REVIEW ARTICLE

Phagocyte subsets and lymphocyte clonal deletion behind ineffective immune response to Staphylococcus aureus

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#Contributed equally to this work.

One sentence summary: This review provides a critical perspective on immune responses to Staphylococcus aureus elicited by infection or vaccination in animal models and humans emphasizing the emerging role of phagocyte subtypes as well as of lymphocyte deletion/anergy processes.

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ABSTRACT

Lack of known mechanisms of protection against Staphylococcus aureus in humans is hindering development of efficacious vaccines. Preclinical as well as clinical data suggest that antibodies play an important role against S. aureus. For instance, certain hypogammaglobulinaemic patients are at increased risk of staphylococcal infections. However, development of effective humoral response may be dampened by converging immune-evasion mechanisms of S. aureus. We hypothesize that B-cell proliferation induced by staphylococcal protein A (SpA) and continuous antigen exposure, without the proper T-cell help and cytokine stimuli, leads to antigen-activated B-cell deletion and anergy. Recent findings suggest an important role of type I neutrophils (PMN-I) and conventionally activated macrophages (M1) against S. aureus, while alternatively activated macrophages (M2) favour biofilm persistence and sepsis. In addition, neutrophil–macrophage cooperation promotes extravasation and activation of neutrophils as well as clearance of bacteria ensnared in neutrophil extracellular traps. Activation of these processes is modulated by cytokines and T cells. Indeed, low CD4+ T-cell counts represent an important risk factor for skin infections and bacteraemia in patients. Altogether, these observations could lead to the identification of predictive correlates of protection and ways for shifting the balance of the response to the benefit of the host through vaccination.

Keywords: Staphylococcus aureus; neutrophils; macrophages; B cells; T cells; correlates of protection; hypogammaglobulinaemia; vaccine

INTRODUCTION

Staphylococcus aureus is one of the most common opportunistic pathogens of humans and causes a wide range of diseases, from mild skin infections to life-threatening diseases such as sepsis, pneumonia, endocarditis and osteomyelitis (Lowy 1998). The burden of staphylococcal disease is increasing due to the ability of S. aureus to acquire resistance to various antibiotics including methicillin and vancomycin (Smith and Jarvis 1999;
Rybak and Akins 2001; Webb et al. 2010; Mishra et al. 2012b). In fact, methicillin-resistant *S. aureus* (MRSA) has been recognized as a major cause of infections in healthcare settings and community environments. For example, in 2011 it was estimated that invasive MRSA infections occurred in 80,461 US subjects, and caused over 11,000 patient deaths in the USA alone (Dantes et al. 2013). The alarming increase in multi-antibiotic resistance of *S. aureus* together with the wide variety and severity of infections pose a threat to public health, and challenge our ability to control the disease. In particular, this is due to the lack of medical treatments alternative to antibiotics (Maskalyk 2002; Shorr 2007). On the other hand, MRSA has been reported to be less virulent than methicillin-resistant *S. aureus* (MSSA) (Pozzi et al. 2012a; Rudkin et al. 2012). MSSA especially in Europe is still responsible for a significant disease burden and associated with high mortality in spite of effective antibiotic treatment (Kock et al. 2010). Therefore, vaccines or new treatment modalities are urgently needed for both MRSA and MSSA.

Although several vaccine candidates have been proposed, and some of them have been tested in clinical trials using both active and passive immunization modalities (Patti 2011), an efficacious vaccine is still missing.

There are several potential reasons behind the disappointing results of clinical trials as already discussed elsewhere (Bagnoli, Bertholet and Grandi 2012). First, vaccine manufacturers likely overestimated preclinical efficacy results. Furthermore, vaccines tested in humans to date comprised single antigens and did not contain new generation adjuvants, and hence were probably disproportionately simple in regard to the complex pathogenic mechanisms of the bacterium. In addition, the lack of known correlates of protection in humans has severely limited the ability to interpret both preclinical and clinical data.

Herein, we review literature on the role of antibodies, phagocytes and T cells with a particular emphasis on clinical data. We shed light on some contradictory and overlooked findings regarding the role of antibodies and phagocyte subtypes against *S. aureus*, and we propose models that recapitulate immune responses elicited in the host by the pathogen.

**ACQUIRED IMMUNITY AGAINST *S. aureus* INFECTIONS**

The role of antibodies

The role of antibodies in preventing or treating *S. aureus* colonization or infection is controversial (Proctor 2012; Spellberg and Daum 2012; Jansen et al. 2013). Patients with X-linked agammaglobulinaemia (XLA), which carry mutations in the gene for Bruton’s tyrosine kinase (Btk), have markedly reduced levels of B cells. As a result, they have an increased susceptibility to a variety of encapsulated bacteria and enteroviruses, but they do not appear particularly prone to *S. aureus* infections (Proctor 2012; Spellberg and Daum 2012). This apparently speaks against a key protective role of antibodies against *S. aureus*. However, Staphylococcus spp. were indeed isolated from XLA patients with pneumonia (Winkelstein et al. 2006). Furthermore, hypogammaglobulinaemia G occurred more often in children with atopic dermatitis who were more susceptible to secondary infections usually caused by *S. aureus* (Ventura et al. 1989). In addition, treatment of a patient with juvenile ptyriasis rubra pilaris associated with hypogammaglobulinaemia and severe *S. aureus* folliculitis and furunculosis with human polyvalent immunoglobulins successfully eradicated *S. aureus* infection (Castanet et al. 1994). Notably, symptoms of patients with persistent cutaneous infection caused by *S. aureus* improved after IgG infusions. Moreover, complications due to infections have a major impact on the clinical course of patients with chronic lymphocytic leukaemia. Infections commonly occur at mucosal sites, especially the respiratory tract, and organisms such as *S. aureus* are frequently isolated. Although the pathogenesis of infection in these patients is multifactorial, systemic hypogammaglobulinaemia is the major immune defect accounting for the increased risk of infection. Finally, a wide variety of rheumatologic disorders also occur in association with hypogammaglobulinaemic states. Among them, septic arthritis associated with *S. aureus* infection has been reported (Lee, Levinson and Schumacher 1993). Therefore, human genetic disorders that affect antibody production may increase the risk of certain staphylococcal infections (Table 1).

Another reason behind the scepticism on the role played by antibodies against *S. aureus* stems from the failure of passive immunization strategies in clinical trials (Spellberg and Daum 2012). It is plausible that passive therapies attempted so far have failed because they all targeted single antigens, therefore inducing insufficient protective immunity (Bagnoli, Bertholet and Grandi 2012). For instance, disappointing results were announced in clinical trials including the following candidate vaccines (reviewed in Proctor 2012): Veronate, based on polyclonal antibodies against the *S. aureus* surface protein ClfA; Alastaph, containing CPS and CP8 antibodies purified from subjects vaccinated with StaphVax; Tefibazumab, monoclonal antibodies against ClfA; and Aurograb, single chain antibodies against an ABC transporter of the pathogen.

On the other hand, there are preclinical and clinical data suggesting that a specific humoral response against the pathogen plays at least a partial role in protecting against the infection. Passive transfer of antibodies raised against different staphylococcal antigens (e.g. ClfA, Hla, IsdA, IsdB, FhuD2) conferred protection against *S. aureus* in animal models (Bubeck Wardenburg and Schneewind 2008; Kim et al. 2010c; Mishra et al. 2012a). In vitro assays have shown that antibodies can have a direct role in inhibiting the function of virulence factors and toxins. For example, antibodies can neutralize the toxicity of Hla, interfere with heme-iron scavenging mediated by IsdB and IsdA, or inhibit bacterial growth as demonstrated with anti-FhuD2 antibodies (Kuklin et al. 2006; Bubeck Wardenburg and Schneewind 2008; Kim et al. 2010c; Mishra et al. 2012a).

In humans, circulating antibodies to several *S. aureus* antigens are commonly found (Dryla et al. 2005; Clarke et al. 2006; Verkaik et al. 2009), particularly in *S. aureus*-colonized subjects and these individuals when systemically infected present milder disease outcomes as compared to non-colonized patients (Wertheim et al. 2004). Indeed, colonized subjects have been shown to have higher antibody titres against several staphylococcal antigens (Verkaik et al. 2009). Along the same line, it has been demonstrated that individuals harbouring TSST-1 producing strains had significantly higher levels of serum antibody to TSST-1 than individuals who carried strains without TSST-1 or non-carriers (Ritz et al. 1984). Carriers with high level of TSST-1 antibodies appeared less prone to toxic shock syndrome (Verkaik et al. 2009). More recently, lower antibody titres against staphylococcal exotoxins, namely Hla, PVI, Hld, SEC-1 and PSM-α3, have been shown to be associated with greater risk of invasive as well as skin infections (Adhikari et al. 2012; Fritz et al. 2013).

Another reason behind the apparent controversial role of humoral response against *S. aureus* is represented by the many immune-evasion mechanisms expressed by the pathogen (Serruto et al. 2010; Kim et al. 2012). Among them are several
Table 1. Human genetic disorders associated with increased risk of S. aureus infection.

<table>
<thead>
<tr>
<th>Pathology/genetic disorder</th>
<th>Disease outcomes</th>
<th>S. aureus infection</th>
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<tbody>
<tr>
<td>Antibodies</td>
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<tr>
<td>Hypogammaglobulinemia (e.g. Btk mutation)</td>
<td>Markedly reduced levels of B cells and immunoglobulins</td>
<td>Pneumonia</td>
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<td>Phagocytes</td>
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<td>Chédiak-Higashi syndrome</td>
<td>Neutropenia</td>
<td>Skin infection</td>
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<td>Mutations in LYST gene, which encodes a cytoplasmic protein involved in vacuole formation and transport of proteins</td>
<td>Defect in the NADPH oxidase system which impairs neutrophils, eosinophils, monocytes and macrophages to mediate killing of phagocytosed microorganisms</td>
<td>Skin infection</td>
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<td>CGD</td>
<td>Functional neutrophils disorder</td>
<td>Skin infection</td>
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<tr>
<td>Leukocyte adhesion deficiency Mutations in the gene encoding CD18 (ITGB2)</td>
<td>Functional neutrophils disorder</td>
<td>Skin infection</td>
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<tr>
<td>Neutrophil-specific granule deficiency (SGD)</td>
<td>Functional neutrophils disorder</td>
<td>Skin infection</td>
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<td>T cells</td>
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<tr>
<td>HIES or Job syndrome</td>
<td>Decreased Th17-cell numbers</td>
<td>Recurrent skin and sinopulmonary infections</td>
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<td>STAT3 and DOCK8 mutations</td>
<td>Deficiency of CD4 memory and Th cells</td>
<td>Eosinophilia</td>
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<td>Eosinophilia</td>
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<td>Atopic dermatitis</td>
<td>Skin barrier defects</td>
<td>Recurrent skin infections</td>
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<tr>
<td>Mutations in the gene encoding filaggrin (FLG)</td>
<td>Decreased levels of antimicrobial peptides</td>
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<td></td>
<td>Increased TH2-cell responses; Decreased TH17-cell responses</td>
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Factors that can block circulating antibodies such as SpA and Sbi (Silverman and Goodyear 2006; Atkins et al. 2008), inhibit development of humoral response by inducing B-cell depletion (SpA) or impair downstream processes mediated by antibodies binding to complement components (e.g. SCIN, Aureolysin, EfB, Ecb, SSL7; Serruto et al. 2010). The presence of such an anti-humoral armamentarium may explain why natural exposure to the bacterium does not elicit a clear antibody profile associated with protection from infection. On the other hand, the great effort of the pathogen to avoid humoral defences may explain another indication that antibodies play an important protective role. The potential protective mechanisms of antibodies against S. aureus infection are summarized in Fig. 1 and discussed further throughout the manuscript.

The role of B- and T-cell clonal deletion and anergy in rendering humoral response to S. aureus non protective

B-cell deletion
As anticipated above, the immune-evasion mechanisms endowed by S. aureus strongly affect the host adaptive response against it with a major role played by SpA. This protein is
Figure 1. Antibody-mediated protective mechanisms against *S. aureus*. (a) Opsonized bacteria by anti-staphylococcal antibodies and serum complement proteins may undergo through the following processes: (b) Phagocytosis or (c) NETosis (although it is not yet clear if for the latter opsonization is required). (b) Through Fc receptors neutrophils phagocytose bacteria. Following phagocytosis neutrophils rapidly undergo apoptosis and clearance by macrophages. (c) Alternatively, neutrophils can assemble NETs that may directly kill the bacteria or ensnare them and then get cleared by macrophages. (d) Staphylococcus aureus toxins and virulence factors (e.g. adhesins) can be neutralized by specific antibodies thus preventing *S. aureus*-mediated diseases.

The outcome of superantigen stimulation depends on the availability of a suitable second signal (e.g. CD40 ligand or IL-4) required for survival and/or cell-cycle progression. Therefore, the in vivo influence of cytokines and/or cognate help from bystander leukocytes may be critical in determining the clonal outcome of B-cell superantigen exposure (Fig. 2).

*Staphylococcus aureus* expresses also T-cell superantigens

*Staphylococcus aureus* expresses also T-cell superantigens (e.g. SEA, SEB, and TSST-1) that bind to a specific subset of the variable Vβ chains of the T-cell receptor (TCR). As a result, powerful polyclonal proliferative responses and clonal deletion of T lymphocytes occurs (O’Hehir and Lamb 1990; McCormick, Yarwood and Schlievert 2001). Furthermore, staphylococcal-specific T cells may become anergic as a consequence of chronic TCR stimulation, cytokine-mediated immunosuppression, superantigen activation or more likely a combination of all those factors that coexist during *S. aureus* infection (Ziegler et al. 2011). Using a long-term *S. aureus* infection mouse model, Ziegler et al. found that bacterial burden was significantly reduced in the kidneys...
Figure 2. Prototypical immune response towards a bacterial antigen compared to a model of the S. aureus-induced responses. (a) Typical immune response to a bacterial antigen. B cell recognizing a given antigen of a bacterium is activated and two different pathways can be induced in the presence or absence of concomitant T-cell help. Without cognate T-cell help, B cells proliferate and generate plasma cells. These cells produce and secrete low-affinity antibodies (mainly IgM). On the other hand, in the presence of T-cell help, B cells undergo through isotype switching and affinity maturation in the germinal centre. From these latter cells, memory B cells and long-lived plasma cells originate. (b) Atypical immune response elicited by Staphylococcus aureus infection. Staphylococcus aureus expresses several immune evasion factors. SpA and T-cell super antigens are among the most important. SpA induces activation of B1 cells. We hypothesize that lack of T-cell help due to anergic and deletion processes mediated by superantigens contributes to activation-induced cell death of SpA-activated B cells. This process may be particularly pronounced for S. aureus antigens expressed simultaneously with SpA. On the other hand, staphylococcal-specific B cells may become anergic after receiving a normal initial stimulus by a staphylococcal antigen, but fail to receive secondary signals that sustain their activation. This phenomenon may be particularly pronounced for antigens non-simultaneously expressed with SpA such as Hla.

Figure 3. Staphylococcus aureus cytotoxins target both innate and adaptive immune cells. Model representing cytotoxins capable of lysing immune cells involved in S. aureus clearance (orange arrows): PMNs, monocytes and macrophages. On the other hand, cytotoxins can also target adaptive immune cells (black arrows): T and B lymphocytes. Finally, cytotoxins can impair the interaction between innate and adaptive immune cells (blue arrows). The cytotoxins are listed on the top panel with different shapes and colours.

**The role of cytotoxins in dampening immune responses**

Staphylococcal cytotoxins are capable of targeting a wide variety of immune cells during staphylococcal infection (Fig. 3). These toxins include leukocidins, phenol-soluble modulins (PSMs) and hemolysins. Leukocidins comprise leukotoxins (LukED and LukAB), PVL and hemolysin gamma (HlgAB, HlgCB). LukAB is extremely effective at killing human polymorphonuclear leukocytes (PMNs) but not murine PMNs (Malachowa et al. 2012), a peculiarity shared with PVL (Löffler et al. 2010). LukAB is also able to kill monocytes, dendritic cells and macrophages (Alonzo and Torres 2013). Recently, the C-C chemokine receptor 5 has been identified as a cellular receptor used by LukED to kill lymphocytes, macrophages and dendritic cells (Alonzo et al. 2013). PSMs are cytolytic for neutrophils in the micromolar range and have a pronounced capacity to kill them after phagocytosis (Cheung et al. 2014). Hla, the best characterized hemolysin, binds to the cellular receptor ADAM10 and targets macrophages, monocytes, neutrophils and T cells (Berube and Bubek Wardenburg 2013). In addition, cytotoxins, by damaging the host cells from within, enable S. aureus to survive within neutrophils after the first 28 days, but then remained constant up to day 56 post-infection. This phenomenon appeared to be associated with the induction of an anergic state of T cells and their consequent reduced responsiveness to antigenic stimulation upon exposure to S. aureus (Ziegler et al. 2011).
phagocytosis, which can function as a Trojan horse to promote bacterial dissemination (Foster 2005).

Thus, cytotoxins although usually not considered among the immune evasion factors can significantly contribute to dampening both innate and adaptive immune responses to S. aureus infection.

**Cumulative effect of B-cell deletion and lack of T-cell help on humoral response to S. aureus antigens**

Considering the different mechanisms affecting B- and T cells mentioned above, in the context of S. aureus infection, SpA-targeted B cells are likely directed to apoptotic death because of the enhanced cytokine requirements of the cells proliferating under the stimulus of SpA with the concomitant impaired T-cell help due to T-cell superantigens and cytotoxins (Fig. 2).

**B-cell anergy**

Development of B cells against certain antigens may be affected less than others by SpA because of the variable expression of S. aureus antigens at different growth phases and site of infection (Fig. 2). For example, it has been demonstrated that SpA is expressed at earlier growth phases while Hla at later phases (Repola and Darfeuille 2009). In addition, Hla is a secreted toxin and may be able to activate specific B cells in locations away from the bacterium. This may explain why humans generally have antibodies against Hla despite most of the circulating strains express SpA. Interestingly, this was also demonstrated in animal infection models where Hla was among the few antigens being recognized by sera of mice infected with S. aureus; on the other hand, most of the other staphylococcal antigens were recognized by the convalescent sera only when the activity of SpA was blocked (Kim et al. 2010b). On the other hand, SpA can also be released from the cell wall of S. aureus (Becker et al. 2014); hence, we do not exclude that the SpA superantigen activity can have effect far from the infection site.

In addition, B cells that escape the SpA activity may fall in a state of lethargy that occurs when B cells mount a normal initial response to antigen but fail to receive secondary signals that sustain their activation. This state, referred to as anergy, is induced by chronic binding of the antigen (Cambier et al. 2007; Andrews and Wilson 2010), a situation that may occur in S. aureus persisting infections and colonization and in the context of the impaired T cell help due to T-cell superantigens and cytotoxins (Fig. 2). This phenomenon may induce a humoral response characterized by low-affinity antibodies.

Altogether, our model shown in Fig. 2 may explain why S. aureus exposure generates antibodies only against some antigens and why these antibodies are not protective against subsequent reinfections. On the other hand, vaccination may provide memory B cells and long-lived plasma cells producing high-affinity antibodies that might be able to induce protective immunity.

**INNATE IMMUNITY AGAINST S. AUREUS**

**The role of phagocytes against S. aureus infection**

Several direct and indirect observations led the scientific community to assume that neutrophils represent the major, if not the only phagocyte being able to contain staphylococcal invasive infections. Indeed, neutrophils represent 60–70% of human white blood cells (Voyich et al. 2005) and are the most numerous phagocytes able to ingest, kill and digest bacteria within minutes (Fig. 1). They are among the first cells to reach the site of infection and kill bacteria with an arsenal of deadly factors such as proteases, lipases, antimicrobial peptides, amides and reactive oxygen species (ROS) that can be used by the phagocytes individually or in combination for a synergistic effect. Patients with quantitative (e.g. chemotherapy-induced neutropenia) or qualitative (e.g. chronic granulomatous disease, CGD) neutrophil disorders are at increased risk of developing S. aureus infections (Table 1). However, these patients are also impaired in other phagocytic functions. For example, CGD patients display a defect in the NADPH oxidase system which impedes neutrophils, eosinophils, monocytes and macrophages to mediate killing of phagocytosed microorganisms (Boxer and Morganroth 1987; Bogomolski-Yahalom and Matzner 1995; Guide et al. 2003), suggesting that the high rates of S. aureus infections in CGD patients might be due not only to neutrophils defects but also to deficiency of different types of leukocytes. A similar consideration applies to chemotherapy-treated patients. For example, cyclophosphamide, a common chemotherapeutic used for cancer patients, ablates most leukocytes.

Although interactions between S. aureus and neutrophils have been extensively studied, comparatively little is known about the contributions of other professional phagocytes, such as monocytes and macrophages (MΦ), in the clearance of staphylococcal infections. Recent publications suggest that MΦ may play a critical role against staphylococcal infections (Murray and Wynn 2011).

**M1 and M2 macrophages**

It is well known that MΦ are vital effector cells that recognize and eliminate invasive pathogens. Despite the heterogeneous phenotypes manifested by MΦ during immune responses, they are generally divided into two broad categories: classically activated MΦ (M1) and alternatively activated MΦ (M2) (Fig. 4) (Gordon and Taylor 2005; Murray and Wynn 2011). M1 mediate defence of the host from a variety of bacteria, protozoa and viruses (Sica and Mantovani 2012). M1 secrete proinflammatory mediators such as tumour necrosis factor, nitric oxide (NO) and IL-1, which participate in the activation of various antimicrobial mechanisms, including oxidative processes.

An adaptive immune response is usually necessary to maintain classically activated macrophages (Fig. 4; Mosser and Edwards 2008). This is typically provided by the sustained production of IFN-γ by T helper 1 (Th1) cells. Activated M1 also release IL-6, IL-12 and IL-23, promoting polarization of Th1 and Th17 cells, which further drive inflammatory responses forward (Fig. 4).

M2 macrophages, on the other hand, are anti-inflammatory and are commonly found in patients with severe infections. The role of these macrophages in host defence and adaptive immunity remains unknown. Although alternatively activated macrophages appear to mediate clearance of helminths and nematodes, there is no clear evidence in support of their direct microbicidal effects (Gordon 2003; Gordon and Taylor 2005; Mosser and Edwards 2008; Murray and Wynn 2011).

As for M1 macrophages, adaptive signals are thought to be the primary pathway for the development and maintenance of M2 cells. However, in this case Th2-type immune responses are involved rather than Th1 (Fig. 4). Indeed, macrophages treated in vitro with IL-4 and/or IL-13 fail to present antigen to T cells, produce minimal amounts of proinflammatory cytokines and are less efficient than M1 at producing toxic oxygen and nitrogen radicals, and at killing intracellular pathogens. Compared to M1, M2 produce less IL-12 and nitric oxide (NO) but more IL-10 (Fig. 4; Murray and Wynn 2011). M2 suppress the generation of M1 as...
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infections comes from studies on staphylococcal biofilm
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and cannot eliminate the invading bacteria, likely because
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be contained by abscess formation causing spread into tissues distal from the
anism of
apoptosis in macrophages infiltrating the abscess, as well as through the anti
NETs by several stimuli, such as antibody/antigen complexes and complement,
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Sheridan
et al.
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M2 macrophages (Katakura
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2011;
K r y s k o
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nations (Sheridan
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2005)
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of cytokine and chemokine production and differed in their
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existence of distinct neutrophil subsets with functional and
phenotypic profiles has not been widely considered or explored
until very recently (Beyrau, Bodkin and Nourshargh
2012). There is significant evidence for the existence of different neutrophil
subsets under both physiological and pathological conditions. Interestingly, S. aureus infection has been shown to induce the
development of different neutrophil populations in S. aureus-susceptible, S. aureus-resistant and naive mice (Tsuda
et al.
2004).
The distinct neutrophil populations showed unique patterns of cytokine and chemokine production and differed in their
during tissue injury is thought to be IL-4, which as mentioned above, drives the development of M2 cells.
Recently, the influence of severe burn injury on abscess formation and bacterial growth at the infection site was investigated in burned mice intradermally infected with S. aureus (Asai
et al.
2010). Differently to healthy mice, in burned mice bacteria did not cause abscess formation after intradermal infection, but spread from local intradermal tissues throughout the whole body. In healthy mice, M1 macrophages were characterized as effector cells for abscess formation. Indeed, M1 cells inoculated in burned mice rescued abscess formation following intradermal infection. These results indicate that M1 are major
receptor cells in the host antibacterial defence against S. aureus. Through abscess formation, M1 inhibit the dissemination of the pathogen, while predominance of M2 cells can result in bacterial spread and sepsis (Fig. 4).
Another evidence of the importance of M1 cells against S. aureus infections comes from studies on staphylococcal biofilm formation. Staphylococcus aureus biofilms were shown to contain macrophages with limited phagocytic capacity and M2 phenotype. Indeed, within biofilm the pathogen appears to skew macrophage differentiation from the proinflammatory and microbicidal M1 phenotype to the anti-inflammatory M2 phenotype, leading to attenuated host inflammatory response and persistence of infection (Thurlow
et al.
2011; Scherr
et al.
2014). Likewise, macrophages co-cultured with S. aureus biofilms in vitro displayed a gene expression profile characteristic of M2 cells (Hanke, Angle and Kielian
2012).
In addition, the exogenous administration of activated M1 directly into sites of biofilm formation was shown to overcome the local immune inhibitory environment and fibrotic barrier associated with biofilms (Hanke
et al.
2013). Intriguingly, the same result was obtained administering the macrophage activating peptide EP67 to facilitate bacterial clearance by inducing a proinflammatory milieu. On the other hand, neutrophil transfer did not attenuate S. aureus biofilm growth even when higher numbers of cells were inoculated (Hanke
et al.
2013). It is now also being elucidated how S. aureus induces a M2 polarization. Indeed, the pathogen induces protein kinase B/Akt1 signalling, which is essential in shifting macrophages from an antimicrobial phenotype M1 to the functionally inert M2 signature (Xu
et al.
2013).
A further indication of the importance of M1 polarization comes from the observation that rhinosinusitis patients with nasal polyp exhibit remarkably increased colonization of S. au-
reus and cannot eliminate the invading bacteria, likely because of malfunctioning M1 and augmented M2 macrophages (Krysko
et al.
2011).

**PMN-I and PMN-II**

The role of neutrophil subtypes against S. aureus has been largely overlooked so far, but is now emerging in the literature. Despite the fact that neutrophil functional heterogeneity has been already described many years ago (Gallin
1984), the potential existence of distinct neutrophil subsets with functional and phenotypic profiles has not been widely considered or explored until very recently (Beyrau, Bodkin and Nourshargh
2012). There is significant evidence for the existence of different neutrophil subsets under both physiological and pathological conditions. Interestingly, S. aureus infection has been shown to induce the development of different neutrophil populations in S. aureus-susceptible, S. aureus-resistant and naive mice (Tsuda
et al.
2004). The distinct neutrophil populations showed unique patterns of cytokine and chemokine production and differed in their

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**Figure 4. Importance of neutrophil and macrophage subsets against S. aureus infection.** (a) Type I response characterized by Th-1. PMN-I and M1 polarization promotes microbicidal activity and containment of S. aureus through abscess formation. Within the abscess, neutrophils can be induced to release NETs by several stimuli, such as antibody/antigen complexes and complement, which might be regulated by vaccination. Inhibition of neutrophil/macrophage cooperation, through release of deoxyadenosine from the NETs, which induces apoptosis in macrophages infiltrating the abscess, as well as through the anti macrophagic activity of Hii, may represent an important immune evasion mechanism of S. aureus. This is another pathogenic process that might be intercepted by vaccination. (b) Type II response characterized by Th-2, PMN-II and M2 polarization has no microbicidal activity. In these conditions, S. aureus may not be contained by abscess formation causing spread into tissues distal from the infection site and sepsis.

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well as various immune reactions. Therefore, M1 are usually not found in hosts where M2 predominate (Katakura
et al.
2004).
Accumulating literature suggests that lack of M1 macrophage development is closely related to ineffective control of S. aureus (Asai
et al.
2010; Krysko
et al.
2011; Thurlow
et al.
2011; Accarias
et al.
2015). Resident macrophages present in healthy donors generally convert to M1 following infection via pattern recognition receptors (Mantovani
et al.
2004). On the other hand, the majority of thermally injured hosts carry alternatively activated M2 macrophages (Katakura
et al.
2005). Indeed, infection is the major cause of morbidity and mortality in severely burned patients and S. aureus is known as a typical pathogen in such infections (Sheridan
2005; Vostrugina, Gudaviciene and Vitkauskiene
2006). Interestingly, one of the first innate signals to be released
expression of Toll-like receptors (TLRs) and CD49d/CD11b integrins. In general, neutrophils isolated from S. aureus-resistant mice (PMN-I) were proinflammatory in nature (i.e. produced IL-12, CC-chemokine ligand 3, CCL3). PMN-I directed a polarization of resident macrophages into M1 macrophages (Sica and Mantovani 2012). Neutrophils from S. aureus-susceptible mice (PMN-II) had an anti-inflammatory phenotype (i.e. produced IL-10, CCL2) (Tsuda et al. 2004), which induce M2 macrophages (Sica and Mantovani 2012).

**Cooperation between PMN and macrophages**

What is probably crucial for a successful response against S. aureus is a concerted action of neutrophils and macrophages (Figs 1 and 4). The two phagocytes share several functionalities, including avid phagocytosis, similar kinetic behaviour under inflammatory/infectious conditions as well as antimicrobial and immunomodulatory activities. However, consequent to specialization during their differentiation, macrophages and neutrophils acquire distinctive, complementary features that originate different levels of antimicrobial capacities and cytotoxicity and different tissue localization and lifespan. The combination of overlapping and complementary characteristics of the two professional phagocytes promotes their cooperative participation as effectors and modulators in innate immunity against infection and as orchestrators of adaptive immunity (Silva 2010). In the concerted activities operating in antimicrobial innate immunity, macrophages and neutrophils are not able to replace each other. Cooperation between the two phagocytes against S. aureus infection has been recently shown (Abtin et al. 2014). Perivascular macrophages were demonstrated to be critical for neutrophil migration into mouse skin infected with S. aureus (Abtin et al. 2014). Neutrophils preferentially extravasated in close physical proximity to perivascular macrophages, which were the dominant source of neutrophil-attracting chemokines.

Staphylococcus aureus has been shown to use different strategies to impair the neutrophil–macrophage cooperation. Hla is able to lyse perivascular macrophages inhibiting the recruitment and extravasation of neutrophils (Abtin et al. 2014). Furthermore, expression of staphylococcal adenosine synthase (AdsA) and nuclease (Nuc) by S. aureus within renal abscesses in mice generates deoxyadenosine from neutrophil extracellular traps (NETs), which in turn induces apoptosis in macrophages infiltrating the abscess (Thamavongsa, Missiakas and Schneewind 2013). This finding suggests that while neutrophils and NETs are important to contain the bacterium forming an abscess, macrophages are important to clear the infection (Fig. 4). Indeed, abscess formation in mice infected with AdsA-deficient S. aureus is significantly reduced (Thamavongsa et al. 2009).

**Neutrophil extracellular traps**

NETs were described for the first time in 2004 (Brinkmann et al. 2004) as structures made of processed chromatin bound to granular and selected cytoplasmic proteins able to kill bacteria. NETs are the result of a unique form of cell death that morphologically is characterized by the loss of intracellular membranes before the integrity of the plasma membrane is compromised. After activation neutrophils flatten and firmly attach to the substratum, the nucleus loses its lobules, the chromatin decondenses, and the inner and outer nuclear membranes progressively detach from each other. Concomitantly, the granules disintegrate, the nuclear envelope disaggregates into vesicles and the nucleoplasm and cytoplasm form a homogenous mass, cell membrane ruptures and the interior of the cell is ejected into the extracellular space, forming NETs (Brinkmann and Zychlinsky 2012). NETs have been shown to act against both Gram-negative and Gram-positive bacteria such as Escherichia coli (Grinberg et al. 2008), Shigella flexneri and S. aureus (Brinkmann et al. 2004). They are also effective in combating parasites such as Toxoplasma gondii (Abi Abdallah et al. 2012) and fungi such as Candida albicans (Urban et al. 2006).

The mechanism of action of NETs is distinct from opsonophagocytosis mediated by neutrophils. Indeed, inhibition of actin polymerization with cytochalasin D blocked opsonophagocytosis but not the ability of NETs to kill bacteria (Brinkmann et al. 2004). On the other hand, when NETs were treated with DNase, killing of bacteria was dramatically reduced. Regarding the mechanism behind NETs bacterial killing, Parker et al. described that S. aureus killing was mediated by myeloperoxidase-generated hypochlorous acid (Parker et al. 2012). In contrast, Menegazzi et al. showed that S. aureus captured by NETs is released and recovered in cell medium upon incubation with DNase suggesting that NETs entrap but do not kill the microorganism (Menegazzi, DeCleva and Dri 2012). However, most of these observations are based on experiments performed in vitro and the role of NETosis in vivo is still not completely elucidated. NETosis has been demonstrated in experimental model of shigellosis in rabbits and in spontaneous appendicitis in humans (Brinkmann et al. 2004) showing that NETs are capable to entrap bacteria also in vivo. Later findings describe that S. aureus injected intradermally in mice can induce the release of NETs structures such as histones and neutrophil elastase within minutes (Yipp et al. 2012). This suggests that in vitro NETosis, which takes hours after the initial induction, may not perfectly mimic the in vivo process. DNase administration to mice after staphylococcal skin infection resulted in a significant increase in bacteraemia and decrease in CFU bacterial counts at the skin lesion indicating that NETs are crucial to contain an acute invasive infection in vivo. However, it is still not clear if NETs act only to ensnare the bacterium or actually kill it. High local concentration of neutrophil granule proteins and antimicrobial peptides in the NETs probably amplify killing effectiveness of these substances (Brinkmann and Zychlinsky 2007). However, clearance of S. aureus cells ensnared within NETs may require macrophages (Farrera and Fadeel 2013). Indeed, as mentioned earlier, the pathogen has been recently demonstrated to inhibit this potential mechanism (Thamavongsa, Missiakas and Schneewind 2013; Abtin et al. 2014). Another aspect that remains to be better understood is how NETosis is induced in vivo. In vitro, NETs are commonly induced with phorbol myristate acetate PMA. However, as already said, this process takes hours and may not resemble physiological inducers. ROS like hydrogen peroxide have been reported as likely physiological inducer of NETs. It has been recently demonstrated that NETs formation can also be stimulated by activated platelets through the release of human β-defensin 10 (Clark et al. 2007; Kraemer et al. 2011). Furthermore, NETs formation can be induced by antibodies, antibody–antigen complexes and complement (Brinkmann and Zychlinsky 2012; Yipp et al. 2012). These observations suggest that the presence of specific humoral response against the pathogen may enhance the formation of NETs.
IDENTIFYING CORRELATES OF PROTECTION AGAINST S. AUREUS

Surrogates of protection

Antibody titre is still the most common biomarker used in clinical studies of experimental vaccine trials or of naturally exposed humans. However, up to date a correlate of protection based on antibody titres against staphylococcal antigens has not been identified. Therefore, research aimed at identifying biomarkers and surrogate assays linked to protection represents a priority for improving the understanding of mechanisms of protection in humans and for vaccine development. Most of such studies have focused so far on opsonophagocytosis. In particular, opsonophagocytic assay (OPA) augmented by antibody and complement deposition onto the bacterial surface has been largely investigated as a surrogate of protection against S. aureus.

For other Gram-positive organisms, such as Streptococcus pneumoniae and Group B streptococcus, opsonophagocytosis is considered a primary mechanism of defence against infection in humans and OPA has been successfully established as a reliable surrogate assay of protection. For these pathogens opsonophagocytic titres correlate with the immunoglobulin G antibody concentrations (Nurkka et al. 2001; Romero-Steiner et al. 2006). HL-60 cell line has been extensively used in OPA (Romero-Steiner et al. 1997). Polar organic compounds, such as dimethylformamide, trigger the differentiation of HL-60 into polymorphonuclear-like cells that possess the receptors, primarily FcγRII, CR1 and CR3, necessary for effective phagocytosis.

In contrast to streptococci, OPA may not represent a reliable surrogate of protective efficacy for S. aureus vaccines. Indeed, while correlation between protective efficacy of streptococcal vaccines in mice and humans and phagocytic titres has been demonstrated (Musher et al. 1993; Johnson et al. 1999), such a correlation is still missing for S. aureus vaccines. A possible explanation behind the unreliability of OPAs used so far for S. aureus is provided by a recent publication which reports that neutrophils in suspension (a condition usually present in standard OPA) provide suboptimal killing activity towards S. aureus when compared to the assay performed using adherent neutrophils (Lu et al. 2014).

Lancefield bactericidal assay or whole blood survival assay (WBA) is an alternative to the traditional OPA (Lancefield 1957). WBA measures bacterial survival in a blood sample and killing capacity is assumed to reflect phagocytic activity driven by neutrophils. The main advantage of the WBA is that the only exogenous component in the assay is the bacterial inoculum and that blood is much more representative of the in vivo situation. Indeed, blood from vaccinated or S. aureus pre-exposed animals or human subjects, contains all the immunological factors (e.g. antibodies, cytokines, neutrophils, monocytes, complement, etc.) likely all necessary to contain the infection. This is particular relevant for correlating the assay with protection against the pathogen.

So far, WBA assays have been mainly used to characterize virulence factors. For example, it has been used to assess the potential anti-phagocytic role of S. aureus virulence factors such as Sbi (Smith et al. 2011), IsdH (Visai et al. 2009) and SpA (Falugi et al. 2013).

Lack of sufficient efforts in developing WBA as a surrogate assay of protection probably stems from limitations in performing the assay within the setting of clinical trials. Indeed, the assay needs to be performed using fresh blood and this means a greater equipment and organizational cost as compared to OPA.

Another line of research aimed at identifying correlates of protection is on mechanisms solely based on antibodies and independent of phagocytosis. Indeed, antibodies by binding to antigens can block their function through allostERIC inhibition or conformational changes. It is therefore necessary to know the function of the target antigens to perform these studies. Classical examples of such functional assays are for the inhibition of toxins and adhesins (Fig. 1). For instance, antibodies raised against Hla can be evaluated in an assay for their capacity to neutralize the hemolytic activity of the toxin (Bubeck Wardenburg and Schneewind 2008; Pozzi et al. 2012b). This assay is usually performed by measuring hemolysis of rabbit red blood cells incubated with the active toxin with and without the test antibodies. Another example is provided by assays using antibodies against clumping factor A and B (ClfA and ClfB). These proteins bind fibrinogen and antibodies elicited against them are used for preventing the binding of S. aureus to fibrinogen immobilized on microtitre plate wells (Hawkins et al. 2012).

Passive immunization is an additional approach that can be considered to measure antibody functionality. As mentioned earlier, it has been reported that antibodies raised against different staphylococcal antigens (e.g. Hla, IsdA, IsdB, ClfA, ClfB, FhuD2) confer protection against staphylococcal infection in animal models (Menzies and Kernodle 1996; Bubeck Wardenburg and Schneewind 2008; Kim et al. 2010c).

The latter two approaches are feasible and relatively easy assays for clinical trials and they can be performed using serum, which can be stocked and frozen.

Finally, research should be done to develop assays in which neutrophils and macrophages differentiated to type I are used. They could be tested either individually or together to exploit their cooperation activity. Given that type I phagocytes have been found to be effective at killing S. aureus, this might represent an important improvement for developing assays to measure antibody functionality.

Cell-mediated immunity

Correlates of protection for S. aureus may also include T-cell responses and cytokotes. The role of specific-pathogenic CD4+ T cells in tailoring the immune response of the host to combat pathogens by differentiating into distinct subsets of effector T helper (Th) cells, characterized by the production of different cytokines, is well described. Accumulating literature indicates that Th cells may play a primary effector role in protection against S. aureus infection both in humans and in mice. Human data are very difficult to interpret because they often come from very complex pathologies like HIV infection or hyper-IgE syndrome (HIES, see Table 1). Although the reasons for the elevated rates of MRSA infections appear to be multifactorial, low CD4+ T-cell counts are a risk factor not only for skin and soft tissue infections (SSTI) but also for S. aureus bacteremia (Shadyab and Crum-Cianflone 2012). HIV-infected persons have a propensity for MRSA SSTI and a high rate of recurrent disease. HIES, or Job syndrome, is characterized by elevated IgE levels, eosinophilia, eczema and recurrent skin and sinopulmonary infections often due to S. aureus (Buckley, Wray and Belmaker 1972).

Chronic mucocutaneous candidiasis is also a hallmark of HIES. Stat3 mutations have been identified in patients with autosomal-dominant HIES (AD-HIES) (Holland et al. 2007; Minegishi et al. 2007). STAT3 is a central regulator of lymphocyte differentiation and function (Kane et al. 2014). Functional STAT3 deficiency compromises the generation of mouse Th2 (Stritesky et al. 2011).
et al. 2011), as well as of human CD4+ memory and T follicular helper cells (Tfh; Cheng et al. 2012). Very recently it has been shown that STAT3-deficient CD4+ mouse T cells have a profound defect in Tfh differentiation, accompanied by decreased germinal centre B cells and antigen-specific antibody production (Ray et al. 2014). This might be the cause of the impaired humoral responses observed in many AD-HIES patients, which may in turn contribute to their S. aureus susceptibility (Leung et al. 1988; Sheerin and Buckley 1991). In addition, Stat3 mutations cause a reduction in STAT3-mediated signalling that results in a failure of Th17–cell differentiation (Holland et al. 2007). Therefore, Th17 cells have been considered as important players in resistance to C. albicans and S. aureus infections (Table 1). Besides Th17, γδ T cells have recently been identified as an important source of IL-17. Although the intrathymic development of γδ T cells is independent of STAT3 (Shibata et al. 2011), most peripheral IL-17-producing γδ T cells respond rapidly to IL-18 and IL-23, which signal through STAT3 (Sutton et al. 2009). Therefore, STAT3 deficiency in HIES syndrome is expected to affect also IL-17 production by γδ T cells. γδ T cells have been implicated in protection in murine models of S. aureus cutaneous infection (Cho et al. 2010), pneumonia (Cheng et al. 2012) and surgical site infection (Maher et al. 2013). Recently, preferential expansion of a population of memory Vγ4+ T cells capable of enhanced IL-17 production during subsequent S. aureus infection has been described. Transfer of S. aureus–primed γδ T cells reduced bacterial burden at the site of infection and bacterial dissemination (Murphy et al. 2014).

IL-17 is involved in neutrophil proliferation and chemotaxis. Impaired neutrophil responses and recruitment to lung and skin account, at least in part, for the recurrent staphylococcal infections observed at these particular sites in humans (Hill et al. 1974; Buckley and Becker 1978; Laan et al. 1999), (Table 1).

IL-17 has been shown to play a role in protection against local but not (or less) systemic S. aureus infections in several mouse models. For example, IiL17a−/−IiL17f−/− mice developed spontaneous mucocutaneous abscesses around the nose and mouth due to S. aureus but were not more sensitive to intravenous infection with the same bacterium (Ishigame et al. 2009). In addition, IiL17a−/− mice had increased synovitis and erosions and locally decreased clearance of bacteria after local but not systemic, S. aureus inoculation (Henningson et al. 2010). IiL17r−/− mice had substantially larger skin lesions with higher bacterial counts and impaired neutrophil recruitment compared with wild-type mice (Kim et al. 2010a). Recently, in a mouse model of S. aureus nasal carriage, it has been shown that IiL17a−/− mice are more prone to S. aureus colonization (Archer, Harro and Shiriliff 2013). Finally, Th17 cells have been shown to play a protective role against S. aureus challenge also following vaccination with the C. albicans adhesion protein Als3p, the S. aureus ClfA or IsdB (Lin et al. 2009; Joshi et al. 2012).

T-cell profiling and cytokine expression is therefore another important area of investigation for identifying correlates of protection and vaccine development.

Making the link with clinical outcomes

An important aspect common to consider to all the approaches described so far is the need of linking the assays, readouts and biomarkers with protective immunity in humans. There are two ways to do that: (i) include the assays within proof of concept trials and efficacy trials to correlate them directly with vaccine efficacy; (ii) use clinical data on natural infections or perform ad hoc studies in which patients are followed during disease progression and relapses. In the first approach, the advantage is that the study will have well-defined control groups and vaccinated subjects that can be compared. However, this approach is valid if the vaccine formulation shows some level of protection in humans. This approach is exemplified by the clinical trials of the S. aureus vaccines StaphVax and V710. In both cases, OPAs were included as secondary endpoints of the trials. Although both studies demonstrated functional activity associated with sera from vaccinated subjects using the two assays, both trials failed to show efficacy against S. aureus infection (Fattom et al. 2004; Harro et al. 2012; Proctor 2012; Fowler et al. 2013). This suggests that the assays, at least as performed during the trials, were not predictive of vaccine efficacy. In the second approach, the disadvantage is the lack of the proper control group and the many confounding factors normally intervening in these studies. These studies can be done comparing healthy individuals with patients affected by staphylococcal infections for the outcome of the selected functional assays. The major difficulty of this approach is to understand if the results of the assays are really associated with protective mechanisms present in the patients or to other factors. Indeed, infected patients may respond differently to antibiotics, they can have different underlying health conditions, they may be infected with staphylococcal strains with different resistance profiles and pathogenic factors, and they may be exposed through different routes to S. aureus (e.g. deep or superficial, surgical seeding, trauma, burn wounds and infection of different layers of the skin). The presence of these confounding factors has so far hindered the development of correlates of protection in the natural infection settings. However, we believe that a prospective clinical study in which a sufficient number of patients and control individuals are monitored from before or at the beginning of the infection throughout the progression and resolution of the infection may lead to important information on mechanisms of protection in humans.

CONCLUSIONS

We believe that lack of a clear association between humoral response and protection against S. aureus in humans is primarily due to B-cell clonal deletion and anergy processes. These are promoted by converging factors that lead to B-cell activation in concomitance with lack of T-cell help due to superantigens and cytotoxins produced by the pathogen during infection. Given these phenomena, humoral response against most S. aureus antigens may be inhibited by SpA activity or characterized by B cells that do develop, but produce low-affinity antibodies due to anergic processes. This complicates interpretation of serological studies performed with the aim of identifying a correlate of protection and may prevent induction of protective immunity following S. aureus infection. The other big issue that hinders the understanding of protective mechanisms against S. aureus is the lack of predictive functional assay. Gold standard OPAs used so far have failed to predict efficacy against S. aureus in clinical trials. Functional assays alternative to OPA are available and, although scarcely adopted so far by investigators, may provide important information on protective mechanisms and may be developed as surrogate assays of protection. Furthermore, we believe that research should be done to set up innovative assays for evaluating the role of neutrophil and macrophage subtypes, as well as their cooperation, NETosis, functional antibodies, cytokines and T cells in promoting bacterial killing. This could be done in vitro by mixing purified components, ex vivo using whole blood from humans or animals or in vivo using animal models. In such an assay, S. aureus antibodies may enhance
phagocytosis as well as NETosis and Th1 cytokines would shift macrophages towards M1 enabling them to successfully clear entrapped bacteria.

We could envisage immune evasion as the central pathogenic mechanism of *S. aureus* with virtually all its virulence factors working in concert. Thus, by dampening humoral response (e.g. SpA, Sbi) and T-cell help (e.g. SEA, SEB and TSST-1), blocking complement factors (e.g. FliP, SCIN) and killing immune cells with toxins (Hla, LukED). This is why we believe that the key to success for developing new efficacious interventions against *S. aureus* is represented by immunological treatments that can block these mechanisms. Prophylactic vaccination can potentially generate *S. aureus* specific memory B cells and long-lived plasma cells producing high-affinity antibodies without being affected by B- and T-cell deletion and anergy processes occurring during infection. Vaccine antibodies against virulence factors together with a Th1/Th17 adjuvant able to efficiently recruit and activate type I macrophages and neutrophils at the site of the infection may successfully block *S. aureus* immune evasion. Indeed, we have recently demonstrated that a vaccine combination targeting different virulence factors formulated with a TLR7 adjuvant stimulating a Th1/Th17 anergy processes occurring during infection. Vaccine antibodies against virulence factors together with a Th1/Th17 adjuvant stimulating a Th1/Th17 response generates unprecedented protective efficacy against *S. aureus* infection in mice (Bagnoli et al. 2015).

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