Analysis of human T lymphocyte-derived immune responses against *Staphylococcus aureus* by delivery of *in vitro* transcribed antigens

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Abbreviations

AMPs	Antimicrobial peptides
AA	Amino acid
AMP	Ampicillin
APC	Antigen-presenting cell
Approx.	Approximately
BCR	B cell receptor
bp	Base pairs
BrdU	Bromdesoxyuridin
BSA	Bovine serum albumin
CA	Community-associated Methicillin-resistant *Staphylococcus aureus*
CD	Cluster of differentiation
cfu	Colony-forming units
Clf	Clumping factor
CNA	Collagen adhesin
DAMPs	Damage-associated molecular patterns
DAPI	4′,6-Diamidin-2-phenylindol
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ds	Double-stranded
e.g.	Example given
ETA/ETB	Exfoliative toxins A/B
EV	Empty vector
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
Fig.	Figure
FnBP	Fibronectin-binding protein
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA-MRSA	Healthcare-associated Methicillin-resistant *Staphylococcus aureus*
HEK293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
Hla	Hemolysin A
hom	Homologous
HS	Human serum
IFN	Interferon
IgM	Immunoglobulin M
IL	Interleukin
IRAK4	Interleukin-1 receptor-associated kinase 4
IsdA/B	Iron-regulated surface determinant protein A/B
IVIg	Intravenous immunoglobulin
ivt	*in vitro* transcription
kDa	Kilo Dalton
LA	Livestock-associated Methicillin-resistant Staphylococcus aureus
LAMP1	Lysosomal associated membrane gylcoprotein 1
lpp	Lipoproteins
LPS	Lipopolysaccharide

LTA	Lipoteichoic acid
luc	Luciferase
MDA-5	Melanoma differentiation- associated gene 5
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cells
MOI	Multiplicity of infection
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MyD88	Myeloid differentiation primary response gene 88
n.s.	not significant
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLR	Nod-like receptor
o/n	overnight
p.t.	Post transfection
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PNAG	Poly-N-acetylglucosamine
PRR	Pattern recognition receptor
PVL	Panton-Valentine leukocidin
RIG-I	Retinoic acid–inducible protein I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RT	Room temperature
S. *aureus*	*Staphylococcus aureus*
Sbi	Second immunoglobulin-binding protein
SCCmec	Staphylococcal cassette chromosome mec
SCIN	Staphylococcal complement inhibitor
SEB	Staphylococcal enterotoxin B
SpA	Staphylococcal protein A
ss	Single-stranded
SSLs	Staphylococcal superantigen-like proteins
Tab.	Table
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{reg}	T regulatory cells
TSST	Toxic shock syndrome toxin
TT	Tetanus toxoid
var	Variable
w/o	Without
WTA	Wall teichoic acid

Summary

Staphylococcus aureus is a harmless commensal of the human mucosa and at the same time the main cause of life-threatening nosocomial infections worldwide. In particular, the growing number of methicillin-resistant *S. aureus* strains, which are resistant to a broad spectrum of β-lactam antibiotics defines the need for alternative treatments such as vaccination. However, so far, all vaccination trials have failed to induce protectivity in patients. Next to successful manipulation of the human immune system by a broad spectrum of pathogen-derived virulence factors, one major issue could lie in insufficient induction of protective T lymphocyte responses.

To analyze T cell immunity against *S. aureus*, the virulence factor protein A (SpA) was selected because of its surface expression and known immunogenicity. SpA is a highly variable Ig-binding protein that allows *S. aureus* to manipulate and evade the human immune response. It consists of a homologous region that harbors the Ig-binding sites and a variable region that determines the *spa* type. To characterize T cell responses to SpA, an mRNA-based approach was chosen because it circumvents the immune response attributable to the Ig-binding properties of SpA. Thus, the gene of interest was cloned into a specialized plasmid, which is suitable for *in vitro* transcription (ivt) and monocyte-derived dendritic cells (MoDC) were transfected with ivt mRNA. Following translation in the cytoplasm, the cells process the antigen and present the peptides via MHC molecules to autologous T cells in co-culture.

Analysis of T cell activation by IFN-γ ELISpot assay revealed the presence of antigen-specific $CD8^+$ and $CD45RO^+$ T cells activated by *spa* mRNA-transfected MoDC, whereas activation of $CD4^+$ T cells was rather weak. Furthermore, donor- and *spa* type-dependent T cell activation was observed, indicating differences in preformed immune memory among donors and in the response to *spa* variants. In average, approximately 0.04% of peripheral $CD8^+$ T cells were activated by *spa* mRNA in healthy donors. This high frequency was comparable to the number of T cells activated by an *Influenza* control antigen. In order to better define the T cell-activating SpA epitopes, mRNA of a SpA Ig-binding mutant and truncated versions of SpA, either lacking the homologous or the variable domain were generated. Interestingly, ELISpot assays demonstrated higher IFN-γ production with the Ig-binding mutant and the truncated *spa* compared to wildtype *spa* mRNA. These results

indicated that all domains elicit T cell responses and reveal an increase in T cell immunogenicity achieved by sequence mutation.

Moreover, the T cell response to mRNA-encoded antigens (*spa*, *mecA* and *sitc*) differed profoundly from that induced by the corresponding protein antigens (SpA, PBP2a and SitC). In contrast to mRNA, protein-based antigen presentation elicited only very low amounts of IFN-γ after overnight incubation. Co-culture assays of mRNA and protein antigens suggested that the mRNA's adjuvant effect is necessary to trigger IFN-γ secretion in *S. aureus* specific T cells. Analysis of cytokine secretion patterns in MoDC:T cell co-cultures after five days of stimulation further revealed that mRNA-encoded antigens elicited a high T_h1-biased immune response characterized by production of IFN-γ and TNF. In contrast, stimulation with staphylococcal proteins led to secretion of low levels of IL-13 and IL-5, indicators for a T_h2-biased immune response. On the contrary to mRNA, SpA protein and, to a minor account, PBP2a induced secretion of IL-2, IL-10 and G-CSF, cytokines that play a role in the differentiation of T regulatory cells. Furthermore, activation of $CD8^+$ T lymphocytes by *spa* mRNA but not by SpA protein triggered degranulation, as suggested by flow cytometric detection of LAMP1.

In conclusion, this study presents mRNA as a novel tool for the analysis and quantification of T cell responses specific for staphylococcal antigens. The results further suggest that this technique represents a promising approach to overcome the T_h2/T_{reg}-dominated skewing of T cell responses to this pathogen.

Zusammenfassung

Staphylococcus aureus ist sowohl harmloser Kommensale der menschlichen Mukosa als auch einer der weltweiten Hauptverursacher lebensbedrohlicher nosokomialer Erkrankungen. Insbesondere die wachsende Zahl Methicillin-resistenter *S. aureus* Stämme, die gegen ein breites Spektrum von β-Lactam-Antibiotika resistent sind, verdeutlicht die Dringlichkeit alternativer Therapiemöglichkeiten wie Vakzinierung. Dennoch konnte bisher in keiner der klinischen Studien Protektivität in Patienten gezeigt werden. Ein Hauptgrund könnte neben der erfolgreichen Manipulation des menschlichen Immunsystems durch eine Vielzahl von *S. aureus* Virulenzfaktoren, eine unzureichend protektive T Lymphozyten-Antwort sein.

Um die T Zell-basierte Immunantwort gegenüber *S. aureus* näher zu untersuchen, wurde der Virulenzfaktor Protein A (SpA) aufgrund seiner Oberflächenexpression und gut beschriebenen Immunogenität gewählt. SpA ist ein hoch variables, Ig-bindendes Protein, welches *S. aureus* eine Manipulation und Evasion der menschlichen Immunantwort ermöglicht. Es setzt sich aus einer homologen Region mit Ig-Bindedomänen und einer hochvariablen Region zusammen, die den *spa* Typen definiert. Um die T Zell-Antwort gegenüber SpA zu charakterisieren und dessen Ig-bindenden Eigenschaften zu umgehen, wurde ein mRNA-basierter Ansatz gewählt. Dafür wurde das zu untersuchende Gen in ein für *in vitro* Transkription (ivt) geeignetes Plasmid kloniert und aus Monozyten generierte dendritische Zellen (MoDC) wurden mit ivt mRNA transfiziert. Nach der Translation im Zytoplasma prozessieren die Zellen das Antigen und präsentieren die Peptide über MHC Moleküle autologen T Zellen in Ko-Kultur.

IFN-γ ELISpot Versuche ergaben das Vorhandensein antigenspezifischer $CD8^+$ und $CD45RO^+$ T Zellen die durch *spa* mRNA-transfizierte MoDC aktiviert wurden. Die Aktivierung von $CD4^+$ T Zellen fiel hingegen verhältnismäßig schwach aus. Außerdem konnte eine spender- und *spa* Typen-abhängige T Zell-Aktivierung beobachtet werden, was Unterschiede in humanen Gedächtnis-Immunantworten gegenüber *spa* Varianten vermuten lässt. Durchschnittlich wurden etwa 0.04% der peripheren $CD8^+$ T Zellen in gesunden Spendern durch *spa* mRNA aktiviert. Diese Anzahl war vergleichbar mit der durch ein *Influenza* Kontrollpeptid aktivierten. Um SpA Epitope, die T Zellen aktivieren besser bestimmen zu können, wurde sowohl mRNA einer SpA Ig-Bindedomänenmutante als auch mRNA trunkierter SpA Varianten, denen entweder die homologe oder die variable Domäne

fehlt, hergestellt. Interessanterweise induzierten sowohl diese Mutante als auch die trunkierten Varianten mehr IFN-γ im ELISpot Versuch als die wildtypische *spa* mRNA. Diese Ergebnisse zeigten auf, dass alle Domänen T Zell-Antworten auslösen und lassen eine Verstärkung der T Zell-Immunogenität durch Sequenzmutationen erkennen.

Zudem unterschied sich die T Zell-Antwort gegen mRNA-kodierte Antigene (*spa*, *mecA* und *sitc*) deutlich von der durch die entsprechenden Proteine hervorgerufenen (SpA, PBP2a und SitC). Im Gegensatz zu mRNA, induzierten Antigene von Proteinen deutlich weniger IFN-γ nach einer Inkubation über Nacht. Eine Ko-Kultur von mRNA mit dem zugehörigen Protein ließ vermuten, dass der mRNA-Adjuvanseffekt Voraussetzung für eine IFN-γ Produktion von *S. aureus* spezifischen T Zellen sein könnte. Untersuchungen zur Zytokinproduktion in MoDC:T Zell Ko-Kulturen nach fünftägiger Stimulation zeigte zudem, dass mRNA-kodierte Antigene eine starke T_h1-gerichtete Immunantwort auslösen, die durch die Produktion von IFN-γ und TNF gekennzeichnet ist. Im Gegensatz dazu, riefen *S. aureus* Proteine eine schwache Sekretion von IL-13 und IL-5 hervor, was wiederum Indikatoren für eine T_h2-basierte Immunantwort sind. Verglichen mit mRNA, induzierten die Proteine SpA und in geringerem Umfang auch PBP2a die Sekretion von IL-2, IL-10 und G-CSF. Diese Zytokine spielen eine Rolle in der Differenzierung von regulatorischen T Zellen. Außerdem führte die Aktivierung von $CD8^+$ T Zellen durch *spa* mRNA- aber nicht durch SpA Protein- zum Auslösen des Degranulierungsprozesses, was durch durchflusszytometrische Messungen der Expression von LAMP1 untersucht wurde.

Zusammenfassend kann festgehalten werden, dass mit dieser Arbeit mRNA als eine neue Methode zur Analyse und Quantifizierung von *S. aureus*- spezifischen T Zell-Antworten vorgestellt wurde. Außerdem ist dies eine vielversprechende Möglichkeit die T_h2/T_{reg}-gerichtete T Zell-Antwort gegenüber diesem Pathogen zu überwinden.

1. Introduction

1.1. The human immune system

The human immune system is a highly complex machinery based on interaction and communication between various specialized cells. It is unique in every individual and formed through the individual´s encounters with microbes and diseases. The immune system can be roughly divided into two major parts: the adaptive immune system and the innate immune system. For both immune sites it is crucial to differentiate between self and foreign antigens. They collaborate to identify invasive microbes and prevent and cure infection.

1.1.1. The innate immune system

Innate immunity provides the early state of defense against microbes and is characterized by a very rapid response. It includes physical and chemical barriers such as epithelia that produce antimicrobial compounds, phagocytic cells as macrophages and dendritic cells, and blood proteins of the complement system to regulate inflammation.

In matters of microbial clearance, professional phagocytes such as macrophages, dendritic cells (DC) and neutrophils build the first line of the innate immunity's defense (1). Engulfment, ingestion and phagosomal degradation of microorganisms by processes such as acidification and enzyme digestion are essential for killing of microbes. Typically, these professional phagocytes are located at sites of microbe exposure such as skin or mucosa. After activation, these cells enter the tissue, which is mediated by integrin binding and chemokine recognition, and migrate to the lymph nodes to activate cells of the adaptive immune system. Hence, antigen-presenting cells (APC) such as DC process antigens and subsequently present peptides via major histocompatibility complex (MHC) molecules. Intracellular antigens (including self-antigens) within the cytosol are processed in the proteasome, transported to the endoplasmic reticulum and loaded on MHC class I molecules to subsequently activate $CD8^+$ T lymphocytes. Extracellular antigens are endocytosed, digested in the lysosome and bind there to MHC class II molecules. Antigens presented this way are recognized by $CD4^+$ T lymphocytes.

Phagosomal degradation allows access to ligands for intracellular pattern recognition receptors (PRR) that recognize a broad spectrum of pathogen- or damage-associated

molecular patterns (PAMPs or DAMPs). Families of PRRs include e.g. cytosolic NOD-like receptors (NLRs) and RIG-like receptors (RLRs) that detect peptidoglycan (PGN) and RNA. However, not all PRR require phagosomal digestion for activation. The most important family of PRR, the Toll-like receptors (TLR) also contains membrane-bound members such as TLR2 that senses di- and triacylated lipoproteins. To date, ten different human TLR are known that recognize LPS (TLR4), flagellin (TLR5), lipopeptides (dimers of TLR1/2 or TLR2/6), dsRNA (TLR3), ssRNA (TLR7 and 8) and unmethylated DNA (TLR9, 2). At present, TLR10 remains an orphan receptor in humans without known function although it has been shown to be involved in influenza infection, asthma, Crohn's disease and bladder cancer (3).

Once activated, all TLR (except TLR3) lead to downstream signaling via myeloid differentiation factor 88 (MyD88), recruitment of interleukin-1 receptor-associated kinase 4 (IRAK4) and other factors followed by the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB). This results in the production of messenger molecules (cytokines) and activation of other immune cells.

1.1.2. The adaptive immune system

The adaptive immune system develops and improves during life. It is composed of two major cell subsets: B lymphocytes and T lymphocytes.

1.1.2.1. B Lymphocytes

B lymphocytes recognize antigens via their antigen-specific B cell receptors (BCR). During development in the bone marrow, their unique BCR is formed by rearrangement of V(D)J genes (4). Upon antigen binding, they start to proliferate and differentiate. This process goes along with the generation of memory B cells and antibody-secreting plasma cells induced by a repeated infection. Secretion of antibodies enables the neutralization of extracellular microbes and toxins and opsonisation to enhance phagocyte binding. High-affinity antibody maturation requires the help of co-stimulatory molecules such as CD40 ligand binding of $CD4^+$ T helper cells and their binding to an antigen epitope presented by the activated B cell. T cell help is also crucial to induce B cell differentiation into long-lived plasma cells, isotype switching and affinity maturation of antibodies. T cell-independent B cell proliferation is accomplished by cross-linking of BCRs by an antigen and/or by triggering of TLR. This process mainly generates plasma cells that secrete immunoglobulin M (IgM) and IgG (4).

1.1.2.2. T Lymphocytes

The effector site of the adaptive immune response is initiated by recognition of antigens by T lymphocytes that develop in the thymus. This cell-mediated immunity is preferentially directed against intracellular microbes. Dendritic cells are the only APC capable of stimulating naive T cells (5). After antigen recognition in the lymphoid organs, T cells undergo clonal expansion and differentiate into effector or memory cells (4). Then, they migrate through blood vessels to peripheral tissues and the site of infection.

1.1.2.2.1. $CD4^+$ Helper T lymphocytes

Effector $CD4^+$ T cells can be further divided into T_h1, T_h2 and T_h17 cells (T helper cells), identified by their cytokine secretion profiles and their expression of surface markers and receptors. They get activated by antigen recognition displayed on MHC II molecules and co-stimulation of CD40 ligand by binding to CD40 on APC having ingested the microbe. T_h cells are essential for the activation and stimulation of innate immune cells, recruitment and activation of more lymphocytes to the site of antigen and the stimulation of phagocytes to eliminate the infected cell or microbe. They are also important players in the regulation of the immune response (4).

T_h1 cells recognizing antigens derived of phagocytosed microbes (6) are often characterized by the secretion of IFN-γ (7), a cytokine which in turn supports killing of the ingested microbes by macrophages and increases antigen presentation. IFN-γ also promotes more Th1 differentiation to amplify the reaction (4). T_h2 cells stimulate IgE- and eosinophil-mediated reactions and are mainly involved in immune responses towards extracellular microbes and helminths (6, 8). There are characterized by secretion of IL-4, the major stimulus for development of T_h2 cells from naive $CD4^+$ helper cells as well as IL-5 and IL-13, which stimulate B cell and eosinophil activation and (6, 9–11). Furthermore, the T_h17 cell subset complements these T_h cell types via induction of the recruitment of neutrophils to the site of infection. By secreting IL-17, IL-22 and IL-21, T_h17 cells mediate clearance of extracellular bacteria or fungi (12).

1.1.2.2.2. $CD8^+$ cytotoxic T lymphocytes

$CD8^+$ cytotoxic T lymphocytes (CTL) are key factors in the eradication of infections with viruses and most intracellular pathogens. This type of antigen-specific T cells also recognizes

transformed, malignant or damaged cells and is specialized on the recognition of peptides derived from microbes located in the cytoplasm. Those peptides are presented to CTL on MHC I molecules, which are expressed on the surface of almost all nucleated cells and enable the T cell to recognize the intracellular antigens. Additionally, activation also occurs by co-stimulatory molecules such as CD80/CD86 (13). Furthermore, CTL are an important source of IFN-γ which, in turn, also stimulates development of $CD8^+$ T cells and T_h1 cells (14).

Upon antigen recognition, CTL attach to the target cell by forming an immunological synapse. After detachment of the CTL, the target cell dies within minutes to a few hours (15). Killing is accomplished by delivering cytotoxic proteins stored within cytoplasmic granules of the CTL. Cytotoxic proteins include perforin and granzymes, which induce perforation of the target cell, caspase activation and therefore apoptosis in the target cell (13). A granule-independent mechanism to induce cell death is binding of the Fas ligand on T cells to the Fas death domain containing receptor of the target cell, which, in turn, also leads to the activation of caspases-3, and 6-10 (13, 16).

1.1.2.2.3. Memory T lymphocytes

In contrast to effector T cells, memory T lymphocytes are long-lived cells with the ability to react rapidly upon antigen re-encounter. Before exposure, the frequency of naive T cells specific for any antigen is 1 in 10^6 lymphocytes. After exposure, this frequency may increase >50.000-fold to 1 in 10 $CD8^+$ lymphocytes and about 1 in 1000 in $CD4^+$ lymphocytes (4). Upon stimulation, most of the expanded effector cells die. However, it is believed that some of the surviving cells develop into memory T cells, which acquire the expression of CD45RO instead of CD45RA expressed on naive T cells. Thus, these markers are used to distinguish these T cell subsets. Alternatively, the branched differentiation model proposes direct differentiation from naive to memory T cells (17, 18). However, details how the development of memory T cells is accomplished remain unclear. Of note, the most important cytokines linked to maintenance, proliferation, survival and differentiation of TCR-activated T cells to memory T cells are IL-2 and IL-7 (17, 19).

1.1.2.2.4. Regulatory T lymphocytes

T regulatory cells (T_{reg}) are subsets of the $CD4^+$ or $CD8^+$ lineage that supress inflammation and maintain tolerance (20, 21). Some T cells that recognize self-antigen in the thymus do not undergo apoptosis but differentiate into so-called central T_{reg} specific for this antigen (22). Contrary to central T_{reg}, peripheral tolerance is a mechanism by which mature T cells that recognize self-antigens become incapable of responding to this antigen (22). This mechanism includes T cell anergy, a tolerance state in which T lymphocytes are alive and functional but remain inactive following antigen encounter (23). T_{reg} are characterized by expression of CD25 and the transcription marker FoxP3. *In vitro*, IL-2 is essential for T_{reg} differentiation and maintenance (4, 24). Furthermore, they secret the anti-inflammatory cytokine IL-10, which is crucial to suppress T cell activation and inhibit T cell effector function (4, 25).

1.2. *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a coccoid, non-motile, non-spore forming, Gram-positive bacterium. It is facultative anaerobic and usually grows in grape-like clusters that gives the genus its name.

1.2.1. *S. aureus* as commensal and pathogen

S. aureus is a commensal of the skin, oral and nasal mucosa and the upper respiratory tract in humans (26). Approximately 30% to 60% of the human population are transiently, about 20% persistently colonized with this bacterium (26, 27). Due to its ability to colonize the skin and mucosa, *S. aureus* causes endogenous infections and is one of the leading and most widespread causes of nosocomial diseases. This includes minor skin infections such as furunculitis as well as life-threatening diseases such as pneumonia, meningitis, endocarditis and sepsis (28, 29). Among staphylococci, the expression of the enzyme coagulase is traditionally used as a criterion to distinguish between harmless and pathogenic bacteria (30). However, in the hospital setting, coagulase-negative staphylococci are also known to cause severe infections (31, 32).

Lately, the growing number of methicillin-resistant *S. aureus* (MRSA) presents an increasing risk for inefficiency of antibiotics. It is estimated that it results in about 290.000 hospitalizations, 12 million medical visits and treatments (33) and the enormous

costs of $478 million to $2.2 billion on third-party payers in the United States alone per year (34). The antibiotic methicillin was launched in the late 1950's and already within one year, first resistances were documented (35). Those strains that can be hospital- (HA-MRSA), community- (CA-MRSA) or livestock-acquired (LA-MRSA), harbor a staphylococcal cassette chromosome *mec* (*SCCmec*) that carries the gene *mecA (36).* This gene encodes an alternative penicillin-binding protein that is less susceptible to modification by a broad spectrum of β-lactam antibiotics and therefore mediates resistance (37). Additional incorporation of other resistance genes decreases susceptibility against antibiotics of the last resort such as linezolid or vancomycin (38, 39).

1.2.1.1. Virulence factors of *S. aureus*

S. aureus expresses a broad spectrum of numerous, strain-dependent, soluble and cell wall-anchored virulence factors. These molecules improve survival under hazardous conditions and facilitate host cell adhesion, invasion and infection as well as evasion from the host immune system.

One important class of virulence factors are adhesins. They mediate the adhesion to different host cells and components. Adhesins can be anchored in the bacterial surface (microbial surface recognizing adhesive matrix molecules-MSCRAMMs) or secreted. Prominent examples are fibronectin-binding proteins (FnBPs), clumping factors (Clfs) and collagen binding protein (Cna). Binding fibronectin and fibrinogen allows a bond between *S. aureus* and host integrins as well as platelet aggregation (33). *S. aureus* secretes a broad spectrum of toxins including enterotoxins, toxic shock syndrome toxin-1 (TSST-1), hemolysins, leukocidins (e.g. Panton–Valentine leucocidin, PVL) and exfoliative toxins (ETA and ETB, (40).

A study by Jenkins *et al.* demonstrated the importance of virulence factor regulation during different bacterial stages of infection. They compared the gene expression of *S. aureus* during nasal colonization, early bacteremia and sepsis. It was shown that genes for adhesion proteins as *clfa* were higher expressed during colonization of the nasal mucosa than during bacteremia or sepsis, when the bacteria are planktonic in the blood stream. In contrast, toxin expression decreased. Expression of hemolysin A (Hla) or the immune evasion protein A was increased

during early bacteremia. However, the regulation of other genes as *ica*, which is crucial in biofilm formation as well as cation transporters was strain-dependent (41).

1.2.1.2. Staphylococcal Protein A

The most prominent virulence factor of *S. aureus* is protein A (SpA). It is a 40-60 kDa large cell-wall structure, which is expressed by 99% of all *S. aureus* strains. SpA harbors a signal sequence and a LPXTG sortase motif by which it is anchored in the bacterial cell wall (42). There, it covers around 7% of the staphylococcal surface, unevenly distributed at two to four foci (43, 44). The expression of SpA is initiated during the exponential growth phase and down-regulated post-translationally (45). SpA consists of five homologous, Ig-binding domains. By binding the Fc part of human immunoglobulin G, *S. aureus* prevents opsonization and Fc receptor-mediated phagocytosis (46–48). The highly variable region X, which consists of a sequence of approximately 24 bp long DNA repeats is located upstream of the gene (Fig. 1.1). The order of those repeats identifies the *spa* type, which is used for the determination and classification of strains in epidemiology (49). The distribution and frequency of *spa* types depends strongly on area and time. For instance, t008 and t002 are among the most common *spa* types with a frequency of more than 50% in tested donors (28, 50, 51). Currently, over 15.000 different *spa* types are described (Ridom SpA Server, at November 5, 2015). Furthermore, due to this high density of short repeats, the variable region is also prone to nonsense mutations and inserted stop codons resulting in even more *spa* types and a protein, which still harbors Ig-binding site but no sortase signal (52, 53).

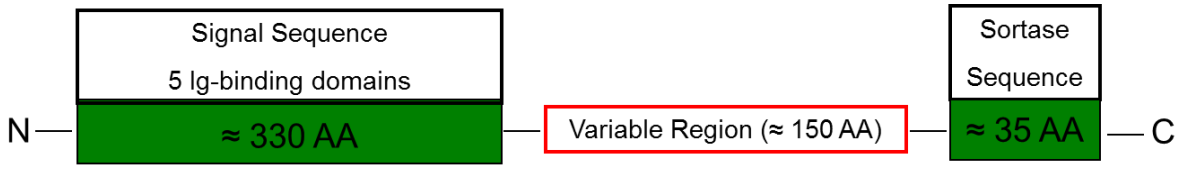


Fig. 1.1: Structure of Protein A. SpA consists of five N-terminal located, homologous Ig-binding domains and a variable region that determines the *spa* type.

In addition to the Fc binding properties, SpA also harbors Fab binding sites for the BCR of V_H3^+ B cells. This results in polyclonal B cell proliferation and apoptosis (48, 54, 55, Fig. 1.2 according to 56). It has been estimated that ~0.01% of human B cells bind a conventional antigen, whereas about ~30% are predicted to bind SpA (57). B cell activation is not only accomplished by SpA alone but also in combination with co-stimulating TLR2 ligands

presented on the bacterial surface, or bound to PGN of cell debris (54). SpA can also bind to the von Willebrand factor and the complement protein C3, increasing bacterial adhesion to platelets (33).

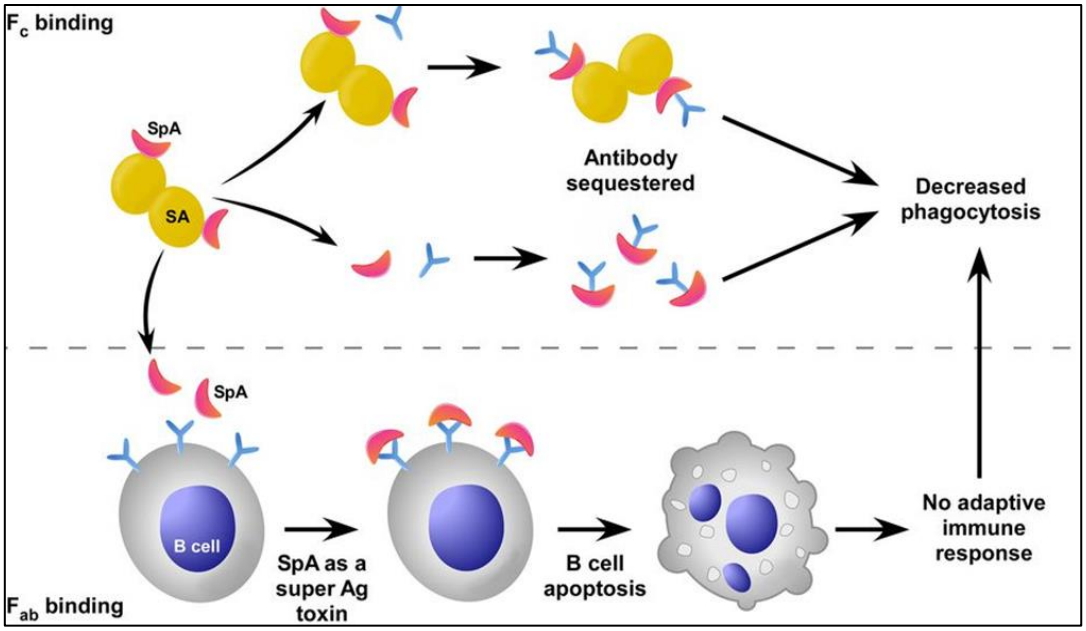


Fig. 1.2: Protein A- mediated immune evasion. SpA binds (soluble or bound to the cell wall of *S. aureus*) to the Fc part of immunoglobulins, which leads to decreased phagocytosis. By binding of the Fab part of the BCR, it acts as superantigen resulting in B cell apoptosis and diminished immune response.

1.2.2. *S. aureus* and the human immune system

To date, very little is known about the factors that lead to colonization and how the immune system distinguishes between the own endogenous strain and a "foreign" strains from outside. Nevertheless, *S. aureus* is one of the most successful manipulators and evaders of the human immune system (Fig. 1.3, according to 58) demonstrated by the increasing number of failing vaccination trials (59).

1.2.2.1. *S. aureus* and the innate immune system

To avoid the innate immune response, *S. aureus* developed numerous strategies that are reviewed elsewhere in detail (60–63). However, regarding the three main branches of innate immunity antimicrobial peptides, the complement system and phagocytosis, *S. aureus* successfully circumvents all three.

Due to expression of Ig-binding proteins as SpA and the second immunoglobulin-binding protein (Sbi) the efficiency of opsonization and phagocytosis by professional phagocytes is reduced (47, 64). Furthermore, *S. aureus*' resistance against lysozyme, the most important enzyme during phagosomal degradation, additionally hampers digestion by phagocytes (65). Plus, the lysozyme resistance results in a suppression of the inflammasome and poor degradation in the phagosome. Thus, *S. aureus* was found to be able to survive up to seven days within rat macrophages and osteoblasts *in vitro* (66, 67). Greenlee-Wacker and colleagues showed that 50% of *S. aureus* survive within the phagosome of neutrophils. These cells upregulated the phosphatidylserine "don't eat me" signal CD47, were therefore inefficiently digested by macrophages and underwent necroptosis (68). Zwitterionic wall teichoic acids (WTA), polysaccharide surface structures of the staphylococcal cell wall, are also known to contribute to lysozyme resistance (69) and mediate nasal colonization by binding to a scavenger receptor (70).

SCIN, the staphylococcal complement inhibitor, is the most efficient complement inhibitor and can block all complement pathways, therefore preventing phagocytosis and killing of *S. aureus* (71). Other factors such as ClfA, SpA and capsular polysaccharides also interfere with complement activation (33). The recognition of PAMPs also activates the production of anti-microbial peptides (AMPs) such as defensins and cathelicidins. *S. aureus* resists α-defensins through expression of staphylokinase (72). LL-37, a potent cathelicidin, is inactivated by the staphylococcal metalloprotease aureolysin (73).

1.2.2.2. *S. aureus* and the adaptive immune system

The staphylococcal superantigens present an important family of immune stimulatory exotoxins of which multiple variant forms exist. They are the most powerful T cell mitogens ever discovered. Less than 0.1 pg/ml are sufficient to stimulate T lymphocytes (74). By directly cross-linking MHC II molecules of ~20% of all T cell receptors they lead to cytokine storm, fever, shock and death (74, 75) and can also T cell anergy (76). Staphylococcal superantigen-like proteins (SSLs) are also described to bind and block TLRs and chemoattractant receptors (33).

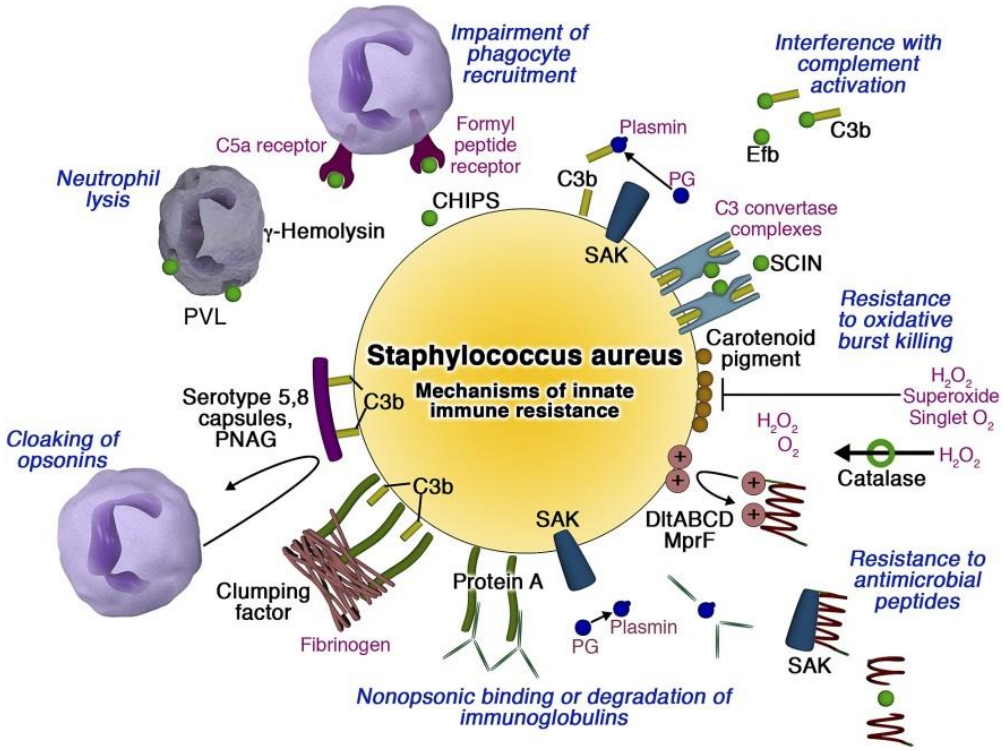


Fig. 1.3: Overview of selected *S. aureus* virulence factors and their influence on the human immune system.

The diverse effects of SpA on the human immune response have been mentioned before. However, it was also demonstrated that SpA activates plasmacytoid dendritic cells (pDC) by bacterial immune complexes with IgG, which is a requirement for the SpA-mediated B cell proliferation followed by anti-inflammatory IL-10 secretion. Therefore, they might act as regulatory B cells, which presents another strategy of *S. aureus* to manipulate the human host (77, 78). Recent studies proposed active release of SpA by a hydrolase after anchoring in the cell wall (79) or before with an unprocessed sorting signal (80). It was also shown that release of SpA increased during early log phase of bacterial growth. This might permit unspecific activation of B cells and hijacking of antibodies when the number of bacteria is still limited (79).

1.2.3. Vaccination trials against *S. aureus*

Currently, systemic *S. aureus* infections lead to a mortality of 20-40% despite appropriate treatment (81). Since *S. aureus* is part of the normal flora, eradication and prevention of infections is hard to achieve. In particular, the rapid spread of MRSA and the lack of therapeutic options define the need for alternative treatments such as vaccination. However, until today all clinical vaccination trials have failed. Most likely, the successful manipulation

of the human host immune response by *S. aureus* is the reason why, so far, no protectivity could be overserved despite promising results in preclinical *in vitro* and *in vivo* experiments (59).

Importantly, active and passive immunization must be distinguished. Active immunization is achieved by a host response to a microbial vaccine antigen, whereas passive immunity is mediated by transfer of sera or specific antibodies (4). Antibodies against e.g. ClfA, lipoteichoic acid (LTA) and types 5 and 8 capsules have been tested for passive immunization (82). Active immunization against a broad spectrum of bacterial surface structures including iron-regulated surface determinant (IsdA-C), staphylococcal enterotoxin B (SEB), ClfA, LTA, PVL, Poly-N-acetylglucosamin (PNAG) and Hla were or are currently at different stages of clinical trials (59, 82, 83). So far, all completed phase II and phase III trials have failed to meet the pre-trial endpoints and did not induce significant efficiency or protectivity (82, 84).

New strategies address the administration of multiple vaccine antigens (85), or multiple specific antibodies in case of passive vaccination as well as antigen delivery by recombinant, non-pathogenic bacteria (86). Recently, Schneewind and colleagues generated a SpA mutant, lacking the Ig-binding properties. Immunization with this mutant protected mice, enhanced opsonization and phagocytosis after bacterial challenge (87). Further, monoclonal antibodies against the Ig-mutant neutralized the wildtype SpA and decreased abscess formation (88). These data clearly demonstrate the important role of SpA in immune evasion of *S. aureus*. In addition to vaccination, a novel approach of antibiotic application has been published recently. Lehar *et al*. developed an antibody-antibiotic conjugate that efficiently kills intracellular *S. aureus*. The antibody binds to the bacterium, which is internalized into the cell. There, the antibiotic becomes active by cleavage from the conjugate in the proteolytic environment of the phagolysosome. This method enables specific and efficient intracellular drug delivery in mice (89) and is therefore an interesting approach. However, the development of a protective *S. aureus* vaccine requires further work.

1.3. mRNA-based vaccines

mRNA-based vaccination presents an effective and interesting alternative for DNA and protein vaccination. Benefits include the rapid translation of genes within minutes after transfection because neither translocation from the nucleus nor transcription is required (90). Furthermore, there is no risk of integration into the host genome and no need for inducible or host cell-specific promotors (91). mRNA production is easy to handle, highly pure and cost-efficient (92). However, the main disadvantage of mRNA transfection is the low stability of mRNA itself, which excludes constitutive expression. Especially regarding therapeutic approaches, the way of mRNA delivery into the cell has to be considered carefully. To improve mRNA stability, adding a poly(A) tail up to 60 AA is beneficial (93). Besides, mRNA can be complexed with protamine, linked to tRNA or be packed within recombinant virus-like particles (93–95). Another recently published approach took advantage of the rolling circle replication method by which extremely long RNA strands can self-assemble into microstructures to enhance stability (96). mRNA can be further protected against nucleases by complexing with nanoparticles. Nanoparticle-based delivery was established some years ago as an alternative approach to the classical transfection protocols as electroporation or lipofection. In particular, mRNA is linked to such non-toxic nanoparticles by ionic or electrostatic interactions and therefore facilitates the uptake by dendritic cells (97–101).

The mRNA that encodes the immunizing peptide is translated intracellularly by antigen-presenting cells. DC are used preferentially, because they have the unique capability to active naive T cells (5). After translation, the processed peptide is presented via MHC molecules and serves a potent T cell activator. Normally, an efficient $CD8^+$ T cell response is generated, whereas the $CD4^+$ T cell response is usually rather weak due to insufficient MHC II presentation (102).

In the past, several protocols were established for vaccination with mRNA-loaded DC in tumor therapy (102–104). mRNA-based approaches have successfully elicited immune responses *in vitro* against viruses as HIV, *Influenza*, Epstein-Barr or papilloma virus (105–108). In 2012, an mRNA-based vaccine against *Influenza* induced long-lasting and protective immunity in young and old mice and pigs (106, 109).

However, some obstacles still have to be overcome before standardized mRNA-based therapy becomes clinical routine. So far, most studies used autologous DC, generated from isolated peripheral blood mononuclear cells (PBMC), that were loaded *ex vivo* with mRNA, tumor cell lysates, proteins or peptides (110). Although clinical trials demonstrated safety, efficiency and feasibility of this approach, *ex vivo* manipulation of DC implies high costs and personalized, time consuming treatment which impedes large scale applications (92). Thus, the administration of naked mRNA offers an alternative, which has been also successfully tested in clinical trials (111).

Hence, delivery methods have to be improved in order to elicit immunity and to maintain DC's viability. Usually, injections are the most suitable delivery method because there are easy to handle and applicable. mRNA can be applied via the subcutaneous, intradermal, intramuscular, intratumoral or intranodal route. However, to induce a strong immune response, injections close to the lymph node or intradermal seem to be preferential because of the rich environment of interacting cells (such as natural killer T cells, fibroblasts, Langerhans cells etc., (110, 111). Intravenous administration may rather send cells to lungs and liver and not to secondary lymph nodes (112). However, also direct antigen delivery by targeting DC with monoclonal antibodies against endocytic receptors and concomitant application of adjuvants or stimulatory factors as GM-CSF should be considered (110, 113). Figure 1.3 presents an overview of the vaccination schemes using either naked or mRNA-transfected DC (according to 111).

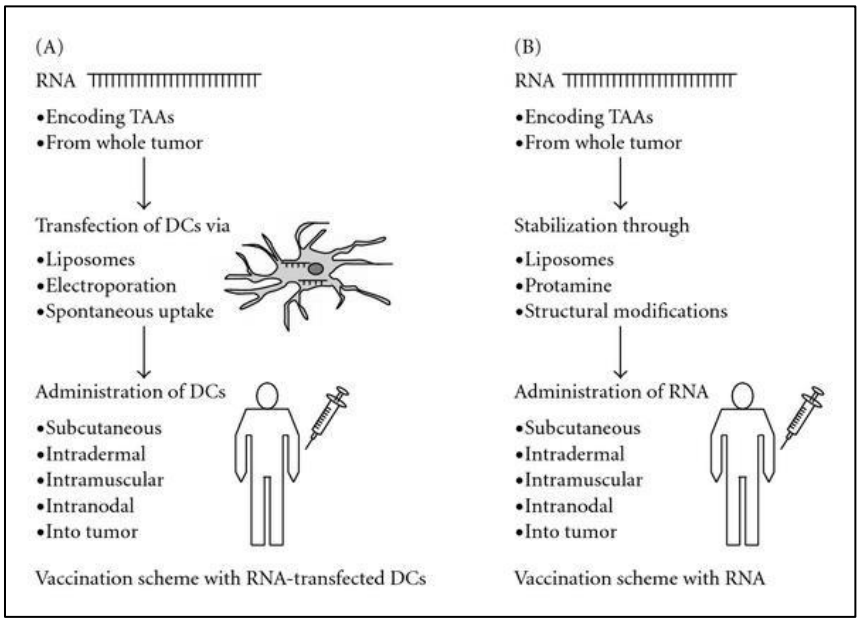


Fig. 1.3: Schematic overview of vaccination approaches using (A) mRNA-transfected DC or (B) naked mRNA. TAA: Tumor-associated antigen.

So far, only a few results of clinical studies of treatment with transfected DC application have been published, including melanoma, prostate cancer, neuroblastoma and adenocarcinoma (111). Application of naked mRNA included trials with patients suffering from melanoma and renal cell carcinoma (111). Most recently, a phase I/IIa study against prostate cancer applied up to 5 times 1250 µg mRNA that encoded multiple tumor antigens intradermal. The study demonstrated the induction of an antigen-specific immune response and a prolonged survival of the patients (114). Even if lots of research is still required, this field of mRNA-based vaccination offers promising new possibilities in the future.

1.4. Aims of the thesis

The strategies of how *S. aureus* evades the human immune response have been investigated in detail. However, in contrast to the humoral response, T-cell mediated immune responses towards this frequent pathogen still remain to be characterized in detail. In light of the numerous vaccination approaches that failed in the past, understanding T cell immunity might represent the key for successful immunization against *S. aureus*.

The aim of the present thesis was to delineate the human T cell response to this bacterium. *S. aureus* protein A was chosen as target antigen due to its known immunogenicity and its well-described strain-dependent variation. SitC, a lipoprotein belonging to the *S. aureus* core genome and the alternate penicillin-binding protein 2a (PBP2a), which confers methicillin resistance, were chosen for comparative studies. T cell responses were analyzed via delivery of mRNA-encoded antigens and pulsing with *S. aureus* derived proteins.

Therefore, the first part of the experiments aimed at the establishment of a protocol for *in vitro* transcription of different *S. aureus* genes, subsequent transfection of mRNA into monocyte-derived dendritic cells and analysis of human T cell responses in MoDC:T cell co-culture experiments. The second part of the thesis compared mRNA and protein-based antigen presentation to T cells by cytokine measurement and flow cytometry and the third part addressed the different SpA domains responsible for T cell recognition.

2. Materials

2.1. Chemicals and Reagents

Reagent	Source
Agarose	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ampicillin	Sigma-Aldrich Chemie GmbH, Munich, Germany
Aqua Molecular Biology Grade	Fisher Scientific, Niederau, Germany
DMSO	Sigma-Aldrich Chemie GmbH, Munich, Germany
EDTA	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ethanol absolute	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ethidium bromide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
FCS	Sigma-Aldrich Chemie GmbH, Munich, Germany
FCS, ultra low IgG	Life Technologies, Darmstadt, Germany
H_2SO_4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
BSA	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Human Serum	Biochrom AG, Berlin, Germany
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Pancoll (1.077 g/ml)	PAN Biotech GmbH, Aidenbach, Germany
PFA	Sigma-Aldrich Chemie GmbH, Munich, Germany
$Tween_{20}$	Sigma-Aldrich Chemie GmbH, Munich, Germany
Triton X-100	Sigma-Aldrich Chemie GmbH, Munich, Germany
Xerum free	TNC Bio, Eindhoven, Netherlands
β-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Munich, Germany

2.2. Buffers and Solutions

Reagent	Composition	Source
1x PBS	80 mM Na_2HPO_4 20 mM KH_2PO_4 1.4 M NaCl pH 7.1	Laboratory
1x TBE	89 mM Tris 89 mM Boric acid 2 mM EDTA	Laboratory
1x TBS	50 mM Tris 150 mM NaCl pH 7.4	Laboratory
AutoMACS Running Buffer		Miltenyi Biotech, Bergisch-Gladbach (Germany)
AutoMACS Washing Solution		Miltenyi Biotech, Bergisch-Gladbach (Germany)

DPBS without Ca^{+} Mg^{+}	Gibco by Life science, Darmstadt, Germany

2.3. Protein Biochemistry

Name	Composition	Source
Acrylamid/Bis-Solution (30% w/v)		Serva, Heidelberg, Germany
APS	(10% w/v) in dH_20	Sigma-Aldrich Chemie GmbH, Munich, Germany
Blocking Solution	1x TBS 0.2% (v/v) $Tween_{20}$ 5% (w/v) milk powder or 5% (w/v) BSA	Laboratory
PageRuler pre-stained Protein Ladder		Fisher Scientific, Niederau, Germany
Pierce BCA Protein Assay Kit		Thermo Scientific, Bonn, Germany
Protran Blotting Membrane 0,2 µm		GE Healthcare, Uppsala, Sweden
RIPA Lysis Buffer	1 mM EDTA (pH=8.0) 0.25% (v/v) Na-Deoxycholate 1% (v/v) NP-40 0.9 g NaCl 0.79 g Tris base ad 100 ml dH_2O Aprotinin, Leupeptin, Pepstatin (all 1 µg/ml) Na_3VO_4; NaF and PMSF (all 1 mM)	Laboratory
SDS PAGE Sample Buffer (4x)	600 mM Tris-HCl (pH 8.8) 15 mM EDTA 0.3% (w/v) bromophenol blue 30% (w/v) sucrose 9% (w/v) SDS 6% (v/v) β-Mercaptoethanol	Laboratory
SDS-PAGE Running Buffer	25 mM Tris-OH (pH 8.3) 192 mM Glycin 10% (v/v) methanol	Laboratory
Semi-dry Blotting Buffer	48 mM Tris 39 mM Glycin 0.00375% (w/v) SDS 20% (v/v) methanol	Laboratory
Separating Gel Buffer	1.5 M Tris-HCl (pH 8.8) 0.4% (w/v) SDS	Laboratory
Separation Gel	1.6 ml H_2O 2.4 ml Acrylamide (30% w/v) 2 ml Separating gel buffer 5 µl TEMED 20 µl APS (10% w/v)	Laboratory

Stacking Gel	722 µl H_2O 270 µl Acrylamide (30% w/v) 1 ml Separating gel buffer 2 µl TEMED 10 µl APS (10% w/v)	Laboratory
Stacking Gel buffer	1 M Tris-HCl (pH 6.8) 0.8% (w/v) SDS	Laboratory
Super Signal West pico		Fisher Scientific, Niederau, Germany
TEMED		Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Washing Solution	1x TBS 0.2% (v/v) $Tween_{20}$	Laboratory
Whatman Filter Paper		Bio-Rad Laboratories GmbH, Munich, Germany

2.4. Molecular Biology

2.4.1. Kits

Kit	Source
Cell Proliferation ELISA, BrdU (chemiluminescent)	Roche Diagnostics GmbH, Mannheim, Germany
Dual-Luciferase Reporter Assay System	Promega GmbH, Mannheim, Germany
mMASSAGE mMACHINE T7	Ambion,Thermo Fisher, Paisley, UK
PCR Purification	Qiagen, Hilden, Germany
QIAquick Gel Extraction	Qiagen, Hilden, Germany
QIAquick Spin Miniprep	Qiagen, Hilden, Germany
Quantifluor ONE ds DNA System	Promega GmbH, Mannheim, Germany
Quantifluor RNA System	Promega GmbH, Mannheim, Germany
UltraClean Microbial DNA Isolation	MoBio, Dianova, Hamburg, Germany

2.4.2. Cytokine measurement

Method	Reagent/Buffer	Composition	Source
ELISpot	Cytokine pair	IFN-γ, IL-10, IL-4	BD Biosciences, Heidelberg, Germany
	Washing Solution	1x PBS pH 7,1 0.05% (v/v) $Tween_{20}$	Laboratory
	Blocking Buffer	1x PBS pH 7.1 10% (v/v) FCS	Laboratory
	AKP Streptavidin		BD Biosciences, Heidelberg, Germany
	Development AP Conjugate Substrate		BD Biosciences, Heidelberg, Germany

ELISA	Cytokine pair	IL-6 IFN-α	BD Biosciences, Heidelberg, Germany eBioscience, Frankfurt, Germany
	Coating Buffer	0.1 M Sodium Carbonate; pH 9.5 ad 1.0 l H_20	Laboratory
	Washing Solution	1x PBS pH 7,1 0.05% (v/v) $Tween_{20}$	Laboratory
	Blocking Buffer	1x PBS pH 7.1 10% (v/v) FCS	Laboratory
	Substrate Solution	BD OptEIA TMB	BD Biosciences, Heidelberg, Germany
	Stopping Solution	1 M H_2SO_4	Laboratory
Bio-Plex Pro Human Cytokine 17-plex Assay			Bio-Rad Laboratories GmbH, Munich, Germany
Milliplex Human T_h17 Magnetic Bead Panel Kit			Merck Millipore, Darmstadt, Germany

2.4.3. Flow cytometry

Reagent	Composition	Source
Blocking Buffer	PBS+ 0.5% FCS	Laboratory
Brefeldin A		eBioscience, Frankfurt, Germany
Calcium Ionophore A23187		Sigma-Aldrich Chemie GmbH, Munich, Germany
Intracellular Fixation and Permeabilization Set		eBioscience, Frankfurt, Germany
Phorbol 12-myristate 13-acetate		Sigma-Aldrich Chemie GmbH, Munich, Germany

2.4.4. Polymerase chain reaction

Reagent	Source
DNA Polymerase Dream Taq + 10 x Buffer	Thermo Fisher, St. Leon-Rot, Germany
dNTPS 10 mM	Thermo Fisher, St. Leon-Rot, Germany
Fast Ruler Middle Range	Thermo Fisher, St. Leon-Rot, Germany
High fidelity DNA Polymerase Phusion + 5 x HF Buffer	Thermo Fisher, St. Leon-Rot, Germany

2.4.5. Endonucleases

Endonuclease	Cutting Site	Source
KpnI	GGTAC'C	Thermo Fisher, St. Leon-Rot, Germany
SpeI	A'CTAGT	Thermo Fisher, St. Leon-Rot, Germany
XhoI	C'TCGAG	Thermo Fisher, St. Leon-Rot, Germany

2.5. Cell culture

Name	Description/Explanation	Source
Blocking Antibodies	Goat IgG Isotype α-IL-2 α-IL-10 α-G-CSF	R&D Systems, Wiesbaden-Nordenstadt, Germany
Cytokines	hIL-4 hIl-7 hGM-CSF hG-CSF hIL-10 hIL-2	Miltenyi Biotech, Bergisch-Gladbach, Germany
FCS		Sigma-Aldrich Chemie GmbH, Munich, Germany
FCS, ultra low IgG		Life Technologies, Darmstadt, Germany
Freezing medium	RPMI 1640 Medium 20% FCS 20% DMSO	Laboratory
HEK293 Medium	RPMI 1640 Medium 10% FCS 1% Pen/Strep 1% L-glutamine	Laboratory
HEPES Buffer	1 M	Biochrom AG, Berlin, Germany
Human Serum		Biochrom AG, Berlin, Germany
L-Glutamine	200 mM	Biochrom AG, Berlin, Germany
Penicillin/Streptomycin (Pen/Strep)	10.000 IU/ml and 10.000 µg/ml	Biochrom AG, Berlin, Germany
RPMI without L-Glutamine 1640 Medium		Gibco by Life science, Darmstadt, Germany
Standard MoDC Differentiation Medium	RPMI 1640 Medium 10% FCS 1% Pen/Strep 1% L-glutamine 1% M HEPES 50 µM β-Mercaptoethanol	Laboratory
Standard Stimulation Medium	RPMI 1640 Medium 5% Xerum free 1% Pen/Strep 1% L-glutamine	Laboratory
Trypan blue solution	0.4%	Gibco by Life science, Darmstadt, Germany
Xerum free		TNC Bio, Eindhoven, Netherlands

2.6. Microbiology

2.6.1. Bacteria

Species	Source
S. aureus SAC	#20372, DSMZ, Braunschweig, Germany
S. aureus USA300	provided by Prof. Friedrich Götz, IMIT, University of Tübingen
S. aureus Rhine-Hesse t002	Clinical isolate, provided by Prof. Gabriele Bierbaum, IMMIP, University of Bonn
S. aureus Rhine-Hesse t003	
E. coli XL10 Gold	Agilent Technologies, Frankfurt, Germany

2.6.2. Agar plates and liquid media

Media	Composition	Source
CHROMagar MRSA/CHROMagar *Staph. aureus*		Mast Diagnostica, Rheinfeld, Germany
Columbia Blood Agar		BD Biosciences, Heidelberg, Germany
Lysogeny Broth (LB)	10 g tryptone 5 g yeast extract 10 g NaCl 15 g/l agar for plates Ad 1 l H_20	Laboratory
Trypticase Soy Broth (TSB)	15 g Tryptone 5 g Soytone 5 g NaCl Ad l H_20	Laboratory

2.7. Disposable plastic ware

Name	Source
BioTix Filtered Tips	VWR, Darmstadt, Germany
Cell culture flask	Greiner Bio-one GmbH, Frickenhausen, Germany
Cell culture plates, sterile (96-,6-well)	Greiner Bio-one GmbH, Frickenhausen, Germany
Combitips (10 ml, 5 ml , 2.5 ml, 1 ml)	Eppendorf, Hamburg, Germany
Cryo tubes	Greiner Bio-one GmbH, Frickenhausen, Germany
ELISA plates, half-area (96-well)	Greiner Bio-one GmbH, Frickenhausen, Germany
ELISpot plates, MultiScreen HTS IP plates, 96 well, 0.45 µm	Merck Millipore, Darmstadt, Germany
Inoculation loops	Sarstedt, Nümbrecht, Germany
PCR reaction tubes (0.2 ml)	Starlab GmbH, Ahrensburg, Germany
Pipette tips TipOne	Starlab GmbH, Ahrensburg, Germany
Plastic pipettes, disposable	Greiner Bio-one GmbH, Frickenhausen, Germany
Reaction tubes (0.5)	BD Biosciences, Heidelberg, Germany
Reaction tubes (2.0 ml, 1.5 ml)	Sarstedt, Nümbrecht, Germany
Reaction tubes (5 ml)	Eppendorf, Hamburg, Germany
Reaction tubes, sterile (50 ml, 15 ml)	Greiner Bio-one GmbH, Frickenhausen, Germany
Round-Bottom Polystyrene Tubes	BD Biosciences, Heidelberg, Germany

2.8. Laboratory Devices

Device	Source
AutoMACS	Miltenyi Biotech, Bergisch-Gladbach, Germany
Cell Counter TC10	Bio-Rad Laboratories GmbH, Munich, Germany
Centifuge 5430R	Eppendorf, Hamburg, Germany
CFX96 Realtime System/ C1000 Touch Thermal Cycler	Bio-Rad Laboratories GmbH, Munich, Germany
ChemiDocTouch Imaging System	Bio-Rad Laboratories GmbH, Munich, Germany
Experion	Bio-Rad Laboratories GmbH, Munich, Germany
Forma Series II Water Jacket	Fisher Scientific, Niederau, Germany
Gel Electrophoresis System	PEQLAB, Erlangen, Germany
iSpot FluoroSpot	Aid Diagnostika, Straßberg, Germany
IX-81 Olympus	Olympus Life science solutions
Laminar flow cabinet	Herasafe, Klasse II, Heraeus Instruments, Hanau, Germany
LSRII SORP	BD Biosciences, Heidelberg, Germany
Magnetic Stirrer IKA® RCT basic	IKA -Labortechnik, Staufen, Germany
MagPix XMAP technology	Luminex, Austin, U.S.
Megafuge 40R	Heraeus Instruments, Hanau, Germany
Microscope ID03	Zeiss, Thornwood, U.S.
Mini-Protean Tetra System	Bio-Rad Laboratories GmbH, Munich, Germany
peqTWIST Vortex Mixer	PEQLAB, Erlangen, Germany
Power Supply PacHC	Bio-Rad Laboratories GmbH, Munich, Germany
QuantusFluorometer	Promega GmbH, Mannheim, Germany
Safire^2 Microplate Reader	Tecan, Wiesbaden, Germany
TE70x Semi-Dry Transfer Unit	Hoefer, Holliston, U.S.
VF2 Magnetic Stirrer	IKA Labortechnik, Staufen, Germany
Wallace Victor^2 1420 multilable counter	Perkin Elmer Labs, Waltham,U.S.

2.9. Software

Software	Soure
Citavi 4	Swiss Academic Software
Codon Code Aligner 5.1.5	CodonCode Corporation
Experion Software	Bio-Rad Laboratories GmbH, München, Germany
Graph Pad Prism 6	GraphPad Software, La Jolla, U.S
Kaluza Analysis Software	Beckman Coulter GmbH, Krefeld, Germany
Leica Application Suite Advanced Fluorescence (LAS AF) software	Leica Microsystems CMS, Mannheim, Germany
Microsoft Office 2010	Microsoft Corporation, Redmond, U.S.
Ridom StaphType	Ridom GmbH, Würzburg, Germany
Wallace Analysis Software	Perkin Elmer Labs, Waltham,U.S.

3. Methods

3.1. Molecular- and Microbiology

3.1.1. Bacterial cultures

If not indicated otherwise, lysogeny broth (LB) - Medium (in some experiments supplemented with ampicillin, AMP) was inoculated with one colony of *S. aureus* or *E. coli* strains, and grown overnight at 37°C and 170 rpm. For culture on plates, one bacterial colony was streaked on LB (± AMP) or Columbia blood agar plates, respectively with an inoculation loop. Plates were incubated overnight at 37°C.

3.1.2. Bacterial storage

Bacteria were stored at -80°C in CryoBank (Copan, Brescia, Italy) cryo tubes, bound on chemically treated ceramic beads.

3.1.3. Isolation of genomic bacterial DNA

2 ml of *S. aureus* SAC, USA300, Rhine-Hesse t002 and t003 overnight cultures were harvested by centrifugation for 5 min, 8000 rpm. Genomic DNA was isolated using the UltraClean Microbial DNA Isolation Kit. Briefly, the culture pellet was resuspended in 300 µl MicroBead solution and transferred to a MicroBead tube. After adding 50 µl of solution MD1, cells were lysed by mechanical disruption on a vortexer for 10 min. Following a quick centrifugation, supernatant was collected and 100 µl solution MD2 were added. The sample was mixed by vortexing and incubated 5 min at 4°C. Next, 900 µl solution MD3 were added. After transfer of the sample to a spin filter, DNA binds to the filter membrane by centrifugation for 30 s at 13.000 rpm. Next, the flow-through was discarded and the filter was washed with 300 µl solution MD4. After a final centrifugation -allowing the filter to dry-, DNA was eluted by adding 50 µl of purified PCR water to the filter centre and final centrifugation for 2 min at 13.000 rpm. DNA was stored at -20°C for further applications.

3.1.4. Plasmid isolation by MiniPrep

5 ml of an *E. coli* overnight culture were centrifuged quickly and the supernatant was discarded. Plasmid DNA was isolated with the QIAquick Spin MiniPrep Kit according to the instructions. Briefly, the bacterial pellet was resuspended in 250 µl buffer P1. Next, 250 µl

buffer P2 were added and cells were incubated until the solution became clear. 350 µl buffer N3 were added and the sample was mixed carefully by inverting. Following centrifugation for 10 min at 13.000 rpm, the supernatant was applied on a spin column. Centrifugation allowed the plasmid DNA to bind to the filter membrane. The supernatant was discarded and the filter was washed twice (500 µl Buffer PB followed by 750 µl Buffer PE). A final centrifugation removed residual wash buffer. DNA was eluted by adding 30 µl of purified water the filter center and centrifuging for 2 min at 13.000 rpm. Plasmid DNA was stored at -20°C for further experiments.

3.1.5. *spa* Typing and full-length *spa* polymerase chain reaction

Polymerase Chain Reaction (PCR) accomplishes the *in vitro* amplification of any DNA template and presents one of the most important tools in molecular biology. For this purpose, two complementary oligonucleotides are needed (called primers), which flank the sequence to be amplified. PCR is performed in three different steps, repeated up to 40 times to allow amplification: during denaturation the double-stranded DNA is disrupted into single-stranded DNA; the annealing steps allow the primers to bind specifically and during elongation the new strand is synthesized by the DNA polymerase.

For the experiments, two different DNA polymerases were used. For clone testing or common genomic PCR, the Dream Taq (elongation speed 1 kb/min) allows reliable amplification. For cloning and insert generation, the faster Phusion polymerase was used, which offers a proof reading ability to minimize error rate during amplification (elongation speed 1 kb/15-30 s).

All primers, purchased from Seqlab (Göttingen, Germany) or Sigma-Aldrich Chemie GmbH (Munich, Germany), are listed in Tab. 3.1 and were used at a working concentration of 10 pm/µl.

Tab. 3.1: Oligonucleotides used in this study.

Oligonucleotide	Sequence	Application
SpA 1514 r	CAGCAGTAGTGCCGTTTGCTT	SpA Typing
SpA113 f	TAAAGACGATCCTTCGGTGAGC	SpA Typing
T7 f	TAATACGACTCACTATAGGG	Sequencing, Clone Testing
T7 Ter r	TAGTTATTGCTCAGCGG	Sequencing, Clone Testing
SpA KpnI f	CCGGGTACCATGATGACTTTACAAATA CATACAGGG	Amplifying full-length *spa*, Cloning *spA* in pT7-CFE-cMyc
Spa XhoI r	CGGCTCGAGTTATAGTTCGCGACGACG TCCAGC	Amplifying full-length *spa*, Cloning *spA* in pT7-CFE-

		cMyc
Luc KpnI f	CCGGGTACCATGGAAGACGCCAAAAAC ATAAAG	Cloning *luc* in pT7-CFE-cMyc
Luc XhoI r	CGGCTCGAGTTACACGGCGATCTTTCCG CCCTT	Cloning *luc* in pT7-CFE-cMyc
USAMecAKpnI	AGGGTACCATGAAAAAGATAAAAATTG TTCCA	Cloning *mecA* in pT7-CFE-cMyc
USAMecAXhoI r	AGCTCGAGTTATTCATCTATATCGTATT TTTTA	Cloning *mecA* in pT7-CFE-cMyc
SitCKpnI f	CCGGGTACCATGAAAAAATTAGTACCT	Cloning *sitC* in pT7-CFE-cMyc
SitCXhoI r	CGGCTCGAGTTATTTCATGCTTCCGTG	Cloning *sitC* in pT7-CFE-cMyc
$SAC^{varReg-}$ XhoI r	TTCTCGAGCTATTCCTCTTTTGGTGCTT GAGCATCG	Cloning *spA* without variable domain in pT7-CFE-cMyc
$SAC^{homReg-}$ KpnI f	AAGGTACCATGGATGCTCAAGCACCAA AAGAGGAAGAC	Cloning *spA* without homologous domain in pT7-CFE-cMyc

Genomic DNA from *S. aureus* SAC, USA300 and Rhine-Hesse strain t002 and t003 was used for SpA typing and amplification of the full-length *spa* gene. PCR was performed as the following with 35 cycles:

Master mix per tube	**Volume (µl)**
H_2O	18.975
10 x Dream Taq Buffer	2.5
dNTPs	0.5
Primer SpA 1514 r/SpA113 f (SpA Typing) SpA KpnI f/Spa XhoI r (full-length *spa*)	0.25 each
Template	2.5
Dream Taq	0.125

Program	
80°C	5 min
94°C 60°C 72°C	45 s 45 s 90 s (1:45 min for full-length *spa*)
72°C	10 min

For *spa* typing, PCR amplification was confirmed by agarose gel electrophoresis (chapter 3.1.6), purified (chapter 3.1.7) and sequenced chapter 3.1.9). The analysis of sequences and the determination of the *spa* type were carried out with the Ridom StaphType Software.

3.1.6. Agarose gel electrophoresis

PCR products were visualized on a 1% agarose gel (in TBE buffer). The Fast Ruler Middle Range ladder was used as size reference. Agarose gel electrophoresis was performed at 130 V for 30 min. Subsequently, the gel was stained in a Ethidium bromide solution (0.5 µg/ml) for 20 min and the amplicons were visualized with the ChemiDocTouch Imaging System.

3.1.7. PCR product purification

For downstream applications as cloning or sequencing, PCR samples were purified to eliminate residual dNTPs and salt from the buffers. For purification, the PCR Purification Kit from Qiagen was used following the manufacturer's protocol.

3.1.8. Quantification of nucleic acids with the QuantusFluorometer

In contrast to common spectrophotometric-based quantification of nucleic acids, the QuantusFluorometer presents a more sensitive method for determination of DNA and RNA concentrations. In principle, a fluorescent dye is incubated with the sample, which binds specifically to either DNA or RNA. Therefore, a contamination by other nucleic acids does not influence the measurement, which is indeed, the case with the spectrophotometric analysis. The fluorescence intensity is then measured by the QuantusFluorometer and an internal standard allows the quantification of sample.

Briefly, after measurement of the standard 1 µl of a DNA sample is added to 199 µl of QuantiFluor ONE dsDNA dye solution in a 0.5 ml reaction tube. For mRNA detection, 100 µl QuantiFluor RNA Dye working solution (QuantiFluor RNA dye 1:200 in 1x TE buffer) was mixed with 100 µl sample solution (1 µl mRNA with 99 µl 1x TE buffer). Following incubation in the dark for 5 min, the samples were measured.

3.1.9. Sequencing

Sequencing according to the Sanger method was carried out by Seqlab and MWG. Samples were prepared according to the companies' guidelines.

3.1.10. Cloning of antigens for *in vitro* transcription into pT7CF1-cMyc

For *in vitro* transcription (ivt) the plasmid pT7CFE1-cMyc (Thermo Fisher, Dreieich, Germany) was chosen carrying an AMP resistance, T7 promotor and terminator, a cMyc tag, an internal ribosome entry site (IRES), a PolyA tail and a multi cloning site (MCS, Fig. 3.1). Genes of interest were full-length SAC (t076) *spa*, USA300 *spa* (t008), Rhine-Hesse *spa* (t002 and t003), *mecA*, *sitC*, *luc*, truncated SAC *spa* w/o hom and SAC *spa* w/o var.

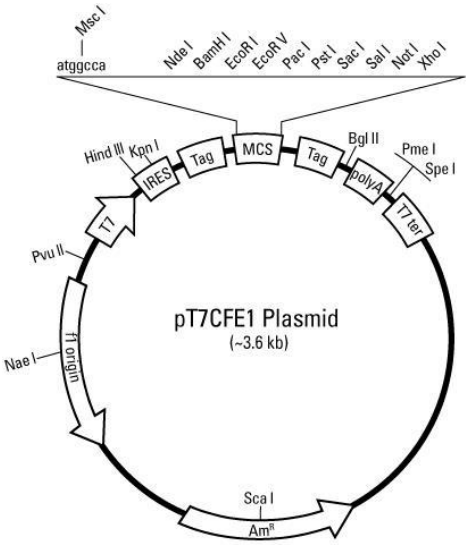


Fig. 3.1. Plasmid map of pT7CFE1-cMyc (map by Thermo Fisher). The plasmid harbors a T7 promotor and a polyA tail crucial for ivt.

3.1.10.1. Insert generation

For insert generation, genes were amplified with primers providing cutting sites for the endonucleases KpnI and XhoI (Tab. 3.1 and Tab. 2.4.5) by PCR, as described below with 35 cycles. PCR products were visualized by agarose gel electrophoresis (chapter 3.1.6), purified (chapter 3.1.7) and sent for sequencing (chapter 3.1.9). DNA concentration was determined by QuantusFluorometer (chapter.3.1.8).

Master mix per tube	Volume (µl)
H_2O	35
5 x HF Phusion Buffer + $MgCl_2$	10
dNTPs	1
Primer f and r	0.5 each
Template	0.5
Phusion	0.125

Program	
98°C	3 min
98°C	10 s
57°C	30 s
72°C	depending on the target
72°C	10 min

3.1.10.2. Digestion of insert and plasmid

The amount of insert used for cloning was calculated using the following formula:

$$\text{ng [insert]} = ((100 \text{ ng [plasmid]} * \text{kb [insert]}) \div \text{kb [plasmid]}) \times 5$$

100 ng plasmid and the calculated amount of insert were digested using 1 µl KpnI, 1 µl XhoI and 10 x FastDigest buffer (all from Thermo Fisher, St. Leon-Rot, Germany), ad 10 µl H_2O. The samples were incubated for 45 min in a 37°C water bath and enzymes were heat inactivated by incubation for 5 min at 85°C in a heat block.

3.1.10.3. Dephosphorylation

To reduce background of self-ligated plasmid, the vector was dephosphorylated following digestion. Thus, 10 µl digestion sample were added to 1 µl Fast AP, 10x buffer AP (Thermo Fisher, St. Leon-Roth, Germany) and filled up to 20 µl with H_2O. Tubes were subsequently incubated for 30 min at 37°C in a water bath and enzyme deactivation was accomplished by incubation for 5 min at 75°C.

3.1.10.4. Ligation of insert and plasmid

To ligate the digested insert with the digested and dephosphorylated plasmid, both samples were mixed together and 0.5 µl T4 DNA Ligase and 10 x T4 Ligase buffer with ATP (Thermo Fisher, St. Leon-Roth, Germany) were added. Preparations were incubated for 1.5 hours at room temperature following heat inactivated for 10 min at 65°C.

3.1.10.5. Transformation of chemically competent *E. coli* XL-10 Gold

For plasmid amplification, the ligation mixture was transformed into *E.coli* XL-10 Gold cells. Bacteria were thawed carefully on ice and 4 µl β-Mercaptoethanol were added to 100 µl cell suspension, mixing carefully. Cells were incubated for 10 min on ice, inverting the tube every two minutes. Next, the ligation mix (50 ng) was added to the bacteria. In addition, depending on the experiment, controls for transformation, dephosphorylation or ligation were prepared. Following incubation on ice for 30 min, cells were heat-shocked for 30 s in a 42°C water bath and immediately put on ice for 2 min. 990 µl pre-warmed TSB medium was added and cell suspension was cultured for 1-2 hours at 37°C and 220 rpm, allowing the bacteria to recover.

Finally, different dilutions (concentrate, undiluted, 10^{-1}) were plated on LB agar plates +100 µg/ml AMP for antibiotic selection and incubated overnight at 37°C.

3.1.10.6. Clone testing

The next day, single colonies were collected and resuspended in 50 µl purified water. Samples were incubated for 10 min at 98°C and centrifuged for 5 min at maximal speed. The supernatant was used as PCR template. For clone testing by PCR, primers were chosen that bind outside the inserted gene of interest to avoid DNA amplification located in the cell cytoplasm and not on the plasmid. Positive clones were distinguished from negative clones by size differences. PCR was performed as described below with 30 cycles:

Master mix per tube	**Volume (µl)**
H_2O	11.37
10 x Dream Taq Buffer	2.5
dNTPs	0.5
Primer T7 f T7 Ter r	0.25 each
Template	10
Dream Taq	0.125

Program	
80°C	5 min
94°C	45 s
57°C	45 s
72°C	1:45 min
72°C	10 min

PCR amplification was visualized by agarose gel electrophoresis. For sequencing, an overnight culture (LB+ 100 µg/ml AMP) was inoculated with a positive clone, the plasmid was isolated the next day by MiniPrep and samples were sent for sequencing as described before.

3.1.11. *In vitro* transcription

The principle of *in vitro* transcription is based on a suitable vector (in this study pT7CF1-cMyc) which carries the gene of interest and links it to a polyA tail. During ivt the DNA is transcribed into RNA and while additionally adding a 5' cap analogue, which allows the mimicry of a eukaryotic mRNA.

Following the transfection of antigen-presenting cells as MoDC, mRNA is translated in the cytoplasm, the protein is processed and the peptides are presented via MHC molecules. In co-culture with T cells, this allows the characterization of an antigen-specific immune response

and the circumvention of interfering protein properties, e.g. Ig-binding capacities in the case of SpA. A technical overview of this method is displayed in Fig. 3.2.

In vitro transcription was performed with mMESSAGE mMACHINE T7 Kit (Ambion, Thermo Fisher, Paisley, UK) following the manufacturer's protocol. Briefly, the pT7CFE1-cMyc plasmid carrying the gene of interest was linearized using Fast Digest SpeI restriction enzyme, and complete linearization was proved via agarose gel electrophoresis. Transcription reaction mix containing cap analogue $m^7G(5')ppp(5')G$ was added to the linearized plasmid and allowed to incubate for 2 h at 37°C. Followed by DNAse treatment for 15 min, mRNA was recovered using lithium chloride precipitation provided with the kit and ethanol precipitated. mRNA quality was checked using the Experion RNA StdSens analysis Kit (chapter 3.1.12) and quantity was measured with QuantusFluorometer (chapter 3.1.8). mRNA was stored in small aliquots at -80°C avoiding to thaw the sample more than three times.

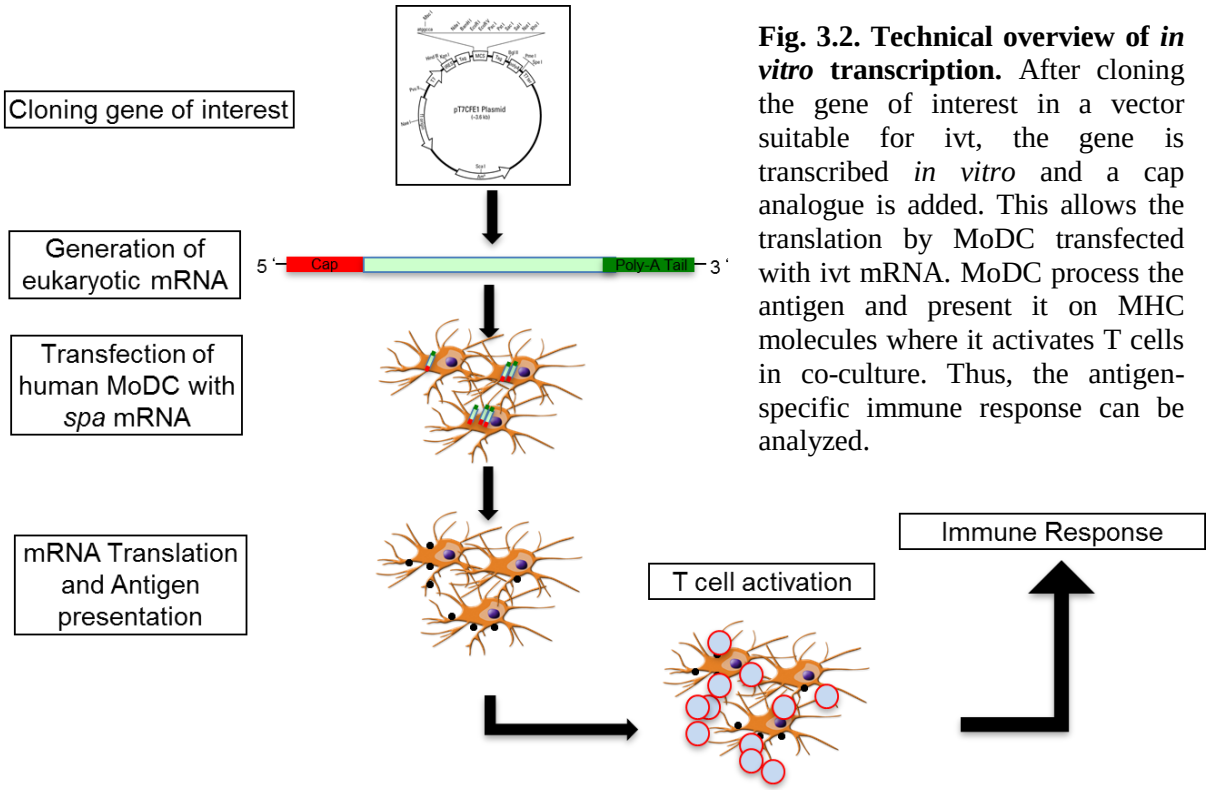


Fig. 3.2. Technical overview of *in vitro* transcription. After cloning the gene of interest in a vector suitable for ivt, the gene is transcribed *in vitro* and a cap analogue is added. This allows the translation by MoDC transfected with ivt mRNA. MoDC process the antigen and present it on MHC molecules where it activates T cells in co-culture. Thus, the antigen-specific immune response can be analyzed.

3.1.12. mRNA quality check by Experion analysis

Following *in vitro* transcription, the quality of the mRNA was checked with the Experion RNA StdSens Analysis Kit (BioRad) and the corresponding Experion system including the electrophoresis station, the priming station, the vortex station and the analysis software. Each different mRNA was checked once according to the manufacturer's instructions. If no degradation or side products were detectable, the sample was used for further experiments.

3.2. Cell Biology

3.2.1. HEK293 cell culture

Human embryonic kidney 293 cells (HEK293) were stored in liquid nitrogen and transferred to a 37 °C water bath to thaw the cells prior seeding. Cells suspension was washed by adding 10 ml HEK293 medium, centrifuged (1300 rpm, 6 min) and the supernatant discarded. The cell pellet was resuspended in 10 ml HEK293 medium and cells were seeded in a 75 cm^2 cell culture flask. Every 2-3 days of incubation, when cells reached approx. 80% confluency, the culture was split. Thus, the medium was carefully removed, adherent cells were washed once with DPBS and incubated with 10 ml 2 mM EDTA for 5-10 min at room temperature. Detached cells were then collected in a 15 ml reaction tube and centrifuged (1300 rpm, 6 min). The pellet was resuspended in 10 ml DPBS, centrifuged again and resuspended in 10 ml HEK293 medium. 2.5 ml of the cell suspension were added to 7.5 ml of fresh HEK293 medium and given into a new cell culture flask. Incubation took place at 37°C in a 5% CO_2 humidified incubator.

3.2.2. Isolation of human PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from Buffy Coats of healthy donors, obtained from the Institute of Experimental Haematology and Transfusion Medicine (University Hospital Bonn) and the Institute for Transfusion Medicine and Immunohaematology (University Hospital, Frankfurt/Main), approved by the local ethics committee. In order to separate PBMC from erythrocytes, standard Ficoll-Paque density gradient centrifugation was performed. Buffy coat was filled up to 100 ml PBS, and 15 ml Pancoll reagent (1.077 g/ml) in four single 50 ml reaction tubes was carefully overlaid with 25 ml of the blood-PBS-mixture. After centrifugation without break (2000 rpm, 30 min), the ring of leukocytes was transferred into a new 50 ml reaction tube and filled up to 50 ml with

washing buffer (PBS+ 5% standard MoDC differentiation medium). Following centrifugation (1600 rpm, 8 min) the supernatant was discarded, cell pellets were resuspended in washing buffer, four single reaction tubes were pooled to two and filled up to 50 ml with washing buffer. This step was repeated twice until all cells were collected in one 50 ml reaction tube. In order to achieve lysis of the remaining erythrocytes, the pellet was resuspended in 0.86% ammonium chloride solution and incubated at room temperature for 10-15 min. Cells suspension was filled up with PBS to 50 ml and centrifuged (1300 rpm, 6min). The cell pellet was resuspended in 50 ml PBS, cells were diluted 1:20 with trypan blue and counted with a Neubauer cell counting chamber with dead cell exclusion.

3.2.3. Freezing and thawing of PBMC

After Ficoll-Paque isolation, $5*10^7$ PBMC were resuspended in 1 ml freezing medium and stored in cryo tubes at -80°C. On day six, cryo tubes were incubated in 37°C water bath allowing the cell suspension to thaw. Next, cells were washed in 10 ml DPBS, centrifuged (1300 rpm, 6 min) and prepared for subsequent experiments.

3.2.4. Isolation of leukocyte subsets

In order to isolate leukocyte subsets, the Automated Magnetically Activated Cell Sorting (AutoMACS) device was used. In principle, magnetic beads are coupled to cell subset-specific antibodies. Following binding to the cell surface, the cell population is separated via adhering of the beads to magnetic columns. AutoMACS Programs with variable flow rate allow the isolation of the cell subset with desired purity. The purity was checked by flow cytometry for subset-specific surface markers (chapter 3.2.11.1).

For the isolation of leukocytes, the amount of magnetic beads (all from Miltenyi, Bergisch-Gladbach, Germany) is depending on the desired yield of cells, the frequency of the cell subsets and the amount of PBMC applicable for isolation (Tab. 3.2). For isolation cells are resuspended in AutoMACS Running Buffer (ration volume of buffer to microbeads 3:1) microbeads were added and the suspension was incubated at 4°C for 15 min. Next, 10 ml AutoMACS Running Buffer were added, cells were washed by centrifugation (1300 rpm, 6 min) and resuspended in 2 ml AutoMACS Running Buffer. For isolation, the possel program was chosen. Cells were counted by trypan blue including dead cell exclusion.

Tab. 3.2: Isolation of leukocytes by AutoMACS

Cell Subset	α-human Microbeads (µl)	Program
T lymphocytes	CD8	Possel
	CD4	
	CD45RO	
Monocytes	CD14	

3.2.5. Generation of MoDC

Following AutoMACS isolation, $1.5*10^6$/ml $CD14^+$ monocytes were seeded in 6-well plates in standard MoDC differentiation medium supplemented with 50 ng/ml GM-CSF and 20 ng/ml IL-4. Cells were incubated at 37°C with 5% CO_2. On day 3, 1 ml standard MoDC differentiation medium with 100 ng/ml GM-CSF and 40 ng/ml IL-4 was added. By washing vigorously, cells were harvested on day six of culture, counted by trypan blue dead cell exclusion and seeded for individual experiments in standard stimulation medium. Differentiation of MoDC was confirmed by flow cytometry (chapter 3.2.11.1).

3.2.6. Stimulation of lymphocytes

If not indicated otherwise, following isolation T lymphocytes were cultured in 200 µl standard stimulation medium in 96-well plates and stimulated with different reagents (Tab. 3.3).

Tab. 3.3: Stimuli used in T lymphocyte culture.

Stimulus	Concentration	Source
CD3/CD28-coated beads	1 µl/well	human T Cell Activation/Expansion Kit, Miltenyi, Bergisch-Gladbach, Germany
LPS (isolated from *E.coli*)	1 µg/ml	Sigma-Aldrich Chemie GmbH, Munich, Germany
Peptide pool *A. fumigatus* Crf1	1 µl/well	Miltenyi, Bergisch-Gladbach, Germany
Peptide pool *C. albicans* MP65	1 µl/well	Miltenyi, Bergisch-Gladbach, Germany
Peptide pool *Influenza* H1N1, MP1	1 µl/well	Miltenyi, Bergisch-Gladbach, Germany
PHA	1 µg/ml	Sigma-Aldrich Chemie GmbH, Munich, Germany
Protein A (SpA)	1 µg/ml	GE Healthcare, Uppsala, Sweden
SitC	1 µg/ml	Provided by Prof. Friedrich Götz, IMIT, University of Tübingen
SpA, recombinant (t008, t002, and t003)	1 µg/ml	Provided by Dr. Gert Bange, LOEWE-Zentrum, Marburg
Tetanus toxoid (TT)	1 µg/ml	Statens serum institute, Copenhagen, Denmark

3.2.7. Endotoxin testing

Contamination of proteins by endotoxins as LPS is a severe problem in cell culture because of its highly cell stimulating activity. This contamination can occur during protein purification from *E. coli* or use of contaminated water or tips.

To avoid unspecific cell activation due to LPS contamination, proteins used for stimulation were tested for endotoxin contamination with the Endosafe-PTS System (Charles River, U.S.) following the manufacturer's protocol.

3.2.8. Monocyte-derived dendritic cell: T cell co-culture

MoDC were generated and harvested as described at 3.2.5. 20.000 MoDC were seeded in 50 µl standard stimulation medium in a 96-well ELISpot plate. In the meantime, PBMC were thawed (chapter 3.2.3) and autologous $CD4^+$ and $CD8^+$ T lymphocytes were isolated by magnetic separation (chapter 3.2.4). Transfection of MoDC was performed as described in chapter 3.2.9.1. The other stimuli (mentioned in Tab. 3.3) were directly added to the cells in 50 µl standard stimulation medium. After one hour of incubation at 37°C 100.000 $CD4^+$,$CD8^+$ and $CD45RO^+$ T lymphocytes, respectively, were added to the MoDC in 100 µl standard stimulation medium.

In the experiments of cytokine blocking, T cells were added to MoDC in 50 µl medium to stick to 200 µl end volume per well. Blocking antibodies (0.5 µg/ml α-IL-2, 1 µg/ml α-G-CSF and/or 1 µg/ml α-IL-10) were added to the MoDC:T cell co-culture after stimulation with proteins in 50 µl standard stimulation medium. The isotype control normal goat IgG was used at a concentration of 2.5 µg/ml according to the highest antibody concentration used in the triple block conditions. 1 ng/ml IL-2, IL-10 or G-CSF were given to the co-culture after transfection of MoDC.

If not indicated otherwise, the cells were incubated overnight at 37°C and in a humidified 5% CO_2 incubator, in a shock-free environment to avoid movement of the cells.

If MoDC were stimulated alone (for multiplex cytokine assays and ELISA), 100.000 cells were stimulated with the indicated concentration of protein or transfected with 100 ng mRNA for one or four days of culture.

3.2.9. Transfection of MoDC and HEK293 cells with ivt mRNA

Delivery of nucleic acids into eukaryotic cells can be achieved in several ways. In addition to electroporation, injection or particle gun delivery, lipofection offers a convenient way of transfection. DNA or RNA are packed into lipophilic complexes that fuse with the cell membrane allowing uptake and dispersion of nucleic acids into the cytoplasm. For transfection of HEK293 cells and MoDC, lipofectamine was used as chemical reagent to form lipoplexes with mRNA. As control served transfection with lipofectamine without mRNA (LF). To avoid RNAse contaminations, all steps were performed using filtered pipette tips.

3.2.9.1. Transfection of MoDC for co-culture with T cells

For co-culture with T cells, 20.000 MoDC were transfected with 100 ng mRNA of different *spa* types, *mecA* or *sitC*. Thus, two different transfection solutions were prepared as a mastermix for the desired number of wells:

1) 100 ng mRNA in 25 µl OptiMEM per well and

2) 0.25 µl lipofectamine in 25 µl OptiMEM per well

Both pre-mixes were mixed carefully and incubated 5 min at room temperature. Next, the premixes were mingled carefully and incubated 20 min at room temperature. Then, cells were incubated with 50 µl of the transfection mix per well for one hour at 37°C, 5% CO_2. Afterwards, either fresh culture medium or T cells were added for ELISpot assays.

Transfection with *gfp* mRNA for evaluation of transfection efficiency, *luc* mRNA for the Luciferase assay or transfection for Western blot lysates was performed following to the same protocol.

3.2.9.2. Transfection of HEK293 cells

For the transfection of HEK293, 50.000 cells were seeded in HEK293 medium and incubated overnight at 37°C, 5% CO_2. Assuming duplication of the cells, the next day 100.000 cells were transfected as described above with the indicated amount of *luc*, *spa* or *gfp* mRNA. After one hour incubation at 37°C, 150 µl fresh HEK293 medium were added to the transfection mix and cells were incubated for additional 24 hours before proceeding with the Dual-Luciferase Reporter Assay.

3.2.10. Luciferase Assay

To confirm successful translation of ivt *luc* mRNA, luciferase activity of 100.000 HEK293 cells and MoDC transfected with varying concentrations of *luc* mRNA, which was quantified at the indicated time points with the Dual-Luciferase Reporter Assay System.

The assay was performed according to the manufacturer's instructions with a few changes: the cellular supernatants were removed but not discarded. Cells were washed once with DPBS and were lysed by adding 40 µl PBL buffer for 15 min, shaking at RT. 40 µl of cell lysate or 100 µl of supernatant were mixed with 40 µl of LARII substrate and light emission was measured immediately with a Victor^2 Wallace reader in white, flat, clear bottom 96-well plates.

3.2.11. Flow cytometry

If cells pass a laser, they emit light when coupled to a fluorochrome. This is the basic principle of fluorescence-activated cell sorting (FACS), a method which is used for cell sorting, counting and characterizing depending on their size (displayed in the forward scatter channel FSC) and granularity (displayed in the side scatter channel SSC).

3.2.11.1. Purity and differentiation analysis

Following isolation or depletion of leukocyte subsets, purity was controlled by staining for surface markers typical for the isolated cells. Purity was always ≥93% for monocytes and T cells (Fig 3.3). Differentiation of monocytes into MoDC was also confirmed by flow cytometry with markers for CD14, CD83, CD11c and HLA-DR (Tab. 3.4 and Fig. 4.5). Cells were stained by adding the antibody in staining buffer and incubating the suspension for 10 min at room temperature. Cells were washed once and either resuspended in 200 µl staining buffer and measured immediately or fixed with DPBS/2% PFA and stored at 4°C until measurement. All antibodies were purchased from BD Biosciences, Heidelberg, Germany.

Tab. 3.4: Antibodies used for purity measurement and MoDC differentiation.

Antibody	Application
α-CD4 PerCP Cy5.5	Purity $CD4^+$ T cells
α-CD8 APC/PE	Purity $CD8^+$ T cells/LAMP Assay
α-CD45RO APC	Purity $CD45RO^+$ T cells
α-CD14 V450	Purity $CD14^+$ monocytes/ differentiation MoDC
CD11c PE	differentiation MoDC
HLA-DR PerCP Cy5.5	differentiation MoDC
α-CD83 APC	differentiation MoDC

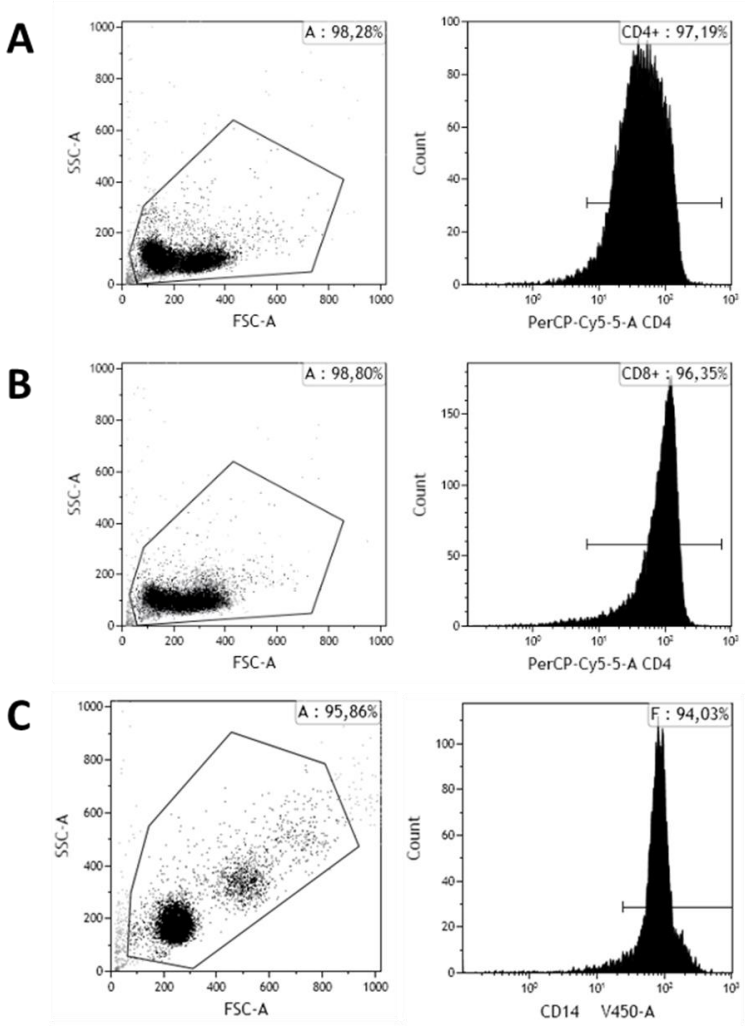


Fig. 3.3. Flow cytometric analysis of purity of leukocyte subsets. Purity of cells was measured following AutoMACS isolation as described in 3.2.4 and Tab 3.4. Dot Blots and histogram of one representative donor per staining are shown of **(A)** $CD4^+$ T cells, **(B)** $CD8^+$ T cells and **(C)** $CD14^+$ monocytes.

3.2.11.2. Staining of LAMP1 as a marker for degranulation

In order to analyse CTL degranulation, LAMP1 staining was performed and $LAMP1^+$ cells were quantified by flow cytometry according to the protocol of Betts *et al*. (115). MoDC were therefor either transfected with 100 ng SAC *spa* mRNA or 100 ng empty vector mRNA or stimulated with 1 µg/ml SpA or 1 µl/ well CD3/CD28 beads. In co-culture with $CD8^+$ cells as

described above, 5 µl α-LAMP1 APC antibody (BD Biosciences, Heidelberg, Germany) were added to 200 µl standard stimulation medium per well. Cells were incubated overnight at 37°C, 5% CO_2. The next day, cells were re-stimulated for 4 hours at 37°C with 5 µg/ml brefeldin A to block Golgi activity and, therefore, increase the fluorescence signal. After washing, the Life/Dead Fixable Aqua Dead cell stain Kit (Life Technologies, Darmstadt, Germany) was used according to the manual to exclude dead cells.

After washing the cells with 200 µl staining buffer, surface staining with α-CD8 PE was performed followed by 30 min incubation at 4°C. Using the Intracellular Fixation and Permeabilization Set, cells were fixed with 100 µl IC fixation buffer for 20 min. After washing the cells with Perm buffer, they were permeabilized by incubation within Perm buffer for 20 min. After a final washing step with staining buffer, cells were resuspended in 200 µl staining buffer and measured by flow cytometry.

3.2.11.3. Intracellular staining of cytokines

To determine the production of cytokines, intracellular flow cytometry was performed to measure IFN-γ and IL-10. MoDC were transfected with 100 ng SAC *spa* mRNA, w/o hom SAC *spa* mRNA, w/o var SAC *spa* mRNA and mRNA of the empty vector. After co-culture with $CD8^+$ T cells for one or three days respectively, cells were re-stimulated with 50 ng/ml PMA, 1 µg/ml ionomycin and 5 µg/ml brefeldin A for 4 h at 37°C, 5% CO_2. Next, cells were washed with staining buffer and surface marker staining was performed with α-CD8 APC antibody for 30 min at 4°C. After washing, cells were fixed with 100 µl IC fixation buffer (Intracellular Fixation and Permeabilization Set) for 20 min at RT. 200 µl Perm buffer were directly added to the cells and after washing, 100 µl Perm buffer including α-IL-10 PE and α-IFN-γ FITC were added for 20 min. Subsequent washing with staining buffer, the sample was resuspended in 200 µl fresh staining buffer and was ready for flow cytometric analysis.

3.2.12. Proliferation assay

Upon stimulation, lymphocytes often respond with proliferation. There are several ways to measure proliferation: to date, radioactive 3_HThymidin labelling is often substituted by Bromdesoxyuridin (BrdU) incorporation, which offers a non-radioactive and convenient assay to quantify proliferative activity. BrdU intercalates in DNA during DNA synthesis and increases with the amount of synthesized DNA. After cell permeabilization and denaturation

of DNA, incorporated BrdU is marked with an α-BrdU antibody labelled with HRP or a fluorophore and can be quantified by chemiluminescence or flow cytometry, respectively.

3.2.12.1. Monocyte:T cell co-culture BrdU ELISA

For determination of T cell proliferation, $CD4^+$ T cells and $CD14^+$ monocytes were isolated by magnetic beads and co-cultured 5:1 in 200 µl RPMI pure+ 1%Pen/Strep/L-Glut supplemented with either 2% HS or 5% XF. Cells were stimulated with 1 µg/ml SpA, TT and PHA and 2 nm BrdU were added per well (part of the BrdU ELISA kit). After incubation for 4 days at 37°C, 5% CO_2 in white, clear bottom 96-well plates, BrdU amplification was determined by the BrdU ELISA kit (chemiluminescent), following the manufacturer's protocol. Briefly, the plate was centrifuged (1300 rpm, 6min) and cell supernatant was discarded. Cells were fixed by blow-drying the plate, and 100 µl Fix/Denat solution was added. Subsequent 30 min incubation at room temperature, 50 µl α-BrdU-POD (diluted 1:100 in antibody dilution solution) were added per well and incubated 90 min at room temperature. Following three washing steps with 200 µl washing buffer each, 50 µl substrate (solution B:A 1:100) were given to each well, incubated 3 min at room temperature and chemiluminescence signal was measured immediately with the Wallace Victor^2 reader.

3.2.12.2. MoDC:T cell co-culture BrdU ELISA

To investigate proliferation capacity, co-cultures of MoDC and $CD4^+$, $CD8^+$ or $CD45RO^+$ T cells as indicated were seeded at a ratio of 5:1 in standard stimulation medium and stimulated with 1 µl *Influenza* MP1 peptide pool, or MoDC transfected with either 100 ng EV or SAC *spa* mRNA.

As indicated in some settings, the co-culture was re-stimulated on day 3 with autologous $CD14^+$ monocytes ($1*10^5$/ml) pulsed with 1 µg/ml SpA or TT overnight. 10 ng/ml hIL-2 were added on day 1 and 6 of culture.

Incubation took place at 37°C, 5% CO_2 for 6, 10 or 14 days and BrdU ELISA was performed as described above.

3.3. Protein Biochemistry

3.3.1. Cytokine measurements

3.3.1.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Detection of cytokine secretion in the supernatants from MoDC stimulated for one or three days, was performed by sandwich ELISA using BD OptEIA kit BD Biosciences, Heidelberg, Germany for IL-6 or Module Set eBioscience, Frankfurt, Germany for IFN-α based on the manufacturer's protocols. Cytokine levels were quantified by absorbance measurement (450 nm) using the Safire^2 Microplate Reader.

3.3.1.2. ELISpot

The main advantage of ELISpot over ELISA for the measurement of cytokines is the high sensitivity of this method. The amount of e.g. antigen-specific T cells is very low and therefore only detectable by ELISpot on a single-cell level. The ELISpot plate is covered with a high binding affinity membrane, which is coated with a detection antibody against the desired cytokine. Cell culture occurs directly within the plate, allowing the low amount of cytokines to bind immediately to the antibody on the membrane. After development, those spots representing one cytokine-secreting cell can be detected by a colorimetric reaction and counted with an ELISpot reader.

ELISpot also facilitates the characterization of T cell responses by the composition of the spots. For quantification of cytokines, the enzymatic activity was preferentially chosen over the amount of cytokine-secreting cells. The enzymatic activity is calculated by the ELISpot software referring to size and intensity of spots and therefore including the amount of secreted cytokine.

For ELISpot assays, MoDC:T cell co-culture was performed in 96 well MultiScreen HTS IP plates (0.45 µm, clear, Merck Chemicals GmbH, Darmstadt, Germany), coated 1:200 with an capture antibody α-IFN-γ, α-IL-4 or α-IL-10 in PBS (100 µl/well) overnight at 4°C. Prior stimulation, the plate was washed two times with culture medium and blocked two hours at room temperature (200 µl medium/well). Cells were seeded and cultured as described earlier (chapter 3.2.8).

After culture, cells were discarded and the plate was washed two times with sterile water followed by three times washing with PBS 0.05% $Tween_{20}$ (200 µl/well, washing solution). Biotinylated detection antibody was added to the cells (1:250 in PBS/10 % FCS, 100 µl/well) and the plate was incubated 2 h at RT. After washing the plate 3 times with washing solution, ARP Streptavidin was added to the wells 1:1000 in PBS/10% FCS (100 µl/well) followed by incubation at RT for one hour. Next, plate was washed three times with washing buffer and two times with PBS. Development of the plate was performed with the AP conjugate substrate kit and stopped by washing the plate thoroughly with water. The plate was dried overnight. Spots and enzymatic activity were quantified with an ELISpot reader (*i*Spot FluoroSpot Reader System).

3.3.1.3. Multiplex Assays

Multiplex assays are based on the binding of a specific antibody to a cytokine within a sample. By coupling the antibody to a fluorochrome and beads differing in size the amount of multiple cytokines can be determined within one single assay by measurement with a MagPix XMAP reader. All multiplex assays were performed following the manufacturer's protocol.

For cytokine measurement of 5 days MoDC:T cell co-culture, supernatants were analyzed by Bio-Plex Pro Human Cytokine 17-plex Assay (Bio-Rad Laboratories GmbH, Munich, Germany).

Milliplex Human T_h17 Magnetic Bead Panel Kit (Merck Millipore, Darmstadt, Germany) was utilized to quantify cytokine production by MoDC culture stimulated with mRNA and proteins after one and four days, respectively.

3.3.2. Confocal Microscopy of HEK293 cells

HEK293 were seeded as quadruplicates at a density of $5*10^5$ cells/ml in 96-well plates in HEK293 medium. Cells were transfected with 100 ng *spa* of 100 ng EV mRNA as described above. After overnight incubation, cells were harvested by PBS/2mM EDTA and 25.000 cells were seeded on adhesion cover slides (Marienfeld-Superior, Lauda-Königshofen, Germany). To allow adherence of the cells, slides were incubated 15 min at 37°C, 5% CO_2. Spots were washed with PBS and cells were fixed with 2% PFA for 10 min. After washing, cells were permeabilized by incubation with PBS/0.2% Triton X-100 for 30 min. Next, cells were washed two times and incubated with α-SpA mouse antibody (Sigma-Aldrich, Darmstadt,

Germany) 1:200 in PBS/2%FCS overnight at 4°C. The next day, cells were washed and stained with Alexa Fluor 594 goat anti-mouse 1:200 in PBS/2% FCS (Jackson ImmunoResearch,UK). After washing and counterstaining the cells with DAPI (Jackson ImmunoResearch,UK) they were covered in mounting medium (Vectashield, Sigma-Aldrich, Darmstadt, Germany), dried overnight, sealed with nail polish and microscope analysis was carried out on a Olympus IX81 microscope.

3.3.3. Protein Lysates

3.3.3.1. Cell lysates

Quadruplicates of 100.000 HEK293 cells were transfected with ivt mRNA of different *spa* types, EV mRNA or LF control as described above. 24 p.t. cells were washed with PBS, lysed with 50 µl 4x SDS Sample Buffer per well and quadruplicates were pooled. For SDS-PAGE, 20 µl of cell lysate was loaded per lane.

For MoDC, 500.000 MoDC were transfected with 1000 ng SAC *spa* mRNA and LF as control. 24 h p.t. cells were washed and lysed in 100 µl SDS Sample Buffer of which 20 µl were loaded per lane for SDS-PAGE. For the detection of protein, 20 µl of supernatant were added to 5 µl SDS Sample buffer and loaded directly on the gel.

3.3.3.2. Bacterial lysates

Bacteria were grown in TSB at an OD_{600}=1 (early log phase). The culture was centrifuged (4000 rpm, 10 min, 4°C) and washed twice with PBS (5 min, 10.000 rpm). The pellet was resuspended in 500 µl freshly prepared RIPA Lysis buffer and cells were additionally shred by mechanical disruption using 0.5 mm glass beads and a Precellys Homogenizer (3x 30 s, 6800 rpm). Between lysing cycles, bacteria were incubated on ice to avoid protein denaturation through heat. Finally, the solution was incubated on ice for 20 min, centrifuged (13.000 rpm, 15 min, 4°C) and protein concentration of the supernatant was quantified by the Pierce BCA protein assay.

3.3.4. Protein quantification

For determination of protein concentration, the Pierce BCA protein assay was performed based on manufacturer's protocol. Briefly, sample were diluted 1:4 in PBS and mixed with 180 µl Pierce Solution (Reagent A: Reagent B 50:1) in a 96-well plate. The plate was

incubated for 30 min at 37°C and measured at 550 nm with a photometer. Protein concentrations were calculated using a standard dilution of BSA.

3.3.5. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

SDS-PAGE is used for protein separation by electrophoresis, which is depending on the protein's size and charge. Following protein denaturation by adding SDS and β-Mercaptoethanol, separation is achieved by different pore size of the poured acrylamide SDS gel. Binding of SDS-anions allows the protein to migrate towards the anode.

SDS-PAGE was performed with an 12% acrylamide gel (1.5 mm, chapter 2.3) and the Mini-Protean Tetra System by Bio-Rad for approx. 2 hours at 120 V. Prior loading, samples were incubated with 4x SDS PAGE Sample Buffer for 10 min at 94 °C. 10 µg of bacterial lysates and 20 µl of cell lysates were loaded per lane. 5 µl of the pre-stained PageRuler Protein Ladder served as a protein size reference. Proceeding, the gel was used for Western Blot analysis.

3.3.6. Western Blot analysis

Western Blot is performed for the detection of minor protein concentrations. In order to analyze the expression of different SpA variants, Western Blot of bacterial lysates was performed. Further, this methods was also used to confirm translation of ivt mRNA in transfected HEK293 cells and MoDC.

During immunoblotting the electrophoresed proteins are transferred onto a nitrocellulose membrane under semi-dry conditions using a TE70x Semi-Dry Transfer Unit with 1 mA/cm^2 for 1 hour.

In order to block unspecific binding sites, the membrane was briefly washed in washing solution (TBS/0.2% $Tween_{20}$, TBS-T) and incubated rotating at room temperature in 10 ml blocking solution with 5% milk powder (for IVIg) or 5% BSA (for α-cMyc antibody). If SpA was detected in bacterial lysates, the blocking solution was additionally supplemented with 1% rabbit serum (RS). Rabbit antibodies have a higher affinity to bind to SpA than human IgG allowing an optimized blocking.

For incubation of primary antibodies, the antibody was diluted in the blocking solution or in TBS-Tween$_{20}$ (TBS-T) and incubated either 2 h at room temperature or in case of weaker binding overnight at 4°C rotating. Table 3.5 summarizes the optimized incubation procedure for different application.

After 3 times washing for 5 min in TBS-T, incubation with the secondary antibody coupled to HRP followed. Finally, the membrane was washed again as before, was incubated with Super Signal West Pico Development Solution to achieved HRP-mediated peroxide cleavage. The resulting light emission was detected by the ChemiDocTouch Imaging System.

Tab. 3.5: Optimized antibody incubation protocols for Western Blots.

Blocking Solution	Primary Antibody	Dilution/Incubation	Secondary Antibody	Application
TBS 0.2%Tween$_{20}$ 5% BSA 1% RS	IVIg (Octapharma GmbH, Langenfeld, Germany)	1:2000 in Blocking solution 2 h RT	1:10.000 total human Ig HRP-conjugated goat α-human Ig (Jackson ImmunoResearch, UK), 2 h in blocking solution	SpA expression in bacterial lysates
TBS 0.2%Tween$_{20}$ 5% Milk Powder 1% RS		1:2000 in TBS-T o/n 4°C	1:10.000 α-total human Ig, 2h in TBS-T	SpA expression in HEK293 cells
	Actin	1:1000 (Cell signaling technology, Danvers, U.S.)	HRP-conjugated goat α-rabbit (Jackson ImmunoResearch,UK) 0.5 h in Blocking solution	
TBS 0.2%Tween$_{20}$ 5% BSA	cMyc (Antikörper-online GmbH, Aachen, Germany)	1:1000 in blocking solution o/n 4°C	HRP-conjugated goat α-rabbit 2h in Blocking solution	SpA expression in MoDC

3.4. Statistics

Statistical analysis of results was carried out using GraphPad Prism 6. If not indicated otherwise, Wilcoxon matched-pairs signed rank test was used to calculated significance of two groups, one-way ANOVA for several groups. Results are presented as mean values ± standard error of the mean and were considered statistically significant at $p \leq 0.05$.

4. Results

The human immune response against *Staphylococcus aureus* has been thoroughly investigated over the last decades. However, many aspects remain unclear and require further, detailed research. Whereas humoral immunity against these bacteria is well established, the role of T lymphocytes was neglected.

In this thesis mRNA based presentation of bacterial antigens was evaluated as a novel tool to detect T cells specific for *S. aureus* antigens. Therefore, the first part of the thesis focused on the establishment and validation of a protocol for *in vitro* transcription (ivt) of staphylococcal antigens and transfection into MoDC.

4.1. Choice of staphylococcal antigens and their cloning for ivt

Three different antigens from the staphylococcal genome were selected for the analysis of T cell responses by ivt. The core gene *sitC* which encodes a lipoprotein, an accessory genetic element *mecA* which encodes a modified penicillin binding protein (PBP2a) conferring methicillin resistance and the variable gene *spa* that serves as an immune evasion factor.

To analyse the variablility of the *spa* gene, the following representative strains were used: *S. aureus* Cowan Strain I (SAC), USA300, the most common MRSA strain in the United States (116) and two different Rhine-Hesse strains (RH t002 and RH t003), which are widely distributed in western areas of Germany (117).

When inoculated on Columbia blood agar, they grow in white-yellowish, circular and convex colonies that are typical for *S. aureus* and vary in their hemolysis activity, depending on the expression of agr (118), a broad regulator for production of exotoxins including hemolysin (Fig. 4.1 A). The methicillin resistance of USA300 and the two Rhine-Hesse strains was confirmed by the growth of mauve colonies on selective MRSA agar plates, whereas SAC is susceptible to β-lactam antibiotics (Fig. 4.1 B).

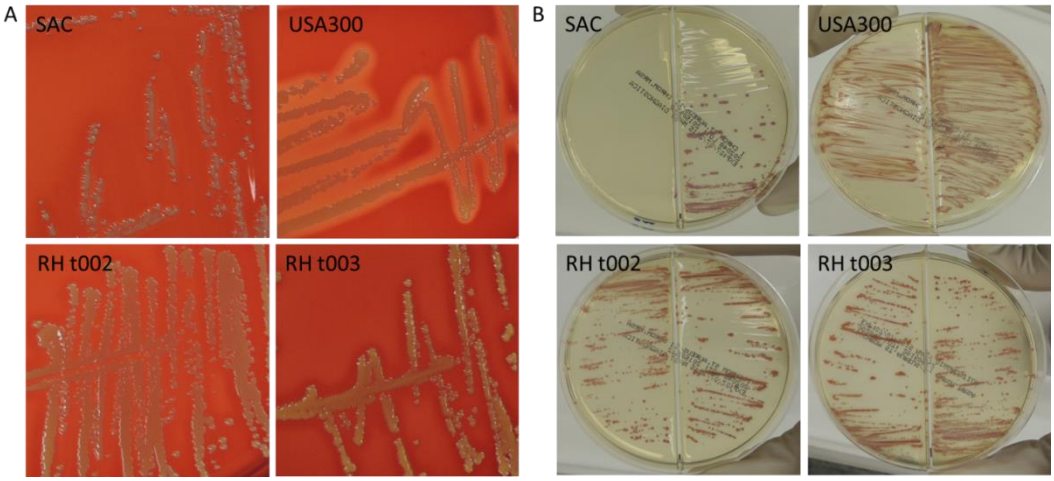


Fig. 4.1: *S. aureus* strains differ in their growth behavior. *S. aureus* SAC, USA300, RH t002 and RH t003 were incubated overnight at 37°C on **(A)** columbia blood agar plates and **(B)** selective MRSA plates. On the right site of those plates, the species *S. aureus* is confirmed by growth of mauve colored colonies, on the left site growth indicates methicillin resistance.

Notably, SpA consists of an N-terminal, highly homologous region that harbors the five immunoglobulin (Ig)-binding domains A-E and the signal sequence, which is in addition to the sortase sequence necessary for the translocation to and anchoring in the cell wall (44, 119). A variable region is located upstream of the homologous region and consists of different DNA repeats, each approximately 24 bp long (49). This region determines the *spa* type, which is utilized for the classification of *S. aureus* strains in hospital epidemiology (120). In the present study, four different *spa* types were used (Fig. 4.2 A, upper panel): t076 (SAC), t008 (USA300), t002 (RH t002) and t003 (RH t003). Alignment of the four different sequences confirmed the schematic structure of *spa*, described above, consisting of a homologous domain followed by a variable region (Fig. 4.2 A, lower panel). The complete sequences of all four *spa* types are listed in the appendix. Furthermore, different *spa* types and the differences in DNA sequence of the variable domain lead to variation in gene (Fig. 4.2 B, left panel) and protein size (Fig. 4.2 B, right panel).

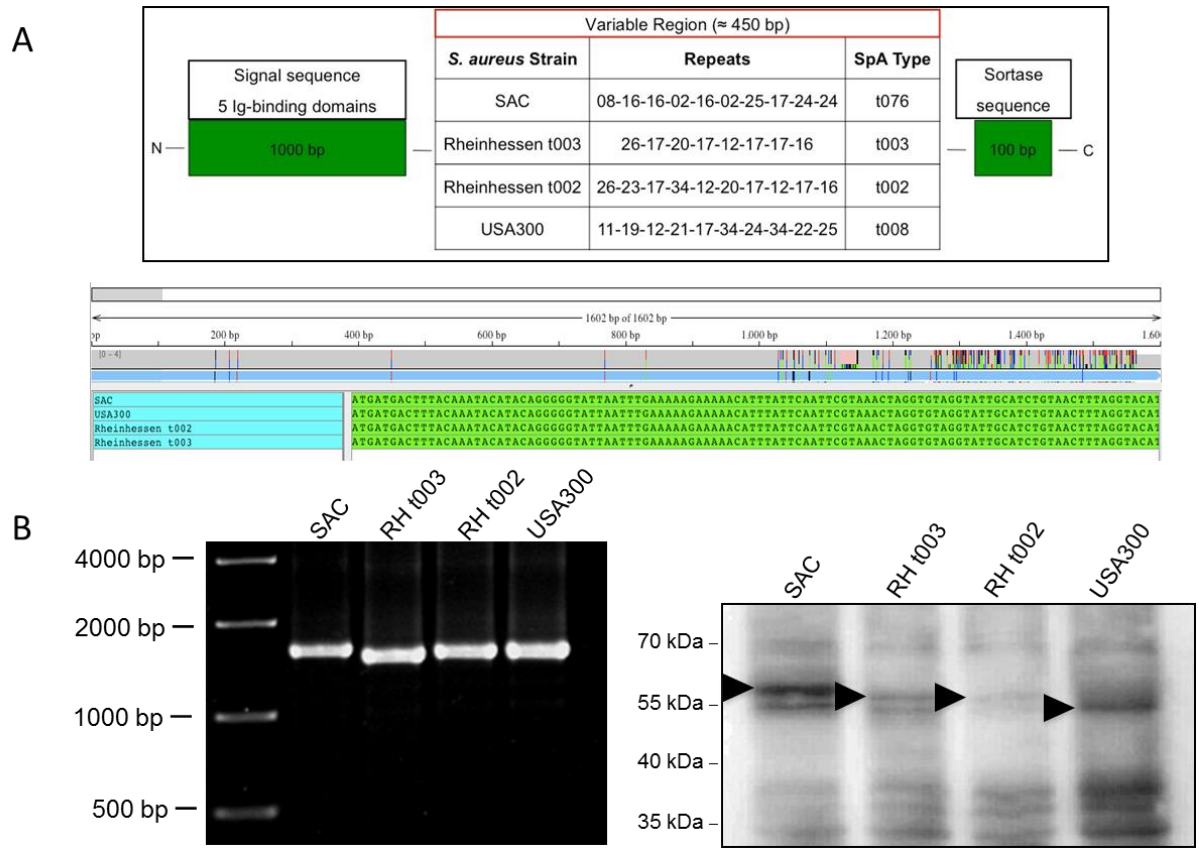


Fig. 4.2: Structural organization of protein A and its consequences for gene and protein size. **(A)** SpA consists of five N-terminal located, homologous Ig-binding domains and a variable region that determines the *spa* type. **(B)** Agarose gel (left) and Western Blot (right, arrows indicate SpA) against IVIg of full-length SpA demonstrate that different *spa* types result in variations of gene and protein size.

For ivt, full-length *spa* of those *spa* types, *mecA* and *sitC* (both from USA300) were cloned into the plasmid pT7CFE1-cMyc, suitable for ivt due to the T7 promotor and the inserted poly(A) tail. All linearized plasmid variants were *in vitro* transcribed, following the manufacturer's protocol of the Ambion mMASSAGE mMACHINE T7 kit. The quality and integrity of every mRNA batch was checked with a Experion RNA StdSense Analysis chip. If no degradation or side products were detected, the mRNA was used for downstream applications (Fig. 4.3).

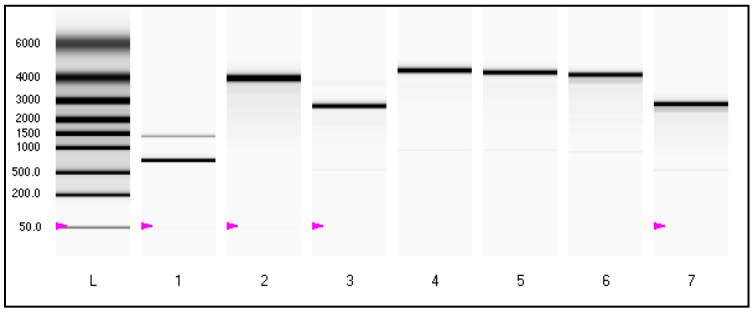


Fig. 4.3: Quality control of ivt mRNA by Experion RNA StdSense Analysis chip. Lanes 1-7 of one representative run show mRNA samples from the empty vector (EV), *mecA*, *spa* t011, *spa* SAC, *spa* USA300, *spa* t002 and *spa* t003.

4.2. Establishment of a transfection protocol for *in vitro* transcribed mRNA

Initially, the homogeneity of the HEK293 cell line was exploit to set the basic transfection protocol of ivt mRNA. Transfection of HEK293 cells with *luciferase* mRNA was employed to confirm the successful translation of ivt mRNA into a structurally intact protein. If the luciferase is active, the added luciferine is cleaved and light emission can be determined.

First, the mRNA concentration-dependent activity of lucierfase was analysed. As shown in figure 4.4, the intensity of light emission was depending on the amount of transfected mRNA and reached its maximum with 500 ng *luc* mRNA 24 h post transfection (p.t) of 100.000 HEK293 cells. This confirmed the integrity of ivt *luc* mRNA and the successful translation in luciferase.

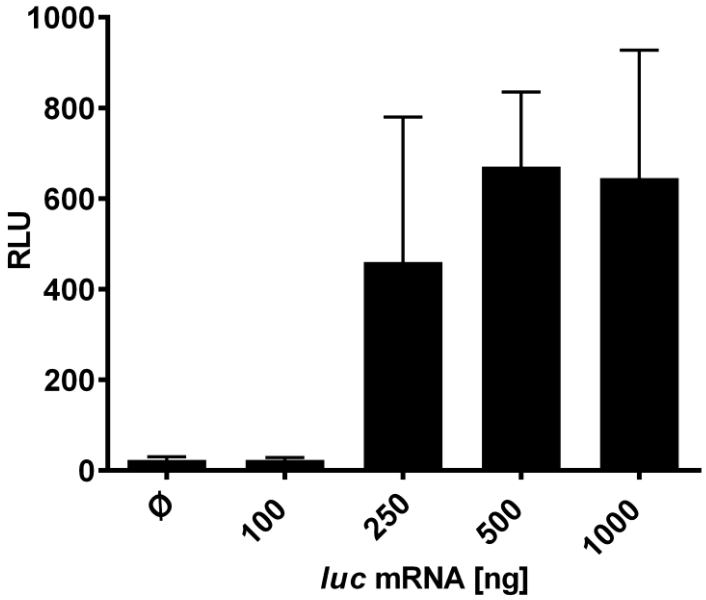


Fig. 4.4: Transfection of HEK293 cell with ivt *luc* mRNA. 100.000 HEK 293 cells were transfected with the indicated amount of *luc* mRNA and light emission was determined 24 h p.t. columns represent the mean ± SEM of n=2 independent experiments.

4.3. Antigen delivery to monocyte-derived dendritic cells

Culture of monocyte-derived dendritic cells (MoDC) was performed in medium supplemented with a serum replacement (xerum free) to avoid serum-derived variability in cell differentiation and GM-CSF and IL-4 as described in chapter 3.2.5**.** Successful differentiation into MoDC, defined as $CD14^-$, $HLA\text{-}DR^{high}$, $CD83^{dim}$ and $CD11c^+$ phenotype, was confirmed by flow cytometry (Fig. 4.5).

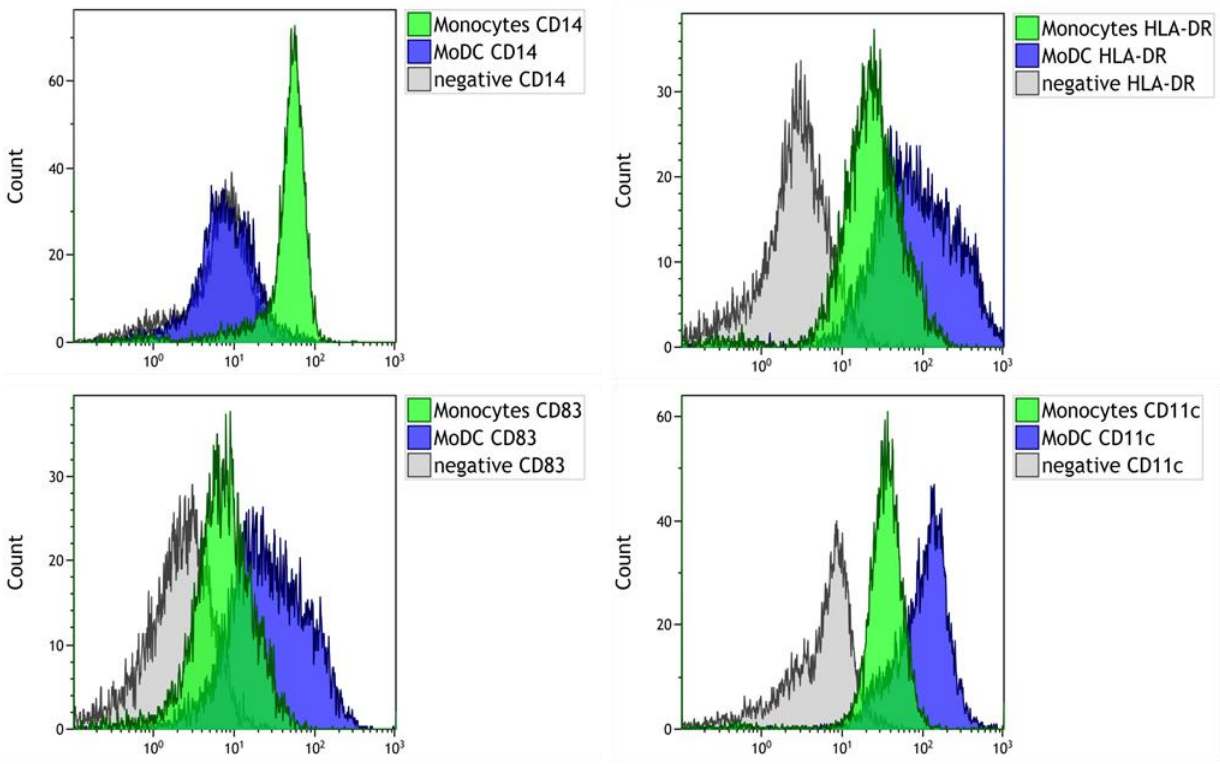


Fig. 4.5: Phenotyping of MoDC by flow cytometry analysis. MoDC (blue) and monocytes (green) of the same donor were stained for CD14 V450, HLA-DR PerCP-Cy5.5, CD83 APC and CD11c PE. For comparison, an unstained control was measured (grey) additionally. Histograms show one representative donor.

In contrast to the homogeneity of the HEK293 cell line, the viability, structure and function of MoDC underlies donor-dependent fluctuations. Thus, different transfection protocols were compared in MoDC based on the results in HEK293 cells.

MoDC cells were transfected with different amounts of *luc* mRNA, complexed with lipofectamine or added directly to the cells. One hour after transfection, the transfection mix was either replaced by 200 µl new culture medium, or 150 µl culture medium or Opti-MEM were added per well (Fig. 4.6 A). Incubating mRNA directly without transfection reagent did

not result in light emission, measured 4 hours p.t. No differences in luciferase activity were detected with different media and 250 ng transfected mRNA. However, when transfecting 500 ng *luc* mRNA, replacing transfection mix or adding new culture medium resulted in slightly higher luciferase activity than adding Opti-MEM.

MoDC process (self)-antigens and present them via MHC molecules. Therefore, the point in time had to be determined at which light emission decreases due to processing of luciferase. As shown in figure 4.6 B, light emission reached its maximum 4 h p.t. of 100.000 MoDC transfected with 250 ng *luc* mRNA.

Further, intensity of luciferase activity was the highest after the transfection of 500 ng *luc* mRNA 4 h p.t. The way of harvesting MoDC was crucial. Only cells harvested by washing and not by scratching showed luciferase activity even though cell viability was confirmed after harvesting by trypan blue staining. No luciferase activity could be detected in the supernatant of the transfected cells (Fig. 4.6 C).

Those results indicate that MoDC translate but do not take up ivt mRNA by endocytosis or macropinocytosis, as described earlier (102, 121) because simple co-incubation of cells with mRNA did not result in luciferase activity. Hence, transfection by a transfection reagent as lipofectamine is crucial. Other reagents or transfection methods as electroporation did not work in our hands (data not shown).

To quantify the efficency of transfection, HEK293 cells and MoDC were transfected with *gfp* mRNA and GFP^+ cells were quantified by flow cytometry after the indicated time. Transfection of 100 or 200 ng *gfp* mRNA resulted in 24% and 52% GFP expressing HEK293 cells 24 h p.t., respectively (Fig. 4.7 A). The percentage of GFP^+ MoDC was lower after transfection with 200 ng mRNA, and reached its maximum with 12% 4 h p.t. (Fig. 4.7 B) indicating that compared to the cell line transfection efficiency of MoDC depends on the donor.

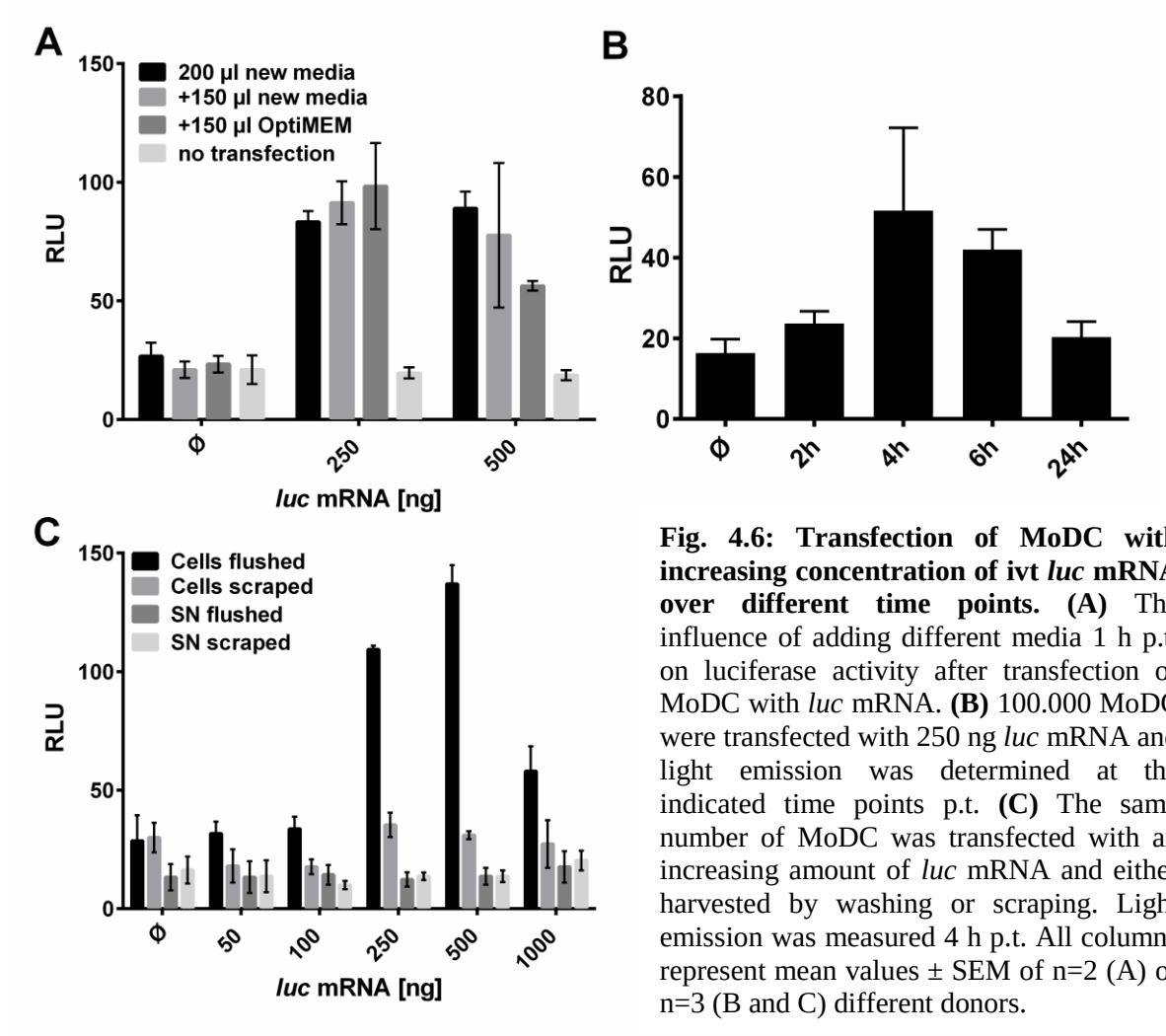


Fig. 4.6: Transfection of MoDC with increasing concentration of ivt *luc* mRNA over different time points. (A) The influence of adding different media 1 h p.t. on luciferase activity after transfection of MoDC with *luc* mRNA. **(B)** 100.000 MoDC were transfected with 250 ng *luc* mRNA and light emission was determined at the indicated time points p.t. **(C)** The same number of MoDC was transfected with an increasing amount of *luc* mRNA and either harvested by washing or scraping. Light emission was measured 4 h p.t. All columns represent mean values ± SEM of n=2 (A) or n=3 (B and C) different donors.

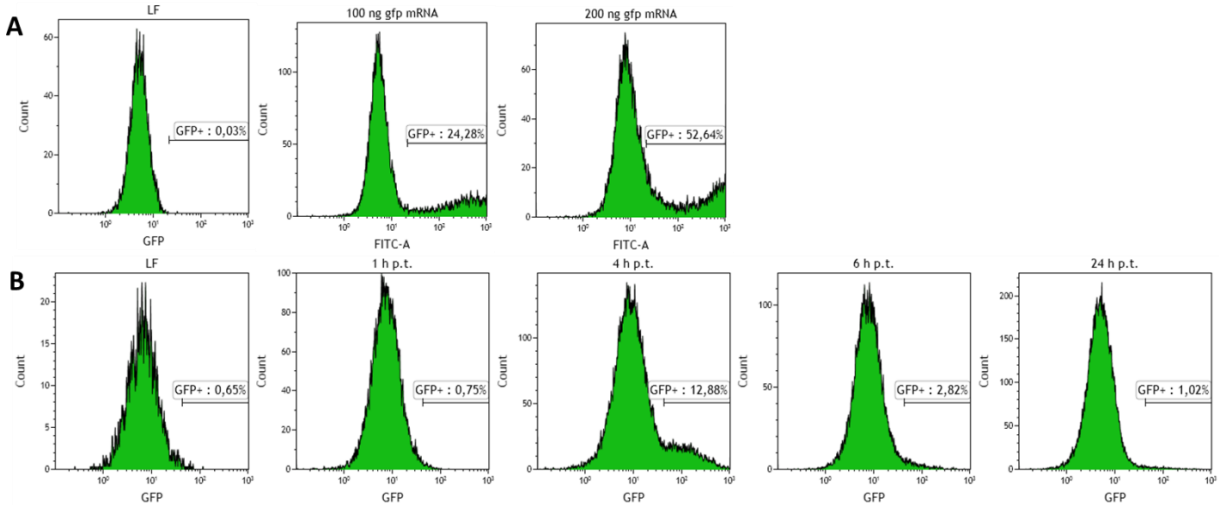


Fig. 4.7: Efficiency of Transfection. (A) HEK 293 cells were transfected with 100 or 200 ng *gfp* mRNA and amount of GFP expressing cells was measured by flow cytometry 24 h p.t. **(B)** MoDC were transfected with 200 ng *gfp* mRNA, harvested and fixed at the indicated time points and GFP^+ cells were quantified by flow cytometry. Histograms show one representative donor out of n=2 independent experiments. As control of transfection lipofectamine (LF) alone was used.

According to this optimized transfection protocol, HEK293 cells and MoDC were transfected with ivt *spa* mRNA of four different *spa* types (described before). Successful translation was proven via confocal microscopy (Fig. 4.8 A) with SpA-specific antibody that detects protein A in the cytoplasm of HEK293 cells, and Western Blot against the cMyc tag of the translated protein in MoDC lysate (Fig 4.8 B and 4.28 for HEK293 cells). In accordance with the transfection of *luc* mRNA, no SpA could be detected in the supernatant of transfected MoDC culture.

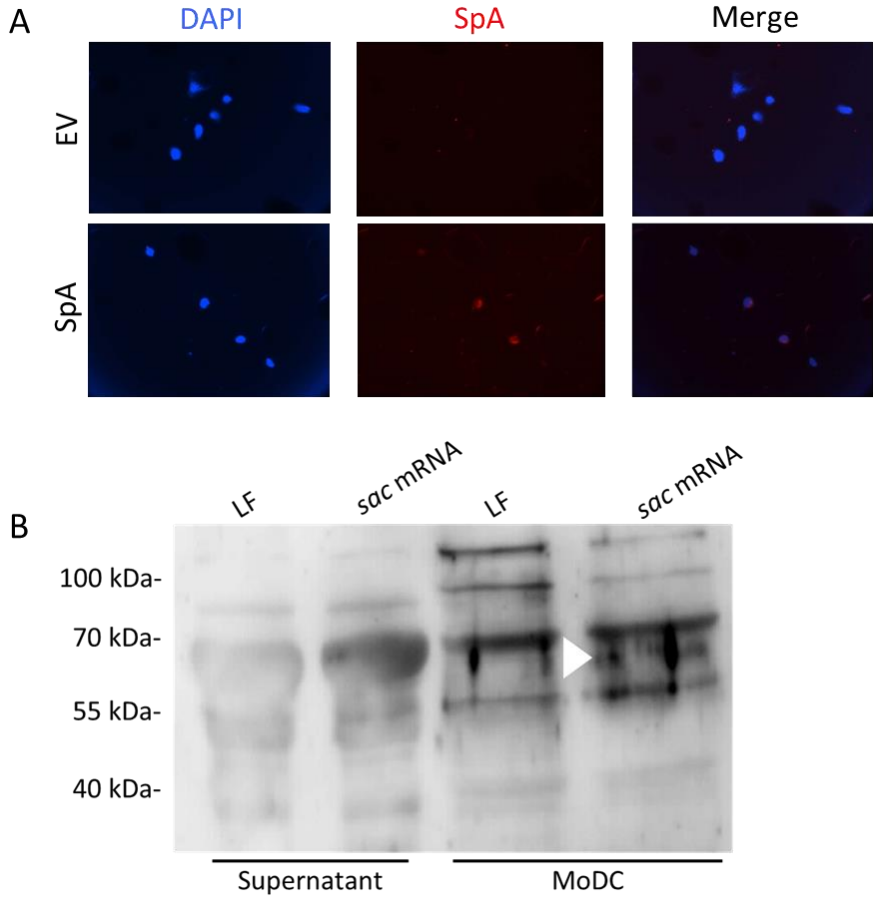


Fig. 4.8: Confirmation of SpA expression in HEK293 cells and MoDC. (A) HEK293 cells were transfected with 100 ng ivt SAC *spa* mRNA and confocal microscopy was performed with a SpA-specific antibody and DAPI for nucleus counterstaining. The empty vector mRNA (EV) was used as control. **(B)** Cell lysates of MoDC transfected with 1000 ng SAC *spa* mRNA overnight were analyzed by Western Blot with an α-cMyc tag antibody. Lipofectamine alone (LF) was used as a control. SpA is indicated by a white arrow.

4.4. Human T cell responses vary towards different *spa* types

To date, little is known about the effect of SpA on T lymphocytes. However, it is published that it acts as a mitogen leading to monocyte-dependent T cell proliferation (122). For further analysis, T cell-monocyte co-culture was stimulated with SpA, tetanus toxoid and PHA for comparison. Cell proliferation in varying media (supplemented with HS or XF) was determined after five days of incubation by BrdU incorporation (Fig. 4.9). Disregarding the higher background in XF-medium, only very weak proliferation was induced by SpA and as expected by TT. PHA is a potent mitogen leading to strong T cell proliferation.

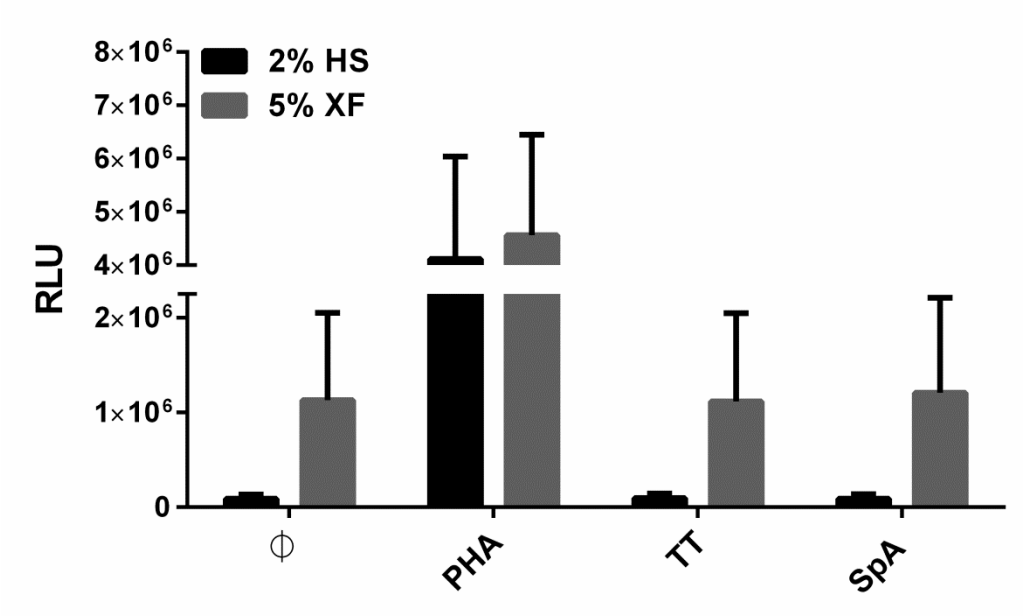


Fig. 4.9: SpA leads to T lymphocyte proliferation. $CD4^+$ T cells were incubated 1:5 with monocytes and stimulated with 1 µg/ml SpA, tetanus toxoid (TT) or PHA in medium supplemented either with 2% Human serum (HS) or 5% Xerum free (XF). Incubation was analyzed after five days by BrdU incorporation quantified by chemiluminescent ELISA. Results are displayed as columns of the mean + SEM of n= 5 different donors.

Next, functionality of MoDC as APC according to uptake, processing and presentation of antigen was tested by pulsing the cells with either tetanus toxoid (Fig. 4.10 A) or peptide pools of antigens derived from *Aspergillus fumigatus*, *Candida albicans* or *Influenza* H1N1 (Fig. 4.10 B). In overnight co-cultures of MoDC with autologous $CD4^+$ T cells, IFN-γ was measured by ELISpot and enzymatic activity was determined with an ELISpot Reader. As shown in figure 4.10 A, all tested antigens elicit IFN-γ release. Furthermore, IFN-γ production increased with an decreasing MoDC:T cell ratio. In conclusion, this strongly indicates that the

generated MoDC are able to take up whole protein, present its epitope and therefore activate T cells to produce IFN-γ.

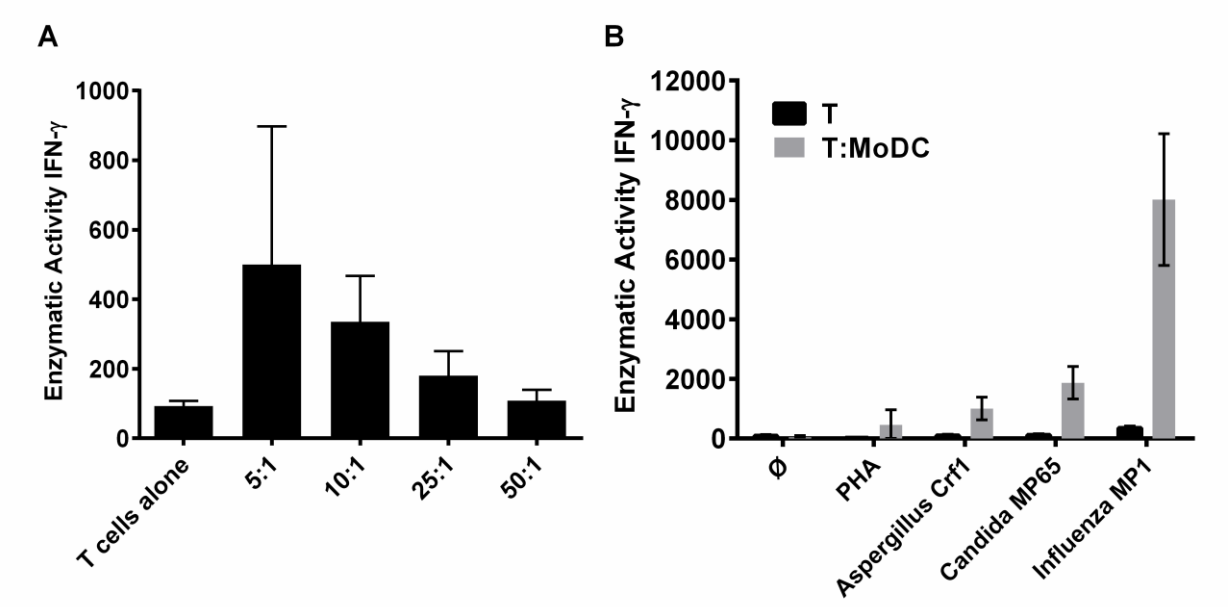


Fig. 4.10: Analysis of functional integrity of MoDC. (A) MoDC were stimulated with 1 µg/ml tetanus toxoid and co-cultured overnight with autologous $CD8^+$ T cells in the indicated T: MoDC ratio. Enzymatic activity of IFN-γ was measured by ELISpot. **(B)** $CD4^+$ T cells alone or in 1:10 co-culture with autologous MoDC were stimulated with 0.1 µg/ml PHA or 3 µl of peptide pool from *Aspergillus* Crf1, *Candida* MP65 or *Influenza* MP1. After overnight incubation ELISpot assay was used for determination of IFN-γ enzymatic activity. All columns represent mean values ± SEM of n=2 donors.

Next, ivt *spa* mRNA was used for antigen delivery to MoDC. To investigate T cell responses towards different *spa* types, ELISpot was chosen as a highly sensitive tool to analyze T cell activation visualized by IFN-γ production. mRNA of *spa* SAC, USA300, RH t002 and RH t003 was transfected into and translated by MoDC and co-cultured with $CD4^+$, $CD8^+$ or $CD45RO^+$ T cell subsets. Non-coding mRNA of the empty vector pT7CFE1-cMyc (EV) served as background control for non-specific T cell activation. Raw data (ELISpot wells) of one representative experiment are shown in figure 4.11. They visualize differences in the activation of different T cell subsets by mRNA. In general, mRNA elicits strong responses in $CD8^+$ T cells and even higher responses in the $CD45RO^+$ T cell fraction, a marker for memory T cells, whereas only low or absent responses in $CD4^+$ T cell fractions were detected. Compared to $CD8^+$, $CD45RO^+$ cell -derived responses are characterized by less, but bigger IFN-γ spots with stronger intensity. EV mRNA elicits highly variable unspecific donor-dependent IFN-γ production. However, *spa* mRNA of all *spa* types led to higher IFN-γ

secretion by $CD8^+$ and $CD45RO^+$ cells. Interestingly, *spa* type-dependent differences in IFN-γ production were detected. Quantitative analysis of secreted IFN-γ by those three T cell subsets is summarized in Fig. 4.12 A.

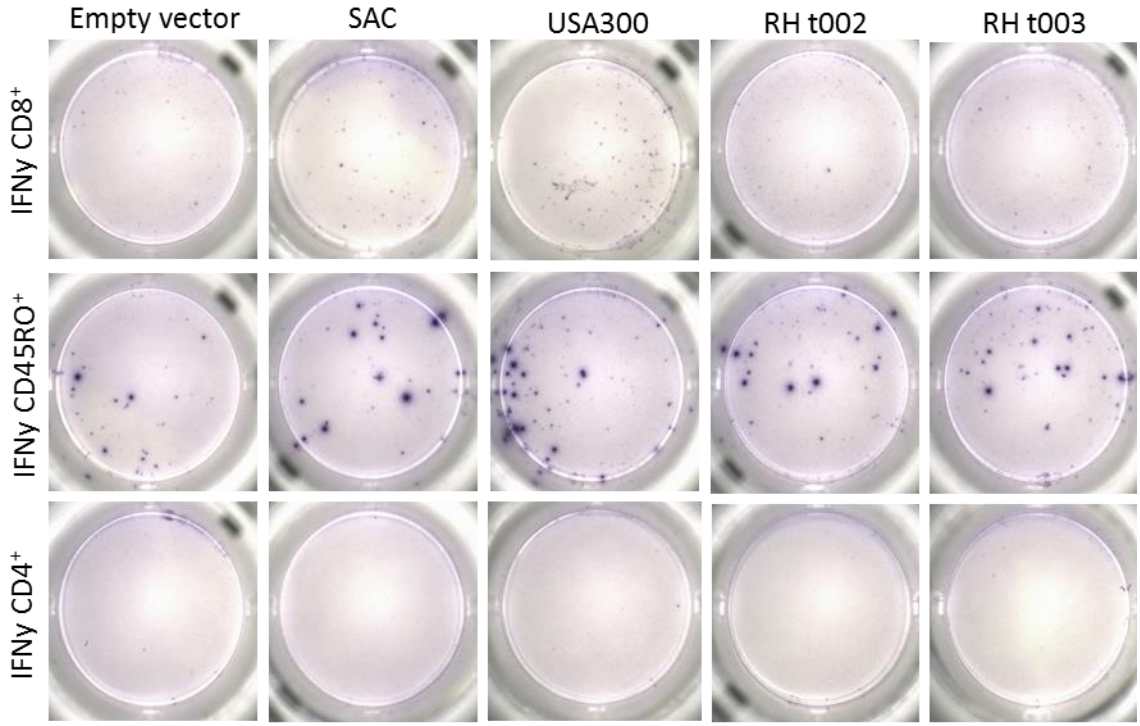


Fig. 4.11: IFN-γ ELISpot of T lymphocytes activated by *spa* mRNA-transfected MoDC. MoDC were transfected with mRNA of the empty vector (EV), SAC, USA300, RH t002 or RHt003 *spa* and co-cultured overnight with $CD8^+$, $CD45RO^+$ or $CD4^+$ isolated cells. IFN-γ was measured by ELISpot assays and wells of one representative experiment are shown.

To analyze the production of other cytokines, secretion of IFN-γ by $CD8^+$ cells was compared to IL-10 and IL-4 in the same donors (Fig. 4.12 B). Alterations in IFN-γ production by different *spa* types were confirmed and no IL-4 was detected. Interestingly, only stimulation with the SAC *spa* mRNA led to secretion of IL-10 levels above the unstimulated and EV background. In conclusion, ivt mRNA preferentially activates $CD8^+$ and $CD45RO^+$ T cells, which strongly indicates a recall response considering the short overnight incubation of cells. Based on these results, IFN-γ production by $CD8^+$ or $CD45RO^+$ cells was chosen for the main analysis of antigen-specific T cell responses.

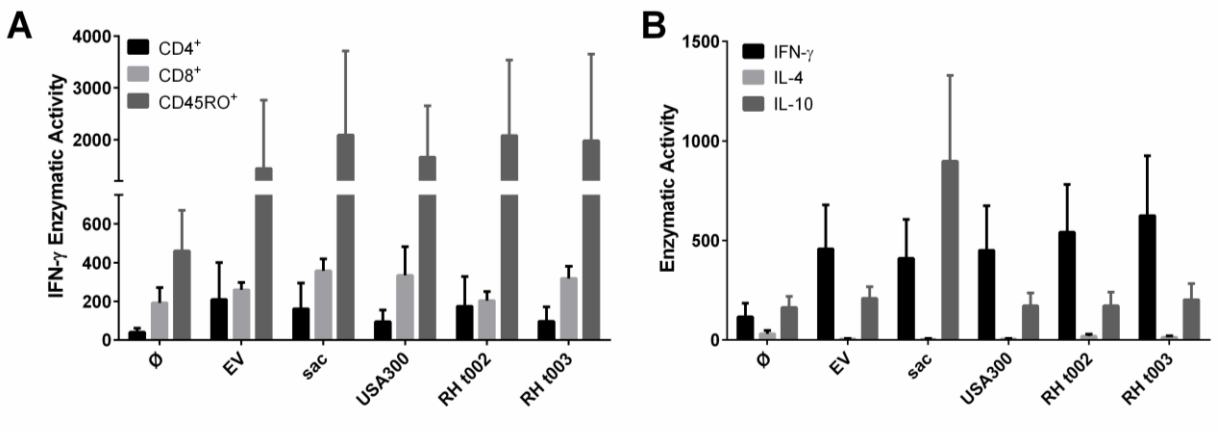


Fig. 4.12: Characterization of T cell responses against SpA by ELISpot. (A) MoDC were transfected with mRNA of the empty vector (EV), SAC, USA300, RH t002 or RHt003 *spa*, co-cultured 1:5 overnight with $CD8^+$, $CD45RO^+$ or $CD4^+$ cells and IFN-γ production was measured by ELISpot. **(B)** Following the same protocol, IFN-γ, IL-10 and IL-4 secreted by $CD8^+$ were quantified by ELISpot assays. Both graphs show enzymatic activity as columns of the mean + SEM of n=3 different donors.

To analyze the *spa* type-dependent differences in IFN-γ production in more detail, seven healthy donors were tested for their $CD8^+$ and $CD45RO^+$ T cell responses towards SpA (Fig. 4.13 A and B). For better comparison, the amount of IFN-γ measured by ELISpot was normalized to the background of the EV control mRNA. However, *spa* mRNA induces up to two times more IFN-γ than the EV control mRNA. As seen before, mRNA of different *spa* types leads to differences in the IFN-γ response in both T cell subsets. A closer look reveals that this effect is highly donor-dependent (lower panel 4.13, indicated by arrows), explaining the big standard deviation of the summarized data (Fig. 4.13 upper panel). Donors, which show IFN-γ levels above the EV, control in response to any kind of *spa* mRNA were defined as "responders" (indicated by solid arrow). In contrast, "non-responders" display the highest or an equivalent response towards the EV background control (indicated by open arrow). Furthermore, the data reveal donor-specific differences in IFN-γ secretion levels depending on the *spa* type in both cell subsets. For instance, with $CD8^+$ T cells, donor 6 only shows an IFN-γ response above the EV-induced level to one *spa* type. In general, frequency of "responders" ranged from 30-50% in all experiments.

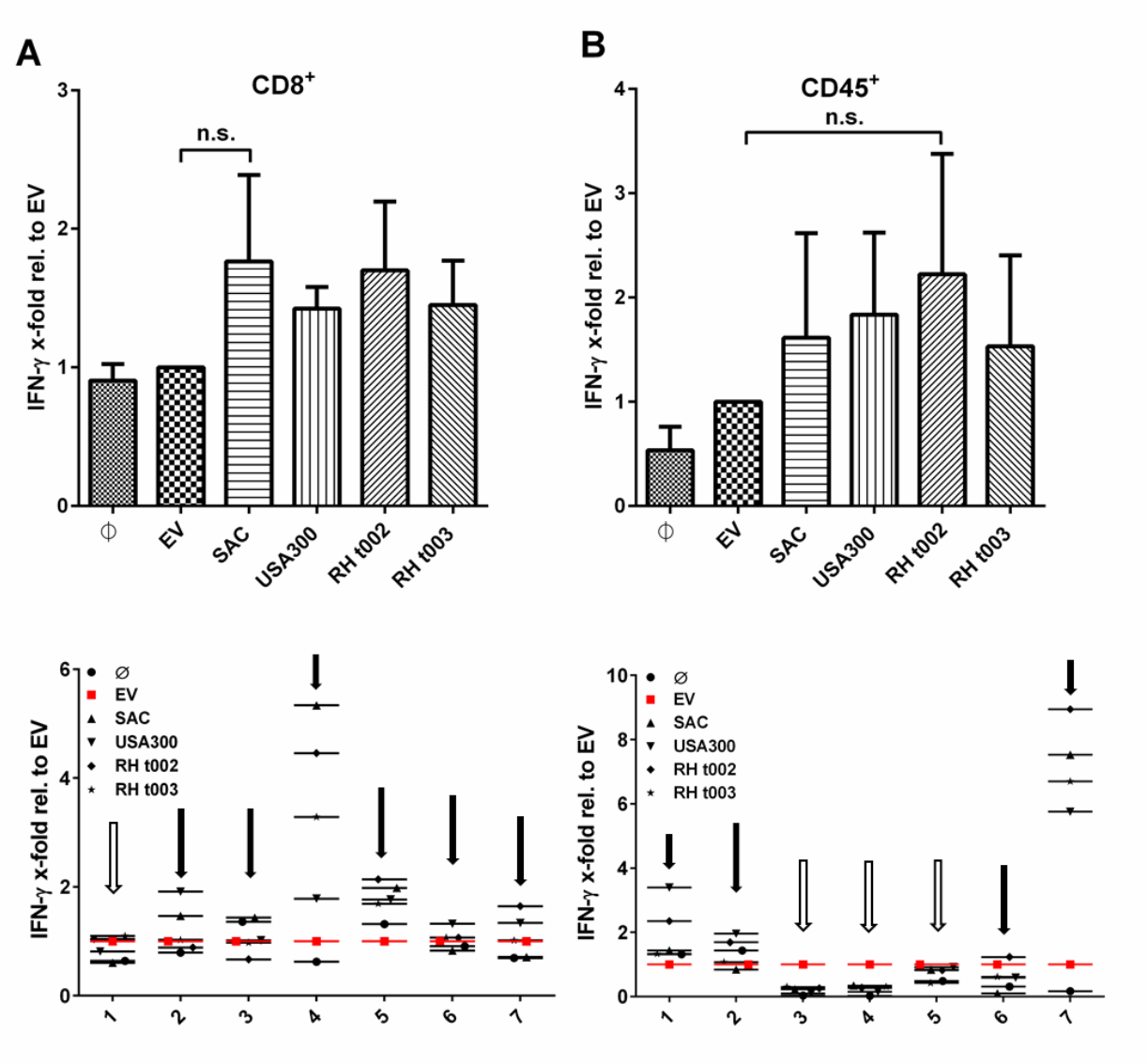


Fig. 4.13: IFN-γ response of CD8$^+$ and CD45RO$^+$ T cells towards different *spa* mRNA. MoDC were transfected with mRNA of the empty vector (EV), SAC, USA300, RH t002 or RHt003 *spa* and co-cultured 1:5 overnight with CD8$^+$ **(A)** or CD45RO$^+$ **(B)** cell and IFN-γ production was measured by ELISpot. Responders are indicated by solid arrows, non-responders by open arrows. In the upper panel, graphs show enzymatic activity of IFN-γ relative to the empty vector (EV) mRNA control as mean + SEM of n=7 different donors. Lower panels show the identical response on single donor level. Wilcoxon matched-pairs signed rank test was used for statistical analysis.

One important characteristic of antigen-specific T cells is the ability to react to re-stimulation with the known antigen with rapid cell cycle entry. To analyze the ability of SpA-specific T cells to proliferate upon antigen stimulation, BrdU incorporation measured by ELISA was performed. At first, CD4$^+$ and CD8$^+$ T cells were stimulated with a peptide pool of *Influenza* MP1 protein and proliferation was measured at the indicated time points (Fig. 4.14 A). Initially, the amount of quantified incorporated BrdU decreased over time in both cell subsets

but regained ($CD4^+$) or surpassed ($CD8^+$) the BrdU level at day 14. In contrast, co-culture of MoDC and $CD45RO^+$ T cells transfected with SAC *spa* mRNA showed no significant BrdU amplification compared to EV mRNA or the unstimulated cells after 6 days (Fig. 4.14 B). Neither in longer culture for 10 or 14 days, nor with re-stimulation of $CD8^+$ T cells by SpA-pulsed monocytes increased, BrdU levels above the unstimulated background could be measured (Fig. 4.14 C). In conclusion, proliferation of SpA-specific $CD8^+$ and $CD45RO^+$ T cells could not be confirmed.

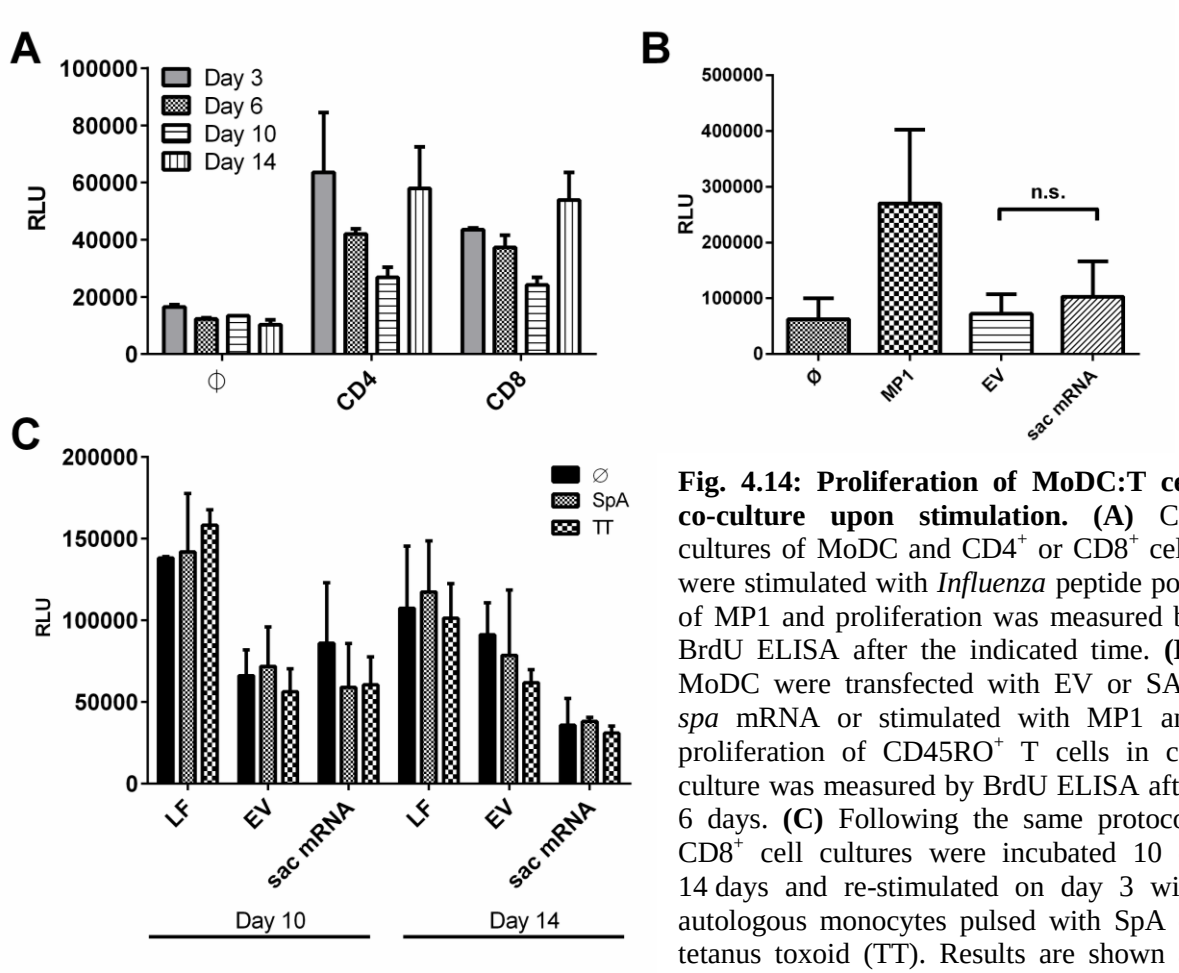


Fig. 4.14: Proliferation of MoDC:T cell co-culture upon stimulation. (A) Co-cultures of MoDC and $CD4^+$ or $CD8^+$ cells were stimulated with *Influenza* peptide pool of MP1 and proliferation was measured by BrdU ELISA after the indicated time. **(B)** MoDC were transfected with EV or SAC *spa* mRNA or stimulated with MP1 and proliferation of $CD45RO^+$ T cells in co-culture was measured by BrdU ELISA after 6 days. **(C)** Following the same protocol, $CD8^+$ cell cultures were incubated 10 or 14 days and re-stimulated on day 3 with autologous monocytes pulsed with SpA or tetanus toxoid (TT). Results are shown as mean + SEM of n=2 different donors.

4.5. Human T cell responses against other staphylococcal antigens

Since the results obtained with ivt mRNA revealed the presence of an IFN- γ response in $CD8^+$ T cells and donor-specific differences in the response to different SpA variants, it was further investigated whether this system could also be used to analyze T cell responses to PBP2a (*mecA)* and SitC (*sitC).*

Following *in vitro* transcription of these two genes, transfection of MoDC and co-culture with T cells was done as described above. Similarly, to the results obtained with *spa* mRNA, mRNA encoding *mecA* and *sitC* activated $CD8^+$ cells stronger than $CD4^+$ cells. Figure 4.15 shows IFN-γ ELISpot wells of three representative donors: a strong responder, a weak responder and a non-responder with similar activation of both T cell subsets (from left to right, Fig. 4.15).

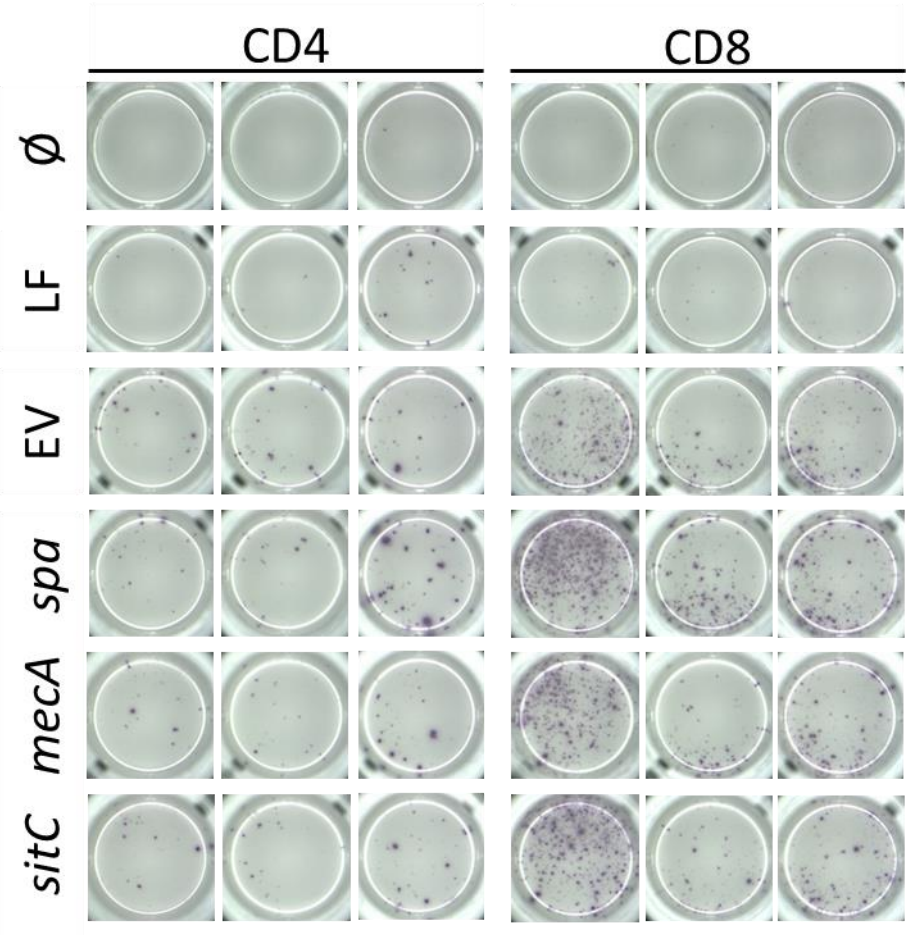


Fig. 4.15: IFN-γ ELISpot of $CD4^+$ and $CD8^+$ T cell cultures stimulated with *mRNA* of staphylococcal antigens. T cells were co-cultures with MoDC, transfected with lipofectamine alone (LF), empty vector (EV), SAC *spa*, *mecA* or *sitC* mRNA overnight. Wells of IFN-γ ELISpot are shown for three representative donors.

Taken together, ELISpot assays of overnight culture of MoDC transfected with *mecA* or *sitC* mRNA and $CD8^+$ cells elicit approximately 2.5-fold (*mecA*) and 1.4-fold (*sitC*) more IFN-γ than the background (EV) control (Fig. 4.16). This indicates the presence of T cells specific to these antigens.

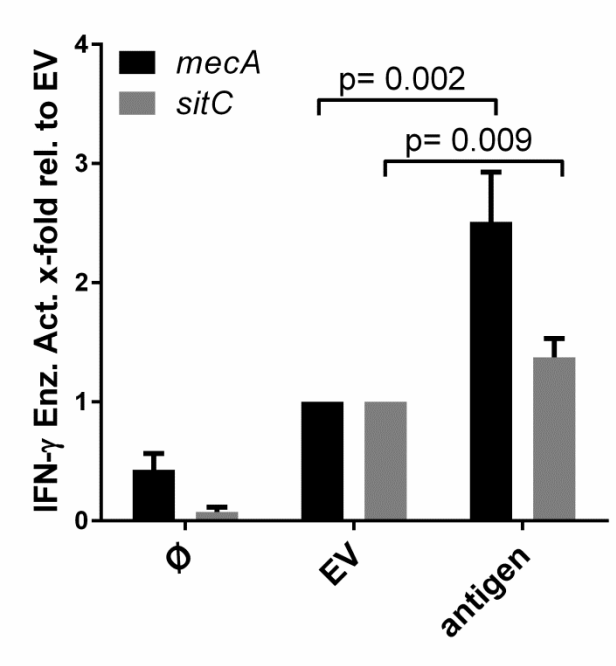


Fig. 4.16: IFN-γ ELISpot of T cell cultures stimulated with *mecA* and *sitC* mRNA. $CD8^+$ cells were co-cultured with MoDC and transfected with empty vector (EV), *mecA* or *sitC* mRNA overnight. IFN-γ is presented as enzymatic activity and relative to EV. Columns show the mean + SEM of n=10 independent donors. Statistical significance was calculated with the Wilcoxon matched-pairs signed rank test.

4.6. Comparison of human T cell responses towards staphylococcal mRNA and protein-derived antigens

Addressing the question whether mRNA-encoded antigens and corresponding proteins differ in their immunogenicity, their ability to stimulate T cells was compared in the second part of this thesis. Therefore, SpA protein purified from SAC, SitC purified from SA113 and recombinant PBP2a were compared to stimulation with mRNA encoding the same antigens. Additionally, recombinant protein A of all *spa* types described above was used to test whether *spa* type-dependent differences in the T cell responses observed with mRNA-mediated antigen delivery also occur when antigens are delivered as whole proteins.

4.6.1. mRNA-encoded antigens elicit higher IFN-γ production than proteins

For determination of an appropriate protein concentration for stimulation, amounts of SpA (from SAC), PBP2a and SitC, were titrated and IFN-γ and IL-6 , one of the main activation marker of DC (123), were measured by ELISpot and ELISA, respectively. Regarding IFN-γ,

no big differences in cytokine production by $CD4^+$ and $CD8^+$ T cells could be detected (Fig. 4.17, upper panel). Both, SpA and PBP2a-induced IFN-γ secretion increased with the amount of protein, although SpA led to higher IFN-γ concentrations in $CD4^+$ cells. Notably, increasing amounts of SitC resulted in less IFN-γ production. Within same donors, high levels of IL-6 were detected in SitC-stimulated cultures, reciprocal to increasing SitC concentration, whereas PBP2a-stimulated cultures showed the exact opposite effect. SpA-induced IL-6 reached the maximum concentration between 0.5 and 1 µg/ml protein. In general, more IL-6 could be detected in $CD4^+$ T cell cultures (Fig. 4.17, lower panel). Considering those results, 1 µg/ml of each protein was chosen for comparison with mRNA's immunogenicity.

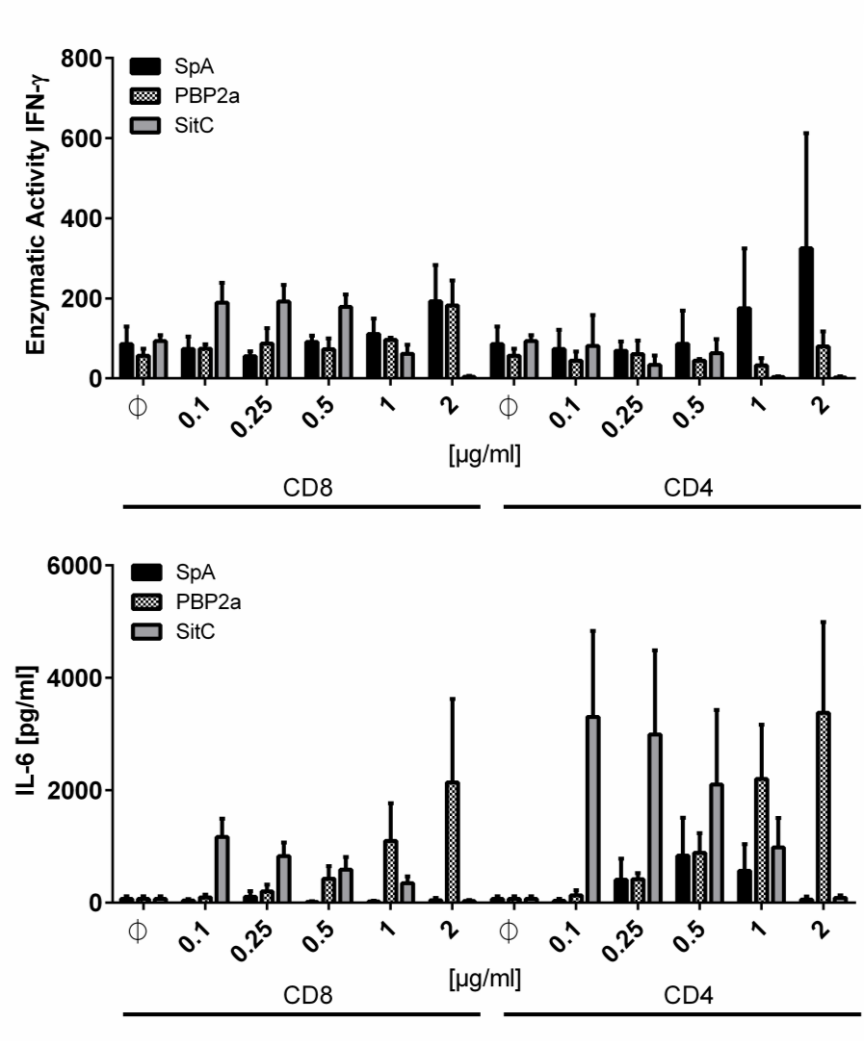


Fig. 4.17: Titration of staphylococcal antigens and determination of IFN-γ and IL-6 levels. **(A)** ELISpot of T cell: MoDC co-cultures stimulated with SpA, PBP2a and SitC were incubated overnight and IFN-γ was measured. **(B)** Following the same protocol, IL-6 concentration was determined by ELISA. Results are presented as columns of the mean + SEM of n=3 different donors.

To compare the ability of mRNA-encoded and protein-derived antigens of *S. aureus* to elicit an IFN-γ response, co-cultures of MoDC and T cells were stimulated overnight in an ELISpot assay. For better analysis, responders and non-responders were distinguished as described

before (see 4.13 and corresponding text). Figure 4.18 shows IFN-γ production of eight responders and non-responders in $CD8^+$ (Fig. 4.18 A) or $CD4^+$ (Fig. 4.18 B) T cells. In both subsets, *spa* mRNA induced significantly more IFN-γ in responders than the background control EV. The other mRNA *sitc* and *mecA* showed the same tendency but the results were not statistically significant. Notably, this could not be seen in non-responders. When compared to mRNA directly, all proteins induced less or no IFN-γ production at all in both T cell subsets. The amount of cytokine resulting from stimulation with antigen MP1 was comparable to the mRNA level in responders, whereas tetanus toxoid only led to a response in $CD4^+$ T cells. In general, as described earlier (Fig. 4.11 and 4.12), $CD8^+$ T cells secreted up to two times more IFN-γ than $CD4^+$ T cells.

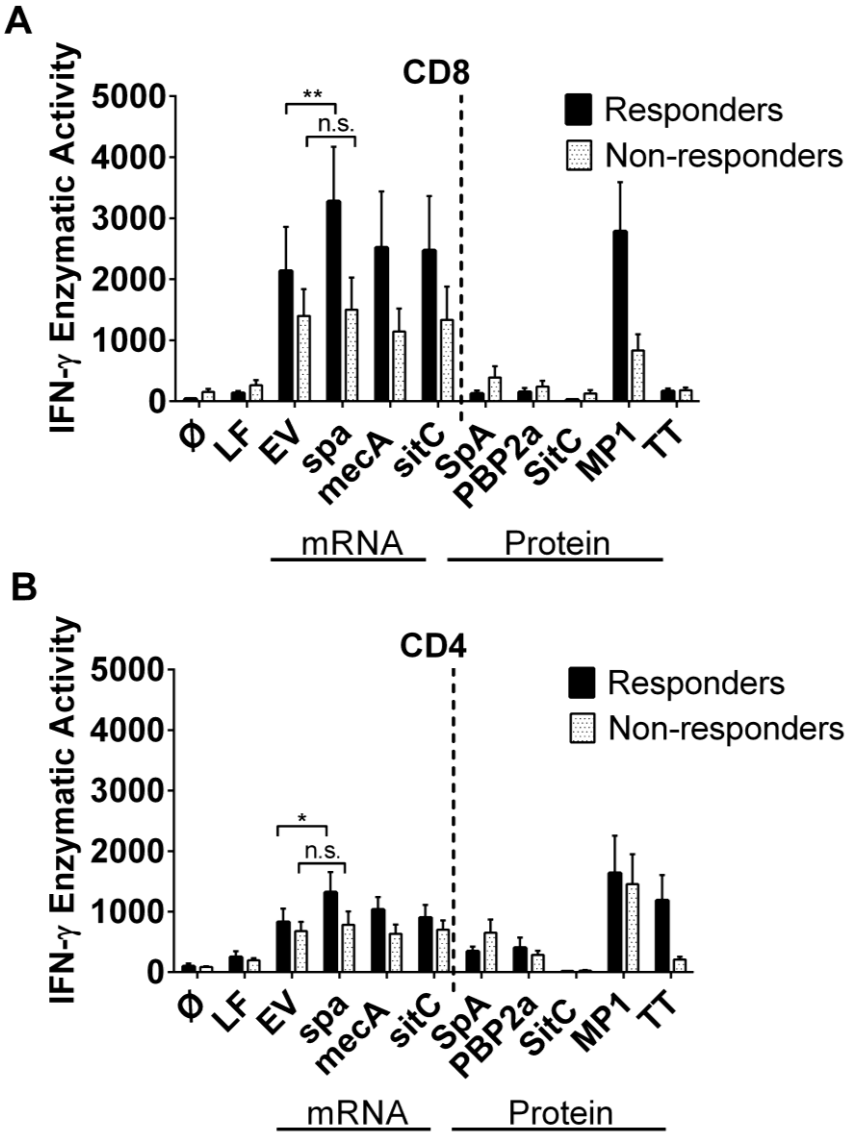


Fig. 4.18: Comparison of mRNA and protein-derived IFN-γ production. ELISpot of **(A)** $CD8^+$ or **(B)** $CD4^+$ T cell: MoDC co-cultures stimulated with 1 µg/ml SpA, PBP2a and SitC or 100 ng mRNA of the same antigens were incubated overnight and IFN-γ enzymatic activity was measured. As controls served mRNA of the empty vector (EV), lipofectamine without mRNA (LF), 1 µg/ml tetanus toxoid (TT) or 1 µl *Influenza* MP1 peptide pool. Columns of the mean of n= 8 different donors + SEM show the results. Statistical significance was calculated with the Wilcoxon matched-pairs signed rank test. p-values are for $CD8^+$ responders EV:*spa* mRNA= 0.0078 and for $CD4^+$ responders EV:*spa* mRNA= 0.0391.

Based on these results, the amount of antigen-specific cells in responders was calculated. Therefore, the number of IFN-γ secreting cells was measured by ELISpot. For mRNA-based stimulation the EV background and for protein-stimulation the unstimulated control was subtracted from this cell number. Taking in account the number of 100.000 T cells seeded per well, percentage of *S. aureus* antigen-specific T cells was calculated (Fig. 4.19). As expected, it is approximately three times higher in $CD8^+$ cells than in $CD4^+$ cells. The number of SpA-specific T cells triggered by mRNA is the highest and ranged from 0.032% to 0.06%. Remarkably, this amount is similar to the one triggered by *Influenza* peptide.

In conclusion, these results clearly demonstrate the different stimulatory capacities of mRNA or protein-derived antigens in terms of IFN-γ secretion by T cells.

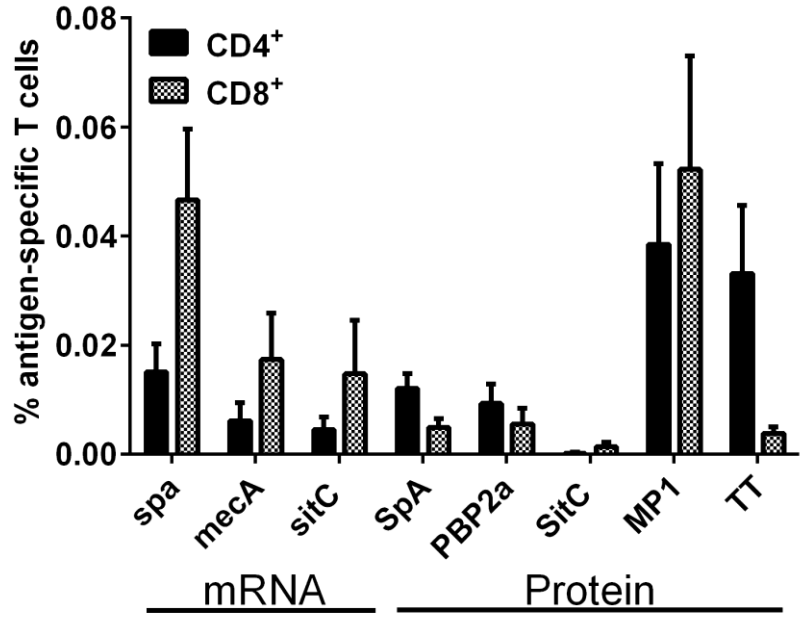


Fig. 4.19: Calculation of antigen-specific T cells for *S. aureus*. Based on the results from Fig. 4.18, the number of IFN-γ secreting cells was used to calculate the percentage of activated $CD8^+$ and $CD4^+$ cells specific for mRNA- and protein-based antigens SpA, PBP2a and SitC. The amount of cells activated by EV was subtracted for mRNA-based, and the unstimulated control subtracted for protein-based stimulation.

4.6.2. The mRNA adjuvant effect is necessary to trigger antigen-specific T cell responses

As one possible explanation for the differences between IFN-γ induction by mRNA and protein-based antigens, the adjuvant's effect of the mRNA itself was considered. To address this question, co-stimulation assays with *mecA* mRNA with and without the corresponding antigen PBP2a were performed and the amount of IFN-γ was determined by ELISpot. Although, it is not statistically significant, *mecA* mRNA led to higher IFN-γ production within

the tested donors as the empty vector control (Fig. 4.20), whereas PBP2a-induced levels are similar to the lipofectamine control. When co-cultured together with mRNA, the amount of secreted IFN-γ was even higher than with mRNA alone. Furthermore, this effect increased significantly, when mRNA encoding for the same antigen was used as co-stimulus compared to EV as unspecific mRNA. These results indicate that mRNA acts as an additional stimulus needed for the activation of T cells and that this might be dependent on the mRNA´s adjuvant effect, e.g. recognition by cytosolic receptors.

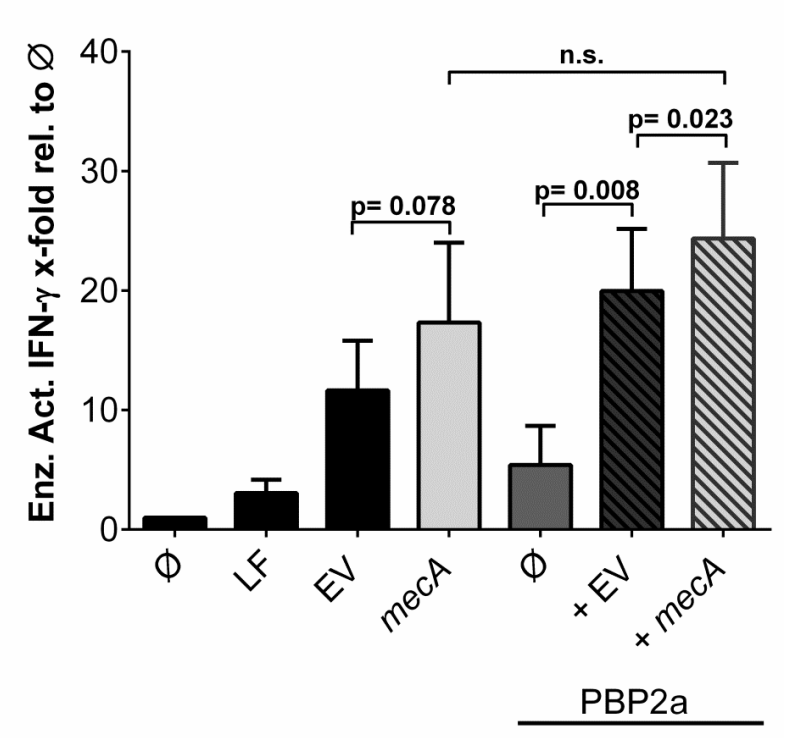
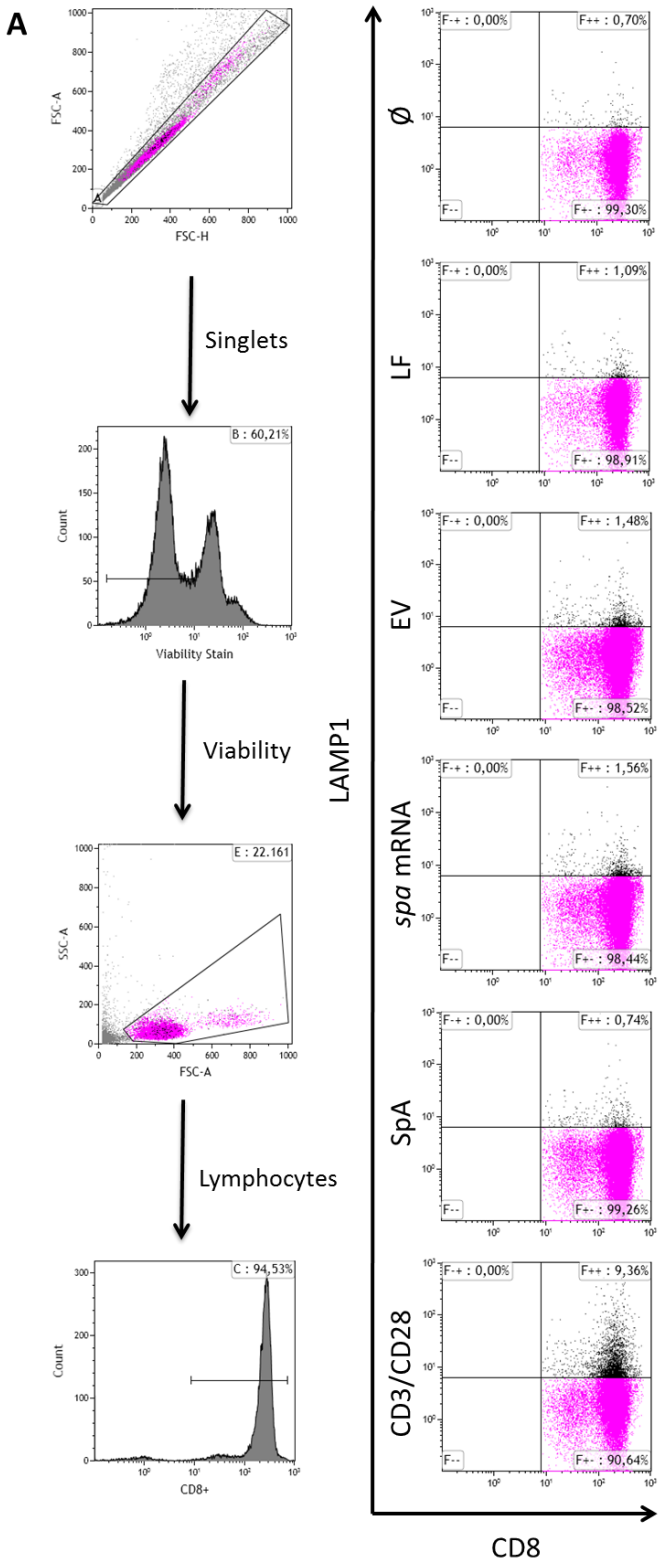


Fig. 4.20: Co-stimulation of mRNA and protein-derived antigen. MoDC were co-cultured with $CD8^+$ T cells and stimulated with either empty vector (EV) mRNA, *mecA* mRNA or 1 µg/ml PBP2a alone, or in combination. ELISpot assays were performed for measurement of IFN-γ after one day incubation. Data is normalized to the unstimulated control and presented as columns of the mean + SEM of n= 8 independent donors. The Wilcoxon matched-pairs signed rank test was performed to evaluate statistical significance.

4.6.3. mRNA-derived antigens activate cytotoxic T lymphocytes

Next, the question was addressed whether besides IFN-γ production mRNA also triggers the degranulation of $CD8^+$ CTL. Therefore, LAMP-1, also called CD107a, as a marker for degranulation was chosen. MoDC:T cell co-cultures were stimulated for one day with either EV mRNA, *spa* mRNA or SpA protein and stained for LAMP-1 for the period of culture. Upon incubation with brefeldin A cells were stained for viability and expression of CD8 and measured by flow cytometry according to the protocol of Betts et al. (115). Stimulation with CD3/CD28 beads served as positive control. Figure 4.21 A shows dot blots of one representative experiment. Data of five responders are presented together in figure 4.21 B. Although not significant, the amount of LAMP-1^+ cells is higher after stimulation with antigen-encoding SAC *spA* mRNA compared to the EV control. In contrast, LAMP-1

expression induced by SpA protein was as low as the unstimulated control. This indicates, that *spa* mRNA, but not SpA protein is inducing CTL degranulation.



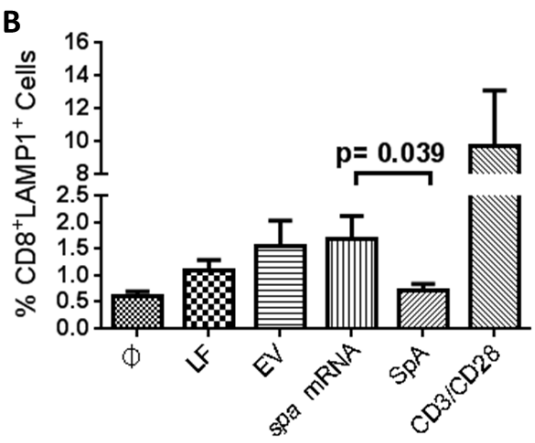


Fig. 4.21: LAMP-1 as an degranulation marker for cytotoxic T cells. MoDC:CD8$^+$ T cells were stimulated with *sac* or EV mRNA, SpA or CD3/CD28 beads and stained for LAMP-1$^+$ cells. **(A)** For the analysis by flow cytometry, cells were gated for singlets, viability and finally CD8$^+$LAMP-1$^+$ expression. Dot blots of one representative donor are shown out of ten independent experiments. **(B)** Percentage of CD8$^+$LAMP-1$^+$ cells measured by flow cytometry of n=5 different donors (all responders). Columns show the mean + SEM. Student's paired t test was used for statistical analysis.

4.6.4. The T cytokine secretion profiles upon stimulation with staphylococcal antigens

For further characterization of the immunogenicity of *S. aureus* antigens and better comparison with T cell co-cultures, a multiplex magnetic bead assay was performed from single culture of stimulated MoDC. High levels of IL-6 and TNF were measured, which are induced by mRNA-encoded antigens, although without significant differences to the EV control (Fig. 4.22). Neither SpA protein nor the control proteins *Influenza* MP1 or tetanus toxoid led to measurable cytokine production. In contrast, IL-6 and TNF were detected after stimulation with PBP2a and SitC (Fig. 4.25). With the same assay, no IL-33 or IL-21 and only very low levels of IL-1β, IL-10 and IL-12p40 were detected. Furthermore, no differences in cytokine production by MoDC between day 1 or 4 of culture were measured (data not shown). ELISA measurement of the same samples revealed high IFN-α secretion upon stimulation with mRNA (Fig. 4.22). Taken together, this data demonstrates profound stimuli-dependent differences in cytokine production by MoDC.

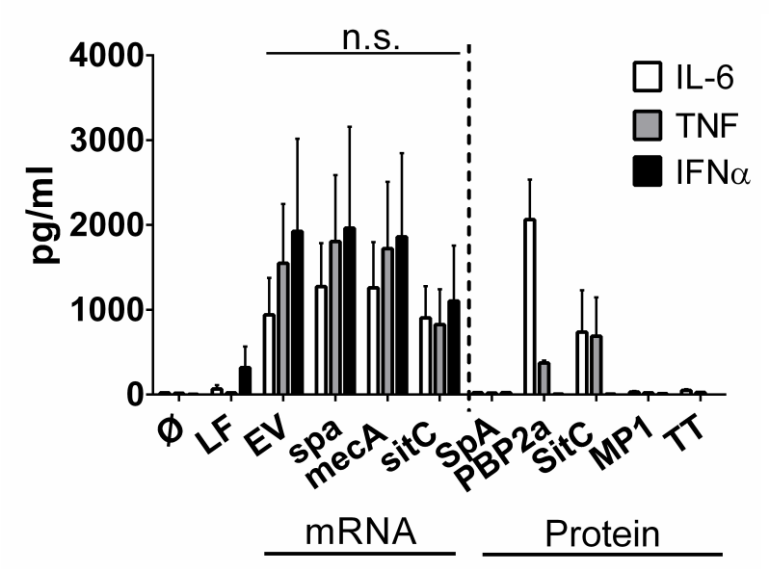


Fig. 4.22: Cytokine production by MoDC. 100.000 MoDC were stimulated with 1 µg/ml SpA, PBP2a, SitC or tetanus toxoid (TT), 1 µl *Influenza* MP1 peptide pool, or transfected with 100 ng *spa*, *mecA*, *sitC* mRNA or lipofectamine control (LF) overnight. Multiplex magnetic beads assay and ELISA were performed and concentrations of measured IL-6, TNF and IFN-α are displayed as columns of the mean + SEM of n= 4 different donors. Statistic was performed using Wilcoxon matched-pairs signed rank test.

Next, T cell cytokine profiles were analyzed using a multiplex magnetic beads performed with supernatants from MoDC:$CD4^+$ and MoDC:$CD8^+$ co-cultures of responders after five days (Fig. 4.23 and 4.24).

In general, the cytokine profile of mRNA-derived antigens was relatively similar, whereas SpA was by far the most immunogenic protein stimulus used. While PBP2a results seemed to be more consistent leading to moderate cytokine production, SitC induced only very low cytokine responses.

Interestingly, the control proteins MP1 and tetanus toxoid caused only low cytokine induction in T cells and the cytokine profiles did not differ strongly between $CD4^+$ and $CD8^+$ T. On the one hand to SpA protein, *spa* mRNA elicited elevated IFN-γ, TNF and IL-1β levels in both T cell subsets. Contrarily, SpA protein induced stronger IL-5 and IL-13 levels, regardless of the T cell subset. Nevertheless, overall, the concentration of cytokines induced by SpA protein was much lower than that induced by *spa* mRNA. Interestingly, the levels of IL-4 remained weak for all antigens. Furthermore, the amount of secreted IL-17A was high in all conditions, but remained rather unspecific due to the high background values (Fig. 4.23).

Outstanding for SpA, high levels of IL-12 were detected. Besides, the cytokine pattern of TNF, as well as IL-6 is similar to the one observed with MoDC alone (Fig. 4.22), only the total amount of cytokine is up to four times higher for TNF and up to ten times higher for IL-6 with T cells in co-culture. Most interestingly, PBP2a led to a strong induction of G-CSF and IL-10, and in case of SpA additionally of IL-2, whereas mRNA-derived antigens elicited these cytokines only slightly above the background level (Fig. 4.24). Furthermore, very high levels of chemokines were measured. Whereas induction of CCL2 was similar in all stimuli, levels of CCL4 were higher after mRNA transfection (Fig. 4.24).

Based on the same assay, high levels of IL-7, IL-8, GM-CSF were detected without strong differences between the different stimuli (data not shown). Taken together, this data demonstrates profound differences in the T cell cytokine patterns by mRNA and protein stimulation.

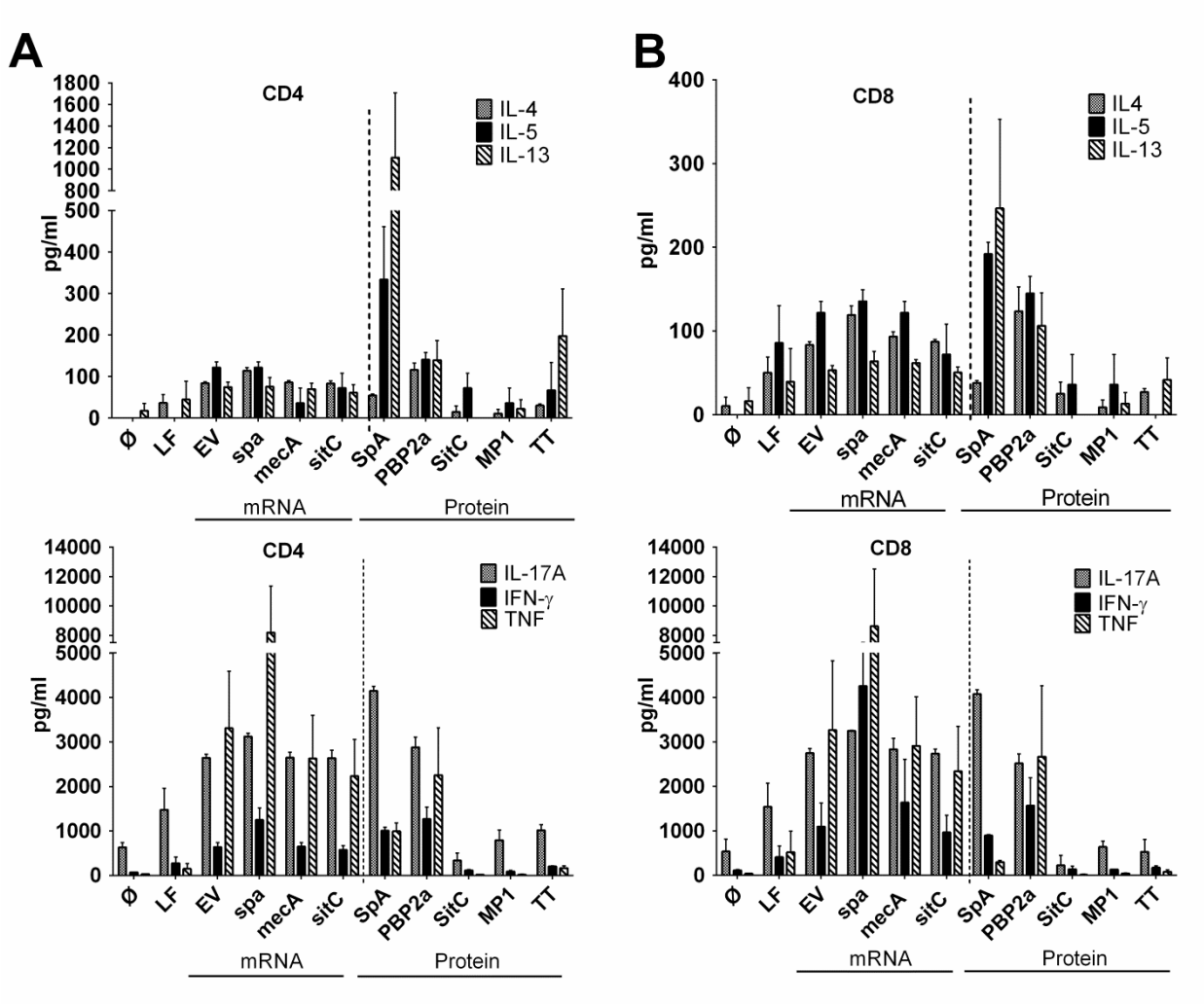


Fig. 4.23: Cytokine secretion by mRNA and protein-stimulated MoDC:T cell co-cultures. Supernatants after 5 days of culture were used to perform a multiplex magnetic beads assay for the analysis of the indicated cytokines. **(A)** MoDC:$CD4^+$ and **(B)** MoDC:$CD8^+$ co-cultures, stimulated with 100 ng mRNA (EV, *spa*, *mecA*, *sitC*), 1 µg/ml protein (SpA, PBP2a, SitC, TT) or 1 µl *Influenza* MP1. Columns represent the mean + SEM of n=3 different donors (responders).

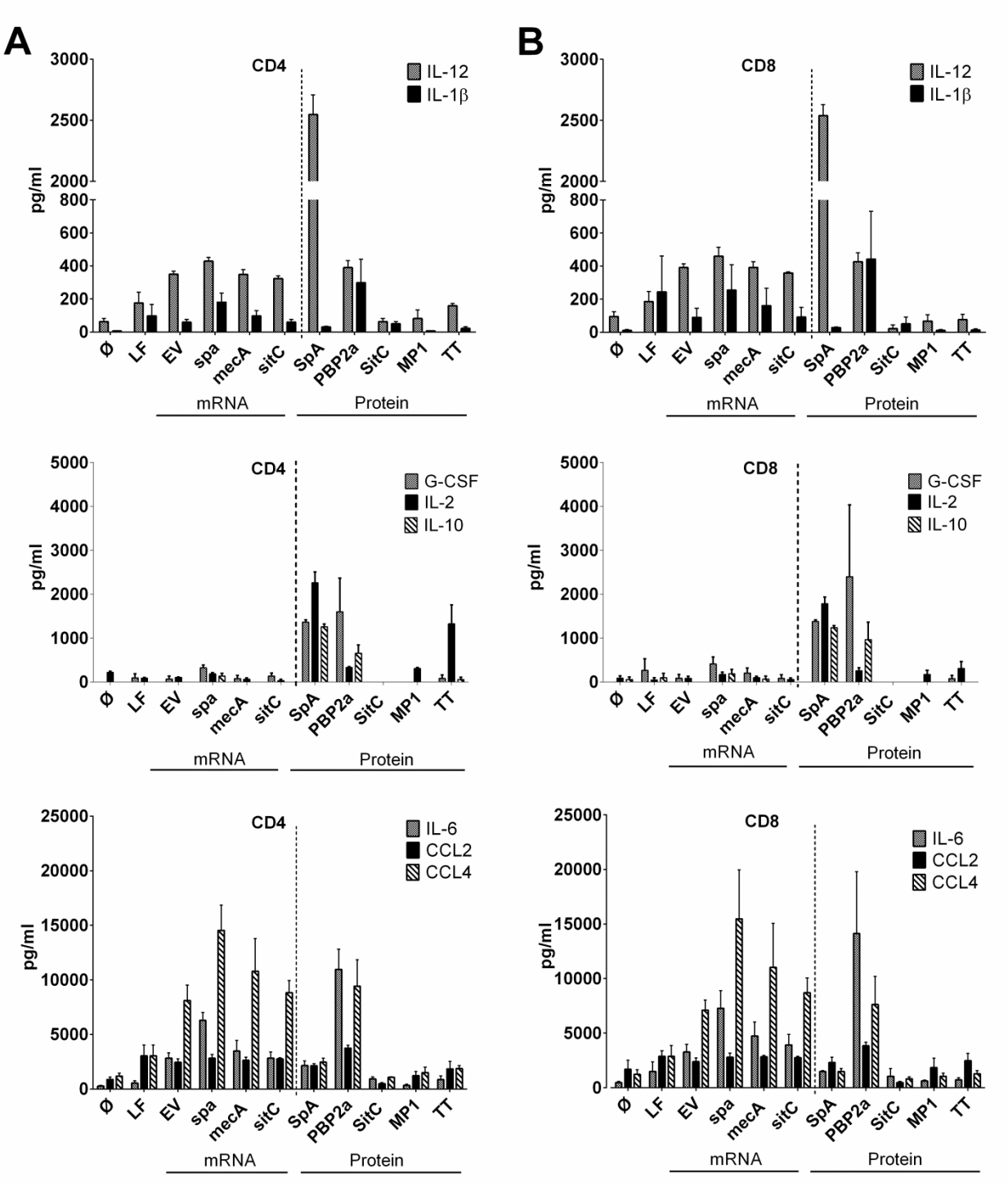


Fig. 4.24: Cytokine secretion by mRNA and protein-stimulated MoDC:T cell co-cultures. Supernatants after 5 days of culture were used to perform a multiplex magnetic beads assay for the analysis of the indicated cytokines. **(A)** MoDC:$CD4^+$ and **(B)** MoDC:$CD8^+$ co-cultures, stimulated with 100 ng mRNA (EV, *spa*, *mecA*, *sitC*), 1 µg/ml protein (SpA, PBP2a, SitC, TT) or 1 µl *Influenza* MP1. Columns represent the mean + SEM of n=3 different donors (responders).

To address the question whether SpA protein induces IL-2, IL-10 and G-CSF in order to suppress pro-inflammatory immune response by IFN-γ, blocking assays were performed. Therefore, cells were stimulated either with SpA protein and G-CSF, IL-10 and/or IL-2 were blocked by antibodies, or those cytokines were added to *spa* mRNA transfected MoDC. Although this was not statistically significant, blocking IL-2, IL-10 and G-CSF alone or in combination resulted in slightly increased IFN-γ production during stimulation with SpA protein compared to the isotype control (Fig. 4.25 A). In contrast, reduction of IFN-γ was only significant when G-CSF was added to *spa* mRNA transfected cells in presence of $CD8^+$ T cells (Fig. 4.25 B). Adding IL-2 resulted in T cell activation and higher IFN-γ secretion. Additionally, supplementing the medium with IL-10 only had a weak impact on INF-γ decrease. Taken together, those results reveal that indeed IFN-γ is indeed, but not exclusively regulated by IL-10 and G-CSF.

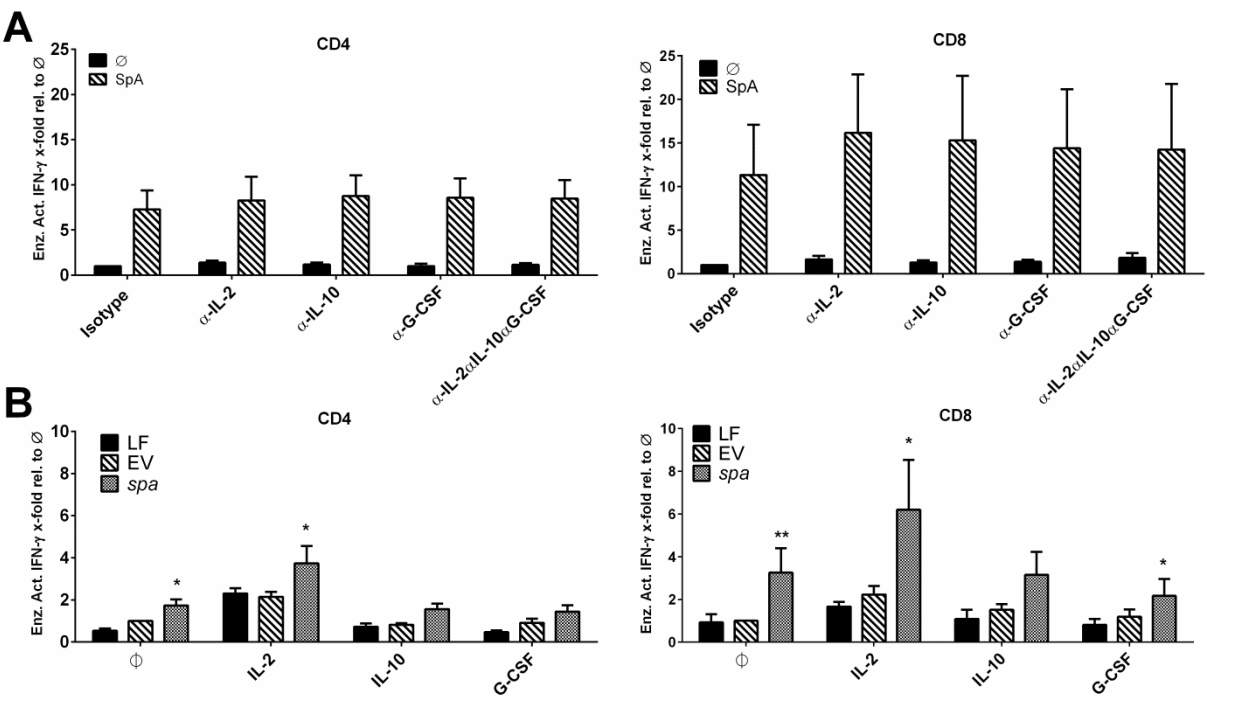


Fig. 4.25: Regulation of IFN-γ by IL-2, IL-10 and G-CSF. MoDC:$CD4^+$ (left panel) or $CD8^+$ (right panel) were **(A)** stimulated with 1 µg/ml SpA and cytokines were blocked by α-IL2, α-IL10 or α-G-CSF antibodies alone or in combination. As control served the isotype Goat IgG. **(B)** Cell cultures were transfected with *spa* mRNA and supplemented with IL-2, IL-10 or G-CSF. Enzymatic activity of IFN-γ was measured after overnight incubation by ELISpot. Results are displayed as the mean + SEM of n= 4 (blocking) or n=8 (adding) different donors and statistical analysis was performed using the Wilcoxon matched-pairs signed rank test. p-values are for $CD4^+$ cells: Ø EV:*spa*= 0.0156, IL-2 *spa*: Ø *spa*: 0.0156; for $CD8^+$ cells: Ø EV:*spa*= 0.0078, IL-2 *spa*: Ø *spa*: 0.015, G-CSF *spa*: Ø *spa*: 0.0156.

4.6.5. Endotoxin contamination in recombinant SpA proteins influences the IFN-γ response

Since SpA of different *spa* types is not commercially available, Protein A of *spa* type t076, t008, t002 and t003 was purified from the cytoplasm of *E. coli*. To compare the different protein's immunogenicity and *spa* type-dependent activation of T cells to mRNA, 1 µg/ml of each protein was used in ELISpot assays. In contrast to previously made results, all SpA proteins elicit strong IFN-γ production, especially in $CD8^+$ T cells (Fig. 4. 26). To exclude a possible contamination of those samples with endotoxins as LPS, endotoxin concentration was determined by Endotoxin-Portable test system (PTS). Thus, the recombinant SpA t076 tested here as representative sample, revealed high concentrations of 234 IU/ml endotoxin in a 1 µg/ml protein working solution. This corresponds to approximately 23.4 ng/ml endotoxin. In contrast, recombinant PBP2a commercially purchased contained only 0.121 IU/ml. As expected, in proteins purified from *S. aureus* no endotoxin was detected (Tab. 4.1).

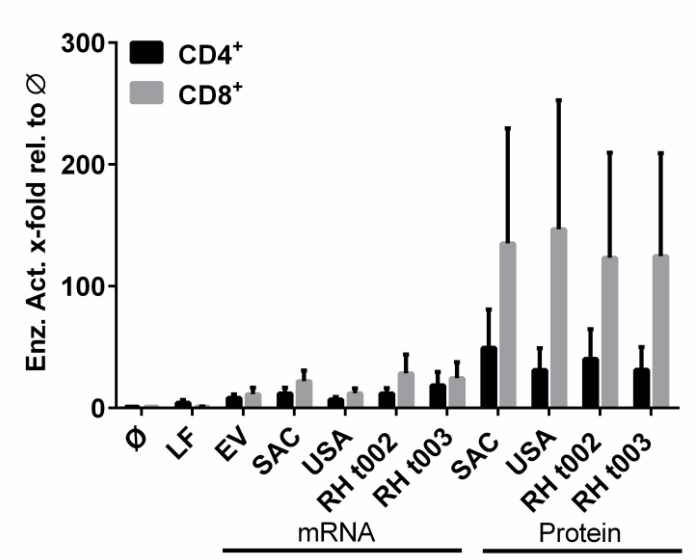


Fig. 4.26: IFN-γ secretion induced by SpA proteins. ELISpot assays demonstrated stronger IFN-γ production by recombinant SpA proteins compared to mRNA of different *spa* types by $CD4^+$ and $CD8^+$ T cells. Results are displayed as columns of the mean + SEM of n= 5 different donors (all responders).

Tab. 4.1: Endotoxin measurement of various *S. aureus* protein batches tested by Endotoxin-PTS.

1 µg/ml tested Protein	Source	Purified from	Endotoxin [IU/ml]
SpA	GE Healthcare	SAC	<0.005
SpA	Lab	*E. coli*	234
PBP2a	Antibodies-online	*E. coli*	0.121
SitC	Lab	SA113	0.006

To analyze the effect of endotoxin on IFN-γ production, $CD4^+$ T cells were either stimulated alone with the detected level of LPS (20 ng/ml) or in co-incubation with SpA. As displayed in

figure 4.27, incubation with LPS induced high levels of IFN-γ. Additional co-incubation with SpA purified from *S. aureus* did not lead to a significantly higher cytokine profile but was still about one third stronger than with LPS alone. Since it could not be distinguished between a synergistical stimulation and a SpA-mediated effect, not commercially produced recombinant SpA proteins were not used for further experiments.

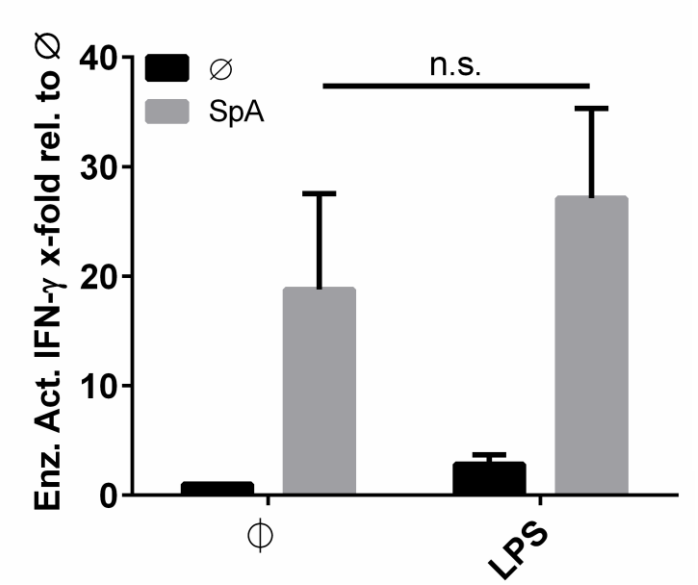


Fig. 4.27: LPS-induced IFN-γ production. In co-culture with MoDC, $CD4^+$ T cells reveal IFN-γ production upon stimulation with LPS derived from *E. coli* and higher cytokine production in co-stimulation with SpA. Columns represent the mean + SEM of n=4 different donors. IFN-γ was determined by ELISpot assays and the data was normalized to the unstimulated control. Statistics were calculated with the Wilcoxon matched-pairs signed rank test.

4.7. Effects of mutation and truncation of protein A on the T cell response

Very little is known about the staphylococcal epitopes that activate antigen-specific T cells and most hypotheses are based on *in silico* predictions (124, 125). Therefore, the third part of this thesis aimed at the characterization of possible SpA epitopes through generation of SpA variants and analysis of their effect on the human T cell response.

4.7.1. SpA Ig-binding mutant induces high IFN-γ and IL-10 production

In a promising approach published by the Schneewind lab in 2010, SpA lacking the ability to bind to Fc or Fab parts of immunoglobulins due to inserted point mutations at all Ig binding sites, was successfully used for immunization of mice (87, 88). Based on this work, our lab generated such an Ig-binding mutant by PCR-based mutagenesis (see Bachelor Thesis of Christoph Stein, 2014 and Fig. 4.28). To further investigate the effect of those mutations, full-length *spa* Ig-mutant mRNA was generated to analyze T cell responses with the established protocol.

Transfection of HEK 293 cells with SAC *spa* wildtype and Ig-mutant mRNA (based on the SAC t076 *spa* type) confirmed successful translation of *spa* and mutation of Ig-binding sites. Western Blot against human IVIg demonstrated that immunoglobulins are bound by all four *spa* types and not by the Ig-mutant (Fig. 4.28).

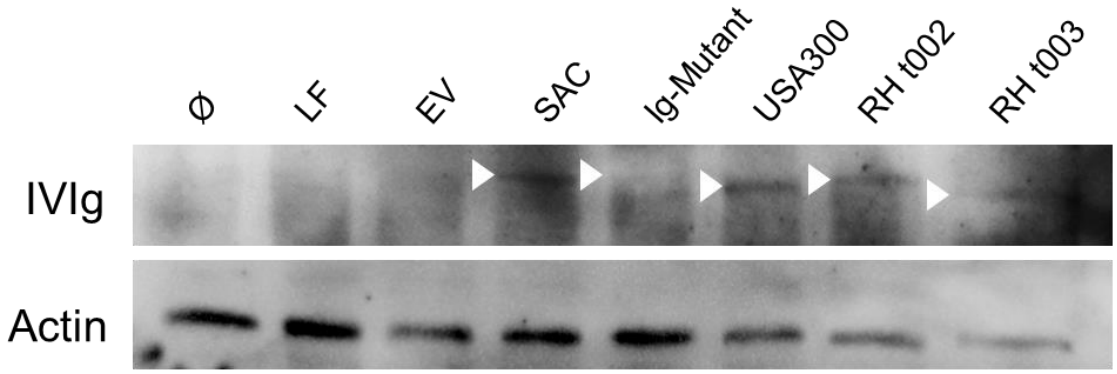


Fig. 4.28: Mutation of Ig-binding sites of SpA. Lysates of HEK293 cells, which were transfected with various *spa* mRNAs and the empty vector (EV) control were used for Western Blot analysis against IVIg. It demonstrated the binding of human immunoglobulins to all four *spa* types, but not the Ig-mutant, LF control and the EV. The housekeeping protein actin served as loading control.

Next, MoDC were transfected with mRNA of the Ig-binding mutant and SAC wildtype *spa* mRNA for comparison, following the established protocol. Interestingly, in co-culture with $CD8^+$ cells, mutation of Ig-binding sites led to significantly more IFN-γ than the wildtype *spa* mRNA. Furthermore, it induced high levels of IL-10, whereas the wildtype *spa* mRNA did not produce IL-10 above the background level (4.29 and also 4.31). This indicates that the Ig-binding sites indeed influence T cell activation.

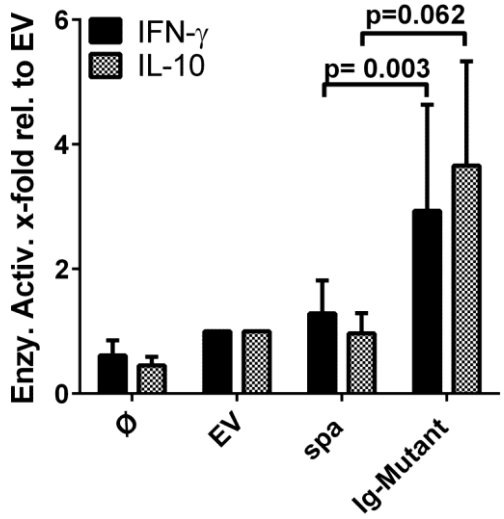


Fig. 4.29: Influence of Ig-binding site mutation of *spa* mRNA on T cell responses. ELISpot of $CD8^+$ or T cell MoDC co-cultures transfected with 100 ng empty vector (EV), *spa* or Ig-Mutant mRNA were incubated overnight and IFN-γ and IL-10 were measured. The data is presented relative to the EV control as columns of the mean + SEM of n= 10 (for IFN-γ) and n=5 (for IL-10) different donors. Statistical significance was calculated with the Wilcoxon matched-pairs signed rank test.

4.7.2. Truncation of SpA increases pro-inflammatory T cell responses

Based on the observation that different *spa* types induce different immune responses, the question, whether the homologous domain is crucial for T cell activation, was addressed. Therefore, truncated *spa* mRNA was generated by insertion of suitable start and stop codons, lacking either the homologous domains or the variable region (Fig. 4.30).

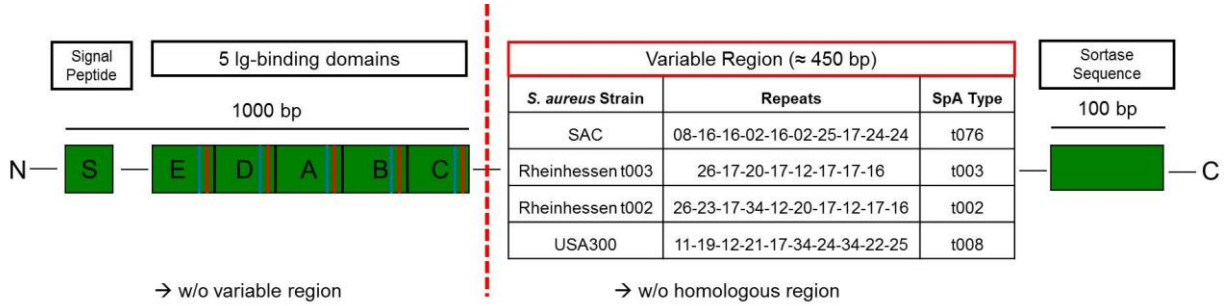
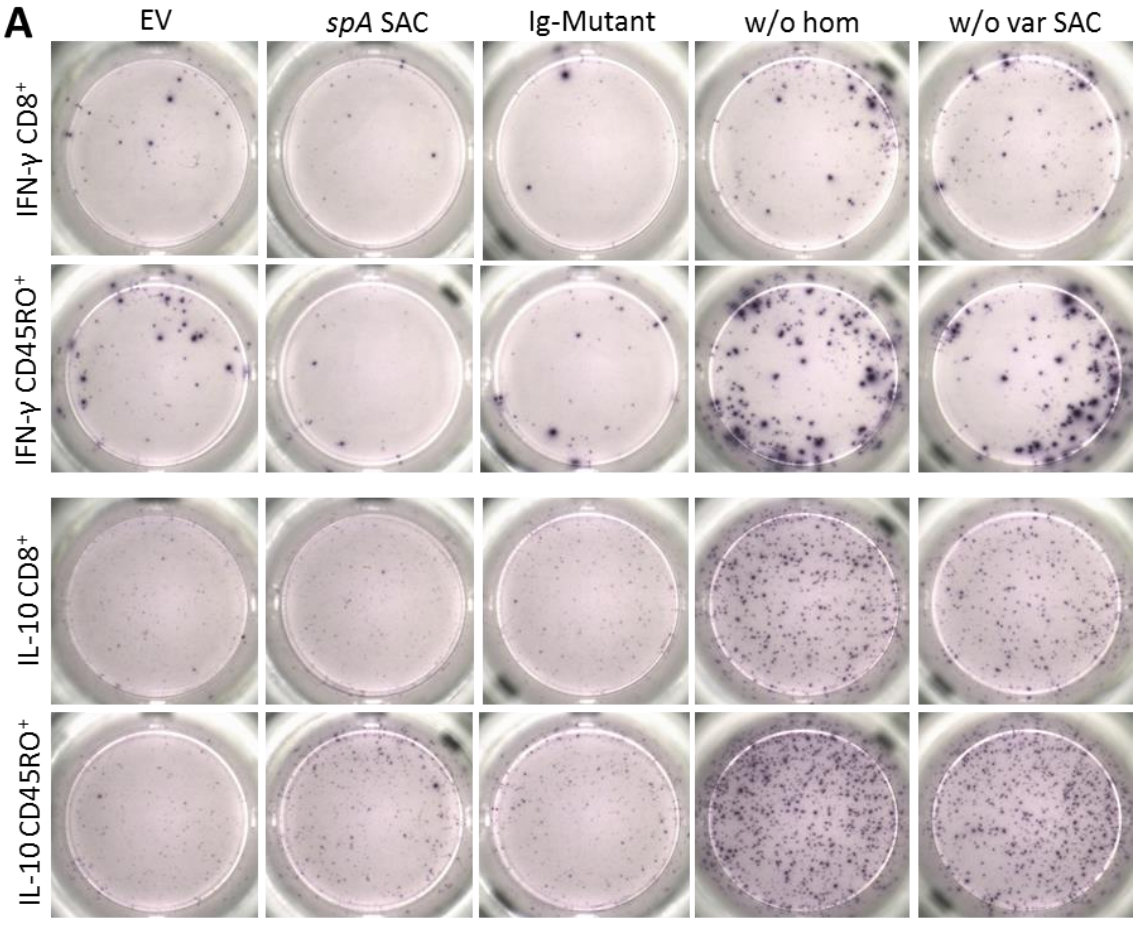


Fig. 4.30: Scheme of generation of truncated *spa* mRNA. Scheme of the *spa* gene shows separation at the red dotted line to create mRNA lacking either homologous or variable region. The solid lines in the Ig-binding domains indicated the point mutation of the Ig-binding sites (blue Fc-biding site, red Fab binding site).

Finally, it was investigated whether truncation of SpA influences T cell responses. Fig. 4.31 A shows IFN-γ and IL-10 ELISpot wells of $CD8^+$ and $CD45RO^+$ cells of one representative experiment after stimulation with truncated *spa* mRNA. Indeed, induction of both cytokines was strongly increased compared to the wildtype *spa* and the Ig-binding mutant mRNA. Whereas IFN-γ secretion by a few cells is intense (indicated by dark color and big size of the spots), IL-10 is secreted by about three times more cells, but in lower concentration (faint color and small spots). This strong cytokine induction of both cytokines by truncation of the variable region was confirmed by ELISpot assays of several donors (Fig. 4.31 B). However, only cutting off the Ig-binding domains of the SAC *spa* type resulted in even higher, although not significant, secretion of IL-10 and a slightly decreased level of IFN-γ compared to SAC without variable domain. With other *spa* types, except RH t002 that induced low amounts of IL-10, no differences were observed compared to the wildtype mRNA. In summary, the data presumes that both SpA domains harbor epitopes crucial for T cell activation and that T cell immunogenicity is increased by sequence mutation.



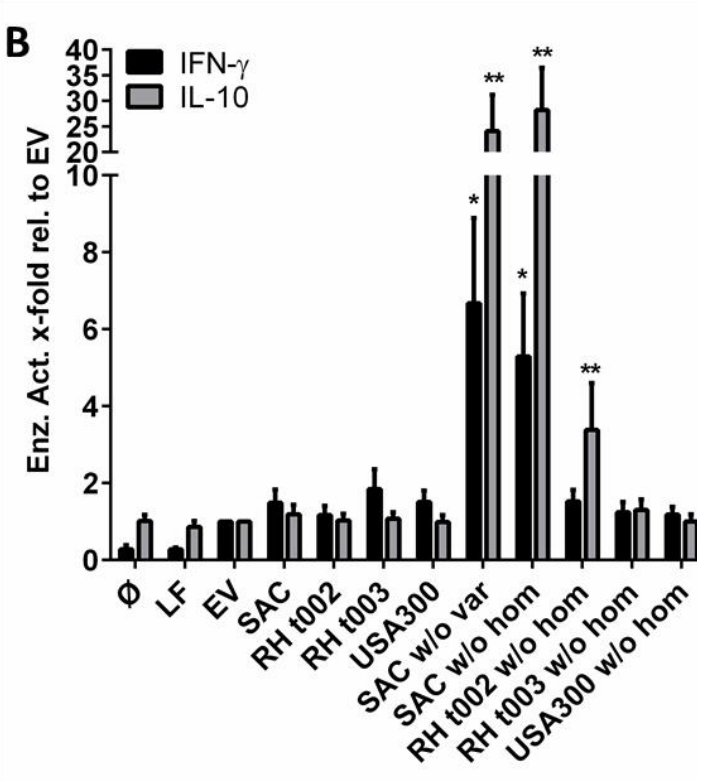


Fig. 4.31: IFN-γ and IL-10 induction by truncated *spa* mRNA. (A) IFN-γ and IL-10 ELISpot wells of one representative donor after stimulation of $CD8^+$ and $CD45RO^+$ cells in MoDC co-culture, transfected with empty vector (EV), SAC *spa*, w/o homologous domain or w/o variable domain mRNA overnight. **(B)** IFN-γ and IL-10 ELISpot of MoDC:$CD8^+$ cell co-culture stimulated with full-length and truncated *spa* mRNA of all *spa* types (SAC, RH t002, RH t003, USA300). As a control served stimulation with lipofectamine alone (LF) and empty vector (EV) mRNA. The data is presented as columns of the mean + SEM of n=10 different donors and put in relation to the EV control. Statistical analysis was performed with the Wilcoxon matched-pairs signed rank test. p-values are for IFN-γ: SAC w/o var: EV= 0.0137, SAC w/o hom: EV= 0.019; for IL-10: SAC w/o var: EV= 0.002, SAC w/o hom: EV= 0.002 and RH t002 w/o hom: EV: 0.001.

To answer the question whether IFN-γ and IL-10, induced by stimulation with truncated *spa* mRNA, derive from the same T cell subset, intracellular FACS staining was performed. Co-cultures of MoDC and $CD8^+$ T cells were stimulated as before and incubated for one or three days, respectively (Fig. 4.32). However, within this representative donor IFN-γ production did not exceed the EV mRNA induced background on day 1. Secretion of IL-10 secretion was induced after stimulation with mRNA lacking the homologous domain. Two days later, IFN-γ secretion increased with the truncated mRNA stimuli, regardless of the higher background, whereas IL-10 could not be detected anymore. However, those assays revealed, that indeed on day 1 both cytokines are secreted by different cell subsets.

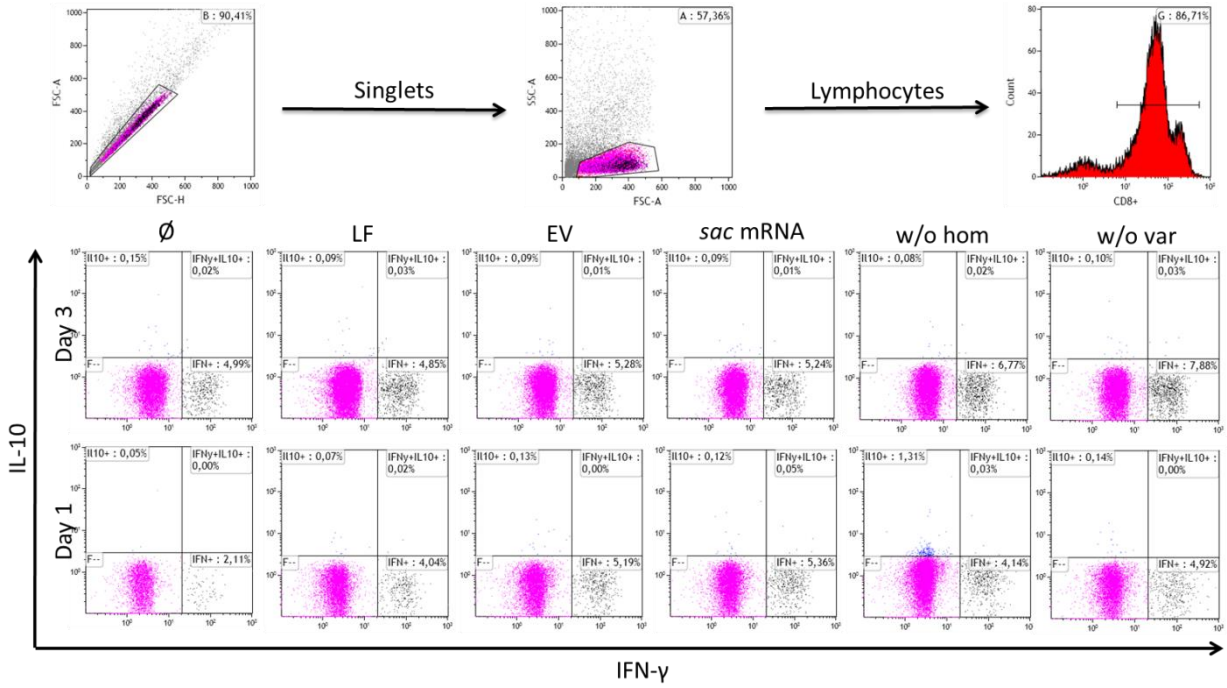


Fig. 4.32: IFN-γ and IL-10 secretion derives by different $CD8^+$ T cell subsets after stimulation with truncated *spa* mRNA. MoDC:$CD8^+$ T co-cultures were stimulated with lipofectamine (LF), empty vector (EV), wildtype SAC *spa*, SAC *spa* without homologous domain (w/o hom) or SAC *spa* without variable domain (w/o var) mRNA for one or three days. After re-stimulation with PMA+ ionomycin and incubation with brefeldin A, cells were harvested and stained. For the analysis by flow cytometry, single, $CD8^+$ cells are gated for IFN-γ^+IL-10^+ expression. Dot blots show one representative donor out of four independent experiments.

5. Discussion

The experimental results of this thesis demonstrate the presence of T cells specific for *S. aureus* antigens within the peripheral blood. They further elucidate that human T cell responses to protein antigens are skewed towards T_h2 and T_{reg} -associated cytokines. This can be overcome by delivery of antigens via ivt mRNA. Donor-specific differences in the responses to variable proteins or the corresponding mRNA sequences indicate that recognition of staphylococcal antigens might be fine-tuned by the recognition of variable epitopes.

5.1. Use of ivt mRNA for delivery of staphylococcal antigens

The successful establishment of a protocol for transfection of ivt mRNA was confirmed by luciferase activity in HEK293 and MoDC cell lysates. No luciferase protein activity was detected in the supernatants of HEK293 or MoDC (Fig. 4.6 and 4.8), although this was suggested as a consequence of secretion of the protein (126, 127). The transfection efficiency of MoDC is donor-dependent and was evaluated by GFP expression (Fig. 4.7). To draw conclusions from the amount of transfected mRNA to the amount of translated protein is difficult. Nevertheless, MoDC are known for their high translation efficiency (128, 129) and it has been reported that already very low amounts of 5 ng mRNA result in protein translation (93).

Stimulation of MoDC: T cell co-cultures with peptide pools and IFN-γ measurement was utilized to confirm the structural integrity of MoDC. Besides, strong IFN-γ production was induced by incubation with peptide pools from matrix protein 1 (MP1) of *Influenza* subtype H1N1, as also published elsewhere (130). Albeit weaker, the peptide pools from *Aspergillus fumigatus* catalase 1 (cfr1) and *Candida albicans* mannose protein 65 (MP65) were also effective T cell stimuli as was also shown in other studies (131, 132). Similar to *S. aureus*, these fungi are either successful commensals of the human flora or we encounter them on a daily basis (133, 134). Thus, IFN-γ secretion upon stimulation with Crf1 and MP65 was comparable to staphylococcal antigens. However, stimulation with MP1 from *Influenza* that is infectious in humans was as strong as with mRNA-derived staphylococcal antigens (Fig. 4.18).

5.2. Comparative analysis of T cell responses towards protein and mRNA-delivered staphylococcal antigens

Since IFN-γ is the main hallmark of T cell activation (7), IFN-γ ELISpot was chosen as a highly sensitive method for the analysis of T cell responses by the established mRNA-based approach. ELISpot assays revealed the activation of antigen-specific $CD8^+$ and $CD45RO^+$ T cells by mRNA-encoded *spa* (Fig. 4.11- 4.13). Dark, big spots are mainly a sign for the activation of memory T cells because they secret high amounts of IFN-γ (17), whereas small, faint spots rather indicate the activation of primary T cells.

However, *S. aureus* is known to survive, persist and replicate in the cytosol or phagosome of professional and non-professional phagocytes as epithelial and endothelial cells, fibroblasts, osteoblasts and keratinocytes (68, 135, 136). Therefore, the activation of $CD8^+$ T cells by mRNA-based stimulation is plausible. Interestingly, also high numbers $CD45RO^+$ T cells were activated which has been postulated before (137, 138). Since the memory of the immune system of every individual is unique, differences in immune activation are immense. This explains particularly alterations in memory T cell activation as well as the appearance of non-responders and responders, defined by higher IFN-γ secretion by any antigen-encoding mRNA compared to the empty vector background control (Fig. 4.13).

Only weak activation of $CD4^+$ cells was observed upon *spa* mRNA transfection. This is a well-known phenomenon in mRNA-based T cell stimulation because the translated antigen is located in the cytosol and therefore mainly presented via MHC class I that preferentially activate $CD8^+$ T cells (102). Although, some publications suggest that the translated antigen is secreted by DC and then taken up by others to be presented via MHC class II (126). There are several approaches how to trigger a $CD4^+$- biased response by mRNA. One is the blockage of the invariant chain of MHC class I molecules in the endosome or Golgi complex to facilitate the loading of MHC class II (139). Another way is the adding of a mannose tag or a leader sequence and a sorting signal to mediate the delivery to the endosomal or lysosomal compartment (140, 141).

Two other antigens were selected to compare the immunogenicity among staphylococcal proteins. Additional to *spa*, a highly variable core gene, *mecA* and *sitC* were chosen.

The core gene for the lipoprotein *sitC* is part of the *sitABC* operon that belongs to a family of ABC transporters and is broadly expressed in *S. aureus* (142). Staphylococcal lipoproteins (lpp) present approximately 2% of the staphylococcal proteome and are regulators of the iron transport through the cytoplasmic membrane (143, 144). These lipoproteins undergo several post-translational modification steps. One is the transfer of a diacylglycerol group by the lipoprotein diacylglyceryl transferase (lgt, 145). Thereby, they are major ligands for TLR2, one of the innate immune receptors that is predominantly expressed on the cell membrane of monocytes, macrophages, DC and lymphocytes (146, 147). TLR2 recognizes di- and triacetylated lipoproteins forming heterodimers on the activation with TLR6 and TLR1, respectively (146). For instance, SitC the most prominent lpp was shown to co-localize with TLR2 and to trigger intracellular TLR2 accumulation in keratinocytes (148). However, this bacteria-dependent post-translational modification is missing when transfecting MoDC with *sitC* mRNA.

The accessory gene *mecA* is located on the staphylococcal cassette chromosome *mec* element and mediates methicillin resistance (149, 150). The arrangement and composition of the *mec* element can vary, but *mecA* itself is highly conserved (151). *mecA* encodes the alternative penicillin-binding protein 2a (PBP2a or PBP'), an enzyme that is anchored on the cytoplasmic membrane and involved in biosynthesis of the cell wall by cross-linking of the glycan chains in peptidoglycan (151). PBP2a is less susceptible to modifications by β-lactam antibiotics and capable of taking over the function of other PBP (37). It therefore mediates resistances and the survival of the bacterium.

SpA is one of the major virulence factors of *S. aureus* and therefore more immunogenic than tested *sitC* and *mecA* mRNA that elicited lower IFN-γ responses by T cell (Fig. 4.18). Besides, SpA is more easily accessible to the human immune system because it is cell wall anchored and most likely actively released by the bacteria in a growth-dependent manner. This enables *S. aureus* to trigger B cell apoptosis without activating the NOD2 pathway by peptidoglycan residues linked to SpA (79, 80).

When an antigen is encountered for the first time, the frequency of T cells specific for this antigen is very low, ranging from 0.01% to less than 0.001% (152). In our study, the number of antigen-specific T cells stimulated by *spa* mRNA was much higher, ranging from 0.032% to 0.06% in $CD8^+$ T cells (Fig. 4.19). This strongly indicates the activation of memory T cells and a recall immune response since IFN-γ could be measured already after overnight stimulation. Interestingly, the amount of cells activated by *Influenza* MP1 was similar and fit to the data published elsewhere (153). Work by the group of Barbara Bröker also revealed unexpected high amounts of staphylococcal-specific peripheral T cells, ranging from 0.2 to 5,7% (154). However, this percentage is based on calculations considering reaction of 80 immunogenic extracellular *S. aureus* proteins in total. Looking at the actual number of proliferating T cells, it ranged between 0.00001% and 0.001% dependent on the antigen. This is consistent with our data obtained with stimulation of SitC (Fig. 4.19). Supporting the hypothesis that SpA is more immunogenic because it is (among other things) easy accessible to the immune system, the Bröker group also obtained higher responses to extracellular antigens (154). Overall, we could show that stimulation by mRNA-encoded antigens activated significantly higher numbers of specific T cells than stimulation with protein.

The data of the work by Barbara Bröker was based on proliferation assays (154). Although in our experiments we could activate $CD45RO^+$ memory T cells that are known for fast proliferation upon re-stimulation, proliferation assays did not work in our hands (Fig. 4.14), disregarding co-culture with essential IL-2 and IL-7 (data not shown). However, one subset of T cells -effector memory T cells- is described to secret effector cytokines as IFN-γ, but lacks the ability for rapid proliferation. They enable a very fast reaction upon antigen stimulation to a repeated exposure to a microbe but complete eradication may require large numbers of those cells (4, 17). An activation of those $CD45RO^+$ effector memory T cells might explain the failure of proliferation assays.

Furthermore, endotoxin contaminations in not commercially produced recombinant proteins were detected (Tab. 4.1) that resulted in synergistically TLR4-mediated T cell stimulation together with SpA. It has been reported that T cells increase IFN-γ production and TLR4 expression upon LPS stimulation (155, 156) leading to a positive feedback loop of stimulation. Hence, the activation of T cells by mRNA for quantification of antigen-specific

T cells allows purer and more transparent analysis than proliferation assays and stimulation with recombinant proteins.

In order to compare the immunogenicity of the same antigens derived by proteins, the immunogenicity of SpA, PBP2a and SitC on MoDC alone and with T cells in co-culture was analyzed. IL-6 and TNF were measured as the main MoDC activation markers (123). SitC is known to induce IL-6 and TNF in murine macrophages and human Mono Mac 6 cell lines by TLR2 activation due to its post-translational modifications by the enzyme lgt (123, 142, 148). In contrast to these studies, IL-6 decreased with increasing protein concentration in our experiments. Interestingly, we saw that PBP2a behaved the opposite way. However, nothing is known about PBP2a's immunogenicity on human immune. Nevertheless, we showed that it induced also high amounts of IL-6 and TNF in MoDC stimulated alone (Fig. 4.22). Although the low contamination by endotoxins (Tab. 4.1) did not influence IFN-γ production it cannot be excluded that weak stimulation via TLR4 led to cytokine production by MoDC as was published before (157). Furthermore, nothing is known about post-translational modification of SpA. In this context, the weak induction of IL-6 and TNF is conclusive. The response against mRNA-encoded antigens (and therefore not post-translational modified) in MoDC alone was high for both cytokines but without antigen-dependent differences. This strongly indicates the activation of antigen-specific T cells since differences in cytokine production could only be seen in the presence of those cells. Potentially, mRNA is inducing cytokine production in MoDC by triggering endosomal TLR7 and cytosolic RIG-I (158, 159) since MoDC generated with GM-CSF harbor higher levels of TLR7 and TLR4 enabling stronger stimulation (160).

Analysis of supernatants after 5 days of MoDC: T cell co-cultures revealed a broad spectrum of secreted cytokines. In comparison to staphylococcal antigens, the T cell response towards the control proteins MP1 and TT was weak. Since most individuals are vaccinated, TT induces a recall and a T_h2-biased immune response, indicated by secretion of IL-2, IL-4, IL-5 and IL-13 (161). It was further reported, that immune responses against *Influenza* are defined by IL-6, IL-1β, TNF along with high IFN-α and monocyte-attracting chemokines production (162). Since stimulation was performed with peptide pools and not with the virus itself, this was not confirmed in our study (Fig. 4.23-24).

The immunogenicity among the protein-derived antigens is similar to the mRNA-derived antigens. SpA protein is the most immunogenic, followed by PBP2a whereas SitC elicits only low cytokine responses (Fig. 4.23-24). In comparison with the corresponding proteins, mRNA-encoded antigens induced much higher IFN-γ and TNF responses. Those are hallmark cytokines of T_h1-biased immune response characteristic for defense against intracellular pathogens as viruses or bacteria (6). In contrast, especially SpA protein induces IL-13 and IL-5 which are characteristic for a T_h2-biased immune response, typical for extracellular pathogens as helminths (6). Nevertheless, regarding the total amount of cytokines, this T_h2-biased immune response is rather weak. In disagreement with the fact that IL-4 is also a hallmark cytokine for T_h2 immune responses (6, 163), we could only detect very low levels. Otherwise, it is known that high IFN-γ levels in turn suppress IL-4 and T_h2 immune responses (6, 9). Besides, there are others who also reported only low amounts of IL-4 induced by staphylococcal antigens (154).

The cytokine IL-12 usually enhances T_h1 responses by increasing IFN-γ, TNF and $CD8^+$ cytotoxicity (164, 165). In contrast to that, SpA induced high levels of IL-12, but only low amounts of IFN-γ and TNF.

Moreover, the pattern of IL-6 and TNF in MoDC single cultures was confirmed and even amplified in co-cultures with T cells, indicating that cytokine secretion is increased by co-stimulation with lymphocytes. IL-6 induces T cell proliferation and differentiation of CTL, which is supported by the high IL-6 levels induced by mRNA in contrast to SpA protein (166).

Most interestingly, high concentrations of IL-17a were detected. Even though *S. aureus* is known to induce a T_h17 biased immune response (167, 168), those levels seemed unspecific due to the high background. Furthermore, some publications presume that T_h17-response is induced by DC-derived cytokines as IL-6, IL-1β, TGF-β and IL-23 (123, 166) but no IL-1β nor IL-23 was measured in MoDC single cultures in our experiments (data not shown). In a different study did not observe changes in IL-1ß or IL-23 production with increasing T_h17 response (168), indicating that this process is more complex. Besides, new studies described trans-differentiation of $CD4^+$ T_h17 cells into T_{reg} during immune response in a mouse model (169). This suggests a new possibility for *S. aureus* to manipulate and down-regulate the human immune response.

There is evidence, that *S. aureus* induces T_{reg} responses in mice and human that are an important feature to maintain the homostasis of the immune system (170, 171). Furthermore, SpA protein and to a weaker extent also PBP2a induced the secretion of IL-2, IL-10 and G-CSF whereas none was detected with mRNA. All three cytokines are directly or indirectly involved in T_{reg} differentiation. G-CSF is capable of inducing IL-10 by DC that implicates the differentiation of tolerogenic DC and T_{regs} in mice and human and to drive cytokine secretion towards a T_h2 phenotype (172, 173). Together with high dose IL-2, G-CSF mobilized human T_{regs} can be expanded up to 40.000-fold with artificial APC *in vitro* (174). It was reported, that IL-2 in turn activates T_{regs}, and induces IL-10 production *in vivo* and *in vitro* (24, 175). IL-10 is long known for its immunosuppressive function and its role in inducing and maintaining T_{regs} after stimulation with various pathogens (25, 176, 177). Moreover, it has been shown that T_{reg}-derived IL-10 signaling is required for the suppression of T_h17-mediated inflammation (178).

To prove the hypothesis whether T_{reg}-associated cytokines can be regulated by IFN-γ, cells were stimulated with either *spa* mRNA or SpA protein and IL-2, IL-10 and G-CSF were added or blocked, respectively. Indeed, blocking assays provided evidence, that IFN-γ production is partially down-regulated by those cytokines (Fig. 4.25 A). Blocking IL-2, IL-10 and/or G-CSF led to higher, but not significantly increased IFN-γ levels. As reported before, G-CSF not only suppresses IL-4 but also IFN-γ production by T cells (179). IL-2 is rather known for inducing T cell proliferation, survival, differentiation and activation (19, 180), therefore it is most likely influencing IFN-γ production. Albeit, IL-2 possess also anti-inflammatory properties since it can constrain IL-17 production (19). The suppression of IFN-γ by IL-10 is well established (181–183). However, the triple block did not result in higher IFN-γ levels and a concomitant effect of all three on IFN-γ regulation has not been reported so far.

In contrast, adding IL-2 to *spa*-transfected MoDC:T cell co-cultures resulted in significantly higher IFN-γ secretion which was more specific in $CD8^+$ cells because of the lower background (Fig. 4.25 B). As mentioned before, this is most probably a consequence of the activating properties of IL-2 (19, 180). Adding IL-10 did not mediate IFN-γ regulation whereas adding G-CSF decreased IFN-γ significantly. Hence, the production of IFN-γ is

negatively but not exclusively regulated by T_{reg}-associated cytokines and requires further experiments.

For further comparison of mRNA and protein's stimulatory capacity, flow cytometric quantification of $LAMP1^+CD8^+$ cells was used as a marker for the degranulation of CTL. Granules that contain cytotoxic proteins are surrounded by a lipid bilayer containing lysosomal associated membrane gylcoproteins (LAMP), including CD107a (LAMP-1, 115). The importance of LAMP-1 in delivery of granzyme B to the target cell has recently been demonstrated (184). Normally, LAMP is not found on the cell surface and is rather proposed to protect against the leakage of the granule (115). Fig 4.21 demonstrated that indeed, cytotoxicity was triggered by *spa* mRNA and not by SpA protein. Conclusive with these results, mRNA encoded antigens induced also more CCL4 (MIP-1β) in $CD8^+$ cells (Fig. 4.24). This chemokine is predominantly expressed by activated CTL to attract monocytes and macrophages (185).

mRNA-protein co-stimulations were performed to explore whether the mRNA's adjuvant effect is necessary to induce T cell-derived IFN-γ. Those assays demonstrated that stimulation with mRNA and the corresponding protein PBP2a (Fig. 4.20) not only produced more IFN-γ than mRNA alone, but also activated more T cells in total (data not shown). One most likely explanation is the adjuvant effect of ivt mRNA (186).

Adjuvants are components that intensify, improve and prolong the immune response towards an antigen by acting as carriers that provide T cell help, active stimulants or vehicles that serve as matrix for antigens (187). Major adjuvant groups are mineral salt adjuvants such as aluminium, bacteria-derived substances such as peptidoglycan or LPS, liposomes, cytokines or naked unmethylated DNA (187). There are several mechanisms how adjuvants enhance the immune response. Aluminium for instance can bind antigens to facilitate their uptake by APC (188). Especially bacterial components act as ligands for innate pattern-recognition receptors, including TLRs, Nod-like receptors (NLRs) and RIG-I-like receptors (RLR). Those receptors mediate the activation of various transcription factors resulting in enhanced cytokine production and the formation of the inflammasome (189).

The immunostimulatory properties of ivt mRNA to provide danger signals were demonstrated before (186). By adding the cap analogue m7G(5')ppp(5')G during ivt, a triphosphorylated

mRNA is generated. Triphosphorylated single or double stranded RNA is sensed by receptors of the RLR family, including Retinoic acid–inducible protein I (RIG-I), melanoma differentiation– associated gene 5 (MDA-5) and LGP2 (190, 191). Even if RIG-I normally recognizes uncapped, short (< 4000 nt) 5'-ppp-RNA, it has been shown that also the usage of cap analogs mediates receptors activation (191). RIG-I is mainly expressed in plasmacytoid dendritic cells, but also in fibroblasts, keratinocytes and MoDC (159, 192, 193). Activation of this receptors, which can be also achieved with ivt mRNA, leads to production of type I interferon that is required for a protective response against viruses (191, 194). In line with that, high levels of IFN-α were detected in mRNA-transfected MoDc single cultures. Furthermore, there is clear evidence that both IFN-γ and RIG-I amplify each others expression (195, 196).

Another pathway which could be triggered by the mRNA is the activation of TLRs. Small amounts of mRNA that do not necessarily result in a strong $CD4^+$ T cell activation, are taken up by MoDC from the environment and act as ligand for endosomal, RNA-sensing TLR7. DC stimulated by TLR7 ligands induces $CD4^+$ T cells to produce IL-17, IFN-γ and in turn were also shown to be involved in upregulation of RIG-I expression (193, 197). Furthermore, activation of TLR7 also induces cross-presentation, a process by which endosomal antigens are presented via MHC class I, which leads to efficient $CD8^+$ T cell activation (198, 199). A recent vaccination trial against *S. aureus*, applying a mixture of surface proteins and secreted factors and obtained better protection in mice with an additional TLR7 agonist (200).

T cell anergy might be an explanation for the low pro-inflammatory response by staphylococcal proteins. Ziegler and colleagues observed decreased T cell activation during *S. aureus* infection in mice when the bacteria switched from acute to a persistent phase of infection. T cell anergy could be reverted by stimulation with PMA indicating that failure of TCR-signalling underlies T cell hyporesponsiveness (201). Further, T cell superantigens as staphylococcal enterotoxin B have been shown to induce T cell expansion and anergy, resulting in downregulation of TCR expression and cytokine storm that prevents specific T cell responses (76, 202). G-CSF, that was strongly induced by SpA stimulation is involved in the development of T cell anergy as well (172).

In summary, mRNA-encoded staphylococcal antigens induce a strong T_h1-biased immune response characterized by high amounts of IFN-γ and TNF. In contrast, stimulation with

corresponding proteins and mainly SpA resulted in a weak T_h2 cytokine profile indicated by IL-5, IL-13 and low levels of IL-4. Besides, SpA led to secretion of T_{reg}-associated cytokines that partially suppress IFN-γ production. Thus, the induction of T_{reg} by staphylococcal antigens represents way of *S. aureus* to evade the human immune response. *spa* mRNA but not SpA protein is inducing degranulation of CTL. Regarding future therapeutic application, taking advantage of the ivt mRNA's adjuvant effect to induce potent immune responses by re-directing tolerogenic T cell responses is a promising approach.

5.3. Specific analysis of T cell responses to variable proteins associated with strain lineage

Only a tiny minority of people is carrying more than one colonizing *S. aureus* strain. Normally, no other exogenous strain takes over the niche (81, 203). Thus, after decolonization carriers get easily recolonized with their prior *S. aureus* in competitive colonization assays, assuming it is "known" to the immune system (26). Until now, there is no convincing genetic trait found that explains carrier status from the side of the host. Even studies with same-sex siblings or twin pairs did not show concordant colonization pattern, suggesting that colonization fate is determined by multiple factors that differ among carriers (81).

However, the immune mechanisms activated by colonization are poorly understood. One study demonstrates that 80% of all bacteremia are caused by the colonizing strain, and carriers have a slightly increased incidence of bacteremia even if mortality was not significantly higher compared to non-carriers (204). Further, persistent carriers also harbor high antibody titers against antigens specific for their endogenous strain that can favor infection outcome (138, 205, 206). Nonetheless, there is this phenomenon of "low-level" antibodies in humans, carriers or non-carriers, against several virulence factors that are considered to be non-protective (59, 81). Conclusive with this hypothesis, B cell-deficient patients and mice do not have increased infection rates (81, 207). Besides, SpA is not only known to induce B cell apoptosis, but also unspecific plasmablast activation. When binding to V_H3^+ plasmablasts, it induces somatic hypermutation and class-switch recombination leading to activation and non-specific antibody secretion. Further, the plasmablast's BCR demonstrated significant tendency towards a V_H3^+ idiotype, facilitating SpA immune dominance (57, 202).

Little is known about T cell immunity. But lately, several studies concentrate on T_h17-biased responses which is also considered to play an important role in nasal colonization (167, 168, 208). Even if other studies could not detect differences in T cell proliferation induced by staphylococcal antigen between carriers and non-carriers (154), our results revealed that about 30-50% of all tested donors were responders. Since those numbers correspond to the percentage of individuals colonized with *S. aureus*, it is indicated that carriers have a better response towards *S. aureus* antigens. Also, we demonstrated the activation of high numbers of memory T cells, presuming a prior contact with staphylococcal antigens.

S. aureus harbors a few surface structures and secreted proteins with lineage- and host-specific variations. This includes variations in functional domains, some are truncated and some are completely missing (209). Considering that the immune system has to distinguish the exogenous from the endogenous strain by a bacterial feature that impairs uniqueness, SpA represents a plausible candidate (203). It is a core gene, but yet highly variable (49). Although some arguments exclude a correlation between polymorphisms or variable regions of hypervariable genes (as *spa* or *fnbp)* and persistence or colonization (210), it has been demonstrated that over 92% off all carriers keep their *spa* type in a long-term study (211). Our results indeed demonstrate a varying T cell response towards different *spa* types, confirming the hypothesis that the T cell response differs towards a variable surface structure. It further supports the theory that SpA is necessary for the immune system to distinguish between "friend" and "foe" *S. aureus*. In contrast, in most donors SpA from SAC was the most immunogenic. While this strain is known for high SpA expression and broad use in the laboratory (54), it is no widespread colonizer and neither is *spa* type t076. It was rather suspected that t002, t003 (Rhine-Hesse-strain) or t008 (USA300) elicit stronger T cell responses because of their broad distribution (28, 51, 212).

To date, only a few *S. aureus* T cell epitopes and antigens have been predicted (124, 125, 154, 213, 214). To address the question whether Ig-binding domains also influence T cell activation, an Ig-binding mutant based on t078 *spa* type from SAC was generated. Since SpA is commercially available from SAC, this *spa* type was used for further evaluation. Based on a protocol of the Schneewind lab, SAC SpA was used to insert point mutations abolishing the Ig-binding properties of the protein (87). Surprisingly, Ig-mutant mRNA led to higher production of IFN-γ than the wildtype SAC *spa* mRNA (Fig. 4.29).

One explanation for this phenomenon might be the presentation of altered epitopes as result of the inserted point mutations. An activation of $CD8^+$ cells by mutation is known in cancer research. CTL detect and recognize tumor cells as "non-self" because of single point mutations (215–217). Proposing that *S. aureus* is recognized as "self-antigen", which could also explain the non-protective immune response in case of infection, the insertion of mutation in SpA would be a similar phenomenon and an explanation for the better recognition by $CD8^+$ T cells. Indeed, *in silico* predictions revealed epitopes with point mutation in the Fab binding site under the top hits whereas Fc binding sites mutations were not displayed (data not shown).

However, not all mutations in CTL epitopes are recognized which therefore represents a successful escape strategy for viruses (213, 218–221). Interestingly, one study showed that codon optimization resulted in $CD8^+$ T cell activation by a cryptic epitope (222). Thus, this issue requires further examination in the future. One approach could be the preparation of peptides from DC and the mass spectrometric analysis as described elsewhere (223, 224). To test whether those mutated epitopes indeed activate T cells they can be coupled to MHC I tetramers linked to fluorochromes and quantified by flow cytometry (225).

Even though, the tumor-associated mutation pattern is unique in each patient, the Ig-mutant mRNA elicited stronger IFN-γ responses in all tested donors and also in memory T cells, that require prior antigen contact. Hence, the second hypothesis suggests that Ig-binding domains under normal circumstances diminish T cell activation by decreasing protein processing or presentation efficiency. This implies a specific mechanism and another sight of the evolution of Ig-binding domains that then indeed, not only manipulate humoral, but also T cell derived responses. Nevertheless, additional experiments and comparison with Ig-binding proteins from other species are necessary.

Based on *spa* type-dependent IFN-γ secretion detected earlier, it was analyzed whether the variable domain of SpA contains T cell epitopes. Truncated mutants were generated, lacking either the variable or the homologous domain. *In silico*, no changes in epitope formation and neither in protein folding (data not shown) were predicted. Interestingly, only truncation of SAC SpA led to significantly higher IFN-γ production compared to full-length *spa* mRNA, whereas the other *spa* types only induced slightly or no increased cytokine levels (Fig. 4.31). Because more IFN-γ was detected without the variable domain this debilitates the hypothesis

that epitopes from this SpA region alone are needed to activate T cells. However, also without the homologous region, the SAC *spa* type elicits higher IFN-γ production compared to the wildtype.

In conclusion, both domains seem to provide important CTL epitopes. Besides, an extra suppressing activity of one or both domains has to be considered and requires further examination. Presuming that *S. aureus* is recognized as "self-antigen" it is also most likely that this activating/suppressing effect is highly donor-dependent and a consequence of the individual immune history.

Non-antigen-specific $CD8^+$ T lymphocytes have been shown to inhibit antigen-specific T cell proliferation *in vitro* by secretion of IL-10 (226, 227) and DC-derived IL-10 can also promote anergy in $CD4^+$ and $CD8^+$ T cells (228). Therefore, the induction of IL-10 by Ig-mutant and the truncated variants (Fig. 4.31 B) is most likely a reaction to the strong pro-inflammatory environment induced by IFN-γ and an attempt to retain homeostasis. Plus, flow cytometric analysis demonstrated that both cytokines are secreted by different cell subsets and that IL-10 was no longer be detected after three days of stimulation (Fig. 4.32).

5.4. Summary

This thesis demonstrates that mRNA-based delivery of bacterial antigens represents a promising approach to circumvent the immunomodulatory effects employed by staphylococcal proteins, including the Ig-binding protein SpA. Further, ivt can be utilized to analyze and quantify antigen-specific T cell responses towards different staphylococcal antigens. Within this study, protocols for efficient *in vitro* transcription, generation of MoDC and transfection with mRNA-encoded antigens were established. The results highlight the potential of mRNAs encoding staphylococcal antigen such as *spa*, *mecA* and *sitC* to induce IFN-γ production by antigen-specific $CD8^+$, $CD45RO^+$ and to minor extent $CD4^+$ T cells. Notably, mRNA-based antigen delivery facilitated IFN-γ secretion, which was only weakly induced by the corresponding proteins. Our data suggest that the potency of the mRNA method lies in its adjuvant properties, i.e. additional mRNA recognition by cytosolic and/or endosomal pattern recognition receptors. In line with these results, cytokine multiplex assays revealed that presentation of staphylococcal proteins triggers the production of T_h2-associated cytokines whereas mRNA-derived antigens induced a strong T_h1-biased response.

Furthermore, MoDC pulsing with SpA protein led to secretion of cytokines associated with the induction of T_{reg}, suggesting that T cell responses to *S. aureus* are strictly regulated. Moreover, *spa* mRNA and not SpA protein induced degranulation of $CD8^+$ T cells suggesting the formation of cytotoxic T lymphocytes.

Lastly, experiments with truncated and Ig-mutant SpA variants provided further evidence that both the variable and the homologous domain contain epitopes recognized by $CD8^+$ T cells. Notably, induction of IFN-γ production increased after mutation of the Ig-binding sites within full-length *spa* mRNA when compared to the wildtype, which suggests that mutation if these sights increases T cell immunogenicity for presently unknown reasons.

5.5. Conclusions and Perspectives

There have been numerous approaches of vaccine development against *S. aureus* during the last decades. Even though there is clear evidence that T cells are important keyplayers in *S. aureus*-derived immunity, they have been neglected in vaccine research so far. Compared to adaptive cellular responses, antibody measurement is uncomplicated and easier to predict because it only depends on conserved protein structures rather than unknown peptide conformation after processing by APC (81). Today, it is known that low Ig-levels against *S. aureus* are not necessarily protective and that the role of protein A in production of unspecific antibodies has been underestimated.

Successful mRNA-based vaccination against viruses has been established during the last years. Including all known advantages of mRNA vaccination mentioned above, we demonstrated that mRNA of various staphylococcal antigens enables activation of specific T cells and CTL degranulation. Further, the mRNA's adjuvants effect might be crucial to overcome T cell anergy and to re-direct anti-inflammatory T_{reg} differentiation induced by *S. aureus* proteins.

Triggering either a strain-specific, pro-inflammatory adaptive immune response by *spa* type specific mRNA, or a broad immune response by mRNA application of various staphylococcal antigens presents a promising novel approach of vaccination against *S. aureus* to circumvent and interrupt immune manipulation. Nevertheless, it will be most likely necessary to trigger

B cell derived immunity in parallel to provide protectivity which can be achieved for instance by application of a SpA Ig-mutant protein (87, 88).

In further experiments, we want to prove our hypothesis that *S. aureus* carriers are better responders to mRNA-encoded antigens. Therefore, cell culture of colonized donors will be stimulated with mRNA encoding their own *spa* type in comparison to an exogenous *spa* type. Furthermore, the activation of antigen-specific T cells will be proven by proliferation assays, tetramer staining or other suitable applications. The induction of T_{regs} by stimulation with SpA will be analyzed by determination of FoxP3 and surface marker by flow cytometry.

To answer the question about the high immunogenicity of the SAC *spa* type and to provide further evidence for SpA T cell epitopes, Ig-mutant mRNA with variable regions of other *spa* types and a truncated Ig-mutant mRNA will be generated and evaluated in T cell assays.

The hypothesis that Ig-binding domains can also suppress T cell activation will be addressed by comparing the immunogenicity of the Ig-mutant mRNA with a non-sense SpA mutant and a SpA mutant with various silent mutations. Plus, we want to mutate the Ig-binding domains of other species as protein G from *Streptococcus* and of Sbi, the other staphylococcal Fc-binding protein.

At last, immunization of mice or pigs with *spa* mRNA and subsequent challenge with *S. aureus* will provide further evidence whether mRNA-based vaccination could elicit protective immunity *in vivo*.

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Publications and Presentations

Publications

1. **Uebele J**, Stein C, Nguyen MT, Schneider A, Kleinert F, Tichá O, Bierbaum G, Götz F and Bekeredjian-Ding I.
   Antigen-encoding mRNA demonstrates $CD8^+$ T cell memory against *Staphylococcus aureus* and shifts endogenous T cell responses from T_h2/T_{reg} to T_h1/T_h17-like patterns. (in preparation)

2. Waller T, Kesper L, Hirschfeld J, Dommisch H, Kölpin J, Oldenburg J, **Uebele J**, Hoerauf A, Deschner J, Jepsen S and Bekeredjian-Ding I.
   Porphyromonas gingivalis outer membrane vesicles induce selective TNF tolerance in a TLR4- and mTOR-dependent manner. (In revision)

3. Bekeredjian-Ding I, Stein C and **Uebele J**.
   The innate immune response against *Staphylococcus aureus*. Current Topics in Microbiology and Immunology, Springer International Publishing AG (In press)

4. Cecil A, Ohlsen K, Menzel T, François P, Schrenzel J, Fischer A, Dörries K, Selle M, Lalk M, **Hantzschmann J**, Dittrich M, Liang C, Bernhardt J, Ölschläger TA, Bringmann G, Bruhn H, Unger M, Ponte-Sucre A, Lehmann L and Dandekar T. Modelling antibiotic and cytotoxic isoquinoline effects in Staphylococcus aureus, Staphylococcus epidermidis and mammalian cells. Int J Med Microbiol. 2015 Jan;305(1):96-109.

5. Ziegler S., Gartner K., Scheuermann U., Zoeller T., **Hantzschmann J**, Over B, Foermer S, Heeg K and Bekeredjian-Ding I.
   $Ca(2^+)$ -related signaling events influence TLR9-induced IL-10 secretion in human B cells. Eur J Immunol. 2014 May;44(5):1285-98.

Presentations

1. Poster: "A new approach to analysis of T cell responses to *S. aureus* in healthy donors"
   Staphylococcal Disease, 14th Gordon Research Conference and Seminar, Lucca, Italy, July 2015

2. Talk: "mRNA as a novel approach of vaccination against *Staphylococcus aureus*"
   Retreat on Biomedical Research, Paul-Ehrlich-Institute, Heidelberg, January 2015

3. Poster: "mRNA as a novel approach of vaccination against *Staphylococcus aureus*"
   ImmunoSensation Cluster Science Day, Bonn, November 2014

4. Talk: "Development and evaluation of an mRNA-based vaccine against *Staphylococcus aureus*"
   66th annual meeting DGHM, Dresden, October 2014

5. Poster: "The effect of different *Staphylococcus aureus spa* types on the human immune response"
   Current Concepts in Immunology, 6th Autumn school, Merseburg, October 2013

Appendix

Amino acid sequences of all *spa* types used in this study. The variable region is underlined. *= Stop Codon

SAC

MMTLQIHTGGINLKKKNIYSIRKLGVGIASVTLGTLLISGGVTPAANAAQHDEAQQNAFYQVL
NMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSQAPKADAQQNNFNKDQQSAFYEIL
NMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPKADNNFNKEQQNAFYEILNMPN
LNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPKADNKFNKEQQNAFYEILHLPNLNEEQ
RNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLTEEQRNGFI
QSLKDDPSVSKEILAEAKKLNDAQAPKEEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKE
DNNKPGKEDGNKPGKEDNKKPGKEDGNKPGKEDNKKPGKEDGNKPGKEDGNKPGKEDGN
GVHVVKPGDTVNDIAKANGTTADKIAADNKLADKNMIKPGQELVVDKKQPANHADANKAQ
ALPETGEENPFIGTTVFGGLSLALGAALLAGRRREL*

USA300

MMTLQIHTGGINLKKKNIYSIRKLGVGIASVTLGTLLISGGVTPAANAAQHDEAQQNAFYQVL
NMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSQAPKADAQQNNFNKDQQSAFYEIL
NMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPKADNNFNKEQQNAFYEILNMPN
LNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPKADNKFNKEQQNAFYEILHLPNLNEEQ
RNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLTEEQRNGFI
QSLKDDPSVSKEILAEAKKLNDAQAPKEEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKE
DGNKPGKEDNKKPGKEDGNKPGKEDNKKPGKEDGNKPGKEDGNKPGKEDGNGVHVVKPG
DTVNDIAKANGTTADKIAADNKLADKNMIKPGQELVVDKKQPANHADANKAQALPETGEE
NPFIGTTVFGGLSLALGAALLAGRRREL*

Rhine-Hesse t002

MMTLQIHTGGINLKKKNIYSIRKLGVGIASVTLGTLLISGGVTPAANAAQHDEAQQNAFYQVL
NMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSQAPKADAQQNNFNKDQQSAFYEIL
NMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPKADNNFNKEQQNAFYEILNMPN
LNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPKADNKFNKEQQNAFYEILHLPNLNEEQ
RNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLTEEQRNGFI
QSLKDDPSVSKEILAEAKKLNDAQAPKEEDNKKPGKEDGNKPGKEDGNKPGKEDNKKPGKE
DGNKPGKEDNNKPGKEDGNKPGKEDNNKPGKEDGNKPGKEDGNKPGKEDGNGVHVVKPG
DTVNDIAKANGTTADKIAADNKLADKNMIKPGQELVVDKKQPANHADANKAQALPETGEE
NPFIGTTVFGGLSLALGAALLAGRRREL*

Rhine-Hesse t003

MMTLQIHTGGINLKKKNIYSIRKLGVGIASVTLGTLLISGGVTPAANAAQHDEAQQNAFYQVL
NMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSQAPKADAQQNNFNKDQQSAFYEIL
NMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPKADNNFNKEQQNAFYEILNMPN
LNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPKADNKFNKEQQNAFYEILHLPNLNEEQ
RNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLTEEQRNGFI
QSLKDDPSVSKEILAEAKKLNDAQAPKEEDNKKPGKEDGNKPGKEDNNKPGKEDGNKPGKE
DNNKPGKEDGNKPGKEDGNKPGKEDGNKPGKEDGNGVHVVKPGDTVNDIAKANGTTADKI
AADNKLADKNMIKPGQELVVDKKQPANHADANKAQALPETGEENPFIGTTVFGGLSLALGA
ALLAGRRREL*

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Bonn, Dezember 2015