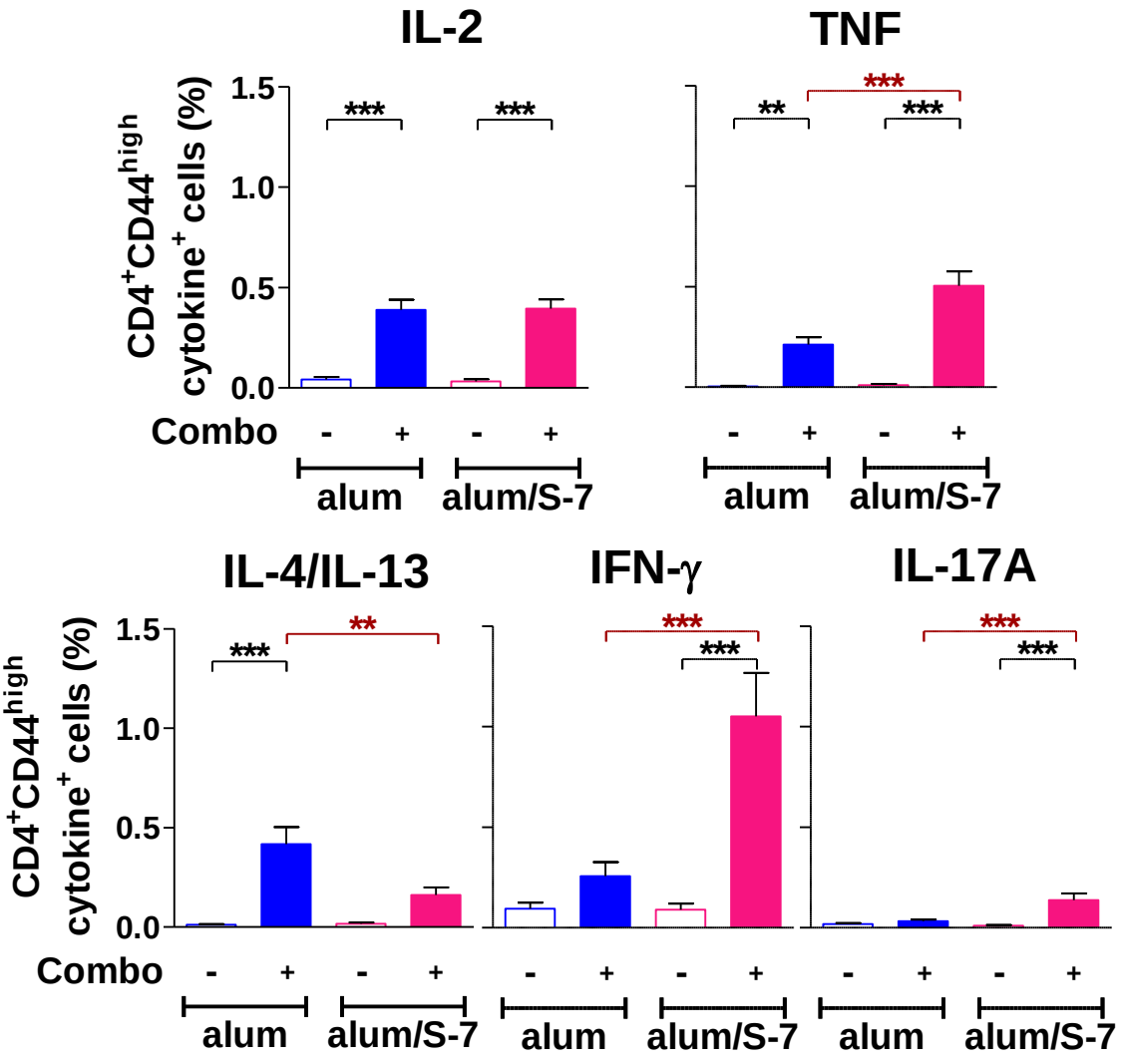


Figure 3.10 Quality of Combo-specific CD4$^+$ T-cell responses in mice vaccinated with Combo+alum or Combo+alum/S-7. BALB/c mice were immunized with Combo+alum (close blue bar) or Combo+alum/S-7 (close pink bar) or, as negative controls, with alum (blue open bar) or alum/S-7 (pink open bar) only. Twelve days after vaccination, mice were sacrificed and splenocytes from each mouse were stimulated or not *in vitro* with Combo for 18 h. The response of non-stimulated cells was subtracted from that of stimulated cells. The graphs show the percentages of CD4$^+$CD44^{high} T cells producing TNF, IL-2, IFN-γ, IL-17A, IL-4/IL-13 in response to Combo stimulation. Bars represent mean±SEM (n=16 from 3 independent experiments). ANOVA and Tukey's post-test correction: *** P<0.001, ** P<0.01, and * P<0.05.

Cytokines were measured also in culture supernatants of splenocytes stimulated or not with Combo. As shown in Figure 3.11, this analysis confirmed that more IFN-γ and IL-17A were produced by cells from mice immunized with Combo+alum/S-7 than from mice immunized with Combo+alum. Comparable IL-2 and IL-4 and higher TNF (not shown) production were also observed in mice immunized with Combo+alum/S-7 *vs*. Combo+alum, in agreement with results obtained by intracellular cytokine staining. The average concentration of cytokines in non-stimulated cell culture supernatants was: 7078.1±7586.8 pg/ml for IL-2, 125.5±135.3 pg/ml for IL-4, 5.2±11.5 pg/ml for IL-13, 0.6±1.1 pg/ml for IFN-γ and 12.6±8.2 pg/ml for IL-17A.

Overall, Combo+alum/S-7 vaccine induced higher frequencies of Combo-specific $CD4^+$ T cells as compared to Combo+alum vaccine, and these T cells were polarized towards Th1 and Th17.

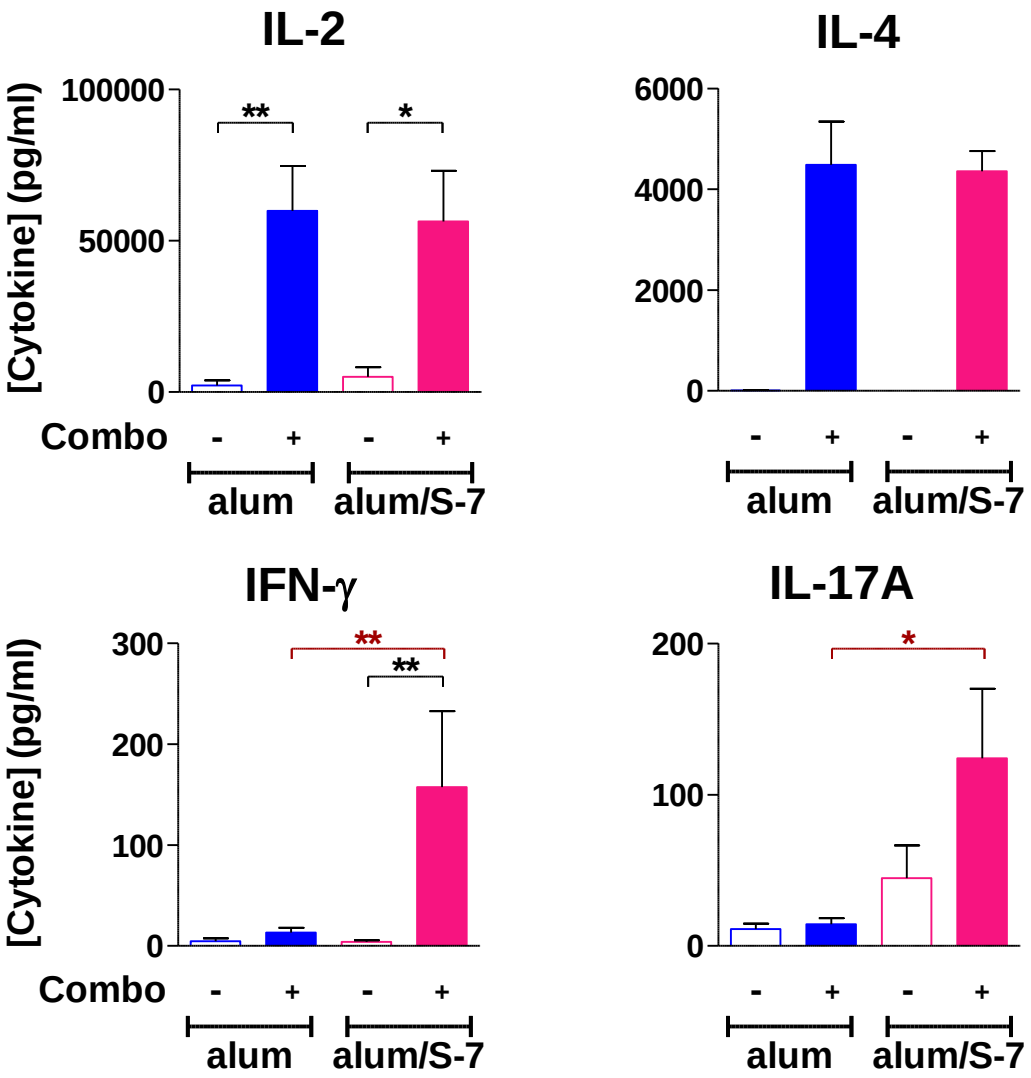


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Figure 3.11 Cytokines secreted by splenocytes from mice vaccinated with Combo+alum or Combo+alum/S-7. Splenocytes were stimulated or not for 48 h with Combo. Supernatants were harvested and stored at -20° C until tested. Cytokine concentration was measured by MSD kit. The response of non-stimulated cells was subtracted from that of cells stimulated with Combo. Bar represent mean±SEM (n=6). Data are representative of 3 independent experiments. ANOVA and Tukey's post-test correction: ** $P<0.01$ and * $P<0.05$.

3.8 Combo-specific IFN-γ–producing CD4$^+$ T cells induced by Combo+alum/S-7 immunization do not produce IL-17A or IL-17F and are RORγt$^-$

Th17 cells have considerable plasticity *in vitro* and *in vivo* and can readily acquire the ability to produce IFN-γ in addition to or instead of IL-17 production [163] [164] [60] [61] [165]. The developmental plasticity of Th17 cells toward a Th1-like phenotype (Th1/Th17 that produce both IL-17 and IFN-γ, and ex-Th17 that produce IFN-γ only) has been well documented in models of autoimmune diseases [166].

Therefore, in the attempt to establish if Combo+alum/S-7 vaccination induced these Th1-like cells, we stained cells from mice vaccinated with Combo+alum/S-7 for IL-17A, IL-17F, IFN-γ, and RORγt, a lineage-specific master regulator of Th17 development that induces the transcription of the genes encoding IL-17A and IL-17F.

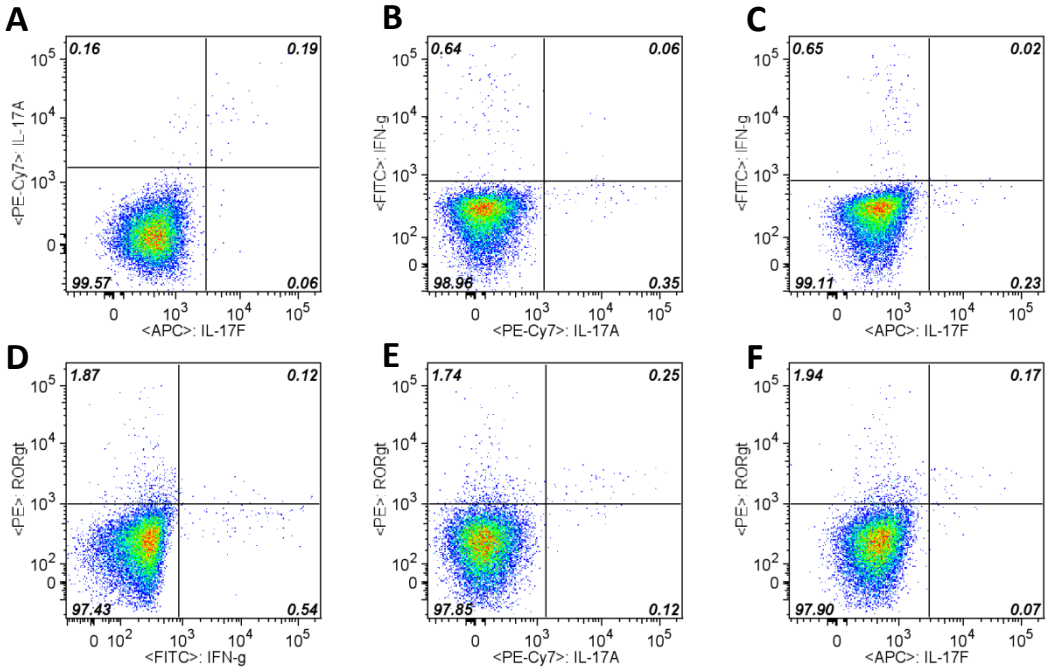


Figure 3.12 (previous page) Intracellular staining of IL-17A, IL-17F, IFN-γ, and RORγt in Combo-specific Th17 cells induced by immunization with Combo+alum/S-7. BALB/c mice (n=5) were immunized with Combo+alum/S-7 or alum/S-7 alone. Twelve days after vaccination, splenocytes were prepared from each mouse and stimulated *in vitro* with medium or Combo. Splenocytes were stained with Live/Dead yellow, fixed, permeabilized, and stained with mAbs directed against CD3, CD4, CD8, CD44, IFN-γ, IL-17A, IL-17F, and RORγt (panel 3). Live cells were identified based on Live/Dead staining. Lymphocytes were identified first based on a morphological gate. Single cells were identified based on forward scatter properties. $CD4^+CD44^{high}$ T cells were then identified based on expression of CD3, CD4, and CD44 markers. Within the $CD4^+CD44^{high}$ T-cell population, cells producing IL-17A, IL-17F, and IFN-γ were identified. RORγt expression was evaluated in IFN-γ–, IL-17A–, and IL-17F–producing cells.

As shown in Figure 3.12, Combo-specific $CD4^+CD44^{high}$ T cells induced by vaccination with Combo+alum/S-7 that produced both IL-17A and IFN-γ were extremely rare (B) and no cells that produced both IL-17F and IFN-γ were found (C). On the opposite, Combo-specific $CD4^+CD44^{high}$ T cells that produced both IL-17A and IL-17F were observed (A). In addition, Combo-specific IFN-γ^+ $CD4^+CD44^{high}$ T cells induced upon immunization with Combo+alum/S-7 were RORγt^- as compared to IL-$17A^+$ or IL-$17F^+$ $CD4^+CD44^{high}$ T cells that were RORγt^+ (D *vs*. E and F), suggesting that they were *bona fide* Th1 cells and not Th1/Th17.

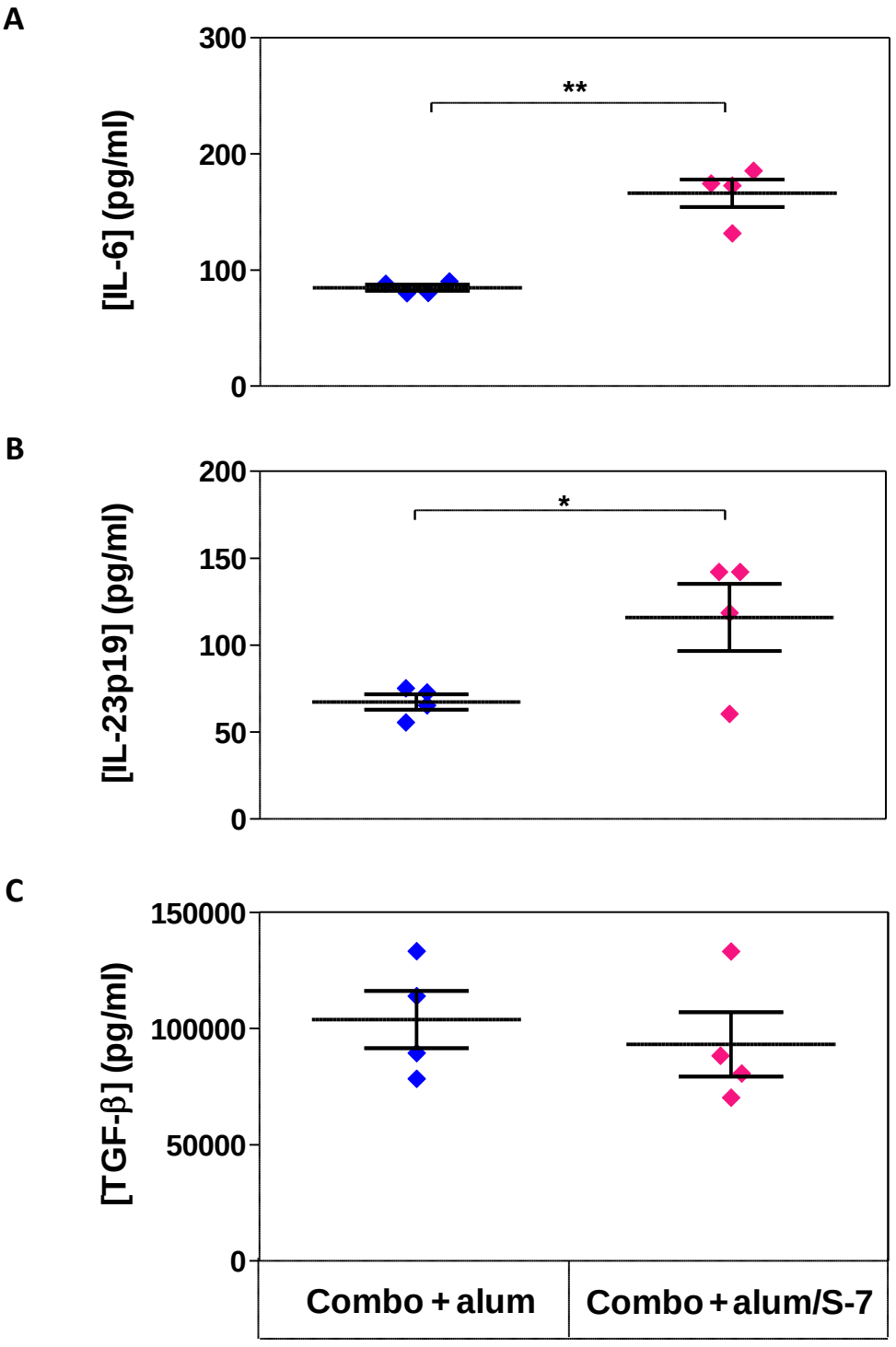
3.9 Th17-promoting cytokines were found in sera of mice vaccinated with Combo+alum/S-7

Th17 differentiation and expansion have been shown to be promoted by TGF-β, IL-6, and IL-23 in mice [42] [43]. Therefore, mice were bled 3 h after vaccination with Combo+alum/S-7 or Combo+alum to assess whether Th17-promoting cytokines could be found in the serum.

As shown in Figure 3.13, higher levels of IL-6 and IL-23, and comparable levels of active TGF-β were found in sera from mice vaccinated with Combo+alum/S-7 compared to mice vaccinated with Combo+alum.

Overall, these data indicated that Combo+alum/S-7 vaccination elicits a Th17-promoting cytokine environment.

Figure 3.13 (next page) Th17-promoting cytokines in mouse serum after vaccination with Combo+alum or Combo+alum/S-7. BALB/c mice were bled 3 h after vaccination with Combo+alum or Combo+alum/S-7. (**A**) IL-6, (**B**) IL-23p19, and (**C**) TGF-β concentrations in serum samples were determined using Bio-Plex Pro or MSD kits. Four independent experiments were performed. Sera from the same immunization group of each experiment were analyzed in pool (n=12/pool, 4 pools). Each dot represents a pool, mean±SEM are also shown. ANOVA and Bonferroni post-test correction: ** $P<0.01$ and * $P<0.05$.


A
[IL-6] (pg/ml)
300
200
100
0
**
B
[IL-23p19] (pg/ml)
200
150
100
50
0
*
C
[TGF-β] (pg/ml)
150000
100000
50000
0
Combo + alum
Combo + alum/S-7

3.10 Combo+alum/S-7 vaccination does not induce IL-17–producing γδ T cells

Recently it has been found that various "innate" subsets of lymphoid cells, like γδ T cells, NK cells, NKT cells, and innate lymphoid cells (ILCs), can produce IL-17 [167] [168] [169] [170]. In particular, γδ T cells can produce high amounts of IL-17 [171] in response to IL-23 in combination with IL-1β or IL-18, but largely independently of T-cell receptor activation [172]. Since we have shown that alum/S-7 immunization induces IL-23 and also some IL-1β (data not shown) production, we investigated whether immunization with Combo+alum/S-7 promoted IL-17 secretion by γδ T cells. For this purpose, splenocytes were prepared from mice vaccinated with Combo alone or formulated with alum or alum/S-7 or, as negative controls, with adjuvant alone, 12 days after immunization. Splenocytes, stimulated or not *in vitro* with Combo, were analyzed by intracellular flow cytometry following the gating strategy reported in Figure 3.14.

As shown in Figure 3.15, there was no difference in the mean percentage of IL-17–producing TCRγδ$^+$ T cells between any groups despite the stimulation with Combo, in line with the fact that IL-17 production by γδ T cells was Ag-independent. The average frequency of γδ T cells was 0.3% of CD3$^+$ cells: 5% of them produced IL-17A and 9% produced IL-17F with or without Combo stimulation.

These data demonstrated that although IL-17–producing γδ T cells were found, they were not induced by vaccination with Combo+alum or Combo+alum/S-7. In addition, these cells produced IL-17 independently of Ag recognition.

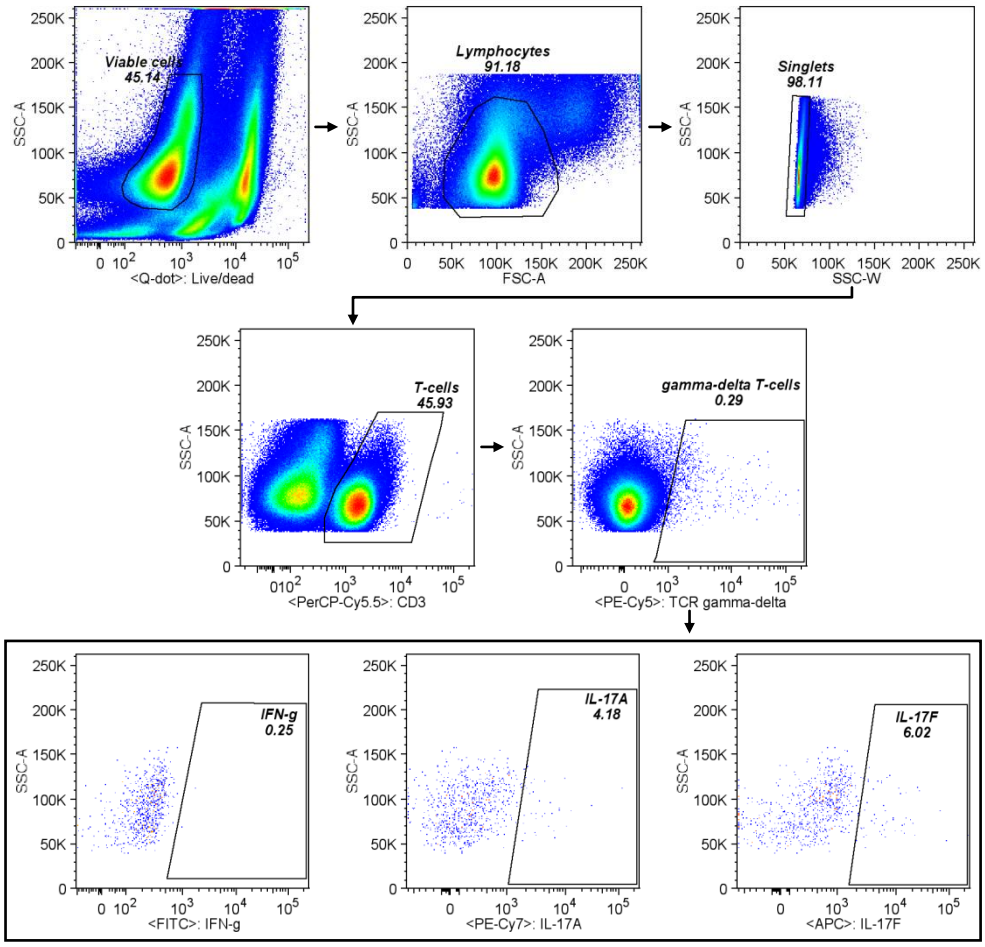


Figure 3.14 Gating strategy for intracellular cytokine staining analysis of γδ T cells. Splenocytes were stained with Live/Dead yellow and mAbs directed against CD3, TCRγδ, IFN-γ, IL-17A, IL-17F, and RORγt (panel 2). Live cells were identified based on the viability marker, lymphocytes were then identified using forward scatter and side scatter, and singlets were identified using forward scatter properties. Then, γδ T cells were positively identified as $CD3^{+}TCR\gamma\delta^{+}$ cells. Within the γδ T-cell population, cells producing IFN-γ, IL-17A, or IL-17F were identified.

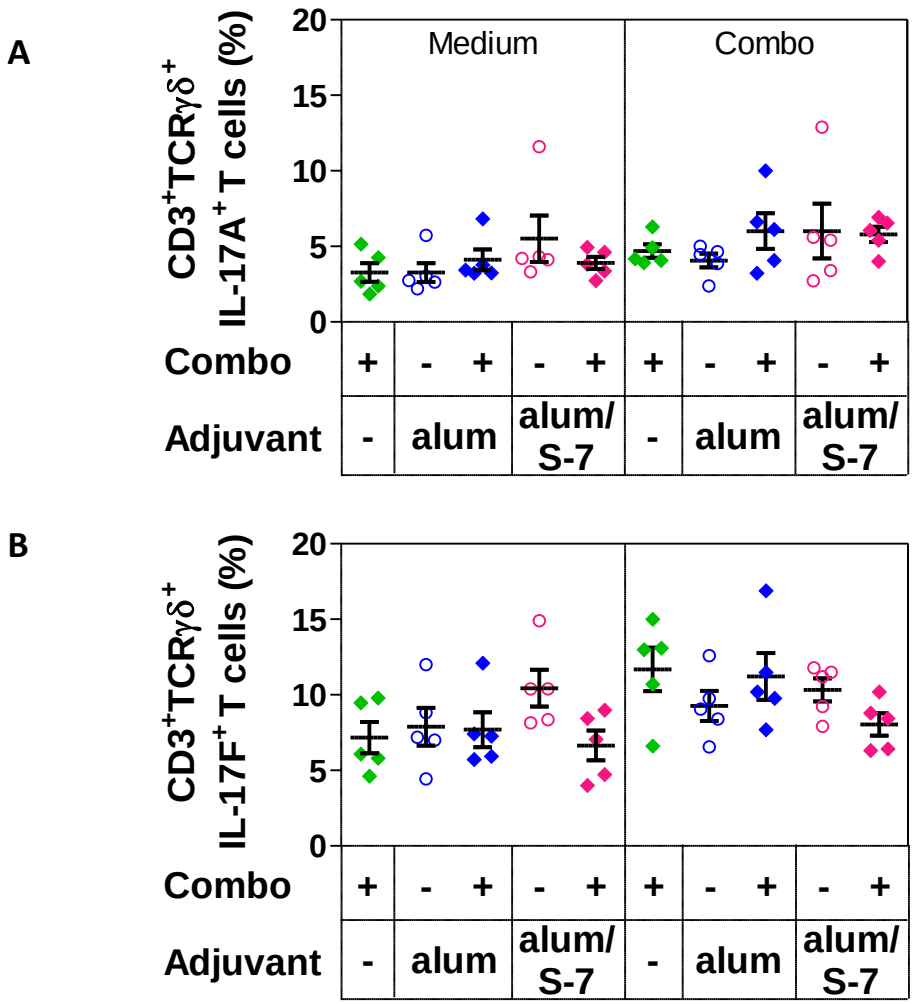
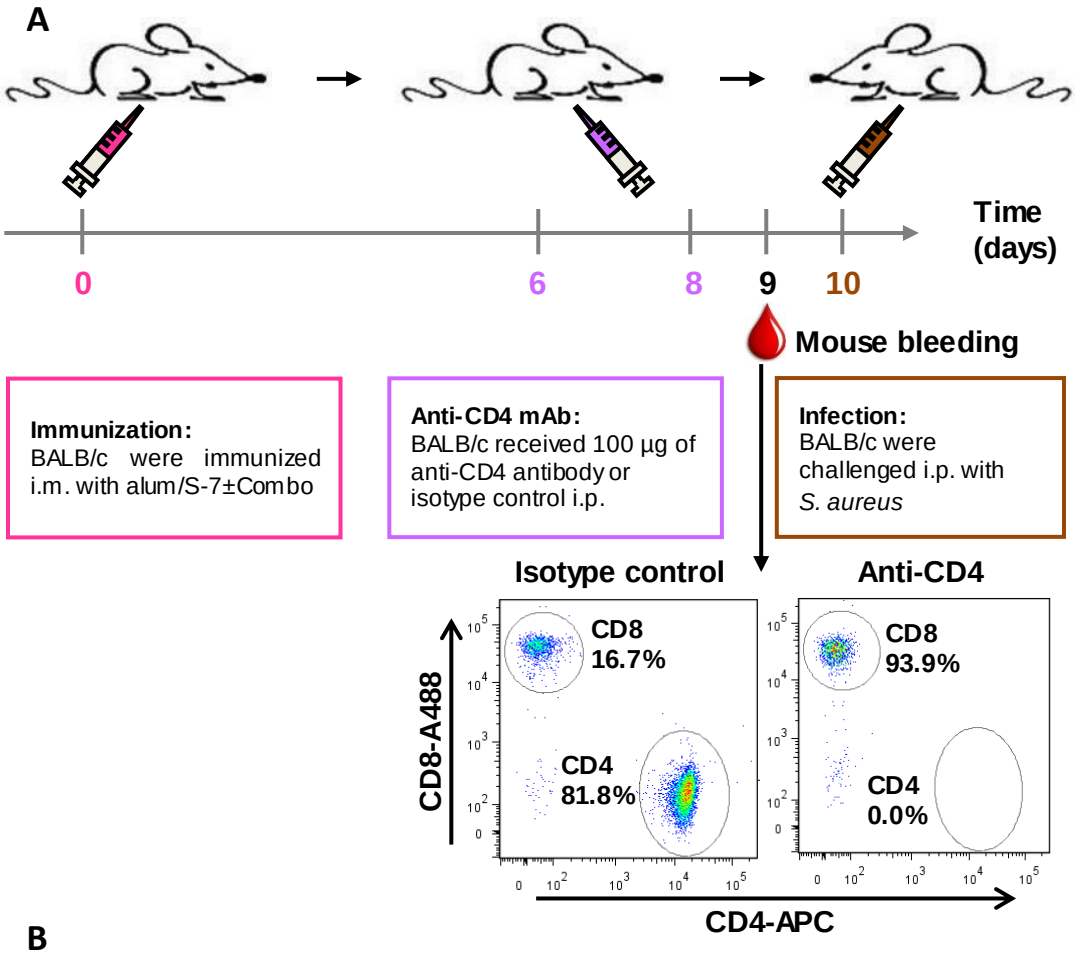


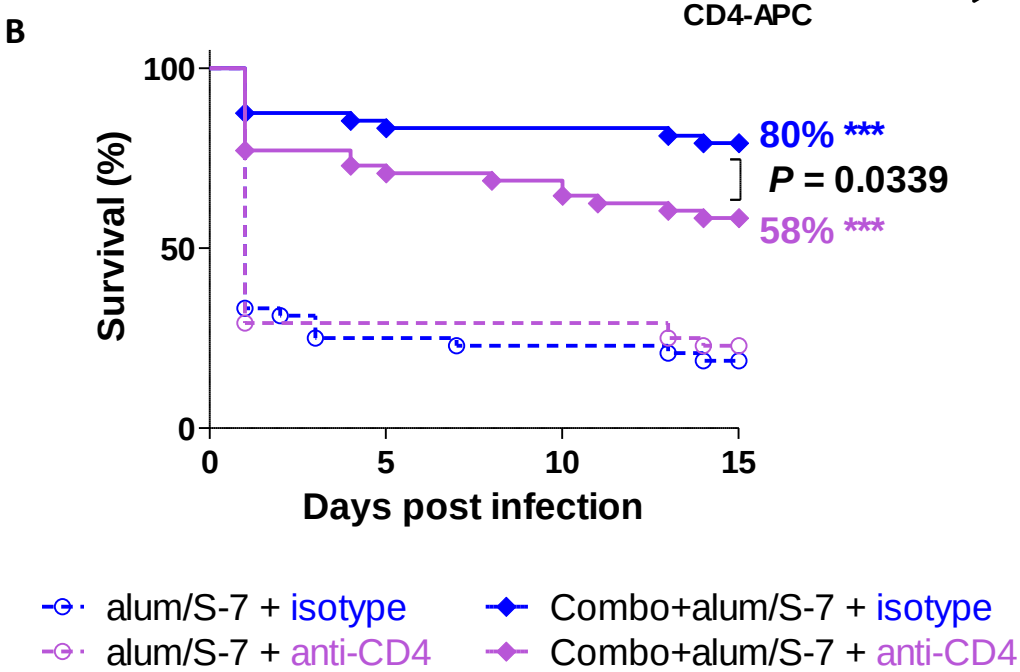
Figure 3.15 IL-17–producing γδ T cells in splenocytes of mice vaccinated with Combo+alum or Combo+alum/S-7. BALB/c mice were immunized with Combo (close green symbol), Combo+alum (close blue symbol), or Combo+alum/S-7 (close pink symbol) or, as negative controls, with alum (blue open symbol) or alum/S-7 (pink open symbol) only. Twelve days after vaccination, mice were sacrificed and splenocytes from each mouse were stimulated or not *in vitro* with Combo for 18 h. Frequencies of TCRγδ$^+$ T cells producing (**A**) IL-17A and (**B**) IL-17F. Lines and error bars represent the mean±SEM (n=5). Data are representative of 5 independent experiments. ANOVA and Tukey's post-test.

3.11 Combo-specific CD4$^+$ T cells have a protective role against *S. aureus*-induced peritonitis

We have shown that Combo+alum/S-7 vaccine induced stronger Combo-specific CD4$^+$ T-cell responses as compared to Combo+alum vaccine, and that the CD4$^+$ T-cell responses were polarized towards Th1 and Th17. Therefore, we further asked whether these CD4$^+$ effector T cells played a role in protection against *S. aureus*-induced peritonitis. To address this question, we depleted CD4$^+$ effector T cells by injecting an anti-CD4 mAb at day 6 and 8 after vaccination with Combo+alum/S-7 or, as control, with alum/S-7 alone. At day 9 after vaccination, mice were bled to confirm that CD4$^+$ T cells were depleted efficiently and that anti-Combo Ab titers in sera of mice depleted of CD4$^+$ T cells were not affected (data not shown). The day after, mice were challenged with *S. aureus* and their survival was monitored for the following 15 days. To control that the effect of anti-CD4 mAb was specific, control groups of vaccinated mice were injected with an isotype-matched irrelevant mAb (Figure 3.16 A).

Figure 3.16 (next page) Effect on survival of CD4$^+$ effector T-cell depletion in mice vaccinated with Combo+alum/S-7. (**A**) On day 6 and 8 after alum/S-7±Combo immunization, 100 µg of anti-CD4 or isotype-matched control mAb were injected in the peritoneum of BALB/c mice. On day 9, mice were bled and the percentage of circulating CD4$^+$ T cells (relative to total CD3$^+$ T cells) was determined by flow cytometry analysis. Results from one representative experiment are shown. On day 10, mice were infected i.p. with *S. aureus* and (**B**) survival was monitored for 15 days after challenge. Data were pooled from 4 independent experiments (n=48). Log-rank (Mantel-Cox) test:
Combo+alum/S-7 + anti-CD4 *vs*. Combo+alum/S-7 + isotype control, * $P<0.05$.
Combo+alum/S-7 + isotype control *vs*. alum/S-7 + isotype control, *** $P<0.001$.
Combo+alum/S-7 + anti-CD4 *vs*. alum/S-7 + anti-CD4 mAb, *** $P<0.001$.


A
Time (days)
0
6
8
9
10
Mouse bleeding
Immunization: BALB/c were immunized i.m. with alum/S-7±Combo
Anti-CD4 mAb: BALB/c received 100 µg of anti-CD4 antibody or isotype control i.p.
Infection: BALB/c were challenged i.p. with S. aureus
Isotype control
Anti-CD4
CD8 16.7%
CD4 81.8%
CD8 93.9%
CD4 0.0%
CD8-A488
CD4-APC


B
Survival (%)
100
50
0
0
5
10
15
Days post infection
80% ***
P = 0.0339
58% ***
alum/S-7 + isotype
alum/S-7 + anti-CD4
Combo+alum/S-7 + isotype
Combo+alum/S-7 + anti-CD4

As shown in Figure 3.16 B, treatment with anti-CD4 mAb resulted in decreased survival of Combo+alum/S-7 immunized mice compared to treatment with isotype control mAb (*P*=0.0339). Treatment with isotype control mAb had no effect on survival of mice vaccinated with Combo+alum/S-7 (not shown).

Mice that survived the infection were subjected to necropsy 15 days after challenge. *S. aureus* bacterial load in kidneys was determined by plating homogenized tissue on agar plates and enumerating the CFU.

As shown in Figure 3.17, mice depleted of $CD4^+$ effector T cells that survived the infection had higher bacterial load in kidneys compared to mice that received the isotype control mAb, although the differences were not statistically significant.

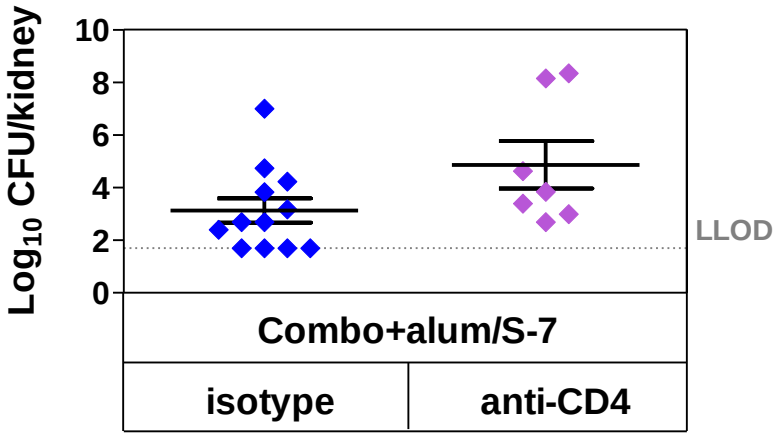


Figure 3.17 Effect of $CD4^+$ effector T-cell depletion on bacterial load in kidneys of mice vaccinated with Combo+alum/S-7. BALB/c mice were immunized with Combo+alum/S-7 and treated with depleting anti-CD4 (pink symbol) or isotype control (blue symbol) mAb prior to challenge with *S. aureus*. Fifteen days after *S. aureus* inoculation, animals were euthanized and bacterial load in kidneys was determined by plating homogenized tissue on agar plates and enumerating the CFU. Lines and error bars represent mean±SEM (Combo+alum/S-7 + isotype: n=12; Combo+alum/S-7 + anti-CD4: n=7). Data are representative of 4 independent experiments. Unpaired *t* test, two-tailed.

These data showed that $CD4^+$ effector T cells contribute to the protection conferred by Combo+alum/S-7 vaccine.

3.12 *in vivo* IL-17A neutralization increased the bacterial load in kidneys of Combo+alum/S-7 immunized mice

We demonstrated that Combo+alum/S-7 vaccine induced Combo-specific IL-17– and IFN-γ–producing $CD4^+$ T cells and that $CD4^+$ effector T cells contributed to the protection conferred by this vaccine. To assess the role of IL-17 and/or IFN-γ, IL-17A and/or IFN-γ were neutralized *in vivo* in vaccinated mice prior to infection with *S. aureus*.

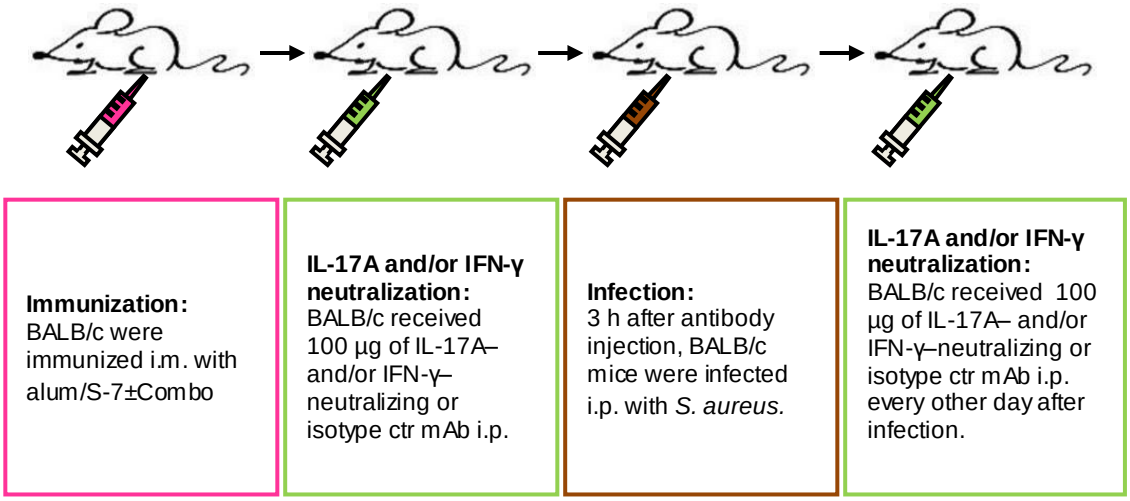
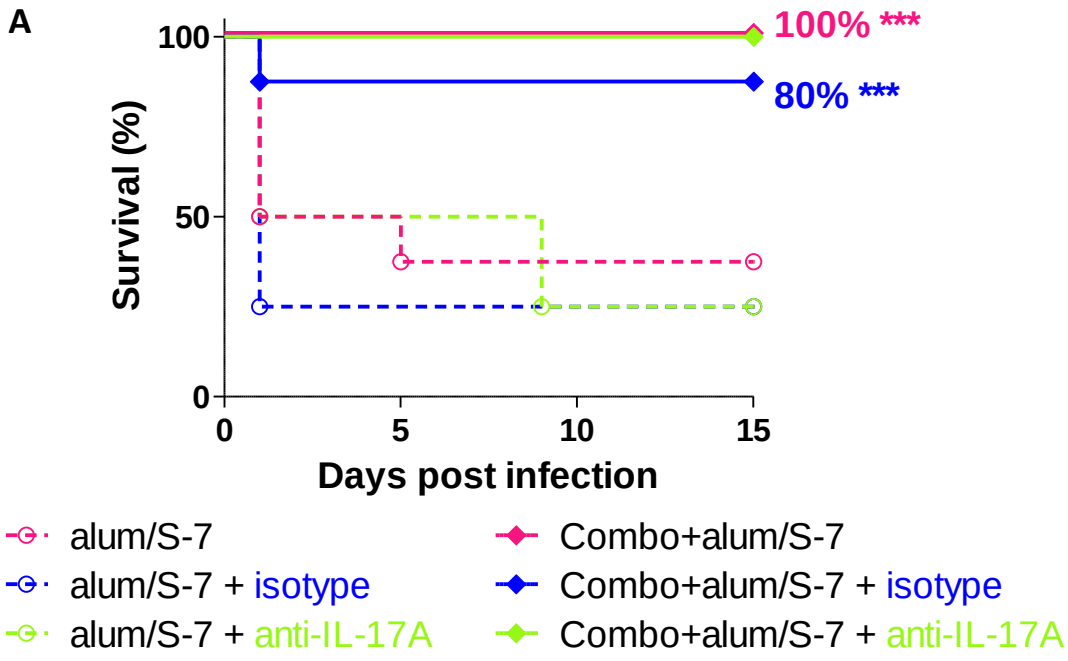


Figure 3.18 Experimental design of IL-17A and/or IFN-γ *in vivo* neutralization. BALB/c mice were treated with IL-17A– and/or IFN-γ–neutralizing or isotype-matched control mAb 3 h prior to infection with *S. aureus*, while control animals were injected with PBS. Mice were treated with mAb every other day for 15 days after infection.


A
100
50
0
Survival (%)
0
5
10
15
Days post infection
100% ***
80% ***
alum/S-7
alum/S-7 + isotype
alum/S-7 + anti-IL-17A
Combo+alum/S-7
Combo+alum/S-7 + isotype
Combo+alum/S-7 + anti-IL-17A

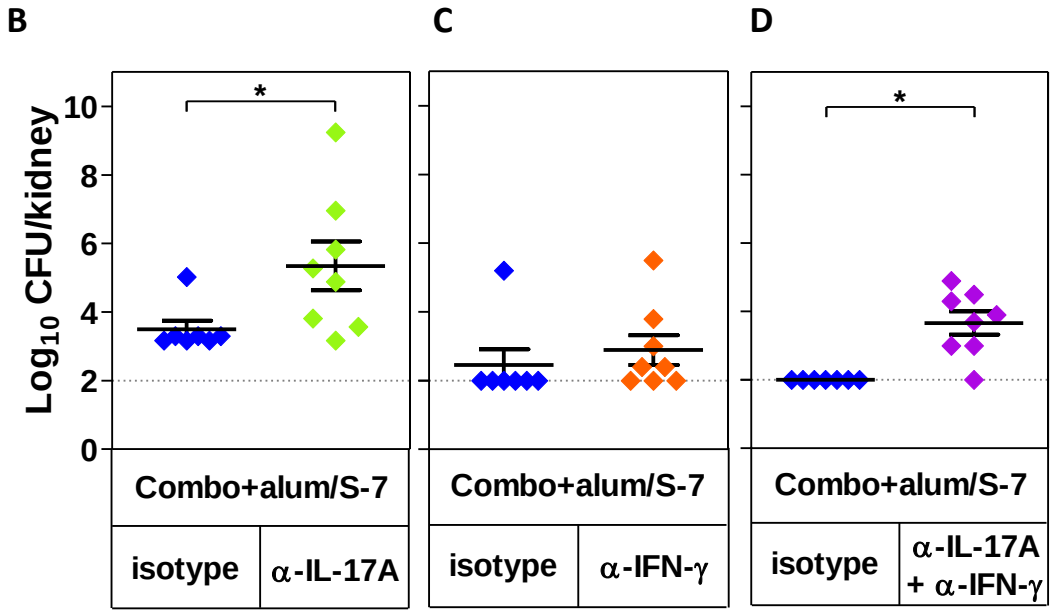

B
C
D
Log10 CFU/kidney
10
8
6
4
2
0
*
*
Combo+alum/S-7
Combo+alum/S-7
Combo+alum/S-7
isotype
α-IL-17A
isotype
α-IFN-γ
isotype
α-IL-17A
+ α-IFN-γ

Figure 3.19 (previous page) Effect of *in vivo* neutralization of IL-17A and/or IFN-γ on survival and bacterial load in kidneys of mice vaccinated with Combo+alum/S-7. Survival of BALB/c mice (n=8) immunized with alum/S-7±Combo and treated with IL-17A– and/or IFN-γ–neutralizing mAb or isotype-matched control mAb was monitored for 15 days after *S. aureus* challenge. (**A**) Survival of alum/S-7±Combo immunized mice injected with IL-17A mAb *vs*. isotype control mAb. Log-rank (Mantel-Cox) test: Combo+alum/S-7 + isotype control *vs*. alum/S-7 + isotype control, *** $P<0.001$. Combo+alum/S-7 + anti-IL-17A *vs*. alum/S-7 + anti-IL-17A, *** $P<0.001$. (**B-D**) Fifteen days after *S. aureus* challenge, survivors were euthanized and bacterial load in kidneys was determined by plating homogenized tissue on agar plates and enumerating the CFU. Lines and error bars represent mean±SEM. Data are representative of 2 independent experiments. Unpaired *t* test, two-tailed: *** $P<0.001$, and * $P<0.05$.

BALB/c mice immunized with alum/S-7±Combo were injected i.p. with 100 µg of IL-17A– and/or IFN-γ–neutralizing or isotype-matched control mAb, every other day starting 3 h before infection. Mouse survival was monitored for 15 days after bacterial challenge.

As shown in Figure 3.19A, treatment of mice immunized with Combo+alum/S-7 with neutralizing mAb specific for IL-17A had no effect on survival compared to treatment with isotype-matched control mAb. No effect on survival was observed either when mice were treated with IFN-γ–specific neutralizing mAb alone or in combination with IL-17A–specific mAb (data not shown). Treatment with isotype control mAb had no effect on survival of mice vaccinated with Combo+alum/S-7.

However, *in vivo* neutralization of IL-17A alone (Figure 3.19 B) or together with IFN-γ (D), but not of IFN-γ alone (C), caused an increase in bacterial load in kidneys of mice that survived the infection compared to survivors injected with isotype control mAb.

Moreover, histopathologic analysis of kidneys from mice treated with IL-17A mAb showed an increased pathology compared to kidneys from mice injected with isotype control mAb (Figure 3.20 B and C *vs*. A). Kidneys from mice treated with IL-17A mAb showed pyelonephritis, characterized by abscesses in the interstitium of renal parenchyma, damaged tubules, and severe unilateral necrosis.

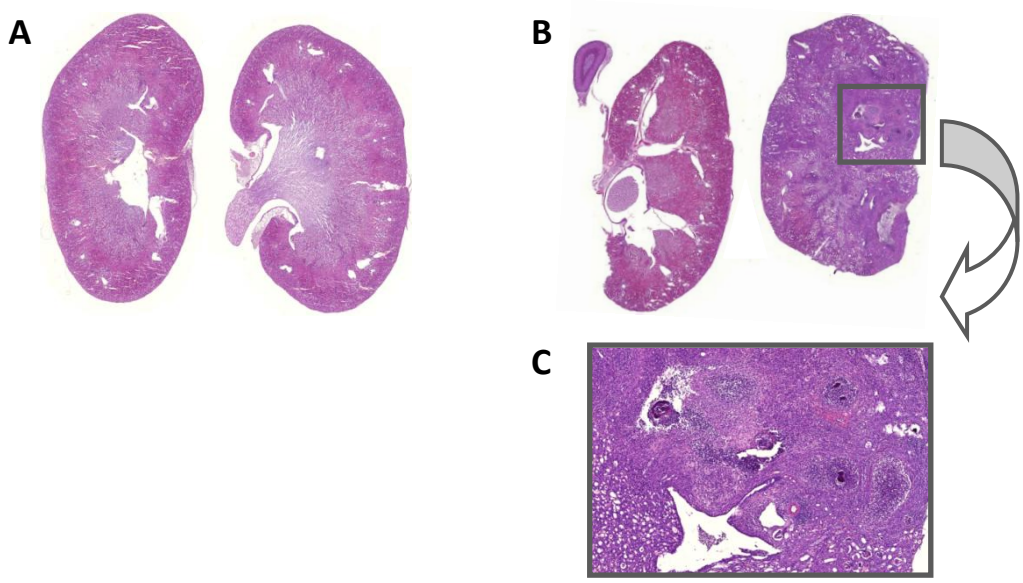


Figure 3.20 Effect of *in vivo* neutralization of IL-17A on kidney histopathology of mice vaccinated with Combo+alum/S-7 after challenge with *S. aureus*. Combo+alum/S-7 immunized BALB/c mice were infected with *S. aureus*. After 15 days, animals were euthanized and the kidneys were excised, fixed in 4% buffered formaldehyde, and processed for paraffin embedding. Kidneys were thin sectioned, stained with hematoxylin and eosin, and analyzed for histopathology. Representative specimens of hematoxylin and eosin-stained kidney of a Combo+alum/S-7 immunized mouse treated with (**A**) isotype control mAb or (**B-C**) IL-17A–neutralizing mAb are shown.

Overall, these data indicated that IL-17A induced upon vaccination with Combo+alum/S-7 is important to control *S. aureus* growth and/or spreading to distant organs.

Chapter 4

Discussion

Staphylococcal disease represents a universal burden including acute, life-threatening infections as well as chronic infections usually associated with foreign materials (e.g., prosthetic devices or catheters), often hospital acquired [173].

Although it has been extensively investigated, natural protective immunity against *S. aureus* is poorly understood. Acute infection with *S. aureus* does not prevent re-infection [174]. Preclinical and clinical data indicate that immunization with intact bacteria induces high serum Ab immune titers to *Staphylococcus*, but does not confer protection against *S. aureus* infection [174] [117].

To date, both active and passive immunization strategies against *S. aureus* have failed. Failures in vaccine clinical trials might be due to a focus on single targets while *S. aureus* has a plethora of pathogenic mechanisms. In addition, the lack of known correlates of protection in humans has severely limited the ability to interpret both preclinical and clinical data. Finally, the vaccines tested in humans so far lacked new generation adjuvants, which may be critical in augmenting Ab production

and steering the T-cell responses toward the proper profile of cytokine production [126].

In the attempt to overcome these limitations, Novartis Vaccines has developed a Combo vaccine against *S. aureus* consisting of four bacterial proteins: Hla_{H35L}, a non-toxic mutant of α-toxin [73], EsxAB, which is a fusion of EsxA and EsxB secreted virulence factors [128], FhuD2, a surface protein involved in iron uptake [129], and Sur2, an internally discovered surface protein whose function is currently investigated.

Recent work has shown that Combo vaccine adjuvanted with alum was more protective than single Ags in the peritonitis, kidney abscess, pneumonia, and skin mouse infection models (Bagnoli et al., manuscript in preparation). In addition, Combo vaccine conferred a broader protection against different staphylococcal strains than single Ags in mice. Combo vaccine with or without alum has proven to be safe in a recent phase I human clinical trial.

The aim of the work described in this thesis was to evaluate if the inclusion of a novel adjuvant, alum/S-7, which consists of a small molecule TLR7a adsorbed to alum, improves the protective efficacy of the Combo vaccine in the mouse peritonitis model and to elucidate the underlying immunological mechanisms of protection.

A single dose of Combo+alum/S-7 significantly increased the percentage of mice that survived to a lethal challenge with *S. aureus* compared to Combo+alum (Figure 3.1A). Of note, Combo+alum/S-7 not only protected the majority of mice from early death caused by *S. aureus*, but also induced sterile immunity in 56% of mice vaccinated with Combo+alum/S-7 *vs*. 31% of mice vaccinated with Combo+alum (Figure 3.1B-C). This result was very important since Rauch *et al.* have recently reported that Hla is a key determinant of early lethal outcomes associated

with i.p. *S. aureus* challenge. Indeed, infection with *hla*^{ko} mutant bacteria caused fewer lethal events, especially within the first 24 h post infection. In addition, vaccination with non-toxigenic Hla_{H35L}, which is one component of Combo vaccine, or treatment with an Hla-neutralizing mAb protected BALB/c mice from death [175]. Nevertheless, mice either infected with *hla*^{ko} bacteria or vaccinated with Hla_{H35L} or treated with anti-Hla mAb before challenge with wild-type *S. aureus* presented peritoneal abscess lesions that persisted for 15 days after infection, similar to animals that had been infected with wild-type *S. aureus* strains. Further, bacterial load within renal abscess lesions was similar for mice infected i.v. with wild-type or *hla*^{ko} strains suggesting that once bacteria have been encapsulated in abscesses they are no longer attacked by anti-Hla Abs. The authors speculated that peritoneal lesions may rupture over time giving rise to new lesions, as observed during human peritonitis and peritoneal abscesses. So, the eradication of *S. aureus* from the peritoneum is likely to be very important for patients that experience intraperitoneal abscess formation as a consequence of continuous peritoneal dialysis for end-stage renal disease [176]. In addition, the capability of the immune system to effectively attack *S. aureus* also once it hides inside an abscess is likely to have an impact in many other *S. aureus*-induced pathologies.

To elucidate the protective mechanisms of Combo+alum/S-7 vaccine, first we characterized Combo-specific Ab responses. Combo+alum/S-7 induced faster and higher Combo-specific Ab titers, and Hla-neutralizing activity compared to Combo±alum. IgG responses against each of the Combo Ags was detected in mice vaccinated with Combo+alum/S-7 by 12 days and increased at day 32 after immunization, as did Hla-neutralizing activity (Figure 3.2 and Figure 3.3).

The requirement of B cells/Abs to confer protection against *S. aureus* in the peritonitis model by Combo vaccination was evidenced by the death of 96% of B-cell ko J_H mice vaccinated with Combo+alum/S-7 or Combo+alum (Figure 3.4).

The protective role of Abs induced by Combo+alum/S-7 vaccination was confirmed by passive immunization experiments, in which serum from actively immunized mice was given to naïve mice before i.p. challenge with *S. aureus*. While 100% of mice passively immunized with Combo+alum/S-7 serum were live 4 days after challenge, only 6% of mice passively immunized with alum/S-7 serum survived at that time. However, passively vaccinated mice then started to die and mice that survived for the 15 day observation time showed increased peritoneal abscesses and bacterial load in kidneys compared to actively vaccinated mice (Figure 3.5 and 3.6). This suggested that either Abs induced by active vaccination were not the only mechanism involved in protection of mice from death and from bacterial spreading or levels of Abs reached with passive transfer declined over the 15 day observation time. Indeed, preliminary data showed that Combo-specific Ab titers were lower in mice that survived *S. aureus* challenge in the passively vaccinated group *vs*. mice actively vaccinated (data not shown) suggesting that Ab titers might have become limiting at later times in our passive transfer experiments. Since the half-lives of serum Igs in mice are 2 days for IgM, 4-6 days for IgG2b, and 6-8 days for IgG1, IgG2a and IgG3 [177], the observed decrease in protection during the 15 day observation period after bacterial challenge could mirror this reduction. Further experiments are planned to address this point.

Another aspect that deserves further investigation is that vaccination with Combo+alum/S-7, but not with Combo±alum, induced Combo-specific Abs

of the IgG2a and IgG2b isotypes (data not shown). These Abs might be functionally more efficient at fighting against *S. aureus*.

Since several lines of evidence support a role for $CD4^+$ T-cell responses to *S. aureus* infection in mice [123], we studied the role of these cells in the protection conferred by Combo vaccine adjuvanted with alum/S-7.

First of all, we showed that vaccination with Combo+alum/S-7 or Combo+alum induced measurable Combo-specific $CD4^+$ T-cell responses. In particular we observed that one vaccination with Combo+alum/S-7 induced an increase in frequency of cytokine-producing (Figure 3.9), but not proliferating (Figure 3.7) Combo-specific $CD4^+$ T cells as compared to Combo+alum vaccination. In addition, Combo+alum/S-7 skewed the T-cell responses towards Th1 and Th17 (Figure 3.10) suggesting that a $CD4^+$ T-cell effector response was induced. The importance of this $CD4^+$ T-cell responses for the protection conferred by vaccination with Combo+alum/S-7 was shown by $CD4^+$ effector T-cell depletion experiments that indicated a role for these cells both in survival (Figure 3.16) and bacterial control (Figure 3.17). Such a fast and strong $CD4^+$ effector T-cell response might be desirable in patients that are scheduled for cardiothoracic surgery since *S. aureus* causes substantial morbidity and mortality following median sternotomy [178].

A recent Phase IIB/III study of V710, a vaccine made of non adjuvanted IsdB, did not induce protection against post-surgery bacteremia or deep sternal wound infection even if an increase in anti-IsdB IgG levels from baseline to day of hospital admission (14-60 days after vaccination) was observed. $CD4^+$ T-cell responses were not reported [179]. IsdB has been shown to be a potential vaccine candidate for the prevention of *S. aureus* infection in mouse challenge models. Importantly, enhanced

protection from lethal i.v. infection was mediated by both IsdB-specific Th17 cells and IsdB-specific mAb [180]. An observed rise in anti-IsdB titers induced by IsdB formulated on aluminum adjuvant correlated with protection against lethal *S. aureus* challenge, indicating that Ab titers may be used as a nominal biomarker for vaccine efficacy [99]. However, the negative results of the efficacy trial strongly suggest that Abs might not be sufficient and that Th17 cells may be important.

There are other several observations that imply that Abs may not be sufficient for protection against *S. aureus* infection. For example, Abs targeted to several *S. aureus* surface Ags have demonstrated preclinical efficacy, including polyclonal anti-capsule type 8 and type 5 [150] [181] [182], and Fab to an ABC transporter [183]. However, human clinical trials resulted in a disappointing lack of statistically significant efficacy [184] [185] [186].

In vivo neutralization of IL-17 and/or IFN-γ in Combo+alum/S-7 immunized mice showed that while these cytokines, either alone or together, had no effect on survival of vaccinated mice, neutralization of IL-17, but not of IFN-γ, caused an increase in bacterial burden in kidneys of vaccinated mice (Figure 3.19). These results indicate a role of Combo+alum/S-7 vaccine-induced Th17 cells in local but not systemic bacterial control. Indeed, Th17 cells are essential for intact mucoepithelial host defense against *S. aureus* both in mice and men. For example, double *il17a*$^{-/-}$ *il17f*$^{-/-}$ mice showed increased susceptibility to mucocutaneous *S. aureus* abscess formation but not to death induced by i.v. *S. aureus* infection [86]. Similarly, patients with hyper-IgE syndrome, despite their systemic Th17 deficiency, are prone to staphylococcal skin and lung abscesses but not to *S. aureus* bacteremia. This might be due to differences in site-specific requirements for production of antimicrobial peptides and neutrophil chemoattractants [96].

Of note, our IL-17 neutralization experiments are likely to have underestimated the role of IL-17 since IL-17A, but not IL-17F, which is also produced by Combo-specific Th17 cells (Figure 3.12), was neutralized. An essential role of IL-17F for *S. aureus* control was shown by the observation that both *il-17a* and *il-17f* gene had to be knocked out to lose control of *S. aureus* [86]. Moreover, IL-17F modest signaling activity may be increased by TNF, which is induced by Combo+alum/S-7 vaccination (Figure 3.10). TNF exhibits potent synergy also with IL-17A and IL-17A/F, probably through cooperative enhancement of mRNA stability of certain IL-17 target genes [84] [88].

Local IL-17 production could be part of a protective early immune response to peritoneal dialysis-related peritonitis since high levels of IL-17 in peritoneal dialysis effluents during the early phase of peritonitis correlated with a favorable outcome [187] [86]. Indeed, mesothelial cells, which are present in the peritoneum, express IL-17RA [188].

IL-17 induces the differentiation of progenitor cells into neutrophils through the regulation of G-CSF in bone marrow stromal cells [189], as well as neutrophil recruitment, via the regulation of CXC chemokines in epithelial cells [190]. IL-17 induces also the production of antimicrobial peptides by epithelial cells [57]. This cooperative induction of neutrophil recruitment and antimicrobial peptide production increases epithelial barrier function and is critical for host defense against *S. aureus* [191].

Phagocyte-mediated killing is a crucial defense mechanism against *S. aureus*. Neutrophils not only represent 60% of the leukocyte population in the blood and are the most important phagocytic cells, but they are the only cells in the immune system that effectively kill staphylococci [192]. In addition, the speed of neutrophil recruitment is an important factor [193].

Interestingly, IL-17A was shown to recruit neutrophils in the peritoneum of mice with zymosan-induced peritonitis [194].

In vitro experiments by Frodermann et al. [195] indicated that TLR2 signaling triggered by *S. aureus* PGN led to predominantly modulatory or pro-inflammatory responses depending on the involvement of Mo/Mϕ or DC, respectively. As a result of the different imprinting by APC (Mo/Mϕ *vs*. DC), the subsequent adaptive response was either an IL-10 response or a Th1/Th17 response. *S. aureus* colonization, primarily occurring in the upper respiratory tract, will be facilitated by the predominant presence of Mo/Mϕ in the submucosa. As a consequence, the vast majority of individuals is expected to develop an IL-10 response towards *S. aureus* infection resulting in inefficient bacterial clearance. On the other hand, our Combo+alum/S-7 vaccine induces a protective Th1/Th17 response.

It is becoming apparent that adaptive immunity to *S. aureus* may extend beyond neutralizing, opsonizing, and/or complement-fixing Ab responses. Vaccines that drive an appropriate cell-mediated immune response, including Th17 and/or Th1/Th17 cells, may also contribute to acquired protective immunity [196]. Combo vaccine adjuvanted with alum/S-7 induces both humoral and cellular immune responses, which may both improve efficient eradication of bacterial infection.

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