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Susceptibility of humanized mice to *Staphylococcus aureus* in a localized deep-tissue abscess model

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“If you dare nothing, then when the day is over, nothing is all you will have gained.”

Neil Gaiman, The Graveyard Book

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List of Abbreviations

ADAM	a disintegrin and metalloproteinase
ANOVA	analysis of variance
AU	arbitrary units
AZ	Arizona
Balb/c	BALB/cJRj
BLI	bioluminescence imaging
BLT	bone marrow, liver, thymus
BM	bone marrow
BRG	Balb/c Rag2−/− Il2rg−/−
C.B-17 scid	CB-17/Icr-Prkdcscid/scid/Rj
CA-MRSA	community-associated MRSA
CCL-2	CC-chemokine ligand 2
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention
CFU	colony-forming units
CIA	collagen-induced arthritis
d2	day 2
d7	day 7
DNA	deoxyribonucleic acid
EBV	Epstein-Barr Virus
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species
ET	exfoliative toxin
EU	European Union
FFPE	formalin-fixed and paraffin-embedded
FL	fetal liver
GM-CSF	granulocyte–macrophage colony-stimulating factor
GVHD	graft-versus-host disease
Gy	Gray
h	human
H&E	haematoxylin and eosin
H2	histocompatibility-2
HAI	healthcare-associated infection

HA-MRSA	healthcare-associated MRSA
HCV	Hepatitis C virus
HIV	human immunodeficiency viruses
HLA	human leukocyte antigen
HLH	hemophagocytic lymphohistiocytosis
HSC	hematopoietic stem cells
Hu	human
huNSG	humanized NSG
huSGM3	humanized NSG-SGM3
IE	infectious endocarditis
IFN-γ	interferon gamma
IgG	immunoglobin G
IL	Interleukin
LA-MRSA	live-stock associated MRSA
m	murine
MA	Massachusetts
MAS	macrophage activation syndrome
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
muNSG	murinized NSG
muSGM3	murinized NSG-SGM3
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NK	natural killer
Nod	non-obese diabetic
NOG	NOD/Shi-scid Il2rg−/−
NSG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ
NSG-SGM3	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ
p.i.	post infection
PBL	peripheral blood lymphocyte
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PMN	polymorphonuclear leukocytes
PRR	pattern recognition receptors
PSMs	phenol-soluble modulins

PVL	Panton-Valentine leukocidin
SAgs	superantigens
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
SCF	stem cell factor
SCID	severe combined immunodeficiency
SE	staphylococcal enterotoxins
SEM	standard error of the mean
sJIA	systemic juvenile idiopathic arthritis
SRC	SCID-repopulating cell
SSSS	staphylococcal scalded-skin syndrome
SSTIs	skin and soft tissue infections
subsp.	subspecies
TCR	T cell receptors
Th1	T-helper type 1
Th2	T-helper type 2
TLR2	Toll-like receptor 2
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin-1
UBC	umbilical cord blood
USA	United States of America
wt	wild-type

1 Introduction

While the headlines of the last years have been dominated by prominent viral epidemics like Covid 19 and Ebola, the danger of bacterial infections, specifically those resistant to common antimicrobial agents, has not abated. *Staphylococcus aureus* finds itself at the forefront here, a bacterium that has colonized around 30 % of the population and can cause a wide array of clinical diseases ranging from simple skin infections to life-threatening ones, such as pneumonia, endocarditis, and bacteraemia (Cheung et al. 2021; Monaco et al. 2017). *S. aureus* possesses a variety of host-specific virulence factors, and a history of antimicrobial resistance that stretches back to the discovery of penicillin (Turner et al. 2019). Today, methicillin-resistant strains (MRSA) represent a particular challenge for healthcare providers and for the society in general, with infections occurring in the healthcare setting (HA-MRSA), the community (CA-MRSA) and livestock (LA-MRSA) (Cuny et al. 2015; Otto 2012). Despite the undeniable need for new therapeutical options, specifically a vaccine, the developments have stalled (Clegg et al. 2021). In particular, there is a prominent discrepancy between promising pre-clinical trials and disappointing clinical outcomes, which can at least in part be explained by the fact that *S. aureus* is equipped with various virulence factors, such as superantigens, that have an enhanced detrimental effect on human immune components. In addition, the murine and human immune systems respond differently to an infection with *S. aureus* (Salgado-Pabón and Schlievert 2014).

One strategy to bridge the gap between the mouse, the preferred laboratory animal in infectious disease research, and the realities of the human host, are humanized mice; immunodeficient mice that are engrafted with human hematopoietic stem cells ($CD34^+$ cells) and subsequently develop a functional human immune system (Shultz et al. 2012). The system is not without flaws, and while a tremendous amount of progress has been made over the last decades, the search for the perfect model is still ongoing. A commonly used strain is the NSG mouse, which supports robust numbers of T and B cells, but is lacking in the myeloid compartment (Coughlan et al. 2016; Shultz et al. 2005). Humanized NSG (huNSG) mice have already been used in a handful of *S. aureus* studies covering peritonitis, skin infection, pneumonia, and osteomyelitis models. Consistently, humanized mice have demonstrated an increased susceptibility compared to the control groups including wild-type mice, murinized NSG mice, and non-engrafted immunodeficient NSG mice (Muthukrishnan et al. 2021; Prince et al. 2017; Knop et al. 2015; Tseng et al. 2015).

To improve the number of myeloid cells, researchers have incorporated three myelo-supportive cytokines into the NSG mice, thus creating the NSG-SGM3 mouse. The transgenic expression of the stem cell factor (SCF), granulocyte–macrophage colony-stimulating factor

(GM-CSF), and Interleukin-3 (IL-3) does have one down-side, though. Long-term engrafted animals develop a variety of symptoms, described as secondary hemophagocytic lymphohistiocytosis (HLH) or macrophage activation syndrome (MAS)-like disease. Nonetheless, considering that neutrophils play a prominent role in the fight against S. *aureus,* this mouse strain offers a promising opportunity to advance our knowledge of the interactions between the pathogen and the human immune system. This thesis is the first published work using NSG-SGM3 mice for *S. aureus* trials.

In this study, NSG and SGM3 mice were humanized and infected locally in the thigh muscle with *S. aureus,* a deep-tissue abscess model which makes it possible to examine both the acute and the chronic stages of the infection. The thesis aimed at elucidating the susceptibility of humanized mice in this model, investigating how the human immune reaction differs from the murine one, and to determine whether their enhanced myeloid immune system protects the huSGM3 mice from the infection better than their NSG counterparts.

2 Literature Review

2.1 *Staphylococcus aureus*

2.1.1 General Characteristics

In 1880, Sir Alexander Ogston first named a round shaped bacterium that liked to form clusters. Burrowing from the Greek, *staphyle,* meaning bunch of grapes, and *kokkos,* grain or berry, the term *Staphylococcus* was born. *Staphylococcus aureus* represents the major pathogen of the genus *Staphylococcus* and is distinguished by its ability to produce coagulase. The bacterium is Gram-positive, facultative anaerobic, non-motile and non-sporing (Gatermann 2012; Humphreys 1998). If grown on blood agar, the colonies usually show a characteristic golden colour (*aureus* meaning golden), due to the carotenoid staphyloxanthin (Pelz et al. 2005). The bacteria range from 0.5 to 1 µM in size (Gnanamani et al. 2017). Some strains of *S. aureus* form a capsule from polymers out of glucosaminuronic acid or mannosaminuronic acid (Gatermann 2016).

S. aureus can be typically found in the nares, with around 20% of the human population consisting of persistent carriers. A further 60% are intermittent carriers and only about 20% of the population never harbour the bacterium (Kluytmans et al. 1997). *S. aureus* is not limited to the nostrils but can also occupy the skin and mucosa of other parts of the body, like the axilla, groin, and perineum. But if the pathogen is eradicated from the nares, it mostly disappears from those other areas as well (Parras et al. 1995).

S. aureus belongs to a group of bacteria that bears responsibility for a big share of nosocomial infections, the so-called ESKAPE pathogens, which are characterized by their antimicrobial resistance. ESKAPE is an acronym standing for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species (Rice 2010). Nosocomial infections are placing a heavy burden on the health system, with 3.8 million patients contracting at least one healthcare-associated infection (HAI) in acute care in the European Union and European Economic Area per year (Kärki et al. 2019).

2.1.2 Virulence Factors

S. aureus is equipped with numerous extracellular and cell-associated factors, that either individually or interactively promote the organism's virulence and survival in the face of host defences (Humphreys 1998; Lowy 1998).

2.1.2.1 Surface proteins

On its surface, *S. aureus* carries various cell-wall associated proteins, which enable the organism to attach and invade host cells, build biofilms, and evade the immune response. The largest class of surface proteins are the **MSCRAMM** (microbial surface components recognizing adhesive matrix molecules), which include among others the fibrinogen-binding proteins clumping factors A and B, the collagen-binding protein C and the fibronectin-binding proteins A and B (Foster et al. 2014; Foster and Höök 1998). **Protein A** is a prominent cell-wall associated protein, which has many functions, such as binding the Fc region of the immunoglobin G (IgG) and the von Willebrand factor (Rigi et al. 2019; Hartleib et al. 2000). It is also the only known B-cell superantigen that *S. aureus* produces (Tam and Torres 2019).

2.1.2.2 Toxins

2.1.2.2.1 Cytotoxins

S. aureus secrets multiple toxins, that prominently influence its virulence. Cytotoxins attack the cytoplasmic membrane through pore formation, which leads to the leakage of metabolites and molecules and ultimately to the lysis of the cell (Otto 2014b). A prominent representative of this group is the **α-toxin**, also called α-hemolysin or Hla. The α-toxin is a beta barrel forming toxin, secreted as a water-soluble monomer by 95% of clinical *S. aureus* strains (Oliveira et al. 2018). Upon binding to the host receptor ADAM 10 on its target cell, the toxin oligomerizes into a heptamer to form a pre-pore. The process is finalized by building a transmembrane channel (Seilie and Bubeck Wardenburg 2017). Hla lyses a variety of cells, ranging from (non-human) erythrocytes to endothelial cells, epithelial cells, and certain leukocytes (Berube and Bubeck Wardenburg 2013).

The **bicomponent pore-forming toxins**, also called leukocidins, share structural similarities with the α-toxin and are relying on a resembling pore-forming mechanism. However, the leukocidins are composed of two compartments, the F- and the S-unit (Tam and Torres 2019). Of this group, five are known to be associated with *S. aureus* infections in humans: LukAB (also known LukHG), LukED, the γ-hemolysins AB and CB (HglAB, HglCB), and LukSF-PV (also known as Panton-Valentine leukocidin, PVL). Three more leukocidins, LukMF', LukPQ and lukF/S-BV are targeting other animals. As their name suggests, mutual targets of these toxins are usually leukocytes (Monecke et al. 2021; Tam und Torres 2019). The most highly virulent *S. aureus* strains infecting humans can produce all five leukocidins, the lesser virulent strains at least three (HlgAB, HlgCB, and LukAB/HG) (Alonzo and Torres 2014).

Phenol-soluble modulins (PSMs) differ from the toxins mentioned above, in that their role is not solely cytotoxic. They also play an important part in inflammation, biofilm development and the surface colonisation by *S. aureus.* PSMs are uniquely found in staphylococci and can be

divided into two groups, the longer β-type and the smaller α-type. The α-PSMs produced by *S. aureus* consist of five PSMs named PSMα1-4 and the long known δ-toxin. PSMβ1 and PSMβ2 make up the β-PSM group (Otto 2014a). Phenol-soluble modulins are amphipathic peptides that bind to the cytoplasmic membrane in a non-specific way, which can lead to the disintegration of the membrane and cell lysis (Oliveira et al. 2018). PSMs kill both erythrocytes and neutrophiles, with especially PSMα3 being highly capable of neutrophil lysis (Cheung et al. 2012; Wang et al. 2007).

In 2015, Merriman et al. coined a new exoprotein, called **ε-cytotoxin**. This toxin kills keratocytes, lyses red blood cells, and acts proinflammatory at subcytotoxic levels (Merriman et al. 2015). Due to its recent discovery, there are still many unanswered questions about the role and regulation of this toxins, but it may play a part in impaired wound healing associated with *S. aureus* infections (Merriman et al. 2015).

2.1.2.2.2 Staphylococcal superantigens

Staphylococcal superantigens (SAgs) are microbial proteins that range in size from 19 to 30 kDA and are characterized by their unique resilience against adverse conditions, like heat, desiccation, and proteolysis (Spaulding et al. 2013). The term “superantigens” was first coined by Marrack and Kappler in the late 1980s and describes toxins that stimulate a substantial percentage of the T-cells (5-30%). This is particularly remarkable in contrast to typical antigens, that only activate <0.01% of T-cells (Krakauer 2019). The reason for this discrepancy can be found in the different binding mechanisms. When peptides are presented by the major histocompatibility complex, it leads to a specific and directed T cell activation. Sags, on the other hand, bind directly to the MHC class II and also build a link with the T cell receptor of the T cells (Rödström et al. 2014) This crosslink leads to a so-called cytokine storm, which is characterized by a massive release of interleukin-1, interleukin-6, interferon-gamma, as well as tumor necrosis factors alpha and beta (Burnham and Kollef 2015). Commonly encompassed by the term of staphylococcal superantigens are the group of staphylococcal enterotoxins, the toxic shock syndrome toxin-1, and the staphylococcal enterotoxin-like proteins (Xu and McCormick 2012).

To date, 23 **staphylococcal enterotoxins (SE)** have been recognized, numbered from SEA to SE1Y (Grispoldi et al. 2019). Only a handful of those have been studied extensively though, with SEA and SEB the most common and best characterized (Pinchuk et al. 2010). Only microgram amounts of staphylococcal enterotoxins are sufficient to induce food poisoning symptoms, like diarrhoea and vomiting, after consumption of contaminated food (Humphreys 1998). These toxins are more resistant to adverse conditions, like heat, freezing, and low pH than the producing bacteria and also withstand the proteolytic enzymes in the digestive tract

(Hennekinne et al. 2012). SEA is the most frequently SE associated with food poisoning, followed by SED (Argudín et al. 2010). SEB has gained particular notoriety, as it does not only cause food poisoning, but can potentially be used as a biological weapon. It can be easily aerosolized, and if inhaled, can lead to extensive lung damage due to the infiltration of immune cells, exceeding cytokine production, and pulmonary edema (Rao et al. 2014).

The **toxic shock syndrome toxin-1 (TSST-1)** might be the most commonly known SAg, due to a toxic shock syndrome (TSS) outbreak in the 1980s, that was associated with the use of tampons (Otto 2014b). Super-absorbent tampons brought oxygen into a usually anaerobic environment, which promoted the proliferation of *S. aureus* and the production of TSST-1. Toxic shock syndrome (TSS) presents a non-specific clinical picture, with patients suffering from fever, gastrointestinal symptoms, diffuse erythroderma, malaise, confusion among others (Burnham and Kollef 2015).

2.1.2.2.3 Exfoliative Toxins

Exfoliative Toxins (ETs), also known as epidermolytic toxins, are highly specific serine proteases that hydrolyse desmosomal proteins in the superficial layers of the skin (Bukowski et al. 2010). ETs have been appropriately described by Nishifuji et al. as “molecular scissors” that separate the connection between keratinocytes, leading to blistering of the skin. This damage can either appear locally as bullous impetigo or generalized as staphylococcal scalded-skin syndrome (SSSS) (Nishifuji et al. 2008).

2.1.2.3 Enzymes

S. aureus produces a variety of enzymes, both for procuring essential nutrients for the bacterial growth from the host and to help the pathogen to evade the immune system. Prominent example is the **coagulase** whose ability to coagulate human plasma was first documented in 1903 and is used today as a mean to classify staphylococci into coagulase-positive, coagulase-variable and coagulase-negative staphylococci (Tam und Torres 2019; Becker et al. 2014). The **staphylokinase** activates the host plasminogen for dissolution of fibrin clots, leading to a dissemination of the bacteria (Algammal et al. 2020). The task of breaking down the host tissue on the other hand, is based on the activity of **nucleases**, **proteases**, **lipases,** and the **hyaluronidase** (Tam and Torres 2019).

2.1.3 Diseases caused by *Staphylococcus aureus*

As an opportunistic pathogen, *S. aureus* can turn from an inconspicuous member of the microbiota to the leading cause of a variety of diseases, ranging from skin and soft-tissue

infections (SSTIs), pneumonia, endocarditis to bacteremia (Paling et al. 2020;Laupland et al. 2013; Talan et al. 2011; Fowler et al. 2005).

A study encompassing medical centers in 16 countries worldwide determined *S. aureus* as the leading cause of **infectious endocarditis (IE)** (Fowler et al. 2005). When the cardiac endothelium is damaged, for example by an intravascular catheter, injected matter from drug use, or inflammation caused by rheumatic heart disease, bacteria can colonize the created niche and multiply. If left untreated, the following endocarditis is lethal (Tong et al. 2015a; Que und Moreillon 2011).

There are three major types of **osteoarticular infections**: osteomyelitis, prosthetic joint infection, and native joint septic arthritis, with *S. aureus* the leading pathogen in all of them (Tong et al. 2015a). Osteomyelitis is an infectious inflammation of the bone which has been classified by Waldvogel et al. into three categories: hematogenous formation, local transmission, and osteomyelitis due to vascular insufficiency (e.g. diabetes mellitus) (Schwarzmann et al. 2016). An acute osteomyelitis becomes chronic in 10 to 30% of patients, with around 75% caused by *S. aureus* and coagulase negative staphylococci (Walter et al. 2012). With a rapidly aging population in many countries and an accompanying rising life expectancy, joint replacements are on the rise. As with every medical procedures, complications can arise, with worldwide 1.4-2.5% of patients with total arthroplasty suffering of prosthetic joint infection (Zardi and Franceschi 2020). Native joints can be infected as well, mostly by bacteria that have descended from the blood stream. Occasionally, direct trauma like an animal bite, or very rarely complications after an arthroscopy, are to blame (Ross 2017; Babcock et al. 2002).

However, not only prosthetic joints are affected, *S. aureus* is very proficient at infecting any **prosthetic device** that has been implanted into the human body, like prosthetic cardiac valves and intravascular catheters. A particular role plays its ability to build biofilms on these foreign bodies, which make an elimination of the infection without removal of the implant extremely difficult (Tong et al. 2015a).

S. aureus **pneumonia** occurs relatively rarely in the community environment, but is linked to a high mortality rate ranging from 48% to 84% (Vlaeminck et al. 2020). In the hospital setting, *S. aureus* takes up a markedly bigger role as major pathogen, as a large cohort study across Europe by Paling et al. has shown: the incidence of pneumonia caused by *S. aureus* lay at 4.9 events per 1000 intensive care unit patients-days, with a 3.6 times higher risk in patients already colonized with *S. aureus* (Paling et al. 2020). *S. aureus* has also lately been identified as an influential part of mortality and complications in patients suffering from pneumonia caused by SARS-CoV-2 (Sharov 2020).

Skin and soft tissue infections (SSTIs) can be benign and superficial, like an infected abrasion and impetigo, or be more complicated such as furuncles, subcutaneous abscesses,

and infected wounds. What combines all of them is *S. aureus* as the leading cause (Krishna and Miller 2012). Importantly, a localized skin infection may progress to an invasive infection encompassing the neighbouring muscle and bone, or even disseminate to the lung and heart (McCaig et al. 2006).

In the industrialized world, the incidence of *S. aureus* **bacteremia** ranges from around 20 to 32 per 100.000 population per year, with the very young and the elderly particularly at risk (Laupland et al. 2013). Usually, the sources of a *S. aureus* blood stream infection are SSTIs, endocarditis, osteoarticular, pleuropulmonary, and vascular catheter-related infections, though in around 25% of cases no original focus of infection can be found (Tong et al. 2015a). Of the affected patients, 10 to 30% will succumb to the disease. A stark decrease compared to pre-antibiotic times, when the chance of survival was as low as 17 to 25% (van Hal et al. 2012).

S. aureus also plays an important role in agriculture as one of the main causes of **mastitis** in dairy cows, goats and sheep (Bergonier et al. 2014; Wilson et al. 1997). The bacteria does not only affect the health of the animal, but also represents a considerable economic burden due to a decreased milk output and treatment costs (Hogeveen et al. 2011).

2.1.4 The Immune Response to *Staphylococcus aureus*

2.1.4.1 The Innate Immune Response

The **skin** is the first barrier encountered by pathogens in the environment. The outermost layer of the skin is the epidermis, which is dominated by keratinocytes that make up 85% of the cells. Keratinocytes take about 14 days to develop from an undifferentiated basal cell to a terminally differentiated keratinocyte of the corneal layer (Joffe et al. 2020). Keratinocytes and mucosal epithelial cells recognize various components of the *S. aureus* cell wall, like lipoproteins and peptidoglycan via pattern recognition receptors (PRR). PRR are present both on the surface of the cell and intracellular, with Toll-like receptor 2 (TLR2) being the major surface receptor in charge of recognizing *S. aureus* and other Gram positive pathogens (Bekeredjian-Ding et al. 2017). If epithelial cells are invaded by *S. aureus,* the peptidoglycan binding receptor Nod2 is activated. A particular strong inflammatory response is triggered by a coactivation of TLR2 and Nod2, led by the release of cytokines, chemokines, and antimicrobial peptides (Brandt et al. 2018; Bitschar et al. 2017).

Mast cells reside mostly in tissues where the body connects with the environment, like the skin or mucosa. While best known for their damaging role in allergies and other inflammatory diseases, mast cells also play a part in fighting bacteria, including *S. aureus* (Johnzon et al. 2016). For this purpose, mast cells are equipped with a range of defence mechanisms, namely degranulation, chemokine secretion, and the release of extra cellular traps that are predominantly relying on reactive oxygen species. The immune cells also don't resort to a 'one

fits all' response, but instead use an individualized response pattern (Garcia-Rodriguez et al. 2020). True to their name, the "big eaters", **macrophages** engulf and digest a wide range of bacteria, apoptotic cells, and other debris. However, phagocytosis is not their only capability. Macrophages secret enzymes, cytokines, complement components, coagulation factors, reactive oxygen intermediates, and arachidonic acid intermediates. Crucially, they also process and present antigens to B- and T-cells (Fox et al. 2010).

Neutrophils hold a prominent role in the defence against *S. aureus*. At 60%, they are the most numerous leukocytes and are equipped with specific granules to kill Gram-positive and Gram-negative bacteria (de Jong et al. 2019). Neutrophils originate in the bone marrow, where they spend the biggest part of their life, with only 2% of the population circulating in the bloodstream. There, their lifespan is short with a half-life of only 6 to 8 hours in humans and 11 hours in mice, which leads to an estimated daily production of $5x10^{10}$- $10x10^{10}$ new neutrophils (Sadik et al. 2011). In response to inflammatory mediators and neutrophil chemoattractants secreted by various host cells, such as endothelial and epithelial cells, mast cells, macrophages, monocytes and keratinocytes, neutrophils exit the blood vessels and enter the infected tissue (Rigby and DeLeo 2012). Incoming neutrophils can recognize bacteria due to pathogen associated molecular pattern, like lipoproteins and lipopolysaccharides (gram-negatives), but ideally, the bacteria is opsonized with complement and antibodies to improve the following phagocytosis (Kobayashi et al. 2015). Neutrophils are capable of phagocytosing intruding pathogens and subsequently killing the incorporated microorganisms by antibacterial proteins or the NADPH oxidase, an enzyme that produces reactive oxygen species. Patients suffering from chronic granulomatous disease, which is characterized by a malfunctioning of the NADPH oxidase, repeatedly catch severe fungal and bacterial infections, including *S. aureus* infections. The antibacterial proteins defensins, cathepsins, lysozyme and lactoferrin can also be released by neutrophils into the extracellular surroundings to eliminate pathogens (Roos 2016; Borregaard 2010) Activated neutrophils can take the extracellular killing of pathogens even further by committing "suicide" to produce NETs (neutrophil extracellular traps) (Brinkmann and Zychlinsky 2007). Upon disintegration of the nucleus, followed by that of the cell membrane, the extracellular traps emerge, consisting of DNA and granular proteins, such as neutrophil elastase, myeloperoxidase, gelatinase, and lactoferrin (Fuchs et al. 2007; Brinkmann et al. 2004).

The characteristic inflammatory lesions of a *S. aureus* infection are **abscesses**, which aim to restrict and eradicate the bacteria. The abscess core contains bacteria, fibrin and live and dead neutrophils, which are surrounded by a fibrin capsule in mature abscesses (Brandt et al. 2018). Skin and soft tissues are typically affected, but deeper layers such as muscles and organs can form abscesses as well (Kobayashi et al. 2015).

2.1.4.2 The Adaptive Immune Response

The main function of **B cells** is the production of pathogen-specific antibodies. By binding antibodies to pathogens, these can be neutralized, efficiently phagocytosed, or destroyed by complement activation (Gulbins and Lang 2019).

Patients suffering from a lack of mature B cells are highly at risk of infection with numerous bacteria and viruses, however, these patients do not have a higher susceptibility to invasive *S. aureus* infections. Similar findings have been made in mouse experiments indicating that antibodies play a negligible role in the fight against *S. aureus* (Karauzum and Datta 2017). On the other hand, Montgomery et al. were able to prove that after a cutaneous *S. aureus* infection, BALB/c mice develop antibodies to protect them from a secondary infection (Montgomery et al. 2014). Also patients with lower antibody levels against staphylococcal exotoxins have shown a greater risk of developing a sepsis than those patients with higher levels (Adhikari et al. 2012).

T cells mature in the thymus and carry unique T cell receptors (TCR) that consist usually of an alpha and a beta chain. The alpha and beta chain each have a constant and a variable part that gives the cell its specificity. TCRs only bind to antigens presented on a major histocompatibility complex (MHC). T helper cells ($CD4^+$ T cells) play a central role in the regulation of the immune response, while $CD8^+$ T cells, also called cytotoxic T cells, kill the infected cell directly (Gulbins and Lang 2019). Considering that *S. aureus* is mainly a extracellular pathogen and $CD8^+$ cells foremost task is to kill intracellular pathogens by eliminating the affected host cell, a decisive role for cytotoxic T cells in a staphylococcal infection has not been described (Karauzum and Datta 2017). When $CD4^+$ cells are activated by antigen-presenting cells (APCs), they multiply and differentiate into various subtypes with individual functions. T-helper type 1 (Th1) cells produce principally interferon gamma (IFN-γ) cytokines, which activate macrophages to stimulate the elimination of intracellular pathogens (Actor 2014). In the case of a *S. aureus* infection, the role of Th1 seems conflicting. There is research showing an increased bacterial clearance due to IFN-γ, but also detrimental effects of an overproduction or indeed Th1 making no contribution to the fight against *S. aureus* (Karauzum and Datta 2017).

T-helper type 2 (Th2) cells produce cytokines, including IL-4, IL-5 and IL-13 and play vital roles in parasite control and tissue repair (Allen and Sutherland 2014). Concerning their roles during a *S. aureus* infection, a similar picture as with Th1 emerges: an overexpression of Th2 cytokines characterises the atopic dermatitis, a widespread inflammatory disease of the skin, which is in most cases accompanied by a *S. aureus* infection. Even so, Th2 could positively influence, for example, chronic infections by balancing inflammatory and anti-inflammatory mechanisms (Karauzum and Datta 2017).

Th17 is a more recently discovered subspecies of T-helper cells that produces interleukin 17a, IL-17f, IL-21, and IL-22. The receptors for IL-17 and IL-22 are widely expressed on many epithelial tissues and play an important role in tissue immunity. Th-17 cells also lead to an enhanced neutrophil recruitment and hold a role in autoimmune disease (Korn et al. 2009). Ishigame et al. have shown that mice deficient in both IL-17a and IL-17f developed spontaneous mucoepithelial *S. aureus* infections. An increased susceptibility against systemic *S. aureus* has not been observed in these mice (Ishigame et al. 2009).

2.1.4.3 Immune Evasion

S. aureus is very skilled at evading the immune system, which is reflected in the approximately 40 so-called immune evasion proteins that the pathogen secretes. That number is very likely an underestimate, as there are still various *S. aureus* proteins which have no known function yet. Several of these evasion proteins target neutrophils, not surprisingly, considering their central status in the defence against *S. aureus* (de Jong et al. 2019). Evasion proteins are active on every step of the neutrophil defence. They block the extravasation through the endothelium, the chemotaxis and the activation of neutrophils, and also help the pathogen to resist opsonization and phagocytosis (de Jong et al. 2019).

The proteins employ various mechanisms to evade the immune system, most importantly the blocking of host factors, such as enzymes, immune receptors, or antimicrobial peptides. Alternatively, *S. aureus* secretes enzymes that degrade host components like DNA, which is part of extracellular traps, immune receptors and complement factors (Koymans et al. 2017). Staphylococcal toxin can kill immune cells directly, both extra- and intracellularly, after the pathogen has been already phagocytosed. Finally, staphylococcal proteins are also able to activate and modulate immune cells, foremost affecting the adaptive immune system (Koymans et al. 2017).

2.1.5 Methicillin-resistant *Staphylococcus aureus*

The history of antimicrobial resistance is nearly as old as the one of antimicrobial agents themselves. While the prognostic outcome of a *S. aureus* infection improved drastically in the early 1940s due to the introduction of **penicillin**, already in 1942 strains resistant to penicillin were recognized (Lowy 2003; Rammelkamp and Maxon 1942). This development started in the hospital setting, where only six years after introduction 25% of the strains were resistant. About fifteen to twenty years later, the same resistance against penicillin could be found in the community isolates. This pattern of antimicrobial resistance emerging in hospitals, under the selective pressure of antibiotics exposure, to be followed by a spreading in the community, is by now well established (Chambers 2001). *S. aureus* bases its resistance against penicillin on

the β-lactamase. This predominantly extracellular enzyme is encoded by the *blaZ* gene and hydrolyses the β-lactam ring, which renders β-lactam antibiotics, like penicillin, useless (Lowy 2003).

Methicillin was introduced at the end of the 1950s, and the first methicillin-resistant *S. aureus* (MRSA) strain was described in 1960, less than a year later. In fact, the first MRSA strain already emerged in the mid-40s, as shown by Harkings et al., using whole genome sequencing. This infers that the introduction of methicillin was not, as previously thought, the driver of MRSA evolution, but rather the widespread use of first generation β-lactams, like penicillin (Harkins et al. 2017). Even though methicillin is not used anymore today, as it has become replaced by less toxic and more stable isoxazolyl penicillins, like oxacillin, flucloxacillin, and dicloxacillin, the term methicillin-resistant *S. aureus* has endured (Lee et al. 2018). In contrast to the resistance to penicillin, which rests on plasmid-borne *blaZ* β-lactamase genes, the resistance to isoxazolyl penicillins of MRSA is mediated in most cases by the *mecA* gene, which encodes a penicillin binding protein, called PBP2' or PBP2a (Katayama et al. 2000; Dyke 1969). This low affinity penicillin binding protein offers a broad β-lactam resistance against penicillins, carbapenems, and cephalosporins (Chambers and DeLeo 2009). It should be noted that most MRSA isolates - at least those from animal sources - carry both ß-lactam resistance genes, *blaZ* and *mecA* (Krüger-Haker et al. 2023). During recent years, another *mec* gene, *mecC*, has emerged in *S. aureus* of human and animal origin (García-Álvarez et al. 2011; Shore et al. 2011). Nowadays, MRSA can be found worldwide, though the distribution rates vary distinctly. Particularly affected is the Asia-Pacific region, with rates over 60%, and parts of South America. In Europe, a clear north-south disparity exists, with rates under 5% in Scandinavia, compared to over 25% in many of the Mediterranean countries (Lee et al. 2018; Diekema et al. 2001).

Mirroring the preceding development of the penicillin resistance, MRSA was mainly a healthcare-associated occurrence until the 90s. Several risk factors, like long hospitalization, central venous catheters, prior antibiotic exposure and surgery, increase the likelihood of hospital-associated MRSA (HA-MRSA) colonisation and infection (Mao et al. 2019; Chambers 2001). The tragic cases of four children in the United States, who succumbed to infections with MRSA at the end of the 1990s, particularly shocked the public. None of the risk factors mentioned above applied, and no family members worked in healthcare or long-term care facilities (Centers for Disease Control and Prevention 1999). This community-associated MRSA (CA-MRSA) can infect healthy individuals and is distinguished from the traditional HA-MRSA strain by its increased fitness and virulence. This is aided by the fact that all CA-MRSA are carrying a novel type of methicillin resistance locus. Furthermore, the addition of specific toxins, like Panton-Valentine leukocidin (PVL) and high production of phenol-soluble modulins (PSMs) from the α-type, are playing important roles (Otto 2013). The most prominent clone of

the community-associated MRSA is called USA300. It is nearly single-handedly responsible for the CA-MRSA epidemic that has gripped the United States. First isolated in 1999, it quickly spread in the country, and by the year 2005, it had become the most common cause of SSTIs (Planet 2017). Only six years later, the clone had become the most common MRSA isolated from infections at all body sites. By this time, USA300 had not only supplanted other *S. aureus* strains, but a simultaneous rise of serious SSTIs indicated that the strain had added to the overall severity of the disease. Despite the widespread prevalence of USA300 in the United States, outside its borders the strain only managed to gain a foothold in the north of South America (Planet 2017). In contrast, CA-MRSA is comparatively rare in most European countries.

One additional source of infection outside the hospital setting is close contact to livestock. The nasal colonisation of workers in MRSA-positive stables lies at 77% to 86% and in Germany, livestock-associated MRSA (LA-MRSA) infections make up 10% of non-healthcare-associated cases. The first reports of LA-MRSA came from conventional pig farms in Europe, with most of these cases attributed to clonal complex CC398 (Cuny et al. 2015). The distribution of LA-MRSA seems dependant on the method of farming and husbandry system, as the prevalence in organic farms is much lower (van de Vijver et al. 2014; Cuny et al. 2012).

At the end of the 1990s the first cases of **vancomycin**-resistant *S. aureus* strains emerged, with the first reported case found in Japan in 1996 (Smith et al. 1999; Hiramatsu et al. 1997). This development is particularly worrisome because of its role as last line of defence against organisms with multiple resistances, including MRSA (Greenwood 1998).

Today, a number of additional antimicrobial agents are available to treat MRSA, but all of them are characterized by existing or emerging resistances (Vestergaard et al. 2019). Examples are daptomycin, a cyclic lipopeptide, teicoplanin as well as linezolid and tedizolid, two oxazolidinones (Brenciani et al. 2022; Schwarz et al. 2021; Gómez Casanova et al. 2017).

2.1.6 Host Specificity

Laboratory animals, specifically rodents, have been extremely important for enhancing our understanding of the *S. aureus* pathogenesis (Thammavongsa et al. 2013; Bubeck Wardenburg et al. 2007). Nevertheless, there are considerable differences between the immune system of mice and humans, not surprising considering that the evolution of both species has diverged between 65 and 75 million years ago (Mestas and Hughes 2004). This means that the immune reaction against invading pathogens, and consequently the outcome, vary substantially depending on the species. For example, the Panton-Valentine leukocidin is highly toxic to human (and rabbit) neutrophils, but not murine cells (Löffler et al. 2010). Murine neutrophils are also impervious to the bicomponent pore-forming toxins γ-hemolysin AB and CB, due to a different set of target receptors (Spaan et al. 2014). Humans are also much more

vulnerable to superantigen effects than mice (Faulkner et al. 2005). The reason for this lies in the higher binding affinity of superantigens to human MHC class II than to its murine counterpart (Krakauer 2005). For mice, an exposure to superantigens at $4x10^6$ µg per kg is not lethal, while for humans, dosages as low as 0.0013 µg per kg are deadly (Salgado-Pabon and Schlievert 2014). This markedly lower sensitivity of mice to *S. aureus* virulence factors combined with very different immune combat strategies against an infection have culminated in the fact that so far no successful animal vaccine trial could translate into a functional human immunization against *S. aureus* (Clegg et al. 2021; Salgado-Pabón and Schlievert 2014)

2.2 Humanized mice

2.2.1 Definition

Humanized mice have been defined by Schultz and co-workers as “immunodeficient mice that have been engrafted with human primary haematopoietic cells and tissues that generate a functional human immune system” (Shultz et al. 2012).

2.2.2 History

For the development of humanized mice, immunodeficiency in the animals is an essential precondition. The research started in the 1960s with the discovery of **nude mice**, who besides lacking fur, were also missing the thymus, which led to extremely low counts of blood leukocytes (Szadvari et al. 2016; Pantelouris 1968). The next significant step in the development of immunodeficient mice emerged about two decades later in the form of the **C.B-17 scid** mouse strain. Due to their severe combined immunodeficiency (scid) phenotype, these mice lack functioning T and B cells (Bosma et al. 1983). Partly driven by the escalating Aids pandemic at the time and the desperate need for better research models, the discovery that these C.B-17 scid mice could be engrafted with human cells and develop a rudimental human immune system, followed shortly after (Shultz et al. 2007; Mosier et al. 1988).

However, these early humanized mouse models were facing several limitations. The engraftment was very low, probably due to the murine innate immune system, which remained unchallenged by the scid mutation. In addition, the successfully engrafted human stem cells were hampered in their development and proliferation by a lack of suitable haemopoietic growth factors. The available murine cytokines were mostly unsuitable owing to a lack of species cross-reactivity. A further concern represented the so called “leaking” of T and B cells which signified a return of the adaptive immunity cells as the mice were aging (Greiner et al. 1998). The backcrossing of the scid mutation on the **non-obese diabetic (nod)** background presented the next advancement. This strain allowed a higher engraftment of human

hematopoietic cells and showed a marked decrease in NK cell activity (Shultz et al. 1995). A major advancement was achieved by the generation of a mutation of the **IL-2R y** chain. This subunit is necessary for the functionality of six cytokine receptors (IL-2; IL-4, IL7, IL9, IL15, IL21) (Manz 2007). A null mutation of this protein, in addition with the NOD-scid background, lead to mice which lack T cells, B cells, NK cells, and do not show the “leakiness” mentioned before. Furthermore, these mice have a normal life span and do not develop thymic lymphomas, in contrast to the NOD-scid strain. Due to a high engraftment of human hematopoietic stem cells, these mice present a very robust model, suitable for a wide range of *in vivo* studies. The three most commonly used strains incorporating the mutation of the *Il2rg* are the so-called NOG (NOD/Shi-scid Il2rg−/−), NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ), and BRG (Balb/c Rag2−/− Il2rg−/−) mice. The latter is not based on the NOD-scid background, but instead bases its immunodeficiency on a mutation in the *rag2* gene (Fujiwara 2018; Manz 2007; Shultz et al. 2005). Of these three strains, **NSG** shows the highest engraftment of hematopoietic stem cells (Brehm et al. 2010; McDermott et al. 2010).

However, these strains still face one major limitation: their grafts consist mainly of human lymphoid cells. To improve the myeloid compartment, a relatively recent development has been the so-called **NSG-SGM3** (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ) mouse. This mouse strain combines the immunodeficiency of the NSG mouse with a transgenic expression of three human myelo-supportive cytokines: the stem cell factor (SCF), the granulocyte–macrophage colony-stimulating factor (GM-CSF), and the interleukin-3 (IL-3) (Wunderlich et al. 2018). SCF, GM-CSF, and IL-3 are characterized by a lack of cross-reactivity between the two species (Manz 2007), and their introduction has opened new opportunities for the field of humanized mouse models. Unfortunately, this improved humanization has come at a price. Several authors have described a clinical phenotype in humanized NSG-SGM3 mice, characterized among others by weight loss, anaemia, splenomegaly, and eventual death. The animals become moribund starting from around 16 to 18 weeks after engraftment, while changes in the blood count, such as decreasing red blood cells are detectable weeks earlier. This complex of symptoms and lesions has been described as secondary hemophagocytic lymphohistiocytosis (HLH) or macrophage activation syndrome (MAS)-like disease (Janke et al. 2021; Yoshihara et al. 2019; Wunderlich et al. 2016). The term MAS is varyingly used synonymously with secondary HLH or described as a form of the latter. Apart from semantics, the principal pathological feature is clear: an unrestrained proliferation and activation of T-cells and macrophages which release large quantities of proinflammatory cytokines culminating in a “cytokine storm”. MAS/HLH can be triggered by rheumatic disorders, such as systemic juvenile idiopathic arthritis (sJIA), malignancies and viral infections (Henderson and Cron 2020; Crayne et al. 2019; Schulert and Grom 2014). In the case of humanized NSG-SGM3 mice, there are several indicators that the

overexpression of human GM-CSF is the driving force behind the secondary MAS/HLH-like disease. Mice with transgenic expression of GM-CSF displayed increased amounts of activated macrophages and died of tissue damages (Lang et al. 1987). Campbell and co-workers showed that injections of GM-CSF led to an aggravated collagen-induced arthritis (CIA) in mice, while GM-CSF knockout mice were nearly immune (Campbell et al. 1998; Campbell et al. 1997). In human patients, a deadly HLH developed after they received GM-CSF along with chemotherapy (Risti et al. 1994). Findings that mice expressing GM-CSF in T-cells develop a histiocytosis with similar symptoms as described above, e.g reduced survival, splenomegaly, and anaemia (van Nieuwenhuijze et al. 2014) underline the connection.
Apart from NSG-SGM3 mice (also called NSGS mice), there exists a number of other so called "next-generation mice", such as the DRAG mice (transgenic expression of HLA class II), the NSG-15 mice (expression of human IL-15), and many more (Gillgrass et al. 2020).

2.2.3 Humanization Process

Researchers who mean to create humanized mice, must not only decide which of the existing immunodeficient mouse strain to choose, but also which of the approaches to engraft a human immune system fits their purpose best.

2.2.3.1 Engraftment Models

Hu-PBL-SCID

In this model, human peripheral blood lymphocytes (PBL) are injected into severe combined immunodeficiency (SCID) mice. There are three ways to administer PBLs: intrasplenic, intraperitoneal, or by an intravenous injection into the tail vein. Human PBLs can be obtained easily from healthy adult blood donors and volunteers (King et al. 2009). This model is very well suited for the study of T cell function, with a fast engraftment of human $CD3^+$ T cells after one week. However, there is only a limited time window due to the development of graft-versus-host disease (GVHD) after four to eight weeks. This is not only to its disadvantage, as this model can be used to study the very disease (Walsh et al. 2017; Shultz et al. 2012).

Hu-SRC-SCID

By injecting SCID repopulating cells, meaning human CD34+ hematopoietic stem cells (HSC), the Hu-SRC-SCID model is created. The HSC can derive from several sources, specifically the bone marrow (BM), fetal liver (FL), umbilical cord blood (UBC), and G-CSF-mobilized peripheral blood. The HSC can be transferred by intravenous or intrafemoral injection. If newborn pubs are used, the hematopoietic stem cells are injected via the liver, the heart, or the face vein instead. This model allows the implanted stem cells to develop into numerous hematopoietic cell lineages including B and T cells, NK cells, and myeloid cells. However,

granulocytes, red blood cells and platelets are circulating in only very low numbers in the blood. Furthermore, thymocytes are educated in the murine thymus and therefore not HLA-, but H2-restricted (Walsh et al. 2017; Shultz et al. 2012).

SCID-Hu

The simultaneous implantation of human fetal thymus and liver pieces under the renal capsule provides a model previously used for the study of HIV infection of the thymocytes. In contrast to the Hu-SRC-SCID model, thymocytes are HLA-restricted. All other hematopoietic cell lineages besides the T cells are underrepresented, with low engraftment in the bone marrow and peripheral tissue (Shultz et al. 2012).

BLT

In this last model, fetal liver and thymus fragments from the same donor are implanted under the capsule of the kidney, and autologous hematopoietic stem cells, isolated from the liver, are additionally injected into the tail vein. The HSC engraft the murine bone marrow, which completes the name of this model, bone marrow, liver, thymus (BLT). The advantages of the BLT mice are numerous. A complete human immune system is engrafted, where all hematopoietic cell lines are developed, and in contrast to the Hu-SRC-SCID model, the T cells are HLA restricted. Importantly, this is the only model that allows the development of a human mucosal immune system. Despite all these merits, there are also serious limitations. The obligatory surgical expertise alone makes this model unattainable for many researchers. The acquisition of the necessary fetal material is difficult and the window for experimentation is limited in many laboratories due to the development of a GVHD-like syndrome of the BLT mice (Walsh et al. 2017; Shultz et al. 2012).

2.2.3.2 Conditioning

One important step to achieve a high rate of human stem cell engraftment is the conditioning of the mice by myeloablative drugs or irradiation prior the HSC injection (Shultz et al. 2005). The level of radiation used in experiments varies depending on the mouse strain and also on individual experience (McDermott et al. 2010). Typical dosages lie between 1 and 2,4 Gray (Gy) (Prince et al. 2017; Bryce et al. 2016; Coughlan et al. 2016; Billerbeck et al. 2011). An alternative to radiation are myeloablative drugs, such as busulfan, which is also used in medicine to prepare patients for stem cell transplantation (McCune and Holmberg 2009). The dosages injected into mice may vary, but lie around 30 to 40 mg/kg (Wunderlich et al. 2014; Hayakawa et al. 2009). Though conditioning before engraftment is common, it can have side effects and, depending on the model and mouse strain, it is not always necessary to achieve adequate human cell chimera (McIntosh et al. 2015; Tseng et al. 2015).

2.2.4 Fields of Research using Humanized Mice

2.2.4.1 Humanized mice and *Staphylococcus aureus*

To date, apart from the studies that are part of this thesis, there are only four published papers that have used humanized mice for *S. aureus* experiments. The first was published by **Knop et al.**, who were able to show that humanized mice have a higher susceptibility towards *S. aureus* (Knop et al. 2015). To create humanized mice, they irradiated newborn pups with 1 Gy and afterwards injected them intrahepatically with $2x10^5$ $CD34^+$ human HSC, isolated from human cord blood (Ernst et al. 2013). After three to five months, the mice were injected intraperitoneally with *S. aureus*. In comparison to the control groups (C57BL/6 wild-type mice, non-engrafted NSG mice, irradiated NSG mice), the humanized mice showed a significantly higher weight loss and a marked increase of mortality. A systemic spreading was also detected, with humanized mice representing the highest bacterial burden, specifically in the lung, spleen, liver, and kidney. Furthermore, the *S. aureus* infection led to increased activation and apoptosis of T cells in humanized, but not in the wild-type mice (Knop et al. 2015).

Tseng et al. published the second study using humanized mice as a model for *S. aureus* infections. As described above, newborn NSG mice were injected intrahepatically with hematopoietic stem cells isolated from human umbilical cord blood. However, in this study the pups were not irradiated before and injected with only $1x10^5$ $CD34^+$cells. As a control group, Tseng et al. also created murinized mice by injecting $1x10^5$ Balb/c bone marrow cells intrahepatically. These two groups, plus Balb/c wild-type mice, were subsequently used for a skin infection model. At 12 to 16 weeks, the flanks of the animals were shaved and a *S. aureus* suspension injected into the subcutis. Humanized mice developed markedly larger lesions of the skin than the control groups, with 10 to 100-fold less bacteria necessary to cause analogous lesions in humanized mice than in the control groups. No significant differences in the bacterial counts could be found (Tseng et al. 2015). In addition, the sizes of the skin lesions of the humanized mice could be correlated with the level of chimerism: the higher the human $CD45^+$ engraftment rate, the bigger the skin lesions. The group also investigated the role of polymorphonuclear leukocytes (PMN) and Panton-Valentine leukocidin (PVL) in skin lesions. Human PMN were transferred in NSG mice and subsequently the mice were infected with PVL-negative *S. aureus* and the wild-type strain. The latter led to markedly bigger skin lesions (Tseng et al. 2015).

In the third study, **Prince et al.** irradiated six weeks old NSG mice with two Gy prior to injecting intravenously $2x\ 10^5$ $CD34^+$ cells isolated from fetal liver. In addition, analogous thymus tissue was transplanted under the kidney capsule. Humanized mice proved again to be more susceptible to *S. aureus* infections than the control groups (wild-type, murinized NSG, non-engrafted NSG, NOG mice), this time in a lung infection model. The bacterial burden in lung

tissue and airways was up to 32-fold higher than compared to the C57BL/6J wild-type mice (Prince et al. 2017). Prince et al. also showed that the Panton-Valentine-Leukocidin (PVL) plays a role in the pathogenesis of the infection, by using mutant strains and PVL antibodies. Humanized mice infected with the wild-type bacterial strain had a 57% higher bacterial count in the lung tissue than in the absence of PVL (Prince et al. 2017). The number of macrophages was also 95% higher in the mice infected with the deletion mutant (Prince et al. 2017).

Like its predecessors, the most recent paper by **Muthukrishnan et al**. also attributes humanized mice with a higher susceptibility towards *S. aureus*. Prior to an intravenous injection with 2x 10^5 $CD34^+$ hematopoietic stem cells, three weeks old NSG mice were radiated with 1 Gy. After 17 to 21 weeks, the humanized NSG mice (huNSG) and two controls groups (non-engrafted NSG and C57BL/J6 wild-type mice) underwent a surgical procedure to implant a transtibial pin that had been inoculated with *S. aureus.* The mice were monitored for 14 weeks before euthanization: the humanized mice lost more body weight, failed to recover the loss over time and appeared sicklier in general. Furthermore, the humanized group showed increases in bacterial dissemination into other organs, peri-implant osteomyelitis, and staphylococcal abscess communities compared to the control groups. Both humanized NSG and non-engrafted NSG mice had a markedly higher bacterial burden in the tibia and the surrounding soft tissue, and to a lesser extent also on the implant, than the wild-type group (Muthukrishnan et al. 2021). The group also investigated the role of T cells during the *S. aureus* mediated osteomyelitis and demonstrated that infected humanized mice showed significantly more $CD3^+$ T cells, including its $CD4^+$ and $CD8^+$ subpopulations, in the spleen than uninfected huNSG. In addition, using multicolour immunofluorescence histochemistry, significant trafficking and induction of T cell clusters close to the staphylococcal abscess communities could be found (Muthukrishnan et al. 2021).

2.2.4.2 Overview of further Applications for Humanized Mice

Their intrinsic traits and qualities make humanized mice an interesting and useful animal model in various fields of research, ranging from the study of graft versus host disease (King et al. 2009), allergy (Ito et al. 2013) to cancer (Choi et al. 2016). The area in which humanized mice have been used most widely are infectious diseases. The research of human specific pathogens can especially profit from this development, particularly as the keeping of and experimenting on primates poses many difficulties, both practically and ethically. In the area of viral infections, those caused by HIV-1, EBV, HCV and dengue are the most extensively studied ones (Fujiwara 2018; Akkina 2013). The applications of humanized mouse models for the research of *S. aureus* infections have been described above, but also for the study of other bacterial infections humanized mice have proved useful. For example Libby et al. were able to show that humanized mice infected with *Salmonella enterica* subsp. *enterica* serovar Typhi,

succumbed to the disease, showing pathological responses resembling human typhoid fever (Libby et al. 2010). Further examples are infections caused by *Mycobacterium tuberculosis* (Heuts et al. 2013), *Streptococcus agalactiae (Ernst et al. 2013)* or *Borelia hermsii* (Vuyyuru et al. 2011).

3 Aims of the Study

This study aims at elucidating whether humanized mice are superior experimental models to investigate the effects of *S. aureus* deep tissue infections. For this purpose, two immunodeficient mouse strains, NSG and NSG-SGM3, were bred, humanized by injecting cord blood isolated hematopoietic stem cells, and finally infected in the thigh muscle with *S. aureus.*

Humanized NSG mice have been used in a small number of *S. aureus* studies before, but none using a thigh infection model, which covers both the acute and chronic stages of the infection. Subsequently, the first aim of this thesis was to see how the human immune system in the humanized NSG mice reacts to a longer infection period, and whether differences between the murine and the human immune reaction can be detected.

Considering the fact that myeloid cells, specifically neutrophils, play an important role in the human defence against *S. aureus,* the question arose whether a more advanced mouse strain, such as NSG-SGM3, which combines the marked immunodeficiency of NSG mice with three added myeloid enhancing cytokines, might be even better suited to represent the condition that prevails during staphylococcal infections in humans. Consequently, the second aim of this thesis was to compare the susceptibility of humanized SGM3 mice to *S. aureus* with that of the humanized NSG mice.

4 Publications

4.1 Publication I

Sophia Hung, Liane Dreher, Joachim Diessner, Stefan Schwarz,
Knut Ohlsen and Tobias Hertlein

MRSA Infection in the Thigh Muscle Leads to Systemic Disease, Strong Inflammation, and Loss of Human Monocytes in Humanized Mice

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Sophia Hung	First author	Performed the experiments, analysed the data, wrote the first draft of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Liane Dreher	Co-author	Contributed to the performance of the experiments and the analysis of the data Contributed to manuscript revision, read, and approved the submitted version

Joachim Diessner	Co-author	Contributed to conception and design of the study, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Stefan Schwarz	Co-author	Contributed to conception and design of the study, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Knut Ohlsen	Co-author	Contributed to conception and design of the study, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Tobias Hertlein	Co-author	Contributed to conception and design of the study, contributed to the performance of the experiments and the analysis of the data, co-wrote parts of the first draft of the manuscript Contributed to manuscript revision, read, and approved the submitted version

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MRSA Infection in the Thigh Muscle Leads to Systemic Disease, Strong Inflammation, and Loss of Human Monocytes in Humanized Mice

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MRSA (Methicillin-resistant *Staphylococcus aureus*) is the second-leading cause of deaths by antibiotic-resistant bacteria globally, with more than 100,000 attributable deaths annually. Despite the high urgency to develop a vaccine to control this pathogen, all clinical trials with pre-clinically effective candidates failed so far. The recent development of "humanized" mice might help to edge the pre-clinical evaluation closer to the clinical situation and thus close this gap. We infected humanized NSG mice (huNSG: (NOD)-*scid IL2R*$_\gamma^{null}$ mice engrafted with human CD34+ hematopoietic stem cells) locally with *S. aureus* USA300 LAC* *lux* into the thigh muscle in order to investigate the human immune response to acute and chronic infection. These mice proved not only to be more susceptible to MRSA infection than wild-type or "murinized" mice, but displayed furthermore inferior survival and signs of systemic infection in an otherwise localized infection model. The rate of humanization correlated directly with the severity of disease and survival of the mice. Human and murine cytokine levels in blood and at the primary site of infection were strongly elevated in huNSG mice compared to all control groups. And importantly, differences in human and murine immune cell lineages surfaced during the infection, with human monocyte and B cell numbers in blood and bone marrow being significantly reduced at the later time point of infection. Murine monocytes in contrast behaved conversely by increasing cell numbers. This study demonstrates significant differences in the *in vivo* behavior of human and murine cells towards *S. aureus* infection, which might help to sharpen the translational potential of pre-clinical models for future therapeutic approaches.

Keywords: humanized mice, MRSA - methicillin-resistant *Staphylococcus aureus*, monocyte, bacterial infection model, inflammation, NSG, staphylocccal infection/epidemiology

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INTRODUCTION

Staphylococcus aureus is one of the most successful pathogens of our time and an efficient colonizer, which can be transmitted in community and healthcare. It causes a wide range of infections from skin and soft tissue infections to life-threatening diseases like pneumonia, endocarditis and bacteremia (1, 2). Its high genetic variability and flexibility as well as its infamous feature of developing or acquiring antibiotic resistance makes this pathogen a major problem for our healthcare systems and causes high costs and many deaths every year. Its Methicillin-resistant variant (MRSA) is the second-most leading cause of attributable deaths to antibiotic-resistant bacteria world-wide (3).

Despite the undeniable and urgent need for new therapeutics, particularly for a vaccine, the development has been hampered by poor correlation of pre-clinical and clinical efficacy (4, 5). Two explanations have been proposed for this discrepancy since most pre-clinical studies were performed in mouse models: (i) *S. aureus* is well known for a long list of virulence factors like bi-component toxins, surface proteins and immunomodulators, that act with different efficiency against human and murine immune components (6–8) and (ii) the human's and the mouse's immune system react differently to an encounter with *S. aureus* (9).

In consequence, the main idea to resolve this problem has been to adapt the pre-clinical models in order to become more representative for the clinical situation in humans. "Humanized mice" – mice with a human immune system – might be a first step to meet these needs. The most widespread type of humanized mice is the NSG ((NOD)-*scid IL2R*γ^{null}) mouse engrafted with human (CD34+) hematopoietic stem cells (huNSG), which constitute a human immune system in this mouse (10, 11). A limited number of earlier studies about *S. aureus* infection in humanized mice have already proven, that these mice are more susceptible to infection with this pathogen (12–15). Since most of these studies focused on the short-term or local effects of *S. aureus* challenge in humanized mice, we wondered how the human immune cells adapt during a longer period of infection and whether we can see differences emerge between the murine and the human immune response over the course of infection.

To this end, we infected humanized mice locally in the thigh muscle with *S. aureus* and monitored the local course of infection as well as the systemic spreading of the bacteria. Furthermore, we correlated the rate of humanization in these mice with the severity of infection. Finally, in an effort to examine the immune system's struggle between fighting the pathogen on one hand and limiting the inflammation on the other hand, we characterized the recruitment of human and murine immune cells from/to bone marrow, spleen and blood, as well as the repertoire of human and murine cytokines in blood and the infected thigh muscle.

MATERIAL AND METHODS

Ethics Statement

All animal studies were approved by the local government of Lower Franconia, Germany (approval numbers 55.2-2532-2-836 and 55.2-2532-2-1129) and performed in strict accordance with the guidelines for animal care and experimentation of the German Animal Protection Law and the DIRECTIVE 2010/63/EU of the EU. The animals were housed in cages under standardized lighting conditions and had *ad libitum* access to food and water. All *in vivo* imaging was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. All experimentations with anonymized and non-traceable human cord blood were approved by the ethics committee of the University Würzburg.

Humanization and Murinization Procedure (Incl. CD34+ Isolation)

Female and male NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice from the Jackson Laboratories (Bar Harbor, ME, USA) or female Balb/c mice (BALB/cJRj, Janvier labs, Le Genest-Saint-Isle, France) were used for all experiments. Human CD34+ hematopoietic stem cells were isolated from human cord blood by magnetic separation (EasySep™ Human Cord Blood CD34 Positive Selection Kit II, STEMCELL technologies, Cologne, Germany) following the manufacturer's protocol and purity controlled by flow cytometry. Humanized NSG (huNSG) mice were generated by engraftment of 100,000 hCD34+ cells (of a donor mix) at the age of 6 – 8 weeks, similar to the procedures described in earlier publications (15–18). Briefly, mice received whole-body irradiation with a sub-lethal dose of 2 Gy and hematopoietic stem cells were administered intravenously 2 hours later. For the generation of murinized NSG mice (muNSG), mice were treated as the huNSG mice, but received 100,000 bone marrow cells from a Balb/c donor instead of human CD34+ cells. The peripheral blood of huNSG mice was analyzed for the presence and frequency of murine CD45+, as well as human CD45+, CD3+ and CD20+ cells at week 18 post engraftment by flow cytometry.

Thigh Infection Model (Incl. Bacterial Burden)

HuNSG mice were infected at 18 weeks post engraftment. Therefore, the left thigh of each mouse was shaved, disinfected and 1 x 10^8 CFU of bioluminescent *S. aureus* LAC* *lux* (19) was injected into the muscle as described before (20). The infection dose was generated from an overnight shaking culture (at 37°C) in B medium by pelleting the bacteria, resuspending them and diluting them to the final concentration in sterile 0.9% NaCl solution. Age-matched muNSG, wild-type NSG and Balb/c mice served as controls to investigate differences during the course of infection. The wellbeing of each mouse was inspected and scored every 12 hours p.i. and the weight measured every 24 hours. Mice were either sacrificed at day 2 or at day 7 p.i. with the exception of seven huNSG mice and one wild-type NSG mouse that reached the humane end point of the experiment prematurely. At each end point, we harvested the infected thigh muscle, kidneys, liver, spleen and heart, as well as peripheral blood and bone marrow from tibia and femur from each mouse. The thigh muscle, kidneys, liver and heart were homogenized in 0.9% NaCl and serial dilutions were plated on B agar plates in order to

determine the bacterial burden. The spleens were homogenized by pressing through a 70 µm filter with 0.9% NaCl. Serial dilutions of this cell homogenate were then plated on B agar plates to determine the bacterial burden. Cell suspensions from the bone marrow were harvested by flushing both femurs and tibias of each mouse with 0.9% NaCl solution.

In Vivo Bioluminescence Imaging (BLI)

All infected mice were monitored by *in vivo* Bioluminescence Imaging (BLI) throughout the course of infection as described earlier (21). Briefly, starting at 5 min p.i. and followed by 24 h inspection intervals, the luminescence signal of each mouse was measured from dorsal view with a Lumina II bioluminescence imager (PerkinElmer, Waltham, MA, USA). The signal of the infected thigh muscle was measured by LivingImage 3.2 software (PerkinElmer, Waltham, MA, USA) within a region of interest with same geometry and size for each mouse and time point (imaging settings: exposure, 120 s; FStop, 1; excitation, block; emission, open; FOV, D; height, 1.5 cm).

Flow Cytometry

All flow cytometric analyses were performed and analyzed on a MACSQuant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Human CD34+ hematopoietic stem cell purity was checked by staining the isolated cells with anti-human CD34 and anti-human CD3 antibodies. Cells were only used for humanization, when purity was > 85% CD34+ cells of all cells and CD3 T cells < 1% of all cells. At the end of the humanization period, the peripheral blood was stained after red blood cell lysis with anti-mouse CD45 and anti-human CD45/CD3/CD19 antibodies to monitor the rate of humanization (which was hCD45+ cells/(hCD45+ cells + mCD45+ cells)). Spleen cell suspensions were stained after red blood cell lysis with anti-human CD45/CD3/CD19 antibodies, bone marrow cell suspensions with anti-human CD45/CD14/CD66b/CD19 antibodies and peripheral blood at the end of the infection experiments with combinations of anti-human CD45/CD3/CD4/CD8/CD19/CD14/CD66b and anti-mouse CD45/Ly6C/Ly6G antibodies. The gating strategy is outlined in **Figure S4**. All antibodies were supplied by Miltenyi Biotec (Bergisch Gladbach, Germany).

Determination of Cytokine Levels and Myeloperoxidase Activity

For determination of cytokine levels and myeloperoxidase activity, the thigh muscle homogenate was centrifuged at 3,000 x g for 5 minutes and the supernatant stored at -80°C until further processing. The peripheral blood samples were agglutinated overnight at 4°C, then centrifuged at 15,000 x g for 15 minutes, the serum harvested and stored at -80°C until cytokine measurement. Levels of human or murine MCP-1, IL-1β, IL-6, IL-10, IL-17A, IFN-γ and TNF-α in the infected thigh muscles or peripheral blood were measured with custom-mixed Luminex assays from Bio-Techne (Wiesbaden, Germany) following the manufacturer's manual. As internal cross-reactivity controls, we applied samples of the cytokine/chemokine standards of the mouse cytokine kit for the measurements with the human cytokine kit and vice versa, but could only detect neglectable cross-reactivity. Myeloperoxidase activity in the infected thigh muscle was measured with small adaptions as described earlier (13). Briefly, 50 µL supernatant of an 1.25% dilution of thigh muscle homogenate in PBS was mixed 1:1 with 3,3',5,5'-tetramethylbenzidine (TMB) (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 15 minutes at room temperature. The reaction was stopped by adding another 50 µL 2N H_2SO_4 to the reaction and the absorbance at 450 nm determined with a microplate reader. Results are expressed as arbitrary units (AU).

RESULTS

Humanized NSG Mice Are More Susceptible to Localized Infection With MRSA

Since a limited number of earlier studies *of S. aureus* infection in humanized mice showed higher susceptibility (12–15), we wondered whether the severity of *S. aureus* infection in our deep-tissue abscess model is increased, too. Therefore, we humanized 6 – 8 weeks old NSG mice by the administration of human cord-blood isolated CD34+ hematopoietic stem cells. After 18 weeks of engraftment, we checked the frequency and number of human CD45+ cells in the blood and included only mice with more than 10% human CD45+ blood cells among all CD45+ cells (humanization rate) into the infection experiments. The average humanization rate for the mice applied in this study was 25.1% (range: 10.9 – 54.5%). We applied three control groups: wild-type NSG mice, wild-type Balb/c mice, which are regularly used to investigate *S. aureus* infection, and murinized NSG (muNSG) mice, which served to exclude a possible influence of the humanization process on the outcome of the infection experiments. The wild-type NSG mouse group was not irradiated since we found no difference in the number of murine neutrophils or monocytes at 18 weeks post irradiation in a pilot experiment (with n = 3), which was in accordance with earlier results by others (22). The murinized mice were generated by the same procedure as humanized mice but received murine bone marrow cells (from Balb/c mice) instead of human CD34+ hematopoietic stem cells. This engraftment resulted in the establishment of a murine instead of a human immune system in otherwise immunodeficient NSG mice.

Applying bioluminescence imaging on a daily basis during the *S. aureus* infection allowed to monitor the bacterial burden and local spreading during the course of the infection (exemplary mice are depicted in **Figure S1**). The humanized mouse group showed a significantly higher signal intensity than all other groups as early as 24 hours p.i. and remained significantly stronger throughout the experiment (**Figure 1A**).

This observation was confirmed by the bacterial burden in the infected thigh muscle (**Figure 1B**). The infected thigh muscles of HuNSG showed an almost 10-fold higher bacterial load at day 7 p.i. compared to all other groups, including immunodeficient wt NSG mice. Of particular interest in this regard is, that while all other mouse groups were able to reduce or control the number of bacteria in the infected thigh, the number of bacteria in the HuNSG group increased significantly ($p = 0.0187$) between day 2 and day 7 p.i. and was at both time points higher than the initial inoculum of 10^8 CFU.

Impaired Survival of HuNSG After Localized Infection and Signs of Systemic Disease

The wellbeing of each individual mouse was judged daily based on a score sheet throughout the whole infection experiment and score points assigned accordingly. The score sheet included physical signs of disease like weight loss (**Figure S2**) or heavy breathing and behavioral signs like reduced activity or hunchback posture. The only signs of disease (besides the abscess formation) encountered in all mice except the huNSG group and one wild-type NSG mouse was a moderate loss of weight, especially in the first two days after infection (**Figure S2**). The huNSG group, in contrast, lost weight continuously and heavily during the whole course of infection and showed additionally severe signs of disease. In consequence, 7 of 20 mice from the huNSG group (and one wild-type NSG mouse) reached the humane end point, which defined the maximum of animal suffering that is acceptable for our experimental purpose. Since these mice had to be sacrificed and removed from the experiment, we designated them as "dead" and plotted them in a survival plot to visualize the impact of localized thighmuscle infection with *S. aureus* for each group (**Figure 2A**). The huNSG mouse group showed significantly inferior survival to all other

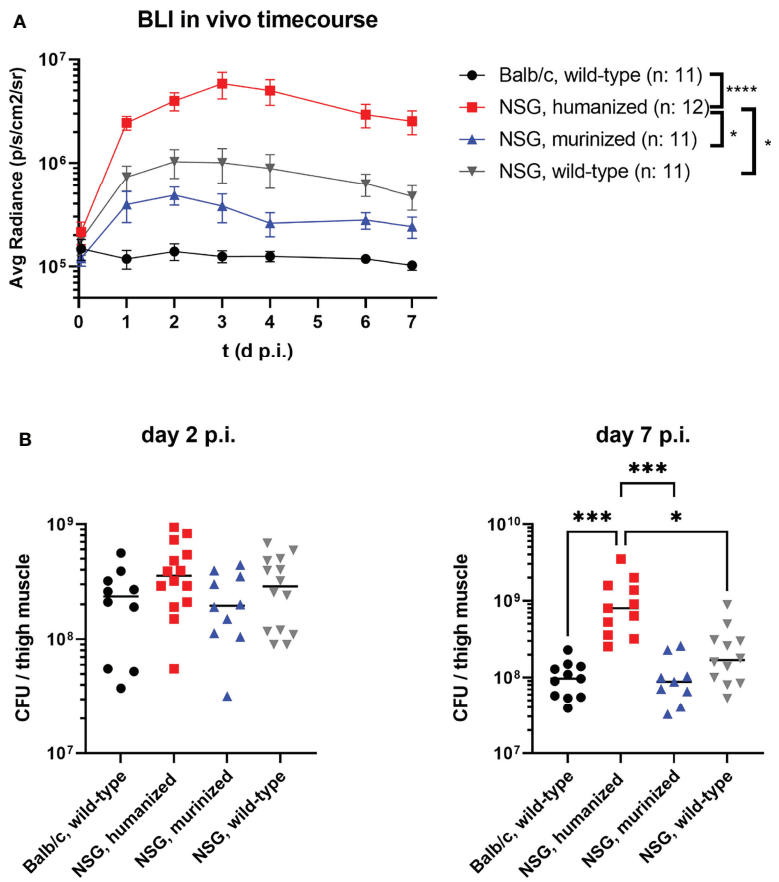

FIGURE 1 | The course of MRSA thigh muscle infection in humanized and control mouse groups. Mice were infected with $1x10^8$ CFU *S. aureus* LAC* *lux* into the left thigh muscle. **(A)** Bioluminescence signal of the luciferase-expressing bacteria at the primary site of infection (thigh muscle) was measured every 24 hours. Depicted are the mean radiance levels +/- SEM for each respective group. Statistical significance was tested for each time point with Kruskal-Wallis test. **(B)** The infected thigh muscles were recovered at either day 2 or day 7 p.i., homogenized and plated in serial dilutions on agar plates to determine the bacterial burden. Shown are the individual colony-forming-units (CFU) per mouse and the corresponding medians per group. Statistical significance was determined by Kruskal-Wallis with Dunn's post-test (*$p \leq 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

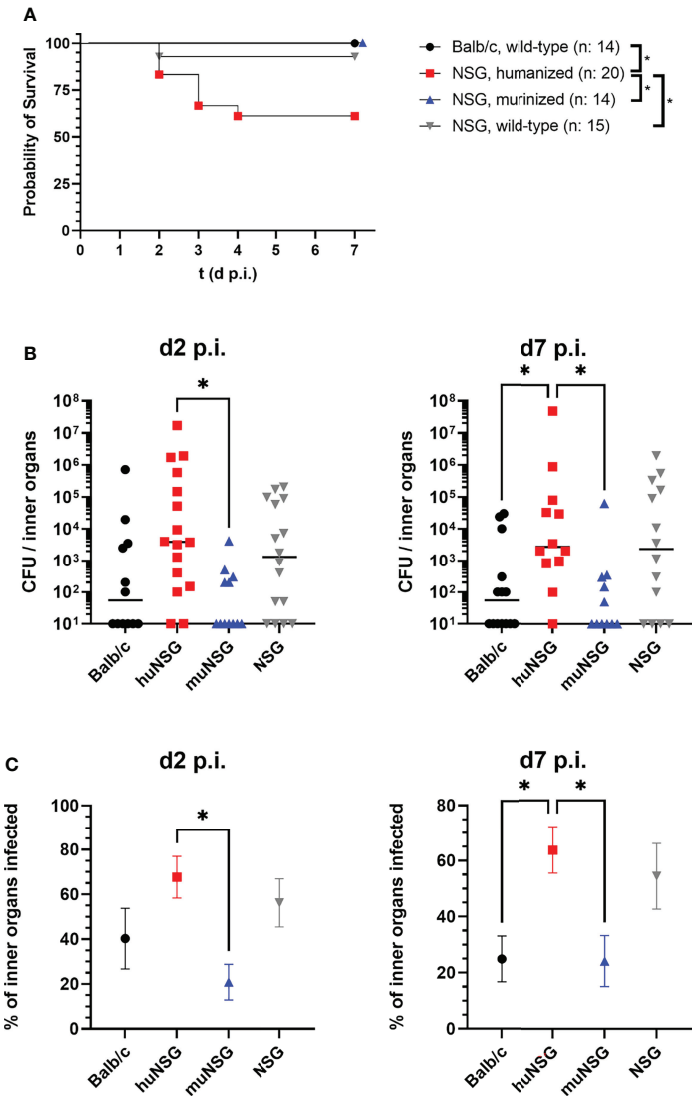

FIGURE 2 | Mortality of the locally infected mice, signs of systemic infection and humanization rates of the humanized mice groups. **(A)** Seven of 20 humanized mice and one of 14 wild-type NSG mice reached the humane end point during the course of infection. These mice were removed from the experiment and counted as "dead". Statistically significant differences between the groups were tested with Log-rank (Mantel-Cox) test. **(B)** The spleen, heart, liver and kidneys were recovered at the indicated time points and the bacterial burden determined. Displayed are the individual values per mouse and the corresponding median per group. **(C)** The percentage of tested inner organs (kidneys, liver, spleen and heart) with bacterial burden per animal was calculated and the mean +/- SEM per group is displayed (n: huNSG = 12; NSG, wild-type = 14; Balb/c = 14; muNSG = 11). Statistical significance was tested with Kruskal-Wallis with Dunn's post-test **(B, C)** (*$p \leq 0.05$).

mouse groups which raised the question, how local *S. aureus* infection in the thigh muscle could cause this dramatic escalation of disease.

A possible explanation for this severity of disease might be spreading of bacteria from the local site of infection to inner organs, thus causing systemic infection. This would be particularly interesting since the applied *S. aureus* USA300 derivate is notorious for causing skin and soft tissue infections, but also for systemic, life-threatening infections (1, 23).

In order to test this hypothesis, we investigated the bacterial burden in liver, kidneys, heart and spleen of all mice. The bacterial colonization of these organs was highly diverse and showed no clear picture for each organ at its own, but gave a distinct pattern, when all investigated inner organs were assumed as one combined system. Adding the numbers of all bacteria in these organs showed a significantly higher bacterial burden in the inner organs of huNSG mice compared to Balb/c and muNSG group mice (**Figure 2B**). Only the wild-type NSG mouse group

showed similarly high numbers of systemic bacteria. However, not only the total number of bacteria in the tested inner organs was higher for huNSG and NSG mice, the percentage of infected inner organs was also significantly elevated (**Figure 2C**).

The Stronger the Humanization Rate the Higher the Chance to Succumb to Infection

The finding, that humanized mice succumbed to infection following a local challenge with *S. aureus* was rather surprising, and exceeded our first hypothesis of humanized mice being more susceptible than non-humanized ones considerably. We wondered whether we could more precisely determine the factors which caused this increased susceptibility and shift from local to systemic infection. The first question we asked in this regard was whether the rate of humanization (hCD45+/(hCD45+ and mCD45+) could serve as a predictor for the severity of infection within the humanized mouse group. Those humanized mice, which were either assigned to the day 7 p.i. group or reached the humane end point during the experiment, were, therefore, grouped into three groups dependent on their rate of humanization prior to infection. And indeed, the group with the highest rate of humanization prior to infection included significantly more mice which reached the humane end point within 7 days post thigh muscle challenge than the other two groups (**Figure 3A**). The rate of humanization was thus from a retrospective point of view a very good predictor for a humanized mouse's chance of survival.

But also reviewing our data from the end-point perspective, namely is there a difference in the rate of humanization between mice which survived until day 7 p.i. (d7 group) and those that reached the humane end point, led to a similar interpretation: the mice that succumbed to infection had an overall higher rate of humanization (prior to infection) than those that survived (**Figure 3B**). Of note, there was no difference in the rate of humanization prior to infection between the day 2 p.i. group (d2 group) and the d7 group or the huNSG that reached the humane end point.

The rate of humanization in blood, spleen and bone marrow was in addition measured at the end point of the d2 and d7

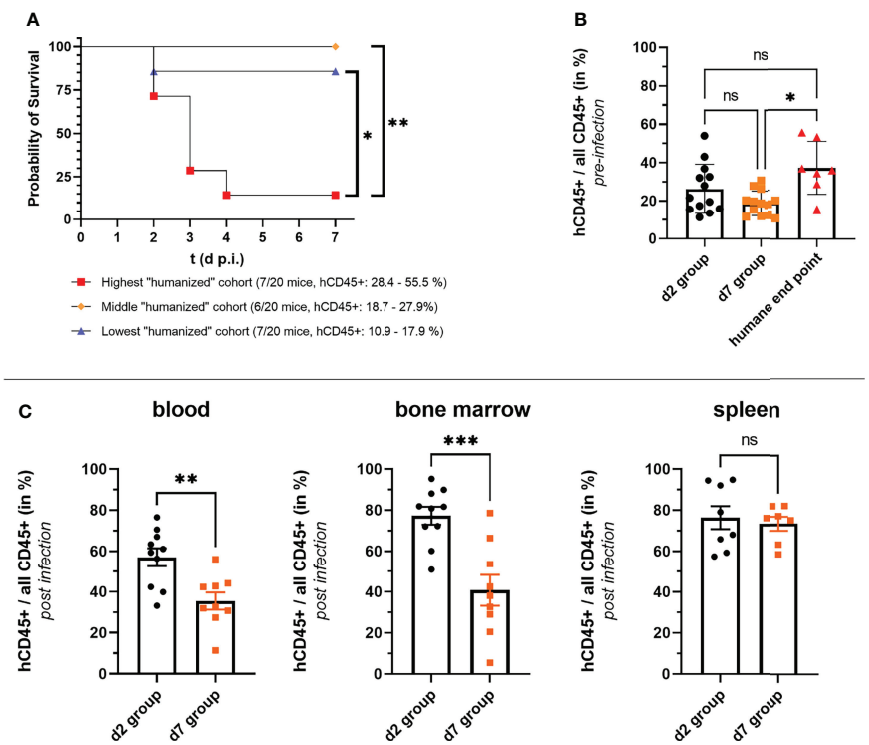

FIGURE 3 | Correlation of rate of humanization (hCD45+ cells/all CD45+ cells) and severity of infection. **(A, B)** The numbers of human and murine CD45+ cells were determined by flow cytometry before mice were infected with *S. aureus*. **(A)** HuNSG mice which either reached the humane end point of the experiment or were assigned to the day 7 p.i. group were ordered dependent on their rate of humanization into three groups. All but one mouse that reached the humane end point was in the group with the highest rate of humanization as depicted in the survival graph. Statistical significance was tested with the Log-rank (Mantel-Cox) test. **(B)** The rate of humanization of each huNSG mice in the experiment is depicted according to the outcome/end point of the experiment. Surviving mice were either assigned to the day 2 or day 7 p.i. end point groups, while the seven huNSG mice which reached the humane end point prematurely between day 2 and day 7 p.i. were assigned to the "humane end point group". **(C)** The rate of humanization at the end of the infection experiment was additionally measured by flow cytometry for the d2 and d7 groups in blood, spleen and bone marrow. Statistical significance was tested by Kruskal-Wallis with Dunn's post-test (ns, not significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

groups. Notably, the mice from the d7 group had a significantly lower humanization rate than the d2 group mice in blood and bone marrow at their respective end points (**Figure 3C**), although their blood humanization rates prior infection were similar (**Figure 3A**). No differences between both groups could be seen in the spleen.

Stronger Inflammation in HuNSG

Since the presence of human immune cells in huNSG was prominently linked to a more severe infection, we asked next, whether there are differences in the human and murine immune response and inflammation. First, we checked whether the myeloperoxidase activity, a main component of neutrophils to combat bacterial infection, was different between the huNSG mice and the control groups in the infected thigh muscle. Since we found no differences in the levels of myeloperoxidase activity between the groups or time points (**Figure S3**), we focused on the next question which was whether the pattern or the levels of human or murine cytokines/chemokines were different.

Therefore, the levels of human and murine MCP-1, IL-1β, IL-6, IL-10, IL-17A, IFN-γ and TNF-α in the blood and the infected thigh muscle were measured at days 2 and 7 p.i. The levels of all tested signaling molecules was in general lower in the blood than at the site of infection (**Figure 4**). The levels of IL-17A and IFN-γ were in all groups either below or at the detection limit. The huNSG mice delivered overall higher levels of murine MCP-1, IL-6 and IL-10 in the blood than the other groups and had in addition similarly high levels of their human counterparts in the blood, too. It is important to note in this regard, that purified human cytokines/chemokines delivered only neglectable signal in the murine Luminex assay and vice versa. The pattern was similar, albeit at much higher levels, for MCP-1 and IL-6 in the

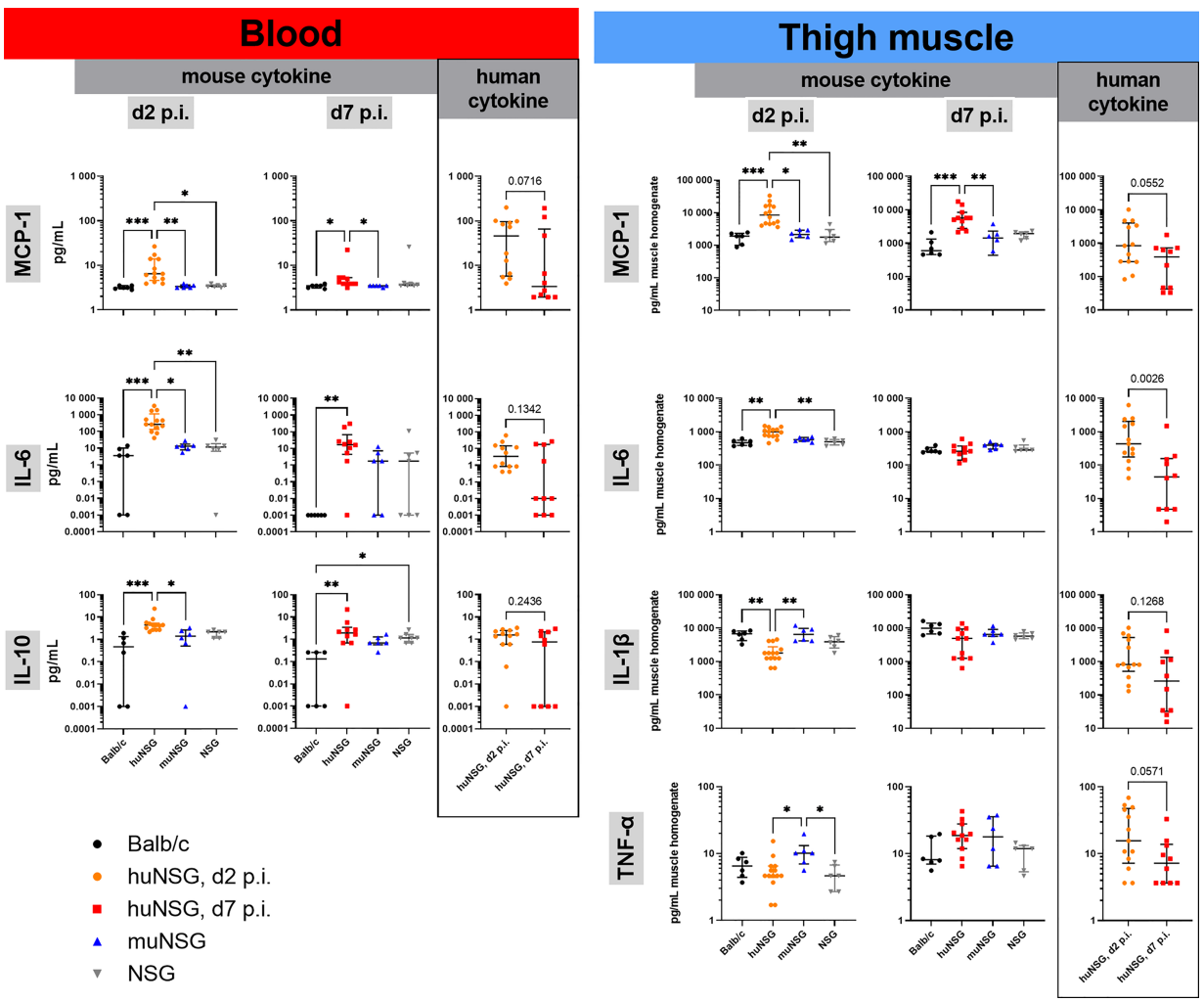

FIGURE 4 | Cytokine levels in blood and infected thigh muscle at early and late time point of infection. Displayed are the individual levels per mouse and the median with interquartile range for each respective group/time point. Levels in blood serum or muscle homogenate were determined with customized Luminex panels. Statistical significance was tested by Kruskal-Wallis with Dunn's post-test for the murine cytokines or Mann-Whitney-test for the human counterparts (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

infected thigh muscle (**Figure 4**). The huNSG mice showed higher levels than all other groups and had in addition human cytokines/chemokines at similar levels than murine ones. The levels of murine IL-1β and TNF-α was slightly lower for the huNSG mice, but the levels were in similar range when their human counterparts were added. The levels of murine MCP-1, IL-10 and IL-1β were in both, blood and muscle at similar levels at day 2 and day 7 p.i., while the level of IL-6 decreased. A completely different pattern could be seen for the human cytokines/chemokines of the huNSG mice. Human MCP-1, IL-1β, IL-6 and IL-10 were all lower at day 7 compared to day 2 p.i. (**Figure 4**), thus indicating a clearly different behavior of the human and the murine immune system, even within the chimeric huNSG mice.

Levels of B and T Cells at Early and Late Infection

This difference in the signaling of human and murine immune systems raised the question whether the immune cell lineages behave differently, too? Since the dominating fraction of the human immune system in huNSG mice consists of T and B cells, we first focused on their numbers in blood, spleen and bone marrow. Since we already knew from the determination of the rate of humanization in the blood, that the surviving huNSG mice (d7 group) showed a lower rate of humanization at day 7 than the d2 group (see **Figure 3C**), we assumed that their prevalence might be lower in the blood at day 7 p.i. However surprisingly, we saw no differences in their overall cell numbers (**Figure 5**). The number of human CD19+ B cells in the bone marrow was significantly lower at day 7 than at day 2 p.i., but unchanged in the spleen. The T cell numbers on the opposite increased in the spleen between both time points. The increase of T cell numbers in the spleen indicates the induction of a T cell response against *S. aureus* as indicated by earlier publications (15).

Decreased Number of Human CD14+ Monocytes in the Blood of Humanized Mice at Later Stage Infection

Since neither the human T nor the human B cell compartment showed differences in their numbers in the blood of huNSG at day 2 and day 7 p.i., we wondered how other immune cell lineages reacted to the challenge with *S. aureus* in the thigh muscle and the development of systemic disease in the humanized mice. We checked the number of neutrophils (CD66b+) and monocytes (CD14+) in the blood and bone marrow. Neutrophil numbers were low in the blood at both time points, with a tendency towards lower numbers at day 7 p.i. Total numbers resembled those of earlier studies in huNSG mice (16). Monocytes on the opposite showed a very distinct pattern for blood and bone marrow. In both organs, the number of monocytes was significantly lower at day 7 than at day 2 p.i. (**Figure 5**). This became even more puzzling when we investigated the numbers of murine monocytes (Ly6C+ Ly6G-) in both, humanized and non-humanized mice for comparison (**Figure S5**). In all investigated mouse groups, the number of murine monocytes increased strongly and significantly between both time points, proving a strong activation. Even in huNSG mice, in which the number of human monocytes dropped severely, the number of murine monocytes increased strongly, reversing the ratio between both from day 2 to day 7 p.i. The drop in blood humanization rates between day 2 and day 7 p.i. (**Figure 3C**) can thus be explained by both, an increase of murine cells and the simultaneous decrease of human monocytes in the blood of huNSG mice.

DISCUSSION

Among the emerging threat posed by antibiotic-resistant bacteria, MRSA holds the pole position as the pathogen with most clinical cases and attributable deaths in the USA, and second in the European Union (24, 25). However, although there is a high urgency and need for novel therapies, many pre-clinically efficient approaches targeting *S. aureus* failed in clinical trials (4, 26). This lack of translational power from pre-clinical models to infection in humans is, at least to some extent, due to the pronounced host tropism of clinical *S. aureus* strains (9). Several ideas to overcome this obstacle have been proposed by the scientific community, with humanized mice being one of the most promising solutions (5, 7).

The basis for humanized mice was the development of highly immunodeficient mouse strains, that lack B, T, NK cells and support human haemato-lymphopoiesis after engraftment with human CD34+ stem cells (27, 28). Humanized NSG mice (huNSG) are the most widely used model in this context and have so far been used to study *S. aureus* in peritonitis, skin infection, pneumonia and osteomyelitis models (12–15). It became obvious in this limited number of recent studies, that huNSG are more susceptible to *S. aureus* than wild-type or "murinized" mice. We wondered how the human immune system adapts to long-term infection with MRSA and whether differences between the murine and the human immune response might surface which might explain this higher susceptibility?

We decided to investigate the behavior of the human immune system during infection with MRSA in the thigh muscle, which resembles a localized, deep-tissue abscess formation and covers both, acute and chronic phases of infection. We found in accordance with the earlier studies of staphylococcal infection in humanized mice, that they were more susceptible to bacterial infection resulting in higher bacterium-derived bioluminescence signal and higher bacterial burden at the primary site of infection. The bacterial load in the infected thigh muscle was significantly higher in huNSG mice than in murinized or wild-type NSG mice, as well as compared to Balb/c mice.

The most stunning observation, nonetheless, was that 35% of the humanized mice reached the humane end point between day 2 and day 7 p.i. (based on the animal welfare score sheet criteria) and had to be removed from the experiment due to critical signs of disease and infection. This was in contrast to all control groups in which only 1 wild-type NSG mouse reached this point. And even those humanized mice that survived until day 7 p.i. showed

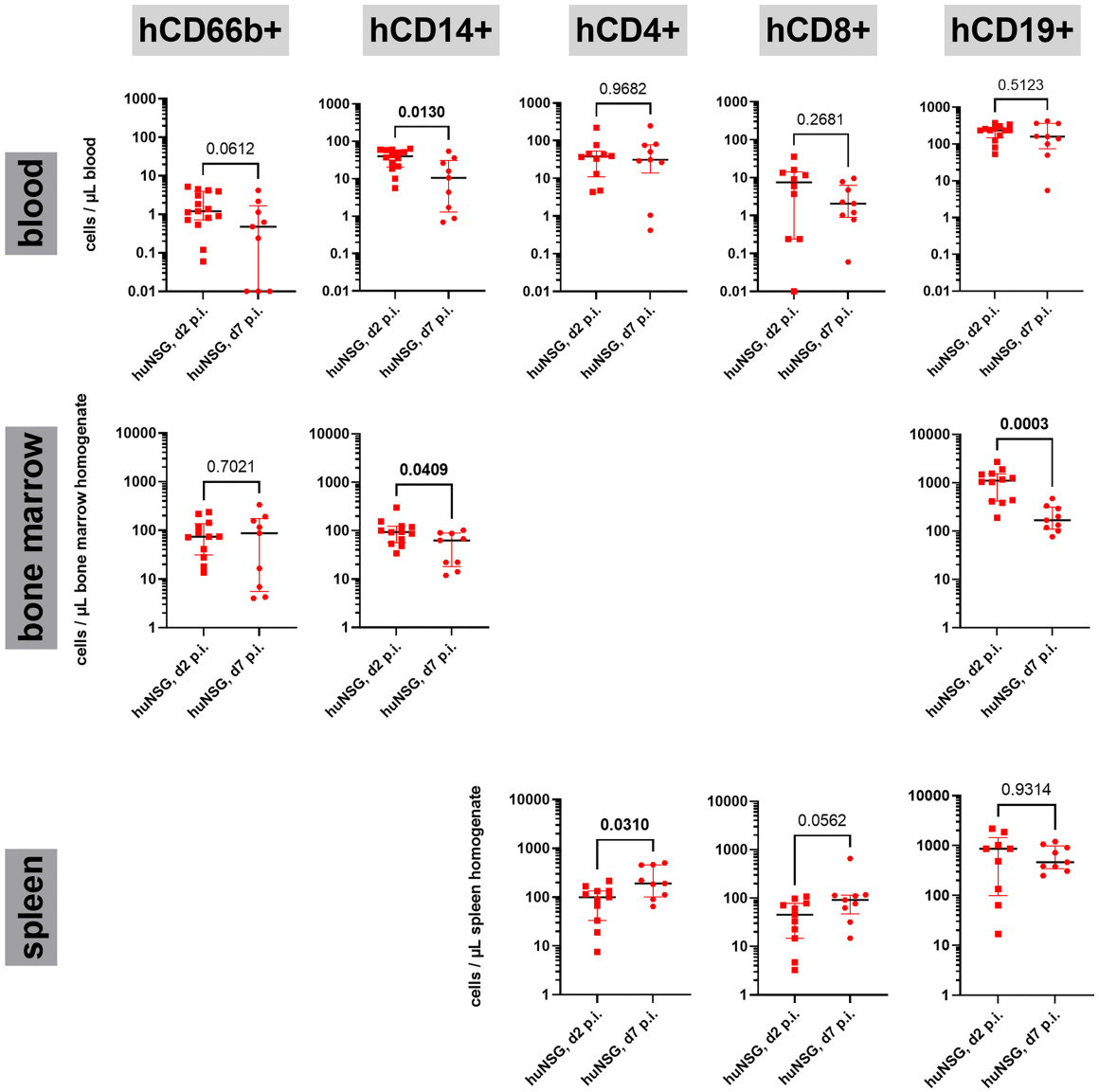

FIGURE 5 | Human immune cell lineages in blood, bone marrow and spleen during the course of thigh infection. Cells were first gated for expression of hCD45 and lack of mCD45 expression, then for the respective lineage markers (hCD66b: granulocytes, hCD14: monocytes, hCD4: T helper cells, hCD8: cytotoxic T cells, CD19: B cells). The gating strategy can be found in **Figure S4**. Statistical significance was tested with Mann-Whitney-test and p-values displayed (significant values ($p < 0.05$) are depicted as bold numbers).

a much higher probability of bacterial spreading from the primary site of infection to the inner organs. When we took a closer look at the correlation of the rate of humanization and the severity of infection, it became clear, that the mice with a high rate of humanization (prior infection) had a strongly reduced chance of surviving local MRSA challenge compared to their less humanized littermates.

This divergence in the outcome of localized *S. aureus* infection between humanized and non-humanized mice asked the question which factors or components are responsible. We approached this question by investigating the immune system's signaling and cell population dynamics during the infection and found significant differences between the human and the murine immune response. First, the cytokine and chemokine levels in huNSG mice were much higher than in the other groups, and we could detect both, human and murine cytokines in these mice. The higher levels of MCP-1, IL-6 and IL-10 suggested a much stronger inflammatory response to the bacterial challenge in the humanized mice, especially at day 2 p.i.

It is well known that several factors of *S. aureus*, that can result in pro-inflammatory cytokine signaling, are much more efficient against human cells than against their murine counterparts, like bi-component toxins, superantigens/enterotoxins or immunomodulators (6, 29–31).

However, in discrepancy to the increase of the bacterial burden in the thigh muscle and the inner organs, we could see lower levels of human cytokines at the later time point of infection. Reasons for this might be either the regulation of the immune response respectively the inflammation or an exhaustion of the immune response or its deployed cells. Either way, it might indicate the failing of the immune system to overcome the bacterial infection and thus explain the decreased survival of the huNSG mice.

Our hypothesis was that if it is exhaustion that leads to the reduced inflammatory status and the impaired survival of the huNSG mouse group, we would see the depletion or reduction of at least one major lineage of human immune cells. In order to investigate this, we took a closer look at the cell numbers of immune cell lineages in the blood, bone marrow and spleen by flow cytometry. We could detect considerable numbers of human B and T cells, as well as monocytes, but only very low numbers of human neutrophils in the blood, as described in an earlier study (16). We observed furthermore, as described for *S. aureus* osteomyelitis infection in huNSG mice (15), a significant increase of human T cell numbers in the spleen, which indicates a pathogen-induced human immune cell response in the animals. On the other hand, we found that the number of CD19+ B cells in the bone marrow decreased dramatically, while their numbers in blood and spleen remained stable. In addition, the numbers of CD14+ monocytes dropped similarly drastically in both, blood and bone marrow, which in turn might be the reason for the lower cytokine levels in blood and thigh muscle at the later time point. Both observations indicate that the human immune system in the huNSG mice gets caught in the maelstrom of local infection and bacterial spreading without being able to reproduce enough immune cells to overcome this challenge. This might, at least, in parts be based on the fact, that some human immune cell lineages are underrepresented in huNSG mice, most notably neutrophils (16), or that others are not terminally differentiated. At the first glance this might be regarded as a drawback of humanized mice in preclinical research for infectious disease. However, from a different point of view, it might even reflect the specific clinical need much better, in which success of therapy is important at most: in patients with an impaired immune response, particularly when neutrophil function is affected like congenital neutropenia or chronic granulomatous disease (32, 33).

The drop of human monocytes and B cells raises additionally the question about the influence of *S. aureus*. Is it simply the overwhelming presence of the bacteria that leads to the depletion of these cell lineages or is *S. aureus* actively depleting these cells by the action of virulence factors? Particularly the differences between human and murine monocytes are striking in this regard. While the numbers of human monocytes are fading away systemically during the course of infection, we could, in huNSG and control mice, measure an increase of the murine ones. Importantly, the fate of the human monocytes remains elusive at this point: future studies are necessary to unravel whether they are migrating to the site of infection, are undergoing apoptosis, or having their reproduction in the bone marrow affected. Furthermore, the dynamics of human and murine immune cells in the infected thigh muscle apart from the cytokine levels were inaccessible in this study, too, and should be investigated on a single cell level in future in order to understand the shift from local to systemic infection. Considering, that several virulence factors of *S. aureus* show higher affinity or activity towards human than murine immune cells *in vitro*, like bi-component toxins, superantigens or immunomodulators (6, 29, 34), it seems reasonable to assume, that humanized mice are indeed representing the clinical course of infection more closely than wild-type mice. On the other hand, limitations of humanized NSG mice like weak human myeloid reconstitution and the lack of human thymus tissue (10, 35) have to be considered when interpreting the observed results in our infection model.

In summary, we could show in this study, that a local infection with MRSA in humanized mice induced systemic spreading of the bacteria and high mortality, while the infection stayed locally in non-humanized mice. We furthermore unraveled grave differences between the human and murine immune response against *S. aureus* in this *in vivo* system, particularly in the cytokine response and the recruitment of monocytes. These results might help to develop better disease-related pre-clinical models for the validation of novel therapeutic approaches and the identification of promising targets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by local government of Lower Franconia, Germany (approval numbers 55.2-2532-2-836 and 55.2-2532-2-1129).

AUTHOR CONTRIBUTIONS

KO, TH, SS, and JD contributed to conception and design of the study. SH, LD, and TH performed the experiments. SH, LD, and TH analyzed the data. TH and SH wrote the first draft of the manuscript. KO, JD, and SS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.892053/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

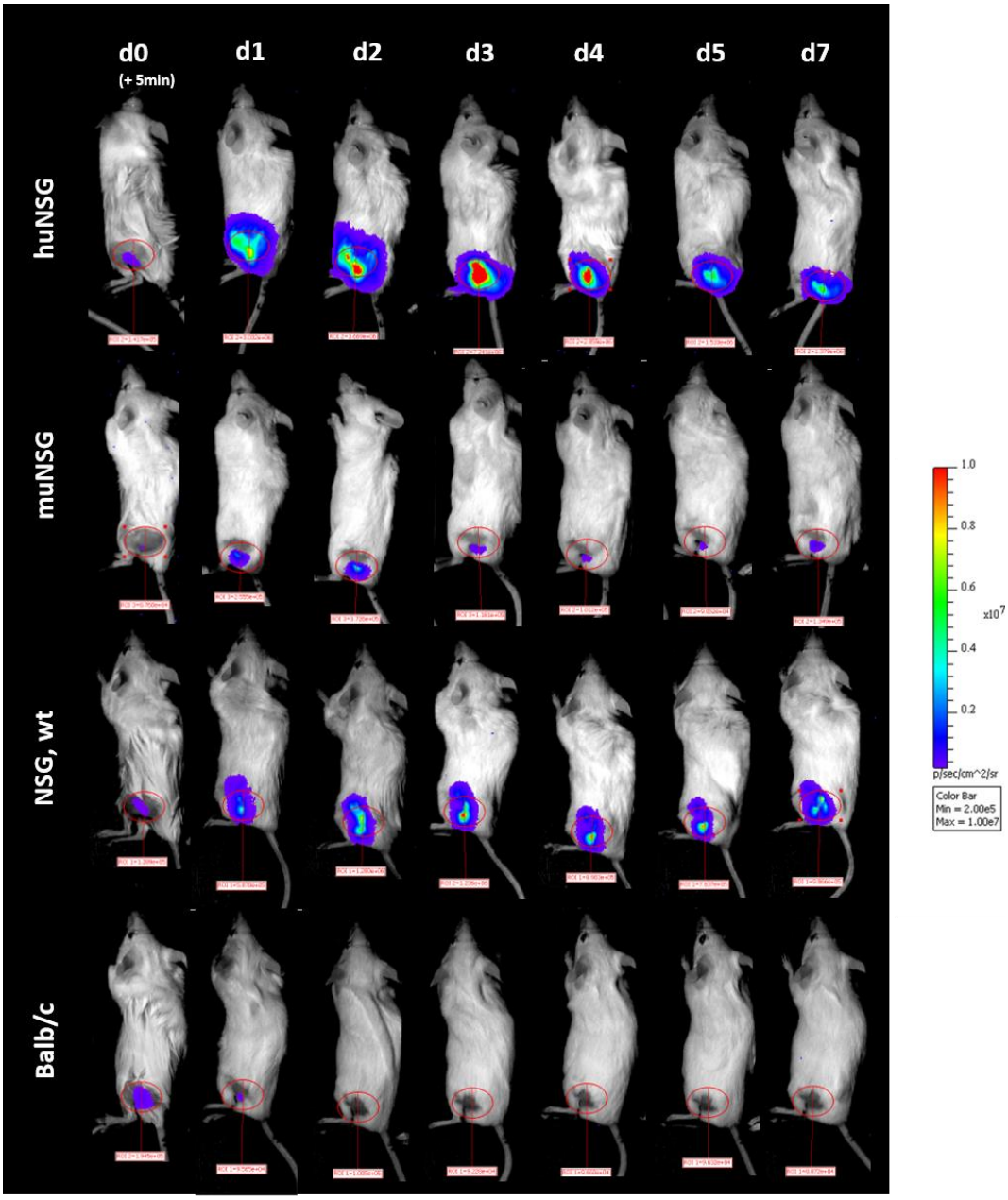

Figure S1: Bioluminescent overlay images of one representative mouse per group during the course of infection. The region-of-interest (ROI) had the same size and geometry for each mouse.

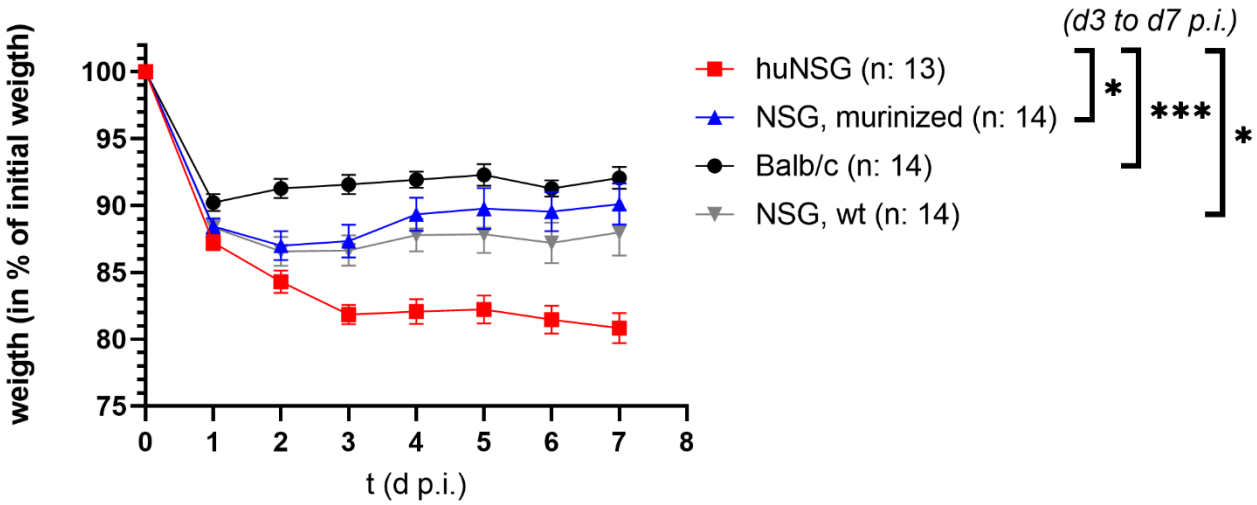

Figure S2: Weight loss during the course of infection. Visualized are the mean values +/- SEM per group of all mice which survived until day 7 p.i.. Statistical significance was tested for each time point with Kruskal-Wallis test.

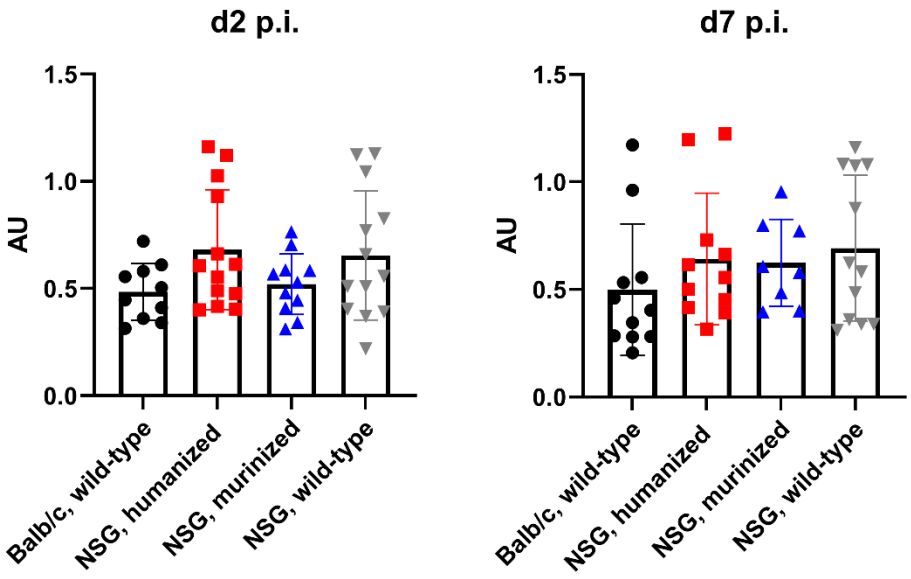

Figure S3: Myeloperoxidase activity in the infected thigh muscle. Infected thigh muscles were recovered at day 2 and 7 p.i., homogenized and the myeloperoxidase activity measured in a 1.5% dilution of this homogenate. (AU: arbitrary units)

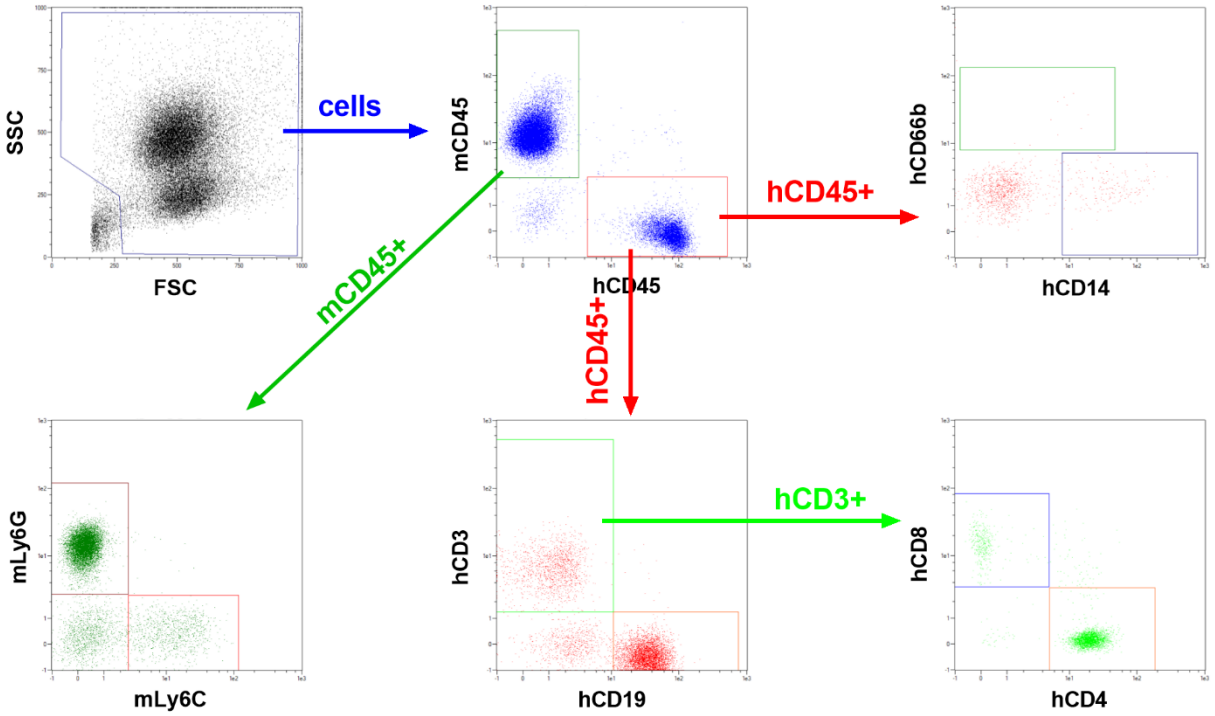

Figure S4: Principal gating strategy for this study. Blood samples and spleen or bone marrow cell suspensions were stained with different combinations of antibodies as stated in the methods section. In general, we first excluded cell debris and then gated for human or murine cells based on species-specific CD45+ expression. The immune cell lineage was then determined for these gated cells based on their expression of following markers: human: CD66b: neutrophils, CD14: monocytes, CD3, CD4 or CD8: T cells, CD19: B cells; or mouse: Ly6G: neutrophils, Ly6C: monocytes.

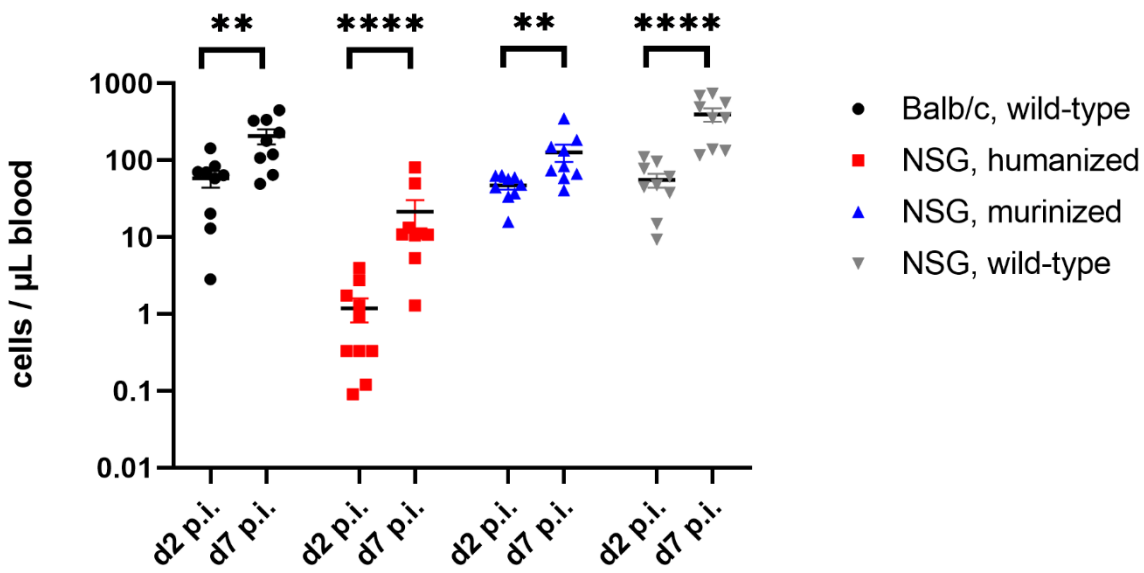

Figure S5: Murine monocytes (mCD45+ Ly6G- Ly6C+) in the blood of infected mice at day 2 or day 7 p.i. analyzed by flow cytometry. Statistical significance was tested with Mann-Whitney-test and p-values displayed (**: $p \leq 0.005$, ****: $p \leq 0.0001$).

4.2 Publication II

Sophia Hung, Amelie Kasperkowitz, Florian Kurz, Liane Dreher, Joachim Diessner, Eslam S Ibrahim, Stefan Schwarz, Knut Ohlsen, Tobias Hertlein

Next-generation humanized NSG-SGM3 mice are highly susceptible to *Staphylococcus aureus* infection

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Author contributions:

Name	Description author	Share of work
Sophia Hung	First author	Performed the experiments and the analysis of the data, wrote the first draft of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Amelie Kasperkowitz	Co-author	Contributed to the performance of the experiments and the analysis of the data, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version

Florian Kurz	Co-author	Contributed to the performance of the experiments and the analysis of the data, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Liane Dreher	Co-author	Contributed to the performance of the experiments and the analysis of the data Contributed to manuscript revision, read, and approved the submitted version
Joachim Diessner	Co-author	Contributed to conception and design of the study, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Eslam S Ibrahim	Co-author	Contributed to the performance of the experiments and the analysis of the data, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Stefan Schwarz	Co-author	Contributed to conception and design of the study, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Knut Ohlsen	Co-author	Contributed to conception and design of the study, co-wrote parts of the manuscript Contributed to manuscript revision, read, and approved the submitted version
Tobias Hertlein	Co-author	Contributed to conception and design of the study, contributed to the performance of the experiments and the analysis of the data, co-wrote parts of the first draft of the manuscript Contributed to manuscript revision, read, and approved the submitted version

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Next-generation humanized NSG-SGM3 mice are highly susceptible to *Staphylococcus aureus* infection

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Humanized hemato-lymphoid system mice, or humanized mice, emerged in recent years as a promising model to study the course of infection of human-adapted or human-specific pathogens. Though *Staphylococcus aureus* infects and colonizes a variety of species, it has nonetheless become one of the most successful human pathogens of our time with a wide armory of human-adapted virulence factors. Humanized mice showed increased vulnerability to *S. aureus* compared to wild type mice in a variety of clinically relevant disease models. Most of these studies employed humanized NSG (NOD-*scid* IL2Rg^{null}) mice which are widely used in the scientific community, but show poor human myeloid cell reconstitution. Since this immune cell compartment plays a decisive role in the defense of the human immune system against *S. aureus*, we asked whether next-generation humanized mice, like NSG-SGM3 (NOD-scid IL2Rg^{null}-3/GM/SF) with improved myeloid reconstitution, would prove to be more resistant to infection. To our surprise, we found the contrary when we infected humanized NSG-SGM3 (huSGM3) mice with *S. aureus*: although they had stronger human immune cell engraftment than humanized NSG mice, particularly in the myeloid compartment, they displayed even more pronounced vulnerability to *S. aureus* infection. HuSGM3 mice had overall higher numbers of human T cells, B cells, neutrophils and monocytes in the blood and the spleen. This was accompanied by elevated levels of pro-inflammatory human cytokines in the blood of huSGM3 mice. We further identified that the impaired survival of huSGM3 mice was not linked to higher bacterial burden nor to differences in the murine immune cell repertoire. Conversely, we could demonstrate a correlation of the rate of humanization and the severity of infection. Collectively, this study suggests a detrimental effect of the human immune system in humanized mice upon encounter with *S. aureus* which might help to guide future therapy approaches and analysis of virulence mechanisms.

KEYWORDS

humanized mice, *Staphylococcus aureus*, MRSA, NSG, NSG-SGM3, staphylococcal abscess, *Staphylococcus aureus* immune response, humanized hemato-lymphoid mice

Introduction

The evaluation of promising new treatments against infectious diseases is challenging and the path to clinical application is plastered with failures. *Staphylococcus aureus* has emerged as a prominent example in this group. This bacterial pathogen causes a wide array of diseases, ranging from superficial skin infections to life threatening bacteremia, endocarditis, pneumonia and osteomyelitis (1–3). Its genetic flexibility, evolution of host-specific virulence factors and notorious acquisition of antimicrobial resistance genes makes it one of the most important bacterial pathogens of our time (4, 5).

Despite huge clinical and economic impact, all immunotherapies - most importantly vaccination attempts - have so far failed (6, 7). Multiple reasons for the lacking efficacy of these approaches during clinical trials, although being effective in pre-clinical models, have been proposed by the scientific community with two standing out: (I) the lack of understanding of host-pathogen interaction during infection in humans and (II) the poor translational power of pre-clinical data (7, 8).

Humanized mice, or humanized hemato-lymphoid system mice, have drawn attention in recent years as a promising solution to at least some of these problems (9). This model is based on highly immunodeficient mouse strains which are engrafted with human hematopoietic stem cells, which in turn differentiate into various human immune cell lineages in these mice (10, 11). This makes it possible to investigate host-pathogen interaction as well as the interplay of different human immune cell populations in a highly complex *in vivo* system.

Recent publications in this field suggest that humanized mice are an interesting and viable option to investigate *S. aureus* infection. They show that humanized mice are much more susceptible to *S. aureus* infection than wild-type, murinized (immunodeficient mice with engrafted murine stem cells) and even non-engrafted immunodeficient mice in models of peritonitis (12), pneumonia (13), skin infection (14), osteomyelitis (15) and deep tissue infection (16). These studies furthermore delivered proof, that some virulence factors of *S. aureus* show much higher activity against human than murine cells and factors during *in vivo* infection (13, 14). Nonetheless, it has to be stated that the humanized mouse models studied thus far harbor a serious drawback, particularly in studying *S. aureus* infection: the numbers of human myeloid and monocytic cells are rather low. This is due to the application of NSG mice, which are widely used in the field, but are unable to sustain high numbers of these immune cells after engraftment due to the lack of necessary human growth factors and cytokines (17, 18). Several strategies have been applied to overcome this problem: the administration of these factors during humanization (19, 20), hydrodynamic injection of human cytokine-encoding plasmids (21) or genetically engineered humanized mice, which are producing these factors (22, 23).

Since neutrophils and the macrophage-monocyte axis play a prominent role during the immune response against *S. aureus* (24–26), we asked whether next-generation humanized mice perform better or worse during *S. aureus* infection than the above-mentioned humanized NSG mice. Therefore, we humanized NSG-SGM3, which provide high numbers of human monocytes and neutrophils due to genetical integration of human SCF, GM-CSF and IL-3 genes (17), and compared their performance during *S. aureus* deep tissue infection with those of humanized NSG mice.

Material and methods

Ethics statements

All animal studies were approved by the local government of Lower Franconia, Germany (approval numbers 55.2-2532-2-836 and 55.2-2532-2-1129) and performed in strict accordance with the guidelines for animal care and experimentation of the German Animal Protection Law and the DIRECTIVE 2010/63/EU of the EU. The animals were housed in IVC cages under standardized lighting conditions and had *ad libitum* access to food and water. All experimentations with anonymized and non-traceable human cord blood was approved by the ethics committee of the University Wuerzburg (approval number 20191212 02).

Humanization and murinization procedure (incl. CD34+ cell isolation)

We included female and male NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) and NSG-SGM3 (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3, CSF2,KITLG)1Eav/MloySzJ) mice from the Jackson Laboratories (Bar Harbor, ME, USA) and female Balb/c mice (BALB/cJRj, Janvier labs, Le Genest-Saint-Isle, France) in all experiments. The human CD34+ hematopoietic stem cells were isolated from human cord blood by magnetic separation (EasySep™ Human Cord Blood CD34 Positive Selection Kit II, STEMCELL technologies, Cologne, Germany) following the manufacturer's protocol. The quality of the cell preparation was controlled by staining for hCD34+ and hCD3+ cell markers by flow cytometry. Only preparations with > 85% hCD34+ purity and < 1% hCD3+ cell content were used for humanization purposes. Humanized NSG (huNSG) and NSG-SMG3 (huSGM3) mice were generated by engrafting 100,000 hCD34+ cells (of a donor mix) at the age of 6 – 8 weeks, similar to the procedures described in earlier publications (15–17, 27). Briefly, after whole-body irradiation with a sub-lethal dose of 2 Gy, mice were injected intravenously with the human hematopoietic stem cells. Murinized NSG-SGM3 mice (muSGM3) received a similar treatment than huNSG and huSGM3 mice, but 100,000 bone marrow cells from a Balb/c donor instead of human CD34+ cells were injected. The peripheral blood of humanized mice was analyzed every two weeks after engraftment for the presence and frequency of murine CD45+, as well as human CD45+, CD66b+, CD3+ and CD20+ cells by flow cytometry.

Determination of blood hemoglobin content and erythrocyte and reticulocyte numbers

Every two weeks during the course of the humanization and at the end of the bacterial thigh muscle infection, peripheral blood was

collected and mixed with EDTA as anticoagulant (pluriSelect Life Science, Leipzig, Germany). The hemoglobin content (Hämoglobin, Diaglobal GmbH, Berlin, Germany) and the red blood cell count (Erythrozyten Gower's Reagenz, Bioanalytic GmbH, Umkirch/Freiburg, Germany) was measured by fluorometric assays following manufacturer's instructions. During the humanization period, we furthermore determined the reticulocyte numbers by streaking blood samples on glass slides and counted the number of reticulocytes among red blood cells after staining (Brillant cresyl blue, Bioanalytic GmbH, Umkirch/Freiburg, Germany).

Thigh infection model (including determination of bacterial burden)

HuSGM3 mice were infected intramuscularly (i.m.) at 12 weeks and the huNSG mice at 18 weeks post hCD34+ stem cell injection as described previously (16). At these points human CD45+ cell numbers reached robust numbers in blood and the number of hCD3+ T cells and hCD19+ B cells were at comparable levels. Balb/c, wild-type NSG-SGM3 and murinized NSG-SGM3 mice were infected at the age of 18 weeks in order to match the age of the humanized NSG-SGM3 mice (engraftment at the age of approximately 6 weeks and 12 weeks of humanization). Briefly, Methicillin-resistant *S. aureus* (MRSA) LAC* *lux* (28) was pelleted after overnight shaking at 37°C in B medium and resuspended in 0.9% NaCl solution. The left thigh of each mouse was then shaved, disinfected and injected with 1 x 10^8 CFU bacteria in a total volume of 50 µL (16, 29). Besides huSGM3 and huNSG mice, we used age-matched murinized NSG-SGM3, wild-type NSG-SGM3 and Balb/c mice as controls. The wellbeing of each mouse was inspected and scored every 12 hours p.i. and the weight measured every 24 hours. Those mice that did not reach the humane end point as defined by the score sheet were either sacrificed on day 2 or on day 7 p.i. Peripheral blood, the infected thigh muscle, kidneys, liver, spleen, heart, lung and bone marrow from tibia and femur were then harvested. The spleens were halved and one part homogenized by pressing through a cell strainer for flow cytometry and bacterial burden determination, while the second half was processed for histological examination. The thigh muscle, kidneys, liver, lung and heart were homogenized in 0.9% NaCl and serial dilutions were plated on B agar plates in order to determine the bacterial burden. Bone marrow was harvested by flushing both femurs and tibias with sterile 0.9% NaCl solution, followed by filtration through a cell strainer.

Histology

Histological sections and immunohistochemical stainings of splenic tissue were performed using formalin-fixed and paraffin-embedded (FFPE) tissue slides according to standard protocols. Briefly, spleens were fixed overnight in 10% neutral-buffered formalin solution, embedded in paraffin and cut to 5 µm slices. After deparaffinization, samples were stained with H&E and immunohistochemistry staining was performed with anti-human CD45 primary antibody (Agilent Dako, Waldbronn, Germany) at the Institute of Pathology at the University Clinics Wuerzburg according to the appropriate protocols within an automated immunostainer (Benchmark Ultra; Ventana/Roche, Tucson AZ, USA). Specimens were then inspected by an experienced pathologist (FK) for the presence and organization of human immune cells in the spleens.

Flow cytometry

The peripheral blood of humanized mice was examined by flow cytometry every two weeks after stem cell administration. Blood samples were therefore stained with anti-human CD45/CD3/CD19/CD66b and anti-mouse CD45. The rate of humanization was calculated as: hCD45+ cells/(hCD45+ cells & mCD45+ cells), as applied previously (16). The peripheral blood, the spleen homogenate and bone marrow were interrogated for the presence of immune cells on day 2 p.i. with combinations of anti-human CD45/CD3/CD19/CD14/CD66b and anti-mouse CD45/Ly6C/Ly6G antibodies after red blood cell lysis. All antibodies were supplied by Miltenyi Biotec (Bergisch Gladbach, Germany). Flow cytometric measurements were performed on a MACSQuant flow cytometer and analyzed with MACSQuantify software 2.6 (Miltenyi Biotec, Bergisch Gladbach, Germany).

Determination of cytokine levels and myeloperoxidase activity

In order to determine cytokine levels in the thigh muscle, homogenate was centrifuged at 3,000 x g for 5 minutes and the supernatant stored at -80°C until further processing. The peripheral blood samples were agglutinated overnight at 4°C, then centrifuged at 15,000 x g for 15 minutes. The serum was harvested and stored at -80°C until cytokine measurement. Levels of human or murine CCL-2, IL-1β, IL-6, IL-10, IL-17A, and TNF-α as well as human IL-8 were measured in the infected thigh muscles or in peripheral blood with a custom-mixed Luminex assays from Bio-Techne (Wiesbaden, Germany) following the manufacturer's manual. In order to test the specificity of the individual assay, we measured the cytokine standards of the human kit with the mouse kit and vice versa and could not detect values above background levels.

Statistical analyses

All statistical analyses were performed with GraphPad Prism (9.1.2) and $p < 0.05$ was considered as significant. The applied statistical tests can be found in the respective figure caption.

Results

Humanized NSG-SGM3 mice show strong human immune cell engraftment and develop anemia during prolonged course of humanization

We generated humanized NSG (huNSG) and NSG-SGM3 (huSGM3) mice by sublethally irradiating the animals and

intravenously injecting human cord blood-derived CD34+ hematopoietic stem cells. The mice were then continuously monitored and weighed over a period of 18 weeks. A divergence between the two groups in terms of body weight change (Figure 1A) became obvious. While huNSG mice continually gained weight, huSGM3 mice initially gained weight, too, but then started to stagnate from week 12 to 18.

Overall, huNSG and huSGM3 mice showed similar developments and levels of humanization rates and individual immune cell populations than described earlier (16, 30). HuSGM3 animals had throughout the whole course of humanization a higher rate of humanization and stronger increase in human immune cells (Figures 1B–F). In particular, the amount of hCD66b+ cells was at all times strongly enhanced compared to huNSG mice.

Since symptoms of secondary hemophagocytic lymphohistiocytosis (HLH) and/or macrophage activation syndrome (MAS) were described for NSG-SGM3 mice after engraftment with human CD34+ stem cells (30–32), we measured the numbers of erythrocytes, reticulocytes and the level of hemoglobin in the blood of the humanized mice every two weeks post engraftment (Figures 1H–J). While all three parameters remained at a comparable level for the huNSG mice, we saw a drop in erythrocyte numbers and hemoglobin levels as well as an increase in reticulocyte numbers in huSGM3 mice during prolonged course of humanization. The development and extend of these changes fits to earlier description and started at 8 to 14 weeks post engraftment (30–32). Of note, this was accompanied by a strong acceleration of hCD3+ T cell numbers in blood. The reticulocytosis implies that this is caused by a decimation or increased usage of erythrocytes, not a dysfunctional production (30, 31).

MRSA thigh infection leads to strongly reduced survival of huSGM3 mice

Based on the results from the humanization phase, we decided to infect huNSG mice at 18 weeks and huSGM3 at 12 weeks after engraftment. HuSGM3 mice showed at this point an overall enhanced level of human immune cells compared to huNSG mice, with slightly reduced levels of hemoglobin and erythrocytes and increased numbers of reticulocytes. Both humanized mouse groups were infected locally with 1 x 10^8 CFU *S. aureus* LAC* *lux* in the left thigh muscle, with wild type NSG-SGM3, murinized NSG-SGM3 (which were treated equally to humanized NSG-SGM3, but received murine bone marrow cells instead of human CD34+ stem cells) and wild type Balb/c mice as controls. This type of infection causes the formation of large deep tissue abscesses in wild-type mice as described earlier (29). Since *S. aureus* utilizes various mechanisms and virulence factors to acquire iron from hemoglobin (33, 34), we decided to track the levels of erythrocytes and hemoglobin in the humanized mouse groups during the course of infection. The overall amounts were, for both factors, lower in huSGM3 mice than in huNSG mice at the start of infection (Figures 2A, B). But the level of erythrocytes in the blood of huSGM3 mice increased in the first 48 hours of infection, reaching the levels of huNSG.

Mice were weighed and inspected every 12 hours throughout the infection experiment and received a score based on weight loss and signs of disease. The humanized mouse groups proved to be much more susceptible to the deep tissue infection with *S. aureus* than the control groups (Figure 2C), which fitted to earlier results in this model (16). Nonetheless surprising was the extreme vulnerability of next-generation humanized (huSGM3) mice which showed early in the experiment very strong signs of disease and weight loss, causing a strongly decreased survival rate compared to all other groups, even significantly inferior to huNSG.

Bacterial burden in the thigh muscle and inner organs of infected mice

The strongly reduced survival rate of huSGM3 mice raised the question of the cause for the enhanced vulnerability, particularly in comparison to huNSG mice. In a first attempt to define the systemic consequences of a local infection with *S. aureus* in the thigh muscle, we analyzed the bacterial burden in various organs on day 2 p.i. Later dates were not accessible since the number of surviving huSGM3 mice was not sufficient for analysis. No difference in terms of bacterial burden could be seen at the primary site of infection (Figure 3A). The analysis of bacterial burden in kidneys, liver, lung, spleen and heart revealed, that humanized mice showed a tendency towards higher numbers of *S. aureus* in the respective organs, although the differences were rather small and the results varied (Figure 3B). The strongest difference could be measured in the lungs with humanized mice displaying significantly higher bacterial numbers than Balb/c mice (Figure 3C). Balb/c mice showed the weakest bacterial spreading and infection of inner organs, while it was similar for all other groups (Figure 3D). Overall, a clear correlation of bacterial burden on day 2 p.i. and survival during the course of infection could not be established.

Stronger response of human immune cells in humanized NSG-SGM3 than in humanized NSG mice

Since the number of bacteria during infection did not coincide with the severity of infection, we next analyzed immune cell types and effector molecules in order to identify differences which might explain the high vulnerability of huSGM3 mice. First, we compared major murine immune cell populations in the blood, spleen and bone marrow of all mice (Figure S1). Both humanized mouse groups had similar numbers of murine CD45+ cells, granulocytes and monocytes, but significantly less than wild type or murinized mouse groups (Figure S2). Immunophenotyping of human immune cells in the blood of huSGM3 and huNSG mice at the start of the infection and on d2 p.i. showed overall higher numbers of all investigated cell types in huSGM3 mice, namely T cells, B cells, granulocytes and monocytes (Figure 4A). The numbers remained stable in huNSG within the first 2 days of infection, but a clearly distinguishable pattern became visible for the huSGM3 mice. While the number of hCD66b+ granulocytes increased between d0 and d2

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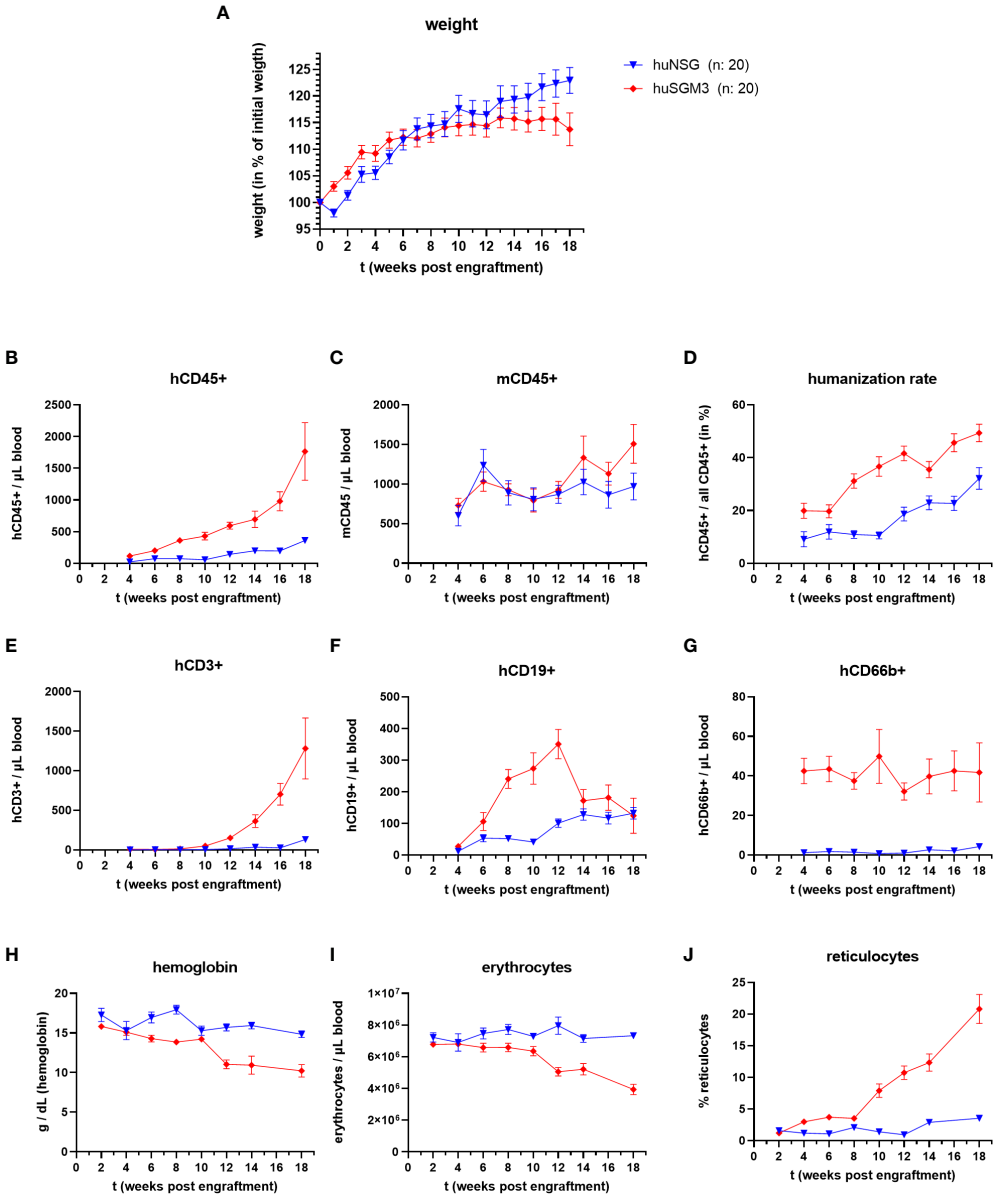

FIGURE 1
Weight, immune cell types and blood cells during the course of humanization of NSG and NSG-SGM3 mice. NSG or NSG-SGM3 mice were humanized by intravenous administration of 100.000 cord-blood derived hCD34+ cells. **(A)** The weight gain of each mouse compared to their weight on the day of engraftment was calculated and the mean values +/- SEM of each group is displayed. Statistical differences between both mouse strains was determined with Mann-Whitney test for each respective point in time. **(B–H)** The blood cell numbers of human and murine CD45+ cells, as well as human T cells **(E)**, B cells **(F)** and granulocytes **(G)** were determined every two weeks post engraftment by flow cytometry. **(D)** The humanization rate was calculated as hCD45+/(hCD45+ and mCD45+) cells. Visualized are the mean values +/- SEM for each group. **(H–J)** The level of hemoglobin, as well as the numbers of erythrocytes and the percentage of reticulocytes was measured bi-weekly and is displayed as mean +/- SEM for each group.

p.i., we could see significant drops of B and T cell numbers in the blood. The pattern of higher human immune cell numbers in huSGM3 mice was interestingly mirrored in the spleen (Figure 4C) but not the bone marrow (Figure 4B) on d2 p.i. The number of human cells in general, as well as of B cells, monocytes and granulocytes in particular, were similar in the bone marrow of huSGM3 and huNSG mice. In contrast to this, we could measure five-fold more hCD45+ cells in the spleens of huSGM3 mice than in huNSG mice, with all investigated human immune cell types represented significantly stronger. Histological examination of the spleens from huNSG and huSGM3 mice showed similar hCD45+ cell patterns with strong accumulation in lymphoid follicles at the periarteriolar lymphoid sheath and associated lymph follicles as well as in the parenchyma (Figure 5).

Next, the levels of murine and human cytokines/chemokines CCL2, IL-1β, IL-6, IL-10 and TNFα, as well as of human IL-8 were determined in the infected thigh muscle and in the blood on d2 p.i., since we hypothesized that the higher immune cell numbers in huSGM3 might be accompanied by higher levels of effector molecules and an elevated inflammatory state. The levels of

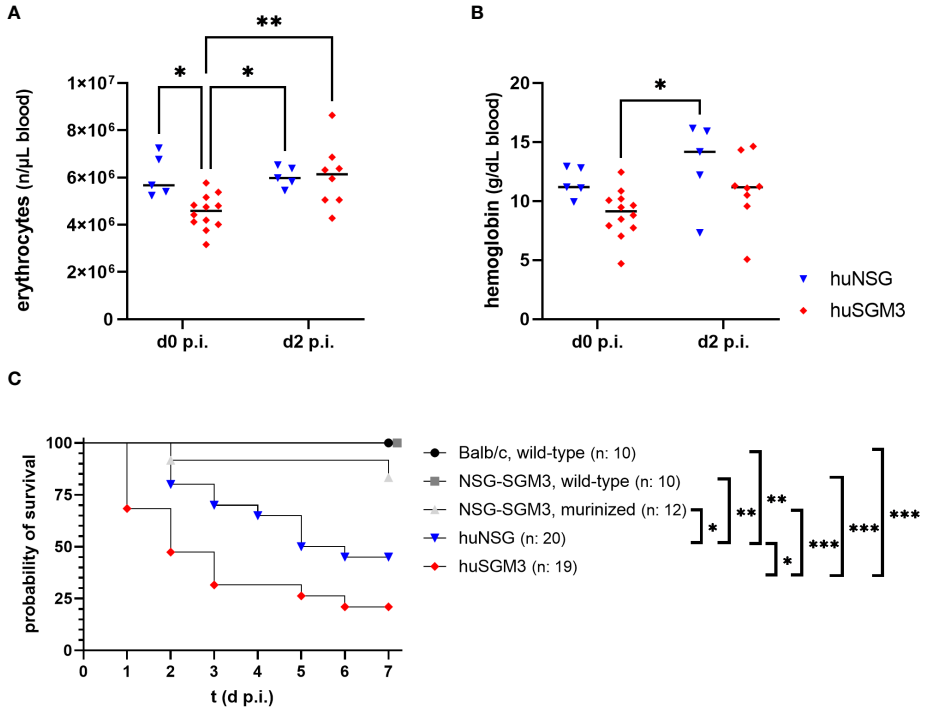

FIGURE 2
Erythrocyte numbers, hemoglobin levels and survival during severe local *S. aureus* infection in the thigh muscle of mice. The number of erythrocytes **(A)**, as well as the amount of hemoglobin **(B)** was measured fluorometrically at the start and on day 2 after infection with 1 x 10^8 *S. aureus* LAC* lux. Individual values per mouse as well as the respective medians per group are displayed. Statistical significance was tested with an ordinary two-way-ANOVA and Sìdàk's multiple comparison test (*p < 0.05, **p < 0.01). **(C)** The survival curve displays the percentage of animals per group which did not reach the humane end point as determined by the score sheet at the respective time. Statistically significant differences between the group survival was determined with Gehan-Breslow-Wilcoxon-test (*p < 0.05, **p < 0.01, ***p < 0.005).

murine cytokines were similar for all groups in both compartments, indicating that the infection with *S. aureus* did not lead to different activation of the murine immune cells (Figure S3). The examination of human cytokine levels revealed that while the levels at the primary site of infection were similar for huSGM3 and huNSG mice, strongly increased levels of CCL2, IL-6, IL-8 and TNF-α could be measured by the Luminex assay in the blood (Figure 6). This indicates a systemic response of the human immune system in huSGM3 mice against the *S. aureus* infection and might furthermore reflect the response to systemically spreading bacteria.

The stronger the humanization, the higher the vulnerability to *S. aureus* infection

The decreased survival of huSGM3 mice following local *S. aureus* infection in the thigh muscle compared to huNSG mice was accompanied by higher human immune cell numbers in blood and spleen, as well as with increased levels of immune effector molecules in the blood, but not with increased bacterial burden. Thus, we hypothesized, that the vulnerability does not originate from the pathogen itself but rather from the human immune system. This leads in consequence to the assumption, that a higher rate of humanization might cause higher susceptibility against *S. aureus*. Comparing the rate of humanization (prior infection) to the time at which each individual mouse reached the humane end point (or survived until the end of the experiment on day 7 p.i.), showed a clear negative correlation between both parameters (Figure 7). In consequence, high numbers of human immune cells prove to be detrimental for the mice during bacterial infection with *S. aureus*.

Discussion

Humanized mice, respectively mice with a humanized hemato-lymphoid system, emerged as a promising model to study infections and their treatment or prevention in recent years. Most commonly, they are generated by engrafting immunodeficient mice with human CD34+ stem cells, which repopulate hematopoietic niches and give rise to various human cell lineages within the mouse (10, 23). These models have proven their worth for the investigation of human-specific or human-adapted pathogens like HIV, *Salmonella enterica* subsp. *enterica* serovar Typhi or *Mycobacterium tuberculosis* (22, 35). *S. aureus* is not limited to its capability to infect humans, but can also infect other mammals or prosper as colonizer on different species (36, 37). However, it has become clear in the last decade, that those strains of *S. aureus* which are exceptionally successful in clinics, have evolved a large repertoire of virulence factors which can be regarded as highly human-specific (24, 38). The idea that these strains might behave differently when challenged with a human rather than with a murine

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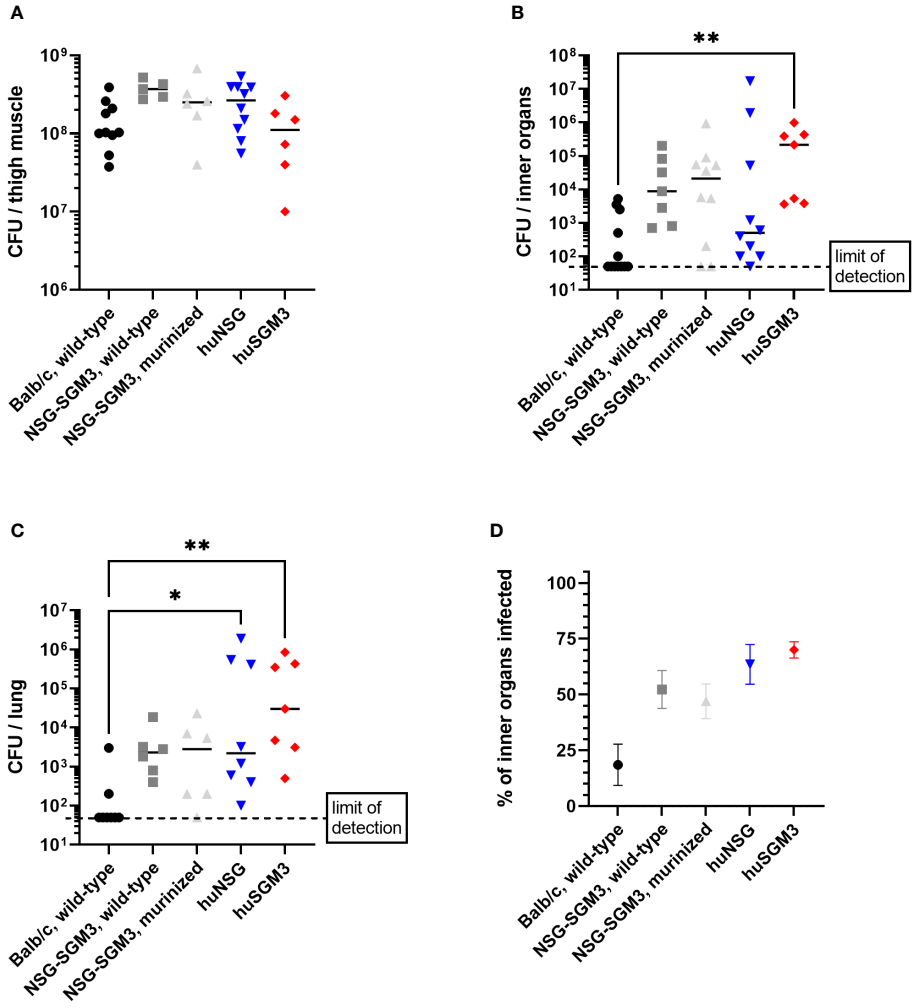

FIGURE 3
Bacterial burden in *S. aureus*-infected mice on day 2 p.i. The infected thigh muscle, as well as liver, kidneys, heart and lung were recovered on day 2 p.i. and homogenized. Serial dilutions were then plated to measure the colony forming units (CFU) of *S. aureus* in the respective organ. Displayed are the individual bacterial burden in the thigh muscle **(A)**, the lung **(C)** or the combined bacterial burden in investigated inner organs (kidneys, liver, heart, spleen and lung) **(B)**. The percentage of infected inner organs was furthermore calculated for each individual mouse **(D)**. Statistical significance was tested with Kruskal-Wallis with Dunn's multiple comparison test (*p < 0.05, **p < 0.01).

immune system during infection, resulted in the application of humanized mice. This hypothesis was recently tested in different experimental models and it could be shown that humanized NSG mice are more susceptible to *S. aureus* infection than wild-type, murinized (immunodeficient mice engrafted with murine stem cells instead of human ones) and even non-reconstituted immunodeficient mice (12–16). These studies implemented disease models of peritonitis, pneumonia, osteomyelitis, skin and deep tissue infections, thus covering a wide range of the clinical manifestations of *S. aureus*. All but one (13) of the above-mentioned studies applied huNSG mice, which are widely used in the community but which have a poor reconstitution of the human myeloid immune cell compartment (17, 23). Since myeloid cells, particularly neutrophils, play a major role in the defense against *S. aureus* (24, 25, 39), we hypothesized that next-generation humanized mice with a stronger reconstitution of the myeloid compartment might even be better suited to investigate *S. aureus* infections. To test this hypothesis, we humanized NSG and NSG-SGM3 mice by administration of human cord blood derived CD34+ hematopoietic stem cells. The genetically integrated human SCF, GM-CSF and IL-3 genes enabled overall higher numbers of human CD45+ cells in the blood of NSG-SGM3 mice, particularly of hCD66b+ granulocytes, similarly to earlier publications (17, 40).

On the other hand, previous studies demonstrated that the expression of the human cytokines/growth factors in NSG-SGM3 mice at supraphysiological levels comes with side effects during humanization, namely a deficiency in hematopoiesis (41, 42) and the development of secondary hemophagocytic lymphohistiocytosis (HLH) and/or macrophage activation syndrome (MAS) (30–32). We, too, could observe hallmarks of a MAS/HLH-like disease starting at week 10 post engraftment, namely a decrease in erythrocyte numbers as well as of hemoglobin content and an increase of reticulocyte numbers in the blood of humanized NSG-SGM3 mice. In order to prevent that these physiological changes

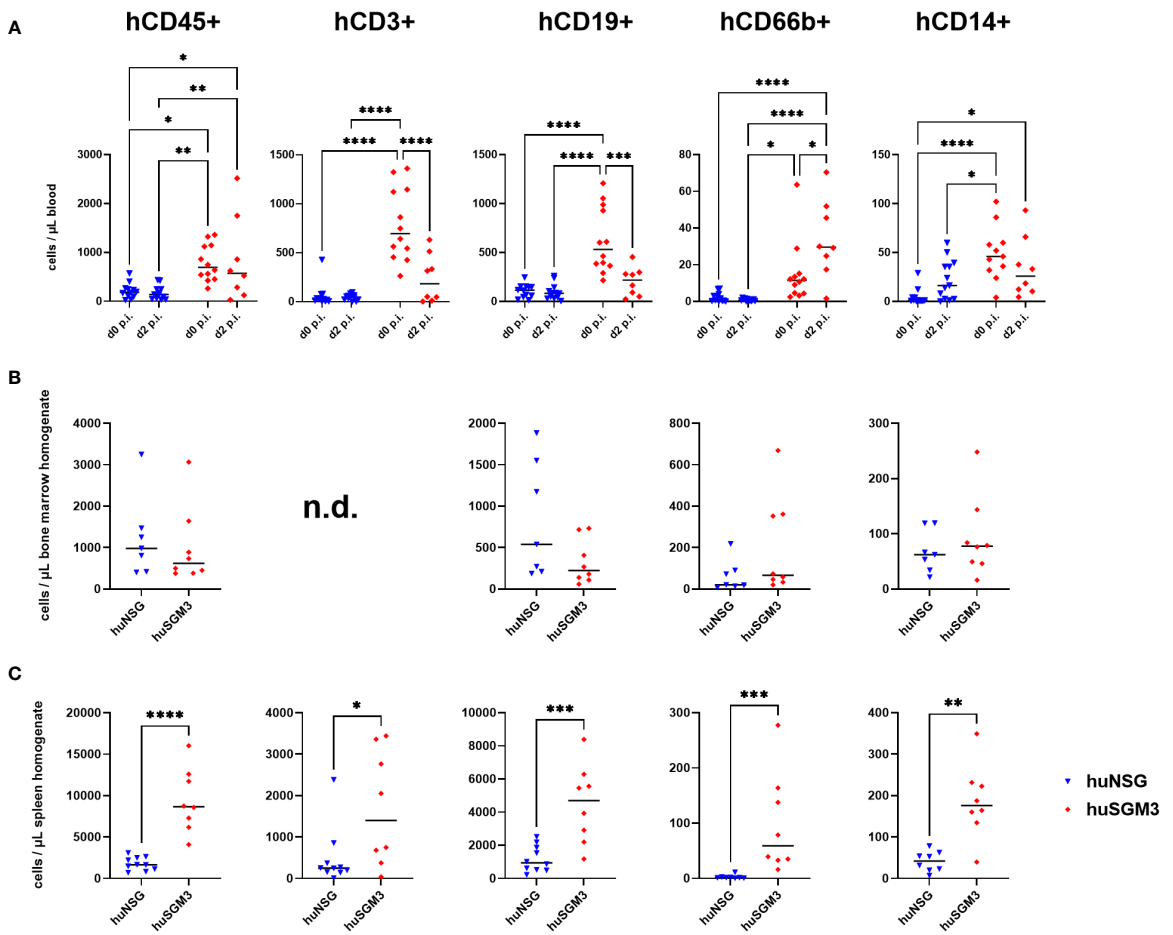

FIGURE 4
Human immune cell types in *S. aureus*-infected huNSG and huSGM3 mice. Immune cells were measured by flow cytometry with antibodies against hCD45, hCD3, hCD19, hCD66b and hCD14. **(A)** Blood samples were drawn at the start point of infection and on day 2 p.i. **(B)** Bone marrow was harvested by flushing tibia and femur on day 2 p.i. **(C)** Spleens were recovered on day 2 p.i. and homogenized by pressing through a 70 µm cell strainer. Displayed are the individual values per mouse as well as the medians per group. Statistical significance was tested with either Kruskal-Wallis with Dunn's multiple comparison test **(A)** or Mann-Whitney-test **(B** + **C)** (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).

alter the outcome of a *S. aureus* infection experiment, we decided to infect huSGM3 mice at 12 weeks post hCD34+ administration with *S. aureus* when signs of anemia were still rather mild but the number of human immune cells significantly higher than in huNSG mice. In order to identify differences in the susceptibility to *S. aureus* infection, we included age-matched Balb/c, wild-type NSG-SGM3 and murinized NSG-SGM3 mice as well as humanized NSG mice at 18 weeks post stem cell administration.

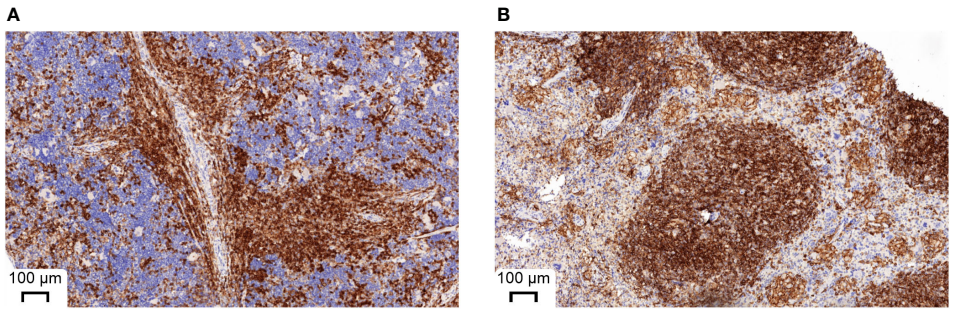

FIGURE 5
Representative histological appearance of spleens from huNSG-SGM3 **(A)** and huNSG mice **(B)** during *S. aureus* infection. Spleens were harvested on day 2 p.i. and processed to formalin-fixed and paraffin-embedded (FFPE) tissue slices. Specimens were then stained with anti-human CD45 and H&E. Strong CD45 expression can be seen at the periarteriolar lymphoid sheath and associated lymph follicles.

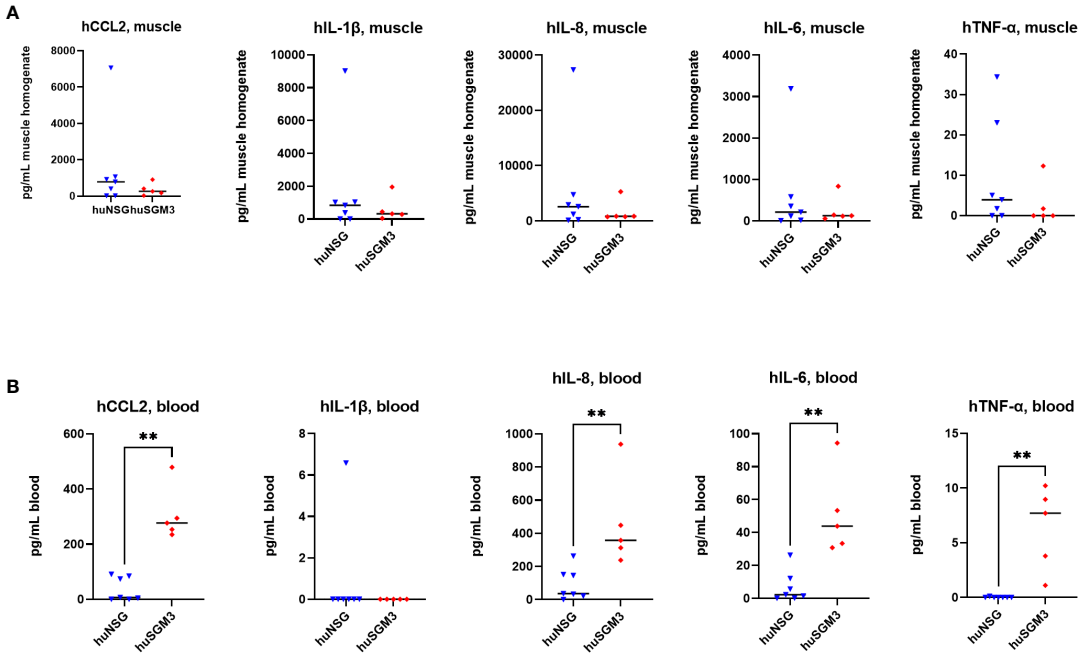

FIGURE 6
Levels of human cytokines in the infected thigh muscle **(A)** and the blood **(B)** of *S. aureus*-infected huNSG or huSGM3 mice on day 2 p.i. **(A)** The infected thigh muscles were recovered and homogenized in sterile PBS. Cytokine levels in filtered homogenate were then determined by a Luminex assay. **(B)** Blood serum was recovered on day 2 p.i. and the cytokine levels measured with a Luminex assay. Displayed are the individual values and the respective median per group. Statistical significance was tested with Mann-Whitney test (**p < 0.01).

When huSGM3 and huNSG as well as the control groups were infected with *S. aureus* into the left thigh muscle, we could observe two outcomes: (I) both humanized mouse groups developed systemic signs of disease and succumbed to the bacterial infection, while wild type, immunodeficient or murinized mice survived and could control the infection and (II) the next-generation huSGM3 mice were significantly more vulnerable than huNSG mice. Interestingly, the impaired survival of humanized mice was not accompanied by an increased bacterial burden in the infected thigh muscle or inner organs, suggesting that the detrimental outcome was not linked to bacteria overwhelming the immune system in the early phase of the infection. The comparison of the rate of humanization and the time at which each individual mouse reached the humane end point demonstrated a clear influence of the humanization on the outcome of the infection. This is in line with earlier studies demonstrating this correlation in huNSG mice (14, 16). On the

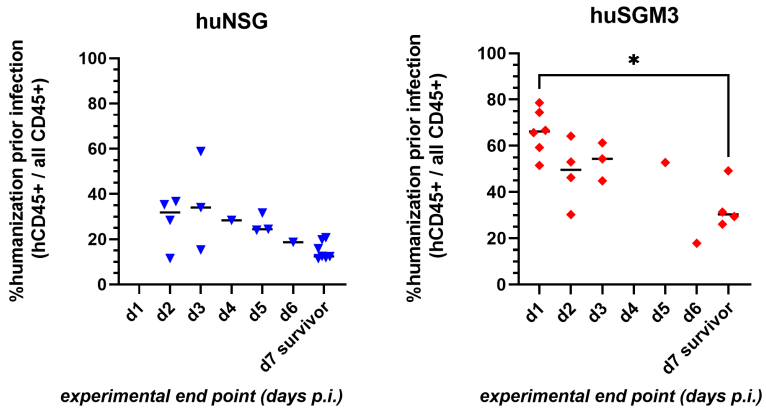

FIGURE 7
Correlation of rate of humanization prior infection and severity of disease during infection experiment. Blood was drawn from each individual mouse prior infection and analyzed by flow cytometry for the presence of human and murine CD45+ cells. The experimental end point was defined by either the mouse reaching the humane end point (according to the score sheet) or surviving until day 7 p.i. (when the experiment was ended). Statistically significant differences between points in time were calculated by Kruskal-Wallis with Dunn's multiple comparison test (*: p < 0.05).

other hand, the humanization of both NSG and NSG-SGM3 mice led to lower numbers of murine Ly6G+ neutrophils and Ly6C+ monocytes, which might as well have impacted the outcome of the infection. Since the number of murine cells was similar for huSGM3 and huNSG, we can assume that the difference in susceptibility between these two groups is not linked to the murine immune system. The repertoire and capability of the human immune system in huNSG and huSGM3 mice on the other hand appeared largely different. HuSGM3 mice had overall higher numbers of human CD45+ cells in the blood and the spleen with particularly higher numbers of myeloid cells. But interestingly, the increased number of human neutrophils in the blood of huSGM3 mice did neither help to control nor limit the bacterial infection compared to the huNSG mice, even though they play a decisive role in the human immune defense against *S. aureus* (24, 25, 39). The higher human immune cell content was accompanied by elevated levels of cytokine in the blood, suggesting strong activation of the human immune system in huSGM3 mice. Increased levels of IL-6, IL-8 and TNFα have been shown to be significantly increased in *S. aureus* patients compared to healthy controls (43) and are associated with a complicated course of infection in *S. aureus* bacteremia patients (44–46). Our data showed significantly higher levels of these cytokines in the blood of huSGM3 than in huNSG mice. This suggests, together with the decreased survival, that huSGM3 mice might reflect the clinical course of severe *S. aureus* infections closer than huNSG or wildtype mice and that they might be a promising model to study cytokine intervention therapy. On the other hand, further efforts to improve the current humanized mouse models might be necessary to close the gap between model and clinics. In particular, because both, huNSG and huSGM3, deviate from the clinical representation by some missing, underrepresented or immature immune cell lineages, impaired antigen-specific adaptive immunity or limited graft-to-host tolerance (23).

We can summarize, that both the increased human immune cell reconstitution, particularly of myeloid cells, and the stronger human immune response in huSGM3 mice failed to control or resolve *S. aureus* infection in this deep-tissue abscess model. Our study suggests on the contrary that the stronger humanization of huSGM3 mice had a detrimental effect on the survival after local infection with *S. aureus*. This might indicate the failure of the human immune system to fight *S. aureus* as efficiently as the murine one and/or the adaption of *S. aureus* to components of the human immune system. Furthermore, humanized mice might help to reveal the pathogenic potential of *S. aureus*, which is impaired during infection in wild-type mice since many of the deployed virulence factors have a high degree of human specificity. It might be very interesting for future studies to reveal whether *S. aureus* adapts its pathogenic strategy and gene expression when infecting humanized mice compared to wild type mice. Taken together, we could show that next-generation humanized mice are more vulnerable to *S. aureus* infection than previous mouse models and might help to understand in the future why and how *S. aureus* became one of the most successful pathogens in humans.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Government of Lower Franconia, Germany.

Author contributions

TH, KO, SS, and JD contributed to conception and design of the study. SH, AK, FK, LD, EI and TH performed the experiments. SH, AK, FK, LD, EI and TH analyzed the data. TH and SH wrote the first draft of the manuscript. AK, FK, JD, EI, SS and KO wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1127709/full#supplementary-material

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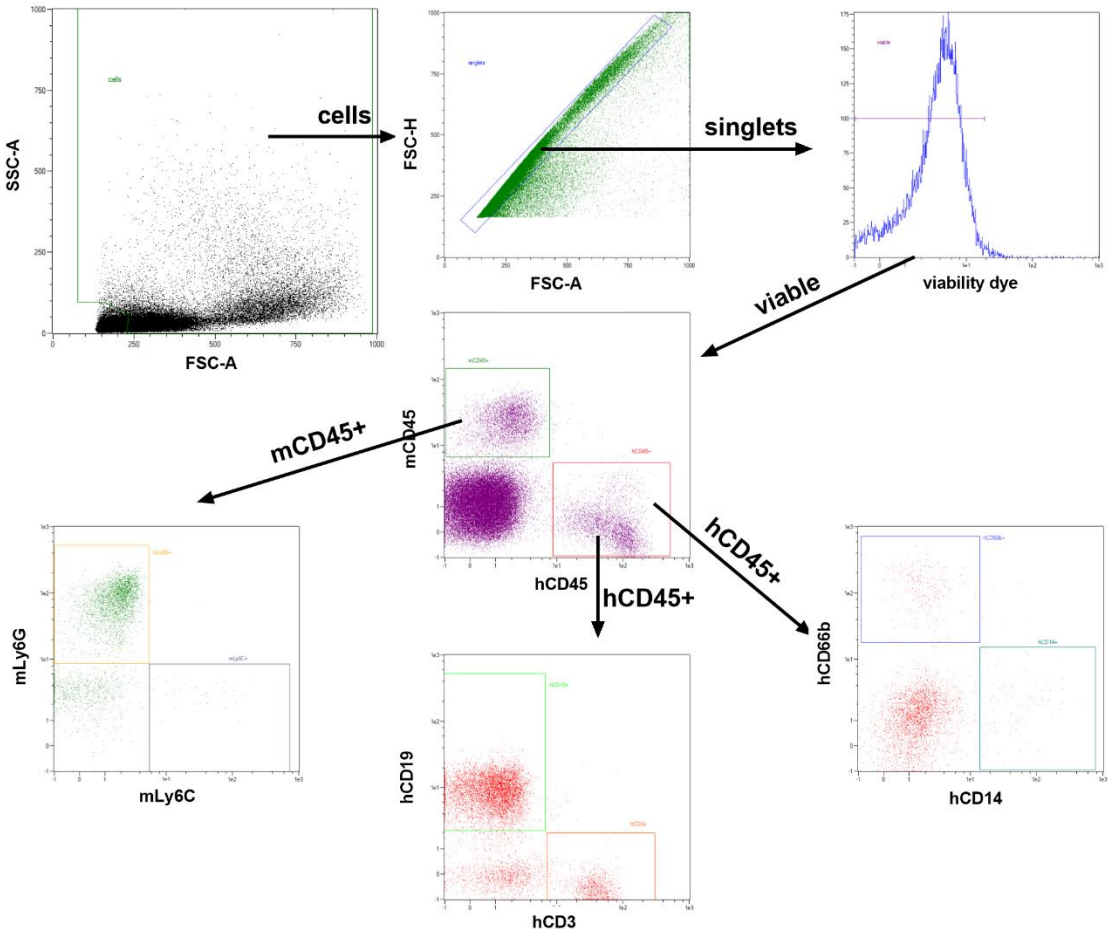

Fig. S1: Schematic representation of the hierarchical gating strategy for the analysis of human and murine immune cells in blood, spleen and bone marrow. Single cell suspensions were stained with viability dye and only viable singlets analyzed for their surface antigen composition.

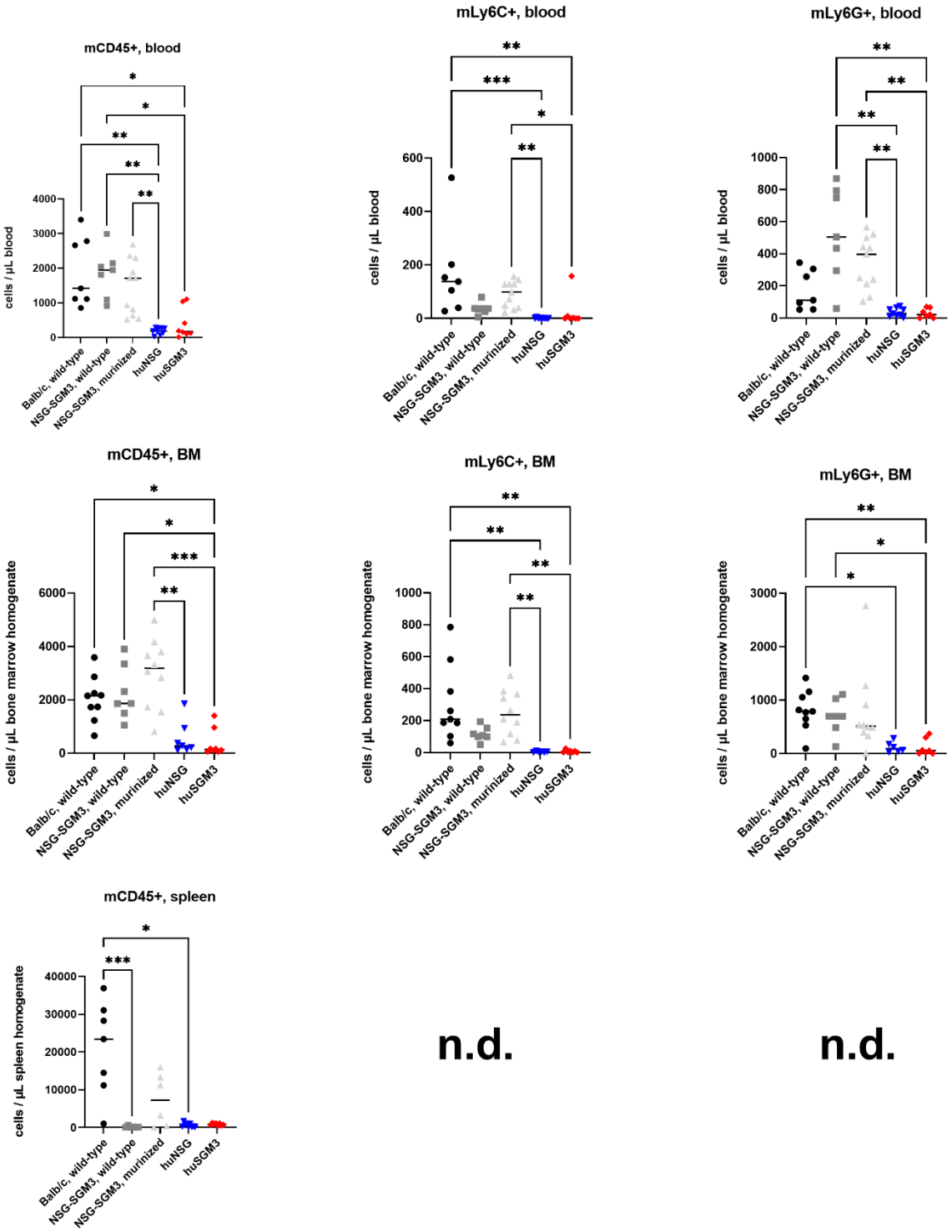

Fig. S2: Murine immune cells in *S. aureus* infected huNSG and huSGM3 mice. Immune cells were measured by flow cytometry with antibodies against hCD45, mCD45, mLy6C, and mLy6G. **(A)** Blood samples were analyzed at day 2 p.i.. **(B)** Bone marrow was harvested by flushing tibia and femur at day 2 p.i.. **(C)** Spleens were recovered at day 2 p.i. and homogenized by pressing through a 70 µm cell strainer. Displayed are the individual values per mouse as well as the medians per group. Statistical significance was tested with either Kruskal-Wallis with Dunn's multiple comparison test (A) or Mann-Whitney-test (B + C) (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$).

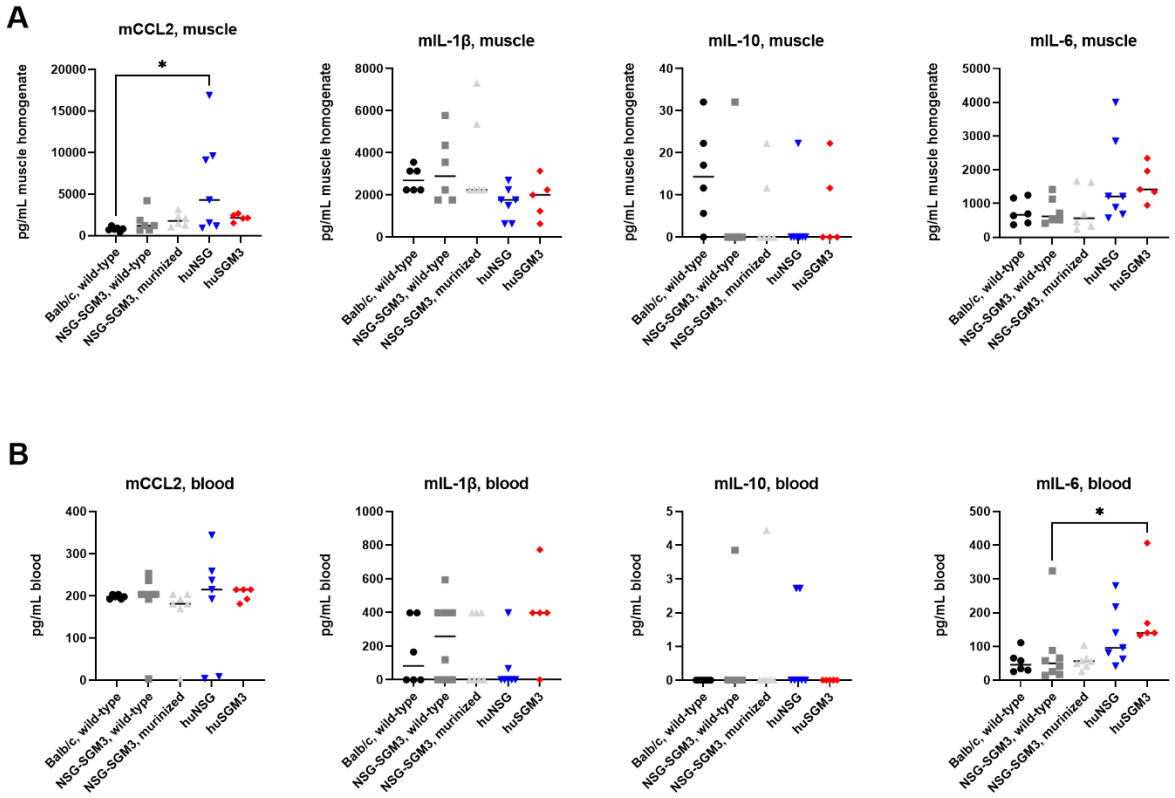

Fig. S3: Levels of selected murine cytokines in the infected thigh muscle (A) and the blood (B) of *S. aureus* infected humanized, wild-type or unreconstituted mice at day 2 p.i.. (A) The infected thigh muscles were recovered and homogenized in sterile PBS. Cytokine levels in filtered homogenate were then determined by a Luminex assay. **(B)** Blood serum was recovered at day 2 p.i. and the cytokine levels measured with a Luminex assay. Displayed are the individual values and the respective median per group. Statistical significance was tested with Kruskal-Wallis with Dunn's multiple comparison test (*: $p < 0.05$).

5 Discussion

The discovery of penicillin ushered in a “golden age”, decades in which dozens of new antibiotics were brought to market and bacterial infections seemed close to defeat (Hutchings et al. 2019). Today, we face a different reality dominated by an increasing antimicrobial resistance and all its consequences (Ventola 2015). A development that is embodied by methicillin-resistant *S. aureus* (MRSA), the pathogen causing the most clinical infections and deaths in the United States, while being the runner up in the European Union (Center for Disease Control and Prevention 2019; Cassini et al. 2019). Accordingly, many attempts have been made to develop new therapies, such as vaccines, but all have failed once they reached the clinical stage (Miller et al. 2020; Fowler, JR and Proctor 2014). The reasons for this are manifold but can at least in part be traced back to limitations of the mouse model, commonly used in pre-clinical trials. The small rodents, while practical in handling, diverge notably from humans in their sensitivity towards a *S. aureus* infection (Salgado-Pabón and Schlievert 2014). To circumvent the marked human host specificity of *S. aureus* and align the pre-clinical results with the clinical reality, humanized mice have emerged as a promising new model (Clegg et al. 2021; Parker 2017). Humanized mice is the umbrella term for a variety of immunodeficient mouse strains that have been engrafted with human tissues and/or human hematopoietic stem cells, resulting in a viable human immune system (Shultz et al. 2012). The research into human-specific pathogens, such as HIV (Abeynaike and Paust 2021) or *Salmonella enterica* subsp. *enterica* serovar Typhi (Libby et al. 2010) has particularly profited from these models, but also for *S. aureus,* equipped with a variety of human-adapted virulence factors, humanized mice could bring new and decisive insights (Clegg et al. 2021). Humanized NSG (huNSG) mice are often used in the field, including a limited number of *S. aureus* studies investigating skin infections (Tseng et al. 2015), peritonitis (Knop et al. 2015), pneumonia (Prince et al. 2017), and most recently, osteomyelitis (Muthukrishnan et al. 2021). Each of these studies attested a higher susceptibility of the humanized NSG cohort to *S. aureus*, than their wild-type, murinized (NSG mice engrafted with murine stem cells) and non-engrafted NSG counterparts.

With these studies focusing primarily on the acute state of infection, the aim of the first publication was to elucidate how a long-term infection with MRSA affects the humanized NSG mice and its influence on the behaviour of the human immune system in the mice. A deep-tissue abscess model was chosen, giving the opportunity to observe both the acute and the chronic stages of a local infection. To achieve this, $1x10^8$ CFU of *S. aureus* LAC* *lux* was injected into the thigh muscle and the animals were removed from the experiment after 2 or 7 days. Similar to the results of the earlier studies mentioned above, the humanized NSG mice proved to be more susceptible to an MRSA infection. This disparity was observed from the

beginning of the experiment. Already 24 hours after the infection, the huNSG mice showed significantly increased bioluminescence signals in the thigh muscle, a trend that was evident throughout the experiment. The humanized mice also had a considerably higher bacterial load in the thigh muscle on day 7 p.i. It is of note, that while the control groups, consisting of Balb/c mice, murinized NSG mice, and non-engrafted NSG mice, were able to reduce or at least maintain the bacterial burden, the amount of bacteria in the infected muscles of the humanized mice rose significantly between day 2 and day 7 p.i.

However, looking at the survival rate, this is where the differences between the humanized mice and the control groups become particularly obvious. Of a group of twenty animals, seven humanized NSG mice reached the humane endpoint between day two and day seven p.i. This means that the animals were so strongly affected by a local tissue infection that they had to be taken out of the experiment. An animal welfare score sheet was used to monitor weight loss, signs of distress like hunchback posture and heavy breathing, among others. Only one wild-type NSG mouse was similarly afflicted, the wild-type Balb/c group and the murinized NSG mice had a 100% survival rate. These findings surpassed our hypothetical assumptions of a higher susceptibility. The literature describes higher mortality in humanized NSG mice, but in a peritonitis model, which differs greatly from our local model (Knop et al. 2015).

So why did a localized deep tissue infection escalate into a lethal disease for these animals? One possibility might be that bacteria ascended from their original site of infection, spreading systemically and infecting inner organs. *S. aureus* is indeed known for its ability to cause life threatening diseases that can stem from local infections such as skin and soft tissue infections (Tong et al. 2015b). And both Muthukrishnan et al. and Knop et al. have shown that humanized mice are more affected by systemic spreading of the pathogen (Muthukrishnan et al. 2021; Knop et al. 2015). Consequently, the bacterial burden of the liver, spleen, heart, and kidneys was analysed. The difference between humanized mice and the control groups was not as clean cut in the inner organs as it was in the muscle, the primary herd of infection. Still, *S. aureus* infected a higher percentage of organs in the humanized NSG mice than in the murinized and the Balb/c group. The humanized mice also had a higher amount of colony forming units in the inner organs, particularly on day 7. Here, only the group of non-engrafted NSG mice did not show significantly lower numbers.

Furthermore, a direct connection between the rate of humanization and the severity of the disease progression was detected. All mice that were designated to the day 7 cohort and those that reached the humane endpoint were arranged into three groups, according to their humanization status. The group that contained the mice with the highest humanization rate

reached the humane endpoint significantly more often than the less humanized groups. In addition, the humanization rate prior infection between the mice that belonged to the 2 day p.i. group and the 7 day p.i. group, and those animals that reached the humane endpoint before they could complete the experiment was compared. Confirming the results above, the humane endpoint group had a significantly higher humanization rate than the day 7 p.i. group. It should be noted that the humanization rate of the day 2 p.i. group did not significantly differ from the day 7 p.i. group or the animals that reached the humane endpoint. This is probably because the day 2 group contains both animals that are highly humanized and, if they had remained in the experiment, would likely have reached the humane endpoint, as well as those that are only moderately or weakly humanized. It is important to always keep in mind when looking at the different results on day 2 p.i. and day 7 p.i., be it CFU numbers or cytokine and immune cell levels, that specifically the day 7 p.i. group only consists of the mice that survived. The animals with the highest humanization rates mostly reached the humane endpoint before and had to be taken out of the experiment. This means in turn, that the day 7 p.i. group consists mostly of mice with lower or medium humanization rates. It is conceivable, that if these mice would have survived and samples could have been taken, that the differences between the humanized mice group and the controls would have been even greater. To state it succinctly, the better the mice are humanized (i.e. the higher the rate of human immune cells of the mice going into the experiment), the lower the likelihood for the mice to survive. This pattern, though with a less severe outcome, has also been detected by Tseng et al. In their study, higher humanization degrees led to bigger skin lesions (Tseng et al. 2015).

Considering the fact that human immune cells so negatively affected the animals, we searched for possible differences in the human and murine immune reaction to the infection. At first, the cytokine patterns in the blood and the thigh muscle were examined. Humanized mice presented noticeably higher levels here than the control groups, including both murine and human cytokines. The huNSG mice had higher levels of murine IL-6, IL-10 and MCP-1, particularly on day 2 p.i., plus an analogous amount of the corresponding human cytokines, indicating that humanized mice react to an infection with *S. aureus* with a stronger inflammatory response. The differences between the murine and the human immune response were underlined by the fact, that while murine cytokines remained relatively stable between day 2 p.i. and day 7 p.i., their human counterparts actually decreased. This dynamic also contrasts with the bacterial burden, which increased in both the thigh muscle and the inner organs between the two points in time. Possible explanations for the drop of human cytokines could be either a downregulation of the inflammation process or a depletion of the immune cells producing the cytokines. In order to investigate the latter possibility, the amount of T and B cells in the blood, the bone marrow and the spleen were measured, as these cell lines make

up the majority of the human cells in engrafted NSG mice (Blümich et al. 2021). In the blood, both cell populations remained relatively stable between day 2 p.i. and day 7 p.i. B cell numbers remained constant in the spleen as well but decreased significantly in the bone marrow. This depletion could be a sign that the immune system of humanized mice is overwhelmed by the infection and unable to supply sufficient new cells to offset the demand. T cells on the other hand increased in the spleen, which has been described before in a osteomyelitis model (Muthukrishnan et al. 2021), likely due to an activation of the T cell response due to the infection. To determine how other immune cell lineages reacted to the infection, the neutrophil and monocyte cells in the blood and the bone marrow were investigated. Neutrophils play an important role in the immune defence against *S. aureus*, but only represent a small percentage of human immune cells in engrafted NSG mice (de Jong et al. 2019; Coughlan et al. 2016). Similarly low numbers were measured, that showed a declining tendency towards day 7 p.i. but no significant differences. Monocytes on the other hand decreased significantly in both organs between the two points in time. This is particularly interesting, because the murine monocytes presented an opposite behaviour, with numbers rising substantially from day 2 to day 7 p.i. This was also true for murine monocytes in the non-humanized mice as well as the humanized ones. The drop in human monocytes could explain the decline in human cytokines discussed above.

To recapitulate, in the humanized NSG mice human $CD19^+$ B cells decreased strongly in the bone marrow in the course of the infection, as did $CD14^+$ monocytes, both in the bone marrow and the blood. This indicates that the human immune system is overwhelmed by the bacterial infection, disseminating from the original injection sites to the inner organs, and unable to keep up with the demand for new immune cells to restrain the pathogen. A reason for this could be that the human immune system is not completely evolved, certain immune cells, such as neutrophils, are barely measurable, others are unable to fully differentiate (Fujiwara 2018; Coughlan et al. 2016). Although this could be seen as a restriction for the use of humanized mice, these animals might actually be able to mimic some of the most vulnerable patients, namely those suffering from a weakened immune system, especially with impaired neutrophil function, like chronic granulomatous disease or congenital neutropenia (Amulic et al. 2012; Winkelstein et al. 2000).

It was outside the scope of this study to examine the behaviour of the immune cells in the infected thigh muscle on a single cell level. This should be considered as important future research in order to determine to determine which cells migrate to the primary herd of infection and how the human and murine cells differentiate here. An increased migration might explain the decimation of the human monocytes, but apoptosis or an affected reproduction in the bone

marrow are also possibilities. It is also important to remember that *S. aureus* is equipped with a variety of virulence factors that have a strong human affinity, such as the Panton-Valentine leukocidin, immunomodulators, and superantigens (Koymans et al. 2017; Löffler et al. 2010; Faulkner et al. 2005). It is conceivable that these factors play a role in the decline of human B cells and monocytes.

Considering all these aspects, it seems likely that humanized NSG mice portray a more accurate human-like clinical picture of a *S. aureus* infection than conventionally used mice. This could offer researchers an alternative model to test new therapeutic approaches before they enter the clinical stages. However, this model has still a number of limitations, such as the missing human thymus, which is important for the development of the T cells (Lee et al. 2019). Moreover, the inadequate engraftment of myeloid cells in the NSG mice might additionally restrict the translational power of our research findings.

Several growth factors and cytokines are required to enable sufficient development of myeloid cell lines in the bone marrow. The signalling molecules available in the mouse often lack cross-reactivity, impeding the complete development of the engrafted human hematopoietic stem cells. To circumvent this issue, the missing factors can be injected, which requires repeated administrations; or the mice can be genetically modified accordingly (Martinov et al. 2021). An example for the latter technique are the NSG-SGM3 mice, that combine the immunodeficiency of the NSG mice with three human myeloid-enhancing transgenes. These next-generation mice are equipped with human SCF, GM-CSF and IL-3 cytokines that markedly improve the reconstitution of the myeloid cell lines (Coughlan et al. 2016). It was of great interest to know whether these human cytokines would influence the course of an *S. aureus* infection and thus making humanized NSG-SGM3 (huSGM3) mice an even better *in vivo* model to study the pathogen than humanized NSG mice.

Consequently, for the second publication, NSG-SGM3 mice were humanized in the same way as the huNSG mice, namely by irradiating the animals at low dosages and subsequently injecting cord-blood derived human stem cells via the tail vein. The expression of the three human cytokines in the huSGM3 mice not only led to dramatically higher neutrophil numbers, but also to an overall increase in human $CD45^+$ cells, which is consistent with previous studies (Coughlan et al. 2016; Billerbeck et al. 2011). During the 18 weeks humanization period, huSGM3 mice outperformed huNSG mice in all humanization aspects with a higher humanization rate and higher numbers of $hCD45^+$, $hCD3^+$, $hCD19^+$, and $hCD66b^+$ cells. However, huSGM3 mice stopped gaining weight at around week 12, while huNSG mice were gaining weight continuously. Earlier publications have attested that long-term humanized

SGM3 mice are affected by a secondary hemophagocytic lymphohistiocytosis (HLH) or macrophage activation syndrome (MAS)-like disease (Janke et al. 2021; Yoshihara et al. 2019; Wunderlich et al. 2016). The term MAS is varyingly used synonymously with secondary HLH or described as a form of the latter (Henderson and Cron 2020; Crayne et al. 2019). HLH can be caused by a genetic predisposition (primary/familial HLH) or arise following other conditions, such as viral infections, malignancies, autoimmune diseases, or immunodeficiencies. Though in some cases of secondary HLH, there is no recognizable trigger (Jordan et al. 2019; Hayden et al. 2016). Clinically, the disease manifests itself among others with fever, cytopenia, hemophagocytosis and splenomegaly, caused by an excessive activation and proliferation of macrophages and T-cells, which release large quantities of proinflammatory cytokines, culminating in a “cytokine storm” (Obayo et al. 2020; Filipovich et al. 2010). A limited number of studies have investigated the syndrome in humanized NSG-SGM3 mice, noticing that huSGM3 mice first started losing weight around 16 weeks after they were first humanized. The animals soon became moribund, and had all died by week 27, at the latest. In contrast, humanized NSG mice and non-engrafted NSG-SGM3 mice were not affected. Blood analysis of huSGM3 mice showed that the animals suffered from anaemia, reticulocytosis, low white blood cells and reduced numbers of platelets (Yoshihara et al. 2019; Wunderlich et al. 2016)

To get a more accurate picture of the health of the huSGM3 mice used in this study, peripheral blood samples were analysed biweekly. The haemoglobin and red blood cell levels started to decline around week 12, while reticulocyte numbers had increased even earlier. The reticulocytosis suggests that, while the erythropoiesis proceeds normally in these mice, the red blood cells are actively decimated or suffer from increased usage (Yoshihara et al. 2019; Wunderlich et al. 2016). In contrast, huNSG mice showed no signs of anaemia and were all healthy until week 18. To determine if the animals were indeed affected by a MAS or secondary HLH-like disease, further investigations would be necessary, particularly histological studies. This was outside the scope of this thesis, but the practical aspect was more important, more precisely, when is the best point of time to infect the humanized SGM3 animals. Balancing the humanization levels with the haematological changes, the huSGM3 mice entered the experiments 12 weeks after the hematopoietic stem cell administration, when the disease was not yet advanced enough to interfere with the results, and the number of human immune cells in the huSGM3 mice was already markedly higher than in the huNSG cohort. Age-matched murinized NSG-SGM3, non-engrafted NSG-SGM3, wild-type Balb/c mice and humanized NSG mice at 18 weeks post $CD34^{+}$stem cell application were included to be able to detect differences in the susceptibility towards *S. aureus.*

All groups were infected with *S. aureus* in the thigh muscle, using the same model as in the first publication. The humanized NSG mice again were more strongly affected by the pathogen than the non-humanized groups, with a substantial number of animals reaching the humane endpoint before the termination of the experiment. The humanized SGM3 mice clearly surpassed these results, with animals also starting to deteriorate much sooner in the experiment. In fact, the first animals reached the humane endpoint on day 1 of the experiment and by day 2 p.i. already around 50 % of the animals had been eliminated. The huNSG mice did not reach this point until day 7 p.i. At this time, only around 25% of the humanized SGM3 mice were still alive, to the effect that not enough animals were available to create meaningful statistics for day 7 p.i.

Striving to pinpoint the reasons for this distinct difference in susceptibility, the bacterial burden in the thigh muscle and the inner organs was measured on day 2 p.i., the only point in time available. At the primary herd of infection, the muscle, no significant differences manifested at all between the different groups. In the inner organs, the only significant difference in colony forming units manifested between the humanized groups and the Balb/c mice. Taking all these results into account, our observations strongly suggest that the survival rate was not directly linked to the bacterial burden and the animals did not succumb to the infection because of an unchecked proliferation and dissemination of the pathogen.

Another possible explanation for the higher vulnerability in humanized mice was a reduced functionality of the murine immune system. The non-humanized groups indeed had higher numbers of murine leukocytes, including murine neutrophils and monocytes; however, there was no difference between the huNSG mice and the huSGM3 mice concerning these cells, which leads to the conclusion that it was not the missing protection by the murine immune system that resulted in their differing survival rate. The situation looked very different when examining the human immune compartment more closely, as the two mouse strains diverged notably here. The huSGM3 mice had significantly more human immune cells in the blood than huNSG mice, including T cells, B cells, monocytes, and particularly neutrophils. The two strains also differed in their dynamics during the infection. Cell numbers remained stable in the huNSG mice between day 0 p.i and day 2 p.i., while T cells and B cells decreased in the huSGM3 on day 2 p.i., with neutrophils numbers rising significantly. The huSGM3 mice also had more human immune cells in the spleen, but, interestingly, no differences between the two strains could be found in the bone marrow. The boosted human immune system in the huSGM3 mice apparently did not enable the mice to curb the *S. aureus* infection, which is surprising considering the relevant role of neutrophils in the human immune defence against the pathogen (de Jong et al. 2019).

It seemed reasonable to suspect, that the high numbers of human immune cells were also accompanied by increased cytokine levels. Although no variances between the two humanized groups in the thigh muscle could be found, in the blood, hCCL2, hIL-8, hIL-6 and hTNF-α numbers were significantly higher in the huSGM3 mice. The elevated cytokine numbers in the blood likely reflected the systemic immune response to the bacteria disseminating from the original herd of infection. The analysed murine cytokines did not exhibit major differences between the groups, including the non-humanized control groups; this suggests that the *S. aureus* infection did not lead to diverse activations of the murine immune cells. In human patients with invasive *S. aureus* infections, Il-6, IL-8 and TNF-α levels have been shown to be elevated compared to healthy controls (Leuzzi et al. 2021). These cytokines have also been linked to adverse outcomes of *S. aureus* bacteremia (Chantratita et al. 2017; Minejima et al. 2016).

Lastly, the question arose whether the humanization rate had an impact on the progress of the infection. When comparing the point at which each mouse succumbed to the infection with the humanization rate before they entered the experiment, a dependence between the two became obvious. In short, the higher the humanization rate of an animal, the sooner they reached the humane endpoint. These results correlate with the findings in the first publication, where strongly humanized huNSG mice had a lower likelihood of survival.

All these results show that humanized SGM3 mice mimic severe *S. aureus* disease progressions in humans better than previous models, including huNSG mice. On the other hand, humanized NSG-SGM3 mice do not embody the human immune system in all its aspects either. Several cell lines are underrepresented and immature in this model as well and the missing human thymus continues to be an issue (Martinov et al. 2021) as does the overexpressing of the three cytokines, which leads to the above-mentioned disordered haematopoiesis. Further improvement and adaptation of the next-generation humanized mice might be necessary.

When examining the results of both publications, one fact is standing out: The more ‘human’ the animals become, the more susceptible they are to a local *S. aureus* infection. Highly humanized NSG mice were more likely to reach the humane endpoint than their less humanized counterparts. HuSGM3 mice, which express more human cells in the blood than huNSG mice, were in turn significantly more affected. And the more human cells these animals carried, the sooner they succumbed to the pathogen. However, it must be noted at this point, that the exact reasons why the animals die, are still unknown. While in the first publication an

overpowering of the human immune system by the systemically spreading bacteria in the huNSG mice was discussed, in the second publication no clear differences between the different groups could be determined in this area. On the other hand, the significant differences of the bacterial burden between the huNSG mice and the control groups only manifested on day 7 p.i., a point in time that was not available for the humanized NSG-SGM3 mice, due to their rapid decline. Though considering the earlier onset of the disease, a higher bacterial count should probably have manifested on day 2 p.i., if this indeed caused their death. In any case, a future step should be to reduce the infection dose, to be able to observe a longer, more chronic development of the tissue abscess model in the huSGM3 mice.

HuNSG mice showed more elevated cytokine levels than the control groups with some cytokines showing significantly higher levels in the huSGM3 mice compared to the huNGS animals. However, it is doubtful, that this could be classified as a 'cytokine storm', which causes life-threatening symptoms (Fajgenbaum and June 2020). Humanized NSG-SGM3 mice also had significantly higher human immune cell levels, including T cells, B cells, monocytes, and, importantly, neutrophils, than humanized NSG mice, but obviously none of these could protect the mice. How exactly this leads to the decreased survival of the mice remains to be determined. There are many questions left to be answered and still a lot of research is required. In addition to the above-mentioned reduced inoculum, single-cell sorting, RNA sequencing, and using knock-out *S. aureus* strains would be reasonable next steps to better understand the processes happening *in vivo*. Particularly the latter one could elucidate the topic of the host tropism of *S. aureus,* which possesses many human-adapted virulence factors (Salgado-Pabón and Schlievert 2014). A prominent example is the Panton-Valentine leukocidin (PVL). Tseng et al. were able to show that PVL^+ strains lead to larger skin lesions than PVL^- *S. aureus* (Tseng et al. 2015). It would be interesting to examine how the different virulence factors impact the infection and how exactly the pathogen adapts itself to the different environment it faces in the humanized mice, compared to the wild-type mice.

In conclusion, humanized mice require substantially more effort than wild-type mice, both financially and labor-wise, and will not be the right model for large-scale studies. Nevertheless, they possess the potential to answer vital questions about the interactions between the human immune system and *S. aureus.* They can be regarded as a chance to contribute to animal welfare by refining experiments and reducing the number of animals needed and might one day actually provide the breakthrough for a much-needed vaccine.

6 Summary

Susceptibility of humanized mice in a localized *Staphylococcus aureus* deep-tissue abscess model

Staphylococcus aureus is a widespread Gram-positive bacterium that colonizes up to 80% of the human population as a facultative pathogen. It can cause a variety of diseases, ranging from mild skin infections to life-threatening endocarditis, pneumonia, and sepsis. *S. aureus* is particularly notorious for its ability to develop resistances against antimicrobial agents, a process that dates back to the discovery of penicillin. Today, methicillin-resistant *S. aureus* presents one of the greatest burdens to our healthcare system. The development of new treatments is therefore of the utmost urgency, especially that of an effective vaccine. So far, however, all promising candidates have failed as soon as they reached the clinical phase. One of the main reasons for this is the species-specificity of *S. aureus*, which is equipped with several virulence factors that are adapted to the human immune system. A promising new method for bridging this translational gap are humanized mice. These are immunodeficient mouse strains engrafted with human stem cells, leading to the development of a viable human immune system. One of the most commonly used strains is the NSG mouse, which was also chosen in the *S. aureus* infection studies conducted so far. These encompass only a small number of models, including peritonitis, skin infection, pneumonia, and osteomyelitis. Humanized NSG (huNSG) mice were more susceptible to the pathogen than the control groups in all of these models.

For this thesis, 6 to 8 week old NSG mice were engrafted with cord-blood derived human stem cells and infected after 18 weeks with *S. aureus* in a localized, deep-tissue abscess model. The huNSG mice and the control groups, wild-type mice (Balb/c), murinized NSG mice (mice implanted with murine bone marrow cells), and non-engrafted NSG mice were weighed every day and their general condition was checked. In addition, the bioluminescence signal in the infected muscle was measured daily. The animals were randomly removed from the experiment after two or seven days, to cover both the acute and the chronic stages of the infection. The huNSG mice consistently showed stronger bioluminescence signals and a higher bacterial burden in the muscle could be detected on day 7. The difference in survival between the groups was particularly striking. All control animals (except one non-engrafted NSG mouse) assigned to the day-7 group survived until the end of the experiment. In contrast, 7 of 20 huNSG animals reached the humane endpoint before. In huNSG mice, the bacteria spread to significantly more inner organs than in the wild-type and the murinized mice, where they also reached a higher bacterial count. Importantly, a direct correlation between the survival probability and the degree of humanization could be determined. HuNSG mice had significantly higher human and murine cytokine levels in the blood and the muscle than the

control groups. The dynamics between the human and murine cytokines also varied greatly. While the levels of murine cytokines remained mostly constant, their human counterparts decreased significantly between day 2 and day 7. The number of human B cells also decreased noticeably in the bone marrow, the same being true for monocytes in the blood and bone marrow. In contrast, murine monocytes increased significantly.

Humanized NSG mice sustain only very small numbers of human neutrophil cells, which play an important role in the human immune defence against *S. aureus*. Consequently, humanized NSG-SGM3 (huSGM3) animals were used for the second trial, as this mouse strain is equipped with three human cytokines that support the formation of myeloid cell lineages. However, overexpression of these cytokines also leads to undesirable side effects in these mice. The animals become anaemic during long-term humanization and eventually die. For this reason, the huSGM3 mice entered the experiments 12 weeks post engraftment. At this time, the animals showed a robust degree of humanization and only slightly decreased erythrocyte and haemoglobin levels. The humanized SGM3 mice were significantly more sensitive in the muscle abscess model than the control groups, including the HuNSG mice. The mice reached the humane endpoint earlier and in such large numbers, that not enough animals were available for the day 7 group. No differences could be detected with respect to the bacterial load in muscle and internal organs that would have explained the death of the animals. In addition, the number of murine immune cells available did not vary between the two humanized groups. In contrast, the huSGM3 mice had significantly more human cells in the blood and spleen; these included B cells, T cells, monocytes, and neutrophils. Individual cytokine levels, namely hCCL-2, hIL-8, hIL-6, and hTNF-α, were also significantly increased in the humanized SGM3 mice. Similar to the first trial, a direct correlation between the degree of humanization and a severe progression of the infection could be found.

Further experiments are needed to fully understand the interactions between the bacterium and the human immune system in the humanized mice. It will be particularly important to characterize the role of virulence factors, such as the Panton-Valentin-Leukocidin, which are characterized by their affinity to human cells. Nevertheless, the results obtained in this thesis represent important new gains in knowledge, which may contribute to the development of an effective vaccine against *S. aureus*.

7 Zusammenfassung

Empfindlichkeit humanisierter Mäuse in einem lokalen *Staphylococcus aureus* Abszessmodell im tiefen Gewebe

Staphyloccous aureus ist ein weitverbreitetes, Gram-positives Bakterium, das als fakultativ pathogener Erreger bis zu 80% der Bevölkerung kolonisiert. Dieses Pathogen kann eine Reihe von Krankheiten verursachen, die von einfachen Hautinfektionen bis hin zu lebensgefährlichen Infektionen wie Endokarditis, Pneumonie und Septikämie reichen. Besonders berüchtigt ist *S. aureus* für seine Fähigkeit Resistenzen gegen antimikrobielle Wirkstoffe zu entwickeln. Ein Prozess, der schon mit der Entdeckung des Penicillins begann. Heute stellen Methicillin-resistente *S. aureus* eine der größten Belastungen für unsere Gesundheitssysteme dar. Die Entwicklung neuer Behandlungsmethoden ist deshalb von großer Dringlichkeit. Dies gilt insbesondere für die Entwicklung eines effektiven Impfstoffes. Bisher sind allerdings alle vielversprechenden Ansätze gescheitert, sobald sie die klinische Phase erreichten. Einer der Hauptgründe hierfür ist die Speziesspezifität einer Reihe von Virulenzfaktoren von *S. aureus,* die humane Immunzellen deutlich stärker schädigen als die gleichen Zelltypen der Maus. Eine vielversprechende neue Methode, um die translationale Lücke zwischen Maus und Mensch zu überbrücken, sind humanisierte Mäuse. Hierbei handelt es sich um immundefiziente Mauslinien, denen humanen Stammzellen implantiert werden, aus denen sich in der Folge ein humanes Immunsystem entwickelt. Eine der hierfür am häufigsten verwendeten Mauslinien ist die sogenannte NSG-Maus, diese wurde auch in den bisher durchgeführten Infektionsversuchen mit *S. aureus* verwendet. In Peritonitis-, Haut-, Pneumonie- und Osteomyelitis-Modellen erwiesen sich humanisierte NSG (huNSG) Tiere deutlich empfindlicher als die Kontrollgruppen. In dieser Dissertation wurde zunächst 6 bis 8 Wochen alten NSG-Mäusen aus Nabelschnurblut isolierte humane Stammzellen injiziert. Die Mäuse wurden anschließend nach 18 Wochen mit *S. aureus* in einem lokalen Abszessmodell infiziert. Die huNSG-Mäuse und die Kontrollgruppen [= Wildtyp-Mäuse (Balb/c), murinisierte NSG-Mäuse (mit murinen Knochenmarkszellen implantierte Mäuse) und unbehandelte NSG-Mäuse] wurden jeden Tag gewogen und ihr Allgemeinbefinden überprüft. Zudem wurde täglich das Biolumineszenz-Signal im infizierten Muskel gemessen. Die Tiere wurden entweder nach zwei oder sieben Tagen aus dem Experiment genommen, damit sowohl die akute als auch die chronische Phase der Infektion beurteilt werden konnte. Die huNSG-Mäuse zeigten durchgehend ein höheres Biolumineszenz-Signal und am Tag 7 konnte auch eine höhere Keimbelastung des Muskels festgestellt werden. Besonders auffällig war aber die unterschiedliche Überlebensrate zwischen den Gruppen. Alle Kontrolltiere (außer einer unbehandelten NSG-Maus) die der Tag-7 Gruppe zugeordnet waren, haben bis zum Experimentende überlebt. Dagegen erreichten sieben von 20 huNSG-Tieren den humanen

Endpunkt zu früheren Zeitpunkten. In den humanisierten NSG-Mäusen zeigte sich eine deutlich stärkere Streuung der Bakterien in die inneren Organe als bei den Wildtyp-Mäusen und den murinisierten Mäusen. Es konnte zudem ein direkter Zusammenhang zwischen der Überlebenswahrscheinlichkeit und dem Grad der Humanisierung festgestellt werden. HuNSG-Mäuse zeigten deutlich höhere humane und murine Zytokin-Mengen im Blut und im Muskel als die Kontrollgruppen. Auch die Dynamik zwischen den humanen und murinen Zytokinen variierte stark. Während die Werte der murinen Zytokine größtenteils konstant blieben, sanken die humanen Zytokine zwischen Tag 2 und Tag 7 deutlich ab. Auch die Zahl der humanen B-Zellen nahm im Knochenmark stark ab, gleiches galt für die Monozyten im Blut und dem Knochenmark. Die murinen Monozyten nahmen im Gegensatz dazu sichtlich zu. Da humanisierte NSG-Mäuse nur sehr geringe Mengen an humanen Neutrophilen bilden, diese jedoch im Menschen eine wichtige Rolle im Kampf gegen *S. aureus* spielen, verwendeten wir in einer zweiten Versuchsreihe humanisierte NSG-SGM3 (huSGM3) Mäuse. Diese Mauslinie ist mit drei humanen Zytokinen ausgestattet, die die Ausbildung myeloider Zelllinien unterstützen. Allerdings führt die Überexpression dieser Zytokine auch zu unerwünschten Nebenwirkungen bei diesen Mäusen. Die Tiere werden im Laufe der Humanisierung immer anämischer und versterben schlussendlich. Aus diesem Grund wurden die SGM3-Mäuse bereits 12 Wochen nach der Humanisierung für die Infektionsversuche verwendet. Zu diesem Zeitpunkt zeigten die Tiere einen robusten Humanisierungsgrad und nur geringfügig erniedrigte Erythrozytenzahlen und Hämoglobinwerte. Die HuSGM3-Mäuse zeigten sich im Muskelabszessmodell deutlich empfindlicher als die Kontrollgruppen, inklusive den HuNSG-Mäusen. Die Tiere erreichten den humanen Endpunkt früher als die HuNSG-Mäuse, so dass nicht ausreichende Tierzahlen für die Tag 7-Gruppe zur Verfügung standen und dieser Zeitraum somit nicht untersucht werden konnte. Bezüglich der bakteriellen Last im Muskel und in den inneren Organen konnten keine Unterschiede zwischen den HuNSG- und den HuSGM3-Mäusen festgestellt werden, die das Versterben der Tiere erklären könnten. Auch die Anzahl der murinen Immunzellen variierte nicht zwischen den beiden humanisierten Gruppen. Dagegen zeigten sich deutlich mehr humane Immunzellen (B-Zellen, T-Zellen, Monozyten und Neutrophile) im Blut und in der Milz der huSGM3-Mäuse. Auch einzelne Zytokine, nämlich hCCL-2, hIL-8, hIL-6 und hTNF-α, waren in den humanisierten SGM3-Mäusen signifikant erhöht. Weiterhin konnte, ähnlich wie im ersten Versuchsteil, ein direkter Zusammenhang zwischen dem Humanisierungsgrad und dem Schweregrad des Infektionsverlauf festgestellt werden.

Es werden noch viele weiterführende Experimente nötig sein, um die Wechselwirkungen zwischen dem Bakterium und den menschlichen Immunsystem in den humanisierten Mäusen genau zu verstehen. So bleibt die wichtige Frage ungeklärt, welchen Einfluss Virulenzfaktoren, die eine ausgeprägte Affinität humanen Zellen gegenüber zeigen, wie zum Beispiel das

Panton-Valentin-Leukozidin, auf den Infektionsverlauf haben. Dennoch stellen die Ergebnisse dieser Dissertation wichtige neue Erkenntnisse dar, die bei der Entwicklung einer funktionierenden Vakzine gegen *S. aureus* genutzt werden können.

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List of Publications

MRSA Infection in the Thigh Muscle Leads to Systemic Disease, Strong Inflammation, and Loss of Human Monocytes in Humanized Mice

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Next-generation humanized NSG-SGM3 mice are highly susceptible to *Staphylococcus aureus* infection

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Interessenskonflikte - Conflict of Interest

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Würzburg, den 20.10.2023 Sophia Hung