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Adaptive immune response in human *Staphylococcus aureus* bloodstream infection

This thesis is submitted to the University of Dublin for the degree of Doctor of Medicine (M.D.)

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. Where any of the content presented is the result of collaborative research, this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own.

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Summary

Staphylococcus aureus bloodstream infection is a common and life-threatening condition. Treatment outcomes are poor, suboptimal clinical management is common, antimicrobial resistance continues to emerge, and there are few new agents in the drug discovery pipeline. There is an urgent need for novel methods of preventing and treating *S. aureus* infection. A lack of understanding of protective immunity or its correlates has greatly impeded progress in the development of rationally-designed anti-*S. aureus* vaccines. This project set out to characterise the pathogen, its clinical impact on patients, and the adaptive immune response it induced in humans, with a particular focus on T lymphocytes.

The clinical characteristics, management and outcomes of adult patients with *S. aureus* and *E. coli* bloodstream infection (SA-BSI and EC-BSI) were contrasted. The bulk of *S. aureus* disease was attributable to methicillin-susceptible *S. aureus* and infection was frequently community-acquired. *S. aureus's* capacity to cause severe disease in otherwise healthy people and to follow a complicated course distinguished it from *E. coli*. Failure, relapse or death was seen in a large proportion of SA-BSI, whereas all *E. coli* infections were easily and rapidly cured. Healthcare resource utilisation was significantly greater in *S. aureus* infection. Of concern, clinical management of SA-BSI was suboptimal in over half of cases, and occurred when patients were not under the care of infection specialists. There was considerable genotypic heterogeneity, particularly in MSSA, in invasive *S. aureus* isolates. Despite this variety in lineage, genes for several cell-wall antigens were present and conserved on next-generation genome sequencing, which may be promising for vaccine development.

The history of anti-*S. aureus* vaccines is littered with failures. A critical problem has been the lack of known mechanisms or correlates of protective immunity to *S. aureus* infection in humans. There is considerable doubt that induction of humoral immunity alone will be sufficient to confer protection against *S. aureus* infection. It is now widely accepted that a vaccine with multiple antigens, that targets both cellular and humoral immunity is required. However, it is unknown which specific cells to target in next-generation vaccines. Clinical observations and data from mouse models support a key role for T helper lymphocytes producing IFNγ or IL-17 in anti-staphylococcal immunity. Despite these clues, it was not known if human T cells could recognise and respond to *S. aureus* antigens, and there had been no previous investigation of human cell-mediated immune response in invasive infection. This project described, for the first time in humans, the adaptive immune response in the early recovery period of *S. aureus* bloodstream infection. In addition to antibody responses, human αβ ($CD4^+$ and $CD8^+$), but not $\gamma\delta^+$ lymphocytes recognised and responded

to *S. aureus* during recovery from bloodstream infection. This response was primarily Th1-mediated, with a lesser contribution from Th17 cells. Th1 immune memory was formed or re-activated during systemic infection. Similar pathogen-specific T helper cell activation was not seen in patients with *E. coli* bloodstream infection, implying a substantial difference between the role of T cells in these two bacterial infections. A *S. aureus* antigen-specific Th1/17 signature thus seems particular to recovery from invasive staphylococcal disease and, similar to animal models, may be a correlate of protective immunity.

The final part of this work successfully developed a model assay to measure T cell immunogenicity of specific *S. aureus* antigens (and T cell-directed adjuvants) using blood donor buffy coats and clumping factor A as a model antigen. This assay can be used to map T helper cell responses over the course of infection in future SA-BSI patients to correlate relative potency of T cell activation with clinical outcome.

These preliminary findings are a substantial step forward in understanding how T cells react during *S. aureus* infection in real patients – knowledge fundamental to manipulating their activity in host-targeted therapeutics.

Acknowledgement

There are many people that deserve my thanks for their contribution to this work.

I would particularly like to thank Professor Rachel McLoughlin for taking a chance on a medic as a novice scientist and for her insightful and encouraging ideas, feedback and constant support. I owe a debt of gratitude too to Professor Tom Rogers for his kind and perceptive advice throughout the years.

To all my colleagues in the McLoughlin Lab Host-Pathogen Interactions group, and in our allied 3.12 labs and its reading room for teaching me how to do everything. Their experience and ability meant that my mistakes were usually caught in time and if not, at least both commiserations and pizza were shared. Dr Micheál Mac Aogáin and Dr Dara O'Halloran are colleagues from other departments who deserve special mention for their assistance with particular experiments.

To the medical scientists and clinical microbiologists in St. James', Tallaght and Beaumont Hospitals, thank you for letting me wander in and out to check the bacteraemia books, sub my plates and for all your courtesy and assistance in this project.

The years between taking my first tentative steps in the lab to completing this thesis have seen many changes in my life – completion of specialist training, moving country (twice), my first appointment as a consultant physician and the addition of a new member of our family. My friends and family have provided a constant quiet support without which none of that could have gone as smoothly as it did.

A final and most important thanks to all the patients who agreed to participate in this study. I hope that their selflessness in contributing to this work will be a small step on the road towards reducing the impact of invasive *S. aureus* infection on future patients.

Abbreviations

AD	Atopic dermatitis
AD-HIES	Autosomal dominant hyper-IgE syndrome
AMR	Antimicrobial resistance
APC	Antigen-presenting cell
BSI	Bloodstream infection
CA	Community-acquired
CA-MRSA	Community-acquired methicillin-resistant *S. aureus*
CC	Clonal complex
CD	Cluster of differentiation
CFU	Colony-forming units
CGD	Chronic granulomatous disease
ClfA	Clumping factor A
CTL	Cytotoxic T lymphocyte
E. coli	*Escherichia coli*
EC-BSI	*Escherichia coli* bloodstream infection
HA	Hospital-acquired
HCA	Healthcare-associated
HKEC	Heat-killed *E. coli*
HKSA	Heat-killed *S. aureus*
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
IL-10	Interleukin-10
IL-17	Interleukin-17
MDSC	Myeloid suppressor dendritic cells
MHC	Major histocompatibility complex
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-susceptible *S. aureus*
OD	Optical density
RC	Research clinician
ROS	Reactive oxygen species
RT	Room temperature

S. aureus	*Staphylococcus aureus*
SA-BSI	*Staphylococcus aureus* bloodstream infection
SEA	Staphylococcal enterotoxin A
SpA	Staphylococcal protein A
ST	Sequence type
TCR	T cell receptor
Th	T helper cell
Th1	T helper cell type 1
Th17	T helper cell 17 (producing IL-17)
Th2	T helper cell type 2
TNFα	Tumour necrosis factor alpha
γδ	Gamma-delta T lymphocyte

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Chapter 1

Introduction

1.1. The pathogen, the host and infectious disease

In 1881, Alexander Ogston, the surgeon who first described *Staphylococcus aureus*, wrote that the bacterium "*has been held to be the most potent factor in producing blood-poisoning and has also been asserted to be an innocent organism*" [1]. This paradox persists today. *S. aureus* colonises a substantial proportion of us. Colonised individuals establish an equilibrium with bacteria and most never develop staphylococcal infection. In apparent contrast, *S. aureus* is also an opportunistic pathogen which frequently causes life-threatening community- and healthcare-acquired infections. Ogston found that experimental infection with *S. aureus* produced "*varying effects*": "*blood-poisoning… suppuration…*" or "*may be resisted by strong individuals under favouring circumstances*" [1]. What factors conspire to determine these outcomes remain largely unknown to this day, and *S. aureus*'s dual identity as innocent bystander and guilty party endures.

The traditional pathogen-centred view, with microbes as the sole perpetrators of infectious diseases, has changed. Increasingly, the concept of a healthy host incorporates a diverse microbiome, including *S. aureus* and other pathobionts. The mere presence of potentially pathogenic bacteria does not inevitably herald clinical infection, largely due to a successful and balanced immune response. Possible outcomes of host-microbe contact are shaped by a complex dance between both parties (Fig. 1.1). Commensalism or colonisation occurs when interaction with a microbe, though it can elicit an immune response, results in no (or clinically inapparent) damage to the host. Infectious disease is a clinical manifestation of damage, which may be pathogen-mediated, host-mediated, or both, and evolves differently among individuals [2]. Ideally, it is resolved with minimal host damage and lasting protective immunity. Alternatively, the pathogen directly damages host tissues, or leads to overwhelming infection. Conversely, an excessive, dysregulated, or misplaced host immune response can lead to overwhelming inflammation.

Encounters with *S. aureus* cannot be fully understood by focussing on the pathogen in isolation. Humans bring different genetic backgrounds, immunological histories and co-morbidities to the point of host-microbe contact. Compelling clinical evidence suggests that development and outcome of human *S. aureus* infection may depend considerably on these host factors [3,4]. Understanding human immunity in parallel with microbiology is essential for development of anti-staphylococcal vaccines and improving management of *S. aureus* disease.

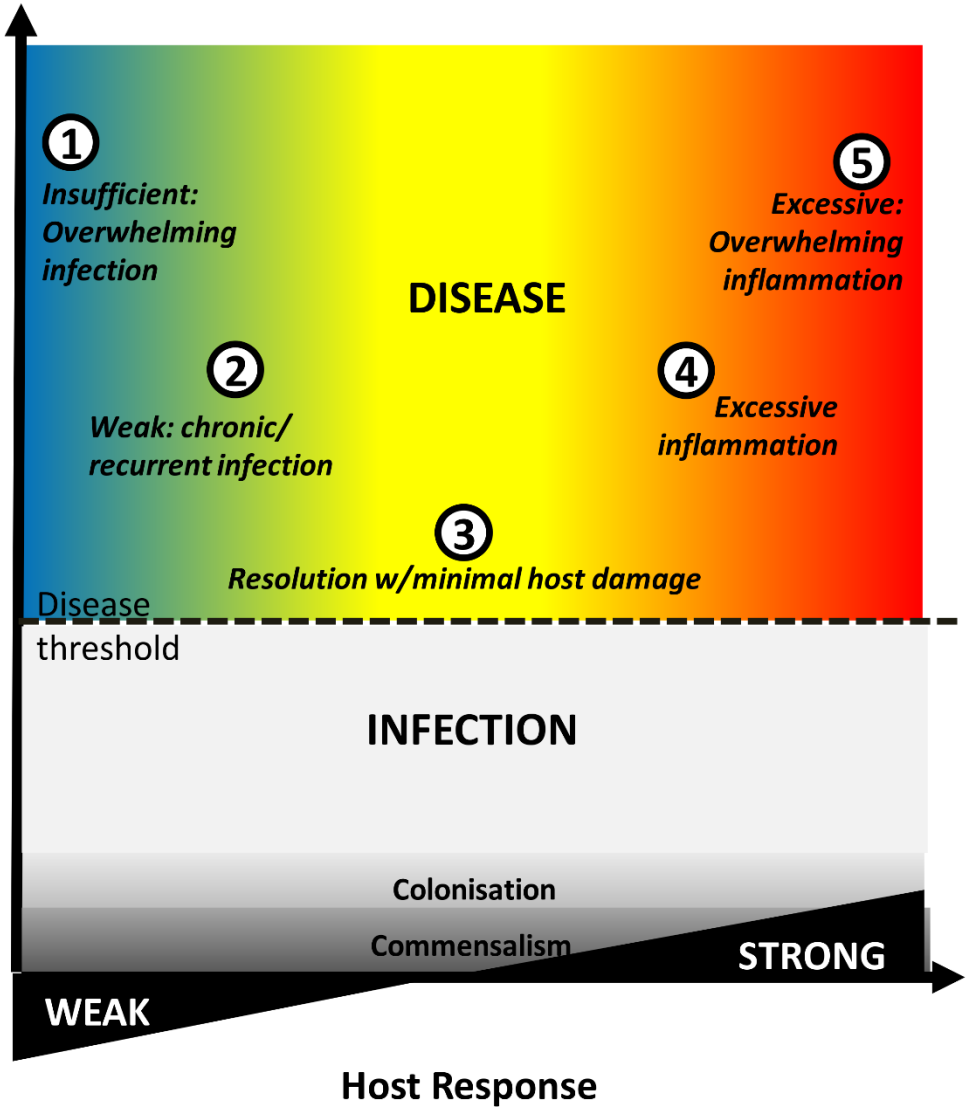

Figure 1.1. Host-pathogen interactions determine disease severity

Commensalism/colonisation occurs when host-microbe contact results in no apparent damage, though it can elicit an immune response. Infectious disease is a clinical manifestation of damage, which may be pathogen-mediated, host-mediated, or both. If immune response is insufficient, overwhelming infection may result (1). Where response is weak, recurrent or chronic infection occurs (2). An ideal response easily resolves infection with minimal host damage (3). A persistently dysregulated immune response leads to chronic inflammation (4). Finally, where host response is entirely excessive, overwhelming inflammation may cause death (5).
Modified from [5].

1.2. *Staphylococcus aureus*

1.2.1. Clinical Significance

Staphylococcus aureus is a species of Gram-positive, coagulase-positive bacteria. A major human pathogen, it causes diverse disease: superficial cutaneous infection (e.g., skin abscess, surgical site infections); systemic disease (e.g., bloodstream infection, endocarditis); serious deep-seated infection (e.g., osteomyelitis, septic arthritis, necrotising pneumonia, meningitis); and toxinoses (e.g., toxic shock syndrome, scalded skin syndrome and food poisoning). Cutaneous infection accounts for the majority of staphylococcal disease, but is rarely life-threatening. Serious invasive *S. aureus* infections, on the other hand, have an enormous impact.

S. aureus is one of the commonest, and the most lethal, causes of bloodstream infection (BSI) worldwide [6]. The incidence of *S. aureus* BSI (SA-BSI) in the UK (19.2 per 100,000) is much greater than that of HIV or tuberculosis, even though these infections receive much more attention and research funding [7]. The problem of serious invasive *S. aureus* infection is growing. In Ireland, between 2012 and 2017, SA-BSI cases increased by 11% (Fig. 1.2) [8]. SA-BSI also increased in Europe by 34% between 2002 and 2009 [9]. Further increases are almost guaranteed, given our ageing population, frequency of invasive procedures and reliance on implantable devices. *S. aureus* is also unusual for its propensity to cause infections among young, otherwise healthy people, without particular risk factors. Even with timely effective antimicrobial therapy and optimum supportive care, up to a third of patients die within a month of diagnosis [10]. This far exceeds the mortality of most other acute medical presentations including myocardial infarction, pulmonary embolism and stroke. Those at the extremes of age and with more complex co-morbidities have a risk of death greater than 50% [3]. *S. aureus* BSI is truly a major world health problem, responsible for a huge burden of global disease, disability and death. While *S. aureus* infection, particularly with antimicrobial-resistant isolates, ranks highly on the public health and research agendas in high-income countries, its profile in the developing world has been largely neglected. SA-BSI-attributable death in developing countries is more than double that seen in Europe, and disproportionately affects neonates [11-13].

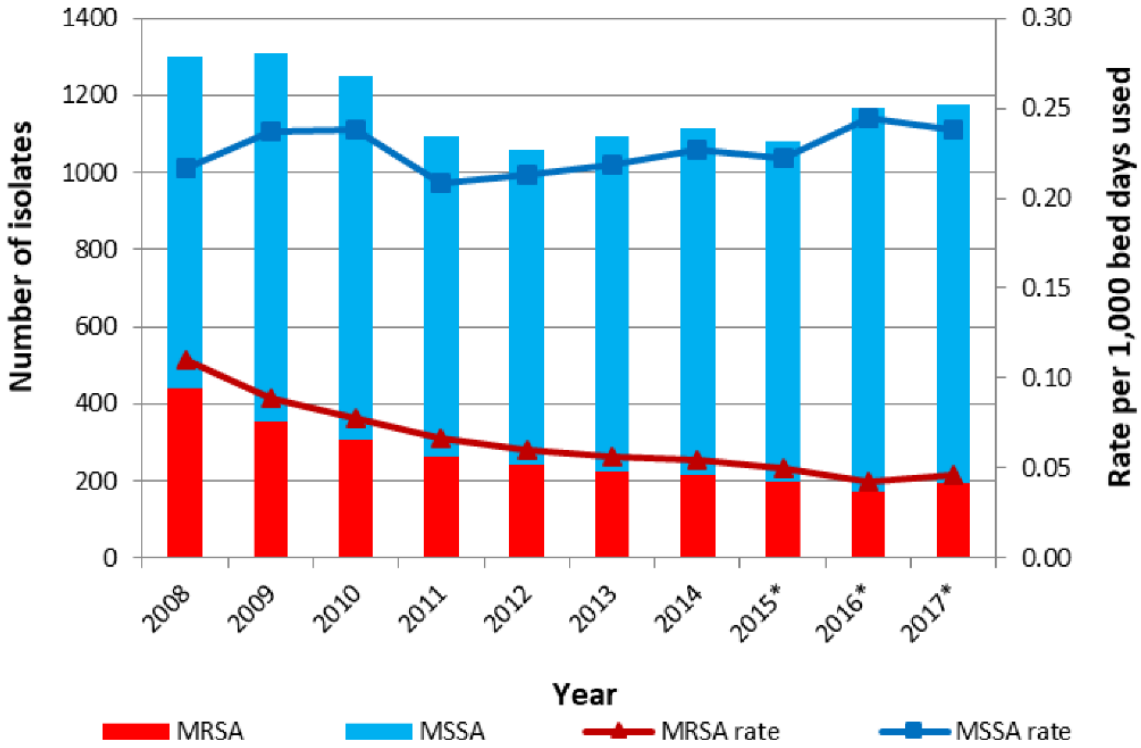

Figure 1.2. National *S. aureus* bloodstream infection rates (2008-2017) for Ireland including methicillin resistance rates

Number (columns) and rates (trend lines) for SA-BSI in Ireland demonstrate a decrease in MRSA (red) rates and slight increase in MSSA (blue) rates over a ten-year period. Figures from Health Protection Surveillance Centre Ireland [8]. MRSA = methicillin-resistant *S. aureus*; MSSA = methicillin-suseptible *S. aureus*.

1.2.2. Epidemiology

Age is a powerful determinant of invasive disease with the highest incidence of SA-BSI occurring at the extremes of life (Fig. 1.3). This is not unique to SA-BSI, but rather a universal finding among all bloodstream infections [14]. Age remains a predictor of increased *S. aureus*-attributable mortality even after correction for co-morbities and other variables [3,15]. Male gender is also consistently associated with increased rates for unknown reasons [16]. While some studies have sugggested females have a higher mortality due to hormonal or health-seeking behaviour differences, larger age-adjusted studies have not borne this out [3]. Lower socioeconomic status is also independently associated with increased incidence of SA-BSI, particularly CA-MRSA. Invasive infection is also seen more frequently among certain ethnicities (including African-Americans, indigenous Australians, Maoris) as compared to Caucasian populations [16]. This finding is not fully explained by socioeconomic disparities between ethnic groups and is not associated with an increased mortality. It may, however, reflect the much greater burden of staphylococcal and other skin disease among indigenous populations [17]. The genetic or immunological basis for any of these host susceptibility differences has not yet been investigated.

Cohorts at highest risk of *S. aureus* bloodstream infection (e.g., dialysis, HIV infection, diabetes mellitus, transplant, cancer) have several risk factors in common – recurrent skin breaks, implanted devices that facilitate biofilm persistence, and frequent healthcare contact [15,16,18]. Increased rates of *S. aureus* nasal colonisation, as well as impaired cell-mediated immunity are also evident in some of these groups [19-21]. The impact of persistent *S. aureus* colonisation on risk for infection and outcome is discussed below (see *1.2.3*). Most analyses of epidemiological risk factors are derived from retrospective observational cohorts of patients who have developed SA-BSI. Population-level prospective studies comparing those who develop SA-BSI directly with those who do not would potentially require thousands of participants and has not yet been attempted. What is known about particular immune dysfunctions which predispose to SA-BSI and correlates of natural immunity is further discussed below (see *1.3* and *1.5.2 Host immunodeficiency and susceptibility to* S. aureus *infection*) [5].

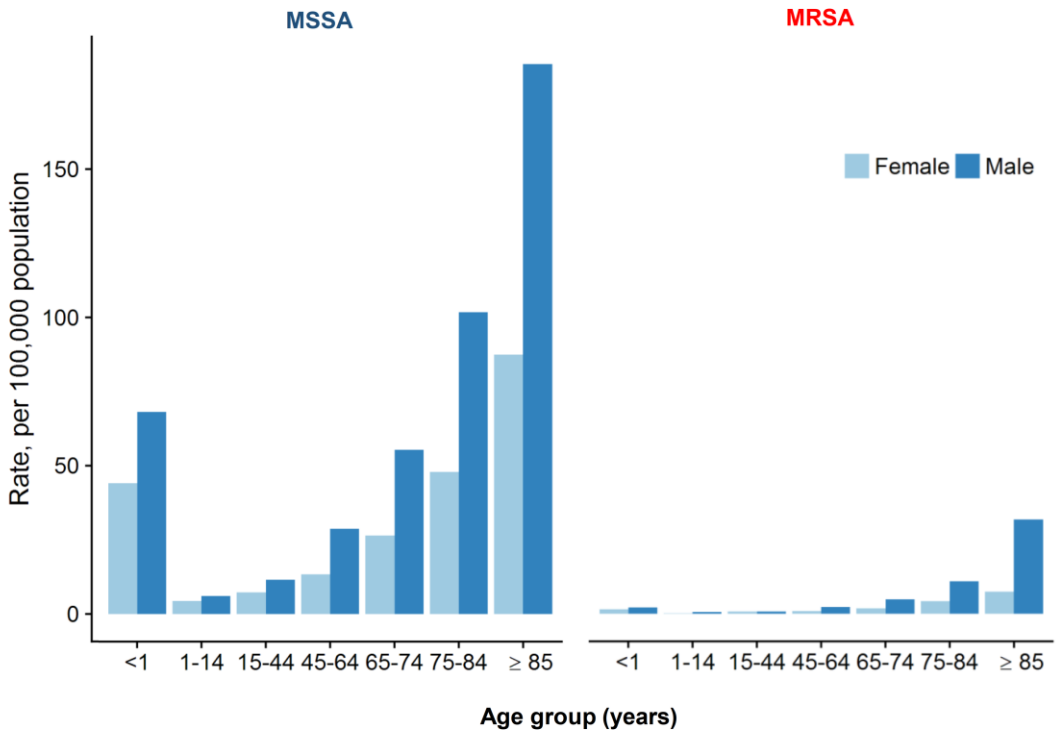

Figure 1.3. National *S. aureus* bloodstream infection rates (2017-2018) for England by age group and gender

Population rates by age group and gender for SA-BSI in England demonstrate the burden of MSSA disease as compared to MRSA infection, as well as an increased incidence in tnfants and older adults. There is also a consistently higher rate ratio for males to females. Figure adapted from Public Health England [22].
MRSA = methicillin-resistant *S. aureus*; MSSA = methicillin-suseptible *S. aureus*.

1.2.3. Colonisation

The primary *S. aureus* reservoir in humans is the anterior nares, although colonisation at other sites is common. Extra-nasal sites include skin, throat, perineum, vagina and gastrointestinal tract [23-25]. Approximately 20% of adults are persistently colonised with *S. aureus*, and the remainder intermittently carry fewer bacteria [26]. Certain populations tend to have higher rates of colonisation, e.g., almost all adults with atopic dermatitis are *S. aureus* carriers [27]. Defining differences between carrier and non-carrier groups has proved difficult. Particular strains of *S. aureus* may be superior colonisers, but predilection for *S. aureus* colonisation seems largely host-determined [19]. Experimental nasal inoculation results in most volunteers returning to their original non-carrier or persistent carrier state [26]. Nasal carriage of *S. aureus* is strongly associated with infection. Carriers have up to a 20-fold relative risk for bloodstream infection [28,29]. Despite this, the vast majority will never develop invasive disease.

Bacterial carriage may also 'prime' the immune system, changing its behaviour – either favourably or unfavourably – during subsequent infection [30-32]. Intriguingly, some data suggest that nasal carriage, while strongly predictive of disease, may be partially protective in invasive infection [30,33]. Among those who developed SA-BSI, carriers had lower all-cause and *S. aureus*-attributable mortality than non-carriers [30]. There were signiciant clinical confounders which may have influenced this difference in outcome – carriers were significantly younger, had fewer cardiac issues but were non-specifically 'immunocompromised'. It is possible that immunocompromise may have dampened detrimental excessive inflammation. Alternatively, a degree of immune tolerance to *S. aureus* may have created a better orchestrated response to invasive disease. Although the mechanism is not known, examining the immune imprint of *S. aureus* colonisation is critical to predicting and influencing response to infection and immunisation in future patients.

1.2.4. Transmission and Infection

Transmission of *S. aureus* occurs almost exclusively as a result of direct skin-to-skin contact, or contact with recently contaminated fomites [34,35]. Hand hygiene is therefore the most effective intervention at preventing spread, reducing transmission by almost 90% [36]. *S. aureus* infections usually arise from autoinfection of colonised individuals or, much less often, from cross-infection (transmission of a new *S. aureus* to an individual that results in

infection) [37]. Determinants of whether a new acquisition will end in eradication, colonisation or infection are unknown.

1.2.5. Genome

S. aureus population structure is largely clonal with infrequent spontaneous mutations giving rise to new lineages. The distribution of these lineages differs geographicallly. *S. aureus* has core and accessory genomes [38]. The core genome contains ~ 75% of genes that encode for essential proteins. Most are highly conserved, and thus a reliable method of inferring evolutionary relationships. 'Core variable' genes – often encoding regulators of virulence genes or surface proteins involved in host interactions – vary between lineages or may even be missing. The accessory genome is largely comprised of horizontally exchanged mobile genetic elements (fragments of DNA encoding toxins, virulence factors, antimicrobial resistance and genes involved in host adaptation).

1.2.6. Antimicrobial resistance (AMR)

The advent of anti-staphylococcal antibiotics revolutionised outcomes for patients with *S. aureus* BSI, reducing mortality by two-thirds [10,39]. This panacea was short-lived. Immense quantities of (often inappropriate) antimicrobials introduced into the clinical and wider environments since the 1940s applied enough selective pressure to bring resistance mechanisms already present in bacteria to the fore. This condensed the emergence and global spread of AMR in staphylococci into a remarkably short timeframe.

Within a decade of the introduction of penicillin, most *S. aureus* strains had acquired enzyme-mediated resistance (Fig. 1.4A). Methicillin (the first penicillinase-resistant penicillin) was introduced in 1961 to combat this problem. Methicillin-resistant *S. aureus* (MRSA) was first reported the same year, mediated by entry of the transferable resistance gene *mecA* into *S. aureus*, probably from a coagulase-negative staphylococcus [40]. Dissemination of a small number of epidemic clones account for the massive global spread of MRSA. Almost half of all *S. aureus* bloodstream isolates in US hospitals are now methicillin resistant [41]. While MRSA BSI proportions are falling across Europe overall, tremendous variation exists between countries due to differing antibiotic usage, infection control practices, health policy and funding (Fig 1.4B) [42]. In addition, MRSA infections, previously

confined to hospitals, are now acquired in the community. Community-acquired MRSA (CA-MRSA) was first described in remote indigenous communities in Western Australia as genetically and phenotypically distinct from healthcare-associated strains, but is now prevalent in many parts of the world [43]. MRSA has added to, rather than replaced, the overall burden of SA-BSI. The problem of beta-lactam resistance is further amplified by *S. aureus* having also demonstrated resistance to every other current anti-staphylococcal drug [40,44,45].

While the antibiotic era was infamously heralded a "*time to close the book on infectious diseases, and declare the war against pestilence won*", the World Health Organisation now describes antimicrobial resistance (AMR) as "*far from being an apocalyptic fantasy… instead a very real possibility for the 21st century*" [46,47]. AMR is a grave challenge to treatment of life-threatening *S. aureus* infections. The World Economic Forum warns that *"a post-antibiotic era means, in effect, an end to modern medicine as we know it"* [48]. The outlook for the future is discouraging given the lack of new classes of antimicrobial agents in the drug discovery pipeline. Development of novel anti-staphylococcal antimicrobials is a lengthy and costly process. Even with recent incentives to support drug discovery, new therapies will have a finite period of utility, limited by the rapid emergence of resistance. This approach is an attempt to win a race that likely cannot be won. As we enter the post-antibiotic era, it is clear that strategies to prevent *S. aureus* disease (regardless of antibiogram) are preferable to chasing diminishing curative options.

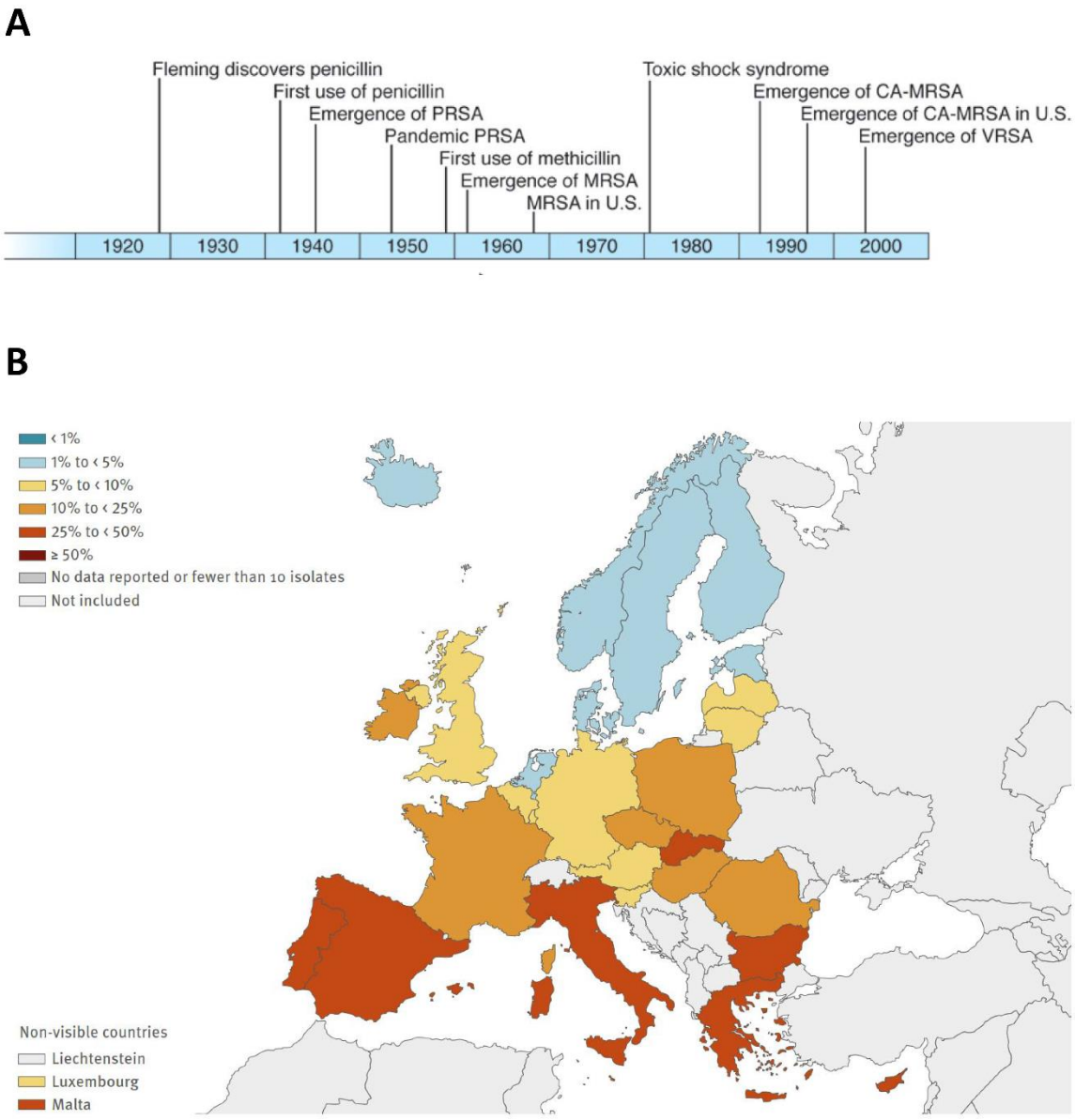

Figure 1.4. Emergence of antibiotic-resistant *S. aureus* and variation in MRSA rates across European countries.

Timeline of the emergence of penicillin, methicillin-, and vancomycin resistant *S. aureus* in the USA (A) [49]. Proportion of invasive *S. aureus* isolates with resistance to methicillin (MRSA) by EU/EEA country 2017 (B). Data from European Centre for Disease Prevention and Control Surveillance Atlas (Antimicrobial Resistance). Available at https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc. [Accessed Oct 2018]

PRSA = penicillin-resistant S. aureus*; MRSA = methicillin-resistant* S. aureus*; VRSA = vancomycin-resistant* S. aureus*; CA = community-acquired*

1.2.7. Virulence Factors

S. aureus's arsenal of pathogenic weapons is extensive: secreted toxins and enzymes, surface-associated adhesins and capsular polysaccharides. Many of these are considered 'virulence factors' (a term derived from the Latin *'virulentus'* meaning 'full of poison'), and increase pathogenesis in animal models. *S. aureus* can also receive mobile DNA, prophages and plasmids that encode for additional pathogenic factors. It produces a variety of potent cytotoxins [e.g., haemolysin-γ (Hlg), Panton-Valentine leukocidin (PVL) and others] that directly lyse cells by forming pores in their membranes [50,51]. Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) are adhesin proteins which mediate attachment of bacteria to host tissue [e.g., clumping factor A (ClfA), fibronectin binding protein A (FnbpA)], providing a critical step to establish infection. While there is extensive knowledge about the activity of individual virulence factors in *vitro* and in animal models, their relative importance in the pathogenesis of clinical infection is, in fact, not well established. Just as no single virulence factor is sufficient to recapitulate a *S. aureus* infection, neutralisation of a single factor is unlikely to disarm the pathogen. Many 'virulence' factors not only directly damage human cells, but also impair or subvert host pathways designed to clear infection (see *1.5.1)*.

1.3. Immune response to *S. aureus* infection

The immune system comprises cells and molecules responsible for protection against infection. It can be divided into two arms which work in synergy. The innate immune system provides an immediate, non-specific response to neutralise or eliminate a pathogen. The adaptive immune system provides a delayed acquired response to specific pathogen elements that, crucially, can form immunological memory. Memory results in faster and more precise attacks on subsequent encounters with the same pathogen, and forms the basis of immunisation.

1.3.1. Innate Immune Response to *S. aureus*

Recognition and initial inflammatory response

Physical barriers (skin or mucous membranes), broad-spectrum antimicrobial peptides (AMPs) and resident microflora are first-line non-specific defences. Intact skin is the most important of these in preventing *S. aureus* infection. Skin trauma (e.g., surgical incision, transcutaneous intravenous catheters) is consistently associated with *S. aureus* infection, and breaches in the skin barrier often precede invasive disease [15,52]. Once general defences are overcome, bacteria are detected via a diverse series of host molecules known as **p**attern **r**ecognition **r**eceptors (PRRs), constitutively expressed on innate immune cells, which recognise highly conserved **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMPs) [53]. A number of PRR families have been identified – Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and RIG-1 like receptors (RLRs). While innate recognition of *S. aureus* is not comprehensively understood, activation of TLR2 by staphylococcal lipoproteins and polysaccharide fragments is probably involved [54]. PAMP:PRR ligation promotes transcription of genes for antimicrobial peptides, cytokines and chemokines to mobilise innate immunity to sites of infection. The initial inflammatory response triggered by pathogen recognition is characterised by the Latin descriptor 'rubor, dolor, calor, tumor' – redness, pain, heat and swelling – resulting from an influx of fluid and cells into infected tissues. This initial signalling also activates the complement system and generates cytokines that begin to activate the adaptive immune system.

Phagocytosis and Antigen Presentation

The professional phagocytes – neutrophils, macrophages and dendritic cells (Fig. 1.5) – are richly endowed with PRRs. They are the most important effector cells of the innate immune system. Phagocytes utilise chemical cocktails to kill engulfed micro-organisms. Dead or dying neutrophils are the major component of the pus so characteristic of *S. aureus* infection. Neutrophils are equipped with three major methods of killing bacteria – production of reactive oxygen species (ROS), release of packaged enzymes via degranulation and NETosis [55,56]. Monocytes leave the circulation to mature into macrophages, the major tissue-resident phagocytes. Activated macrophages act as scavengers, ingesting pathogens and dying cells, to clear infection and limit inadvertent tissue damage [57]. Although neutrophils are conventionally thought of as the primary protective phagocyte in *S. aureus* infection, human macrophages show comparable intracellular killing of *S. aureus* [58]. Phagocytes recruit other inflammatory cells via chemokine signalling. Many are also professional antigen-presenting cells (APCs) that serve as the crucial link between innate and adaptive immunity. These migrate to regional lymph nodes and present fragments of digested bacteria (peptide:MHC complexes) along with co-stimulatory molecules to expand and polarise antigen-specific T lymphocytes and trigger adaptive immunity (Fig 1.6). Dendritic cells (DCs) are the primary APCs of the immune system. A new antigenic challenge cannot induce a T cell response without DC involvement.

Clinical and experimental data supports an important role for phagocytes in defence against *S. aureus* infection. Surprisingly, SA-BSI is actually less common in neutropaenic patients [59]. However, functional neutrophil deficiency is probably more relevant. In inherited leukocyte adhesion deficiencies, neutrophils are unable to migrate out of blood vessels to the site of infection [60]. Chronic granulomatous disease (CGD) is characterised by defective generation of ROS [61]. Neutrophils from CGD patients successfully phagocytose *S. aureus*, but are unable to achieve killing. Inherited macrophage disorders are overwhelmingly typified by susceptibility to mycobacterial infection, although *S. aureus* infections are also observed [62]. Experimental data is uncovering an increasing role for macrophages in *S. aureus* infection. Depletion of macrophages prior to systemic *S. aureus* challenge was lethal in 50% of animals [32]. Depletion of murine DCs before intravenous *S. aureus* challenge increased bacterial burden and accelerated mortality; whereas adoptive transfer of DCs improved control of infection [63].

In summary, the innate immune system recognises *S. aureus*, actively attempts to clear infection and facilitates activation of *S. aureus*-specific adaptive immunity .

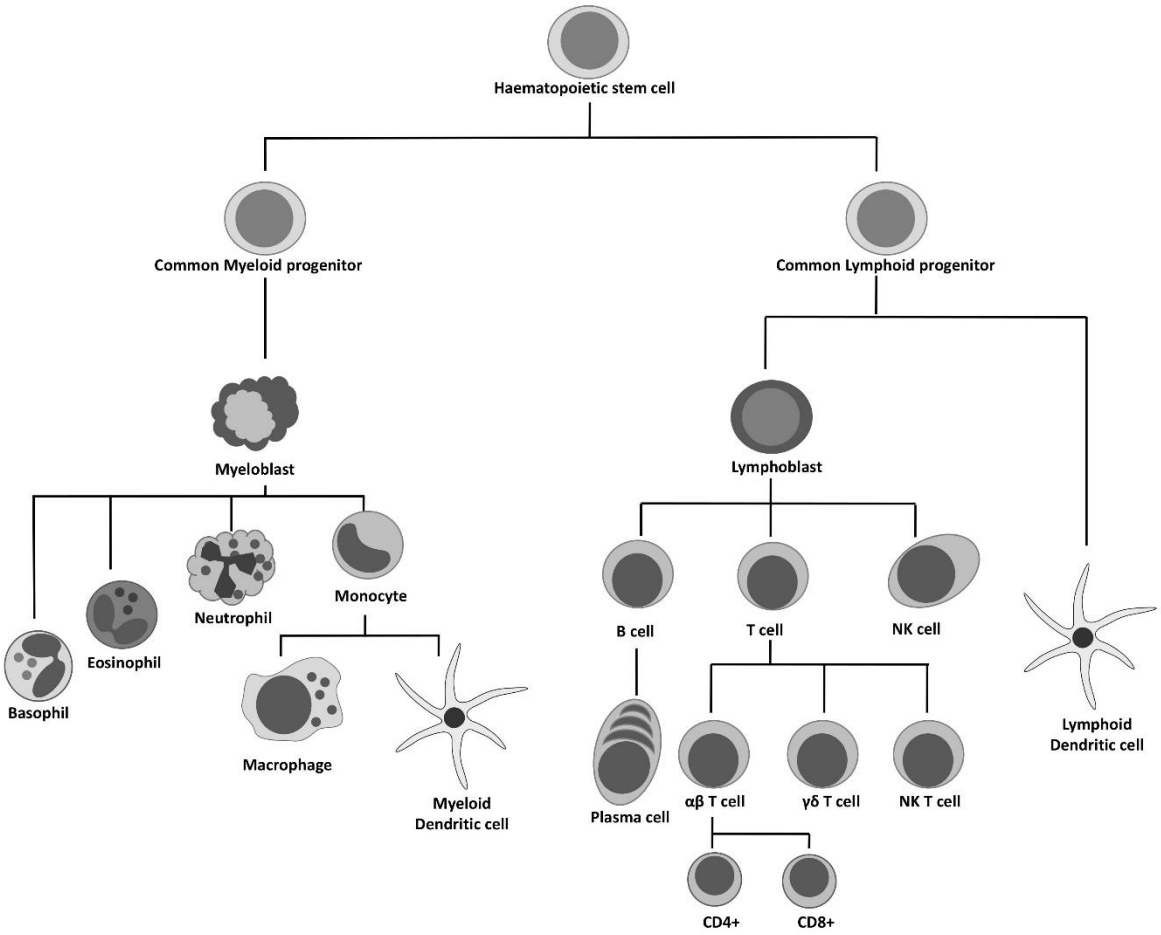

Figure 1.5. Immune cell lineage.

Immune cells are derived from haematopoietic stem cells. Myeloid cells comprise the cellular component of the innate immune system. They contain microbicidal substances to directly kill microbes, and some act as phagocytes and antigen presenting cells. Lymphoid cells comprise the cellular component of the adaptive immune system. B cells mature in the bone marrow, T cells in the thymus and NK cells in the bone marrow, lymph nodes, spleen or thymus. Once activated, B cells differentiate into antibody-producing effector cells known as plasma cells. T cells can differentiate into αβ, γδ or NK T cells, which carry out a diverse range of effector functions, often by secreting cytokines to direct the immune response.

1.3.2. Adaptive immune response to *S. aureus* infection

The adaptive immune system is essential for eliminating pathogens that have not been cleared by more primitive defences. It constructs receptors specific to individual pathogen elements, allowing them to be targeted with much greater precision and, critically, generating memory. The antigen-specific cells formed during primary infection persist as memory cells that react more readily and effectively on re-infection. It comprises humoral (antibodies) and cellular (lymphocytes) components.

1.3.2.1. B lymphocytes and S. aureus *infection*

B lymphocytes recognise extracellular antigens and become efficient antibody-producing cells, with assistance from helper T lymphocytes. Antibodies may have neutralising or opsonising functions. Immunoglobulin (Ig) M is the first class of antibody produced, followed by IgG. IgG-opsonised bacteria engage phagocyte receptors to mediate phagocytosis. B cell cytokines also polarise T helper cells towards specific functions (Fig. 1.7).

There is little evidence that B cell deficiency states in humans (or in mice) result in greater incidence or severity of invasive *S. aureus* disease [15,64]. Healthy children and adults already have a multitude of anti-*S. aureus* antibodies [65]. During *S. aureus* infection, B cells readily generate antibodies against multiple *S. aureus* components. These antibodies appear functional and potentiate opsonophagocytosis *in vitro*. However, it has proved difficult to consistently correlate presence or titre of anti-staphylococcal antibodies with improved clinical outcomes [66-68]. The role of B cells in defence against *S. aureus* is further complicated by staphylococcal protein A, which so effectively disables the humoral immune response during infection (see *1.5.1*). Finally, neither passive immunisation, nor active immunisation with vaccines that successfully produced robust humoral responses, have prevented or attenuated the course of infection in clinical trials (Table 1.1).

1.3.2.2. T Lymphocytes and *S. aureus* infection

T cells are identified by the surface marker CD3, and are categorised on the basis of T cell receptor (TCR; e.g., αβ or γδ). Intravenous *S. aureus* challenge in T cell knockout mice is rapidly fatal [64].

1.3.2.2.1. αβ T lymphocytes

Conventional (αβ) T cells comprise the majority of circulating lymphocytes. CD4 and CD8 co-receptors further categorise αβ T cells that play distinct roles in infection. $CD4^+$

lymphocytes are helper cells which produce cytokines to promote other immune cell functions. $CD8^+$ cells can directly kill compromised or infected cells. T cell response is orchestrated by the APCs of the innate immune system by three signals – processing and presenting of antigens (signal 1), co-stimulation (signal 2), and secretion of cytokines to direct lymphocyte differentiation (signal 3) (Fig. 1.6). Subsets of T helper cells and their roles in *S. aureus* infection are reviewed below.

$CD4^+$ T cells and S. aureus

The primary function of $CD4^+$ T cells is to produce cytokines to help other immune cells. $CD4^+$ subsets are characterised by expression of specific transcription factors, signal transduction pathways and cytokine secretion profiles (Fig. 1.7).

Th1 cells

Th1 cells produce IFNγ as their signature cytokine. IFNγ activates macrophage phagocytosis and ROS formation. It enhances antigen presentation by upregulating expression of MHC. Finally, IFNγ promotes immunoglobulin switching to IgG1/3, which bind avidly to phagocyte Fc receptors and activate opsonophagocytosis [69]. There is accumulating clinical and experimental evidence that Th1 cells play important roles in protection against *S. aureus*, particularly in systemic infection. Patients with acquired impairment of Th1 function (e.g., HIV infection, end-stage renal disease and diabetes mellitus) have the greatest relative risk for *S. aureus* bloodstream infection [18,20,21]. Murine studies also imply a protective role for these cells. Th1-skewed C57BL/6 mice were significantly more likely than Th2-skewed Balb/c to survive i.v. inoculation [70]. Adoptive transfer of *S. aureus*-specific Th1 cells led to accelerated clearance of bacteria [32]. Th1 cells are a dominant source of IFNγ, and exogenous IFNγ administration increased phagocytosis, bacterial clearance and survival in systemic infection [70,71]. Deficiency in IFNγ or its receptor was associated with worse survival [72,73]. Th1 response seems most beneficial early in the course of systemic infection, by aiding phagocytic clearance of extracellular *S. aureus*, and activating phagocyte killing of intracellular staphylococci.

The roles of Th1 cells and IFNγ are less clear in non-systemic infection, where they may even be detrimental. Th1-derived IFNγ in skin infection promoted a neutrophil reservoir in which *S. aureus* could persist intracellularly [74]. Thus, IFNγ may have different roles depending on

the site and stage of infection, and needs regulation to avoid counterproductive inflammation (see *1.4. Immune Regulation*).

Th2 cells

Th2 cells produce IL-4 as their signature cytokine. They promote B lymphocyte proliferation, antibody production, isotype switching to IgG2/4 and IgE, and activation of eosinophils to control large extracellular pathogens like helminths and other parasites. Th1 and Th2 lymphocytes are antagonistic. Th1-derived IFNγ directly suppresses IL-4 secretion, inhibiting differentiation of naïve T cells into Th2 cells. Th2-derived IL-4 suppresses secretion of IL-12 and IFNγ, blocking Th1 development [69]. Th2 cells drive antibody production in *S. aureus* infection.

Th17 cells

Th17 cells produce IL-17 as their signature cytokine and are important in mucosal protection against extracellular bacterial and fungal infections [75]. IL-17 recruits neutrophils to sites of infection. It may prevent haematogenous dissemination from mucosal *S. aureus* infection [76]. Mice deficient in IL-17A/F are more susceptible to mucocutaneous, but not to systemic, *S. aureus* infection than wild type mice [77,78]. Adoptive T cell transfer from Th1-deficient *S. aureus*-exposed mice lessened severity of *S. aureus* skin infection – an effect thought due to transfer of IL-17-producing T cells. Blocking of IL-17 abrogated this protection [79]. Gamma-delta T cells (discussed below) seem the predominant source of IL-17 in mice [31,80,81].

Data from the clinic supports the hypothesis that IL-17-producing T cells are essential to mucocutaneous immunity, although $CD4^+$ (Th17), not $\gamma\delta^+$, T cells are the primary source. Patients with the rare autosomal dominant hyper-IgE syndrome (AD-HIES) are prone to recurrent staphylococcal skin and lung abscesses [82]. Mutations in their signal transducer and activator of transcription 3 (STAT3) genes result in impaired Th17 cell development [83,84]. AD-HIES patients are not more prone to *S. aureus* bloodstream infection, supporting evidence that IL-17 response is critically important at skin and respiratory sites only [85]. Chronic mucocutaneous candidiasis (CMC) is a syndrome characterised by recurrent mucocutaneous *S. aureus* and *C. albicans* infections. A spectrum of inborn errors of IL-17 function (impaired Th17 differentiation, deficient IL-17F production or absence of IL-17 receptor) have been described in CMC patients [86,87].

CD8⁺ T cells and S. aureus

$CD8^+$ T cells are cytotoxic T lymphocytes (CTLs) that interact with MHC I molecules on the surface of compromised host cells, such as those infected or transformed by malignancy. CTLs produce cytokines (e.g., IFNγ and TNFα) and/or release of cytotoxic granules to stimulate apoptosis. CTLs are particularly effective in defence against viruses, but also play a role in some, particularly intracellular, bacterial infections (e.g., *Mycobacterium tuberculosis*). $CD8^+$ T cells have not been demonstrated to play a significant role in defence against *S. aureus* infection. Adoptive transfer of vaccine-induced *S. aureus*-specific $CD8^+$ T cells did not demonstrate protection in murine experiments and a CTL response was not generated to any of four *S. aureus* antigens in a human vaccine study [88,89] .

1.3.2.2.2. Gamma-delta (γδ) T cells and *S. aureus*

Gamma delta T cells are at a crossroads between adaptive and innate cells, and express TCRs composed of γ and δ chains. They are a minority population in the blood, but are abundant at epithelial sites, where they contribute to mucosal immunity. In addition to their γδ TCR, they can also be activated by PRR ligation, cytokine stimulation and/or specific antigens [90]. Gamma-delta cells can be directly cytotoxic, or may act as phagocytes, cytokine-secretors or APCs. There are three major human γδ subsets. (Vγ9) Vδ1 cells are primarily distributed in skin and mucosa, Vδ2 in peripheral blood, and Vδ3 are a scanty population found in gut and liver. Human γδ cells have significant plasticity, and can readily assume Th1-, Th2-, Th17- or Treg-like functions [91].

Staphylococci can activate murine $\gamma\delta^+$ cells. In cutaneous and pulmonary *S. aureus* infection, $\gamma\delta^+$ cell IL-17 production is important in early containment and clearance of staphylococci, presumably by recruiting and activating neutrophils [81,92]. In a peritonitis/systemic infection model, $\gamma\delta^+$ lymphocytes producing IL-17 (but not IFNγ) accelerated bacterial clearance, and adoptive transfer of these *S. aureus*-primed cells was similarly protective [31]. This IL-$17^+\gamma\delta^+$ signal has only been described animals. Murine γδ subsets are not directly analogous to human subsets, which limits the utility of comparative studies (e.g., $V\delta2^+$ cells are only found in primates) [93]. Human $V\delta2^+$ cells were not activated by supernatants from neutrophils that had phagocytosed *S. aureus* [94]. Interestingly, transfer of pamidronate-activated $V\delta2^+$ cells improved bacterial clearance and survival in humanised mice after i.v. *S. aureus* challenge – effects associated with IFNγ production [95]. Thus, γδ protection may not

be truly pathogen-specific but rather related to global γδ activation. No study has attempted to characterise $\gamma\delta^+$ T cells in human *S. aureus* infection.

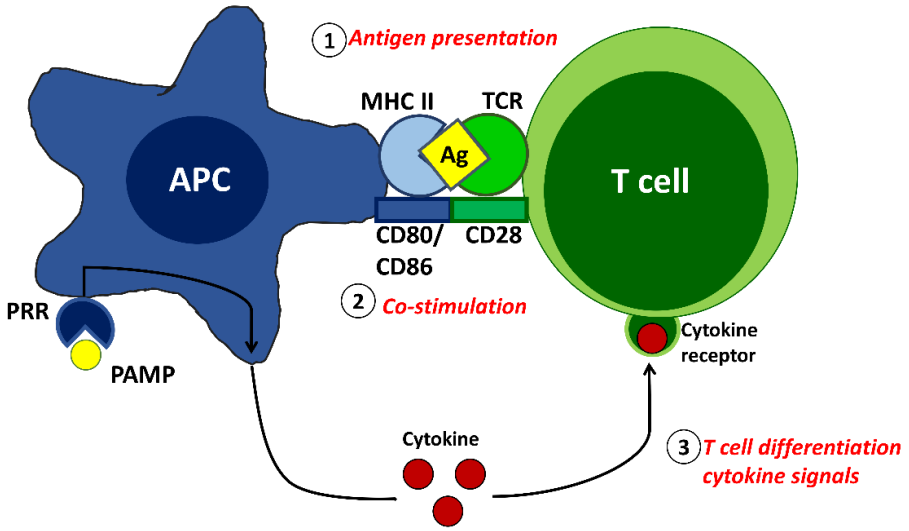

Figure 1.6. T lymphocyte activation.

Complete activation of naïve T lymphocytes requires 3 signals delivered by the APC. Signal 1 (antigen presentation) is delivered via the TCR when it binds a matching peptide–MHC complex on the APC surface. MHC class I molecules are constitutively expressed by all nucleated cells, and report on intracellular events, such as infection, damage or malignant transformation. Surface peptide:MHC I complexes act as alarm 'flags' or 'self-destruct' signals to attract and direct $CD8^+$ cytotoxic T cells (CTLs) to destroy compromised cells. Expression of MHC II molecules is restricted to professional APCs. MHC II presents processed pathogen fragments to $CD4^+$ helper T cells (Th). Signal 2 (co-stimulation) is an accessory signal (typically CD28:CD80/CD86 binding) that induces clonal expansion. Signal 3 (T cell differentiation) comprises the cytokine signals delivered from APC (principally DCs) to T cell that determine its differentiation into an effector cell subset (e.g., Th1, Th2, Th17, CTL).

APC = antigen-presenting cell; TCR = T cell receptor; MHC = major histocompatibility complex; CD = cluster of differentiation; Th = $CD4^+$ T helper cell; CTL = cytotoxic T lymphocyte; DC = dendritic cell.

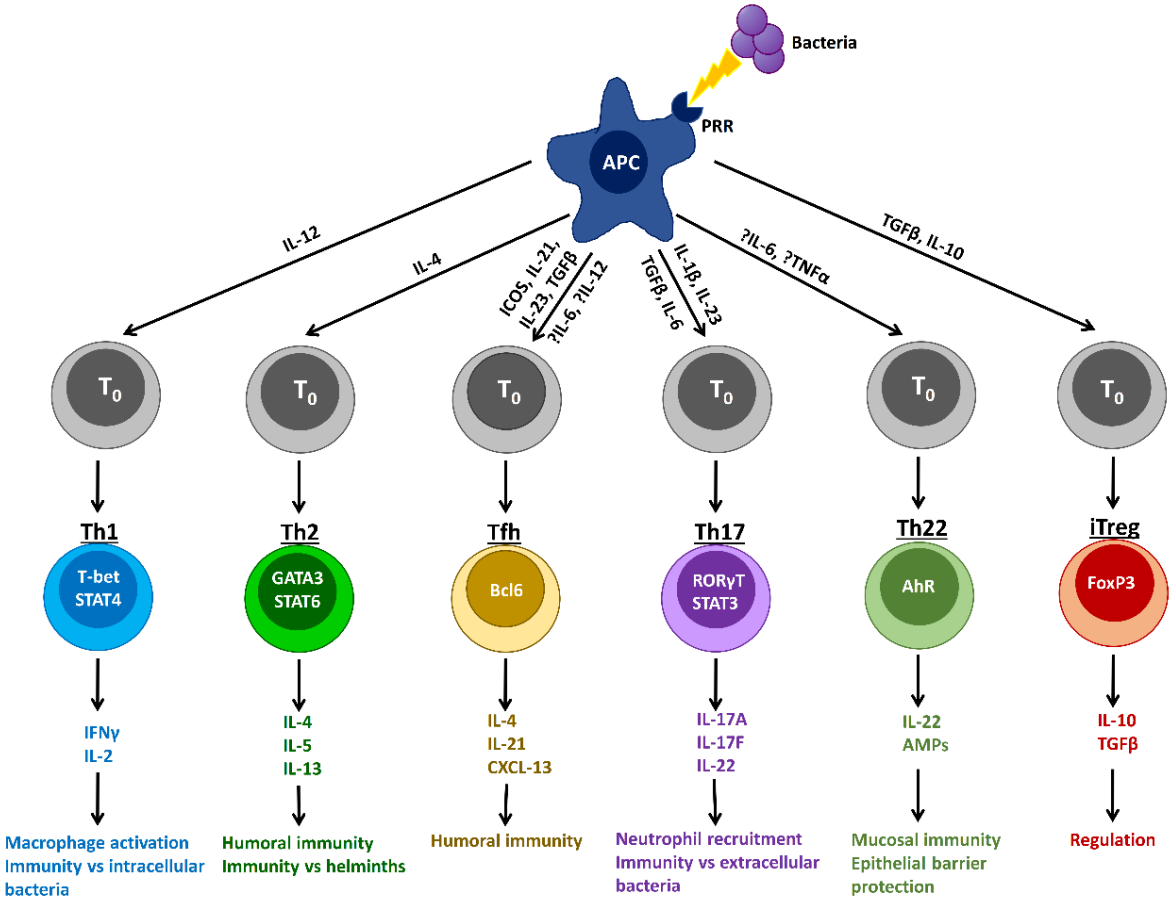

Figure 1.7. CD4⁺ T cell differentiation depends on the cytokine environment (Signal 3)
Following antigen presentation by the antigen-presenting cell, naive CD4⁺ T cell precursor cells can differentiate into different effector T cells: T helper (Th) 1, Th2, Th17, or Th22 cells, T follicular helper cells (Tfh) or inducible T regulatory T cells (iTregs). Subset differentiation is driven by selective cytokines and transcription factors. Each subset produces signature cytokines and carries out specialised immune functions during the inflammatory response to pathogens and/or resolution of inflammation.

1.4. Immune Regulation in *S. aureus* infection

While the immune response is critical to pathogen clearance and host survival, it must also be under tight control to limit inflammatory collateral damage. The ability of immunity to self-regulate is essential, and accomplished by a repertoire of innate and adaptive regulatory cells: tolerogenic DCs, alternatively activated (M2) macrophages, myeloid suppressor dendritic cells (MDSCs), regulatory T (Tregs) and B lymphocytes (Bregs) [96-98]. Their mechanisms include direct cell-contact and production of anti-inflammatory cytokines (e.g., IL-10 and TGFβ). *S. aureus* can facilitate its own survival by inhibiting expansion of pro-inflammatory cells [99]. IL-10 was beneficial in localised *S. aureus* infection by constraining excessive inflammation and tissue destruction, without affecting bacterial clearance [100,101]. However, in systemic infection, limiting inflammation may be detrimental. During persistent intravenous challenge, murine T cells became irreversibly hyporesponsive (anergic) to staphylococcal antigens, largely due to the inhibitory effect of MDSCs [102,103].

The effect of immune regulation on human *S. aureus* bloodstream infection is unknown, although early elevated serum IL-10 levels are associated with higher mortality [104]. A predominantly immunomodulatory response in the bloodstream may explain the tendency for *S. aureus* BSI to persist and establish deep-seated or metastatic infection. The optimal balance between pro- and anti-inflammatory responses for *S. aureus* is unknown, but likely to be tailored to the site of infection.

1.5. Why Immunity Fails

1.5.1. Immune Evasion by *S. aureus*

Many virulence factors not only directly damage human cells (see *1.2.7*), but also impair or subvert host pathways designed to clear infection. *S. aureus* is a master manipulator of both innate and adaptive immunity, and its primary danger may lie in this 'anti-immunity'. A striking feature is that *S. aureus* often has multiple mechanisms to subvert the same defence.

S. aureus evades innate defences by lysing immune cells with toxins, avoiding phagocytic killing, and persisting in metabolically inactive biofilms. Ideally, neutrophils ingest and kill bacteria before undergoing apoptosis to be cleared by macrophages. *S. aureus* subverts this at multiple steps. Up to half of phagocytosed *S. aureus* can survive and replicate in neutrophils, macrophages or dendritic cells, and later orchestrate cell lysis to ensure bacterial release [58,63,105]. Viable bacteria may then be trafficked to distant sites inside these 'Trojan horse' phagocytes to cause metastatic infection [106]. Staphylococci may also invade and persist in non-immune cells, particularly vascular endothelial cells [107]. Intracellular survival protects the organism from host defence and antimicrobial drugs, and has been proposed as a major contributor to metastatic disease and persistent or recrudescent infection requiring prolonged therapy. The particular problem of *S. aureus* biofilms deserves special mention. Staphylococci in biofilms are buried in matrix, making them inaccessible for phagocytosis and other host immune responses. Conventional antibiotic therapy has little activity against quiescent non-dividing *S. aureus* in biofilm phase. In device-related infections, biofilms pose such a significant problem they usually require device removal to achieve cure.

S. aureus also thwarts development of an effective adaptive response, or eliminates such responses once they have formed. Its most noteworthy weapons are worthy of special mention – staphylococcal protein A (SpA) which targets B lymphocytes, and superantigens which target T lymphocytes. Staphylococcal protein A disables antibodies formed against the bacteria during infection. Cell wall-anchored SpA binds to Fcγ domains of human IgG, coating the bacteria in incorrectly-orientated antibodies to inhibit opsonophagocytosis [108]. Free SpA is also released from the cell surface to capture Fcγ portions of circulating IgG in the same way. SpA binds up to 30% of B cell-anchored IgM antibodies in a non-antigen-specific manner, creating an immunodominance leading to activation, expansion, anergy and ultimately apoptosis of B cells. During *S. aureus* infection, the B cell pool is depleted and

completely unresponsive to staphylococcal antigens other than SpA itself [109,110]. Neutralisation of SpA would enable an effective humoral response to many staphylococcal antigens, and is a target for future vaccines, but wild-type SpA is not a suitable vaccine component due to its B cell superantigen effect.

Superantigens (SAgs) are secreted toxins which activate T lymphocytes in a pathological way, independent of antigen processing and MHC-dependent presentation. They can activate 10-30% of the T lymphocyte pool by directly cross-linking the TCR and MHC II. This precipitates a massive polyclonal proliferation and release of pro-inflammatory cytokines. The primary function of staphylococcal SAgs may be to prevent normal development of *S. aureus* antigen-specific effector and memory T cells.

1.5.2. Host immunodeficiency and susceptibility to *S. aureus* infection

Immunodeficiency is a congenital or acquired quantitative or qualitative deficiency of element(s) of the immune response. Certain immunodeficiencies are associated with increased acquisition or severity of *S. aureus* infection, and may give clues as to which arms of the immune system to target for future immunisation and therapeutic strategies. While much research focus to date has been on pathogen attributes, there is compelling clinical evidence that the outcome of human *S. aureus* infection may, in fact, be more dependent on host than microbial factors [3,4,111].

Human genome-wide screening has failed to identify polymorphisms specifically associated with acquisition, or severity, of *S. aureus* bloodstream infection [112]. The most crucial defence is the skin. Some atopic dermatitis (AD) patients have reduced antimicrobial peptide (AMP) activity [113]. Rare but classical inborn errors in the phagocyte:T helper cell axis are associated with increased risk of *S. aureus* infection and have been discussed in greater detail above (see *1.3.1* and *1.3.2*). Briefly, impaired neutrophil chemotaxis (leukocyte adhesion deficiencies) or functional phagocyte deficiencies (e.g., chronic granulomatous disease, Chédiak-Higashi syndrome) lead to increased incidence of skin or respiratory *S. aureus* infections. Congenital errors in Th17 cells – as seen in the rare autosomal dominant hyper-IgE syndrome (AD-HIES) – have been implicated in cutaneous and mucosal immunity to *S. aureus*. Conversely, the role of antibodies in protection against *S. aureus* is much less convincing (see *1.3.2.1*). B cell deficiency is not associated with increased incidence or severity of invasive *S. aureus* disease [15]. Anti-*S. aureus* antibodies are widespread and functional

(i.e., potentiate opsonophagocytosis) in the healthy population, but boosting their titres has not prevented or attenuated the course of infection [65-68].

While congenital immunodeficiencies are more clearly defined, acquired immmunodeficiency and other risk factors overwhelmingly predominate in clinical practice. Male patients and those at the extremes of age experience most infections, while older age and cumulative co-morbidities are associated with the worst outcomes [3,13]. The greatest risk factor for *S. aureus* infection is undoubtedly persistent nasal carriage, and invasion of the colonising strain accounts for most infections [30,37]. Higher colonisation rates are seen in atopic dermatitis, diabetic, dialysis-dependent patients and intravenous drug users [29]. Host factors determining colonisation are unknown, although polymorphisms in genes encoding for IL-4, mannose-binding lectin, TLR2, C-reactive protein and AMP genes have all been associated with nasal carriage [19]. Those at highest risk of *S. aureus* bloodstream infection (e.g., dialysis, HIV infection, diabetes mellitus, transplant, cancer) have recurrent skin breaks, implanted devices that facilitate biofilm persistence and frequent healthcare contact in common, as well as impaired cell-mediated immunity [15,18,20,21]. These risk groups are becoming more prevalent with advances in healthcare.

1.6. Immunisation and Immune Memory

Immunisation is the administration of specific, but relatively harmless, antigenic components to induce protective immunity against an infectious agent. It is one of the greatest triumphs of modern medicine, and a single human disease (smallpox) has been eradicated in this way. Throughout the mid-20th century, it led to widespread control of diphtheria, tetanus, pertussis, *Haemophilus influenzae* type b, poliomyelitis, measles, mumps, rubella and typhoid. In the past 30 years, significant reductions in death and disease attributable to meningococcal and pneumococcal disease, hepatitis A and B, influenza and rotavirus have resulted from vaccine development. Finally, vaccines against oncogenic viruses (hepatitis B and human papilloma virus) have heralded a new era of immunisation to prevent cancer.

Effective vaccines for many diseases remain elusive. Pathogen factors such as antigenic drift, serotype variability, redundancy of virulence factors and immune evasion pose ongoing challenges. Human factors, such as immunocompromise due to extremes of age or medical therapy remain difficult barriers. In addition, there is an ongoing battle for public opinion to keep vaccination rates sufficiently high to maintain population-level protection. Vaccinology has also been challenged by organisms which do not produce disease via a single mechanism (e.g., toxin that can be easily neutralised), which persist intracellularly, or where natural infection does not produce sterile immunity. The failure to develop vaccines against diseases such as human immunodeficiency virus (HIV) and malaria despite decades of effort has underscored the need to better understand mechanisms of protective immunity. It is now clear that the immune system has evolved different responses to protect against different pathogens, and these are informing the movement from empiric to rational vaccine design.

Most vaccines were developed empirically, with little understanding of mechanisms of immune protection. Extant vaccines surreptitiously replicated the sterile protection induced by natural infection by producing plasma cells clones to make neutralising or opsonising antibodies. Rationally-designed vaccines are comprised of antigens, effective delivery systems and adjuvants, adminstered via an optimal route, to predictably elicit protective responses against specific pathogen epitopes. The explosion of immunological knowledge amassed over the last few decades has renewed interest in T cell-inducing vaccines to fill current gaps in the arsenal, particularly against more complex pathogens. Because of how antigens are processed and presented to T cells, epitopes in such vaccines would not be universal, but HLA-restricted, and candidate peptide:HLA multimers must activate a large proportion of diverse humans. Adjuvants enhance adaptive immune responses toward a co-administered

antigen, and direct the quality of that response. Aluminium-based adjuvants are most common and preferentially induce type 2 responses. TLR agonists induce mainly type 1 responses [114]. Novel delivery systems such as attenuated viral vectors have also entered the field. Viral-vectored vaccines with replication-deficient viruses can express complete antigens, eliminating the requirement to define epitopes for multiple HLA types. They also produce sustained T cell responses [115]. Route of administration (intramuscular, subcutaneous, intradermal, mucosal) may also play a role in the character of immune response and its concentration in certain tissue compartments.

Knowledge of the most effective immune response against a pathogen enables selection of vaccine antigens, adjuvants and delivery systems that best invoke those responses. With improved immunological methods we now have an unprecedented opportunity to obtain a more comprehensive understanding of adaptive immunity in invasive *S. aureus* infection, to avoid repeating the disappointments of past vaccine efforts.

1.6.1. Clinical experience with anti-*S. aureus* vaccines

Passive and active immunisation strategies have been evaluated in *S. aureus* infection, using a variety of antigens. Passive immunisation administers functional antibodies to those at immediate risk of infection or to immunocompromised patients who cannot mount an independent response. Two candidates have reached phase III, either as adjunctive therapy during bloodstream infection, or as prophylaxis against BSI in high risk neonates. Both failed to demonstrate protection (Table 1.1). This is perhaps not surprising, given the doubt over the role of antibodies in anti-staphylococcal immunity discussed above (see *1.3.2.1.*). In addition, protein A enables the organism to capture and neutralise exogenous antibodies and render them ineffective. Two active anti-*S. aureus* vaccines have progressed to ultimately disappointing phase III clinical trials, and those that have completed phase II to date have not shown signs of efficacy. The protection demonstrated in animal challenge models failed to translate to clinical studies, despite producing appropriate antibody responses in human participants. Clinical studies are summarised in Table 1.1, and notable vaccines are discussed below.

Surface Polysaccharides:

Capsular polysaccharides (CP) inhibit opsonophagocytosis and increase lethality in animal models. Active or passive immunisation of mice with CP 5 and 8, or anti-CP IgG resulted in reduced bacterial burden and improved survival in systemic infection [116]. However, adjunctive infusion of polyclonal anti-CP 5 and 8 IgG failed to improve outcome in adult *S. aureus* bloodstream infection [117]. It also failed to prevent bloodstream infection in very low birth weight neonates [118]. Active immunisation with CP 5 and 8 (StaphVax®) was tested in adult haemodialysis patients. Despite high antibody titres (and 80% of *S. aureus* isolates expressing vaccine-type CP) a reduction in BSI was not observed [119]. More recently, these polysaccharides have been included in multivalent candidate vaccines, SA4Ag and GSK2392103A.

Clumping factor A and other adhesins:

The ubiquitous cell wall-anchored protein clumping factor A (ClfA) has been included in candidate multivalent vaccines. Administered to mice, alum-adjuvanted ClfA showed protection that was probably IL-17-dependent, in *S. aureus* arthritis and systemic infection [120,121]. Intranasal ClfA nanoparticles also produced antibody-independent benefit in systemic infection [122]. NDV-3 is an alum-adjuvanted vaccine based on an antifungal molecule structurally similar to ClfA (rAls3p-N). It conferred Th1/Th17 cell-mediated protection against invasive *S. aureus* infection in mice [64]. By driving Th17 response, it effectively contained cutaneous infection, reducing systemic dissemination [76]. While it also induced functional anti-rAls3p-N antibodies, the mechanism of protection was antibody-independent [123]. Despite these signals of ClfA-induced cellular immunity, in humans mainly humoral strategies have been pursued. Tefibazumab (a monoclonal anti-ClfA antibody) as adjunctive therapy during adult BSI failed to show a benefit [124]. Passive immunisation with anti-ClfA antibodies (Veronate®) as prophylaxis against *S. aureus* BSI in neonates, failed to show differences in sepsis incidence or mortality [125]. NDV-3 is the first candidate where cell-mediated responses have been evaluated. Phase I studies showed effective induction of humoral and cellular (Th1/17) responses in the majority of volunteers [126]. A phase II study to prevent incident *S. aureus* nasal colonisation in military recruits is currently ongoing.

IsdB immunisation:

Antibody titres against the highly conserved iron-scavenging protein iron-regulated surface determinant (IsdB) increase significantly in patients recovering from SA-BSI, suggesting it is expressed *in vivo* [127]. Immunisation with IsdB improved survival in a murine i.v. *S. aureus* challenge, where protection correlated with anti-IsdB antibody titres [128]. Immunisation of mice also expanded an IsdB-specific Th17 cell population, adoptive transfer of which was protective [88]. This dual activation of humoral and cellular immunity made it a highly attractive molecule. In the phase III trial, ~8000 pre-cardiothoracic surgery patients were vaccinated with IsdB (V710) without adjuvant. Anti-IsdB titres and opsonophagocytic activity increased, but the vaccine failed to reduce *S. aureus* BSI, deep sternal wound infections, or all-cause mortality [129]. Moreover, the trial was terminated early due to a potential safety signal. Although overall mortality did not differ between groups, the risk of death in patients who developed post-operative *S. aureus* infection was five-fold higher in vaccine than in placebo recipients (23 vs 4 per 100 person-years, p=0.001). Although a causal relationship was not established, concerns were raised about vaccine-induced 'immune priming' being detrimental to outcome of subsequent infection (see *Chapter 5*) [130]. Development of V710 was halted.

Protein A

Staphylococcal protein A (SpA) disables B cells and antibodies. 514G3 is an investigational IgG3 monoclonal antibody against SpA [108]. 514G3 binds to and opsonises *S. aureus*, to facilitate immune-mediated clearance. Both as monotherapy and in combination with vancomycin, it reduced mortality from *S. aureus* BSI in mice, and is currently being evaluated as adjunctive therapy in humans [131].

Multivalent antigens

Given *S. aureus*'s arsenal of pathogenic mechanisms, multivalent antigens have recently gained attention over single component vaccines. GSK2392103A includes CPs 5 and 8 conjugated to tetanus toxoid, inactivated α-toxin and ClfA with a squalene-based adjuvant AS03B. In phase I, this induced humoral immunity (although pre-vaccination baseline titres were already high) and low-level Th0/Th1 responses to ClfA and α-toxin [89]. SA4Ag is another candidate vaccine comprising CP5, CP8, ClfA and MntC. It has demonstrated functional antibody production in healthy volunteers and is now being tested in elective spinal fusion patients [132].

Whole killed organism:

Killed whole *S. aureus* cells provide a multitude of antigens. Repeated intradermal vaccination with formalin-killed bacteria did not influence antibody production or disease in a small sample of chronic furunculosis patients [133]. Most of these were nasally colonised, and already had pre-vaccination anti-*S. aureus* antibodies. Protection was not seen in mice using irradiated whole *S. aureus* cells [134].

Anti-toxin strategies

S. aureus produces an arsenal of cytolytic toxins and active immunisation with toxoid antigen(s) may block pathogenesis. This has reduced severity of disease in animals. A recombinant staphylococcal enterotoxin B (SEB) vaccine and an anti-SEB monoclonal antibody are being pursued, but mainly as anti-biological warfare strategies [135,136]. Two monoclonal antibodies against α-haemolysin are currently under investigation for prevention of (MEDI4893) and as adjunctive therapy (AR-301) for *S. aureus* pneumonia (Table 1.1).

Table 1.1. Clinical trials of anti-*S. aureus* vaccines

Vaccine	Antigen(s)	Adj.	Phase	Endpoint	Result	Ref
Passive Immunisation						
Aurograb®	Anti-GrfA mAb	–	III	Adult inpatients with deep-seated MRSA infection. Adjunctive therapy with vancomycin.	*Unpublished (NCT00217841)*	[137]
Pagimaximab	Anti-LTA mAb	–	III	Neonates preventive vs BSI	No efficacy	[138]
Altastaph®	Anti-CP5 and anti-CP8 IgG (polyclonal serum from individuals immunised with StaphVax®)	–	II	Adult BSI, adjunctive therapy; Neonates preventive vs BSI	No efficacy, or difference in adverse events	[117,118]
Tefibazumab	Humanised anti-ClfA mAb	–	II	Adult BSI adjunctive therapy	No efficacy in BSI outcome. Reduction in nasal carriage.	[124]
Veronate®	Pooled anti-adhesin IgG (anti-ClfA; FnbP; SdrG)	–	II	Neonates preventive vs BSI	No efficacy	[125]
MEDI4893	Anti-HLA mAb	–	II	*S. aureus* colonised mechanically-ventilated adults. Prophylaxis vs *S. aureus* pneumonia	*Unpublished (NCT02296320)*	[139]
AR-301	Anti-HLA mAb	–	II	Adult ICU patients with *S. aureus* pneumonia. Adjunctive therapy with standard-of-care antibiotics	*Unpublished (NCT01589185)*	
514G3	Anti-SpA IgG3 mAb	–	II	Adult BSI adjunctive therapy	*Unpublished (NCT02357966)*	[131]

Table 1.1. (continued)

Active Immunisation						
StaphVax®	CP5, CP8 (conjugated to pseudomonal exotoxin A)	–	III	(1) Adult HD, preventive vs BSI. (2) Reduction in nasal colonisation	(1) No efficacy (2) No efficacy	(1) [119,140] (2) [141]
V710	IsdB	–	III	Adult cardiothoracic surgery patients, preventive vs BSI and/or sternal wound infection	No efficacy, increased mortality among vaccines who developed *S. aureus* infection	[129]
SA4Ag	CP5, CP8, ClfA, MntC conjugated to CRM197	–	I/IIb	I: Healthy adults IIb: Pre-spinal fusion, preventive vs SSI	I: Immunogenic (humoral) *IIb: Unpublished (NCT 02388165)*	[132,142]
RSAV (recombinant *S. aureus* vaccine)	IsdB, rAT, SpA5, SEB, MntC	Alum	I	Healthy adults	*Unpublished (NCT02804711)*	[143]
GSK2392103A	CP5, CP8 (conjugated to TT), rAT, ClfA	AS03B	I	Healthy adults	Immunogenic (humoral and cellular)	[89]
Toxoids	rLuks-PV, rAT	Alum	I	Healthy adults	Immunogenic (humoral)	[144]
NDV-3	rAls3p (ClfA analogue)	Alum	I	–	Immunogenic (humoral and cellular)	[126]
STEBVax	SEB	Alum	I	Healthy adults	Immunogenic (humoral)	[136]
SA75	Chloroform-inactivated whole *S. aureus* cells	–	I	Healthy adults	Immunogenic (humoral)	[145]

Adj. = adjuvant; GrfA = ABC transporter component; mAb = monoclonal Ab; BSI = bloodstream infection; CP = capsular polysaccharide; ClfA = clumping factor A; FnbP = fibrinogen-binding protein; SdrG = serine-aspartate repeat-containing protein G precursor; HLA = α-haemolysin; LTA = lipoteichoic acid; SpA = staphylococcal protein A; IsdB = iron-regulated surface determinant; MntC = manganese transport protein C; (r)AT = (recombinant) alpha toxoid; SEB = monoclonal staphylococcal enterotoxin B; TT = tetanus toxoid; AS03 = adjuvant system 03 (α-tocopherol, squalene and polysorbate emulsion); rLukS = recombinant LukS subunit of Panton-Valentine leukocidin. As is standard, no Phase II studies were designed with enough statistical power to truly evaluate efficacy due to small participant numbers, so the outcomes quoted are based on the results and statistical analysis presented in each study.

1.6.2. Challenges in anti-*S. aureus* vaccine development

Vaccine triumphs of the past – where antibodies neutralise dangerous toxins (e.g., diphtheria or tetanus) or opsonise bacteria for phagocyte or complement clearance (e.g., pneumococcus or *H. Influenzae* type b) – have seduced vaccinologists into a continued focus on humoral immunity. Replication of this strategy has failed so far in the case of *S. aureus*. The scientific challenges to development of an anti-*S. aureus* vaccine are daunting. Namely, the organism's multiple mechanisms of pathogenesis, its status as a commensal, difficulties with translational immunology and lack of known correlates of human immunity.

In contrast to many other bacteria, the elements essential to pathogenesis in *S. aureus* infection are unknown – there is no single toxin, adhesin or immune evasion factor that causes disease when present, and fails to cause disease when absent. Attempts to identify a limited range of particularly 'virulent' strains associated with invasive infections have largely failed [146,147]. Its multiple redundant virulence and immune evasion factors means that a vaccine that neutralises a single one is unlikely to impact on bacterial replication, invasion or evasion.

S. aureus's status as a commensal organism complicates vaccine design and testing. Whatever their colonisation status at the time of enrolment in a clinical study, human participants, unlike their laboratory animal counterparts, are not immunologically naïve to *S. aureus* [65,148]. A lifetime's *S. aureus* exposure may alter the immune system's response to subsequent infection or immunisation. A vaccine must have a clear purpose. Would it aim to eliminate colonisation or simply protect against invasion? What effect would a vaccine influencing colonisation (by design or by accident) have on the balance of the microbiome? There has been almost no examination of these issues to date.

S. aureus is a species-restricted organism, colonising and causing natural infections in a limited range of mammals and poultry. It has evolved specific human-adapted virulence and immune evasion factors [149,150]. The mice used as models for infection are very different to humans in immunobiology and lifetime history of microbial exposures [151]. Humanised mice exhibited greater mortality, morbidity and lymphocyte death in systemic infection than wild type mice [152]. Neutrophils are the predominant human leukocytes, making up ~60% of white cells, while lymphocytes comprise ~30%. In mice, this ratio is reversed, which may give a different flavour to the principal immune responses in infection [153]. The diversity of MHC (HLA) types in humans is not replicated in inbred mice, and remains an obstacle for epitope-

based vaccines. The mouse breed used (e.g., Th1-skewed C57BL/6 or Th2-skewed BALB/c) influences experimental findings and clouds translation to a balanced human immune system [70]. These mice require large bacterial inocula to establish infection with *S. aureus*. In contrast, bacterial concentration in the blood in human *S. aureus* BSI is typically < 10 CFU/ml and even in endovascular infections is < 500 CFU/ml [154,155]. As such, the reduction in organ bacterial loads reported as efficacious in vaccinated animals may be completely irrelevant for human infections, and even in murine experiments bacterial load does not necessarily correlate with mortality [155-157]. Direct extrapolation of protection from animals to humans cannot be expected and there must be renewed focus on human immunology.

Currently effective vaccines for other pathogens induce a similar antibody response to that of natural infection to produce long-lived protective immunity. However, natural *S. aureus* infection does not result in sterile protection, and relapse and recurrent infection are common [158,159]. Anti-staphylococcal antibodies are common but not protective, and boosting their titres does not improve protection. Phagocytosis does not necessarily result in killing, and may facilitate dissemination of infection [160,161]. Despite extensive knowledge about microbial attributes, our knowledge of human correlates of immunity in *S. aureus* infection is scant. Given this significant knowledge gap, deciding which antigen to target, or which type of immunity to induce in vaccination strategies is incredibly challenging. However, based on the clinical signals and experimental animal data outlined in this chapter, it seems that T cells, in addition to antibodies, may be required for protection against *S. aureus*. The role of T lymphocytes in patients with invasive *S. aureus* infection has yet to be examined.

1.5. Project aims

This project sets out to characterise the pathogen, its clinical impact on patients, and the human adaptive immune response to it. The first section aims to describe and compare the clinical course of patients with *S. aureus* and *E. coli* bloodstream infections and microbiologically characterise their *S. aureus* isolates. The second aims to characterise the adaptive immune response in the early recovery period of *S. aureus* bloodstream infection. More specifically: (i) to examine acute leukocyte dynamics; (ii) to determine whether particular lymphocyte subsets are activated; (iii) to test for *S. aureus* antigen-specific T cell responses for the first time in a patient cohort; (iv) to characterise selected humoral adaptive responses, and (v) to develop a model assay to test T cell responses to a specific *S. aureus* antigen.

Chapter 2

Materials & Methods

2.1. Materials

2.1.1. General Reagents

Table 2.1. General Reagents

Reagent	Supplier
10X RBC Lysis Buffer	eBioscience
Brefeldin A	Sigma Aldrich
Columbia blood agar plates	St James's Hospital Microbiology
CpG	Oligos Etc.
Fibrinogen	Enzyme Research Laboratories
Ionomycin calcium	Sigma Aldrich
Lymphoprep	Axis-Shield
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich
Phosphate Buffered Saline (PBS)	Sigma Aldrich
Protect Microorganism Preservation System	Technical Service Consultants Ltd
RPMI	Sigma Aldrich
Staphylococcal Enterotoxin A	Sigma Aldrich
Vybrant® CFDA SE Cell Tracer Kit [Carboxyfluorescein diacetate succinimidyl ester (CFSE)]	Invitrogen

2.1.2. Custom Reagents

Table 2.2. Custom (Mixed) Reagents

Reagent	Components
1% Bovine Serum Albumin	2.5 g Bovine Serum Albumin lyophilised powder (Sigma Aldrich) 250 mL 1X PBS
10X PBS	80g NaCl (Sigma Aldrich) 11.6g Na_2HPO_4 (Sigma Aldrich) 2g KH_2PO_4 (Sigma Aldrich) 2g KCl (Sigma Aldrich) pH 7.2-7.4

Table 2.2. (continued)

cRPMI Media	500 ml RPMI (Sigma Aldrich) 10% (v/v) Fetal Bovine Serum (FBS; Biosera); heat-inactivated at 56°C x 1 h 100mM L-Glutamine (Gibco) 100µg/ml penicillin/streptomycin (Gibco)
FACS Buffer	1X PBS (Sigma Aldrich)
MACS Buffer	0.5% (w/v) BSA 2mM EDTA (Sigma) 1X PBS pH 7.2

2.1.3. Protein Assay Reagents

Table 2.3. Protein Assay Reagents

Pierce BCA Protein Assay Kit (Thermo Scientific)	
Standard	2mg/mL bovine serum albumin in 0.9% saline and 0.05% sodium azide
Working Reagent	50 mL Reagent A 1 mL Reagent B
BCA Reagent A	500mL (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide)
BCA Reagent B	25mL (4% cupric sulfate)

2.1.4. FACS antibodies and Reagents

Table 2.4. FACS antibodies and Reagents

Marker	**Clone**	**Supplier**
Extracellular		
CD3	OKT3	eBioscience
CD4	RPA-T4	eBioscience
CD8a	RPA-T8	eBioscience
CD45RO	UCHL1	eBioscience
CCR7	3D12	eBioscience

Table 2.4 (continued)

Gamma delta TCR	B1.1	eBioscience
TCR Vδ2	B6	eBioscience
Intracellular		
IFN gamma	4S.B3	eBioscience
IL-10	JES3-9D7	eBioscience
IL-17AF	20LJS09	eBioscience
TNF alpha	MAb11	eBioscience
Permeabilisation Kit		
DakoCytomation Intrastain Kit		Alere
Viability Dyes		
Live/Dead Aqua		Invitrogen
Proliferation Tracker Dye		
Vybrant® CFDA SE Cell Tracer Kit (CFSE)		Invitrogen
Compensation		
OneComp eBeads		eBioscience

2.1.5. ELISA kits

Table 2.5. ELISA Ready-SET-Go!® kits

Cytokine	Supplier	Capture Ab Dilution	Standard cytokine dilution	Detection Ab dilution
IFNγ	eBioscience	1:250	1:2000	1:250
IL-5	eBioscience	1:250	1:2000	1:250
IL-10	eBioscience	1:250	1:3000	1:250
IL-17A	eBioscience	1:250	1:2000	1:250

2.1.6. ELISA Reagents

Table 2.6. ELISA Reagents

	Components
Coating Buffer	10mL 10X PBS ELISA Coating buffer (eBioscience) 90mL dH_20
Assay Diluent (PBS/Fetal bovine serum/1% ProClin preservative)	10mL 5X Assay Diluent (eBioscience) 40mL dH_20
Wash Buffer	1L 10X PBS 9L dH_20 5ml Tween-20 (Sigma Aldrich)
Substrate solution	Tetramethylbenzidine (1X TMB, eBioscience)
Stop Reagent (1M H3PO4)	34mL 85% phosphoric acid (Sigma Aldrich) 466mL dH_20
Detection enzyme	Streptavidin-HRP (Sigma Aldrich) diluted 1:250 in Assay Diluent (eBioscience)

2.2. Clinical Study Methods

2.2.1. Study Design and Recruitment

Adult inpatients from three acute tertiary care centres in Dublin with bloodstream infection due to *S. aureus* or *E. coli* who met defined inclusion criteria (Table 2.7) were invited to participate. These criteria were designed to collect data on the 'normal' immune response to infection without confounding by iatrogenic immunosuppression. As such, groups of clearly immunosuppressed patients (particularly those with severe functional lymphocyte deficits) were excluded. Prior to commencing recruitment at each site, an email was sent to all clinical consultants explaining the study and inviting any objections to patient inclusion (none were received). New cases of bloodstream infection were prospectively identified by checking blood culture bench or clinical bacteraemia logbooks during regular visits to the participating clinical microbiology laboratories by the research clinician (RC).

If potentially suitable for inclusion, inpatients were approached by the RC for further assessment. During this visit the study was explained, Patient Information Leaflet (PIL, Appendix A) provided for review and any questions were answered. PILs were tailored to each hospital site so that on-site clinical contact names and numbers were correct, but other text remained the same. At a single study site, assent could be obtained from next-of-kin, who received a modified Patient Representative Information Leaflet (Appendix B), for cases where patients were unable to consent themselves (e.g. critical care patients). Patients willing to participate provided a sample on the same day (rarely) or more often within the following 1-2 days, to allow a period of reflection. Consent forms (Appendix C) or assent forms (Appendix D) were signed on the day of sampling. On recruitment, participants were assigned a unique identifier (UI). Patient medical record numbers were recorded alongside this study UI on the Recruitment Record (Appendix E). Clinical isolates of *S. aureus* and *E. coli* were coded numerically to correspond with source patient numbering. The recruitment record was kept in the study file. Consent forms and the recruitment record were the only items with identifiable data (patient signatures and medical record numbers) and will be retained for 10 years before being destroyed in line with standard practice.

Onset of bloodstream infection was taken as onset of symptoms. Attending clinicians took blood cultures when indicated by fever or rigors. In *S. aureus* bacteraemia, symptom onset is acute and typically severe; consequently all patients in this study sought medical attention within 24 h. For *E. coli*, presentation is more variable, and patients presented within 12-48 h

of symptom onset. On day 7 ± 2 post-onset of bacteraemia, 40ml venous blood was collected by the RC. Relevant clinical data on all patients was collected by history-taking during the initial RC visit and accessing the medical record. This was recorded by the RC on a standardised Case Report Form (CRF, Appendix F) and subsequently entered on a password-protected database. At the end of the study, electronic medical records were accessed once more by the RC to determine longer-term clinical outcomes. Despite the Irish acute hospital system depending mostly on paper-based records for contemporaneous clinical entries, re-admissions, deaths, subsequent microbiology or radiology results, and summaries of clinical information in subsequent discharge or outpatient letters are captured electronically. These would generally be expected to capture significant longer-term patient outcomes, including relapses and deaths, as long as patients re-attend the same hospital. Due to the absence of national-level patient unique identifiers and recording of multi-centre patient episodes, mining these records at the recruitment sites only, may not accurately track outcomes for patients who subsequently present at other healthcare institutions. Healthy volunteers were recruited from staff of participating institutions (School of Biochemistry & Immunology, Trinity College Dublin and Departments of Microbiology at clinical sites) by email notice and word of mouth. Two healthy family members of recruited patients also volunteered to donate blood samples. Healthy volunteers also received the information leaflet and formally consented to participate.

Power calculations were based on results of a pilot study (Dr R McLoughlin, unpublished data). This study compared *S. aureus* antigen-specific T cell proliferation (as assessed by thymidine incorporation stimulation index) in *S. aureus*-colonised and non-colonised individuals. Antigen-specific proliferation in colonised individuals was greater by 40 units (SI=66.58 vs.SI =24.94; SD ± 45). Thus, to obtain 80% power, using alpha = 5%, a number of 19 patients per group is required. Although the planned method used to measure proliferation in this study differs (CFSE labelling versus thymidine incorporation), the outcomes are comparable.

Table 2.7. Inclusion and Exclusion Criteria

Inclusion Criteria Patients must satisfy <u>all</u> the following criteria to be eligible for the study:
* Age ≥ 18yrs * Confirmed *S. aureus* or *E.coli* bloodstream infection, i.e., isolation of the organism from ≥ 1 set of blood culture bottles where blood has been collected by use of standard aseptic technique * Able and willing to give informed consent[‡]
Exclusion Criteria Patients may not enter the study if <u>any</u> of the following apply:
* Age < 18yrs * Mixed bacteraemia * Known active infection with blood-borne viruses: o HIV Ab/Ag positive o Hepatitis C RNA positive o Hepatitis B sAg positive * Active haematological malignancy * Active solid organ malignancy * Solid organ transplant recipient * Current Immunosuppressive therapy: o Prednisolone therapy > 30mg/day for ≥ 1 month or alternative steroid equivalent o Cytotoxic immunosuppressant therapy: o Calcineurin inhibitors, e.g. cyclosporine, tacrolimus o Antiproliferative agents, e.g. azathioprine, cyclophosphamide, methotrexate, chlorambucil, mycophenylate mofetil o Immune-active monoclonal antibodies, e.g. adalimumab, alemtuzumab, belimumab, golimumab, infliximab, muromonab-CD3, natalizumab, ofatumumab, rituximab, tocilizumab, tocitumomab.

[‡]At one of the study sites, approval was also later given for next-of-kin/representative assent if patients were unable to give informed consent.

2.2.2. Clinical Definitions

The following standard clinical definitions were used to classify bloodstream infections (BSI) [162,163].

Community-acquired BSI

- index positive blood culture collected < 48 h after hospital admission

Healthcare-acquired BSI

- index positive blood culture collected ≥ 48 h after hospital admission, and no signs or symptoms of such infection noted at time of admission.

OR

- Index positive blood culture collected < 48hrs after hospital admission if any of the following criteria are met:
    - Received intravenous therapy in an ambulatory setting in the 30 days before onset of BSI
    - Attended a hospital clinic or haemodialysis in the 30 days before onset of BSI
    - Was hospitalised in an acute care hospital for ≥ 2 days in the 90 days prior to onset of BSI
    - Resident of nursing home or long-term care facility

***S. aureus* bloodstream infection-specific definitions**

S. aureus BSI is classified as complicated or uncomplicated. These designations have significant implications for the extent and type of diagnostic evaluation, duration of antibiotic treatment, risk of relapse or failure and overall prognosis [164-167]. As with all bloodstream infections, SA-BSI (complicated or uncomplicated) may result in concurrent sepsis, but this does not form part of the classification system [168].

Complicated SA-BSI

Presence of any ONE of the following

- Persistent fever or bacteraemia at 72 h
- Community-acquired infection
- Metastatic or endovascular infection
- Presence of prosthetic material /device
- Skin lesions consistent with acute systemic infection

Uncomplicated (i.e., catheter-related) SA-BSI

To qualify as uncomplicated, a case must fulfil ALL of the following criteria:

- Intravenous catheter identified as source of infection and promptly removed
- Negative follow-up blood culture at 72 h
- Clinical defervescence within 72 h
- No indwelling prosthetic devices
- No symptoms suggestive of metastatic or endovascular infection

SA-BSI Recurrence:

The return of *S. aureus* bloodstream infection after documentation of negative blood cultures and/or clinical improvement, within 12 weeks of completing a course of anti-staphylococcal therapy [169].

***Escherichia coli* bloodstream infection-specific definitions**

No distinction between complicated or uncomplicated bloodstream infection is made in *E. coli* BSI.

2.2.3. Sample Handling

Phlebotomy was carried out by the RC using aseptic technique and Vacuette safety blood collection sets. 10mL of blood for serum was collected directly into Serum Gel tubes (Vacuette) containing clot activator and gel. 20-30mL blood for PBMCs was collected directly into sodium heparin (Vacuette) tubes. Tubes were gently inverted 5 times to ensure adequate mixing and labelled with patient details at the bedside. Samples were transported at room temperature and processed within a maximum of 3 hours of collection.

2.2.4. Ethical approval

The study was approved by the St James's and Adelaide Meath National Children's and Federated Dublin Voluntary Hospitals Joint Research Ethics Committee (2011/35/04) and the Beaumont Hospital Research Ethics Committee (13/101). Written informed consent was

obtained from all patient participants or their next of kin (if patients were unable to give consent).

2.2.5. Blood donors

Anonymised buffy coats were obtained from healthy blood donors at the Irish Blood Transfusion Service, Dublin. All donors have given written approval for the use of cells for scientific purposes.

2.2.6. Funding

The project was supported primarily by a Health Research Board Health Research Award (HRA_POR/2012/104), a Wellcome Trust Research Career Development Fellowship (WT086515MA) to Prof McLoughlin. The funders had no role in study design, data collection or analysis.

2.3. Microbiological Methods

2.3.1. Bacterial identification and Antimicrobial Susceptibility Testing

Blood was inoculated into aerobic and anaerobic BacT/Alert bottles (Biomérieux, France) at the bedside by attending clinicians as part of standard patient care, transported to the clinical microbiology laboratory and incubated in an automated detection system. Positive isolates were identified as either *S. aureus* or *E. coli* in accordance with CLSI (Clinical & Laboratory Standards Institute) standard methodology. Antimicrobial susceptibility testing was performed with direct disc diffusion testing on agar plates and secondary confirmation with Vitek2 (Biomérieux, France) system according to the manufacturer's instructions.

2.3.2. Genetic characterisation of *S. aureus* strains

Next-generation whole genome sequencing (NGS) of 25 *S. aureus* bloodstream isolates from 24 recruited patients and both reference strains (PS80 and SH1000) of *S. aureus* was performed by Dr Micheál Mac Aogáin at Sir Patrick Dun Translational Research Laboratory, School of Medicine, Trinity College Dublin, St James's Hospital, Dublin, after design of experiments was discussed with the RC. Bacteria were grown from frozen stocks on CBA incubated for 18 to 24 h before sweeps of colonies were taken. DNA was extracted from bacteria using the Qiagen DNeasy blood and tissue kit (Qiagen, West Sussex, UK). Nextera XT library preparation reagents (Illumina, Eindhoven, The Netherlands) were used to generate multiplexed paired end sequencing libraries of *S. aureus* genomic DNA. Genomic libraries were sequenced on an Illumina MiSeq instrument achieving an average raw read depth of 130.6 ± 14.7 fold. Paired end reads (2 x 250 bp) were mapped to the *S. aureus* TW20 reference genome (NC017331) with the Burrows-Wheeler Aligner (BWA) and nucleotide variant analysis was performed using the SAMtools analysis suite [170,171]. PhyML was used to generate a Maximum-Likelihood tree based on variant sites detected across the genomes of all 24 strains, applying the Jukes-Cantor model of nucleotide substitution (JC69) [172]. Results of NGS were used to infer clonal complex and sequence type. Allelic profiles for each isolate were submitted to the database (http://saureus.mlst.net/) to obtain a ST number, through an automated comparison to a collection of reference strains previously characterised by MLST [38,173]. Sequences not in the database were assigned a new allele number and profile.

2.3.3. Virulence and immune evasion genes in *S. aureus* isolates

Whole genome sequences of *S. aureus* isolates generated as above were also interrogated for the presence of genes encoding selected staphylococcal antigens of potential vaccine interest. Raw sequencing reads were trimmed with Trimmomatic and assembled de-novo using the SPAdes assembler with default parameters for paired end data [174,175]. This was performed by Dr Micheál Mac Aogáin at Sir Patrick Dun Translational Research Laboratory, School of Medicine, Trinity College Dublin, St James's Hospital, Dublin. Antigens for investigation were chosen by the RC based on those that have been included in previous *S. aureus* vaccines; those that produced humoral response in the study patient cohort; and immune evasion molecules known to directly interfere with lymphocyte function. Antigens are listed in Table 2.8. The presence of these genes in each *S. aureus* strain was determined using BLASTN (Basic Local Alignment Search Tool: Nucleotide), a web-based programme that finds and compares regions of nucleotide sequence similarity to draft genome assembly databases [176]. Reference strains TW20 and Newman, whose complete genomes have been published, were used for gene identification. Where genes were absent from TW20 or Newman strains, other published reference strains were used to determine relevant sequences (Table 2.8). Where target genes were highly homologous to other *S. aureus* genes, each hit with < 90% homology were manually inspected to ensure correct identification.

Table 2.8 Genotypic analysis sources for selected *S. aureus* antigen genes

The presence of 17 selected genes in 22 *S. aureus* bloodstream isolates from patients included in the study and 2 laboratory strains (PS80 and SH1000) was investigated, following next generation sequencing of their genomes. Reference strains TW20 (NC017331) and Newman (NC009641.1), whose complete genomes have been published, were used to identify gene sequences of target antigens. Where genes were absent from TW20 or Newman strains, other published reference strains were used to determine relevant sequences. Genomes are held by National Center for Biotechnology Information and are freely available online at URLs shown. The presence of genes encoding antigens of interest in each strain was determined using BLASTN to query draft genome assembly databases for their presence. Where target genes were highly homologous to other *S. aureus* genes, each hit with <90% homology were manually inspected to ensure correct identification.

Antigen	Gene	*S. aureus* TW20	*S. aureus* Newman	Other strain
Published reference strain complete genome sequences				
		NCBI Reference Sequence FN433596.1 (https://www.ncbi.nlm.nih.gov/nuccore/FN433596.1) [177]	*NCBI Reference Sequence* NC_009641.1 (https://www.ncbi.nlm.nih.gov/nuccore/NC_009641.1) [178]	
Vaccine antigens in previous clinical trials				
ClfA	*clfA*	✓	✓	
IsdB	*Isdb*	✓	✓	
CP5*		–	✓	***Strains**: Newman and Reynolds* (http://www.ncbi.nlm.nih.gov/nuccore/U81973)
CP8*	*cap8H-K*	✓	–	***Strain**: Becker* (http://www.ncbi.nlm.nih.gov/nuccore/U73374)
YkpA	*ykpA*	–	–	***Strain**: MRSA252* https://www.ncbi.nlm.nih.gov/nuccore/BX571856.1
MntC	*mntC*	✓	✓ (hypothetical protein NWMN_0601)	
LTA	*dltD*	✓	✓	
PNAG$	*icaR*	✓	✓	
PVL	*pvl*	*Non-PVL producing strain*	*Non-PVL producing strain*	***Strain**: USA300_FPR3757* (https://www.ncbi.nlm.nih.gov/nuccore/NC_007793.1)
HLA	*hla*	✓	✓	

Table 2.8 (continued)

Immunogenic B cell antigens (in patient cohort)				
IsaA	*isaA*	✓	–	
IsdA	*isdA*	✓	–	
ClfB	*clfB*	✓	✓	
Antigens implicated in directly evading lymphocyte immunity				
Protein A	*spa*	✓	✓	
Map	*mapW*	✓	✓	
LukED	*lukE*	–	✓	
SEA	*sea*	✓	✓	

ClfA = clumping factor A. ClfB = clumping factor B. IsdB = iron-regulated heme-iron binding protein B. IsdA = iron surface determinant A. IsaA = immunodominant staphylococcal antigen A. Cp5, 8 = capsular polysaccharides 5 and 8. MntC = manganese transporter C. LTA = lipoteichoic acid. PNAG = poly-N-acetyl glucosamine. YkpA = ABC transporter ATP-binding protein ykpA. PVL = Panton-valentine leucocidin. HLA = alpha-toxin. Map = MHC Class II analog protein. LukED = leukotoxin ED

[$]PNAG is coded by the *ica* (intercellular adhesion) operon.

2.3.4. Preparation of heat-killed *S. aureus* and *E. coli*

Biosafety Level 2 precautions were employed for all bacterial work. Four reference *S. aureus* strains were used along with each patient's infecting *S. aureus* strain. PS80 and SH1000 are laboratory strains described previously [179,180]. Two invasive clinical *S. aureus* strains from St. James's Hospital patients recruited to this study – one methicillin-sensitive (SJH.MSSA-1; CC7) and one methicillin-resistant (SJH.MRSA-1; CC22) –were also included to represent currently circulating clones. A clinical *E. coli* strain CFT073 (a clinical isolate from St. James's Hospital, Dublin in 2012) was used as a non-staphylococcal comparator. Patients' own *S. aureus* isolates were subcultured from the clinical laboratory nutrient agar slopes and cultivated overnight at 37°C on Columbia blood agar (CBA) plates. Reference strains of *S. aureus* and *E. coli* were cultivated from frozen stocks in the same way.

All bacteria were heat-killed for T cell assays. For heat-killing, bacterial suspensions were prepared in sterile PBS at OD_{600nm} of 1.0. CFUs were verified by plating serial dilutions onto CBA and recorded for each batch to allow later estimation of CFUs per mL by protein content. Staphylococcal bacterial suspensions were heat-inactivated at 90°C for 45 minutes and *E. coli* suspensions at 70°C for 30 minutes in microcentrifuge tubes. Killed bacteria were repeatedly centrifuged at 17000 g and washed in sterile PBS to remove secreted proteins. Killed PS80 was confirmed to be superantigen-free by comparing proliferation of T lymphocytes in response to supernatants of heat-killed or live PS80. The latter induced massive proliferation after only 3 d. Finally, bacteria were resuspended in 200 µL PBS and the contents of 5 microcentrifuge tubes were pooled to give a 5X concentration. Gram staining was performed to confirm that heat-killed *S. aureus* remained intact and could therefore be phagocytosed and re-presented by antigen presenting cells in the usual manner (Fig 2.1). Gram staining heat-killed *E. coli* similarly showed intact Gram negative bacilli. Killing was verified by plating on CBA and incubating at 37°C for 5 d. Dead bacteria were used to allow standardised comparison of lymphocyte proliferation and cytokine response, which may have been skewed due to differences in growth between live strains, as well as to avoid overgrowth and death of PBMCs at later time points. A 100 µL aliquot of heat-killed bacteria was used to quantify total protein concentration with Pierce BCA Protein Assay (Thermo Scientific) kit. Strains were then adjusted to a concentration of 25 µg total protein per mL (approximately 1 x 10^8 CFU/ml), and stored at 4°C for up to 4 weeks. Frozen stocks were prepared from each patient isolate by suspending with Protect Microorganism Preservation System (Technical Service Consultants Ltd.) beads, which were stored at -80°C.

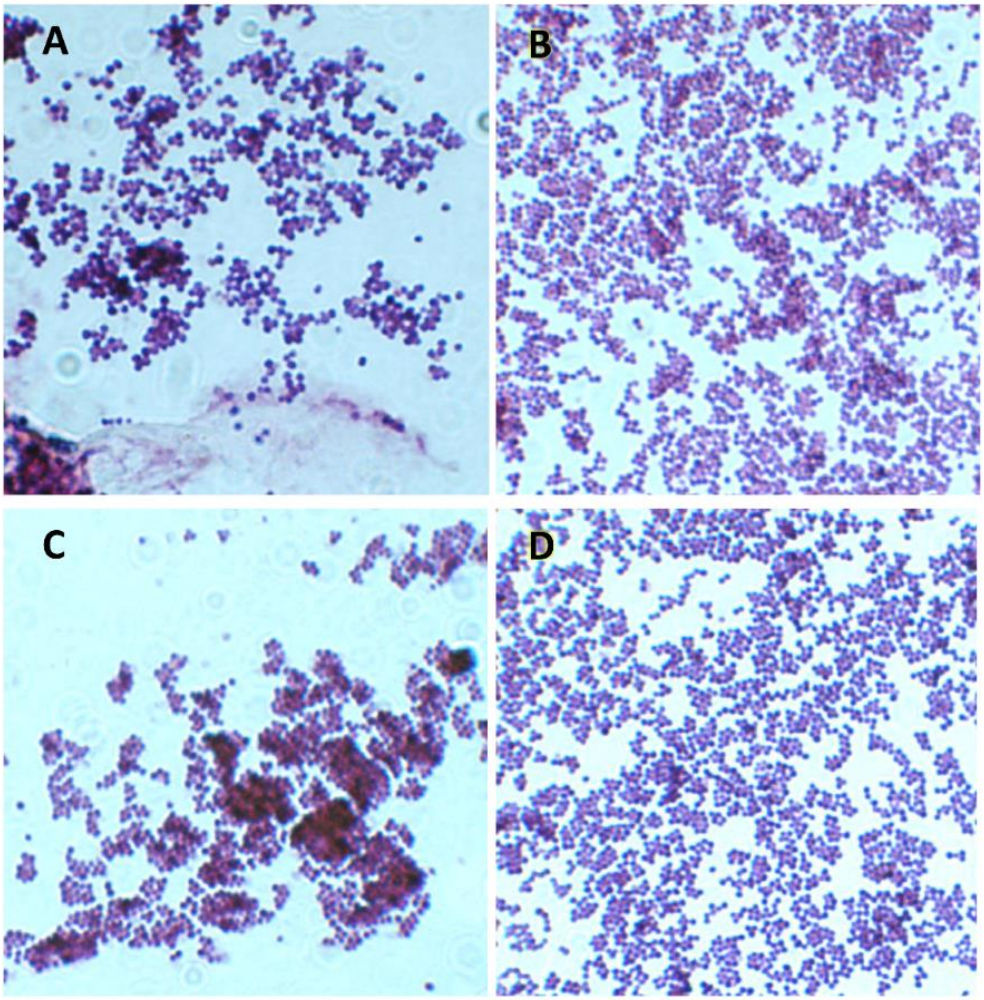

Figure 2.1. Gram staining and microscopy of *S. aureus* before and after heat inactivation.

Bacterial suspensions were heated at 90°C for 45 minutes, after which killing was verified by plating on CBA and incubating at 37°C for 5 d. Gram staining was performed before and after killing of staphylococci to confirm that heat-killed bacteria remained intact. Microscopy is shown at 100x. Live PS80 (A). Heat-inactivated PS80 (B). Live SH1000 (C). Heat-inactivated SH1000 (D).

2.3.5. Protein Quantification

Standards at serial dilutions (Table 2.3, ThermoFisher) and samples were added to a non-binding flat-bottomed 96-well plate (Greiner bio-one) 25 µL/well in duplicate. Working reagent (WR, Table 2.3, ThermoFisher) was added (200 µL/well) to standards and samples and mixed for 30 seconds on a plate shaker. Plates were covered and incubated at 37° C for 30 min before cooling to RT. Absorbance was measured at 562nm using a micro-plate reader and Softmax Pro software.

2.3.6. Expression of clumping factor A in *S. aureus* isolates before and after heat-inactivation

Expression of clumping factor A (ClfA) in reference and clinical strains, and in heat-killed *S. aureus* was assessed by Western blot. This work was performed by Dara O'Halloran in the Geoghegan laboratory, Department of Microbiology, Moyne Institute of Preventative Medicine, School of Genetics and Microbiology, Trinity College Dublin. *S. aureus* strains were grown in brain heart infusion broth with shaking at 37°C, washed and adjusted to OD_{600nm} of 10. *S. aureus* SH1000 *clfA clfB*::Em^r *fnbA*::Em^r *fnbB*::Tet^r, a strain lacking fibrinogen-binding proteins was described previously [181]. Expression of ClfA was confirmed by Western immunoblotting as previously described [182]. Briefly, proteins were separated on 7.5% (w/v) polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Roche) and blocked in 10% (w/v) skimmed milk proteins. Blots were probed with polyclonal anti-ClfA A domain IgG (1:5,000 or 1:500) and bound antibody detected using horseradish peroxidase-conjugated protein A (1:500, Sigma). Reactive bands were visualised using the LumiGLO reagent and peroxide detection system (Cell Signalling Technology).

2.3.7. Bacterial adherence to fibrinogen before and after heat-inactivation

100µL of Fibrinogen (Enzyme Research Laboratories) in PBS was added to wells of a flat-bottomed 96-well plate at two concentrations (0.5µg/mL and 5µg/mL) in triplicate, and incubated at 4°C overnight. Plates were washed with PBS and blocked with sterile 5% (w/v) BSA (100µl/well) for 2 h at 37°C. *S. aureus* SH1000 from frozen stock was grown to stationary phase overnight in tryptic soy broth (TSB), and cultured overnight on CBA plates (to replicate the bacterial method used for cellular assays). SH1000 *clfA clfB*::Em^r *fnbA*::Em^r *fnbB*::Tet^r, a

mutant strain of SH1000 with fibrinogen binding elements (clumping factors A and B, fibronectin binding proteins A and B) deleted, was also grown in TSB [181]. Live bacterial suspensions were washed and resuspended in PBS to an OD_{600nm} of 1 before being added to the wells of the fibrinogen-coated plate (100µl/well). Previously heat-killed SH1000 was washed and resuspended in PBS to an OD_{600nm} of 1 before being similarly added (100µl/well). Plates were then incubated for 90 min at 37°C. The fibrinogen-coated plate was repeatedly washed. To fix any fibrinogen-bound bacteria, 100µl of 25% (v/v) formaldehyde was added to wells for 20 minutes at RT before repeat washing and addition of crystal violet (100µl) for 1 min. After washing, 100µl of 5% (v/v) acetic acid was added for 5 minutes with shaking to solubilise the crystal violet. Absorbance was measured at 570nm on a microplate reader (Labsystems).

2.3.8. Purification of Clumping factor A

Clumping factor A was kindly provided by the Geoghegan laboratory, Department of Microbiology, Moyne Institute of Preventative Medicine, School of Genetics and Microbiology, Trinity College Dublin. Recombinant ClfA N123 (amino-acids 40-559) was cloned and expressed from genomic DNA of *S. aureus* strain Newman and purified from *E. coli* by Ni^{2+} affinity chromatography as previously described [183]. Endotoxin was removed by the Adjuvant Research Group, School of Biochemistry & Immunology, Trinity College Dublin, using Detoxi-Gel Endotoxin Removing Columns (Thermo Scientific).

2.4. Immunological Methods

2.4.1. Isolation of sera and cells

Serum tubes were spun at 900g x 10 min before serum was collected with a transfer pipette and stored at -80° C. PBMCs from BSI patients were isolated, within 3 h of venepuncture, by density centrifugation of PBS-diluted fresh heparinised blood (dilution 1:1) over a Lymphoprep gradient (Axis-Shield). These were centrifuged at 900g for 20 min with the brake off before the buffy coat was transferred to fresh PBS and re-spun at 650g for 10 min. Cells were resuspended and spun for a third time at 320g for 7 min to remove platelets. Mononuclear cells were then counted in a haemocytometer using Trypan Blue (Sigma). If there were visible erythrocytes after PBMC isolation, cells were resuspended in 3mL 1X RBC lysis buffer (eBioscience) for 3 min. Cells were resuspended to a concentration of 1 x 10^6 per mL before either being CFSE-labelled or used unlabelled in relevant assays.

2.4.2. Short '*ex vivo*' re-stimulation with heat-killed bacteria

PBMCs from healthy volunteers, SA-BSI and EC-BSI patients were prepared as described above and immediately transferred to a 96-well round-bottomed plate (1x10^5/well, Grenier bio-one). Cells were cultured with cRPMI alone (negative control), staphylococcal enterotoxin A (100 ng/ml, Sigma [positive control]), heat-killed *E. coli* (1µg/ml), and various strains of heat-killed *S. aureus* (PS80, SH1000, SJH.MSSA-1, SJH.MRSA-1; at 1 µg/ml total protein; approximately 1-2 x 10^7 CFU/ml). Brefeldin A (Sigma) working stock of 25 µg/mL was prepared from frozen stock in cRPMI and added (100 µL/well) to test wells to give a final concentration of 5 µg/mL. Plates were incubated overnight (~16 h) at 37°C and 5% CO_2. The following morning, supplementary positive control wells (cells without antigen) were stimulated with PMA (50ng/ml), ionomycin (500ng/ml) and Brefeldin A (5µg/ml) for the final 4 h to produce polyclonal cytokine stimulation. This would allow for accurate fluorescence minus ones (FMOs) for gating of cytokine positive cells.

2.4.3. CFSE staining

The succinimidyl ester of carboxyfluorescein diacetate (CFSE) is used for the analysis of cellular proliferation. CFSE spontaneously and irreversibly couples to both intracellular and

cell surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, CFSE labelling is distributed equally between the daughter cells, which are each half as fluorescent as parent cells. This halving of cellular fluorescence intensity marks each successive generation in a population of proliferating cells and is readily followed by flow cytometry. A 10 mM CFSE stock was first reconstituted from the Vybrant CFDA SE Cell Tracer Kit (Invitrogen) by dissolving 500µg of CFDA SE lyophilised powder in 90 µL of DMSO (dimethyl sulfoxide; Invitrogen). This stock was frozen at -20° C. For each assay, fresh 10µM CFSE working stock was made by adding 1µL to 999µL warm PBS. This would ultimately create cells labelled with 5µM CFSE when added to an equal volume of cells 1:1. The process was performed as previously described [184]. CFSE work took place in low light with aluminium foil covering cell tubes. PBMCs were resuspended in 1 mL warm PBS/5% heat-inactivated FCS, and transferred to fresh Falcon tubes for staining. Tubes were laid on their sides while 1 mL 10µM working stock was added to top of each tube, avoiding contact with the cell suspension. Tubes were quickly capped and inverted on a vortex before being covered and incubated at room temperature x 5 min. Cells were washed in 10 mL warm PBC/5% FCS and spun at 300g for 5 min twice before resuspension in cRPMI at 1 x 10^6 cells/mL. A small number were left unstained as autofluorescence control.

2.4.4. Lymphocyte proliferation assay

A schematic for investigation of *S. aureus* antigen-induced T cell proliferation and cytokine production in bloodstream infection patients is shown in Fig. 2.2. PBMCs from BSI patients were isolated and labelled with 5µM CFSE as described above. CFSE-labelled PBMCs ($2x10^5$/well) were cultured with cRPMI alone (negative control), staphylococcal enterotoxin A (100 ng/ml, Sigma [positive control]), heat-killed *E. coli* (1µg/ml), and 5 strains (4 reference strains as above and patient's own infecting strain) of heat-killed *S. aureus* at 1µg/ml total protein concentration (approximately 1-2 x 10^7 CFU/ml) in a 96-well round-bottomed plate (Grenier bio-one). cRPMI comprised RPMI (Sigma), 10% v/v fetal calf serum (Biosera), 100mM L-glutamine (Gibco) and 100µg/ml penicillin/streptomycin (Gibco). Sterile PBS was added to empty outer wells to prevent evaporation. All assays were performed in quadruplicate and plates were incubated at 37°C and 5% CO_2 for 10 days, in line with similar studies [185]. A plate map is shown in Fig. 2.3. For intracellular cytokine staining, Brefeldin A (5 µg/ml, Sigma) was added to test wells for the final 16 h of culture, while secondary positive control wells (without antigen) were stimulated with PMA (50 ng/ml), ionomycin

(500 ng/ml) and Brefeldin A (5μg/ml) for the final 4 h. Cells were harvested by centrifugation before FACS analysis.

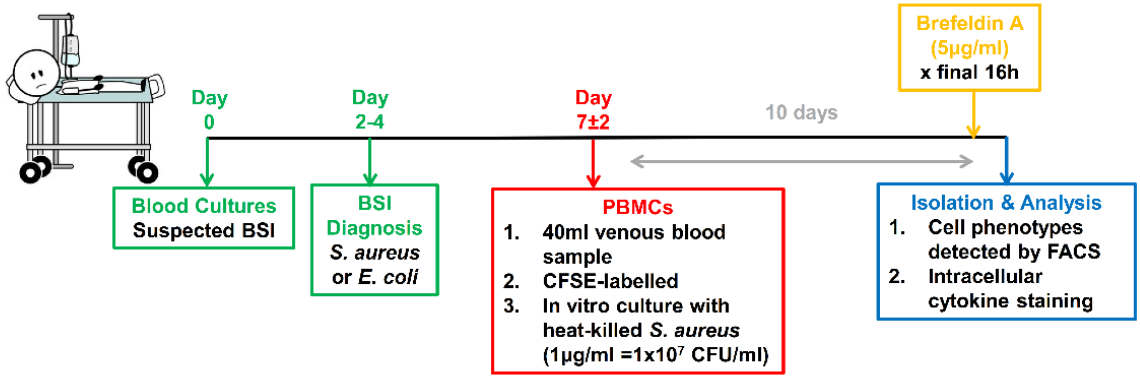

Figure 2.2. PBMC assay for investigation of *S. aureus* antigen-induced T cell proliferation and cytokine production in bloodstream infection patients

Patients with suspected bloodstream infection (BSI) had clinically-indicated blood cultures drawn on day 0. Organisms from positive cultures were identified within the first 2-4 days after which suitable patients could be assessed and approached for recruitment. Consenting patients had blood drawn on day 7 ± 2 post-initial bacteraemia for whole PBMC assays and serum cytokine measurement. PBMCs were stained with CFSE and cultured with heat-killed bacteria for 10 days. Brefeldin A (5 µg/mL) was added for the final 16 h before cells were evaluated by extra- and intracellular flow cytometry.

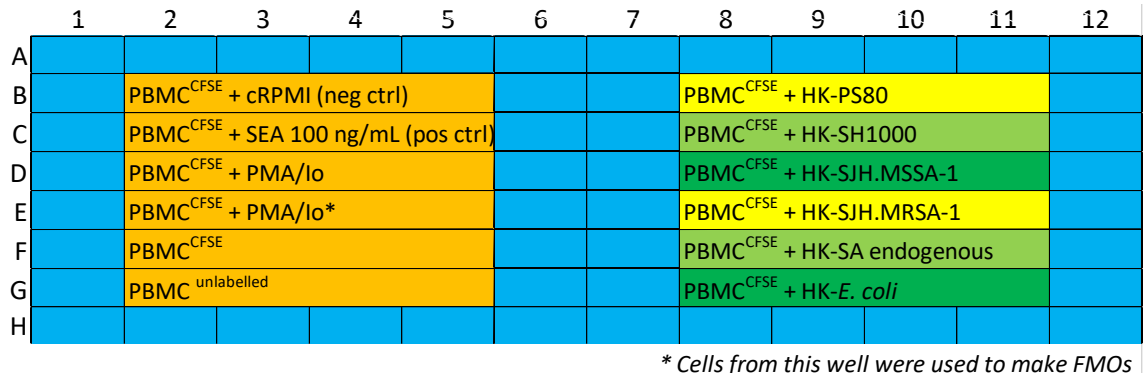

Figure 2.3. Plate map PBMC *S. aureus* antigen-specific proliferation assay

CFSE-labelled PBMCs were cultured at 2 x 10^5 cells per well with control and test antigens for 10 d before Brefeldin A (5 µg/mL) was added for the final 16 h. Sterile PBS was added to empty outer wells to prevent evaporation. Control wells are indicated in orange. Sample wells are indicated in yellow/green. Heat-killed bacterial antigens were added at 1 µg/mL. 'Endogenous' HK-SA indicates each *S. aureus* bloodstream infection patient's own infecting isolate.

SEA = staphylococcal enterotoxin A (100 ng/mL); PMA = Phorbol 12-myristate 13-acetate (50 ng/mL); Io = ionomycin (500 ng/mL); FMO = fluorescence minus one.

2.4.5. T cell-enriched lymphocyte proliferation assay

A schematic for investigation of antigen-induced T cell proliferation and cytokine production in healthy blood donors is shown in Fig. 2.4. To test the T cell-activating potential of purified ClfA, human PBMCs were isolated from healthy blood donor buffy coats in the same way as described above (see *2.4.1*). After separation of PBMCs, they were divided into three batches – those that would act as antigen-presenting cells (APCs, 1-2 x 10^7 cells), those for $CD4^+$ T cell purification (1 x 10^8 cells) and those to act as autofluorescence control (1 x 10^7 cells). PBMCs to act as APCs were gamma-irradiated at 30 Gy with a ^{137}Cs source (Gammacell 3000, Best Theratronics). Irradiated PBMCs were centrifuged at 300g for 5 min and washed twice with cRPMI before being counted and CFSE-labelled (as above). $CD4^+$ T cell purification of PBMCs by magnetic activated cell sorting (MACS) increases T:APC ratio approximately four-fold as compared to physiological concentration (13:1 versus 3:1). A human $CD4^+$ T cell Isolation Kit (Miltenyi Biotec) was used which allows for negative selection of $CD4^+$ T cells. A cocktail of biotin-conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD123, TCRγ/δ and CD235a (Glycophorin A) bind non-target cells and retain them on a MACS column in the magnetic field of a MACS Separator. The unlabelled helper T cells pass through the column. PBMCs for $CD4^+$ purification were centrifuged at 300g for 5 min and re-suspended in cooled MACS buffer (Table 2.2, 40 µL per 10^7 cells). T cell Biotin-Antibody Cocktail (10 µL per 107 cells) was added. The cell suspension was mixed thoroughly and incubated at 4°C for 5 mins. After incubation, MACS buffer (30 µL per 10^7 cells) and human $CD4^+$ T cell MicroBead Cocktail (20 µL per 10^7 cells) were added, before mixing and incubating at 4°C for 10 mins. After incubation the cells were added to an LS column, (a column suitable for the depletion of strongly magnetically labelled cells) before being placed in the magnetic field of the MACS Separator. The column was first prepared by washing it with 3mL of MACS buffer. The cell suspension was applied to the column and the flow-through containing the unlabelled cells was collected. The column was washed with a further 3mL of MACS buffer to complete collection. Purified $CD4^+$ T cells were then washed in cRPMI before counting and labelling with CFSE (as above).

CFSE-labelled $CD4^+$ cells (1 x 10^5 per well) and CFSE-labelled irradiated PBMCs (1 x 10^5 per well) were co-cultured in 96-well round-bottomed plates and stimulated with ClfA (5 µg/mL, 10 µg/mL), CpG (10 µg/mL, Oligos Etc.), ClfA + CpG (5 µg/mL + 10 µg/mL) or heat-killed *S. aureus* PS80 (1µg/ml total protein concentration). Control wells had cells cultured in cRPMI alone (negative control), staphylococcal enterotoxin A (100 ng/ml, Sigma [positive control]),

lipopolysaccharide (LPS, 1 μg/mL, Sigma). Purified $CD4^+$ cells and irradiated 'APC' PBMCs were also each cultured separately with individual antigens to control for HLA II-restricted antigen presentation. Two plates were prepared for each sample to allow cell culture supernatants to be collected on day 4 for cytokine analysis by ELISA (plate A), and later $CD4^+$ T cell proliferation to be assessed on day 10 (plate B). For intracellular cytokine staining, Brefeldin A (5 μg/ml, Sigma) was added to test wells for the final 16 h of culture, while supplementary positive control wells (without antigen) were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5μg/ml) for the final 4 h before harvesting for flow cytometry.

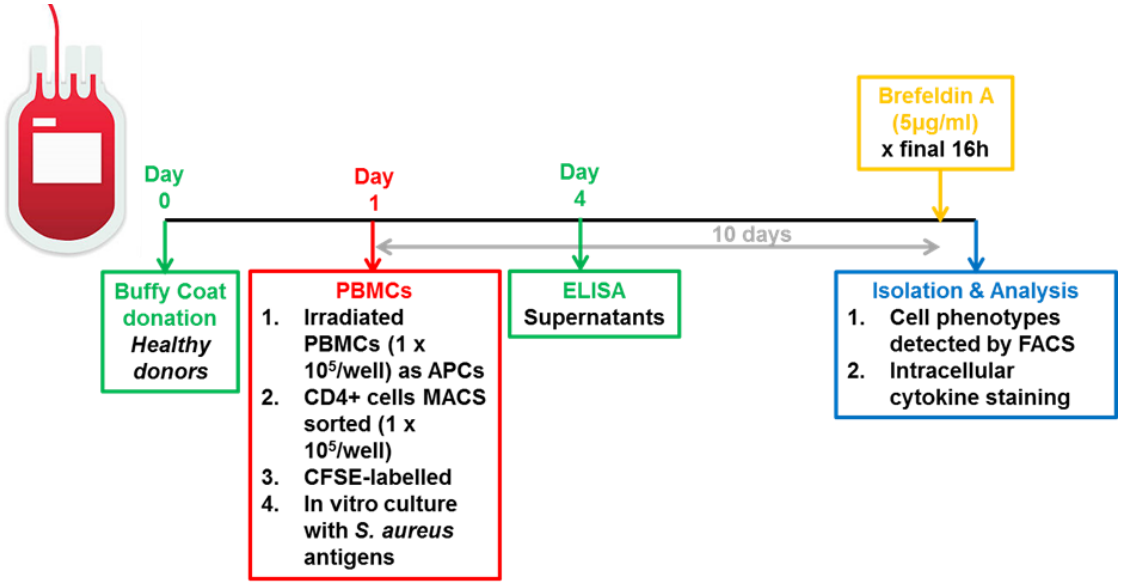

Figure 2.4. CD4⁺ T cell-enriched assay for investigation of antigen-specific T helper proliferation

PBMCs were isolated from the buffy coats of healthy blood donors. 1-2 x 10^7 cells were irradiated to act as antigen-presenting cells (APCs). CD4⁺ T cells were negatively selected from 1 x 10^8 PBMCs by retention of non-target cells in a magnetic field column. APCs and CD4⁺ T cells were then CFSE-labelled before being co-cultured in a 1:1 ratio (1 x 10^5 cells of each/well) with staphylococcal or adjuvant antigens: Clumping factor A (ClfA) 5 µg/mL and 10 µg/mL, CpG (10 µg/mL), ClfA (5 µg/mL) + CpG (10 µg/mL), heat-killed *S. aureus* PS80 (1 µg/mL) for 10 days. Brefeldin A (5 µg/mL) was added for the final 16 h before cells were evaluated by flow cytometry for CD4⁺ T cell proliferation and intracellular cytokine production.

2.4.6. Surface and intracellular cytokine staining (ICS)

Short re-stimulation assay:

After overnight incubation, cells were centrifuged at 300g for 10 min and cell pellets re-suspended in round-bottomed 96-well plates in PBS (100 µL/well) before repeat centrifugation and surface staining as below.

Proliferation assay:

After 10 days incubation, cells from quadruplicate wells were pooled in microcentrifuge tubes and centrifuged at 300g for 10 min. Cell pellets were re-suspended in round-bottomed 96-well plates in PBS (100 µL/well). After further centrifugation at 300g for 3 min, cells were resuspended in Live/Dead Aqua stain (50 µL/well, Invitrogen), diluted to a working concentration of 1:1000 in PBS, for 20 mins at 4°C.

All assays:

Cells were washed again in PBS and centrifuged at 300 g for 3 mins before being re-suspended in a final concentration of 1:100 of specific cell surface antibodies (50 µL/well) diluted in FACS buffer (Table 2.2). Samples were incubated in the dark at room temperature for 15 min. Using the Dakocytomation Intrastain Kit (Alere) to stain for intracellular cytokines, 50µl of Intrastain Reagent A, a fixative reagent (contains 5-10% formaldehyde) was added to each well, before again being incubated in the dark at RT for 15 min. Samples were then washed in FACS buffer and centrifuged at 300xg for 3 mins. Next, 50µl/well of Intrastain Reagent B, a permeabilisation reagent, containing fluorescence conjugated antibodies specific for intracellular cytokines (Table 2.4) was added. Cells were re-suspended in a final concentration of 1:100 intracellular antibody solution diluted in Reagent B and incubated for 15 mins at RT in the dark. Samples were washed again and re-suspended in FACS tubes (BD Biosciences) in PBS.

2.4.7. Flow cytometry

Flow cytometry analysis was performed on freshly stained cells with BD FACSCanto II or BD LSRFortessa using FACS DIVA software (BD Biosciences) and further analysed with FlowJo (Treestar, Inc.). Gating strategy for the proliferation assay is shown in Fig 2.5. Events were gated on lymphocytes using forward and side scatter properties, with a loose gate to ensure inclusion of larger dividing lymphoblasts. Live cells were identified by viability staining. Proliferation was assessed by gating on $CD3^+CD4^+CFSE_{lo}$ cells in the $CD4^+$ population (or the

$CD4^+CD45RO^+$ population for assessment of memory cell proliferation). Antigen-specific proliferation was calculated by subtracting any background $CFSE_{lo}$ proportions in the negative control wells (i.e. cRPMI media only) from test $CFSE_{lo}$ populations. Antigen-specific cytokine responses in proliferating cells were assessed by gating on $IFN\gamma^+$, TNFα, or $IL\text{-}17^+$ $CFSE_{lo}$ cells within the $CD4^+$ population. Antigen-specific cytokine production was similarly calculated by subtracting any background cytokine production seen in negative control wells. Gates were set on respective Fluorescence Minus One (FMOs).

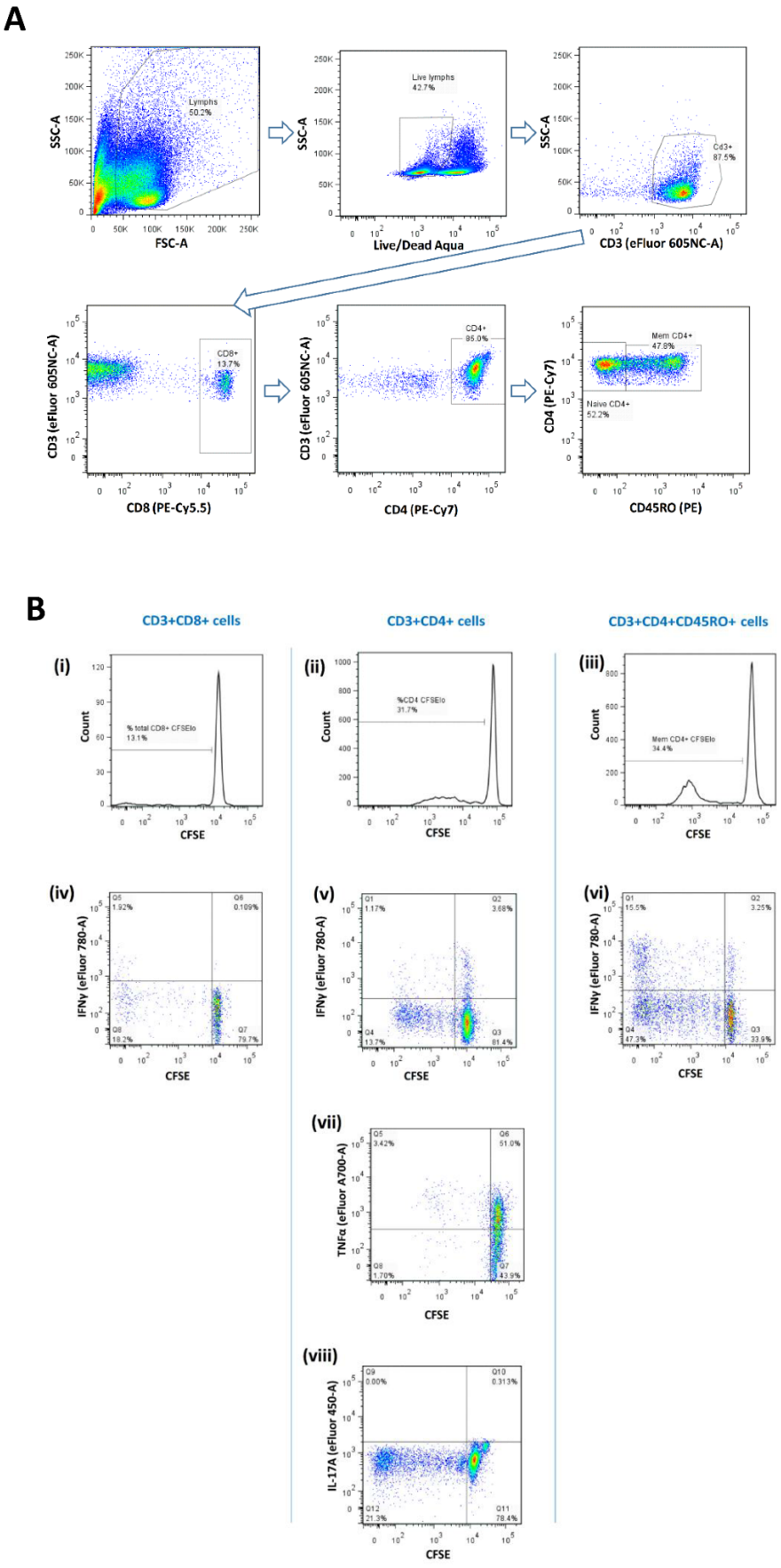

Figure 2.5. Fluorescence-activated cell sorting (FACS) gating strategy for proliferation assay

Figure 2.5 (continued)

After 10 d culture with heat-killed bacteria, CFSE-labelled PBMCs were further stained with FACS antibodies. Representative plots are shown to illustrate stepwise analysis from top left (A). Events were gated on lymphocytes using forward scatter (FSC-A) and side scatter (SSC-A) areas, using a relatively loose gate to ensure inclusion of dividing immature blast lymphocytes. Live lymphocytes were then identified by viability staining with Live/Dead Aqua. $CD3^+$ staining within this population identified T lymphocytes. $CD8^+$ and $CD4^+$ cells were then selected within the $CD3^+$ population. Memory ($CD45RO^+$) and naïve (CD45RO-) $CD4^+$ cells were further distinguished within the $CD4^+$ population. Lymphocyte proliferation was assessed by gating on $CFSE_{lo}$ cells within the $CD8^+$ [B(i)], $CD4^+$ [B(ii)] and memory $CD4^+CD45RO^+$ [B(iii)] populations. Undivided lymphocytes were $CFSE^{hi}$. Antigen-specific proliferation was calculated by subtracting background $CFSE_{lo}$ proportions in the negative control cells (i.e. media only) from that of test cells. IFNγ responses in proliferating cells were assessed by gating on $IFN\gamma^+CFSE_{lo}$ cells in the $CD8^+$ [B(iv)], $CD4^+$ [B(v)] or memory $CD4^+CD45RO^+$ [B(vi)] populations. TNFα [B(vii)] and IL-17A [B(viii)] responses were similarly assessed by gating on $CFSE_{lo}$ cells in the $CD4^+$ population. Antigen-specific cytokine production in proliferating cells was calculated by subtracting any background cytokine production seen in $CFSE_{lo}[cytokine]^+$ quadrants of the negative control. All gates were set on respective Fluorescence Minus One (FMOs).

CFSE = carboxyfluorescein succinimidyl ester.

2.4.8. ELISA

ELISAs for IFNγ, IL-5, IL-10 and IL-17A (eBioscience) were performed in duplicate on human serum and human cell culture supernatants, as per the manufacturer's instructions (Table 2.5). Supernatants were collected at relevant time points and stored at -80°C until assayed. Supernatants for IFNγ measurement generally required 1:10 dilution with cRPMI before addition to wells to bring values within detection range (top working standard 500 pg/mL). Dilution was generally not required for other cytokine supernatant measurements or for serum ELISAs. A high affinity protein-binding 96-well flat-bottomed plate was coated with capture antibody (diluted to working concentration in 1X coating buffer, 50µl/well) and was incubated at RT overnight. Wells were washed in ELISA wash buffer (Table 2.6) 3 times and incubated with 1X assay diluent (AD, 200µl/well) for 1 h at RT to block non-specific binding. Wells were again washed and cytokine standards and samples (50µl) were added to the appropriate wells for 2 h at RT or overnight at 4°C. Following sample incubation, wells were washed and detection antibody solution, diluted to a working concentration in AD (50µl/well), was added for 2 h at RT. After washing, 50µl streptavidin-horse radish peroxidase (HRP) solution, diluted to a working concentration (Table 2.6, 1:250) in RD, was added to the wells for 30 mins at RT in the dark. After washing, substrate solution (Table 2.6, 50µl/well) was added to the wells for 15 mins at RT in the dark. The reaction was stopped using ELISA stop reagent (Table 2.6, 25µl/well). Absorbance was measured at 450nm using a micro-plate reader and Softmax Pro software.

2.4.9. Antibody Assays

Antibody assays were carried out by the van Wamel laboratory, Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center Rotterdam. To study the humoral immune response in healthy volunteers, SA-BSI and EC-BSI patients, the total IgG levels against 35 (10 cell-wall and 25 secreted proteins) purified staphylococcal antigens were measured in serum using a bead-based flow cytometry technique (xMAPH; Luminex Corporation), as previously described [186]. Briefly, serum samples were diluted 1:100 in PBS and secondary phycoerythrin (PE)-labelled goat anti-human antibodies against total IgG were diluted 1:200. Purified non-staphylococcal bacterial proteins were coupled to beads as negative controls. The purified *S. aureus* antigens used were His-tagged recombinant proteins coupled to xMAPH beads (Luminex Corporation, Austin, TX, USA): Luk = leukocidins D, E, S; HlgB = haemolysin γ; IsaA = immunodominant staphylococcal antigen A;

SE = staphylococcal enterotoxins A, B, C, D, E, H, I, J, M, N, O, Q; SCIN = staphylococcal complement inhibitor; NUC = thermonuclease; CHIPS = chemotaxis inhibitory protein of *S. aureus*; HLA = α-haemolysin; Clf = clumping factor A and B; Efb = fibrinogen-binding protein E; LytM = peptidoglycan hydrolase; SAK = staphylokinase; ET = exfoliative toxin A and B; SdrE = (serine-aspartate repeat protein) bone sialoprotein binding factor; Isd = iron-regulated surface determinants A and H; Fnbp = fibrinogen-binding protein A and B; Sdr = serine-aspartate repeat protein D and E; SasG = *S. aureus* surface protein G. All measurements were performed in duplicate and the median fluorescence intensities (MFIs), a semi-quantitative measure of antibody levels, were averaged. All measurements were corrected for non-specific background signal by subtracting the MFIs of control beads not coupled to any protein.

2.5. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Categorical differences between groups were analysed using Fisher's exact test. Normality was tested using a D'Agostino-Pearson test. Non-parametric analyses were used unless otherwise stated, as results followed a non-normal distribution. Non-categorical differences between non-normally distributed groups were analysed using a Mann-Whitney U test or Kruskal-Wallis test with Dunn's multiple-comparison post-test. Correlation was assessed using the Pearson correlation coefficient test for normally-distributed data. A p value <0.05 was considered significant.

Chapter 3

Clinical and Microbiological characterisation of *S. aureus* bloodstream infection

3.1. Introduction

"The simple things must be known first. We must first of all know the properties of the different organisms and the power of each one to bring about pathological processes, before we are in a position to understand the complicated picture of so-called blood poisoning."

– Alexander Ogston, 1880

A fascination for what exactly it is about *S. aureus* that can lead to such deadly disease has occupied science since the organism was first described [1,187,188]. The identification of dominant invasive strains of other pathogens has successfully focussed immunisation efforts. For instance, pneumococcal and meningococcal vaccines only target the serogroups responsible for most invasive disease. Protective immunity that follows infection with, or immunisation against, these pathogens is thus strain-specific rather than species-specific. Identifying *S. aureus* clones particularly refined for transmission or pathogenicity may similarly help to target them in immunisation strategies.

S. aureus evolved primarily by infrequent mutations. This resulted in a relatively clonal population structure – a feature which should be conducive to vaccine development. Roughly ten lineages predominate among human isolates [189]. As molecular techniques have developed, more complex interrogation of *S. aureus* genotypic and phenotypic profiles has become possible. A stable highly conserved 'core' genome accounts for ~75% of genes and is the most reliable method of inferring evolutionary relationships. The combination patterns of seven 'housekeeping' genes in this core genome are used to classify *S. aureus* into multi-locus sequence types (MLST or 'ST') and clonal complex (CC) families [38]. Clonal distribution of invasive *S. aureus* isolates differs around the world (Fig. 3.1). Predominant European clones are CC5, CC22, CC8 and CC30 whereas, in the USA, SA-BSIs are dominated by CC8 and CC5 with much less variability, probably due to the greater contribution of MRSA clones [190]. However, efforts to genotypically define *S. aureus* lineages associated with severe infection have had underwhelming results thus far. It has not been consistently shown that strains associated with invasive disease or poor clinical outcomes have particular characteristics [147,191-193].

The more variable accessory genome encodes for resistance and virulence factors, often located on mobile genetic elements that can be transferred horizontally [189]. The most notable consequence of this genome interchange is the rise of epidemic strains of methicillin-resistant *S. aureus* (MRSA). The frequency of methicillin resistance varies significantly around the world, from less than 1% of all invasive *S. aureus* isolates in the Netherlands to 46% in the USA [11,41]. The spread of epidemic and pandemic MRSA clones, particularly adapted to

transmission in healthcare environments, is alarming. The current predominant MRSA strain in Europe is EMRSA-15 (ST22-MRSA-IV), which emerged in the UK in the 1980s from a community-associated MSSA population [193,194]. The EMRSA-15 clone spread globally at a rate far higher than the spread of its parent MSSA-ST22 population, and has gone on to acquire multi-resistance to a range of other antibiotic classes. The more recent proliferation of community-acquired (CA-MRSA) clones in North America has been an additional cause for concern. These CA-MRSA strains are most commonly associated with skin and soft tissue infections, but can also cause bloodstream infection [195].

Patients with bloodstream infections present acutely and unpredictably, and confirmation of diagnosis is not available for several days. Prospective clinical research in such patients poses unique challenges. The time window to identify and approach participants is narrow, patients often lack autonomous capacity to consent due to illness, dropout rates may be high due to mortality, and obtaining time-sensitive research samples additional to routine care is onerous. For these reasons, patients with acute illness are grossly under-represented in clinical research [196]. This delays identification of potentially effective, ineffective, and harmful interventions. The level of evidence informing optimum management of *S. aureus* bloodstream (SA-BSI) infection is poor. Fewer than 2000 patients have been enrolled in controlled trials of antimicrobial therapy [197,198]. Consequently, clinical practice is driven by observational data and personal experience. There is no national clinical guidance in Ireland on SA-BSI management [199]. Where guidelines do exist, they frequently only refer to management of MRSA infection [200-202]. There is substantial variance in expertise and clinical practice, between and within countries, in managing SA-BSI, with uncertain consequence for patients [13,197,203]. Efforts to enrol patients with SA-BSI in the acute phase are essential to audit and inform clinical practice and to develop novel strategies to prevent and tackle this life-threatening infection.

The first part of this chapter aims to describe and contrast the clinical characteristics and course of *E. coli* and *S. aureus* bloodstream infection patients. The second part will characterise invasive *S. aureus* strains, their expression of potential vaccine antigens and consider the implication of this for vaccine development, using clumping factor A as a model.

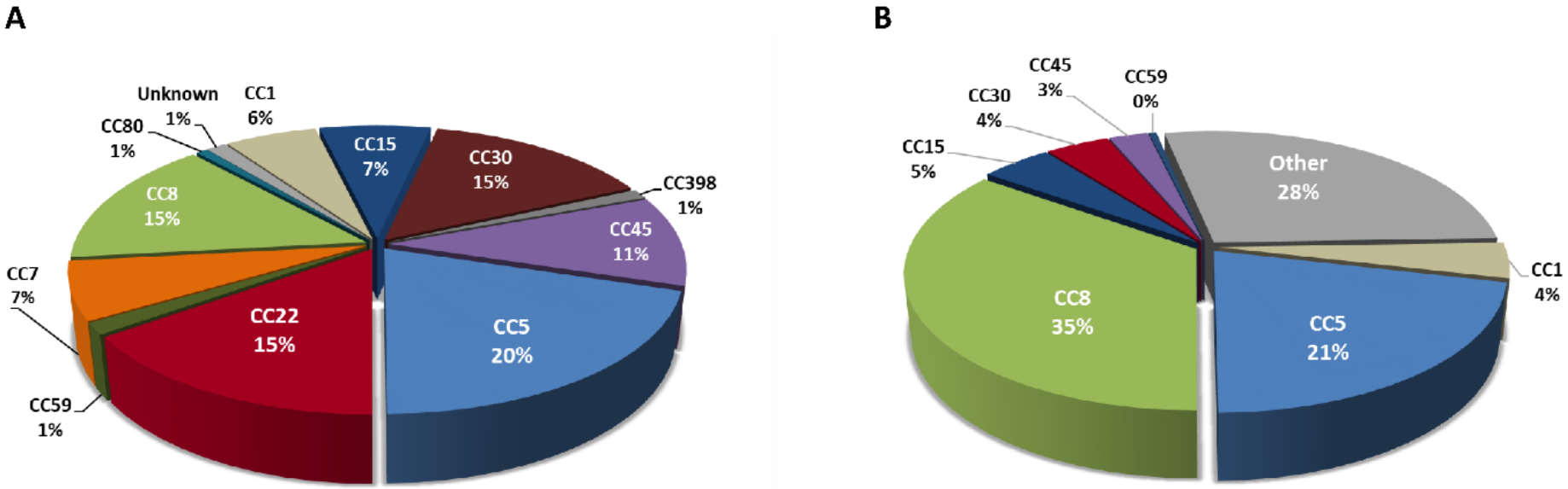

Figure 3.1. Clonal complexes of *S. aureus* bloodstream infections in Europe and USA.

Clonal complex (CC) distribution of European (A) and U.S invasive *S. aureus* isolates [190,193]

3.2. Results

3.2.1. Recruitment and clinical course of patients with *S. aureus* and *E. coli* bloodstream infections

3.2.1.1. Patient Identification and Study Recruitment

Two hundred and fifty six patients with bloodstream infections were prospectively assessed for eligibility to be included in the study – 116 *S. aureus* bloodstream infection (SA-BSI) patients and 140 *E. coli* bloodstream infection (EC-BSI) patients. Inclusion and exclusion criteria are listed in Table 2.7. Reasons for non-enrolment are illustrated in Fig. 3.2. Final numbers included in each analysis are reported in Fig. 3.3. Thirty-five SA-BSI patients were excluded for medical reasons (malignancy = 16; infection with HIV, hepatitis B and/or hepatitis C = 13; transplant or other immunosuppression = 6) Eleven had polymicrobial infection. Twenty-two were unable to consent due to lack of capacity (intubated and sedated, significant delirium, or cognitive impairment). In light of this, a later amendment to the ethics submission allowing assent for participation by next-of-kin or other representatives for patients lacking capacity to consent was submitted and approved at one site. A single SA-BSI patient declined to participate. Thirteen had died, been discharged, self-discharged against medical advice or been transferred to another hospital before they could be approached for inclusion. Five were more than 7±2 days post-initial bacteraemia. One patient had been transferred from another hospital without the bacterial isolate being available and two could not have sufficient study blood samples taken for inclusion. Of patients with EC-BSI, 55 were excluded for medical reasons (malignancy = 50; infection with hepatitis B = 1; transplant or other immunosuppression = 4). Ten had polymicrobial infection. Forty-two were unable to consent due to lack of capacity (intubated, significant delirium, dementia or cognitive impairment). Four EC-BSI patients did not consent to participate. Thirteen had died, been discharged or been transferred to another hospital before they could be approached for inclusion, and 5 were more than 7±2 days post-initial bacteraemia.

Overall, 35 immunocompetent adult patients with 36 episodes of bloodstream infection (BSI) were recruited between February 2013 and November 2014 from three tertiary care centres – 24 *S. aureus* (SA-BSI) and eleven *Escherichia coli* (EC-BSI). This represented 21% of SA-BSI episodes and 8% of EC-BSI initially evaluated for potential inclusion. A single SA-BSI patient (SA003) was recruited a second time with a relapsed infection 11 months after the initial episode.

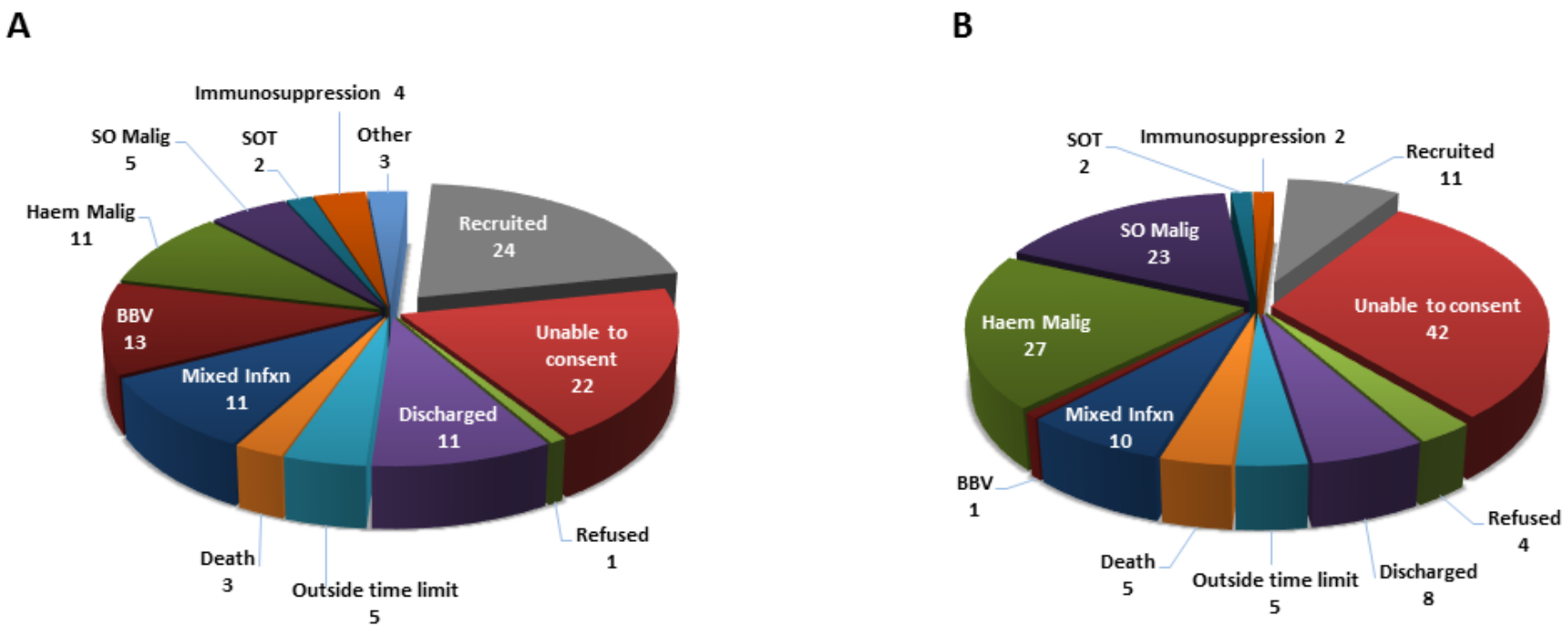

Figure 3.2. Reasons for non-recruitment of *S. aureus* or *E. coli* bloodstream infection patients.

Reasons for exclusion or non-recruitment of *S. aureus* BSI (A), n=116 and *E. coli* BSI (B), n=140 patients. Totals exceed number of patients assessed as 7 SA-BSI and 4 EC-BSI patients had more than one exclusion criteria.

BSI = bloodstream infection; mixed infxn = polymicrobial BSI; BBV = blood-borne viruses (human immunodeficiency virus, hepatitis B and/or hepatitis C); Haem malig = haematological malignancy; SO malig = solid organ malignancy; SOT = solid organ transplant.

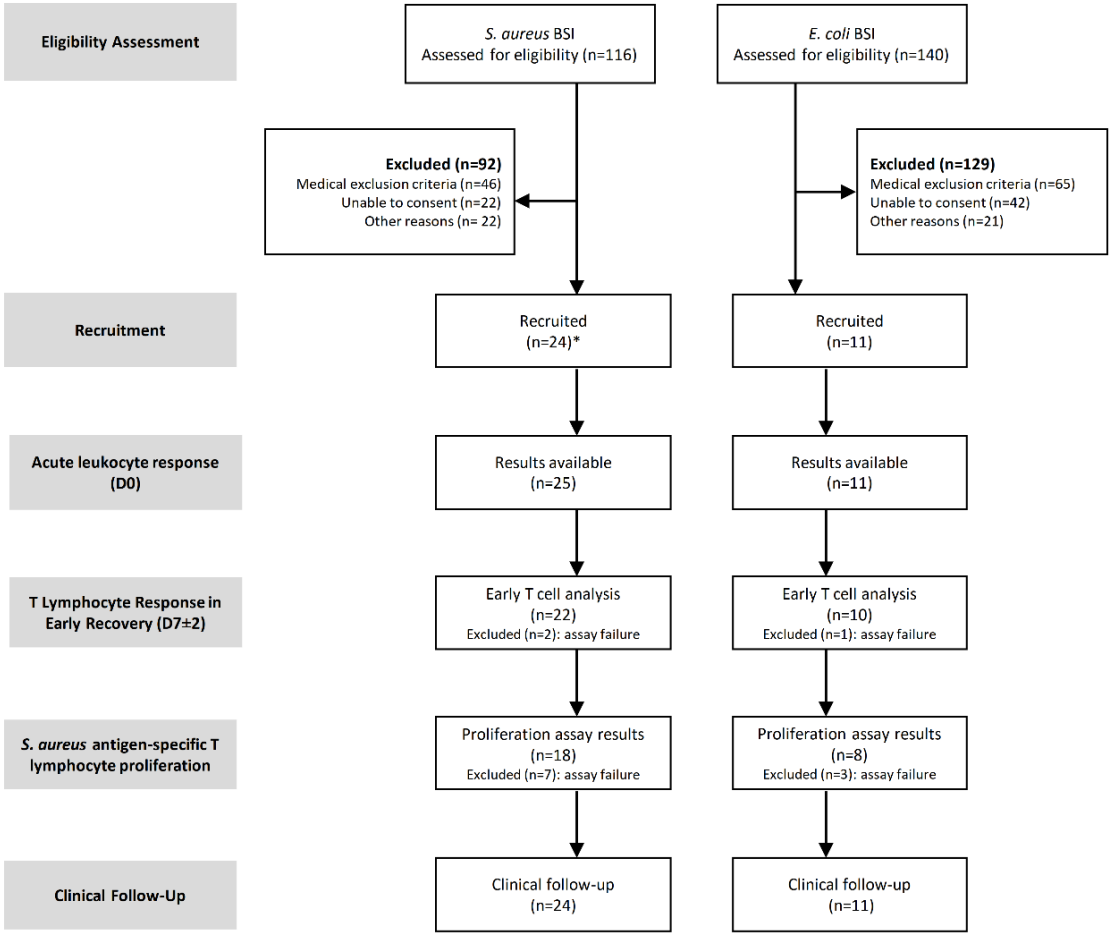

Figure 3.3. Eligibility assessment, recruitment of patient groups and inclusion in subsequent analyses.

Numbers of patients with *S. aureus* or *E. coli* bloodstream infections assessed for eligibility for inclusion, reasons for exclusion, and final recruitment.

*A single *S. aureus* bloodstream infection patient was recruited twice; first with the initial episode, and 11 months later with a relapse, meaning that 24 patients were recruited with 25 episodes of *S. aureus* bloodstream infection. All subsequent figures relate to availability of individual assay results. Reasons for assay failures include incomplete FACS compensation (n=3), incomplete antibody panel (n=1), failure of CFSE staining (n=3), cell culture media batch contamination (n=6).

3.2.1.2. Clinical Characteristics, Management and Outcomes

Demographics of SA-BSI and EC-BSI patients are listed in Table 3.1 and Table 3.2 respectively and compared in Table 3.3. While both organisms infected a wide range of age groups and the overall difference was not statistically significant (*S. aureus* median 47.5, range 22-96 years vs *E. coli* median 75; range 27-96 years; p = 0.06), SA-BSI disproportionately affected younger patients (Fig. 3.4A). Over twice as many SA-BSI patients were younger than 60 years [14 (58%) SA-BSI versus 3 (27%) EC-BSI patients]. There was a male predominance in the SA-BSI group [n=17 (71%) vs 3 (27%) EC-BSI].

Acquisition

Half of SA-BSI episodes were healthcare-associated[1]. Six (25%) were secondary to intravenous catheters newly inserted during hospital admission (3 peripheral and 3 central) and another 4 (17%) related to long-term haemodialysis catheters. A further 3 were associated with diabetic foot ulcers with recent healthcare contact. The remaining twelve 12 SA-BSI episodes were community-acquired. Seven (29%) of these patients presented with primary bacteraemia (of unknown source) and the others were associated with injecting drug use (Table 3.1). The majority [n=9 (82%)] of *E. coli* BSI episodes were community-acquired, with the urinary tract the source for all but two patients [n=9 (82%)]. Patient EC011 had a pyogenic liver abscess in the setting of a metastatic upper GI adenocarcinoma unknown at the time of recruitment and diagnosed several months later, and the source for another (EC008) was unknown (Table 3.2). EC011 was retained despite the later diagnosis of malignancy and deemed a minor protocol deviation given that the original rationale for excluding solid organ malignancy patients was on the basis that they would mostly be subject to severe iatrogenic lymphocyte-specific immunosuppression as part of treatment.

[1] Healthcare-associated infections were defined as (i) index positive blood culture collected ≥48hrs after hospital admission, and no signs or symptoms of the infection noted at time of admission; OR (ii) index positive blood culture collected <48hrs after hospital admission if any of the following criteria are met: received intravenous therapy in an ambulatory setting in the 30 days before onset of BSI, attended a hospital clinic or haemodialysis in the 30 days before onset of BSI, hospitalised in an acute care hospital for ≥ 2 days in the 90 days prior to onset of BSI, resident of nursing home or long-term care facility [see *2.2.2*, p48-49].

Complexity of Infection

SA-BSI episodes were overwhelmingly (88%) complicated[2]. Nineteen (79%) episodes had clear evidence of deep-seated focal or distant metastatic infection, especially at bony or endovascular sites (Table 3.1, Figs 3.4B and 3.5). Bone infection, either contiguous (n=3, 12%) or haematogenous (n=7; 28%) was seen in 10 (40%) SA-BSI episodes. The axial skeleton was more commonly involved in haematogenous cases (n = 5 axial versus n = 2 appendicular skeletal infection). Three SA-BSIs were secondary to chronic diabetic foot ulcers, with *S. aureus* causing contiguous chronic osteomyelitis of the underlying bone in all cases. Persistent endovascular infection was seen in 10 (40%) of SA-BSI patients – 4 regional septic thrombophlebitis, 6 distant septic emboli, 2 right-sided endocarditis and 1 left-sided endocarditis. Even amongst haemodialysis catheter-associated SA-BSI episodes (n=5), most (n=4) were deemed complicated due to clear evidence of metastatic infection. Only 3 episodes of SA-BSI were uncomplicated, all related to temporary intravenous line insertion. There were no cases of toxic shock syndrome. In contrast, most (73%) of the EC-BSI patients followed a largely uncomplicated clinical course. Two EC-BSI patients (18%) presented with septic shock, requiring an initial 12-24 hours of organ support, and one with obstructive nephrolithiasis requiring nephrostomy and ureteric stenting.

Resource Utilisation

Median length of stay was significantly longer for *S. aureus* than for *E. coli* BSI [23.5 days (IQR 16-54.5) vs 10 (IQR 7-15) respectively; p=0.0007) despite the SA-BSI population being generally younger and healthier (Fig. 3.6A). Full treatment details were available for 31 episodes (20 SA-BSI and 11 EC-BSI). Antimicrobial consumption was significantly greater in *S. aureus*-infected patients [median 87 (IQR 49-132) versus 14 defined daily doses (IQR 12-21) for SA- and EC-BSI respectively; p <0.0001] (Fig. 3.6B). Defined daily doses (DDD) are the World Health Organisation's assumed average maintenance dose per day for a drug used for its main indication in adults [204]. Antimicrobial consumptiion for SA-BSI is shown by agent in Table 3.4. Flucloxacillin was the most used therapy [1551 DDDs (1504 intravenous, 47 oral)], followed by daptomycin (266 DDDs). Four SA-BSI patients had their treatment completed with outpatient antimicrobial therapy (OPAT). Oral adjunctive or post-IV

[2] Complicated *S. aureus* BSI is defined as an episode meeting any of the following predictors: delayed clearance (i.e., a positive follow-up blood culture at 48–96 h); persistent fever at 72 h; community-acquired infection; systemic skin lesions. Uncomplicated SA-BSI cases must fulfil all of the following: catheter-associated infection with removal of the catheter; negative follow-up blood culture at 48–96 h; defervescence within 72 h; normal transoesophageal echocardiogram; absence of prosthetic material in joints or intravascular space; and no symptoms suggestive of metastatic infection [see *2.2.2*, p48-49].

consolidation therapy (rifampicin, flucloxacilllin, coamoxiclav, doxycycline, cotrimoxazole and ciprofloxacin) was used for 324 days in 8 patients.

Clinical Management

All EC-BSI patiens received optimal management. The management of SA-BSI is more complex. Infection specialists were involved in the management of all *S. aureus* BSIs – 12 episodes were managed with Clinical Microbiology telephone advice, 3 with Clinical Microbiology bedside review and 10 under direct care of Infectious Diseases physicians. Two patients took their own discharge, truncating their intravenous treatment. Eleven (46%) SA-BSI episodes were not managed in accordance with published quality standards [205]. These deviations occured when patients were not under the care of an infection specialist. Delayed diagnosis, delayed source control, failure to collect repeat cultures, subtherapeutic glycopeptide drug levels [troughs < 15 μg/mL in 2 of 5 (40%) patients who received vancomycin for > 5 days], delayed introduction of beta-lactam therapy and/or inappropriately short therapy were all seen. For instance, SA006a was treated with vancomycin for MSSA infection due to a reported vague penicillin allergy. The research clinician was able to quickly exclude this by confirming with the patient's GP that amoxicillin had been tolerated previously. SA006's vancomycin trough levels remained subtherapeutic for the first 5 days of therapy. Even when dosed appropriately, vancomycin is strongly associated with inferior treatment outcomes in MSSA BSI, compared with beta-lactam therapy [206]. Furthermore, telephone advice was given to add rifampicin for 4 SA-BSI patients (1 with uncomplicated infection, none of whom had indwelling prosthetic devices). These recommendations for rifampicin were advised over the telephone without checking concomitant medications. Two SA-BSI patients had significant drug:drug interactions due to rifampicin administration; with methadone (precipitating opiate withdrawal symptoms) and warfarin (leading to an inability to achieve adequate anticoagulation for deep vein thrombosis).

Outcome

Follow-up clinical data was available for 24 SA-BSI and 11 EC-BSI episodes (Tables 3.1 and 3.2). All EC-BSI patients improved during their initial hospital admissions and were discharged well. None had recurrent *E. coli* infection or died within the in-hospital or follow-up period (8-18 months). One (EC003) was readmitted with candidaemia related to indwelling ureteric stents. A second patient (EC011) was readmitted with advancing malignant disease (underlying carcinoma involving the gallbladder) while undergoing palliative chemotherapy. Fifteen (60%) SA-BSI patients improved without readmission in the

follow-up period. Three patients (12%) died within 90 days of SA-BSI. Causes of death were aspiration pneumonia (SA001), multi-organ failure (SA019) and pulmonary oedema (SA023), of which only the latter was considered unrelated to *S. aureus* infection. Six patients (25%) had recurrent *S. aureus* disease within the follow-up period, all of whom had initially had complicated infection. One patient (SA002) presented 11 months later with MSSA soft tissue infection (shoulder abscess requiring debridement and skin grafting), most likely secondary to ongoing injecting drug use. Three (SA003, SA021 and SA022) had recurrent BSI (likely relapses), related to haemodialysis access and/or diabetic foot infection. SA003 initiallly had a dialysis line-related SA-BSI complicated by septic arthritis. Management was suboptimal given the line was changed over a wire, there was no line-free period and vancomycin rather than beta-lactam therapy was given [207,208]. This patient represented 11 months later with an identical bloodstream isolate on whole genome sequencing, a lead vegetation and right-sided endocarditis. SA021, another haemodialysis patient with a history of recurrent MRSA line infections and diabetic foot infections presented with recurrent MRSA BSI complicated by a mycotic aortic aneurysm 5 months after initial recruitment, despite optimal management. SA022 presented with recurrent BSI 16 days after completion of treatment of a persistent diabetic foot infection. Three of the 4 patients with diabetic foot osteomyelitis (SA004, SA016 and SA022) ultimately required surgical cure with transmetatarsal or below-knee amputations, having failed prolonged antimicrobial treatment.

Table 3.1. Clinical characteristics and outcomes of *S. aureus* bloodstream infection patients

ID	Age	Sex	Acquisition	Source Known	Source	Complicated	Metastatic Infection	Major deep-seated infection site	Additional Clinical Information	Outcome
SA001	89	M	CA	N	-	Y	Y	**Bone** (lumbar discitis)	Admitted with new onset back pain, LLL weakness. No blood cultures. Developed fever and hypotension 4 weeks later during admission. Discitis evident on imaging.	**In-hospital death** day 52: ?aspiration pneumonia
SA002	31	M	CA	Y	IDU	Y	Y	**Endovascular** (femoral septic thrombophlebitis)	Groin pain and inflammation. Re-using needles. Fever, rigors.	**Recurrence:** MSSA SSTI after 10 months (shoulder abscess)
SA003(i)†	67	M	HA	Y	Tunnelled CVC-HD	Y	Y	**Endovascular** (infected vascath) AND **Bone** (shoulder septic arthritis)	Presented with fever, rigors and shoulder pain.	**Recurrence**: MSSA BSI after 11 months
SA003(ii) †	68	M	HA	Y	Tunnelled CVC-HD	Y	Y	**Endovascular** (lead and tricuspid vegetation)	Presented with fever and rigors.	Improved
SA004 MRSA	62	M	HCA	Y	DFI	Y	N	**Bone** (contiguous forefoot osteomyelitis)	Worsening chronic forefoot ulcer. Fever and rigors.	**Failure**: transmetatarsal amputation after 10 months
SA005A	31	M	CA	N	-	Y	Y	**Bone** (epidural abscess)	Presented with fever, rigors and cauda equina. Epidural abscess. Prior distant lumbar spine injury (gymnast). CT-guided drainage = MSSA.	Improved. OPAT
SA006A	42	F	CA	Y	IDU	Y	Y	**Endovascular** (R sided endocarditis with TV vegetation and perforation, femoral septic thrombophlebitis, septic pulmonary emboli)	Presented with fever and rigors	Improved

Table 3.1. (continued)

ID	Age	Sex	Acquisition	Source Known	Source	Complicated	Metastatic Infection	Major deep-seated infection site	Additional Clinical Information	Outcome
SA007	26	M	CA	Y	IDU	Y	Y	**Endovascular** (femoral septic thrombophlebitis)	Fever, rigors, groin pain.	Self d/c. No readmission to initial hospital.
SA008	23	F	CA	Y	IDU	Y	Y	**Endovascular** (femoral septic thrombophlebitis, septic pulmonary emboli)	**Septic shock.**	Self d/c. No readmission to initial hospital.
SA009	96	F	HA	Y	PVC	N	N	-	Admitted with lower GI bleed. Fever.	Improved
SA010	84	M	HA	Y	Non-tunnelled CVC	N	N	-	Aortic valvuloplasty complicated by pulmonary oedema requiring IV diuresis and vasodilators.	Improved
SA011	70	F	CA	N	-	Y	Y	**Bone** (cervical epidural abscess)	Presented with upper back pain and fever. Cervical epidural abscess decompressed.	Improved
SA012	30	M	CA	N	-	Y	Y	**Bone** (haematogenous sternal osteomyelitis)	Recurrent SSTI. Presented with fever and chest pain.	Improved
SA013	40	F	HA	Y	PVC	N	N	-	Alcohol withdrawal seizure. Line site cellulitis/phlebitis	Improved
SA014	36	M	HA	Y	Non-tunnelled CVC-HD	Y	Y	**Endovascular** (septic pulmonary emboli)	AKI post-colectomy requiring CVC for HD. MSSA at line site. Fevers persisted after line removal. Septic pulmonary emboli.	Improved
SA015 MRSA	71	M	HCA	Y	Tunnelled CVC-HD	N	N	-	**Septic shock** causing AKI. Improved but ongoing need for HD via tunnelled CVC. Recurrent sepsis 4 weeks after initial presentation. Tunnelled line removed and dialysed via temporary line.	Recruited episode was **recurrence**. Subsequent improvement after treatment of second BSI.

Table 3.1. (continued)

ID	Age	Sex	Acquisition	Source Known	Source	Complicated	Metastatic Infection	Major deep-seated infection site	Additional Clinical Information	Outcome
SA016	53	M	HCA	Y	DFI	Y	N	**Bone** (contiguous forefoot osteomyelitis)	Admitted with necrotic R 5th toe ulcer and OM 5th MT head for regional amputation. Recurrence of prior staphylococcal + streptococcal OM same site 4 months previously despite 6 wks IV beta-lactam.	**Failure** despite OPAT 6 wks: below-knee amputation after 2 months
SA017	39	M	HA	Y	Non-tunnelled CVC	Y	Y	*IVC filter in situ, not overtly infected*	Trauma. R femoral ORIF. Post-op bilateral pulmonary emboli with IVC filter placement.	Improved
SA018	35	M	CA	Y	IDU	Y	Y	**Endovascular** (septic pulmonary emboli)	Admitted with groin abscess	Improved
SA019	78	F	CA	N	-	Y	Y	**Endovascular** (mitral and aortic valve endocarditis, septic cerebral emboli)	Prosthetic mitral valve. Primary bacteraemia. Attempted medical management.	**In-hospital death** day 27: multi-organ failure
SA020	22	M	CA	N	-	Y	Y	**Bone** (hip septic arthritis, femoral osteomyelitis and quadriceps pyomyositis)	Athlete with prior groin injury.	Improved. OPAT
SA021 MRSA	53	M	HA	Y	Tunnelled CVC-HD	Y	Y	**Bone** (haematogenous acromioclavicular joint septic arthritis)	ESRD diabetic nephropathy. Known MRSA colonised with previous line sepsis. Presented with fever, rigors and shoulder pain.	**Recurrence**: MRSA BSI after 5 months, complicated by mycotic aortic aneurysm requiring endovascular repair.
SA022	77	F	HCA	Y	DFI	Y	N	**Bone** (midfoot contiguous osteomyelitis)	Recurrent polymicrobial OM L forefoot. Admitted with nausea, fever, rigors.	**Recurrence**: MSSA BSI 16 days after stopping short-course (2 wk) therapy. **Failure**: Transmetatarsal amputation 3 months after initial BSI despite OPAT

Table 3.1. (continued)

ID	Age	Sex	Acquisition	Source Known	Source	Complicated	Metastatic Infection	Major deep-seated infection site	Additional Clinical Information	Outcome
SA023	61	M	HA	Y	PVC	Y	N	*ICD in situ, not overtly infected*	Admitted with decompensated heart failure. PVC for diuresis. Noted erythema around site.	**In-hospital death** day 90: end stage CCF (not infection-attributable)
SA024	30	M	CA	N	-	Y	Y	**Endovascular** (bilateral upper limb thrombophlebitis)	Atopic dermatitis. Prior SA-BSI and pectoral pyomyositis. Presented with arm pain.	Improved

† = Patient SA003 was initially recruited in February 2013 during a first episode of *S. aureus* BSI and again in February 2014 during a *S. aureus* BSI relapse.
CA = community-acquired; HA = hospital-acquired; HCA = healthcare-associated. IDU = injecting drug use; CVC-HD = central venous catheter for haemodialysis; DFI = diabetic foot infection; PVC = peripheral venous catheter; CVC = central venous catheter: a jugular, subclavian or femoral central line inserted acutely for a short period; ICD = implantable cardiac device

Healthcare-associated infections were defined as (i) index positive blood culture collected ≥48hrs after hospital admission, and no signs or symptoms of the infection noted at time of admission; OR (ii) index positive blood culture collected <48hrs after hospital admission if any of the following criteria are met: received intravenous therapy in an ambulatory setting in the 30 days before onset of BSI, attended a hospital clinic or haemodialysis in the 30 days before onset of BSI, hospitalised in an acute care hospital for ≥ 2 days in the 90 days prior to onset of BSI, resident of nursing home or long-term care facility [209].

Table 3.2. Clinical characteristics and outcomes of *E. coli* bloodstream infection patients

ID	Age	Sex	Acquisition	Prior EC disease	Source Known	Source	Additional Clinical Information	Outcome
EC001a	83	F	CA	N	Y	Urinary	Septic shock requiring inotropes	Improved
EC002	57	F	CA	N	Y	Urinary	Septic shock	Improved
EC003	96	F	CA	N	Y	Urinary	Obstructive nephrolithiasis requiring nephrostomy and ureteric stenting	Readmission with stent-related candidaemia
EC004	43	M	CA	N	Y	Urinary	Pyelonephritis	Improved
EC005	68	M	HA	N	Y	Urinary	Recent insertion of prostatic brachytherapy seeds	Improved
EC006	92	F	CA	N	Y	Urinary	-	Improved
EC007	61	F	CA	N	Y	Urinary	-	Improved
EC008	79	M	HA	N	N	-	Traumatic extradural lumbar haematoma with post-decompression lumbar surgical site collection (pseudomeningocoele). Source unclear	Improved
EC009	27	F	CA	Y	Y	Urinary	APCKD pyelonephritis	improved
EC010	84	F	CA	Y	Y	Urinary	-	Improved
EC011	75	F	CA	N	Y	Intraabdominal	Pyogenic liver abscess, underlying upper GI malignancy	Progressive malignant disease

CA = community-acquired; HA = hospital-acquired; APCKD = autosomal dominant polycystic kidney disease

Table 3.3. Comparison of *S. aureus* and *E. coli* bloodstream infection patient demographics and infection source

Data are displayed as median (interquartile range) and number (percentage). *P* values are calculated by Mann-Whitney and Fisher's exact test respectively.

	S. aureus *(n=24)*	***E. coli*** *(n=11)*	**p-value**
Age	48 (31-71)	75 (57-83)	0.06
Male	17 (71%)	3 (27%)	**0.03**
Co-morbidities			
Haemodialysis*	4 (17%)	0 (0%)	0.28
Diabetes mellitus	6 (25%)	1 (9%)	0.39
Alcohol dependence	2 (8%)	1 (9%)	1.00
Acquisition[a]			
Healthcare-associated	12 (50%)*	2 (18%)	0.14
Source			
Unknown	7 (29%)	1 (9%)	0.39
Central venous catheter	6 (25%)	0 (0%)	0.15
Peripheral venous catheter	3 (13%)	0 (0%)	0.54
Injection drug use	5 (21%)	0 (0%)	0.16
Diabetic foot ulcer †	3 (13%)	0 (0%)	0.54
Urinary tract	0 (0%)	9 (82%)	**<0.001**
Biliary tract	0 (0%)	1 (9%)	0.31

*Patient SA003 was a haemodialysis patient recruited twice; first with an initial episode *S. aureus* BSI, and 12 months later with a relapse, but is counted as a single patient for the purposes of this comparison. All haemodialysis patients recruited dialysed via catheters rather than arteriovenous fistulae.

[a] Healthcare-associated infections were defined as (i) index positive blood culture collected ≥48hrs after hospital admission, and no signs or symptoms of the infection noted at time of admission; OR (ii) index positive blood culture collected <48hrs after hospital admission if any of the following criteria are met: received intravenous therapy in an ambulatory setting in the 30 days before onset of BSI, attended a hospital clinic or haemodialysis in the 30 days before onset of BSI, hospitalised in an acute care hospital for ≥ 2 days in the 90 days prior to onset of BSI, resident of nursing home or long-term care facility.

† Three patients had chronic diabetic foot ulcers as a source of their *S. aureus* BSI, and in all cases the contiguous underlying bone was also found to be infected.

MRSA = methicillin-resistant *Staphylococcus aureus*. NA = not applicable. BSI = bloodstream infection.

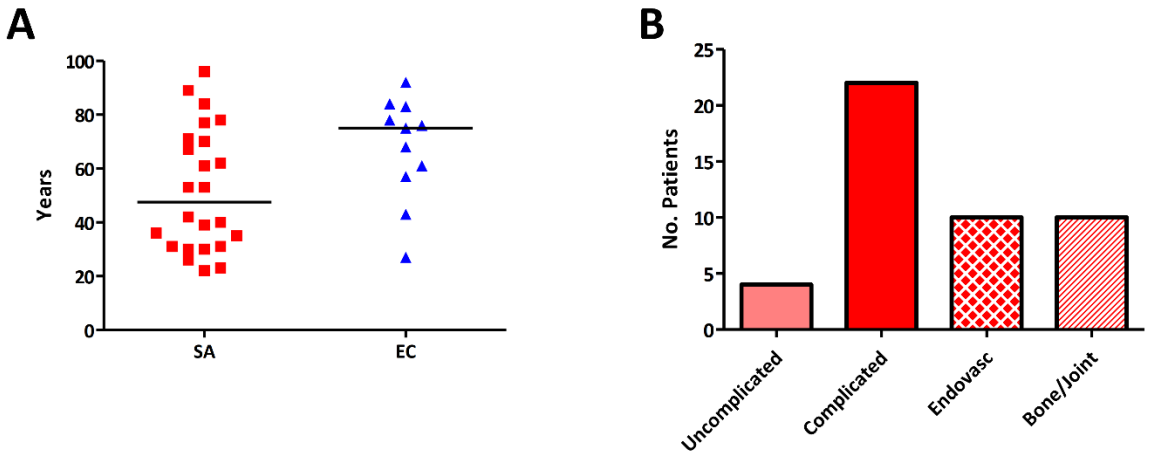

Figure 3.4. Age (*S. aureus* and *E. coli* patients) and complexity of *S. aureus* bloodstream infection.

Ages of adults with *S. aureus* (SA) and *E. coli* (EC) bloodstream infections (BSI) did not differ significantly, although there was a trend towards younger age in SA-BSI (A). Most *S. aureus* BSI episodes were complicated infections and the major sites of metastatic infection were endovascular or skeletal (B). Graphs show individual data points and a median bar. Medians were compared using Mann-Whitney tests.
SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infections
***p<0.001.

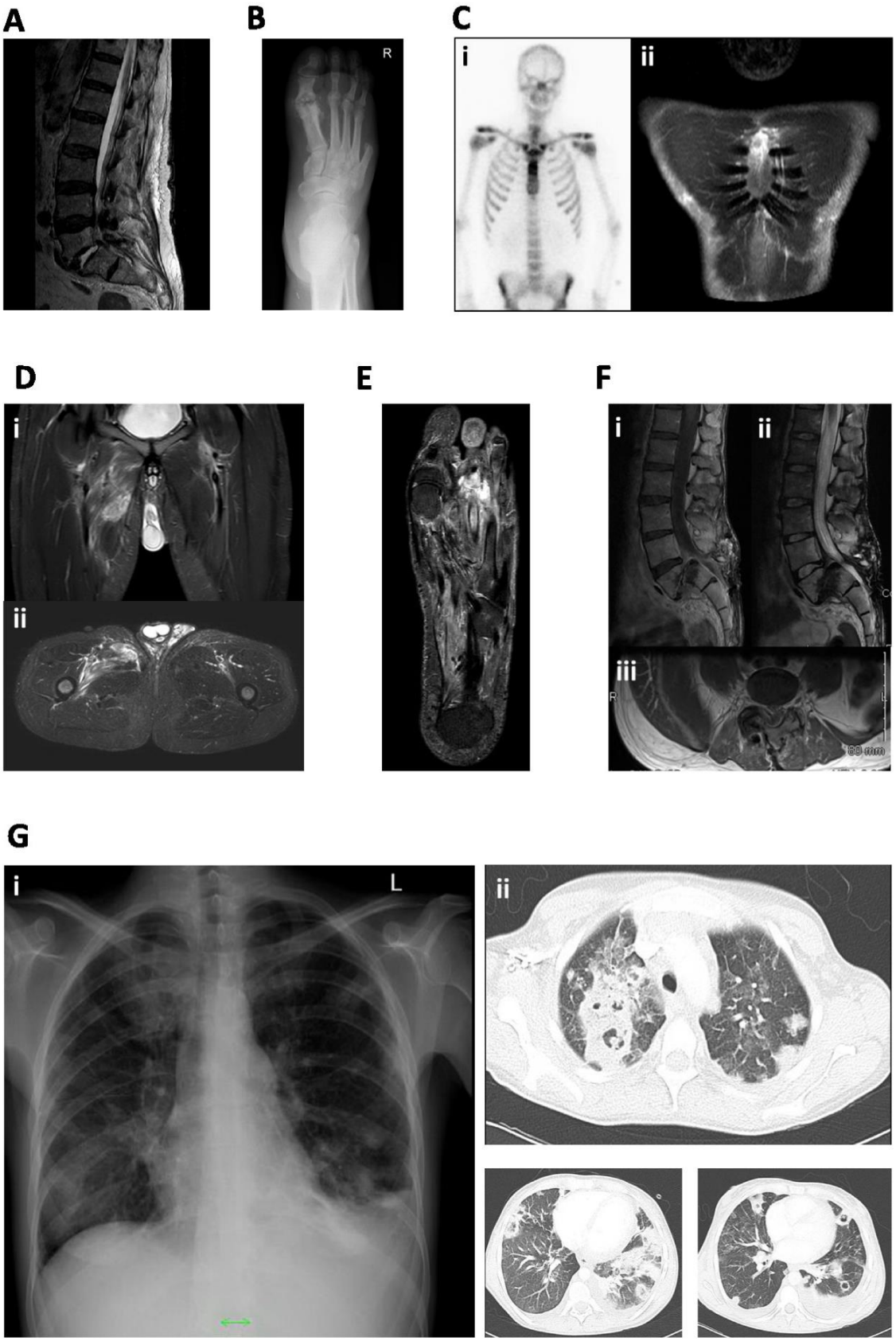

Figure 3.5. Sites of metastatic infection in selected patients with complicated *S. aureus* bloodstream infection.

Figure 3.5 (continued from previous page)

SA01: T2-weighted sagittal MRI image showing high signal in L5/S1 intervertebral disc, with fluid tracking posteriorly into the spinal canal consistent with infective discitis (A).

SA016: X-ray right foot demonstrating advanced destruction of the first metatarso-phalangeal (MTP) joint at the site of an overlying chronic diabetic foot ulcer, with a florid periosteal reaction in the surrounding bones, and less marked destruction of the second MTP joint, indicating septic arthritis and osteomyelitis (B).

SA012: Bone scan (i) showing increased uptake within the superior body of sternum on 3rd phase images, and (ii) coronal MRI slice demonstrating intense enhancement of the sternum and surrounding soft tissue below the sterno-manubrial joint, both consistent with haematogenous sternal osteomyelitis (C).

SA018: Coronal (i) and axial (ii) MRI images of lower limbs showing increased signal intensity around 2 small focal abscesses in the right-sided adductor compartment adjacent to the site of injecting drug use (D).

SA04: T2-weighted axial MRI image demonstrating high signal throughout the 3rd metatarsal bone and proximal phalanx of the 3rd toe, enhancing significantly post-contrast, as well as a joint effusion around the 3rd metatarsophalangeal joint. This communicated to the skin via a chronic diabetic foot ulcer on the plantar surface (E).

SA05a: Sagittal T1- (i) and T2-weighted (ii) MR images showing extensive signal abnormality within the L5/S1 vertebral endplates and a ring-enhancing fluid collection within the anterior L5-S2 epidural space, consistent with an epidural abscess compressing the thecal sac at the level of the cauda equina. Axial (iii) slices also show extensive ill-defined enhancement in the posterior paraspinal soft tissues and muscles at L5-S1 (F).

SA08: CXR (i) demonstrating multiple foci of opacification throughout both lungs, consolidation in the right upper zone with probable cavitation within this, and a lesion in the left lower zone with an air-fluid level. Concurrent CT pulmonary angiogram (ii) showing multiple peripheral patchy areas of consolidation throughout both lungs, many of which demonstrate cavitation. There are also multiple subcentimetre mediastinal and hilar lymph nodes and small pericardial and pleural effusions. This was a consequence of injecting drug use introducing endovascular *S. aureus* infection in the context of a deep vein thrombosis (G).

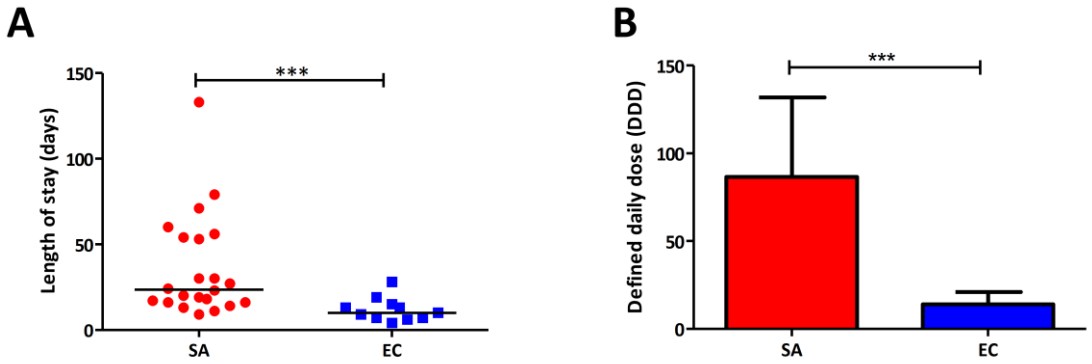

Figure 3.6. Resource utilisation for *S. aureus* and *E. coli* BSI patients.

Length of hospital stay was significantly longer for SA-BSI than EC-BSI patients (A). Antimicrobial consumption per patient episode in defined daily doses for SA-BSI was significantly greater than for EC-BSI (B). Graph A shows individual data points and a median bar, B shows median and interquartile range. Medians were compared using Mann-Whitney tests. SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infections
***p<0.001.

Table 3.4. Antimicrobial consumption in *S. aureus* bloodstream infection patients

	DDDs	Median DDD (IQR)	Patients (n)
Flucloxacillin	1551	84 (47-116)	17
Daptomycin	266	84 (82-100)	3
Rifampicin	105	25 (7-48)	4
Doxycycline	84	84	1
Vancomycin	77	4 (2-12)	10
Co-trimoxazole	42	42	1
Ciprofloxacin*	42	42	1
Coamoxiclav	42	42	1
Cefazolin	28	28	1
Ceftriaxone	14	14	1
Piperacillin-tazobactam	14	5 (2-5)	3
Meropenem	6	6	1

*One patient received ciprofloxacin for a polymicrobial DFI (*S. aureus* + *E. coli*) that had resulted in a monomicrobial *S. aureus* bloodstream infection

DDD = defined daily doses

3.2.2 Genotypic and phenotypic characterisation of *S. aureus* bloodstream isolates

3.2.2.1. Multilocus sequence typing (MLST) and clonal complex (CC) distribution

MLST (multilocus sequence typing) exploits the limited variability of the core genome to classify *S. aureus* into genetically related populations by determining allelic profiles of seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi*, and *yqiL*). Twenty-two (88%) of 25 *S. aureus* bloodstream isolates from 24 recruited patients were available for genomic analysis. DNA was extracted from these and from and 2 laboratory reference (PS80 and SH1000) strains of *S. aureus*. Genomes were sequenced, and sequence type (ST) was inferred by automated comparison to reference strains TW20 or Newman in the *S. aureus* database (http://saureus.mlst.net/). This was performed in Sir Patrick Dun Translational Research Laboratory, School of Medicine, Trinity College Dublin, St James's Hospital, Dublin. Eleven different ST types were represented (Table 3.5 and Fig 3.7A), one of which (SA016) did not match any previously known MLST profile in the database, but had some similarities to ST5. All MRSA isolates were healthcare-associated and belonged to ST22, accounting for 3 (60%) of the 5 ST22 strains. This is consistent with the known dominant European epidemic healthcare-associated MRSA clone ST22-MRSA-IV/EMRSA-15 [191,210]. Two of the 5 ST22 strains (40%) in this cohort were methicillin-susceptible.

Clonal complexes are defined as groups of STs sharing 100% identity at ≥ 5 of 7 loci. The 11 STs found were divided into 9 clonal complex 'superfamilies' by comparison with known isolates in the database. (Table 3.5). Two groups (CC5 and CC22) accounted for 52% of bloodstream isolates (Fig 3.7B). Isolates from patient SA003 in 2013 and 2014 were genetically identical, representing a true relapsed infection. Otherwise, the clinical *S. aureus* bloodstream isolates displayed notable genetic diversity, and also differed from the two laboratory reference strains. A maximum-likelihood neighbour-joining tree was generated based on 109,533 variant sites identified through comparative analysis of whole-genome sequence data (Fig 3.8). There was no clustering of clonal complexes by hospital site (Fig 3.9), acquisition risk (Fig 3.10), or complexity of *S. aureus* infection (Fig 3.11). Taken together, there was considerable genotypic heterogeneity in invasive *S. aureus* isolates, particularly MSSA, despite the small size of the study population and the geographic proximity of study sites. This confirms our inability to define *S. aureus* genetic lineages consistently associated with severe infection. Perhaps it is not surprising that relatively conserved non-virulent 'housekeeping' enzymes essential for bacterial survival and basic function do not correlate

with clinical manifestations of infection. It is thus important to further examine microbial factors more likely to confer pathogenicity, as these may be more promising vaccine targets.

Table 3.5. Characteristics of invasive *S. aureus* isolates.

Molecular analysis of *S. aureus* bloodstream isolates was performed using next-generation sequencing to determine MLST type and clonal complex by automated comparison to reference strains in the *S. aureus* database (http://saureus.mlst.net/). Methicillin resistance was determined by phenotypic testing after culture on solid media to determine cefoxitin disc diffusion zones with respect to CLSI clinical breakpoints.

Strain	ST Type	Clonal Complex	Methicillin resistance
SA01	NT	NT	MSSA
SA02	ST7	CC7	MSSA
SA03ǂ	ST5	CC5	MSSA
SA04	ST22	CC22	**MRSA**
SA05a	ST188	CC1	MSSA
SA06a	ST1	CC1	MSSA
SA07	ST5	CC5	MSSA
SA08	ST15	CC15	MSSA
SA09	ST5	CC5	MSSA
SA10	ST22	CC22	MSSA
SA11	ST6	CC6	MSSA
SA12	NT	NT	MSSA
SA13	NT	NT	MSSA
SA14	ST30	CC30	MSSA
SA15	ST22	CC22	**MRSA**
SA16	ST**	CC5	MSSA
SA17	ST5	CC5	MSSA
SA18	ST5	CC5	MSSA
SA19	ST508	CC45	MSSA
SA20	ST5	CC5	MSSA
SA21	ST22	CC22	**MRSA**
SA22	ST59	CC59	MSSA
SA23	ST22	CC22	MSSA
SA24	ST1	CC1	MSSA

NT = not tested. SA01, SA12 and SA13 isolates were not viable for analysis (dry slopes) from the clinical laboratory.
ǂ = Strains SA03i and SA03ii were genetically indistinguishable, indicating true relapse in patient SA03.
ST = ST type not matching previously reported types in PUBMLST database (similar to ST5).**

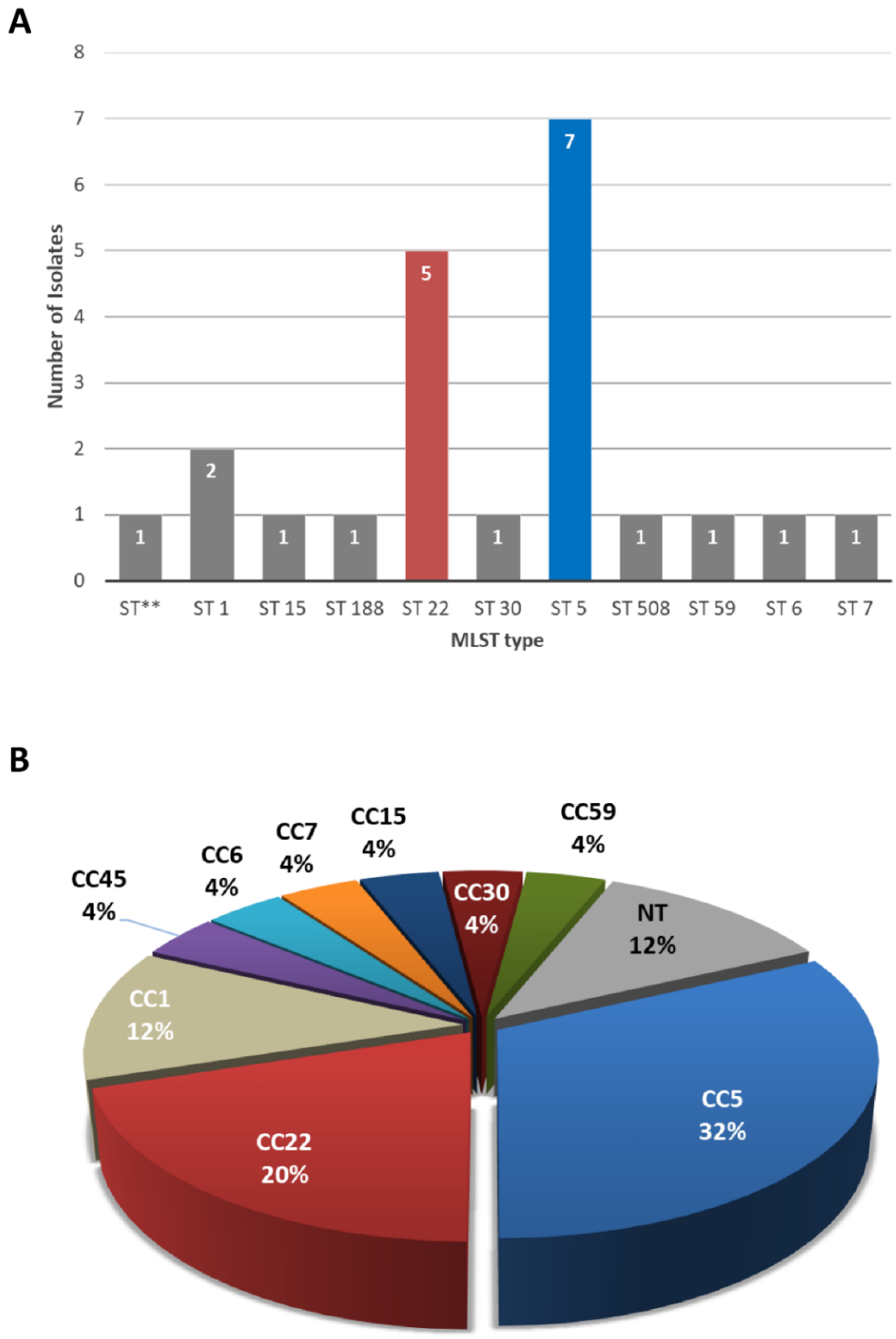

Figure 3.7. MLST type and clonal complex distributions of *S. aureus* bloodstream isolates

Twenty-two of the 25 clinical isolates were available for next-generation sequencing. DNA was extracted from 23 invasive clinical and 2 laboratory reference (PS80 and SH1000) strains of *S. aureus*. Genomes were sequenced and mapped to the *S. aureus* TW20 reference genome (NC017331). Results were used to infer sequence type and clonal complex (CC) by automated comparison to reference strains in the *S. aureus* database (http://saureus.mlst.net/). Allelic profiles of seven housekeeping genes were assigned to ST types by comparison with known isolates in the database (A). ST** denotes an isolate (SA16) that did not match any previously reported ST type, but was similar to ST5. ST types identical at ≥ 5 of the 7 'housekeeping' gene loci were grouped into clonal complexes. Distribution of clonal complexes among *S. aureus* bloodstream isolates is shown (B).

CC = clonal complex; NT = not tested (not available).

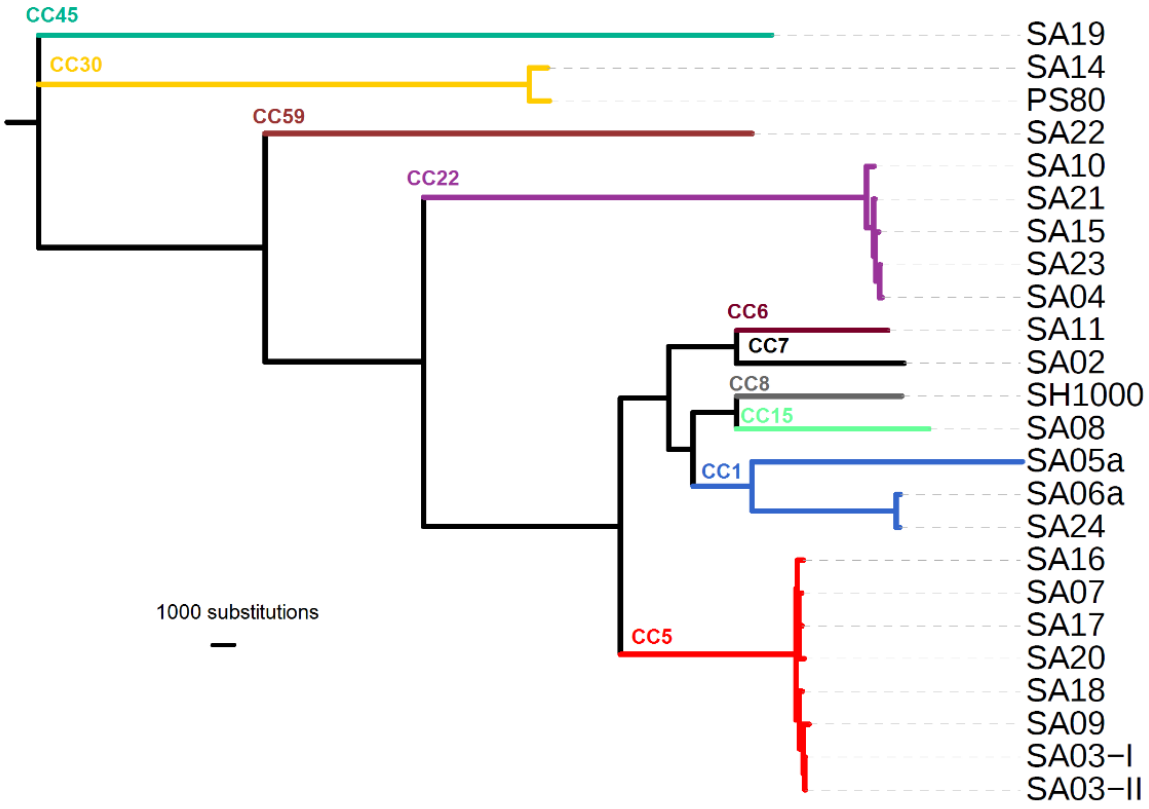

Figure 3.8. Whole genome sequencing demonstrates significant genetic diversity among invasive *S. aureus* isolates.

DNA was extracted from 22 invasive clinical strains and 2 laboratory reference strains (PS80 and SH1000) of *S. aureus* and sequenced before mapping to the *S. aureus* TW20 reference genome. A maximum-likelihood neighbour-joining tree was generated based on 109533 variant sites identified through comparative analysis of whole-genome sequence data. Branch colours correspond to *S. aureus* clonal complex (CC).
Figure courtesy of Dr Micheál MacAogáin.

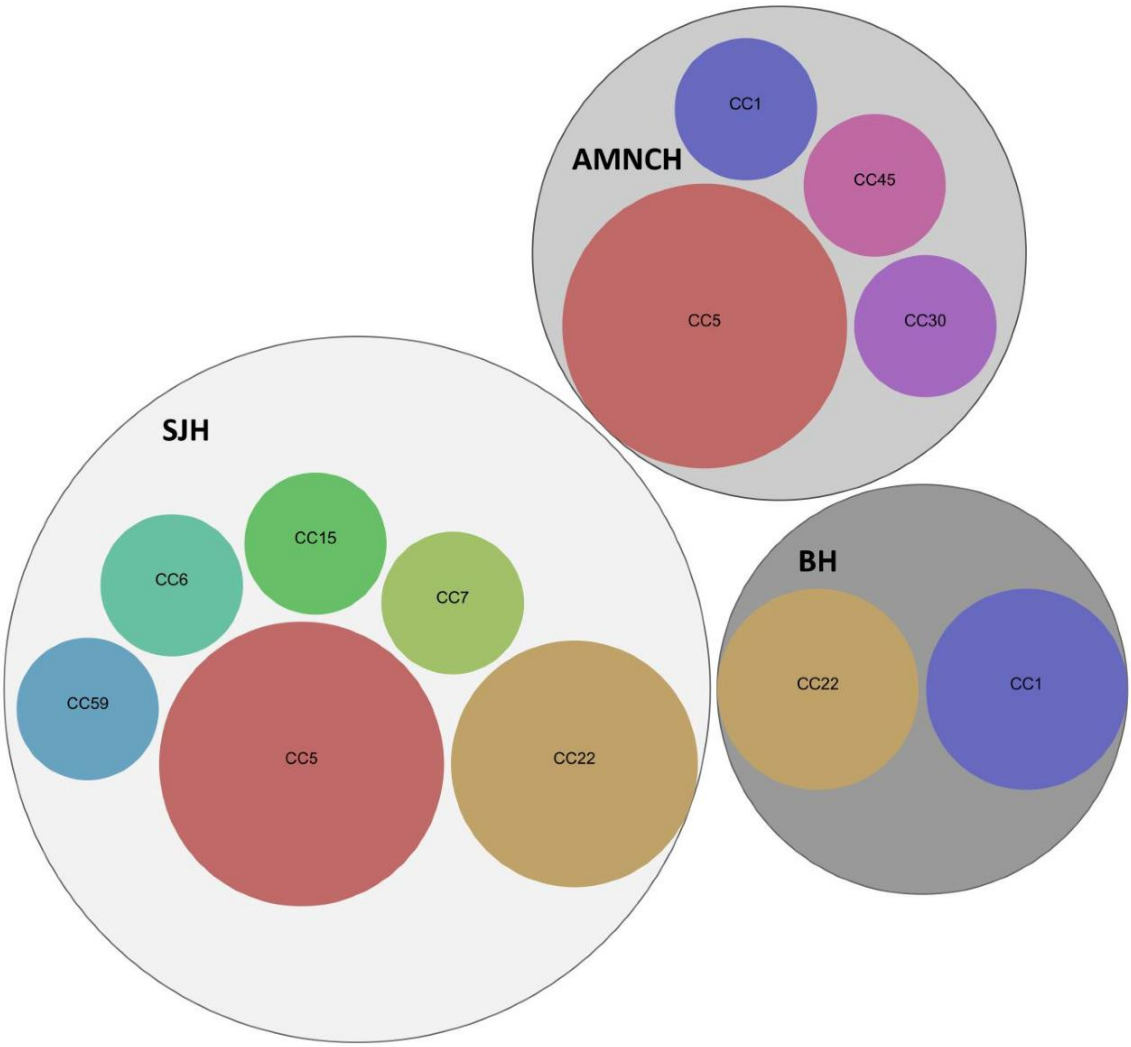

Figure 3.9. Lineages of bloodstream *S. aureus* strains do not cluster by site

Clonal complex distribution is shown by hospital site. Data is shown with circle-packed enclosure diagrams, where the area of each clonal complex circle is proportional to the number of isolates included.
CC = clonal complex; SJH = St James's Hospital; AMNCH = Adelaide, Meath & National Children's Hospital; BH = Beaumont Hospital.
Chart created with RAWGraphs open access at http://app.rawgraphs.io/.

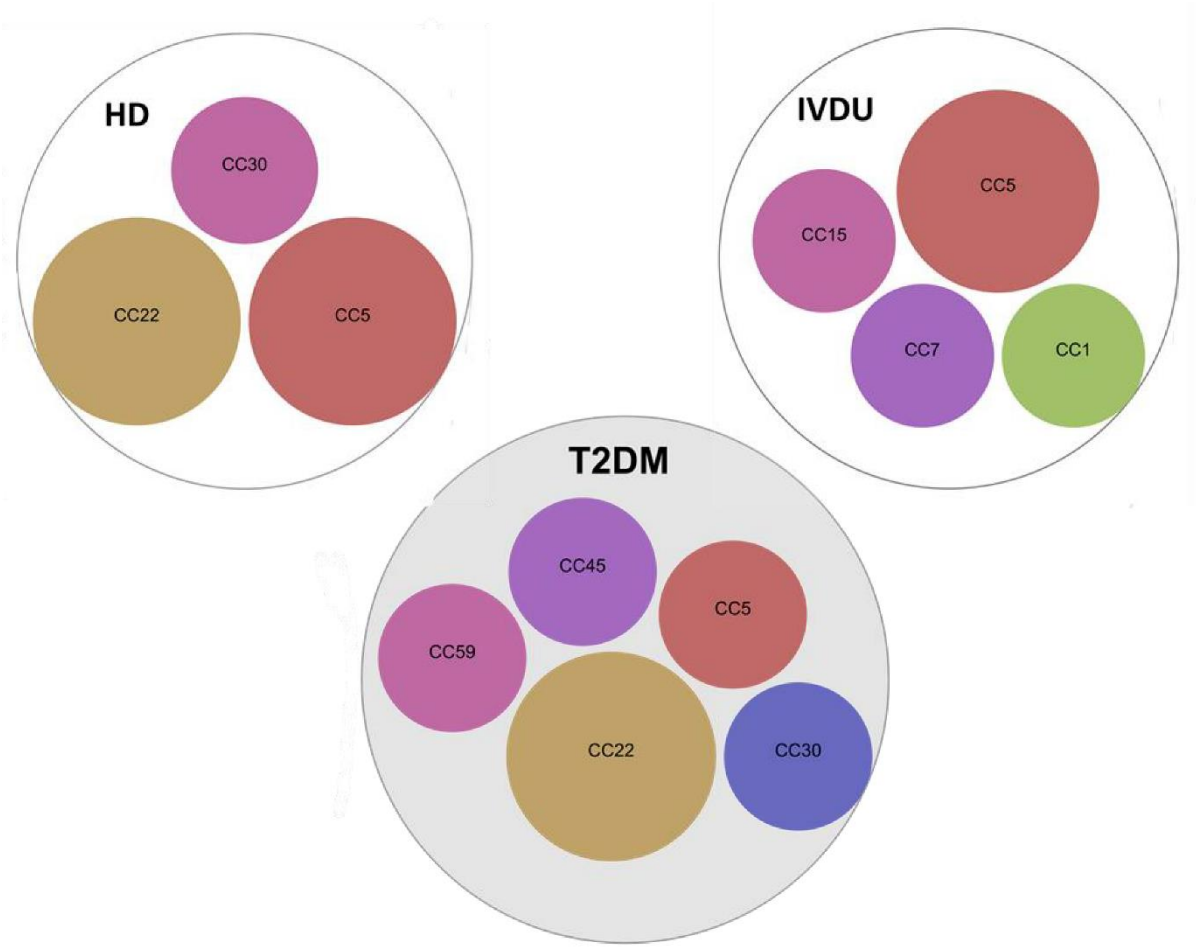

Figure 3.10. Lineages of bloodstream *S. aureus* strains do not cluster by acquisition risk.

Clonal complex distribution is shown by acquisition risk factors. Data is shown with circle-packed enclosure diagrams, where the area of each clonal complex circle is proportional to the number of isolates included.
CC = clonal complex; HD = haemodialysis; IVDU = intravenous drug user; T2DM = type 2 diabetes mellitus.
Chart created with RAWGraphs open access at http://app.rawgraphs.io/

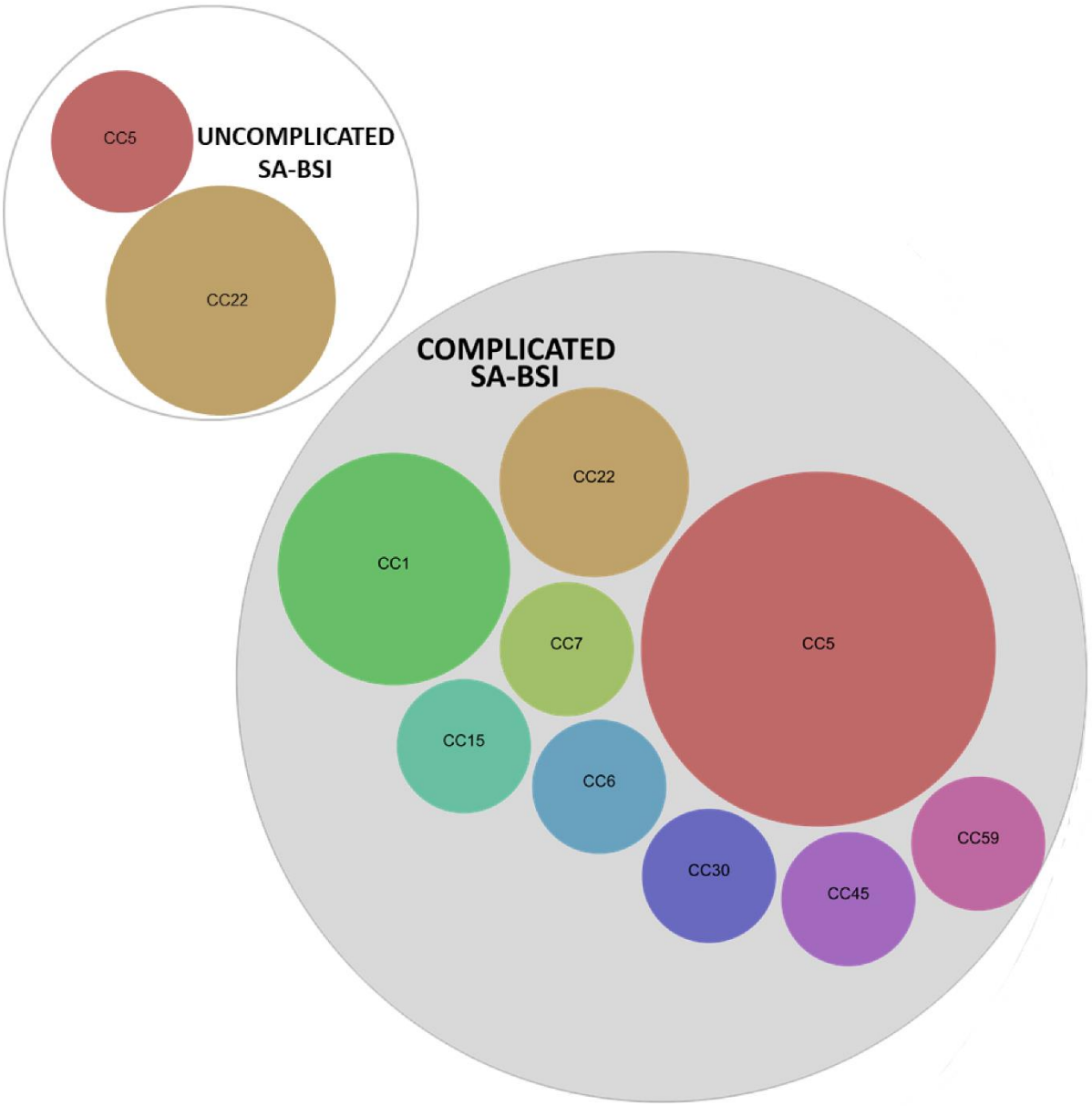

Figure 3.11. Lineages of bloodstream *S. aureus* strains do not cluster by complexity of infection.

Clonal complex distribution is shown by severity of infection as defined by a diagnosis of complicated *S. aureus* bloodstream infection. Data is shown with circle-packed enclosure diagrams, where the area of each clonal complex circle is proportional to the number of isolates included.

CC = clonal complex; SA-BSI = S. aureus *bloodstream infection.*

Chart created with RAWGraphs open access at http://app.rawgraphs.io/.

3.2.2.2. Prevalence of Virulence and Immune Evasion Factor genes in S. aureus *bloodstream isolates*

Having seen that ancestral staphylococcal lineage is not associated with invasive infection, the next step was to interrogate isolates to see if particular staphylococcal attributes are over-represented in invasive strains instead. Certain protein or polysaccharide products of *S. aureus* may be more likely to cluster among pathogenic isolates. Such molecules may therefore be more promising vaccine antigens. Whole genome sequencing to analyse for the presence of genes encoding antigens of interest was performed. Antigens chosen for this analysis have either been included in previous *S. aureus* vaccines; produced humoral response in the study patient cohort; or are known to directly interfere with lymphocyte function. Previous vaccine antigens included clumping factor A (ClfA), iron-regulated heme-iron binding protein B (IsdB), capsular polysaccharides 5 and 8 (CP5 and CP8), ABC transporter ATP-binding protein ykpA (YkpA), manganese transporter C (MntC), lipoteichoic acid (LTA), poly-N-acetyl glucosamine (PNAG), Panton-valentine leucocidin (PVL), α-haemolysin (HLA). Antigens to which an antibody response was demonstrated in our patients (see *4.2.8*) included immunodominant staphylococcal antigen A (IsaA), iron surface determinant A (IsdA) and clumping factor B (ClfB). Antigens implicated in directly evading lymphocyte immunity include staphylococcal protein A (SpA), MHC Class II analog protein (Map), leukotoxin ED (LukED) and staphylococcal enterotoxin A (SEA).

Genes were present in all isolates for cell wall components ClfA, ClfB, PNAG, LTA, IsdB, IsaA, IsdA, Map, YkpA and MntC. All strains had either capsular polysaccharide 5 or 8 genes, polysaccharides included in previously-tested vaccines. Twelve of the clinical isolates (57%) encoded for CP5 and the remaining 43% for CP8. As for secreted toxins, genes encoding α-haemolysin were present in all strains, SEA in 5 (24%), LukED in 13 (62%). No bloodstream isolates contained the gene for Panton-Valentine leukocidin toxin (PVL). Although universally present, the Map and protein A genes showed greatest variability, with 71% and 43% of isolates respectively, showing < 90% sequence homology to the reference genome. These results are summarised in Fig 3.12. In summary, all surface and several secreted staphylococcal components were consistently represented in invasive isolates with a high degree of genetic homology. These molecules may have promise as vaccine antigens.

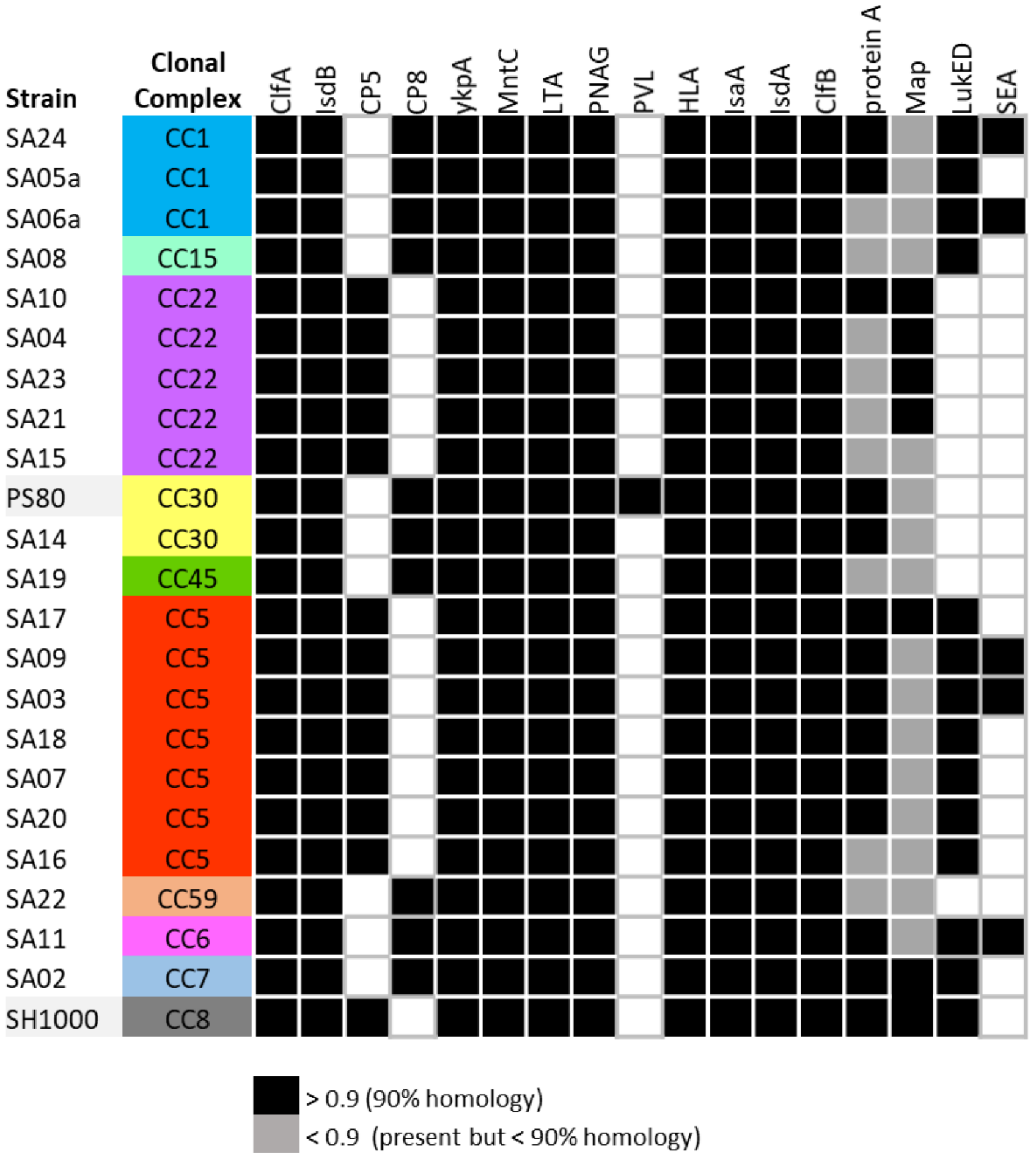

Figure 3.12. Presence of virulence and immune evasion genes in clinical and laboratory *S. aureus* isolates.

DNA was extracted from 23 invasive clinical and 2 laboratory reference (PS80 and SH1000) strains of *S. aureus*. Genomes were sequenced on an Illumina MiSeq instrument and mapped to the *S. aureus* TW20 reference genome (NC017331). Results of next-generation sequencing were used to infer sequence type and clonal complex (CC) by automated comparison to reference strains in the *S. aureus* database (http://saureus.mlst.net/). The presence of genes encoding antigens of interest (virulence or immune evasion factors) in each strain was determined using BLASTN to query draft genome assembly databases for their presence. Isolates are shown grouped by their respective clonal complexes. Patient SA03 was recruited twice after two bloodstream infection episodes separated by 11 months. Both isolates (SA03i and SA03ii) were genetically identical, so only one is shown here. Black boxes denote presence of alleles at relevant loci with >90% homology to reference *S. aureus* strains TW20 or Newman, grey boxes indicate the presence of genes with <90% homology (due to gene variation or sequencing artefact) and white boxes indicate that genes were absent in the tested strains.

ClfA = clumping factor A; IsdB = iron-regulated heme-iron binding protein B; CP5, CP8 = capsular polysaccharides 5 and 8; YkpA = ABC transporter ATP-binding protein ykpA; MntC = manganese transporter C; LTA = lipoteichoic acid; PNAG = poly-N-acetyl glucosamine; PVL = Panton-valentine leucocidin; HLA = alpha-haemolysin; IsaA = immunodominant staphylococcal antigen A; IsdA = iron surface determinant A; ClfB = clumping factor B; Map = MHC Class II analog protein; LukED = leukotoxin ED; SEA = staphylococcal enterotoxin A.

Figure courtesy of Dr Micheál MacAogáin.

3.2.2.3. Clumping factor A illustrates limited antigenic variability of candidate vaccine antigens.

Having interrogated invasive *S. aureus* isolates for conserved and potentially immunogenic antigen genes, several were demonstrated with a high degree of genetic homology between strains. However, even if a gene is present in multiple *S. aureus* strains, it may not be universally transcribed. If transcribed, its degree of expression on the bacterial surface may vary. Finallly, small differences in the genetic code may lead to altered epitopes. This may have implications for vaccine immunogenicity. One potential vaccine antigen will now be examined in further detail. Clumping factor A (ClfA) is a major adhesin that facilitates bacterial binding to host fibrinogen [211]. It has been associated with passive and active protection in several animal models and, as a result, been included in some current candidate vaccine formulations [89,212]. ClfA was present in all clinical and laboratory isolates, and alleles were >90% homologous to *clfA* of reference *S. aureus* strains TW20 or Newman (Fig 3.12).

Whole genomes of 24 (22 bloodstream isolates and 2 reference, PS80 and SH1000) *S. aureus* strains were interrogated for the non-repetitive fibrinogen-binding domain of the *clfA* gene [213]. Thirteen *clfA* allelic variations were identified across the 11 ST types, some differing only by a single point deletion. Variability mostly occurred between, and not within, clonal complex groups (Fig 3.13). CC5 isolates (n=8) possessed either *clfA 002* or *clfA 036*, CC22 (n=5) isolates all had *clfA 022*. However, CC1 isolates (n=3) all possessed different *clfA* alleles. Such allelic variation, albeit limited, may have implications for induction of pan-species immunity.

Strain	Clonal Complex	*clfA* Allele
SA06a	CC1	*clfA 012*
SA05a	CC1	*clfA 019 E139_E169del*
SA24	CC1	*clfA 042 E139_E169del*
SA08	CC15	*clfA 015*
SA10	CC22	*clfA 022*
SA04	CC22	*clfA 022*
SA23	CC22	*clfA 022*
SA21	CC22	*clfA 022*
SA15	CC22	*clfA 022*
PS80	CC30	*clfA 004*
SA14	CC30	*clfA 004*
SA19	CC45	*clfA 009*
SA17	CC5	*clfA 002*
SA07	CC5	*clfA 002*
SA20	CC5	*clfA 002*
SA16	CC5	*clfA 002*
SA09	CC5	*clfA 036*
SA03	CC5	*clfA 036*
SA18	CC5	*clfA 036*
SA22	CC59	*clfA 014*
SA11	CC6	*clfA 019*
SA02	CC7	*clfA 018*
SH1000	CC8	*clfA 010*

Figure 3.13. Allelic variation in clumping factor A (*clfA*) genes of clinical and laboratory *S. aureus* isolates.

DNA was extracted from 22 invasive clinical and 2 laboratory reference (PS80 and SH1000) strains of *S. aureus*. Genomes were sequenced on an Illumina MiSeq instrument and mapped to the *S. aureus* TW20 reference genome (NC017331). Results of next-generation sequencing were used to infer sequence type and clonal complex (CC) by automated comparison to reference strains in the *S. aureus* database (http://saureus.mlst.net/). The presence of the non-repetitive fibrinogen-binding domain of the clumping factor A (*clfA*) gene in each strain was determined using BLASTn to query draft genome assembly databases for its presence. The *clfA* gene was present in all strains and sequenced. Isolates are shown grouped by their respective clonal complexes with corresponding alleles of the *clfA* gene. Two observed 93bp deletions in *clfA* corresponding to 31-residue in-frame deletion at the protein level (E139_E169del) are noted. Alleles cluster by clonal complex and limited allelic variation is present within clonal complexes. Where within-complex variation exists alleles are underlined.
BLASTn = Nucleotide basic local alignment tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

3.2.2.4. Antimicrobial resistance in S. aureus *bloodstream isolates*

All 25 *S. aureus* bloodstream isolates from 24 recruited patients underwent antimicrobial susceptibility testing as part of routine clinical care. Six SA-BSI isolates (25%) were penicillin-susceptible. All of these were community-acquired. Sixteen (64%) of SA-BSI isolates were penicillin-resistant but methicillin-susceptible. Overall, therefore, 22 isolates (88%) from 21 patients (a single patient had a late relapse) were methicillin-susceptible. Three isolates (12%) were methicillin-resistant (MRSA) and were healthcare-associated (Fig 3.14). All isolates were vancomycin susceptible. One MRSA isolate (SA021) had a vancomycin minimum inhibitory concentration (MIC) at the clinical breakpoint of 2 in the context of significant previous vancomycin exposure. All isolates tested (n=14) against daptomycin and linezolid (n=21) were susceptible.

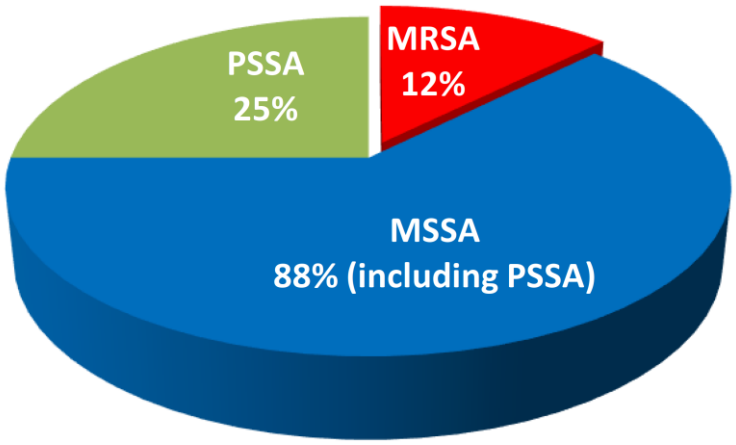

Figure 3.14. Methicillin resistance in *S. aureus* bloodstream isolates from recruited adult patients.

Blood culture isolates were plated on Columbia blood agar. Methicillin resistance was inferred from cefoxitin disc inhibition zones ≥ 21 mm. Rates of methicillin resistant (MRSA) and methicillin-susceptible (MSSA) are shown. Total reported methicillin susceptibility rates include penicillin-susceptible *S. aureus* (PSSA). n=24

3.3. Discussion

The evolutionary strategy of *S. aureus* is driven by clonal expansion of a limited number of highly successful, independent lineages, which should lead to relatively low antigenic diversity. The only prior published typing of Irish SA-BSI isolates has been restricted to the MRSA that accounts for the minority (<20%) of invasive infections [214,215]. MRSA tends to be less diverse, being restricted to epidemic or pandemic clones. Herein, the invasive isolates of the entire patient cohort were analysed, 88% of which were MSSA. *S. aureus* strains in this study were found to be largely concordant with the distribution of ST types in Europe, whereby 2 clonal complex groups (CC5 and CC22) accounted for 52% of bloodstream isolates [193]. There was considerable genotypic heterogeneity in invasive *S. aureus* isolates, particularly MSSA, despite the small size of the study population and the geographic proximity of study sites. Most bloodstream isolates originate from colonising *S. aureus* in the nares [37]. Repeated microbiological characterisations have failed to demonstrate consistently invasive or pathogenic strains [216,217]. Genetic lineage does not predict *in vivo* phenotype. Toxins, virulence or immune evasion proteins do not cluster by clonal complex. Perhaps it is not surprising, then, that the relatively conserved 'housekeeping' genes used in bacterial phyllogenetics do not correlate with clinical severity. Shifting the focus from ancestral relationships to microbial pathogenic elements may be more likely to help identify disease-causing *S. aureus* strains.

An ideal vaccine antigen would be critical to pathogenicity and be present in multiple strains. In this study, all bloodstream isolates were either capsular polysaccharide type CP5 or CP8, and contained genes encoding for all cell wall components tested. Secreted virulence factor genes were much less uniformly present. Immune evasion protein genes (SpA and Map) – although present – displayed significant degrees of sequence variability. Critically, we do not know the relative importance of staphylococcal proteins – whether certain secreted toxins or surface components are crucial for pathogenicity. Although conserved, cell wall components' role in causing disease is not conclusive. Secreted toxins and immune evasion proteins are probably more pathogenic but their allelic variability or complete absence from many strains is problematic for vaccine development. *S. aureus* has an entire arsenal of redunant virulence and immune evasion factors at its disposal, and these weapons can be dynamically expressed [218,219]. It therefore seems unlikely that neutralising a single virulence determinant will disarm this organism.

Even if a single clearly pathogenic and well-conserved antigen could be discovered, allelic sequence variability raises the possibility that transcribed proteins may differ sufficiently to restrict vaccination to strain-specific protection only. There were 12 allelic variants in the *clfA* genes of 22 bloodstream isolates (representing 9 clonal complexes) in this study. There is a relatively low level of sequence diversity in the protein, and most variance occurred between, rather than within, clonal complex groups. Similarly low allelic variance has also been noted in other clinical studies, implying a single or small number of ClfA variants could induce protection against multiple strains of *S. aureus* [213,220]. Whether such polymorphisms result in structural differences sufficient to alter epitope binding sites and hinder cross-protection has yet to be explored in detail. Experimental anti-ClfA antibodies can bind to ClfA variants from multiple *S. aureus* strains. However, the affinity of this antibody binding is weaker than to the parent ClfA, and the opsonic killing activity also differs between *S. aureus* isolates, suggesting a significant element of strain-specific protection [221,222]. Even successful generation of antigen-specific immune responses does not automatically result in sterile protection. A series of experimental models demonstrated that *in vitro* responses to ClfA immunisation were strain-specific, but largely did not translate into improved clinical outcomes [222]. These findings illustrate the complexity of engineering a protective multi-strain response against a single variable antigen.

Antimicrobial resistance drives an urgent need to develop anti-staphylococcal vaccines and novel antimicrobials. The MRSA rate in this study (12%) was relatively concordant with the reported national percentage of MRSA in *S. aureus* bloodstream isolates in Ireland (19.4% in 2014) [223]. One of the most striking things about the rise of MRSA is that it has not replaced MSSA infection at all. Rather, it has added to the overall burden of SA-BSI [3,224]. Hospital-acquired catheter-associated MRSA BSI – while it claims most public and scientific attention – has decreased by over 80% in England in recent years and only accounted for 0.3% of all SA-BSI in 2014-2015 [7,225]. Even in the USA, hospital-acquired MRSA BSI has halved in the last decade [41,226]. Ireland has seen a 21% decrease in the number of MRSA BSI between 2012 and 2017, while MSSA BSIs increased by 20% [8]. In fact, community-acquired methicillin-susceptible disease has always accounted for the bulk of SA-BSI and its incidence continues to increase, even while healthcare-associated and resistant infections decline [9]. One quarter of all *S. aureus* bloodstream isolates in this patient group remained penicillin-susceptible, over twice the proportion of MRSA, and there is a suggestion of an ongoing increase in penicillin susceptibility in other parts of the world also [227,228]. These patients' infections were no less severe. While initially MRSA infections were thought to result in

poorer outcomes as a result either of increased virulence or inferior antimicrobial treatment options, this has not been convincingly borne out. After correcting for other confounders, MRSA infections do not consistently differ from MSSA infections in treatment failure or death rates [3,224,229]. In this context, an excessive focus on resistance distracts from the problem of tackling *S. aureus* infections as a whole.

A descriptive account of epidemiology, clinical management and outcome of *S. aureus* BSI patients in Ireland including both MSSA and MRSA BSI has not previously been published. *S. aureus* was not a more opportunistic pathogen than *E. coli* in this study, given that proportions excluded from each group for reasons of potential severe immunosuppression were not significantly different between SA-BSI and EC-BSI groups [35/116 (30%) vs 55/140 (39%), p=0.80]. Indeed, many of those excluded for reasons of malignancy or blood-borne viral infection also had co-existing conventional risk factors for SA-BSI, e.g., indwelling IV catheters and injecting drug use. Non-recruitment for reasons of inability to consent may have excluded the sickest patients in each group [22/116 (19%) and 42/140 (30%) for SA-BSI and EC-BSI respectively, p=0.04]. However, certainly among the EC-BSI group, most of this was due to pre-existing, rather than infection-attributable, cognitive impairment. Consistent with globally observed trends, in this cohort, *S. aureus* disproportionately affected younger adults and men [16]. Despite the group being younger and healthier than the *E. coli* BSI group, the clinical course of SA-BSI was overwhelmingly complicated. Failure, relapse or death was seen in 40%. The economic impact was also significantly greater for SA-BSI patients. Their length of stay was over twice as long, and antimicrobial consumption more than six times that of the EC-BSI group. Most SA-BSI therapy was administered intravenously, with all its associated costs – in-patient stay, radiological placement of long intravenous lines, outpatient parenteral antimicrobial therapy, community ambulatory healthcare personnel, and regular outpatient clinic visits. In contrast, there were no failures of therapy, deaths or recurrent infections in the *E. coli* group. *S. aureus*'s capacity to cause severe disease in otherwise healthy young people, to commonly relapse or fail to respond to therapy distinguishes it from most other bacterial infections. SA-BSI is recognised as such a uniquely destructive infection that it is the only bloodstream infection considered a distinct indication for U.S. Food & Drug Administration approval of new antimicrobial drugs [230]. *S. aureus* has previously been ranked among the top ten most burdensome infectious diseases in high-income settings, accounting for a significant proportion of premature mortality or reduced functioning [231]. Its contribution to direct healthcare costs and to indirect loss of economic contributions due to disability are substantial.

There has been increasing attention on infection prevention in recent decades. Here, injecting drug users accounted for 5/25 (20%) of SA-BSIs. Increased availability of needle exchange, supervised injection sites and education on safer injecting practice has had some success in reducing BSIs in this population [232-234]. The incidence of catheter-related BSI (CR-BSI), in particular with MRSA, following intravenous catheter insertion has dropped precipitously in the UK since the introduction of line care bundles, national surveillance and financial penalties [7,225]. However, just 2/25 (8%) of this study's SA-BSIs were healthcare-associated MRSA line infections. Rather, MSSA accounted for 22/25 (88%) and 12/25 (48%) of SA-BSIs were community-acquired infections. Despite clear success in reducing healthcare-associated MRSA infection, analogous declines have not been seen in MSSA BSI incidence – whether healthcare-associated or community-acquired [22]. Thus only a minority of this group's SA-BSIs are likely to have been preventable by current best practice.

Implementation of evidence-based quality-of-care indicators in SA-BSI (early source control; early beta-lactam use for MSSA; vancomycin dose adjustment as per levels; follow-up blood cultures; echocardiography in selected patients; and appropriate duration of therapy according to the complexity of infection) have led to improved patient outcomes [205,235]. Clinical management did not meet these indicators in 46% of our SA-BSI cases, and remote advice to add therapy in a further 8% resulted in additional toxicity. These failures overwhelmingly occurred when patients were not under the care of infection specialists. Infection physician-led management more than halves mortality in SA-BSI, by improving classification and management of infection, even after adjustment for patient characteristics and other confounders [235-238]. Clinicians lacking specific training in SA-BSI may be less likely to follow recommendations due to different knowledge, attitude, beliefs and experience (often underestimating the disease and overestimating their own knowledge of its management) [239,240]. The mode of delivery of specialist advice is also critical. There is rarely enough information at the time of communicating an initial laboratory diagnosis of SA-BSI to formulate a comprehensive management plan. Rather, serial bedside assessments are required to detect metastatic infection, optimise antimicrobial therapy, exclude serious drug allergies, arrange source control interventions, monitor clinical response and deal with later complications of therapy. Remote management of complex infections, and particularly SA-BSI, also introduces potential error due to incomplete, incorrect or second-hand information [241]. Bedside specialist care is consistently superior to telephone advice [237,242].

S. aureus bloodstream infection is a life-threatening and life-changing disease. Even a minimally effective anti-*S. aureus* vaccine would be strongly cost-effective in high-risk populations [243,244]. Now, faced with an inability to completely prevent infection, the reality of suboptimal clinical management, frequent failure of current treatments and increasing resistance, the need for a strategy to prevent *S. aureus* infection outright – namely immunisation – has a renewed urgency. Efforts to prevent infection in this way predated the introduction of antimicrobial therapy [187].

After decades of microbial focus, the role of the host in infection and its outcome is now being revisited. Microbial factors initially thought to influence outcome in SA-BSI are no longer significant after adjustment for patient characteristics [3,217,229]. Host factors (e.g., age and co-morbidities) are much more powerful predictors of poor prognosis [218,245,246]. The importance of patient differences was noted over 100 years ago when clinician scientists observed that *S. aureus* infection produced disease in some, but not in others [1,247]. Since then, little more has been discovered about true correlates of protection against *S. aureus* infection. While continuing to search for the staphylococcal elements which contribute the most to disease, we should also acknowledge that human factors are in many ways more important in determining the course of infection. We must now turn our attention to the host and take the first steps in understanding immune response to invasive *S. aureus* infection.

Chapter 4

Adaptive immune responses in human *Staphylococcus aureus* bloodstream infection

4.1. Introduction

Any *S. aureus* strain may cause severe disease, but host characteristics determine infection and its outcome [3,217]. Correlates of human immunity to *S. aureus* infection are largely unknown. Colonisation and natural infection do not result in future sterile protection. On the contrary, relapse and recurrent infections are common [159]. Recognising which arms of immune defence are key responders in invasive disease is a first step in understanding what promotes clearance, or even prevention, of infection. This knowledge must form the foundation of any potential vaccine strategies. Investigation of adaptive immunity to *S. aureus* infection in humans has thus far been limited to humoral responses. Crucially, B cell deficiencies in humans are not associated with increased infection rates, and do not worsen outcomes in animal challenge models [15,64,248]. Neither active immunisation with vaccines that produced robust humoral responses, nor passive immunisation with anti-staphylococcal antibodies, have prevented or attenuated infection in clinical trials. There is therefore real doubt over the importance and longevity of humoral anti-*S. aureus* immunity.

In contrast, the role of cellular immunity is potentially more promising. Significant animal, and limited clinical, data suggest T cells may be vital to *S. aureus* immunity. While T cells do not directly kill bacteria, some can kill infected cells, or importantly – through their cytokines – can orchestrate downstream effects to enhance phagocyte activity [249]. Intravenous challenge in T cell knockout mice results in rapid death, while B cell-deficient mice fare no worse than wild-type animals [64]. T cells may be an important target for next-generation *S. aureus* vaccines. However, it is currently unclear which cell type(s) should be targeted. At the time of commencing this project, mechanistic data from animal experiments supported protective roles for IFNγ- and IL-17-producing T lymphocytes in particular. However, none of this was validated in humans.

In mice, IFNγ from type 1 T helper (Th1; $CD4^+$ $IFN\gamma^+$) lymphocytes activates macrophages for antigen presentation, phagocytosis and microbial killing. Death rapidly follows intravenous *S. aureus* challenge in IFNγ-deficient mice [64,72,73]. Adoptive transfer of *S. aureus*-antigen specific Th1 $IFN\gamma^+$ lymphocytes accelerates clearance of bacteria in systemic infection by enhancing macrophage responses [32]. IL-17 helps to contain and clear staphylococci by recruiting and activating neutrophils, enhancing epithelial defences and reducing haematogenous dissemination of bacteria [76,77,92,250]. Mice deficient in IL-17 are more susceptible to mucocutaneous *S. aureus* infection, but no difference is observed in systemic disease [77,78]. IL-17 in mice is produced mainly by $\gamma\delta^+$ cells, and their adoptive transfer (but

not that of $CD4^+$ Th17 cells) accelerated bacterial clearance in various *S. aureus* infections [31,32,80,81,92,250]. Finally, $CD8^+$ cytotoxic T cells (CTLs) are important in defence against intracellular pathogens, while *S. aureus* is conventionally considered an extracellular bacterium. However, it is now evident that *S. aureus* is wholly competent at establishing intracellular infection and persisting in endothelial cells and phagocytes [107,251]. It may even use these cells as 'Trojan horses' to traffic to otherwise protected or distant sites, resulting in metastatic infection [106]. $CD8^+$ CTLs may thus have a role to play in attenuating or protecting against disseminated infection by targeting infected blood vessel or mobile cells harbouring viable staphylococci for destruction. CTLs have not been examined in anti-staphylococcal immunity.

It is entirely unknown as to whether the protective roles of these lymphocytes in mice could translate to humans. Clinical observations do indirectly support key roles for IFNγ, IL-17 and an intact Th1/17:phagocyte axis in protection against human *S. aureus* infection. Co-morbidities associated with impaired Th1 ($CD4^+$ $IFN\gamma^+$) responses (e.g., renal failure, HIV infection, and diabetes mellitus) confer the highest relative risk for *S. aureus* bloodstream infection [18,20,21,159]. Patients with inborn errors of IL-17 function (e.g., chronic mucocutaneous candidiasis) or who cannot produce Th17 cells (e.g., autosomal dominant hyper-IgE syndrome) suffer from repeated *S. aureus* mucocutaneous (but not bloodstream) infections [82,86,252]. This apparent contradiction between global Th17 deficiency and the skin/lung-restricted susceptibility to staphylococcal infections is explained by human keratinocytes and bronchial epithelial cells having greater dependence on Th17 (IL-17 and IL-22) cytokines than other cell types for effective production of antimicrobial peptides and neutrophil chemotaxins [85]. Unlike in mice, Th17 ($CD4^+$ $IL\text{-}17^+$) cells are the major human source of IL-17. A role for $\gamma\delta^+IL\text{-}17^+$ cells in human *S. aureus* infection is not established and it remains unclear whether *S. aureus* can activate human $\gamma\delta^+$ cells [94]. Translation of murine $\gamma\delta^+$ lymphocyte findings to humans is difficult as there is extreme divergence in γ- and δ- TCR repertoires and no clear correlation between primate and rodent subpopulations. Nonetheless, regardless of primary cell population, there is a strong signal that IL-17 plays a key role in mucocutaneous *S. aureus* immunity. Patients with normal T helper cell activity, but defects in downstream phagocytes also present with recurrent *S. aureus* infections [253]. For example, phagocytes from patients with chronic granulomatous disease (CGD), are unable to generate reactive oxygen species. This markedly impairs their ability to eliminate certain pathogens, including *S. aureus* [254,255].

Despite clinical observations, and animal findings implying that particular T cell subsets play key roles in anti-staphylococcal immunity, there had been no studies examining the role of T lymphocytes in patients with invasive *S. aureus* infection prior to this work. It had not been established whether human T lymphocytes can recognise and respond to *S. aureus* antigens, and few *S. aureus*-specific T cell epitopes were identified [256-259]. A first translational step was to determine whether T lymphocytes expand at all in invasive *S. aureus* infection and, if so, which subpopulations do so. The aims of this arm of the project were to characterise the adaptive immune response in the early recovery period of *S. aureus* bloodstream infection and to develop a model assay to test T cell responses to specific *S. aureus* antigens.

4.2. Results

4.2.1. Leukocyte dynamics and acute phase response in *S. aureus* bloodstream infection

The first step in characterising immune response in the early recovery period of *S. aureus* bloodstream infection was to examine white blood cell dynamics over the initial days of infection. Results of blood tests, performed as part of routine clinical care, were retrospectively analysed. Comparing responses of infected patients with those of healthy controls only would make it difficult to differentiate results indicative of illness from those particular to *S. aureus* infection. *E. coli* bloodstream infection patients were therefore chosen as a more appropriate comparator group, to take account of known leukocyte disturbances in severe infection [260,261]. This is further discussed with regards to known effects on specific lymphocytes below (see *4.2.5*).

Thirty-six patient samples from 24 SA-BSI and 11 EC-BSI patients were analysed for C-reactive protein (CRP) and full blood count (FBC) at time of initial bloodstream infection (Day 0) and in the early recovery period (Day 7±2). Day 0 CRP was elevated (NR 0-5 mg/L) in 96% of SA-BSI and 100% of EC-BSI patients and remained elevated by Day 7±2 in the same proportions (Fig 4.1). Initial CRP was also significantly more elevated in complicated as compared with uncomplicated SA-BSI [median 170 (IQR 127-231) vs 66 (27-112); p=0.04]. Total white blood cell concentration did not differ between groups at either D0 or D7±2. Neutrophilia was seen in both groups at D0 [median 8.5 (IQR 7.4-13.4) vs 9.9 (8.4-13.7) for SA-BSI and EC-BSI respectively] (Fig. 4.2A). Neither absolute nor percentage neutrophil counts differed significantly between SA-BSI and EC-BSI cohorts at either timepoint (Fig 4.2A and 4.2B). Marked reduction in lymphocyte number (Fig. 4.3A) and proportions (Fig. 4.3B) were seen at the time of onset of bloodstream infection in both groups. In the early recovery period, however, an expansion was seen in the SA-BSI patients to restore lymphocytes towards normal, while EC-BSI patients remained lymphopaenic. This difference in lymphocyte recovery was significant [median increase 8.8% (IQR 2.4 – 22.8) vs 0.4% (0.2 - 0.9) for SA-BSI and EC-BSI patients, respectively] (Fig. 4.3C), and did not correlate with patient age (Fig. 4.3D), suggesting that lymphocyte expansion may play a role in recovery from SA-BSI.

Taken together, these findings suggests that innate responses (CRP and neutrophils) are heightened during initial (Day 0) response to bloodstream infection, and that adaptive immune responses (lymphocytes) are relatively suppressed. SA-BSI and EC-BSI patients do

not significantly differ at this early timepoint. In contrast, lymphocyte restoration in SA-BSI patients is striking during the early recovery period (7±2 days post-BSI symptom onset). This may infer *S. aureus*-specific activation of the adaptive immune response is a critical part of immune control of this infection. However, the full blood count is a relatively crude tool by which to measure immune response. Deeper analysis of lymphocyte subsets and their antigen-specific responses to *S. aureus* BSI is required.

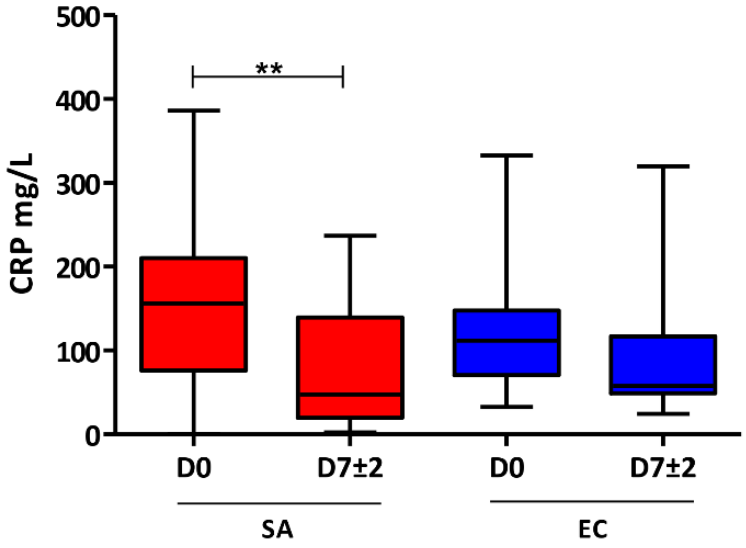

Figure 4.1. Non-specific inflammatory marker C-reactive protein is similar in initial and early recovery periods of *S. aureus* or *E. coli* bloodstream infection.

Serum samples were taken from *S. aureus* and *E. coli* bloodstream infection patients at time of bacteraemia/symptom onset (Day 0) and during the early recovery period (Day 7±2) to measure C-reactive protein, which remained markedly elevated above normal (NR 0-5 mg/L), but did not differ between groups at the same timepoints. Results shown as box-and-whiskers plots where the horizontal line indicates the median, boundaries of the box the IQR, and whiskers indicate the highest and lowest values of the results.
SA = *S. aureus* bloodstream infection; EC = *E. coli* bloodstream infection. EC, n = 11; SA, n= 24.
**p<0.005.

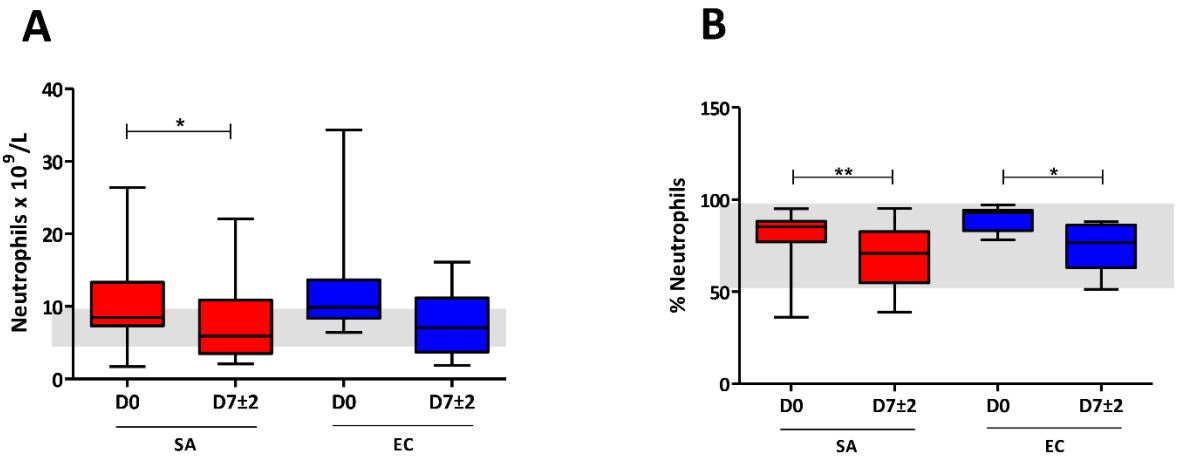

Figure 4.2. Absolute neutrophil count and proportion are similar in initial and early recovery periods of *S. aureus* or *E. coli* bloodstream infection.

Blood samples were taken into EDTA from *S. aureus* and *E. coli* bloodstream infection patients at time of bacteraemia onset (Day 0) and during the early recovery period (Day 7±2). Neutrophil cell concentrations (A) and neutrophils as a proportion of total white blood cells (B) did not differ between groups at the same timepoints. Results shown as box-and-whiskers plots where the horizontal line indicates the median, boundaries of the box the IQR, and whiskers indicate the highest and lowest values of the results. Normal ranges in healthy adults are indicated by shaded grey boxes.

SA = *S. aureus* bloodstream infection; EC = *E. coli* bloodstream infection; EDTA = Ethylenediaminetetraacetic acid. EC, n = 11; SA, n= 24.

* $p<0.05$, ** $p<0.005$.

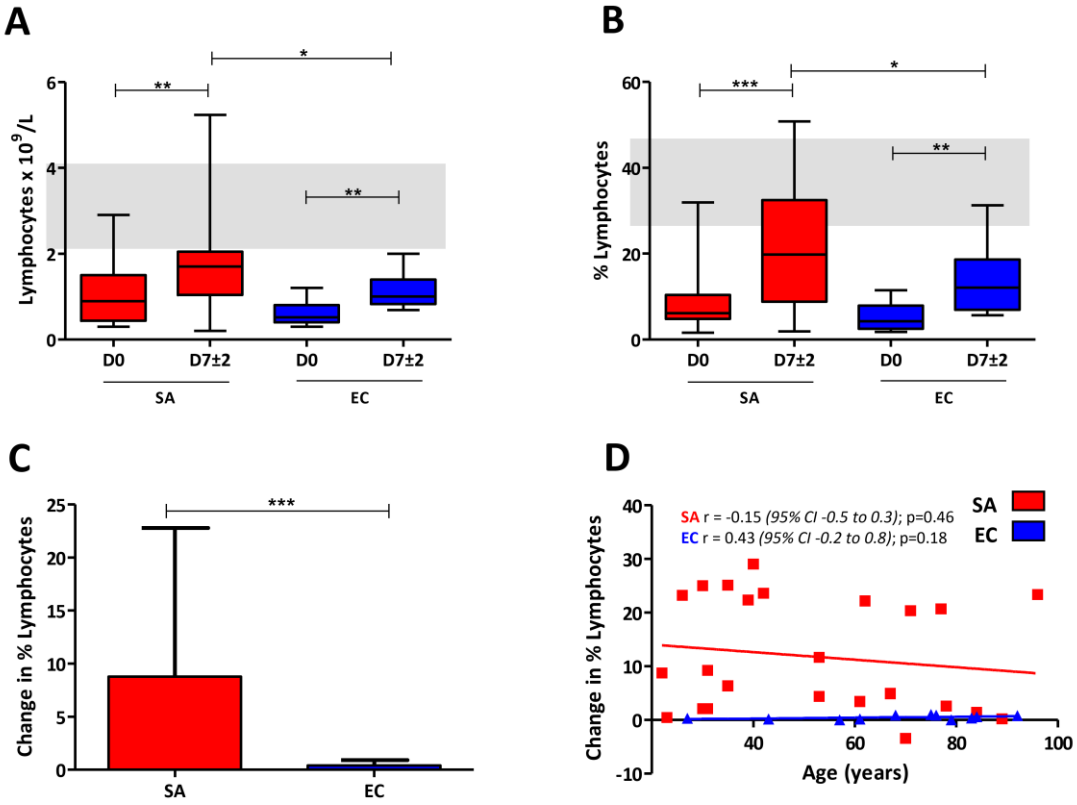

Figure 4.3. Lymphocyte recovery differs in *S. aureus* and *E. coli* bloodstream infection.

Blood samples were taken into EDTA tubes from *S. aureus* and *E. coli* bloodstream infection patients at time of bacteraemia onset (Day 0) and during early recovery period (Day 7±2). Marked reduction in lymphocyte number (A) and lymphocyte proportion of white blood cells (B) was seen in both groups at D0. Lymphocyte restoration was much greater in SA-BSI patients over the first 5-9 days of recovery (C). There was no correlation between patient age and lymphocyte restoration for either SA-BSI or EC-BSI (D). Results shown as box-and-whiskers plots where the horizontal line indicates the median, boundaries of the box the IQR, and whiskers indicate the highest and lowest values of the results (A, B) or as median indicated by upper border of box and upper whisker the IQR (C). Normal ranges in healthy adult humans are indicated by shaded grey boxes. Individual patient lymphocyte recovery data points are shown in (D) along with a best-fit line, Pearson correlation coefficient (r) and associated 95% confidence interval (CI).

SA = *S. aureus* bloodstream infection; EC = *E. coli* bloodstream infection; EDTA = Ethylenediaminetetraacetic acid. EC, n = 11; SA, n= 24.

* p<0.05, **p<0.005, ***p<0.001.

4.2.2. T lymphocyte subset proportions remain within normal ranges during early recovery from *S. aureus* bloodstream infection

The marked restoration of lymphocytes seen in SA-BSI, but not EC-BSI, patients during early recovery did not determine which lymphocyte population(s) might account for this expansion. Blood from 22 SA-BSI and 10 EC-BSI patients (and from 17 healthy volunteers) was taken 7±2 days after symptom onset to examine whether lymphocyte subsets were abnormally expanded or contracted during recovery from illness. PBMCs were isolated, stained and T lymphocyte subpopulations were evaluated by flow cytometry.

Neither infection resulted in expansion of T ($CD3^+$), T helper ($CD3^+CD4^+$) or cytotoxic ($CD3^+CD8^+$) lymphocytes outside of normal ranges [262]. Furthermore, proportions of each of these lymphocyte subsets did not differ between SA-BSI and EC-BSI groups, nor when compared with healthy volunteers (Fig 4.4A-C). Gamma-delta T cells were variably present in patient samples and also did not differ between groups, whether based on pan-γδ TCR staining (n=28) or on more specific Vδ2 T cell receptor staining (n=21) (Fig 4.4D-E). However, even massive increases (such as a doubling of T lymphocyte number) may remain within the wide normal range (NR = 1.5 - 3.5 × 10^9 /L). As antigens activate only a small proportion of T cells, changes in number of entire cell populations are usually not seen [263]. Evidence of antigen-specific signals must be sought by other means.

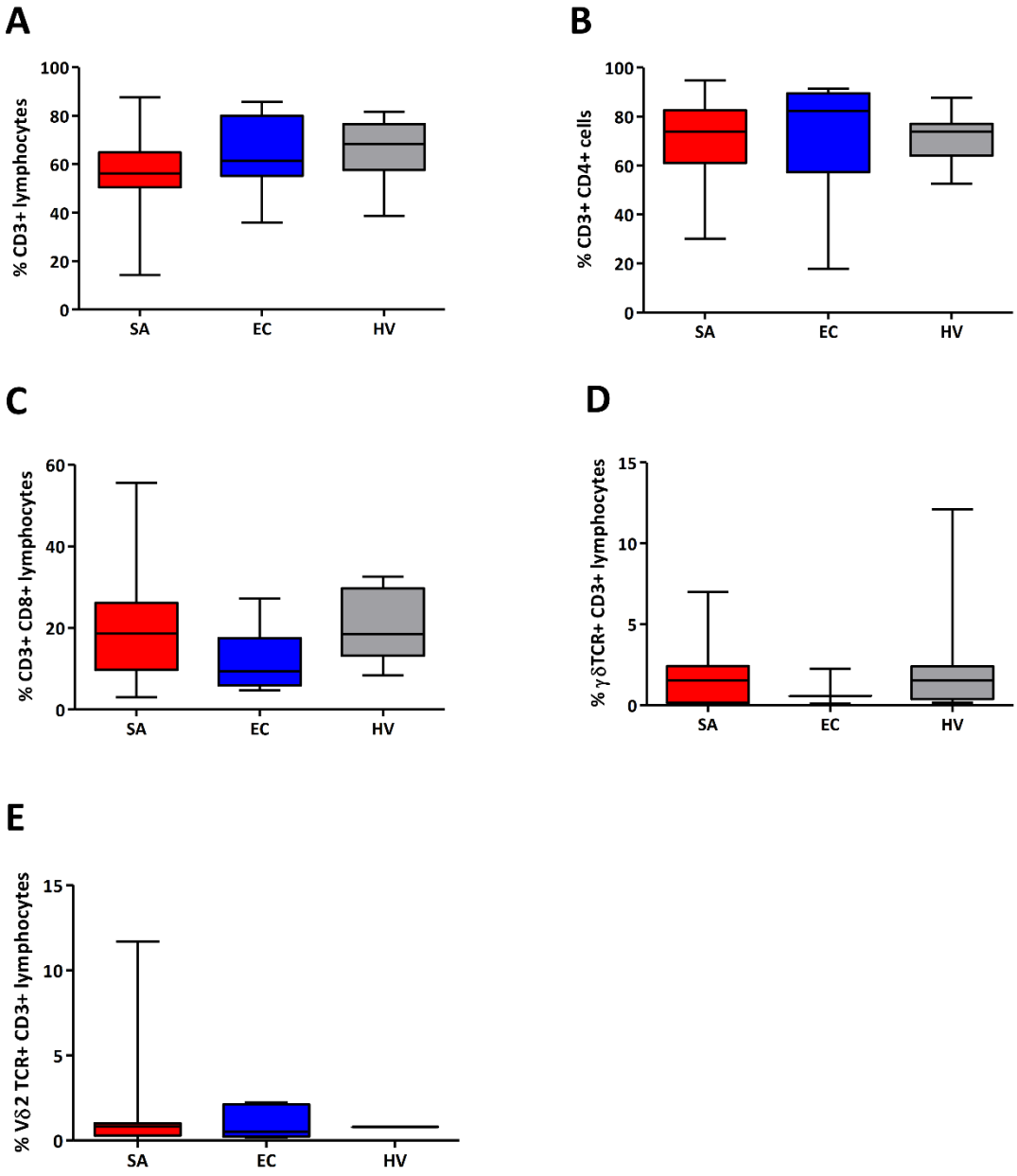

Figure 4.4. T lymphocyte subset proportions remain within normal ranges during early recovery from *S. aureus* or *E. coli* bloodstream infection.

PBMCs were isolated from fresh blood samples taken on day 7±2 following *S. aureus* (SA, red boxes) or *E. coli* (EC, blue boxes) bloodstream infection, or from healthy volunteers (HV, grey boxes), and evaluated by flow cytometry. Events were gated on lymphocytes using forward and side scatter properties and $CD3^+$ to identify T cells. $CD4^+$, $CD8^+$ or $\gamma\delta^+$ T cells were identified by gating for these markers within the $CD3^+$ population. $CD3^+$ cells (A) are expressed as a proportion of total lymphocytes. $CD4^+$ (B), $CD8^+$ (C), $\gamma\delta TCR^+$ (D) and $V\delta2^+$ (E) cells are expressed as a proportion of the $CD3^+$ lymphocyte population. Frequencies were compared between patient groups with the Mann-Whitney test. Results shown as box-and-whiskers plots where the horizontal line indicates the median, boundaries of the box the IQR, and whiskers indicate the highest and lowest values of the results.
SA n=22, EC n=10, HV n=17.

4.2.3. *S. aureus* bloodstream infection is associated with elevated serum IFNγ, but not other signature T lymphocyte cytokines

T helper cell types are characterised by their production of different signature cytokines in response to antigen, and these cytokines may be detectable in serum. The signature Th1 cytokine IFNγ was detectable in 60% (n = 15) of serum taken from SA-BSI, patients on day 7 ± 2 after onset of bloodstream infection a significantly greater proportion than in healthy volunteers or EC-BSI patients (p=0.03 and p=0.03 respectively). IFNγ levels were also significantly higher in serum from SA-BSI patients than from EC-BSI patients [44 pg/mL (IQR 0-109) versus 0 pg/mL (IQR 0-0), respectively; p=0.03] (Fig. 4.5A). Low levels of IL-17A and IL-10 were detectable in sera from a minority of patients, but did not differ significantly between groups (Fig. 4.5B and C). Interestingly, IL-5 levels were lower among recovering bloodstream infection patients than in healthy volunteers (Fig. 4.5D), which may suggest a skewing towards a Th1 response. To explore this further, IFNγ:IL-5 ratios were calculated for all patient groups. This method specifically suggested a Th1 skewing (with concomitant Th2 suppression) in recovery from SA-BSI, but not EC-BSI (Fig. 4.5E). There was a weak inverse correlation (r = -0.49; 95% CI -0.7 to -0.1) between IFNγ level and age in the SA-BSI group, although the implication of this is uncertain as it was not consistently seen across all three patient groups, which may further suggest that the Th1 skewing is *S. aureus* BSI-specific (Fig. 4.5F).

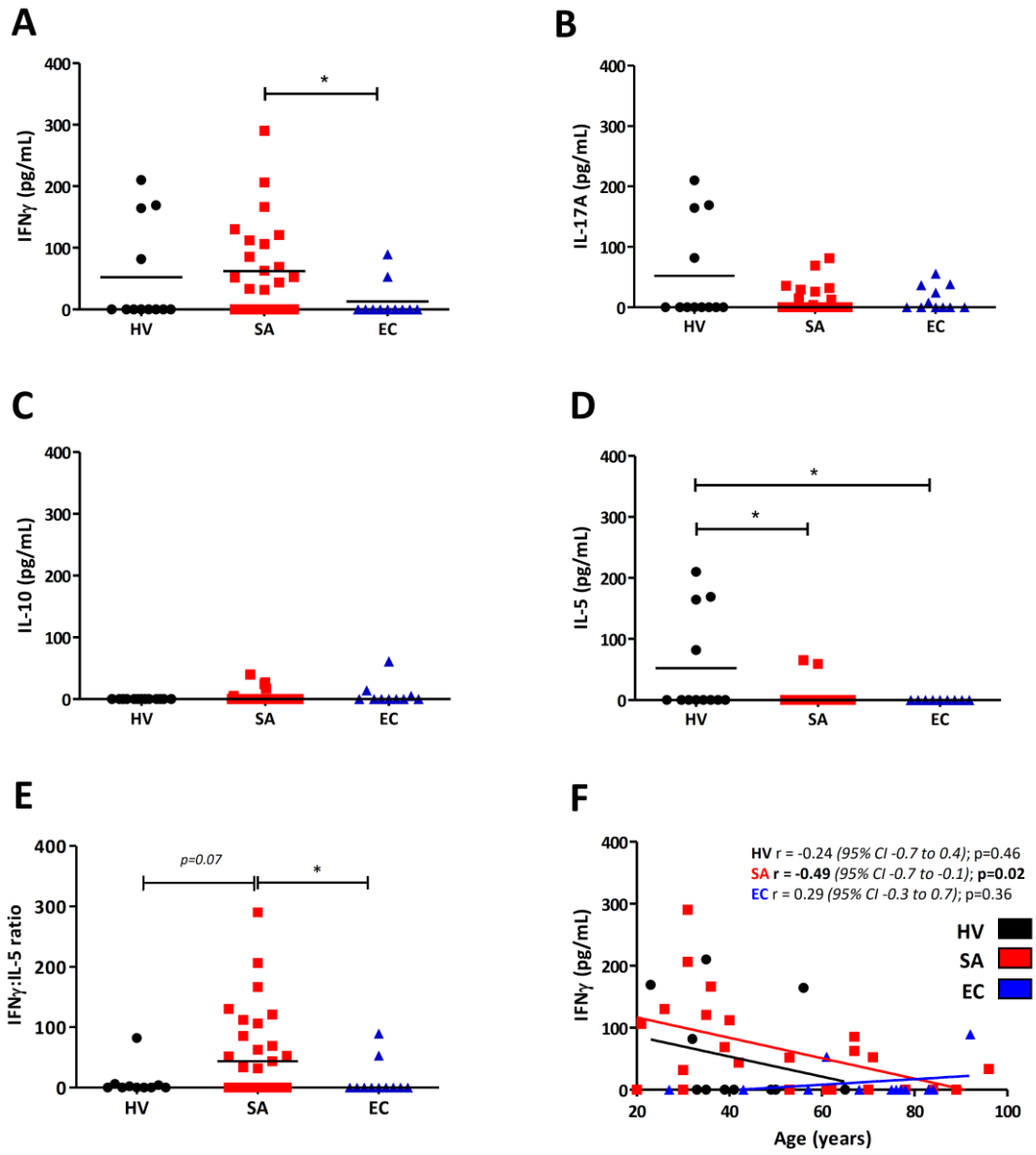

Figure 4.5. *S. aureus* bloodstream infection is associated with elevated serum IFNγ and Th1-skewed response

Blood samples were taken on day 7±2 following *S. aureus* (SA, red boxes) or *E. coli* (EC, blue boxes) bloodstream infection or from healthy volunteers (HV, black dots). Serum was separated by centrifugation in clot separator tubes. Levels of IFNγ (A), IL-17A (B), IL-10 (C) and IL-5 (D) were determined by ELISA and compared between patient groups. IFNγ and IL-5 ratios were compared to assess degree of Th1-skewed response (E). Serum IFNγ response was correlated with age (F). Graphs A-E show individual data points and a median bar. F shows individual data points along with a best-fit line, Pearson correlation coefficient (r) and associated 95% confidence interval (CI).

SA n=24-25, EC n=9-11.

*p<0.05

4.2.4. *S. aureus* bloodstream infection is associated with IFNγ responses from antigen-specific CD4$^+$ and CD8$^+$, but not γδ$^+$ T lymphocytes during the early recovery period

Having observed that the Th1 signature cytokine IFNγ was significantly elevated in the serum of SA-BSI patients (see *4.2.3*), the next steps were to establish if this IFNγ response was staphylococcal antigen-specific and to identify its lymphocyte source(s). PBMCs from healthy volunteers (n=12), SA-BSI (n=13) and EC-BSI (n=6) patients were incubated overnight with heat-killed *S. aureus* (HKSA) or *E. coli* (HKEC). This short stimulation aimed to provide an *ex vivo* snapshot of effector T cells circulating during the early recovery period (day 7±2) of BSI, as cytokine production occurs prior to detectable proliferation of new daughter cells. Heat-killed bacteria provided a source of multiple potential antigens and (in the absence of known T cell epitopes) was considered the most appropriate strategy to evaluate whether human T cells could recognise and respond to *S. aureus* in the first instance. Several strains (PS80, SH1000, SJH.MSSA-1, SJH.MRSA-1) of HKSA, and a single strain of HKEC were used. Antigen-specific cytokine production by T cells was then measured by flow cytometry and normalised to background by subtracting cytokine levels of unstimulated PBMCs.

Starting with CD4$^+$ T helper cells, an antigen-specific IFNγ (i.e., Th1) response to any strain of HKSA was detectable in more SA-BSI than EC-BSI patients, or healthy volunteers (58% vs 40%; p=0.063 vs 33%; p=0.19, respectively). The magnitude of this CD4$^+$ IFNγ$^+$ response to all HKSA strains was also greater among SA-BSI patients (Fig 4.6A). Differences in IFNγ responses to each HKSA strain were not observed (p=0.85; Fig 4.6B), possibly because many identical antigens are presented in each. Rather than report each strain individually, therefore, responses to the various strains of HKSA were combined for clarity and increased power in subsequent analyses. There was a significantly greater overnight CD4$^+$ IFNγ$^+$ response to *S. aureus* antigens in recovering SA-BSI patients than in either EC-BSI patients or healthy volunteers (Fig 4.6C), which did not correlate with age (Fig. 4.6D). There was no difference in CD4$^+$ IFNγ$^+$ response to heat-killed *E. coli* among groups (Fig 4.6E).

CD8$^+$ cytotoxic T lymphocytes (CTLs) from SA-BSI patients also demonstrated greater IFNγ secretion in response to HKSA (Fig 4.6F), but γδ$^+$ cells did not (Fig 4.7A). Interestingly, γδ$^+$ T cells from EC-BSI patients did produce significant IFNγ in response to HKEC (Fig 4.7B). This was the only noteworthy lymphocyte response to *E. coli* seen in EC-BSI patients.

With regard to other effector T cells circulating during the early recovery period, this short stimulation did not detect antigen-specific IL-17 or IL-10 from T lymphocytes in any of the groups. These findings indicate that the increased IFNγ seen in sera of SA-BSI patients may have been secreted by *S. aureus* antigen-specific $CD4^+$ T helper and/or $CD8^+$ cytotoxic T cells, but not from $\gamma\delta^+$ T lymphocytes.

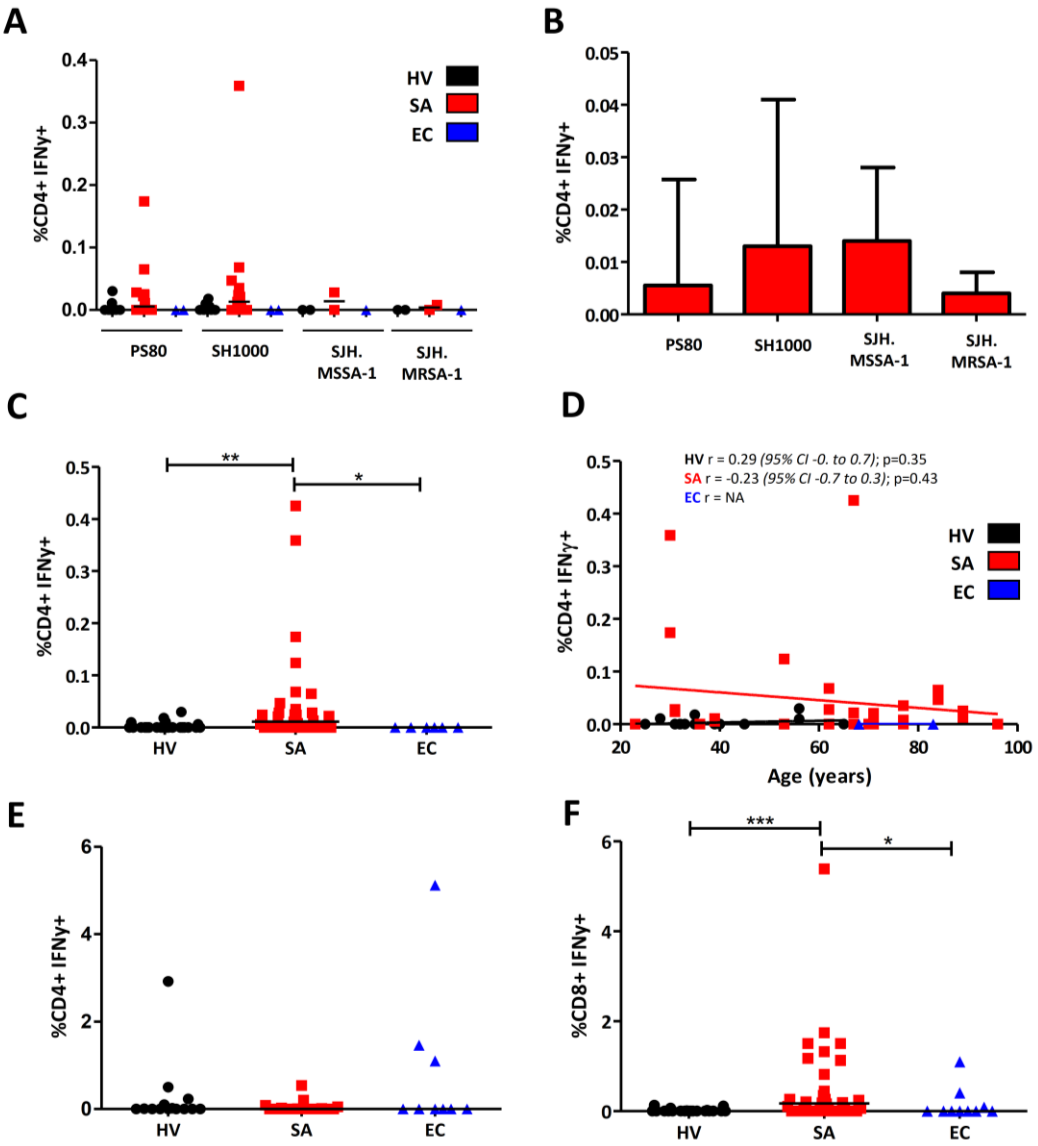

Figure 4.6. IFNγ responses from *S. aureus* antigen-specific CD4⁺ and CD8⁺ T lymphocytes are evident in *S. aureus* bloodstream infection

Blood samples were taken on day 7±2 following *S. aureus* (SA, red boxes, n=13) or *E. coli* (EC, blue boxes, n=6) bloodstream infection or from healthy volunteers (HV, black boxes, n=12). Fresh PBMCs were isolated and incubated overnight with 2 laboratory (PS80 and SH1000) and 2 clinical (SJH.MSSA-1 and SJH.MRSA-1) strains of heat-killed *S. aureus* (HKSA) and a single strain of heat-killed *E. coli* at standard concentration of 1µg total protein per mL (~ 1 x 10^7 CFU per mL *S. aureus*; ~ 8 x 10^6 CFU per mL *E. coli*). Brefeldin 5µg/mL was added for 16 hours to allow assessment of intracellular cytokine production by flow cytometry. Antigen-specific responses were calculated for each patient by subtracting any background cytokine production seen in negative control wells. $CD4^+IFN\gamma^+$ response to HKSA strains was greater in SA-BSI patients (A). As there were no significant differences in $CD4^+IFN\gamma^+$ responses between individual HKSA strains (PS80, SH1000, SJH.MSSA-1, SJH.MRSA-2) in SA-BSI patients (B), responses to all HKSA were combined for comparisons between patient groups. A significantly increased *S. aureus* antigen-specific $CD4^+IFN\gamma^+$ response was seen in *S. aureus* bloodstream infection patients (C). This *S. aureus* antigen-specific $CD4^+IFN\gamma^+$ response did not correlate with age (D). Correlation could not be calculated for the EC-BSI group given there was no detectable IFNγ response to *S. aureus* antigens. In contrast, frequency of $CD4^+IFN\gamma^+$ response to heat-killed *E. coli* was similar across groups (E). Cytotoxic T cells demonstrated an antigen-specific $CD8^+IFN\gamma^+$ response to HKSA in SA-BSI patients (F). Graphs A, C, E and F show individual data points and a median bar; B shows median with IQR, D shows individual data points along with a best-fit line, Pearson correlation coefficient (r) and associated 95% confidence interval (CI).
*p<0.05, **p<0.005, ***p<0.001

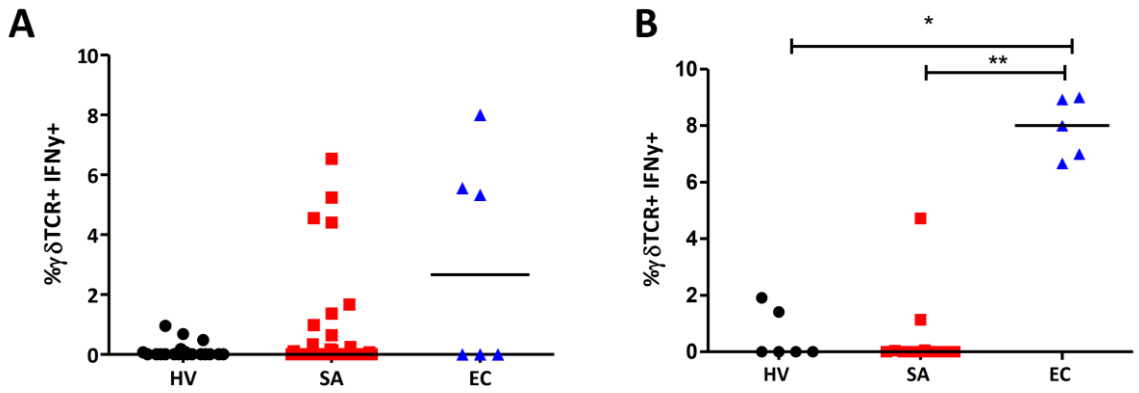

Figure 4.7. *E. coli* bloodstream infection induces pathogen-specific IFNγ production in γδ⁺ lymphocytes, but *S. aureus* bloodstream infection does not

Blood samples were taken on day 7±2 following *S. aureus* (SA, red boxes, n=13) or *E. coli* (EC, blue boxes, n=6) bloodstream infection or from healthy volunteers (HV, black boxes, n=12). Fresh PBMCs were isolated and incubated overnight with 2 laboratory (PS80 and SH1000) and 2 clinical (SJH.MSSA-1 and SJH.MRSA-1) strains of heat-killed *S. aureus* (HKSA) and a single strain of heat-killed *E. coli* at standard concentration of 1µg total protein per mL (~ 1 x 10^7 CFU per mL *S. aureus*; ~ 8 x 10^6 CFU per mL *E. coli*). Brefeldin 5µg/mL was added for 16 hours to allow assessment of intracellular cytokine production by flow cytometry. Antigen-specific responses were calculated for each patient by subtracting any background cytokine production seen in negative control wells. Gamma-delta (γδ) T cell antigen-specific IFNγ⁺ response to heat-killed *S. aureus* was similar across patient groups (A), but a significant antigen-specific IFNγ response to heat-killed *E. coli* was seen in γδ⁺ lymphocytes from *E. coli* bloodstream infection patients (B). Graphs show individual data points and a median bar.
*p<0.05, **p<0.005

4.2.5. Human *S. aureus* bloodstream infection expands a population of *S. aureus* antigen-specific T helper cells

Having observed an IFNγ signal in serum (see *4.2.3*), and demonstrated *S. aureus*-specific IFNγ production from $CD4^+$ and $CD8^+$ T lymphocytes in SA-BSI patients after overnight re-stimulation (see *4.2.4*), we next used a longer assay to determine if antigen-specific T helper cell populations could be expanded by *S. aureus* infection. This would definitively establish – for the first time – whether human lymphocytes can proliferate in response to *S. aureus*, and suggest a role for such an expansion in the immune control of infection. Ability to induce antigen-specific proliferation is a prerequisite for any T cell-based vaccine.

PBMCs from SA-BSI and EC-BSI patients were labelled with CFSE (see *2.4.3*) before being cultured with several strains of heat-killed *S. aureus* (4 reference strains: PS80, SH1000, SJH.MSSA-1, SJH.MRSA-1 and each patient's own infecting strain in the case of the SA-BSI group) and a single strain of heat-killed *E. coli*. Antigen-specific T cell proliferation was determined by measuring decreased fluorescence ($CFSE_{lo}$) of daughter cells by flow cytometry after 10 days incubation, as this duration is required to assess proliferation to conventional T cell antigens (see Fig 2.2). A potential problem with interpreting results of such an assay is that T cell proliferation is significantly reduced following sepsis, regardless of its aetiology [260,261]. Lymphocytes from healthy volunteers not subject to this immunoparesis show much greater proliferation to *in vitro* stimulation. To confirm this, we measured proliferation of $CD4^+$ lymphocytes from bloodstream infection patients and healthy volunteers, in response to a T cell superantigen (staphylococcal enterotoxin A, SEA). Proliferation was indeed significantly reduced in SA- and EC-BSI patients (Fig 4.8). Comparing lymphocyte proliferation of recently infected patients with those of healthy controls would therefore be misleading, indicative of illness rather than of pathogen-specific response. An *E. coli* bloodstream infection cohort was therefore used as a more appropriate comparator group. An additional advantage was that, while both organisms have the potential to cause serious invasive disease, they are also members of normal flora, to which the adult human immune system will not be naive.

S. aureus antigen-specific $CD4^+$ T cell proliferation was generally greater among SA-BSI than EC-BSI patients. $CD4^+$ proliferation to each strain of HKSA is shown by patient group in Fig. 4.9A and by individual SA-BSI patient in Table 4.1 and Fig. 4.9B. Despite stimulation with multiple strains (representing four or five different clonal complex types) of *S. aureus* per patient, each individual's $CD4^+$ cell proliferation did not significantly differ between strains

(p=0.29; Table 4.1 and Fig 4.9B), again possibly due to the presence of several near-identical antigens in all strains. In fact, inter-individual (i.e., between SA-BSI patients) differences in proliferation were more evident than intra-individual differences (between HKSA strains). Individual patients were low-, moderate- or high proliferators to *S. aureus* in general, suggesting a host-determined, rather than strain-specific immune effect. Therefore, later results present combined $CD4^+$ responses to all strains of heat-killed *S. aureus* by patient group for clarity and increased power [4.8% (0.89-17.06) vs 1.78% (0-8.52) $CD4^+CFSE_{lo}$ cells, in SA-BSI and EC-BSI patients, respectively; p=0.04] (Fig. 4.10). This *S. aureus* antigen-specific $CD4^+$ lymphocyte proliferation was particular to SA-BSI patients and did not correlate with age (Fig. 4.10A,C).

There was no significant *S. aureus* antigen-specific $CD8^+$ cell proliferation in the 10-day assay (Fig 4.11A). Interestingly, there was no difference in $CD4^+$ T cell proliferation in response to heat-killed *E. coli* between the patient groups (Fig 4.11B). This suggests that, in contrast to SA-BSI, T helper cell expansion is not critical in immune response to EC-BSI.

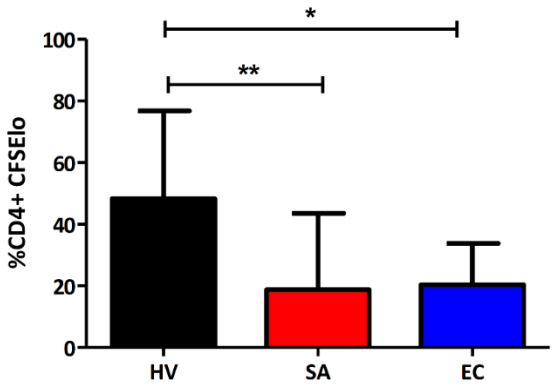

Figure 4.8. Non-specific proliferation of $CD4^+$ T lymphocytes is significantly reduced in bloodstream infection patients as compared with healthy volunteers.

PBMCs were isolated from healthy volunteers and bloodstream infection patients at day 7±2 post-onset of bacteraemia. Cells were CFSE-labelled and incubated with the superantigen staphylococcal enterotoxin A (100ng/l) as a positive non-specific control for 10 d before assessing proliferation by gating on live CFSElo $CD4^+$ T lymphocytes using flow cytometry. Recently infected patients have significantly reduced capacity for $CD4^+$ proliferation as compared with healthy volunteers. Results expressed as median ± interquartile range. HV = healthy volunteers; SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infection; CFSE = carboxyfluorescein succinimidyl ester. HV n=15, SA n=17, EC n=8.
*$p<0.05$, **$p<0.005$.

Table 4.1. Intra-individual variation in $CD4^+$ T cell proliferation to heat-killed *Staphylococcus aureus* strains

Patient	PS80	SH1000	SJH.MSSA-1[A]	SJH.MRSA-1[B]	Endogenous	*p*-value[C]
	(%)	(%)	(%)	(%)		
SA-02	38.4	0	18	-	18[A]	*0.39*
SA-03[1]	69.5	40.5	-	-	82.3	*0.37*
SA-03[2]	9.3	3	8.1	8.5	22	*0.41*
SA-04	38.8	0	-	43.3[B]	43.3[B]	*0.39*
SA-05	24.3	26	21.2	25.9	30.5	*0.41*
SA-06	5.2	0	0	0	0.6	*0.41*
SA-07	47.7	6.9	8.9	21	18.1	*0.41*
SA-08	8	12	27.6	32.5	8.1	*0.41*
SA-11	8.5	4.2	3.9	4.3	2.7	*0.41*
SA-13	23.5	13.5	9.8	4.8	7.1	*0.41*
SA-14	5.9	3.4	19.4	20.3	9.7	*0.41*
SA-17	3.5	1.5	3.1	3.3	4.2	*0.41*
SA-18	0	0	0.9	0	0	*0.41*
SA-19	13.8	8.2	13.5	14.2	5.8	*0.41*
SA-20	1.1	0	0	0	0	*0.41*
SA-22	4.2	0.9	2.2	2.7	2.7	*0.56*
SA-23	0	0	0	.72	0.38	*0.41*

[A] SJH.MSSA-1 strain was the endogenous infecting strain of patient SA-02 and used as a reference strain for subsequent patients.
[B] SJH.MRSA-1 strain was the endogenous infecting strain of patient SA-04 and used as a reference strain for subsequent patients.
[C] *P* values are calculated by Kruskal-Wallis test.
[1,2] Patient SA-03 had recurrent *S. aureus* BSI (device-associated) 12 months after recovery from initial endovascular infection and was recruited on both occasions.
Endogenous = patient's own infecting *S. aureus* strain. MSSA = methicillin-sensitive *Staphylococcus aureus*. MRSA = methicillin-resistant *Staphylococcus aureus*. BSI = bloodstream infection.

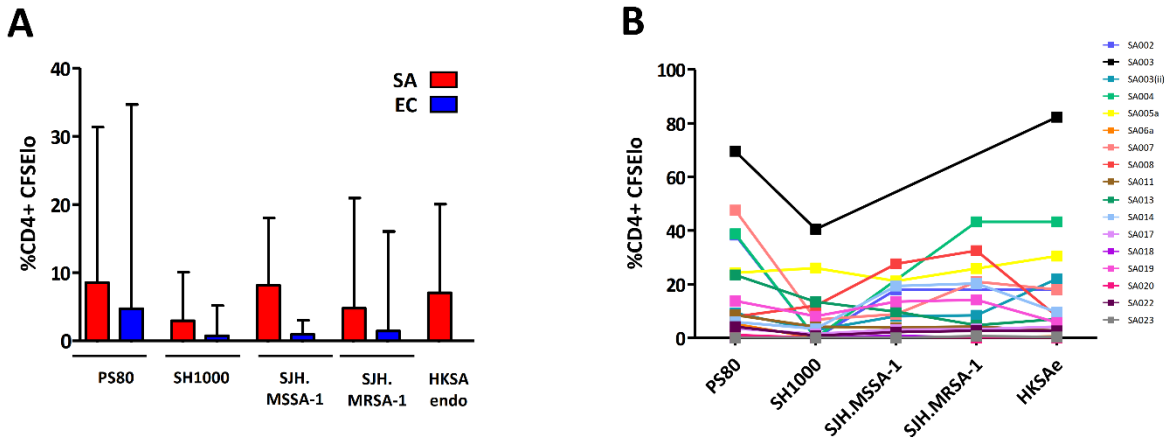

Figure 4.9. CD4⁺ T lymphocytes from *S. aureus* bloodstream infection patients show greater *S. aureus* antigen-specific proliferation than cells from *E. coli* bloodstream infection patients, and this proliferation is patient-specific rather than strain-specific.

PBMCs were isolated from bloodstream infection patients, CFSE-labelled and incubated with various strains of *S. aureus* (HKSAe = endogenous infecting strain; PS80, SH1000, SJH.MSSA-1; SJH.MRSA-1) for 10 d before assessing proliferation by gating on CFSElo CD4⁺ cells using flow cytometry. *S. aureus* antigen-specific CD4⁺ proliferation was generally greater among SA-BSI than EC-BSI patients but did not differ significantly between strains (A). Differences were more evident between SA-BSI patients than between *S. aureus* strains (B). All values are corrected for background proliferation in response to negative control. Results expressed as median ± interquartile range (A) and individual data points (B). SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infection; HKSAe = endogenous infecting strain; PS80, SH1000, SJH.MSSA-1; SJH.MRSA-1 = reference *S. aureus* strains; CFSE = carboxyfluorescein succinimidyl ester. SA n=17, EC n=8.
*p<0.05, **p<0.005.

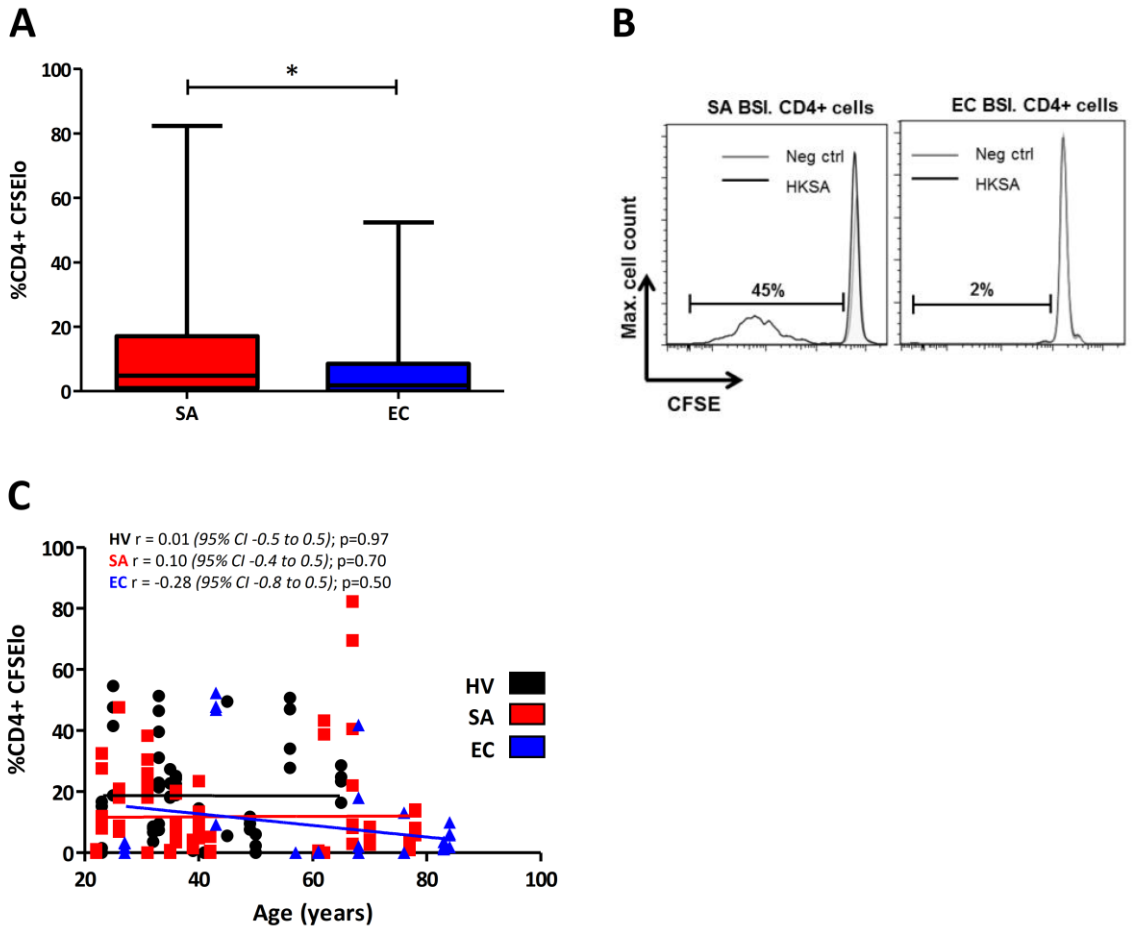

Figure 4.10. CD4⁺ T lymphocytes from *S. aureus* bloodstream infection patients proliferate significantly more than CD4⁺ cells from *E. coli* bloodstream infection patients in response to *S. aureus* antigens.

PBMCs were isolated from bloodstream infection patients, CFSE-labelled and incubated with various strains of *S. aureus* (endogenous infecting strain; PS80, SH1000, SJH.MSSA-1; SJH.MRSA-1) for 10 d before assessing proliferation by gating on CFSElo CD4⁺ cells using flow cytometry. *S. aureus* antigen-specific CD4⁺ proliferation was significantly greater among recently infected SA-BSI patients than among EC-BSI patients (A). Histograms gated on live CD4⁺ cells of representative patients from each group demonstrate measurement of proliferated $CFSE_{lo}$ CD4⁺ cells (B). *S. aureus* antigen-specific CD4⁺ lymphocyte proliferation did not correlate with age in any group (C). All values are corrected for any background proliferation in response to negative control. Results expressed as median ± interquartile range (A) or individual data points along with a best-fit line, Pearson correlation coefficient (r) and associated 95% confidence interval (C).

SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infection; CFSE = carboxyfluorescein succinimidyl ester. SA n=17, EC n=8.

*p<0.05.

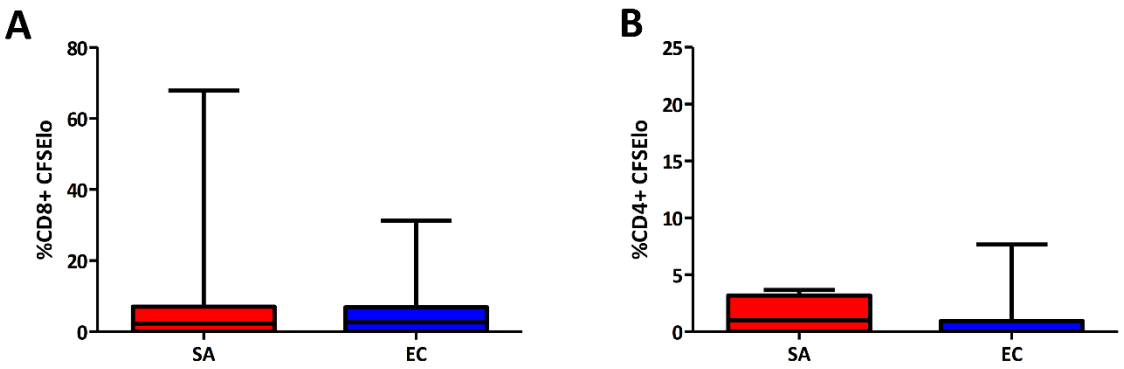

Figure 4.11. *S. aureus*-specific CD8⁺ proliferation and *E. coli*-specific CD4⁺ proliferation is not significantly different between *S. aureus* and *E. coli* bloodstream infection patients

PBMCs were isolated from bloodstream infection patients, CFSE-labelled and incubated with heat-killed *S. aureus* or *E. coli* for 10 d before assessing proliferation by gating on CFSElo CD4⁺ cells using flow cytometry. *S. aureus* antigen-specific CD8⁺ proliferation was no different among recently infected SA-BSI patients than among EC-BSI patients (A). There was no significant difference in the proliferative response of CD4⁺ cells to heat-killed *E. coli* (1µg/ml) between patient groups (B). All values are corrected for background proliferation in response to negative control. Results expressed as median ± interquartile range. SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infection; CFSE = carboxyfluorescein succinimidyl ester. SA n=17, EC n=8.
*p<0.05, **p<0.005.

4.2.6. *S. aureus* antigen-specific lymphocytes are primarily Th1 and Th17 cells

Having found significantly increased *S. aureus* antigen-specific $CD4^+$ T cell proliferation in recently infected SA-BSI patients, the next step was to further characterise these daughter cells by defining their cytokine profiles. To capture lymphocyte cytokine production, Brefeldin A was added for the final 16 h of the 10-day proliferation assay (see *2.4.4* and *4.2.5* above). Most (>90%) proliferating antigen-specific daughter ($CD4^+$ $CFSE_{lo}$) cells did not produce any of the measured cytokines (IFNγ, IL-17, TNFα, IL-10) after 10 days incubation with HKSA, but a minority produced IFNγ or IL-17.

The frequency of $CD4^+$ T cells both proliferating and making IFNγ (Th1) was significantly greater in SA-BSI than EC-BSI patients in response to every *S. aureus* strain (Fig 4.12A). Again, individual SA-BSI patients had broadly similar 'patient-determined' Th1 responses to all strains of HKSA (Fig 4.12B). Interestingly, approximately 10% of these proliferating Th1 ($CD4^+CFSE_{lo}IFN\gamma^+$) cells from SA-BSI patients were also producing TNFα (Fig 4.13C), suggesting that polyfunctional T cells may be involved in immune response to *S. aureus* infection. A *S. aureus* antigen-specific Th1 response was almost completely lacking in EC-BSI patients (Fig 4.13A and B). $CD8^+$ cells showed minimal antigen-specific proliferation and IFNγ production in either patient group (Fig 4.13B). These *S. aureus* antigen-specific Th1 (Fig. 4.13E) and Th17 (Fig. 4.13F) expansions were not correlated with patient age. As for reaction to heat-killed *E. coli*, no substantial Th1 ($CD4^+IFN\gamma^+$) response was seen in either group [0.12% (0-0.6) vs 0% (0-0.09) $CD4^+CFSE_{lo}$, for SA-BSI and EC-BSI patients respectively; p=0.11]. Although an early *E. coli*-specific $\gamma\delta^+IFN\gamma^+$ signal was noted among EC-BSI patients in the shorter overnight stimulation experiment (see *4.2.4*), gamma-delta T lymphocytes were not detectable in culture at all by day 10.

Despite the fact that serum IL-17 levels in both groups were low or undetectable, flow cytometry was used to evaluate T lymphocyte IL-17 production at the single-cell level. *S. aureus* antigen-specific Th17 cell expansion after 10 days culture with HKSA was small in comparison to the predominant Th1 expansion already described, but these Th17 cells were also preferentially expanded in SA-BSI patients (Fig 4.13D).

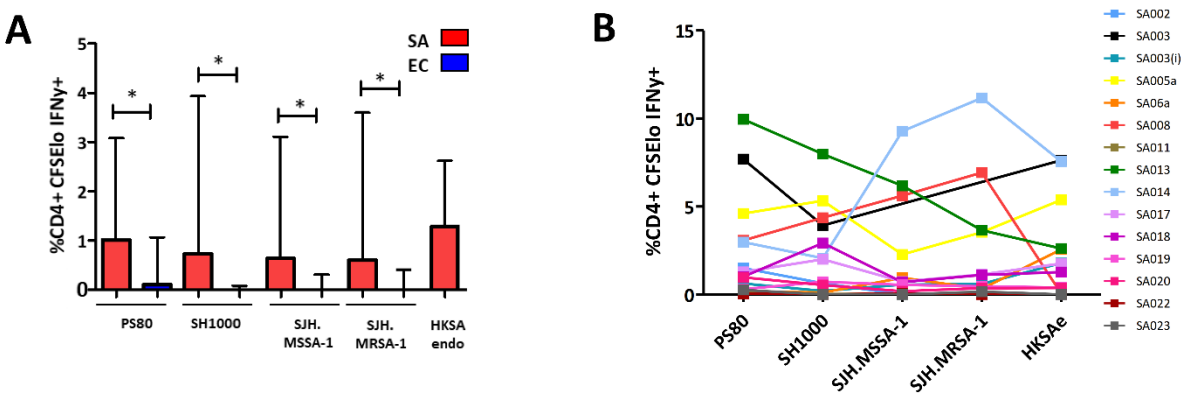

Figure 4.12. *S. aureus* antigen-specific Th1 expansion is evident only in CD4⁺ T lymphocytes from *S. aureus* bloodstream infection patients, and does not differ between bacterial strains.

PBMCs were isolated from *S. aureus* and *E. coli* bloodstream infection patients, CFSE-labelled and incubated with various strains of heat-killed *S. aureus* (HKSA) (1μg/ml; HKSAe = endogenous infecting strain; PS80, SH1000, SJH.MSSA-1; SJH.MRSA-1) for 10 d before assessing proliferation and cytokine production by gating on cytokine-producing CFSElo CD4⁺ cells using flow cytometry. Expressed as a percentage of live CD4⁺ lymphocytes, *S. aureus* antigen-specific CD4⁺ Th1 (IFNγ⁺) proliferation was markedly increased in SA-BSI patients as compared with EC-BSI patients for all strains of HKSA (A). *S. aureus* antigen-specific Th1 proliferation showed variation between individuals, but did not differ significantly between staphylococcal strains for each SA-BSI patient (B). All values are corrected for background proliferation in response to negative control. Results expressed as median ± interquartile range (A) and individual data points (B). Kruskal-Wallis test was used to compare responses. SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infection; HKSAe = endogenous infecting strain; PS80, SH1000, SJH.MSSA-1; SJH.MRSA-1 = reference *S. aureus* strains; CFSE = carboxyfluorescein succinimidyl ester. SA n=17, EC n=8.

*p<0.05.

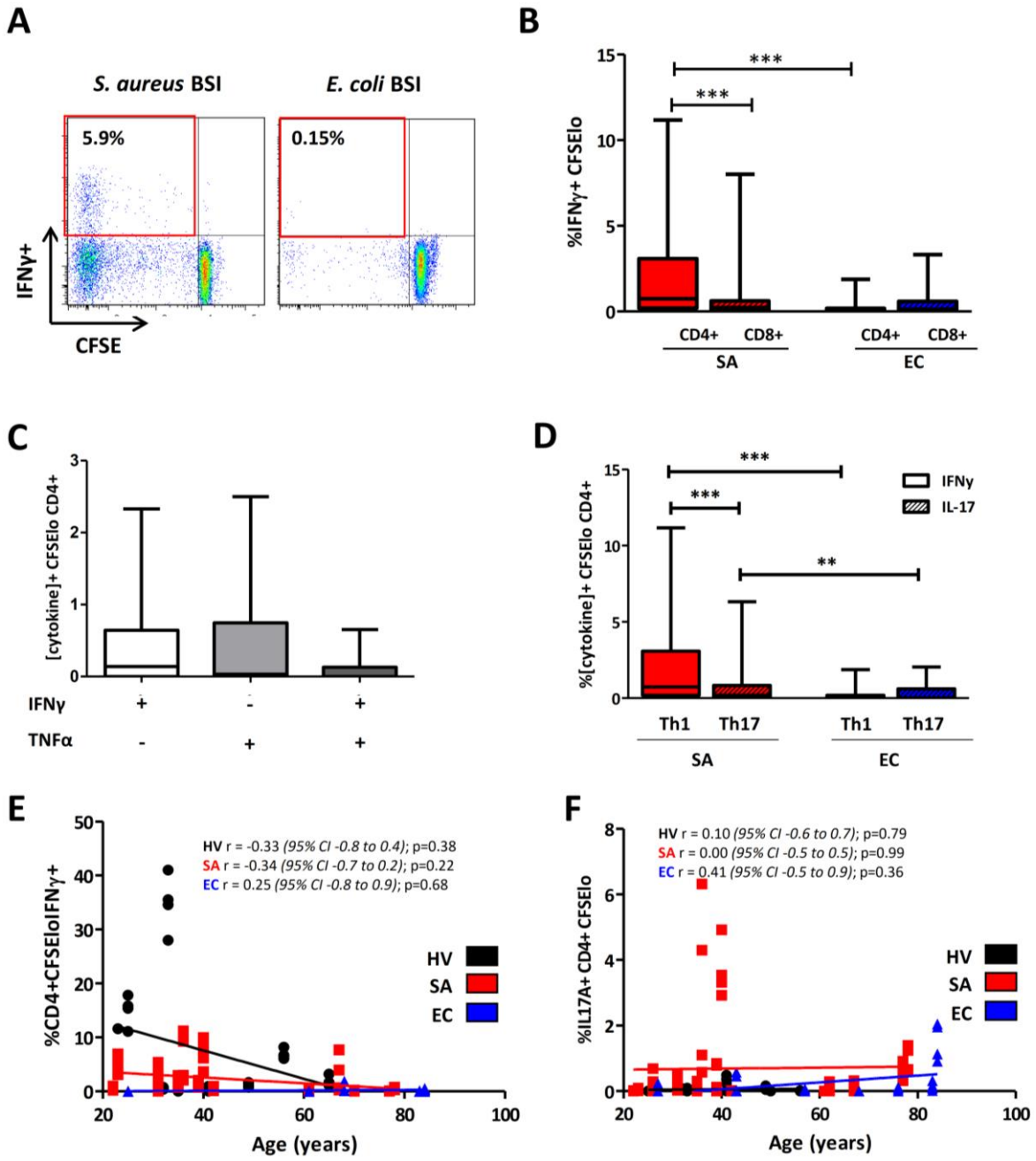

Figure 4.13. *S. aureus* antigen-specific Th1 expansion is the major T lymphocyte response in *S. aureus* bloodstream infection patients.

PBMCs were isolated from *S. aureus* and *E. coli* bloodstream infection patients, CFSE-labelled and incubated with various strains of heat-killed *S. aureus* (HKSA) (1μg/ml; HKSAe = endogenous infecting strain; PS80, SH1000, SJH.MSSA-1; SJH.MRSA-1) for 10 d before assessing proliferation and cytokine production by gating on cytokine-producing CFSElo CD4+ cells using flow cytometry. Representative plots gated on live CD4+ lymphocytes from patients recently infected with either *S. aureus* or *E. coli* showing antigen-specific proliferation ($CFSE_{lo}$) and IFNγ production in response to HKSA (A). Expressed as a percentage of live CD4+ lymphocytes, expansion of *S. aureus* antigen-specific CD4+ Th1 (IFNγ+) cells is markedly increased in SA-BSI patients as compared with EC-BSI patients, whereas CD8+ cytotoxic T cell responses are not different between groups (B). Among SA-BSI patients, a proportion of CD4+ cells having undergone *S. aureus* antigen-specific proliferation (CFSElo) showed Th1 cytokine production, either IFNγ or TNFα alone or both (C). Th1 (IFNγ+) expansion is greater than Th17 (IL-17+) expansion among SA-BSI patients, while Th17 expansion was only significant in SA-BSI patients (D). Expansion of *S. aureus* antigen-specific Th1 (E) or Th17 (F) populations did not correlate with age. All values are corrected for any background proliferation in response to negative control. Results (B-D) expressed as median ± interquartile

range where Kruskal-Wallis test was used to compare responses. Graphs E and F show individual data points along with a best-fit line, Pearson correlation coefficient (r) and associated 95% confidence interval (CI).
SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infection; CFSE = carboxyfluorescein succinimidyl ester. SA n=17, EC n=8. **p<0.005, ***p<0.001.

4.2.7. *S. aureus* bloodstream infection can induce antigen-specific Th1 immune memory

The final step was to establish if these proliferating *S. aureus* antigen-specific T helper cells induced by SA-BSI could form immune memory, essential for protective immunity. Memory cells express CD45RO (a marker of previously activated or antigen-experienced T cells). Most $CD4^+$ T cells proliferating in response to HKSA were $CD45RO^+$ (Fig 4.14A), and these memory cells produced most of the IFNγ (Fig. 4.14B). This population of *S. aureus* antigen-specific memory Th1 cells was minimal in EC-BSI patients ($CD4^+CFSE_{lo}CD45RO^+IFN\gamma^+$ [4.3% (0.87-10.23) vs 0.86% (0.23-4.2); p=0.02 in SA-BSI and EC-BSI respectively] (Fig. 4.14B and C). Fig. 4.14D shows this data alternatively, with a representative plot gated on 'daughter' dividing antigen-specific cells ($CD4^+$ $CFSE_{lo}$) to demonstrate that the majority are memory ($CD45RO^+$) cells, and that these are the only cells to produce IFNγ. In contrast, a representative plot from EC-BSI demonstrates little proliferation or cytokine production in response to heat-killed *S. aureus*.

Taken together, these results demonstrate for the first time that invasive *S. aureus* infection in humans predominantly expands a population of Th1 and Th17 cells (to a lesser extent), and that T helper cell immune memory is formed. These cells are primed and expanded *in vivo* in patients with recent bloodstream infection and thus may play a role in immune control of, and recovery from, infection.

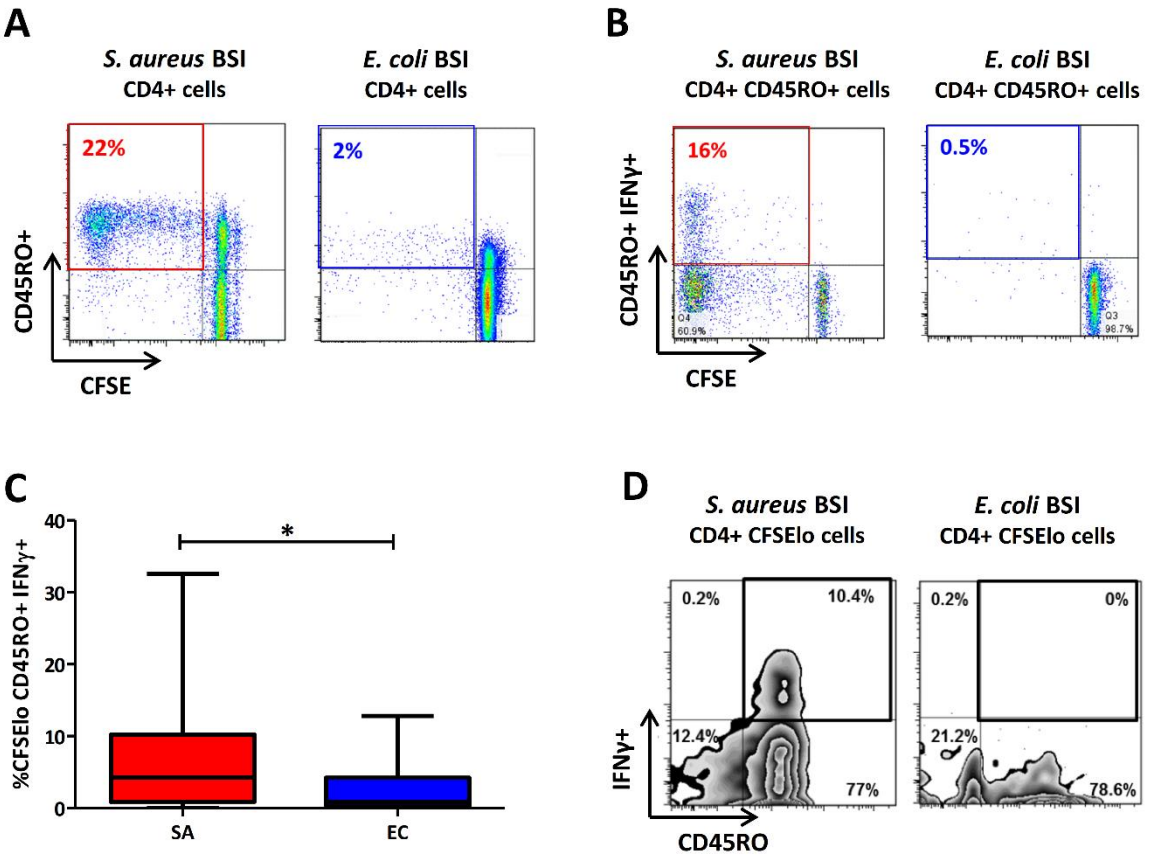

Figure 4.14. *S. aureus* bloodstream infection induces significant *S. aureus* antigen-specific IFNγ response exclusively from CD4⁺CD45RO⁺ memory T lymphocytes.

PBMCs were isolated from bloodstream infection patients, CFSE-labelled and incubated with various strains of heat-killed *S. aureus* (HKSA 1μg/ml; HKSAe = endogenous infecting strain; PS80, SH1000, SJH.MSSA-1; SJH.MRSA-1) for 10 d before assessing proliferation and cytokine production using flow cytometry. Representative plots gated on live CD4⁺ lymphocytes from patients recently infected with either *S. aureus* or *E. coli* showing antigen-specific proliferation ($CFSE_{lo}$) among memory (CD45RO⁺) and naïve (CD45RO⁻) cells (A). Representative plots gated on live memory CD4⁺ cells (CD4⁺CD45RO⁺) from *S. aureus* or *E. coli* BSI patients demonstrate that IFNγ is produced by memory lymphocytes (B), and expansion of *S. aureus* antigen-specific memory Th1 (CD4⁺CD45RO⁺IFNγ⁺) cells is markedly increased in SA-BSI patients as compared with EC-BSI patients (C). Representative plots gated on daughter proliferating cells ($CD4^+CFSE_{lo}$) demonstrate that most proliferation is by memory (CD45RO⁺) cells and that a subset of these produce IFNγ in SA-BSI patients in response to heat-killed *S. aureus* antigens, whereas this pattern is not seen in EC-BSI patients (D). All values are corrected for any background proliferation in response to negative control. Results expressed as median ± interquartile range. Kruskal-Wallis test was used to compare responses. SA = *S. aureus* BSI; EC = *E. coli* BSI; BSI = bloodstream infection; CFSE = carboxyfluorescein succinimidyl ester. HV n=15, SA n=17, EC n=8.
*$p<0.05$.

4.2.8. *S. aureus* bloodstream infection is associated with anti-staphylococcal humoral response

T cell findings alone do not fully characterise the adaptive immune response in invasive *S. aureus* infection, and next-generation anti-*S. aureus* vaccines may need to target both B and T cells. To this end, sera from 34 BSI patients (23 SA-BSI and 11 EC-BSI on day 7±2 post-initial bloodstream infection), as well as from 12 healthy volunteers were interrogated for antibodies of interest. This was carried out by collaborators at the van Wamel laboratory, Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam. Levels of IgG against 35 staphylococcal antigens (10 cell-wall and 25 secreted proteins) were measured in serum using a bead-based flow cytometry technique, and reported as median fluorescence intensity (MFI), a semi-quantitative measure of antibody levels (see *2.4.9*) [186].

IgG MFI levels to all *S. aureus* antigens were greater among SA-BSI patients, followed by healthy volunteers, while EC-BSI patients had the lowest humoral response to *S. aureus* antigens (Figure 4.14). The most 'immunogenic' B cell antigens (those producing the highest IgG MFI) were leukocidins E, D and S (LukE, LukD, LukS), γ-haemolysin (HlgB), immunodominant staphylococcal antigen A (IsaA), staphylococcal enterotoxin C (SEC) and iron-regulated surface determinant A (IsdA). However, high titres against these proteins were also found in healthy volunteers, indeed greater than those seen in EC-BSI patients. Given that pre-infection sera were not available, IgG levels for each antigen were compared between all three patient groups to determine whether some might discriminate for active staphylococcal infection. Significantly increased IgG response was seen to 5 antigens among SA-BSI patients, as compared with both EC-BSI patients and healthy volunteers – LukE, LukS, IsdA, HLA (α-haemolysin) and staphylococcal enterotoxin N (SEN) (Figure 4.15). Taking absolute and relative humoral responses together thus identified 5 staphylococcal B cell antigens that may be specific to active or recent systemic *S. aureus* infection.

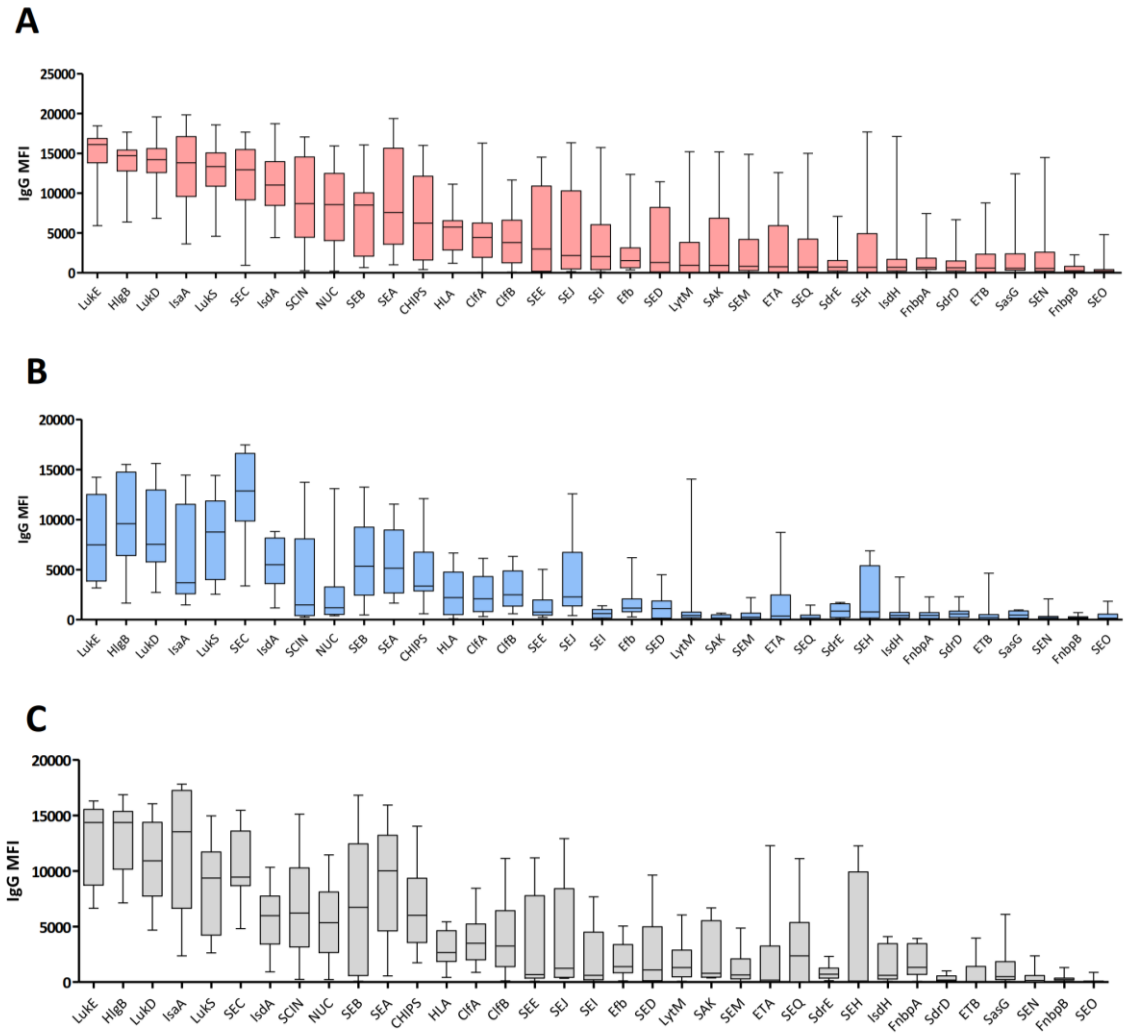

Figure 4.15. Anti-staphylococcal antibodies in health and disease.

Serum from *S. aureus* BSI patients (A), *E. coli* BSI patients (B) on day 7±2 post-onset of bacteraemia, and healthy volunteers (C) was evaluated for IgG binding to 35 purified staphylococcal antigens by averaging duplicate measurements of median fluorescence intensity in a bead-based flow cytometry system. IgG level for each antigen is shown as box-and-whiskers plots where the horizontal line indicates the median, boundaries of the box the IQR, and whiskers indicate the highest and lowest values of the results. SA n = 24; EC n = 11; HV n = 12.
MFI = median fluorescence intensity; BSI = bloodstream infection; Luk = leukocidin; HlgB = haemolysin γ; IsaA = immunodominant staphylococcal antigen A; SE = staphylococcal enterotoxin; SCIN = staphylococcal complement inhibitor; NUC = thermonuclease; CHIPS = chemotaxis inhibitory protein of S. aureus; HLA = α-haemolysin; Clf = clumping factor; Efb = fibrinogen-binding protein E; LytM = peptidoglycan hydrolase; SAK = staphylokinase; ET = exfoliative toxin; SdrE =(serine-aspartate repeat protein) bone sialoprotein binding factor; IsdH = iron-regulated surface determinant H or haemoglobin-binding surface protein; Fnbp = fibrinogen-binding protein; SdrD = serine-aspartate repeat protein D; SasG = S. aureus *surface protein G.*

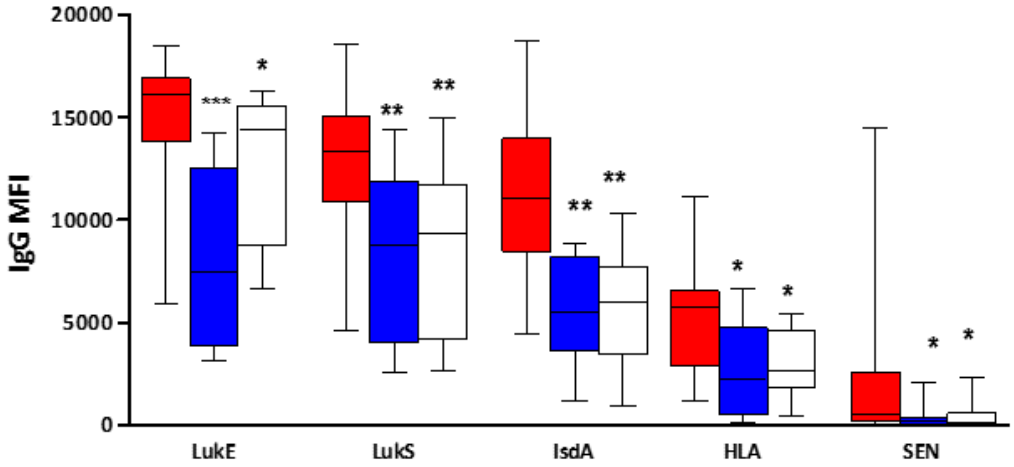

Figure 4.16. Anti-staphylococcal antibodies that discriminate active *S. aureus* infection.

Serum IgG levels to all 35 *S. aureus* antigens (as measured by median fluorescence intensity in a bead-based flow cytometry system) were compared between patient groups [SA-BSI (red), EC-BSI (blue) patients and healthy volunteers (white boxes)]. This was done by Mann-Whitney test, using SA-BSI IgG levels for each antigen as the reference point. This identified five humoral responses significantly different in active *S. aureus* infection. IgG level for each antigen is shown as box-and-whiskers plots where the horizontal line indicates the median, boundaries of the box the IQR, and whiskers indicate the highest and lowest values of the results. SA n = 24; EC n = 11; HV n = 12.

*p<0.05, **p<0.005, ***p<0.001

MFI = median fluorescence intensity; BSI = bloodstream infection; LukE = Leukocidin E; LukS = leukocidin S; IsdA = immunodominant staphylococcal antigen A; HLA = α-haemolysin; SEN = staphylococcal enterotoxin N.

4.2.9. Staphylococcal Clumping factor A produces antigen-specific Th1/17 responses in healthy human CD4$^+$ lymphocytes

Having successfully demonstrated that, in addition to selected antibody responses, invasive *S. aureus* infection in humans produces antigen-specific Th1/17 activation, the concept of pursuing T cell-based vaccination strategies is for the first time supported by clinical evidence. However, the optimum antigen(s) or adjuvant that might produce such a response is unknown. While response to heat-killed whole *S. aureus* was a logical starting point, identification of individual staphylococcal T cell antigens is essential to producing a vaccine. Developing a method to test *S. aureus* vaccine candidate peptides for T cell-activating capacity was the final aim of this project.

The staphylococcal cell wall-anchored protein clumping factor A (ClfA) is one antigen that has been proposed as a promising target for inclusion in multivalent vaccines given its importance as a virulence factor, its ubiquitous expression among clinical isolates and its confirmed expression *in vivo* [212,264]. Serum anti-ClfA antibody titres were higher in the SA-BSI than in the EC-BSI group, and were also detectable in healthy volunteers, validating its humoral immunogenicity (Fig 4.16). Whole-genome sequencing confirmed the presence of the non-repetitive fibrinogen-binding domain of the *clfA* gene in all *S. aureus* bloodstream isolates in the patient cohort (see *3.2.2.3*, Figs 3.12 and 3.13). ClfA may have contributed to the *S. aureus* antigen-specific T helper cell responses already described in the proliferation assay, which used heat-killed bacteria as a source of multiple potential antigens. To examine this possibility, we examined its expression on the bacterial surface and the effect of heat-killing on this expression. Western blotting established that ClfA was expressed *in vitro* by the four reference *S. aureus* strains used in the PBMC T cell proliferation assay (PS80, SH1000, SJH.MSSA-1, SJH.MRSA-1), albeit in varying quantities (Fig 4.18A). ClfA remained present on the bacterial cell surface after killing, although it was not completely unchanged by the process (Fig 4.18B). Live SH1000 showed substantial binding to fibrinogen. A mutant strain (SH1000 *clfA clfB*::Em^r *fnbA*::Em^r *fnbB*::Tet^r) with fibrinogen binding elements (clumping factors A and B, fibronectin binding proteins A and B) deleted, showed minimal binding as expected [181]. Heat-killing significantly impaired SH1000 fibrinogen binding, reducing it almost to that of the mutant strain lacking *clfA* (Fig 4.19). Thus, even though ClfA remains present after heat-killing, fibrinogen-binding capacity was considerably impaired.

To confirm that ClfA itself (in its unaltered form as expressed on live bacteria), may act as a true human T cell antigen, a CD4$^+$ T cell-enriched proliferation assay was designed. CD4$^+$ T

cells were isolated from buffy coats of 9 healthy adult blood donors, magnetically sorted, CFSE-labelled and co-cultured with autologous irradiated APCs in a 1:1 ratio in the presence or absence of purified ClfA (5 or 10μg/mL)or heat-killed PS80 *S. aureus* (1μg/mL). This was similar to the PBMC proliferation assay with heat-killed bacteria, but 'enriched' with a higher-than-physiological ratio of $CD4^+$ T cells – a method particularly useful for detecting small populations of rare antigen-specific cells [263]. An advantage of this model assay is that it can test both antigen and adjuvant effects on T cells *in vitro*. CpG is a TLR9 agonist proposed as a Th1-driving vaccine adjuvant and was evaluated here for its effects on $CD4^+$ cells. Proliferation and cytokine production were assessed by flow cytometry after 10 days. Most healthy individuals had detectable $CD4^+$ proliferation to heat-killed PS80 *S. aureus* (88%) and/or ClfA (78%). Stimulation with purified ClfA at 5 or 10μg/mL induced antigen-specific $CD4^+$ T cell expansion of a similar order to that of HKSA [4.09% (0.96-21.7) and 2.9% (0.57-26.99) vs 3.6% (1.3-5.81), respectively; p=0.98] (Fig 4.20A). There was no significant ClfA dose-response difference in proliferation. Addition of CpG to ClfA significantly boosted $CD4^+$ proliferation compared with ClfA antigen alone (Fig. 4.20A). These proliferating antigen-specific T cells were then further assessed for signature cytokine production. Similar to the patient PBMC results (see *4.2.6*), a proportion of $CD4^+$ T cells were both proliferating and producing IFNγ in response to ClfA (Fig. 4.20B). Addition of CpG boosted this Th1 expansion above that seen with ClfA antigen alone, although this did not reach statistical significance (p=0.11) in this small sample size. Even more ClfA-specific proliferating cells produced TNFα, further supporting evidence that ClfA induces effector Th1 cells (Fig. 4.20C). A small amount of Th17 expansion ($CFSE_{lo}$ IL-17A$^+$) was seen after 10 days, but did not differ by stimulus (Fig. 4.20D).

These findings demonstrate that ClfA – with or without adjuvant – has the capacity to activate healthy human Th1 (and to a lesser extent Th17) lymphocytes, by inducing cell expansion and cytokine production, and that this effect is boosted by CpG adjuvant. Aside from its ubiquitous bacterial expression and implication in virulence, ClfA is now supported as a potential candidate human T cell anti-staphylococcal vaccine antigen by these preliminary human findings. Furthermore, the use of buffy coats with this methodology presents a valuable opportunity to screen staphylococcal antigens and adjuvants for T cell-activating capacity.

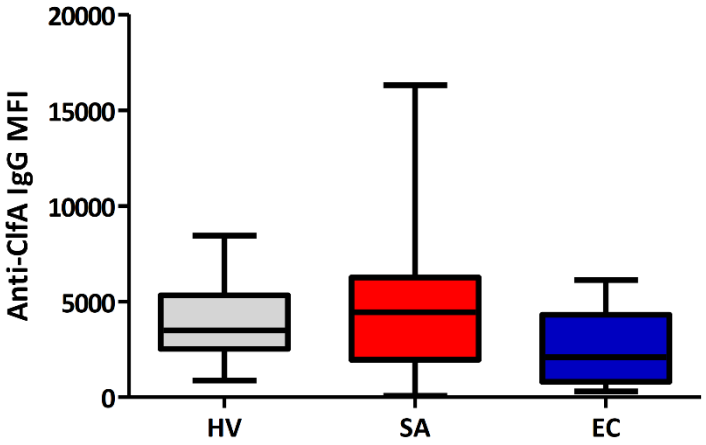

Figure 4.17. Anti-Clumping factor A IgG antibodies are detectable in *S. aureus* bloodstream infection patients and healthy volunteers

IgG antibody binding to ClfA was measured in patient and healthy volunteer sera using a bead-based flow cytometry technique, which estimates antibody titre by median fluorescence intensity (MFI). Results shown as box-and-whiskers plots where the horizontal line indicates the median, boundaries of the box the IQR, and whiskers indicate the highest and lowest values of the results. Kruskal-Wallis test was used to compare groups. HV = healthy volunteers, SA = *S. aureus* bloodstream infection, EC = *E. coli* bloodstream infection. n = 11–24 per group.

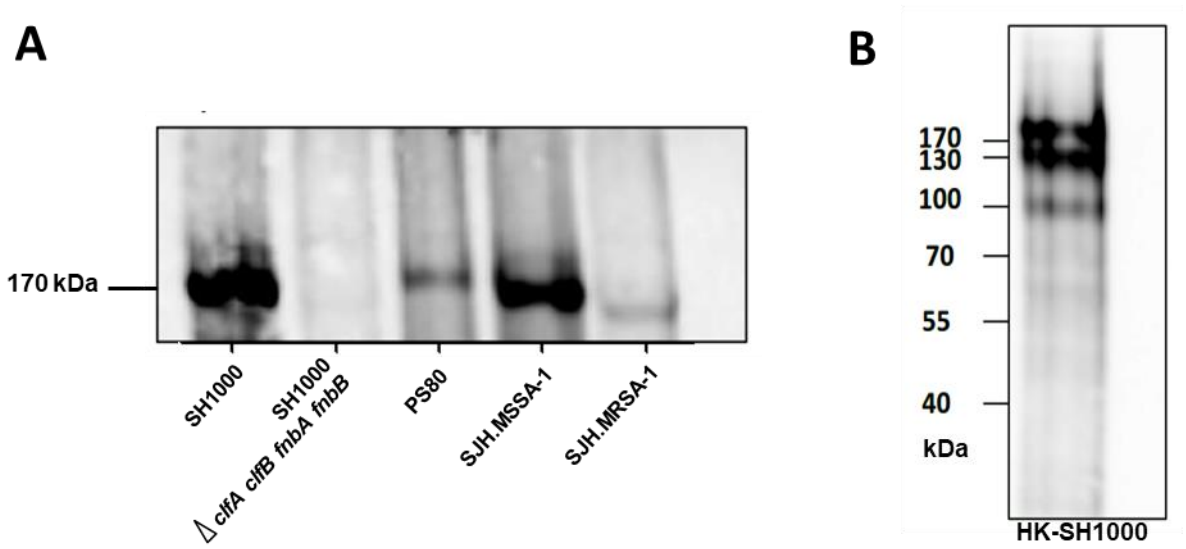

Figure 4.18. Expression of ClfA is conserved across strains and remains present on the cell surface after heat-killing

Reference strains of *S. aureus* (PS80, SH1000, SJH.MSSA-1, SJH.MRSA-1) and a ClfA-deficient mutant (SH1000 clfA clfB::Emr fnbA::Emr fnbB::Tetr) were grown in brain heart infusion broth with shaking at 37°C, washed and adjusted to OD_{600nm} of 10. Cell wall extracts were then prepared. Western blots were probed with rabbit anti-ClfA IgG and bound antibody was detected using protein A peroxidase (A). Differences in band size indicate varying quantities of ClfA production and different numbers of repeats in the C-terminal region, commonly seen with clinical isolates. Cell wall extract from heat-killed SH1000 (HK-SH1000) was treated as above (B). The upper band represents full-length ClfA and the lower band a breakdown product where the N1 subdomain has been removed by proteolytic cleavage.

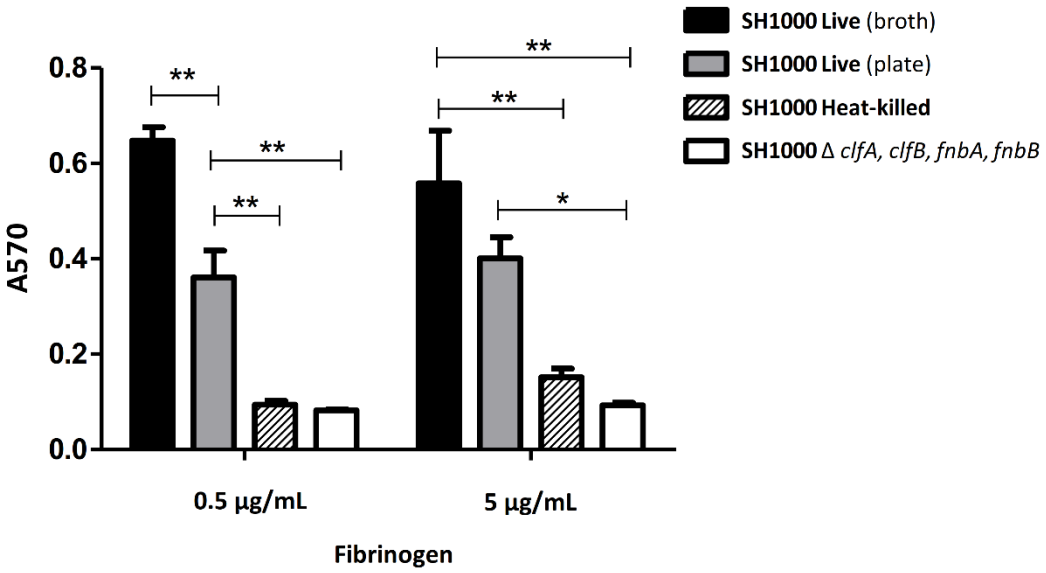

Figure 4.19. Heat inactivation of *S. aureus* significantly impairs binding to fibrinogen

Bacterial suspensions of live and dead *S. aureus* SH1000 at OD_{600nm} were added to wells with plate-bound fibrinogen (0.5 µg and 5 µg/mL). Formaldehyde was added to fix bound bacteria before staining with crystal violet and measuring absorbance at 570nm. SH1000 strains were as follows: (i) Live SH1000 cultured in tryptic soy broth; (ii) Live SH1000 cultured on Columbia blood agar; (iii) SH1000 heat-killed at 90°C x 45 min; (iv) SH1000 *ΔclfA,clfB,fnbA,fnbB* mutant cultured in tryptic soy broth. *p<0.05, **p<0.005

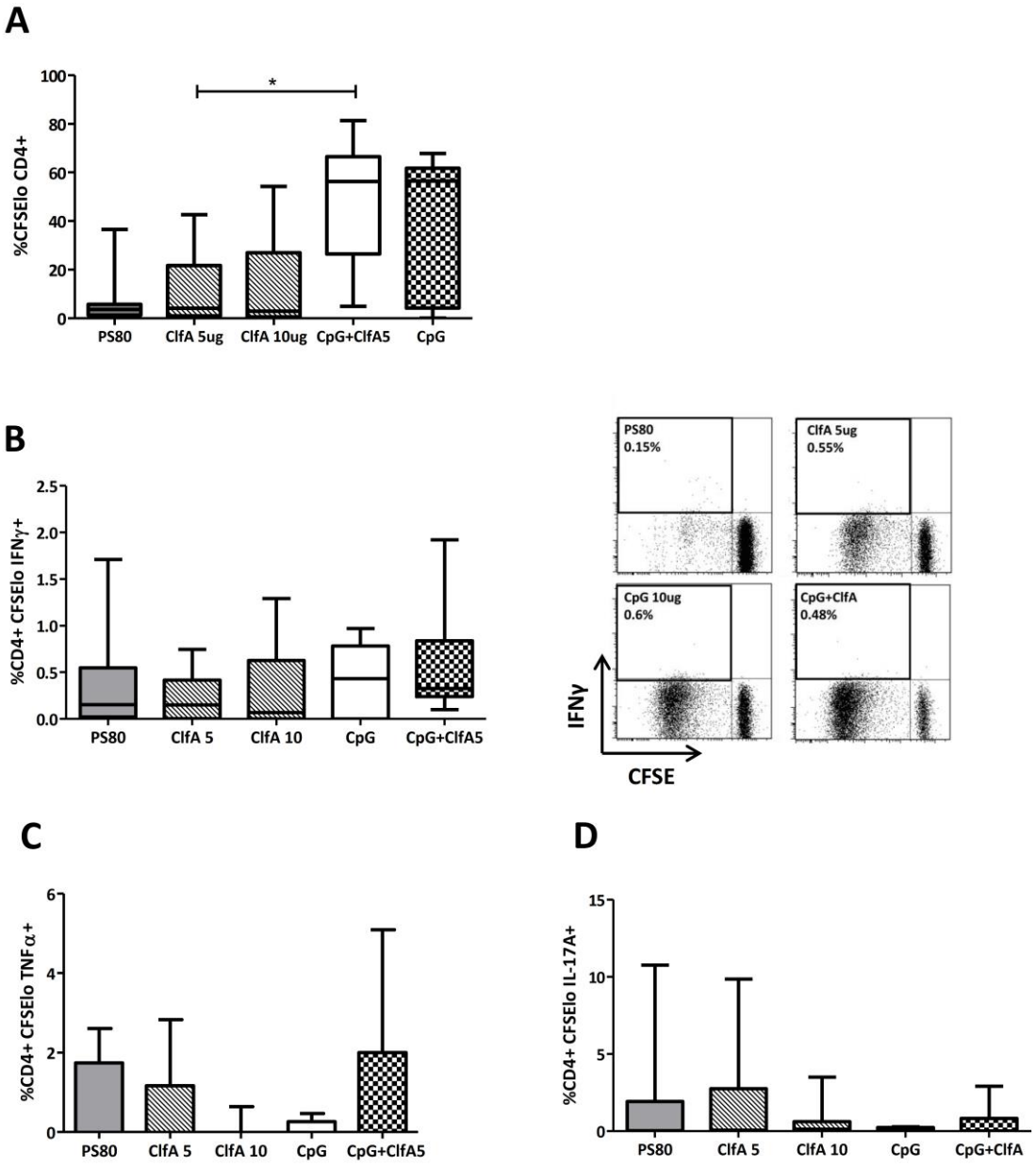

Figure 4.20. Healthy human CD4⁺ cells show ClfA-specific Th1 responses.

Magnetically purified CD4⁺ T lymphocytes were CFSE-labelled and co-cultured 1:1 with autologous irradiated APCs from buffy coats of healthy blood donors along with ClfA (5μg/mL and 10μg/mL), heat-killed *S. aureus* PS80 strain (1μg/ml total protein ≈ 1 x 10^7 CFU/ml), CpG (10μg/mL), CpG (10μg/mL) together with ClfA (5μg/mL) or media alone. After 10 d, antigen-specific proliferation was assessed by gating on CFSElo cells in the live CD4⁺ population using flow cytometry (A). ClfA-specific Th1 and Th17 cell expansion was assessed at the same timepoint by gating on CFSElo IFNγ⁺ (B) and CFSElo TNFα⁺ (C), or CFSElo IL-17A⁺ (D) cells in the live CD4⁺ T lymphocyte population. Representative FACS plots showing ClfA-specific Th1 (IFNγ⁺) expansion are also shown in (B). All values are corrected for any background proliferation in response to negative control. Results expressed as median ± interquartile range. Kruskal-Wallis tests were used for comparisons. n = 9 for all except (D) where n=3.
*p<0.05.

4.3. Discussion

The critical factors that have impeded *S. aureus* vaccine development to date have been absence of known correlates of protective immunity and a lack of translation of animal findings to patients. Prior to this work, there was virtually nothing known about the human T lymphocyte response to *S. aureus*. This study sought to characterise adaptive responses during early recovery from *S. aureus* bloodstream infection, with a particular focus on T lymphocytes, by comparing with *E. coli* bloodstream infection patients. For the first time, it was shown that, in addition to antibody responses, human αβ ($CD4^+$ and $CD8^+$), but not $\gamma\delta^+$ lymphocytes, have the capacity to recognise and respond to *S. aureus* during invasive infection. This response is primarily IFNγ-mediated (Th1), with a lesser contribution from Th17 cells, and is associated with immune memory. In marked contrast, similar *E. coli*-specific T helper cell findings were not seen in patients recovering from *E. coli* infection, nor did they have a *S. aureus* antigen-specific memory lymphocyte population. This infers that Th1/17 responses may be a critical part of immune response to recent invasive *S. aureus*, but not *E. coli*, infection.

The elevated serum IFNγ, *S. aureus* antigen-specific IFNγ response from $CD4^+$ and $CD8^+$ lymphocytes, and memory Th1 expansion, in SA-BSI patients aligns with previously described evidence supporting a protective role for type I immunity, IFNγ, and particularly for Th1 cells, in systemic *S. aureus* infection in mice [32,70-73]. Much of the protection afforded by IFNγ is thought to be due to its effect on phagocytes, particularly macrophages. Th1 cell-derived IFNγ can stimulate macrophage microbicidal activity, upregulate expression of MHC molecules to enhance antigen presentation, and promote immunoglobulin isotype switching to high-affinity IgG1/IgG3 [69]. Accelerated bacterial clearance after adoptive transfer of *S. aureus* antigen-specific Th1 cells was associated with increased macrophage (but not neutrophil) MHC expression and ROS activity, whereas depletion of macrophages was lethal in half of mice [32]. In patients with chronic granulomatous disease, IFNγ has been used successfully as prophylaxis to reduce frequency and severity of *S. aureus* infection. The mechanism of protection was unclear for many years, as it does not restore bacterial killing or superoxide production in defective neutrophils [265]. Recently, IFNγ was found to increase macrophage NADPH oxidase activity and accelerate killing of *S. aureus* in cells from healthy individuals [58,266]. The Th1:macrophage axis may therefore be a more important element of anti-*S. aureus* immunity than previously thought. Given the propensity of *S. aureus* to survive, replicate in, disseminate and subsequently escape from immune cells, mechanisms

that restore or augment phagocyte function (or their upstream helper lymphocytes) represent attractive therapeutic targets [58,106]. Finally, a cytotoxic T lymphocyte ($CD8^+$ $IFN\gamma^+$) recall response to *S. aureus* was seen among SA-BSI patients in the short stimulation assay, inferring that these may be circulating effector cells in the early recovery period. However, experimental data translating $CD8^+$ T cell recognition of *S. aureus* into a truly protective role is less convincing [88,267,268]. No antigen-specific proliferation of $CD8^+$ cells was evident here in our 10-day assay, nor was a CTL response generated to any of four *S. aureus* antigens in a human vaccine study, so the role of CTLs remains unclear [88,89].

An early IL-17 response was not evident in serum, nor after overnight stimulation with heat-killed *S. aureus*. This may be a real absence or potentially due to sampling methodology. An IL-17 response is particularly important for early protection at mucosal surfaces, and may prevent haematogenous dissemination of *S. aureus* from these sites [76,85]. Given this context, an $IL\text{-}17^+$ population may not have been so evident by sampling only peripheral blood at a relatively late collection timepoint (7 ± 2 days post-initial bacteraemia). However, a small population of $CD4^+$ Th17 (but not $IL\text{-}17^+$ $\gamma\delta^+$) cells did expand in the longer proliferation assay, exclusively among SA-BSI patients – results which support a potential role for IL-17 in human systemic *S. aureus* infection.

The significant proliferation of *S. aureus*-responsive Th17 and memory ($CD45RO^+$) Th1 cells suggests that these populations are created or re-expanded *in vivo* during *S. aureus* bloodstream infection and thus may be an appropriate target for vaccines. Model vaccines driving these cells have succeeded in generating antibody-independent protection against systemic infection in mice [64,122]. Both are routinely produced during systemic infection, but only deficiency in IFNγ results in increased mortality [72,77,269]. Synergy between Th1 and Th17 cells may, however, be necessary to mediate protection in systemic infection, at least when infection is introduced transmucosally [31,72,121]. A short-lived IL-17 response from tissue-resident (Th17 or $\gamma\delta^+$) T cells at mucosal surfaces may prevent local invasion and dissemination of infection by driving neutrophil recruitment to eliminate extracellular staphylococci. A sustained Th1 response driving enhanced phagocyte responses to eradicate intracellular staphylococci may be critical to ensure clearance of systemic infection.

Convalescent (day 7±2) timing of sampling was thought to be both necessary and sufficient to allow development, or expansion, of an early adaptive immune response. All recruited patients had relatively acute presentation following onset of symptoms (see *2.2.1*). Increasing age is a risk factor for *S. aureus* (and all other) bloodstream infections. The specific

immunological reasons for this are unknown and likely multifactorial [14]. Although the age difference between groups was non-significant, its potential impact on these results was considered. Aside from an increase in memory T cells, total number and percentages of B and T lymphocytes do not change with aging. Surprisingly, capacity for IFNγ and IL-4 production from both $CD4^+$ and $CD8^+$ lymphocytes is significantly increased in older patients [270]. This enhanced cytokine production in the elderly may imply that immunosenescence is more to do with defects in the function of downstream target cells of these cytokines. T lymphocyte proliferation to specific and non-specific stimulation falls with advancing age [271]. The findings here (elevated serum IFNγ, *S. aureus* antigen-specific early IFNγ response from αβ lymphocytes, and memory Th1 expansion) in SA-BSI patients are thus, at least partially contrary to what would be expected by the effect of age alone. Furthermore, age did not consistently correlate with lymphocyte recovery between days 0 and 7±2, serum IFNγ level, early IFNγ from $CD4^+$ and $CD8^+$ lymphocytes, or Th1/17 proliferation to *S. aureus* antigens. This strongly suggests the results were truly infection group-specific, rather than age-specific.

The responses of patients recovering from *E. coli* bloodstream infection imply a substantial difference in the roles of T cells in these two bacterial infections. Total lymphocyte restoration was not seen among recovering EC-BSI patients, nor did they have a pathogen-specific T helper cell response to re-stimulation with heat-killed *E. coli*. This was in marked contrast to the clear *S. aureus* antigen-specific Th1/17 expansion in SA-BSI patients. Gamma-delta T cells from EC-BSI patients produced significant IFNγ (but not IL-17) in response to *E. coli* antigens. This population did not expand in the 10-day proliferation assay. This was the only noteworthy lymphocyte response to *E. coli* seen in EC-BSI patients. *E. coli* expresses HMB-PP [(E)-4-hydroxy-3-methyl-but-2-enylpyrophosphate], a potent phosphoantigen that activates human $V\delta9^+V\gamma2^+$ T lymphocytes to produce IFNγ [272]. This *in vitro* finding has not been correlated with clinical outcome in *E. coli* infection. *S. aureus* does not produce HMB-PP and did not activate $\gamma\delta^+$ cells from the SA-BSI cohort.

A clinically useful anti-*S. aureus* vaccine must include antigens effective against multiple strains. *S. aureus* epitopes that might activate human T cells were essentially unknown prior to this work, and there is a lack of high-throughput approaches to identify such antigens. 'Antigen soups' (laboratory and clinical strains of heat-killed washed whole staphylococci) were used to provide a large pool of possible surface protein/polysaccharide epitopes, as a first step in determining whether human T cells could recognise and respond to *S. aureus* at all. Dead bacteria were necessary to allow standardised comparison of lymphocyte

proliferation and cytokine response, which may have otherwise been skewed due to differences in growth between live strains, as well as to avoid bacterial overgrowth and death of PBMCs at later time points. However, this use of washed heat-inactivated bacteria will have prevented exposure of PBMCs to secreted antigens they would otherwise be challenged with during *in vivo* infection, as well as potentially denaturing surface proteins sufficiently to change the mechanism or character of the host immune response. For instance, heat-inactivation was shown here to affect fibrinogen binding to ClfA, while not altering its structure enough to preclude detection by Western blot. However, these difficulties would also be expected with alternative methods of bacterial inactivation, e.g., UV irradiation, gamma irradiation or formalin inactivation. Significant differences in survival following challenge infection, and in post-immunisation antibody response, have been observed with different methods of inactivating a single *S. aureus* strain [256,273]. Despite being challenged with different clonal complexes with some variation in surface proteins (see *3.2.2.2* and *3.2.2.3*), a striking finding of this study was the uniformity of each SA-BSI patients' (i.e., intra-individual) T cell cytokine and proliferative responses to multiple *S. aureus* strains. In fact, inter-individual (i.e., between SA-BSI patients) differences were more evident, suggesting a host-determined, rather than strain-specific immune effect. Similar to the findings in the previous chapter, this may suggest some T cell epitopes are highly conserved across strains – promising for the discovery of a pan-species vaccine. Alternatively, it may suggest that responses are peptide:HLA-restricted. This host-specificity of immune response adds to compelling clinical evidence that the outcome of human *S. aureus* infection is more dependent on host than microbial factors, and in particular, on T cell-mediated immunity [3,4,111].

Antibody levels to 5 staphylococcal antigens discriminated for recent infection among SA-BSI patients, as compared with EC-BSI patients and healthy volunteers. Apart from IsdA (a non-secreted surface adhesin which facilitates binding to epithelium), these (LukE, LukS, α-haemolysin, staphylococcal enterotoxin N) are pathogenic toxins, superantigens or immune evasion molecules secreted during infection. Toxin neutralisation is an essential defence mechanism and pre-formed or newly re-expanded anti-toxin IgG is protective against toxic shock syndrome [274]. Ideally, antibody titres should also be compared pre- and post-bloodstream infection. Thus, while the 5 'discriminating' antibodies for recent infection are statistically significant, and a toxin neutralisation function is biologically plausible, their true clinical implication is unknown. *S. aureus* infection produces a measurable B cell response, but its protective efficacy is far from established [68,275]. Antibodies are probably crucial in

preventing or attenuating toxin-driven disease, but have less effect on acquiring infection and on disease caused by other mechanisms. Of course, there is no simple dichotomy of lymphocyte function. B and T cells in fact have constant interplay, mutually influencing each other's functions. It is likely that humoral responses play, at least, a partial role in protection against *S. aureus*, but cellular responses may well hold more promise.

The above description of *S. aureus* antigen-specific adaptive immunity (particularly the first report of adaptive cellular immune response) in the early recovery period of bloodstream infection is considerably important even in the face of several limitations. The most notable of these were the lack of pre-morbid colonisation screening and of 'baseline' blood samples with which to compare immune responses pre- and post-infection. Both would have been logistically impossible for a project like this. Ascertaining true colonisation status would have necessitated serial nasal and extranasal sampling over a period of months to years prior to presentation with bloodstream infection [26]. Blood sampling prior to infection would have allowed quantitation of the actual change in antibody titre or effector T cell response induced by infection at a patient-specific level, as a more accurate measure of infection-attributable changes in *S. aureus* bloodstream infection. However, even among higher risk populations, onset of acute bloodstream infection is so unpredictable that pre-infection testing would be prohibitive. Inclusion criteria were chosen in an effort to investigate the 'normal' T cell response during recovery, and avoid significant confounding by iatrogenic severe lymphocyte-specific immunosuppression. Recruitment of such acute patients given the limited eligibility, narrow time window from presentation to sample collection and necessity for fresh analysis of cells presented several logistical challenges. Despite this, the numbers of *S. aureus* BSI patients recruited here compares favourably to larger scale multicentre studies with significantly longer recruitment periods [129]. The cohort is also biased towards those SA-BSI patients who survive the first 24-48 hours of infection, given that it takes at least this period to confirm the diagnosis. Those patients who present *in extremis* and rapidly deteriorate may have a very different T cell response. It is unclear whether the variance in clinical management described in Chapter 3 may have had any impact on the immunological findings. Nonetheless, the findings presented here represent a substantial step forward in our understanding of adaptive responses in real patients.

Having demonstrated the novel finding that invasive *S. aureus* infection in humans produces a T helper cell response to a pool of multiple undefined *S. aureus* antigens, developing a method of screening prospective vaccine antigens was the next step. Despite significant

investment in anti-*S. aureus* vaccines, there has been virtually no pre-clinical work examining candidate antigens' induction of cell-mediated responses [89,126]. Screening with buffy coats is a practical methodology for testing human T cell immunogenicity prior to clinical trials. Clumping factor A (ClfA) is a promising target for inclusion in multivalent vaccines and induced humoral responses in this cohort and others following *S. aureus* bloodstream infection [186]. Active immunisation with clumping factor A (ClfA) conferred antibody-mediated protection in animal models of *S. aureus* disease [120,276]. Importantly, ClfA also appears to have some T cell-activating capacity in animals [121]. Immunisation with a structurally similar fungal protein (rAls3p-N) produced Th1/Th17 cell-mediated protection in mice against multiple strains of *S. aureus* [64]. While it also induced functional anti-rAls3p-N antibodies, these were a surrogate marker, and the mechanism of protection was antibody-independent [123]. RAls3p-N also activates Th1 and Th17 cells in healthy humans [126]. However, prior to this study, it was not known that ClfA itself is an effective human T cell antigen. It induced significant $CD4^+$ T cell proliferation and IFNγ/TNFα production in the majority (>70%) of healthy adult samples, with a lesser Th17 expansion.

Adjuvants can be a powerful tool to enhance vaccine efficacy by directing the nature of the response to immunisation, and distinct T cell subsets may be explicitly targeted by careful adjuvant selection [277]. Prior *S. aureus* vaccines have either used no adjuvant, conjugation to toxoid (which drives B cell response), or alum (which promotes Th2 and B cells) [278]. CpG is a promising vaccine adjuvant that acts as a TLR9 agonist and potent inducer of Th1 responses [277,279]. Immunisation with a model ClfA+CpG vaccine, which induced both humoral and type I immune responses in mice, demonstrated enhanced clearance of systemic *S. aureus* infection [32]. Addition of CpG to ClfA increased proliferation of healthy human $CD4^+$ cells *in vitro* compared with antigen alone in this study, supporting its inclusion in anti-*S. aureus* vaccines targeting Th1 induction. These preliminary results support the use of buffy coats to screen staphylococcal antigens (and adjuvants) for T cell-activating capacity.

Taken together, the overall findings presented in this chapter indicate for the first time, that – in addition to antibody responses – human $CD4^+$ and $CD8^+$ lymphocytes have the capacity to recognise and respond to *S. aureus* during human invasive infection. This response is primarily T helper lymphocyte and IFNγ-mediated, with a lesser contribution from Th17 cells. Similar pathogen-specific T helper cell activation is not seen in patients with *E. coli* bloodstream infection, and suggests that such a response is particular to immune control of invasive staphylococcal disease. *S. aureus* antigen-specific memory Th1 cells are created or re-

activated during systemic infection. This cellular immune response corresponds to the protective immunity seen by similar cell populations in animal models. It is still unclear in humans, however, whether a strong Th1/17 response is just a marker of immune recognition, or whether it is favourable or detrimental to clinical outcome. Some clinical data suggests that a pro-inflammatory response early in the course of *S. aureus* bloodstream infection may be beneficial. Elevated initial serum IL-1β was associated with accelerated clearance of *S. aureus* bloodstream infection, whereas elevated IL-10 was predictive of death [104]. Inability to maintain a pro-inflammatory state during early recovery (as inferred by higher IL-10:TNFα ratios 72 h after onset) predicted failure to clear bacteraemia and death [280]. IFNγ was not measured in either study, and IL-17 levels were not associated with outcome. Finally, the ClfA buffy coat assay presented here is a new method to identify candidate vaccine antigens and/or assess adjuvant effects. Once staphylococcal T cell antigens are identified, this is a model assay for future work correlating T cell response to these antigens with morbidity and mortality over the course of a bloodstream infection. This may be a powerful way to inform therapeutic immunomodulatory targets. Despite its limitations, studies such as this are vital to advance the knowledge of immune responses during recovery from acute infections in real patients.

Chapter 5

General Discussion

5. 1. Summary of Results

The aims of this project were fourfold. Firstly, to describe the genotypic and phenotypic characteristics of invasive *S. aureus* isolates from adult patients with bloodstream infection across 3 acute hospitals in Dublin, including their expression of potential vaccine antigens and the implication of this for vaccine development, using clumping factor A (ClfA) as a model antigen. Secondly, to compare and contrast the clinical characteristics, management and outcomes of *S. aureus* and *E. coli* bloodstream infection. Thirdly, to develop a model assay to test T cell responses to a specific *S. aureus* antigen. The final aim was to describe for the first time in humans, the adaptive immune response in the early recovery period of *S. aureus* bloodstream infection. The findings of these objectives and their place in current and future approaches to invasive *S. aureus* infection are discussed below.

Genotypic and phenotypic characteristics of invasive S. aureus *isolates*

Prior to this work, published typing of SA-BSI isolates in Ireland had excluded the MSSA that accounts for most (>80%) invasive infections. The distribution of ST types in this study mirrored that of the rest of Europe, with CC5 and CC22 accounting for most isolates [193]. There was considerable genotypic heterogeneity, particularly in MSSA isolates, despite the small size of the study population and the geographic proximity of study sites. The identification of dominant invasive serogroups has facilitated strain-specific immunisation efforts against other pathogens, e.g., pneumococcus and meningococcus, but even much larger-scale efforts than this work to identify pathogenic *S. aureus* strains have been less successful [216,217,281]. Most bloodstream isolates originate from colonising *S. aureus* in the nares, and multiple strains can cause devastating invasive disease [37,147,281]. Such heterogeneity makes the organism much harder to target for immunisation.

An ideal vaccine antigen should be critical to pathogenicity and be present in multiple strains. Genome interrogation revealed that cell wall components, e.g., clumping factor A (ClfA) were more uniformly present and displayed less sequence variability than genes for immune evasion or secreted virulence factors. However, even the limited allelic variation shown in the *clfA* gene has unknown implications for T cell processing and thus induction of epitope-specific immunity. These findings illustrate the complexity of engineering a protective response against variable antigens in multiple strains, and supports the argument that multiple antigens targeting multiple immune responses should be included in anti-*S. aureus* vaccines to achieve anything other than narrow strain-specific, immunity.

Clinical characteristics and course of S. aureus *and* E. coli *bloodstream infection*

The bulk of *S. aureus* disease was attributable to MSSA and infection was frequently community-acquired. Its incidence continues to increase, even while healthcare-associated and resistant infections decline [9]. An excessive focus on resistance has distracted from the problem of tackling *S. aureus* infections as a whole. Most SA-BSI in this study were not preventable by current means. Major success in reducing healthcare-associated MRSA infection has not been replicated in MSSA. There are currently few effective community-based interventions to reduce risk of *S. aureus* infection.

S. aureus's capacity to cause severe disease in otherwise healthy people and to follow a complicated course distinguishes it from *E. coli* BSI. All *E. coli* infections were easily and rapidly cured. In contrast, failure, relapse or death was seen in 40% of SA-BSI. The classification of *S. aureus* bloodstream infection is complex, complications are myriad and challenging to detect, management is patient-specific and requires substantial expertise. Where SA-BSI guidelines exist, they frequently only refer to management of MRSA infection, and there are few formal guidelines encompassing care of all patients with SA-BSI [167,200-202,282]. Irish guidance on MRSA briefly refers to device removal, glycopeptide use and consideration of infection specialist input "as necessary", in a small section on management of invasive MRSA infection [200]. Clinical management was suboptimal in over half of cases in this study when measured against indicators proven to improve outcome in SA-BSI [205,235]. These deviations occured when patients were not under the care of infection specialists. Similar findings have been described from other international centres over the past two decades [283,284]. Despite the fact that bedside infection specialist intervention and adherence to specialist advice is associated with a reduction in mortality by half, it is not currently mandated, resourced or supported on a systems-wide level. A first step may be to develop a comprehensive national clinical guideline on the diagnosis, investigation and management of SA-BSI, against which patient care should then be audited. In comparison with the UK's National Health Service, Ireland also has less embedded national processes for mandatory external reporting on clinical infection rates, benchmarking against similar institutions or financial penalties for avoidable harms, and has only recently introduced multiprofessional post-infection reviews [285-289].

Evidence-based priority setting is increasingly important for rationally distributing scarce health resources and guiding future health research. The level of public concern, media attention and resource allocation for many conditions is disproportionate to their actual disease burden. For instance, the annual incidences of ST-elevation myocardial infarction

(STEMI) and SA-BSI in Ireland are similar, while the risk of death from SA-BSI is approximately five times higher than that from STEMI. Nonetheless, a national clinical programme for standardisation of management of acute coronary syndrome and allocation of resources with the aim of further reducing mortality has been established [290]. There is currently no national clinical programme for infectious diseases, and there has has yet to be an examination of the population burden of infectious diseases or determination of where they rank in comparison to other conditions in the Irish setting. In other high-income settings, the impact of infectious diseases on health-adjusted life years is estimated to be one-quarter that of all cancers, and *S. aureus* is ranked among the top ten most burdensome infections [231]. Compared with bloodstream infection caused by any other pathogen, SA-BSI is associated elsewhere with increased length of stay, investigation and treatment costs, risk of relapse, and infection-attributable mortality – regardless of methicillin resistance. Healthcare cost estimates per episode of SA-BSI range from €9,500 to €110,000 in other countries [6,291,292]. A detailed pharmacoeconomic study on SA-BSI should be undertaken in Ireland, to assess cost-effectiveness of interventions including specialist input.

Methodology for screening candidate staphylococcal vaccine components

There are few known *S. aureus* T cell antigens. Given the mechanism of HLA-restricted antigen presentation to T cells it is difficult to infer potential antigens from translational models. A practical method of screening proteins for human T cell immunogenicity was successfully developed using blood donor buffy coats and clumping factor A (ClfA) as a model antigen. The effect of adjuvants in directing the nature of the T cell response can also be measured in this way. ClfA was shown to be an effective human T cell antigen, inducing significant $CD4^+$ T cell proliferation and IFNγ/TNFα production in the majority of healthy adult samples, with a lesser Th17 expansion. Since this work was completed, colleagues have shown that ClfA subdomains differed in their activation of Th1 and Th17 cells [293]. *S. aureus* T cell antigens should be identified before examining which of their component peptides are most immunogenic with epitope mapping [294]. This should be worked out prior to these antigens entering clinical trials.

Adaptive immune response in S. aureus *bloodstream infection*

There is considerable doubt that induction of humoral immunity alone will be sufficient to confer protection against *S. aureus* infection [248]. Clinical observations and data from experimental models also support a key role for IFNγ, IL-17 and an intact Th1/17:phagocyte axis in anti-staphylococcal immunity, in that patients with impairment of these mechanisms

have an increased risk of *S. aureus* infection [18,252,253]. Notwithstanding these clues, there has been no previous investigation of human cell-mediated immune response in invasive *S. aureus* infection. This work described, for the first time that, in addition to antibody responses, human αβ ($CD4^+$ and $CD8^+$), but not $\gamma\delta^+$ lymphocytes, from SA-BSI patients (in comparisons with both healthy volunteers and EC-BSI patients) can recognise and respond to *S. aureus* during early recovery from bloodstream infection. This response was primarily Th1-mediated, with a lesser contribution from Th17 cells. Th1 immune memory was formed or re-activated during systemic infection. Similar pathogen-specific T helper cell activation was not seen in patients with *E. coli* bloodstream infection. This implies a substantial difference between the role of T cells in these two bacterial infections, and suggests that this Th1/17 signature is particular to recovery from invasive staphylococcal disease. Th1/17 response corresponds to the protective immunity conferred by similar cell populations in animal models, bearing in mind the caveats of translation [31,32,64,122]. An IL-17 response from tissue-resident (Th17 or $\gamma\delta^+$) T cells at mucosal surfaces may act primarily to prevent local invasion and dissemination of infection by driving neutrophil recruitment. A Th1 response driving enhanced macrophage responses may be vital to ensure clearance of systemic infection.

Critically, it is still unclear from these findings whether a strong Th1/17 response is just a marker of immune recognition, or whether it might influence clinical outcome in systemic infection – either favourably or unfavourably. Anti-*S. aureus* antibodies are common, functional, and crucial in preventing or attenuating toxin-driven disease, but have less effect on acquiring infection and on disease caused by other mechanisms. No convincing correlation has been shown between humoral immunity and outcome of bloodstream infection, and increasing antibody titres post-vaccination has not led to protection. Of course, B and T cells are not independent, but rather co-operate to influence each other's functions. Humoral responses play at least a partial role in protection against *S. aureus*, but cellular responses may well hold more promise. Since this work, it has been shown that most healthy adults also possess significant levels of circulating *S. aureus* antigen-specific memory T cells [148]. However, the case for cell-mediated immunity being more than just a bystander in *S. aureus* immunity is significantly stronger than that for humoral response, with a robust biological hypothesis of phagocyte activation that is supported by clinical and experimental data. Nonetheless, there could be an argument that bacteraemia by definition signals a failure of anti-staphylococcal immunity and that SA-BSI patients are not the population of interest to define true correlates of protection. Further comparisons with uninfected healthy subjects – both nasal carriers of *S. aureus* and non-carriers – may yield more useful information. Once a panel of T cell epitopes are established, Th1/17 and other (e.g., regulatory T cell) responses

in SA-BSI patients and comparator groups should be mapped over the course of infection in order to correlate relative potency of T cell subpopulations with clinical outcome. Some data suggests that an early pro-inflammatory response in *S. aureus* bloodstream infection may be beneficial [104,280]. After completion of this work, others have shown that higher neutrophil:lymphocyte and Th17:Th1 ratios predict mortality in SA-BSI [295].

The optimal cells to target for immune response in systemic *S. aureus* infection is likely to be different from that required at other sites of infection [71,74,296]. It is likely that the most effective site-specific immune response will be finely balanced between pro- and anti-inflammation, and dynamic over the course of infection – to have the right cells in the right place at the right time for the right duration. We are only in the very early stages of understanding this process for *S. aureus* infection. Nonetheless, these preliminary findings are a substantial step forward in understanding how T cells react during *S. aureus* infection in real patients – knowledge fundamental to manipulating their activity in host-targeted therapeutics.

5.2. Host-Targeted Therapeutics for invasive *S. aureus* infection

Decades of antimicrobial development following penicillin's introduction and best supportive care have not led to an ongoing fall in risk of death from SA-BSI. There is a renewed urgency for novel strategies. Immune response in *S. aureus* infection is patient-specific and largely unaltered by differences in bacterial strains [245]. Host factors are far more critical to outcome of SA-BSI than are microbial factors [3,217,245,246,297]. These observations were first made over 100 years ago [1]. After decades of microbial focus, medicine is returning to this tenet that the immune system largely dictates the outcome of infectious diseases. Host-targeted therapies may either prevent infection via immunisation or modulate the immune response during infection. Manipulation of immunity with vaccination was arguably the single greatest achievement in the history of medicine. Further understanding of the complex interaction between *S. aureus* and the immune system is likely to pave the way for a revolution in therapeutic and preventative strategies.

5.2.1. Immunomodulatory Therapy for *S. aureus* infection

A vaccine that confers protective immunity would be the ultimate realisation of an ambition to combat *S. aureus* infection and antimicrobial resistance. In its absence, bespoke

therapeutic immunomodulation – targeting the host in order to somehow 'boost' desirable and suppress inappropriate immune responses during infection – would still have a considerable impact on morbidity and mortality. A recent explosion of knowledge has led to revolutionary progress in immunomodulation. Initially, monoclonal antibodies were used for targeted immune blockade (e.g., anti-CD3 in organ transplantation) [298]. More recently, a paradigm shift has occurred. Immune-activating therapies (e.g., CTLA-4 and PD-1 inhibitors) utilise the power of the immune system through checkpoint inhibition, directing it against haematological and solid organ cancers [299]. Immune-activating therapies have not yet been developed for infection. Understanding the immune changes during infection and their impact on prognosis is vital before attempting to manipulate this response. The ability to measure a patient's real-time immune phenotype during infection will also be required to deliver a nuanced, personalised 'tweaking' of immune response, rather than a universal 'one-size-fits-all' intervention.

5.2.2. The Future for Next-Generation anti-*S. aureus* vaccines

All infections which have been eradicated, eliminated or controlled to date included an effective immunisation [300]. A vaccine of even very limited efficacy (≤ 10%) would significantly decrease the incidence of invasive disease in high risk populations [243,244,301]. For pathogens where natural infection does not induce long-lived immunity, however, sterile protection post-vaccination has yet to be achieved. Recurrent infections and failure of previous vaccines imply that immune response induced by *S. aureus* infection is insufficient or becomes dysregulated. We do not currently know what the optimal immune response to *S. aureus* might be. Given this significant knowledge gap, deciding which antigen to target, or which type of immunity to induce in vaccination strategies is incredibly challenging. It is now widely accepted that a vaccine with a cocktail of antigens, that neutralises toxins, targets both cellular and humoral immunity and can tailor immune response to tissue site is required; although it remains unknown whether any of these things would be completely or partially protective in humans. Vaccines in development – albeit no longer single-antigen – look depressingly similar to previous failures. No new anti-*S. aureus* vaccines were registered for clinical trials at the time of writing. The field needs an exciting leap forward, one which may be provided by next-generation vaccines which include T cell targets. Along these lines, a MVA-based (modified vaccinia virus, Ankara) vaccine with unknown *S. aureus* antigen(s) is reportedly planned for human trials soon [302]. A precise understanding of what drives 'appropriate' immunity to staphylococcal infection must provide the immunological

framework for vaccine design in terms of antigen and adjuvant selection for optimum target cell response.

In the era of modern immunology and rational vaccine design, the approach whereby candidate *S. aureus* vaccines continue to be brought to clinical trial without knowing correlates of immune protection or accounting for the potential immune imprint of prior exposure seems a flawed one. Moreover, vaccine development when immunity is incompletely understood can result in unintended deleterious consequences, and should not be seen as a risk-free endeavour. Though a seeming paradox, worse outcomes after vaccination have occurred. Natural infection after vaccination has resulted in vaccine-enhanced immunopathology or, conversely, induced anergy, by affecting either innate or adaptive immune memory [303]. Respiratory syncytial virus (RSV) infection following immunisation with inactivated virus resulted in severe, and in some cases fatal, disease, mediated by distinct subsets of $CD4^+$ Th1 and Th2 memory cells [304]. More recently, the first licensed dengue virus vaccine was found to have differential performance in individuals previously infected by dengue virus (seropositive), as compared with dengue-naive (seronegative) individuals. Vaccine efficacy was much lower among seronegative participants, but of more concern, immunisation increased their risk of severe disease upon subsequent natural infection with dengue. In 2018, the World Health Organisation therefore recommended that only dengue seropositive individuals should be vaccinated [305]. The opposite effect has been described with other organisms, where prior exposure that induces immunosuppressive memory cells has impeded development of protective Th1/Th17 responses, and contributed to vaccine failure or increased infection risk [306-308]. Whether or not such effects may occur for putative *S. aureus* vaccines is completely unknown.

Although infection does not result in long-lived protective immunity, *S. aureus* exposure from infection, colonisation, or vaccination does leave its trace on the immune system [30-32]. Such exposure may 'prime' the immune system, changing its behaviour during subsequent infection. Some evidence suggests that nasal carriage (although strongly predictive of invasive disease) may result in partial protection against death in invasive infection [30]. The mechanism behind this protection is unknown. In contrast, the increased risk of death among V710 vaccine recipients with serious *S. aureus* infections raised concerns about vaccine-induced 'immune priming' being detrimental, rather than protective, in subsequent infection [140]. While IsdB immunisation induced an antigen-specific protective Th17 response in *S. aureus*-naïve mice, it appears that impaired T cell responses in some vaccine recipients prior to vaccination may actually have contributed to mortality [130]. Low serum IL-2 and IL-17A levels (before and after immunisation) predicted death in vaccine (but not in placebo)

recipients [130]. This cytokine profile might suggest a failure to mount an appropriate Th1/17 response and, consequently, misplaced and counterproductive Th2-dominant, or even Treg-dominant, *S. aureus* antigen-specific immunity, recapitulated on subsequent infection.

Immune history, as opposed to genetics, primarily determines response to immunisation [309]. Such responses may not be predicted by pre-clinical work with naïve animals raised in relatively pathogen-free environments [310]. 'Priming' with natural exposure and 'boosting' with immunisation may be the preferable approach. This could be because antigen-specific anti-inflammatory (as well as pro-inflammatory) responses to multiple antigens are induced by natural exposures, whereas vaccines have yet to be designed with this balance in mind. T cells producing both IFNγ and IL-17 will likely be required to confer maximum protection against *S. aureus,* but these responses will need to be tightly regulated. We are on the brink of a new era of targeting the host to prevent or influence *S. aureus* infection. The findings in this thesis play a small part in this endeavour.

5.2.3. A Roadmap for *S. aureus* Vaccine Clinical Trial Design

Anti-*S. aureus* vaccine clinical trial design has to-date been extremely poor. A standardised trial design and clinical endpoints (analagous to the WHO malaria vaccine technology roadmap) would allow easier interpretation and comparison between candidate vaccines [311]. Early clinical trial phases of future vaccines should establish and account for colonisation status and its effect (if any) on immunogenicity. Changes in immune response after repeat immunisation should be documented. Phase II/III trials are best conducted in cohorts at high risk for invasive infection (e.g., haemodialysis, drug users, orthopaedic or cardiovascular surgery patients) and controlled for placebo, adjuvant-free and antigen-free variants. Baseline and post-vaccination measures should routinely extend beyond antibody titres to include changes in frequency or phenotype of specific T lymphocyte populations. Results should be controlled for clinical confounders, to guarantee an accurate description of infection-attributable response, how this differs after immunisation and its effect on clinical endpoints. The variability in clinical management of SA-BSI observed here and elsewhere is a substantial confounder with the potential to under- or over-estimate the effect of any vaccine candidate. Therefore, ensuring adherence to clinical management quality-of-care indicators should be integral to trial design [205]. Development of a vaccine should not replace ongoing quality improvement in clinical care, rather support it to optimise patient outcomes.

5.3. Future Directions

The pursuit of novel therapies and vaccines should not detract from attempts to improve failings in implementing current best practice. Improved awareness, antimicrobial stewardship, incentivised infection prevention measures, and specialist-led clinical management of SA-BSI cases should be prioritised in healthcare systems. Rapid diagnostics and novel therapeutic agents (e.g., phage therapy, quorum sensing inhibitors and competing probiotics) may become additional strategies [312]. Future *S. aureus* infection may be substantially reduced if technological innovations relieve our dependence on temporary or implanted devices on which *S. aureus* can hide from antimicrobial therapy and immune clearance [313].

In summary, this thesis has described the diversity in *S. aureus* bloodstream isolates, outlined the complicated clinical course of this infection and highlighted a role for specialist physician-led management to optimise patient care. In addition, it has answered some fundamental questions about T cell behaviour during systemic *S. aureus* infection in humans. Namely, whether there are staphylococcal T cell epitopes, which type of immune cells are primarily expanded and whether memory is formed during infection. The results add the first report of human *S. aureus*-specific T lymphocyte responses to the previous indirect evidence on the importance of these cells in protection against *S. aureus*. This should serve as a platform for future studies to identify potential vaccine antigens and correlate antigen-specific responses with patient outcome. Further studies such as this in real patients with acute infections are vital. The work presented here represents a small but significant step in informing the development of host-targeted anti-*S. aureus* therapies.

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Appendices

Appendix A. Patient Information Leaflet

[HOSPITAL SITE MASTHEAD]

Human Immune Response to Invasive *Staphylococcus aureus* and *E. coli* Infection

Information for Patients

Study Investigators:

Principal investigator: Dr Rachel McLoughlin[1]

Co-investigators: [NAMED CLINICAL CONSULTANT SITE CO-INVESTIGATOR][2]
[NAMED SCIENTIST SITE CO-INVESTIGATOR][2] (if applicable)
Dr Aisling Brown[1]

1. School of Biochemistry and Immunology, Trinity College, Dublin, Ireland
2. Department of Clinical Microbiology, [ACUTE HOSPITAL SITE], Dublin, Ireland

Contact details:

Dr Rachel McLoughlin	Tel:	(01) 8961608
[NAMED CONSULTANT]	Tel:	(01) XXXXXXX
[NAMED SCIENTIST]	Tel:	(01) XXXXXXX
Dr Aisling Brown	Tel:	(01) 8962736
	Email:	brownai@tcd.ie

Date: January 2014 **Version:** 2

Introduction

You are being invited to take part in a clinical research study being carried out at a number of hospitals including [XXXX] Hospital. It is important for you to understand why the research is being done and what it will involve. You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as 'Informed Consent'. Please take time to read the following information and ask any questions you may have before agreeing to become involved.

You don't have to take part in this study. If you decide not to take part it won't affect your future medical care. You can change your mind about taking part in the study any time you like.

1. Why is this study being done?

Blood poisoning caused by infection with the bacteria *Staphylococcus aureus* ('*S. aureus*') or *Escherichia coli* ('*E.coli*') account for most bloodstream infections in Irish hospitals. These conditions can be potentially very serious and require prompt expert treatment. There are currently no vaccines available to prevent disease caused by these bacteria.

This study aims to understand the response of your immune system to bloodstream infections. Many healthy people are 'colonised' with *S. aureus* – meaning it is living harmlessly in their nose or on their skin without causing infection, and all of us carry *E.coli* in our bowel. Some of these people may go on to develop serious infections where the bacteria get into their bloodstream. How the immune system fights back to clear this infection is poorly understood.

Some special cells of the immune system known as T-cells can 'remember' bacteria they have seen before and this 'memory' means they can fight those bacteria much more effectively. This study aims to find out if these memory T-cells recognise and fight *S. aureus* or *E.coli*. If they do, we hope that these cells could be targeted to try to make a vaccine to prevent these infections from happening.

2. Why am I being asked to take part?

You are being asked to take part because you have been diagnosed with a bloodstream infection with *S. aureus* or *E.coli* bacteria. The immune cells in your blood are busy fighting and clearing infection and are now at the height of their activity against these bacteria. This gives us the best chance of finding out if these cells are important and figuring out how they work in fighting this infection.

These immune cells from patients who have recent *S. aureus* infection will be compared with those from patients with *E.coli* bloodstream infections, and also to healthy non-infected volunteers. We hope to see if this 'memory' effect directed against *S. aureus* is active during recovery from infection and later, if it is still present

at 6 and 12 months following infection.

3. How will the study be carried out?

If you decide to participate in the study the study doctor will take a blood sample from you while you are in hospital and take note of some of your medical details and blood results. Other patients with bloodstream infections such as yours in other Dublin hospitals during 2013 and 2014 are also being asked to take part.

4. What will happen to me if I agree to take part?

If you agree to take part you will have an extra **blood sample** taken by the study doctor while you are in hospital. The volume of blood needed is approximately 30-40mL (7-8 teaspoons). Giving this sample takes approximately one minute. This blood will be transported to the laboratory in Trinity College Dublin and will be used to assess your immune T-cells. If there are extra blood cells left over, these may be frozen and stored in the laboratory for up to 10 years. These may be retested in the future in similar tests for immune cells or signals which may become relevant once we have analysed the results of the initial samples. No genetic testing will be done on these cells.

You will be asked to sign a **consent form** confirming that you agree to participate. The study doctor will also fill in an information form, called a **case report form** (CRF) to record details of your medical history, blood test results and medications. This form will not have any information that could be used to identify you on it, such as your name or medical record number. These forms will be stored in a secure office in Trinity College Dublin for up to 10 years

Consenting now also means you may be contacted by telephone in 6-12 months' time to consider giving another blood sample. This later sample is to see if your immune cells remember fighting the *S. aureus* bacteria and behave the same way as they did during the first bloodstream infection. This may be taken at your follow-up clinic visits at the hospital or by special arrangement with you in the blood-taking facility in Trinity College Dublin. You are not obliged to agree to giving this second sample.

You don't have to take part in this study. If you decide not to take part it won't affect your future medical care which is given by your main hospital doctors.

5. What are the risks of participating in this study?

As part of this study, you will have a blood sample taken. This procedure may be uncomfortable but rarely results in any significant problems. Side effects from drawing blood may include feeling light-headed or faint, bruising and/or infection at the site of the needlestick.

6. What are the possible benefits of participating in this study?

There are no direct benefits to your care if you agree to participate in this study. Your participation may however benefit future patients with *S. aureus* or *E.coli*

bloodstream infections.

7. Will I receive expenses for participating in this trial?

There is no reimbursement for participating in this study.

8. Is the study confidential?

Only the research study doctor will look at your medical records. This information will be kept private and confidential. Any data about your infection and blood results taken from these records for the case report form will not have your name, medical record number or other identifying information. This information will be stored in a locked office at Trinity College Dublin in a password-protected database for up to 10 years, after which time the forms will be shredded. The research blood sample will be handled confidentially and will not be identifiable as coming from you.

9. What will happen to the results?

The final results will be presented at medical/scientific conferences, in medical journals and as part of a thesis. No information that could identify you will appear in any of these. The results of your sample will not impact on your care as an individual patient, but the results of patients recovering from these infections as a group may show whether or not these cells will be important to target in vaccine research.

10. Who is funding this study?

Trinity College Dublin was awarded a grant by the Health Research Board to conduct this study. The Schools of Biochemistry & Immunology and Clinical Microbiology are conducting the research.

11. Who has approved this study?

This study has been approved by the [XXXX] Hospital Research Ethics Committee.

12. Where can I get further information?

Your participation in this study is entirely voluntary. If you have any further questions about the study or if you want to opt out of the study, you can rest assured it won't affect the quality of treatment you get in the future.

If you need any further information now or at any time in the future, please contact:

- **Dr Aisling Brown** on **01 8962736** (Trinity College Dublin).

  Email: brownai@tcd.ie

- **[NAMED CONSULTANT]** on **01 XXXXXXX** [(XXXX Hospital)]

Appendix B. Patient Representative Information Leaflet

[HOSPITAL SITE MASTHEAD]

Human Immune Response to Invasive *Staphylococcus aureus* and *E. coli* Infection

Information for Patient Representatives

Study Investigators:

Principal investigator: Dr Rachel McLoughlin[1]

Co-investigators: [NAMED CLINICAL CONSULTANT SITE CO-INVESTIGATOR][2]
[NAMED SCIENTIST SITE CO-INVESTIGATOR][2] (if applicable)
Dr Aisling Brown[1]

1. School of Biochemistry and Immunology, Trinity College, Dublin, Ireland
2. Department of Clinical Microbiology, [ACUTE HOSPITAL SITE], Dublin, Ireland

Contact details:

Dr Rachel McLoughlin	Tel:	(01) 8961608
[NAMED CONSULTANT]	Tel:	(01) XXXXXXX
[NAMED SCIENTIST]	Tel:	(01) XXXXXXX
Dr Aisling Brown	Tel:	(01) 8962736
	Email:	brownai@tcd.ie

Date: January 2014 **Version:** 1

Introduction

Your next-of-kin may be eligible to take part in a research study being carried out at a number of hospitals including [XXXX] Hospital. However, at present, they may be too sick, confused or fatigued to be able to fully understand and decide whether or not they would participate for themselves. You have been identified as a person who may act as a 'representative' for a patient by virtue of your family relationship with them. You know them well and probably have an idea of what decision they would make were they able to decide for themselves.

It is important for you – on their behalf – to understand why the research is being done and what it will involve. You should clearly understand the risks and benefits of taking part in this study so that you can help make a decision that is right for you and for your next-of-kin. This process is known as 'Informed Consent'. Please take time to read the following information and ask any questions you may have before deciding on whether to become involved.

Your next-of-kin does not have to take part in this study. Deciding not to take part will not affect their future medical care. You or your next-of-kin can change your mind about taking part in the study any time you like.

1. Why is this study being done?

Blood poisoning caused by infection with the bacteria *Staphylococcus aureus* ('*S. aureus*') or *Escherichia coli* ('*E.coli*') account for most bloodstream infections in Irish hospitals. These conditions can be potentially very serious and require prompt expert treatment. There are currently no vaccines available to prevent disease caused by these bacteria.

This study aims to understand the response of the immune system to bloodstream infections. Many healthy people are 'colonised' with *S. aureus* – meaning it is living harmlessly in their nose or on their skin without causing infection, and all of us carry *E.coli* in our bowel. Some of these people may go on to develop serious infections where the bacteria get into their bloodstream. How the immune system fights back to clear this infection is poorly understood.

Some special cells of the immune system known as T-cells can 'remember' bacteria they have seen before and this 'memory' means they can fight those bacteria much more effectively. This study aims to find out if these memory T-cells recognise and fight *S. aureus* or *E.coli*. If they do, we hope that these cells could be targeted to try to make a vaccine to prevent these infections from happening.

2. Why is my next-of-kin being asked to take part?

Your next-of-kin may take part because he/she has been diagnosed with a

bloodstream infection with *S. aureus* or *E.coli* bacteria. The immune cells in their blood are busy fighting and clearing infection and are now at the height of their activity against these bacteria. This gives us the best chance of finding out if these cells are important and figuring out how they work in fighting this infection.

These immune cells from patients who have recent *S. aureus* infections will be compared with those from patients with *E.coli* bloodstream infections, and also to healthy non-infected volunteers. We hope to see if this 'memory' effect directed against *S. aureus* is active during recovery from infection and later, if it is still present at 6 and 12 months following infection.

3. What is my role as 'representative' or 'next-of-kin'?

Medical decisions are made in your next-of-kin's best interest and nobody can consent or refuse consent to treatment on their behalf. However, **research** may involve additional tests which are **not essential** to your next-of-kin's medical care, although they may help patients who find themselves in the same situation in the future. As such, it is even more important that we are guided by those who know the patient best to let us know what decision you believe he/she would make if asked to participate in the full of his/her health.

Because we need to capture the immune cells at the height of their activity, and at the same time in everyone (between 5 and 9 days after the bloodstream infection), not all of our patients are well enough at this stage to consent for themselves. You are being asked to consider participation of your next-of-kin on their behalf if they are not back to full capacity within this time.

There are no legal obligations or consequences on you as a result of your guidance in this matter. On their recovery, the study doctor will also approach them to explain what has happened, and the reasons why we are doing the study. Should they not want their results to be included in the study at that time, they will be withdrawn.

4. How will the study be carried out?

If you think your next-of-kin would decide to participate in the study, the study doctor will take a blood sample from them while in hospital and take note of some of their medical details and blood results. Other patients with the same bloodstream infections in other Dublin hospitals during 2013 and 2014 are also being asked to take part.

5. What will happen if I agree for my next-of-kin to take part?

If you agree for your next-of-kin to take part they will have an extra **blood sample** taken by the study doctor while in hospital. The volume of blood needed is approximately 30-40mL (7-8 teaspoons). Giving this sample takes approximately one minute. This blood will be transported to the laboratory in

Trinity College Dublin and will be used to assess their immune T-cells. If there are extra blood cells left over, these may be frozen and stored in the laboratory for up to 10 years. These may be retested in the future in similar tests for immune cells or signals which may become relevant once we have analysed the results of the initial samples. No genetic testing will be done on these cells.

You will be asked to sign a **consent form** confirming that you agree for them to participate. The study doctor will also fill in an information form, called a **case report form** (CRF) to record details of their medical history, blood test results and medications. This form will not have any information that could be used to identify your next-of-kin on it, such as their name or medical record number. These forms will be stored in a secure office in Trinity College Dublin for up to 10 years.

Consenting now also means your next-of-kin may be contacted by telephone in 6-12 months' time to consider giving another blood sample. This later sample is to see if their immune cells remember fighting the *S. aureus* bacteria and behave the same way as they did during the first bloodstream infection. This may be taken at a follow-up clinic visit at the hospital or by special arrangement with him/her in the blood-taking facility in Trinity College Dublin. They are not obliged to agree to give this second sample.

You don't have agree for your next-of-kin to take part in this study. If you decide they would not agree to take part it won't affect their future medical care which is given by their main hospital doctors.

6. What are the risks of my next-of-kin participating in this study?

As part of this study, they will have a blood sample. This procedure may be uncomfortable but rarely results in any significant problems. Side effects from drawing blood may include feeling light-headed or faint, bruising and/or infection at the site of the needlestick.

7. What are the possible benefits of my next-of-kin participating in this study?

There are no direct benefits to your next-of-kin's care if you agree for them to participate in this study. Their participation may, however, benefit future patients with *S. aureus* or *E.coli* bloodstream infections.

8. Will my next-of-kin receive expenses for participating in this trial?

There is no reimbursement for participating in this study.

9. Is the study confidential?

Only the research study doctor will look at your next-of-kin's medical records. This information will be kept private and confidential. Any data about their infection and blood results taken from these records for the case report form will not have their name, medical record number or other identifying information. This information will be stored in a locked office at Trinity College Dublin in a password-protected database for up to 10 years, after which time the forms will be shredded. The research blood sample will be handled confidentially and will not be identifiable as coming from your next-of-kin.

10. What will happen to the results?

The final results will be presented at medical/scientific conferences, in medical journals and as part of a thesis. No information that could identify patients will appear in any of these. The results of your next-of-kin's sample will not impact on their care as an individual patient, but the results of patients recovering from these infections as a group may show whether or not these cells will be important to target in vaccine research.

11. Who is funding this study?

Trinity College Dublin was awarded a grant by the Health Research Board to conduct this study. The Schools of Biochemistry & Immunology and Clinical Microbiology are conducting the research.

12. Who has approved this study?

This study has been approved by the [XXXX] Hospital Research Ethics Committee.

13. Where can I or my next-of-kin get further information?

Participation in this study is entirely voluntary. If you have any further questions about the study or if you want to opt out of the study, you can rest assured it won't affect the quality of treatment you or your next-of-kin get in the future.

If you need any further information now or at any time in the future, please contact:

- **Dr Aisling Brown** on **01 8962736** (Trinity College Dublin).

  Email: brownai@tcd.ie

- **[NAMED CONSULTANT]** on **01 XXXXXXX** [(XXXX Hospital)]

Appendix C. Consent Form

CONSENT FORM

Human Immune Response to Invasive *Staphylococcus aureus* and *E.coli* Infection

Participant number: ☐☐☐☐☐☐

I have read and understood the **Information Leaflet** about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that I don't have to take part in this study and that I can **opt out** at any time. I understand that opting out won't affect my future medical care.	**Yes** ☐	**No** ☐
I am aware of the potential **risks** of this research study.	**Yes** ☐	**No** ☐
I give permission for researchers to look at my **medical records** to get information. I have been assured that information about me will be kept private and **confidential**.	**Yes** ☐	**No** ☐
I have been given a **copy of the Information Leaflet** for my records.	**Yes** ☐	**No** ☐
I agree to **give a blood sample** or samples for this research project. I understand that giving a blood sample or samples for this research is my own decision.	**Yes** ☐	**No** ☐
Storage and future use of biological material: I give permission for my samples and information collected about me to be **stored** for possible future research studies but only if the research is approved by a Research Ethics Committee.	**Yes** ☐	**No** ☐

Patient name (BLOCK CAPITALS) **Patient Signature** **Date**

Statement of Investigator:

I, the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them. I believe that the participant understands my explanation and has freely given informed consent.

Signature of Investigator (Doctor/Phlebotomist/Study Co-ordinator)	Please PRINT name	Date

Name of sponsor: Trinity College Dublin
Name of Principal Investigator: Dr Rachel McLoughlin
Contact telephone number: 01 896 1608

Appendix D. Assent Form

CONSENT FORM
Patient Representative

Human Immune Response to Invasive
Staphylococcus aureus Infection

Participant number: ☐☐☐☐☐☐

I have read and understood the **Information Leaflet** about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that my next-of-kin does not have to take part in this study.	**Yes** ☐	**No** ☐
I have been made aware of the potential **risks** of this research study.	**Yes** ☐	**No** ☐
I give permission for researchers to look at my next-of-kin's **medical records** to get information. I have been assured that this information will be kept private and **confidential**.	**Yes** ☐	**No** ☐
I have been given a **copy of the Information Leaflet** and consent form for my records.	**Yes** ☐	**No** ☐
I agree for my next-of-kin to **give a blood sample** or samples for this research project.	**Yes** ☐	**No** ☐
Storage and future use of biological material: I give permission for my next-of-kin's samples and information collected about him/her to be **stored** for possible future research studies but only if the research is approved by a Research Ethics Committee.	**Yes** ☐	**No** ☐

_____ _____ _____

Name of next-of-kin/proxy (BLOCK CAPITALS) **Proxy Signature** **Date**

(if participant currently unable to consent)

Relationship of representative to participant _____

Statement of Investigator:

I, the undersigned, have taken the time to fully explain to the patient or next-of-kin the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them. I believe that the participant/next-of-kin understands my explanation and has freely given informed consent.

Signature of Investigator (Doctor/ Study Co-ordinator)	Please PRINT name	Date

Name of sponsor: Trinity College Dublin
Name of Principal Investigator: Dr Rachel McLoughlin
Contact telephone number: 01 896 1608

Appendix E. Recruitment Record

The Impact of *Staphylococcus aureus* Infection on Host Immune Response

On recruitment to the study, participants are assigned an appropriate unique identifier code based on their diagnosis (prefix SA for *S. aureus*, EC for *E. coli*, HV for healthy volunteer) and chronological order of recruitment. SA-BSI participants are coded SA001, SA002; EC-BSI participants EC001, EC002 etc,; healthy volunteers HV001, HV002 and so on. Medical record numbers are recorded alongside this study ID. Lab isolates of *S. aureus* and *E. coli* are named BACSA001, BACSA0002, BACEC001 etc. with coding corresponding with source patient numbering. The master copy of the Recruitment Record is kept in the study file.

RECRUITMENT RECORD

Study ID	Med Record No.	Bac ID
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Appendix E. Case Report Form

Human Immune Response to invasive *Staphylococcus aureus* Infection

CASE REPORT FORM

PARTICIPANT STUDY ID NUMBER ☐☐☐☐☐☐☐

PATIENT INFORMATION

Age _____ Gender M☐ F☐

Relevant comorbidities/risks

Diabetes Y☐ N☐

IDU Y☐ N☐

ESRD Y☐ N☐ PD ☐ HD ☐

[HD Vascath ☐ AVF ☐]

Other ☐ _____

Other relevant clinical information (procedures/risks/skin disease/history of infection/presentation etc.)

Prior colonisation status (MSSA/MRSA) Y☐ N☐ Unknown☐

BSI DIAGNOSIS

S.aureus ☐ *E.coli* ☐

Acquisition & Classification:

Community ☐ *OR* Healthcare☐

(for SA-BSI)

Complicated ☐ *OR* Uncomplicated☐

Documented prior *S. aureus* disease Y☐ N☐ _____

In case of repeated episodes *S. aureus* BSI (if known)

Relapse ☐ *OR* Reinfection ☐ *OR* Unknown ☐

Duration known bacteraemia

☐ <48 hrs ☐48-96 hrs ☐>96 hrs ☐ No repeat sample

Source/Focus

Known☐ *OR* Unknown☐ _____

Indwelling central vascular access Y ☐ N ☐ _____
Peripheral venous access Y ☐ N ☐ _____
IVDU Y ☐ N ☐ _____
Prosthetic material Y ☐ N ☐ _____
Native bone/joint Y ☐ N ☐ _____
Skin/soft tissue/SurgSI Y ☐ N ☐ _____
Urinary Tract Y ☐ N ☐ _____
Intraabdominal Infection Y ☐ N ☐ _____
Instrumentation/Procedure Y ☐ N ☐ _____
Other Y ☐ N ☐ _____

Notes

Metastatic Infection Y ☐ N ☐ Site _____
Toxic Shock Syndrome Y ☐ N ☐

LABORATORY PARAMETERS (within 24hrs of initial positive blood culture sampling time)

WCC _____ Lymp _____
Neuts _____ CRP _____

LABORATORY PARAMETERS (within 24 hrs of study blood sampling time)

WCC _____ Lymp _____
Neuts _____ CRP _____

TREATMENT

Duration of treatment at time of sampling (days) _____
Documented clearance of bacteraemia at time of sampling Y ☐ N ☐
Clinical defervescence at time of sampling Y ☐ N ☐

Antibiotic Rx

MICROBIOLOGY INFORMATION

Isolate Lab ID _____

Time to positivity (hrs) _____

Antibiogram Info