

**Neutrophil antimicrobial defense
against *Staphylococcus aureus***

Contribution of Cathelicidin and the NADPH oxidase

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1. Summary

Neutrophils are among the most important components of the innate immune response, which provides the first line of host defense. The antimicrobial potential of neutrophils has been traditionally divided into either non-oxidative or oxidative mechanisms. Two of the most important antimicrobial systems of these mechanisms are granule-associated antimicrobial proteases and peptides and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase generating reactive oxygen species (ROS). In the past, studies often focused on the effects of either non-oxidative or oxidative mechanisms and decades of research have provided a detailed understanding of the regulation, generation and actions of these processes alone. Recent evidence challenged the established view of two independent mechanisms and proposed the cooperation of the NADPH oxidase with granule proteases in the killing of microorganisms. Furthermore, a novel phagocytosis-independent antimicrobial mechanism was found by the discovery of neutrophil extracellular traps (NETs). The formation of NETs was found dependent on NADPH oxidase activation and the production of ROS but NETs are believed to kill entrapped pathogens by NETs-associated granule proteases and peptides. Consequently, NETs present additional evidence for the interaction of non-oxidative and oxidative killing mechanisms. Together, these findings opened a new field of investigation with many controversies to be elucidated and underscored that we need further insight into the mechanisms by which neutrophils specifically recognize and overcome pathogens.

In this thesis, we followed the question whether the murine antimicrobial peptide cathelin-related antimicrobial peptide (CRAMP) is an important component of the

non-oxidative arm of neutrophil defense against *S. aureus*. This was motivated because little is known about cathelicidin function and activity in neutrophils and seems of crucial interest since mice lack the major constituent of human neutrophils - the α -defensins. We further aimed to specify the relationship between the NADPH oxidase and CRAMP with focus on the antimicrobial activity of CRAMP in association with NETs and in NADPH oxidase-deficient mice (gp91^{phox}^{-/-}). As a result, we could demonstrate a previously unknown intracellular antimicrobial activity of CRAMP against *S. aureus*. Specifically, CRAMP colocalized with *S. aureus* in phagolysosomes and we showed first evidence for the presence of intracellular active CRAMP. Most interestingly, phagolysosomal localization and intracellular activity of CRAMP was found independent of a functional NADPH oxidase controversially to our expectations. Investigation of NET-dependent killing of *S. aureus* revealed a negligible role for CRAMP due to inactivation of the peptide in association with NETs. This point is of particular relevance and should be considered in the current opinion of NETs-mediated antimicrobial activity. In summary, our data provided deeper knowledge about one specific member of the non-oxidative killing mechanism and gives reason to reconsider the controversial results about interaction of NADPH oxidase in activating non-oxidative mechanisms.

In addition, we followed the question whether recognition of *S. aureus* by TLR2 regulates the induction of non-oxidative and oxidative killing responses as well as the induction of NETs. The background of this study is based on several previous reports. First, results of our group evidenced a relationship between TLR2-mediated staphylococcal killing by neutrophils and the susceptibility of *S. aureus* to cationic antimicrobial peptides. Additionally, TLR2 activation has been shown to up-regulate

cathelicidin expression. Second, TLR2 was demonstrated to induce phosphorylation of p47^{phox} and up-regulation of p47^{phox} mRNA in macrophages. However, little attention has been paid to similar studies in neutrophils. Third, recognition of pathogens by TLRs was hypothesized to induce formation of NETs but there is as yet no evidence. First results unraveled a role for TLR2 in rapid induction of the NADPH oxidase, whereas TLR2 signaling had no influence on CRAMP activity. Interestingly, pathogen sensing for the induction of NETs formation did not depend on TLR2-MyD88 signaling. Taken together, the results demonstrate a role for TLR2 in mediating rapid killing of *S. aureus* by accelerating the activation of the NADPH oxidase complex possibly by influencing assembly.

Further studies of the mechanisms underlying the relationship between pathogen sensing and non-oxidative and oxidative killing mechanisms would contribute greatly to our understanding of how the innate immune system resolves bacterial infections and will help in the development of therapeutic strategies to assist in clearance of pathogenic bacteria.

2. General Introduction

2.1. Neutrophil Biology

Neutrophils emerge from pluripotent hematopoietic stem cells in the bone marrow. They are released into the peripheral blood and circulate for 7-10 hours before migrating into the tissues, where they have a life span of only a few hours. Neutrophils leave the bone marrow as terminally differentiated cells synthesizing low amount of RNA and protein. In humans roughly 100 billion neutrophils enter and leave circulating blood every day.

Neutrophils are the first immune cells recruited from the blood stream to the site of infection thereby building the first line of defense against invading microorganisms such as bacteria, fungi, and protozoa. The neutrophil-mediated inflammatory response is a multi-step process involving initial adhesion of circulating neutrophils to activated vascular endothelium, the subsequent extravasation and migration of neutrophils towards the site of infection and finally the elimination of invading microorganism. Most of these processes involve the mobilization of cytoplasmic granules and secretory vesicles. We here focus on the events involved in microbial clearance by neutrophil-mediated killing. On encountering the microbe, neutrophils take them up by phagocytosis. The phagosome fuses with cytoplasmic neutrophil granules to form a phagolysosome, wherein the bacteria are killed by non-oxidative and oxidative killing mechanisms. Non-oxidative killing is mediated by the antimicrobial arsenal stored in the neutrophil granules discussed in sections 2.1.1 and 2.1.2, whereas oxidative killing depends on the generation of reactive oxygen species (ROS) described in section 2.1.3.

2.1.1. Neutrophil Granules

Non-oxidative killing of invading pathogens is mediated by the fusion of neutrophil granules with the phagosome and the subsequent release of antimicrobial granule contents from the granule proteoglycan matrix into the phagolysosome. Four types of granules have been described and can be discriminated according to their protein contents (Figure 1).

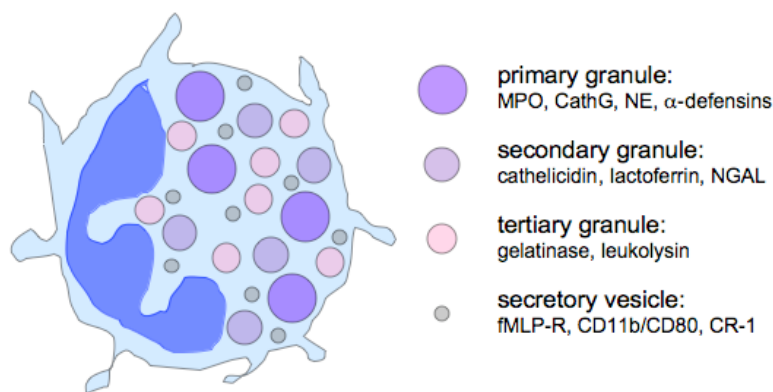


Figure 1. The four types of neutrophil granules. The contents of the neutrophil granule subsets encompass the non-oxidative killing mechanisms of neutrophils. Primary, secondary, and tertiary (gelatinase) granules, as well as secretory vesicles contain characteristic proteases, antimicrobial proteins, and receptors. CathG, cathepsin G; NE, neutrophil elastase; R, receptor; CR-1, complement receptor 1. Other abbreviations as explained in the text.

Primary granules contain acidic hydrolases and antimicrobial proteins. Myeloperoxidase (MPO), the defining protein of primary granules (1-3), reacts with hydrogen peroxide (H_2O_2), formed by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, to form hypochlorous acid (HOCl), which is highly toxic for microorganisms. MPO is released to the phagosome or the extracellular space upon neutrophil activation (4). In humans, the major constituents of primary granules are small cationic antimicrobial peptides named α -defensins (5). α -defensins have antimicrobial activity against a broad range of bacteria, fungi, enveloped viruses,

and protozoa (5-7). They exert antimicrobial function by forming multimeric transmembrane pores (8). Interestingly, murine granulocytes lack α -defensins (9). In addition, primary granules contain three structurally related proteases: neutrophil elastase, cathepsin G, and proteinase-3 (10-12). Neutrophil elastase and cathepsin G double knock-out mice have a severe defect in clearance of infections (13) suggesting an important role of granule proteases in efficient microbial killing. Proteinase-3 is responsible for processing of the secondary granule protein human cathelicidin protein-18 (hCAP-18) into its antimicrobial active form LL-37 (14) indicating interaction of primary and secondary granule contents.

The proteins of secondary granules encompass several potent AMPs including lactoferrin, hCAP-18 (human) and cathelin-related antimicrobial peptide (CRAMP, murine), neutrophil gelatinase-associated lipocalin (NGAL), and lysozyme. Lactoferrin and NGAL are iron-binding proteins and impair bacterial growth by iron sequestration (15-21). hCAP-18 and its murine homologue CRAMP are 18 kDa proteins belonging to the cathelicidin family of AMPs (22-25). The function of hCAP-18 and CRAMP will be discussed in detail in section 2.1.2. In addition to AMPs, the transmembrane units of NADPH oxidase gp91^{phox}/p22^{phox} (cytochrome *b*₅₅₈) are constituent of the secondary granule membrane. It is recruited to the phagosome after neutrophil activation and initiates the oxidative burst (26, 27).

In contrast to secondary granules with their high content of antimicrobials, gelatinase or tertiary granules contain the metalloproteases gelatinase (MMP-9) and leukolysin (MMP-25). They are stored as proforms and are proteolytically activated following exocytosis (28-32). The metalloproteases are believed to be of central importance in the degradation of vascular basement membranes during neutrophil extravasation due to degradation of extracellular matrix components (28, 33, 34).

The membranes of secretory vesicles comprise a reservoir of receptors, which are incorporated in the plasma membrane during exocytosis and are important in pathogen recognition: CD11b/CD18, complement receptor 1 (CR1), formylmethionyl-leucyl-phenylalanine (fMLP)-receptors, CD14, and the Fc γ III receptor CD16 (35-38).

2.1.2. Cathelicidins of Men and Mice

Cathelicidins are small cationic antimicrobial peptides (CAMPs). While human and mice each express a single cathelicidin, hCAP-18 and CRAMP respectively, other mammals express several different cathelicidins. All cathelicidins are produced as inactive precursors consisting of an N-terminal signal peptide, a prosequence named cathelin (cathepsin L inhibitor) domain and a structurally variable C-terminal antimicrobial domain (Figure 2) (39). The signal peptide is cleaved off after targeting the cathelicidin to secondary granules. The 14 kDa cathelin domain is evolutionary highly conserved, whereas the C-terminal antimicrobial domain shows little sequence homology among species. The inactive 18 kDa proproteins are stored in the secondary granules of neutrophils (40). Proteolytic cleavage of the inactive precursor to release the active C-terminal antimicrobial peptide is mediated by proteinase-3 in humans and an unknown protease in mice upon neutrophil activation and degranulation (14). Intracellular cleavage and release of the mature peptide into the phagolysosome has not yet been found. The mature human peptide LL-37 has a length of 37 amino acids; the mature murine peptide CRAMP is 34 amino acids long. Both peptides have predicted amphipathic α -helical structure and the mechanism of bactericidal activity assumes intercalation and assembly of the peptide within bacterial membranes to disrupt membrane integrity (41-44).

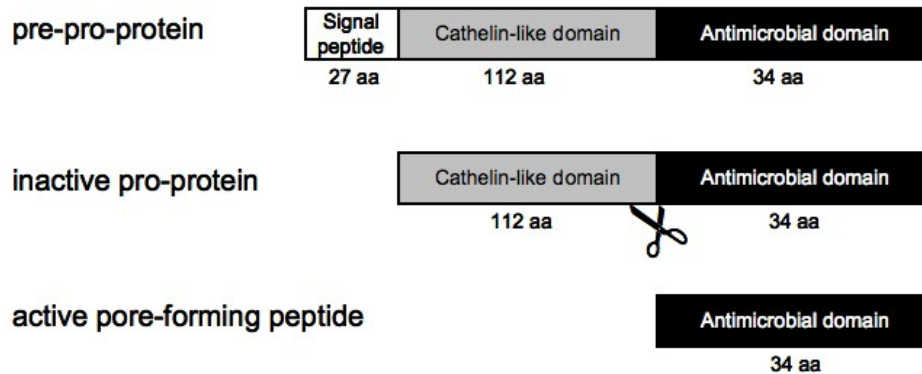


Figure 2. Schematic representation of CRAMP pre-protein and its processing site. The murine cathelicidin contains an N-terminal signal peptide, a highly conserved cathelin-like domain in the middle and a highly variable C-terminal antimicrobial domain.

In addition to neutrophils, hCAP-18 and CRAMP have been found in mast cells, macrophages, lymphocytes, and epithelial cells (45-49). In macrophages and epithelial cells, only low levels of cathelicidin are expressed but become strongly up-regulated after infection or injury. Interestingly, the antimicrobial active form can be found in these cells, but how they are processed remains unknown.

Purified or synthetic LL-37 and CRAMP have a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria. *In vitro*, Gram-negative bacteria are susceptible to lower concentrations of cathelicidin than Gram-positive bacteria. Lately, the generation of CRAMP-deficient mice underscored the importance of CRAMP against invading Gram-negative as well as Gram-positive pathogens *in vivo*. Experimental infection studies in CRAMP-deficient mice have demonstrated a critical role of CRAMP in defense against *Streptococcus pyogenes* skin infections (50), *Escherichia coli* urinary tract infections (51), and *Neisseria meningitides* bacteremia (52). So far, studies investigating the protective effect of cathelicidins mainly focused on epithelial cells and little is known about their function and activity in neutrophils.

2.1.3. The NADPH Oxidase

As mentioned before, oxidative killing of invading pathogens depends on ROS generated by the NADPH oxidase. The NADPH oxidase is a multicomponent enzyme that transfers electrons from cytoplasmic NADPH onto extracellular or intraphagosomal molecular oxygen (O_2), thereby generating superoxide (O_2^-). In resting cells, the NADPH oxidase is unassembled and inactive, with several protein components segregated into membrane and cytosolic locations. The membrane-bound subunits gp91^{phox} and p22^{phox} form the cytochrome *b*₅₈₈. Cytochrome *b*₅₈₈ is incorporated into the membranes of secondary granules and secretory vesicles in resting neutrophils, whereas the subunits p47^{phox}, p67^{phox}, p40^{phox}, and Rac2 reside in the cytosol (Figure 3). During neutrophil activation and recruitment of secondary granules and secretory vesicles, cytochrome *b*₅₈₈ locates to the phagolysosomal membrane or the cell surface. Then, the cytosolic p47^{phox} is rapidly phosphorylated and the cytosolic subunits p47^{phox}, p67^{phox}, and p40^{phox} translocate as a complex to the membrane (53-55). There, p47^{phox} is further phosphorylated and associates with cytochrome *b*₅₈₈. Additionally, p67^{phox} interacts with cytochrome *b*₅₈₈ and binds to the small GTPase Rac2, which translocated independently to the assembling oxidase (56-58). The phosphorylation is assumed to be mediated by protein kinase C (PKC); however, several PKC isoforms have been found to phosphorylate p47^{phox} (59-64).

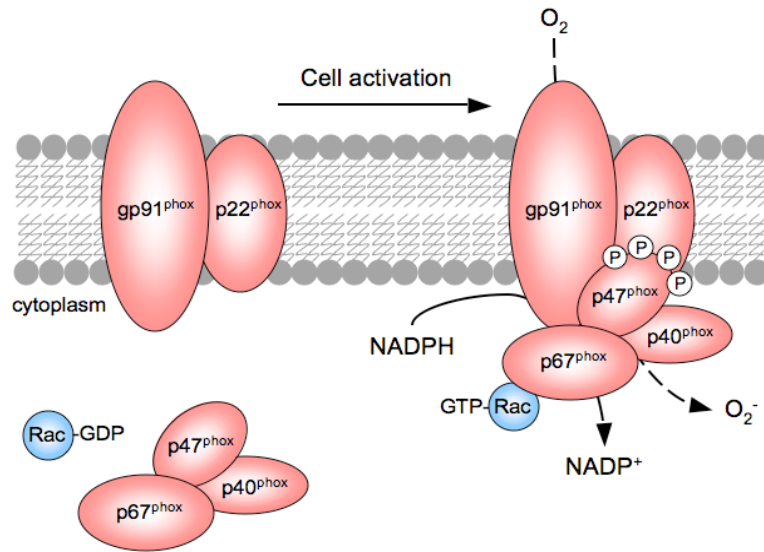


Figure 3. Model illustrating the assembly of the NADPH oxidase. In resting neutrophils, the NADPH oxidase is unassembled and inactive. The subunits are segregated into membrane and cytosolic locations. Upon cell activation, the cytosolic regulatory components p47^{phox}, p67^{phox}, and p40^{phox} translocate as a complex to the membrane-associated gp91^{phox}/p22^{phox} (cytochrome *b*₅₅₈). The small GTPase Rac translocates independently to the assembling oxidase.

Once assembled, the NADPH oxidase generates O₂⁻. However, O₂⁻ itself has low bactericidal potency. Within the phagosome, superoxide is rapidly converted into H₂O₂ by superoxide dismutase (SOD). H₂O₂ reacts to form other ROS, such as singlet oxygen and hydroxyl radical (OH[•]), which can efficiently kill bacteria. In addition, MPO can catalyze the H₂O₂-dependent oxidation of halides to form toxic hypohalous acids, mainly HOCl. The importance of NADPH oxidase is illustrated by the fact that persons who lack a functional oxidase have chronic granulomatous disease (CGD) and suffer from repeated life-threatening bacterial and fungal infections. In most cases of CGD the gene for gp91^{phox} is mutated resulting in total absence of both gp91^{phox} and p22^{phox} (65-67). Interestingly, deficiency of MPO is common but seldom leads to serious defects in microbial killing questioning the importance of ROS in bacterial

clearance. Indeed, bactericidal activity of ROS is low when assessed under conditions found in the neutrophil phagolysosome (68). These findings raised doubts about the role of ROS in efficient microbial killing.

Besides electron transfer, the NADPH oxidase also transfers protons to compensate for charge separation (69). Unexpectedly, the intraphagosomal pH does not decrease, but initially increases shortly after phagosome formation. Part of the charge compensation was shown to be due to influx of potassium ions instead of protons. Additionally, these cations were instrumental in liberating proteases from their acidic proteoglycan matrix in the granules (13). Thus, Segal and colleagues proposed the concept that the NADPH oxidase is primarily involved in the liberation of granule proteases into the phagosome. Their concept challenges the generally accepted view that oxidants directly confer microbial killing and needs to be further validated. However, this theory takes not into account the possibility that the activation of cathelicidin, which depends on active granule proteases, consequently might be affected as well.

2.1.4. Neutrophil Extracellular Traps

In 2004, a novel phagocytosis-independent killing mechanism, the formation of neutrophil extracellular traps (NETs), was discovered (70). In contrast to active phagocytosis and intracellular killing by AMPs and ROS, NETs provide an extracellular site for microbial killing. NETs consist of a backbone of nuclear chromatin-DNA that is decorated with AMPs and enzymes. Pathogens trapped by NETs are killed by high local concentrations of AMPs and enzymes similar to the killing in the phagolysosome; however, histones are also involved (71).

Lipopolysaccharide (LPS), phorbol myristate acetate (PMA), IL-8, and interferons have been shown to induce NETs to some extent as well as bacteria directly (70, 72, 73). Pattern recognition receptors such as Fc γ receptors and Toll-like receptors (TLRs) are speculated to be involved in sensing and signaling and coincide to activate PKC, which induces assembly of the NADPH oxidase. Interestingly, the generation of ROS was found to be essential for NET induction (72). This ROS-mediated induction of NETs formation was termed NETosis (74) which points out that NETs are formed in a process that is neither apoptotic nor necrotic (75). However, the exact signaling events leading to NET formation are still unclear and need further investigation.

NETs are able to entrap Gram-positive and Gram-negative bacteria as well as pathogenic fungi. Surprisingly, the mechanism of trapping has not yet been studied. Speculations about a charge-mediated mechanism whereby CAMPs in NETs trap negatively charged microbes exist. Consideration of the negative charge of the DNA backbone itself indicates that the positive net charge of CAMPs could also be neutralized by NETs-association alone. There is evidence that association of CAMPs with DNA reduces their bactericidal activity pointing towards neutralization of charge and thereby abolishing binding to the bacterial surface (76, 77). Consistently, no significant differences in the trapping by NETs of *Streptococcus pneumoniae* that had different surface charges were observed (78). It remains open what effectively attaches the microbe to NETs.

Most bacteria are killed after they become trapped, but some bacteria are equipped with weapons against NETs. The most studied evasion mechanism is the production of an extracellular DNase for degradation of the DNA backbone. Other possible mechanisms might involve bacterial cell wall modifications to repel NETs or the

granule proteins, inhibition of NETs formation by blocking ROS, and secretion of proteases that degrade NET-associated granule proteins.

2.2. Toll-like Receptors on Neutrophils

Microbial infections are sensed by specific innate immune receptors, which recognize conserved patterns derived from pathogenic and non-pathogenic microbes. TLRs play a crucial role in the immediate detection and control of invading microbes. To date, 11 mammalian and 13 murine TLRs have been described (79). TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor, termed the Toll/IL-1R homology (TIR) domain. TLR1, 2, 4, 5, and 6 are expressed at the cell membrane whereas TLR7, 8, and 9 reside in subcellular compartments such as the endosome. Neutrophils express all of the TLRs with the exception of TLR3 (80). Individual TLRs recognize distinct microbe-associated molecular patterns (MAMPs) that have been evolutionary conserved in specific classes of microbes. The recognition of their unique ligands is mediated through diversification of LRR motifs, cooperative interactions between different TLRs, and the use of co-receptors or accessory molecules.

Upon ligand binding, TLRs dimerize and undergo conformational changes. TLR2 was shown to form heterodimers with either TLR1 or TLR6 depending on the nature of the ligand (81). Conformational changes lead to the association of the TIR domains with either myeloid differentiation factor 88 (MyD88) or TIR domain-containing adaptor protein inducing interferon- β (TRIF). MyD88 is critical for the signaling of all TLRs, except TLR3. Downstream signaling of MyD88 involves IL-1 receptor-

associated kinase-4 (IRAK-4) and IRAK-1, which recruit tumor necrosis factor receptor-associated factor-6 (TRAF6). The cascade results in activation of nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinases to induce pro-inflammatory genes and an antimicrobial response. TRIF signaling leads to the induction of a signaling cascade activating IRF-3 and IRF-7 and expression of type I interferons.

2.3. *Staphylococcus aureus*

Staphylococcus aureus is a facultative anaerobic Gram-positive bacterium colonizing the human skin and mucous membranes as a commensal. About 20% of the human population is persistently colonized, 60% are intermittent carriers, while 20% never carry the pathogen (82). When crossing the skin barrier, *S. aureus* causes abscess formation and has the possibility to disseminate and cause severe systemic infections. In the tissue, *S. aureus* is rapidly phagocytosed by neutrophils and exposed to the intracellular killing mechanisms described in the previous chapters. But, *S. aureus* is well equipped with several resistance mechanisms to survive in the phagosome. On the one hand, AMP resistance is due to natural modifications of teichoic acids and membrane phospholipids and secretion of proteases to disarm AMPs. On the other hand, *S. aureus* can resist oxidative killing by radical scavenging and removal of superoxide.

Recognition of *S. aureus* by neutrophils inducing subsequent activation of antimicrobial defense and its resistance mechanisms against killing will be briefly outlined in the following sections.

2.3.1. Recognition of *S. aureus* by TLR2

Sensing of *S. aureus* is mediated by staphylococcal lipoproteins and to a lesser extent by lipoteichoic acids, which are recognized by TLR2 and its co-molecules CD14 and CD36 (83-87).

Activation of TLR2 by staphylococcal peptidoglycan and lipoproteins increased cathelicidin mRNA in murine fibroblasts (88) and cathelicidin protein in human corneal epithelial cells (89). The importance of TLR2 in mediating inflammatory and antimicrobial response against *S. aureus* is evidenced in TLR2-deficient mice, which are highly susceptible to *S. aureus* septicemia (90). Taken together, these results indicate a possible interaction between TLR2 and CAMP-mediated host defense.

It remains to be elucidated whether TLR2 activates either a non-oxidative or oxidative antimicrobial response against *S. aureus* with further interest on the involvement of NADPH oxidase and the induction of NETs.

2.3.2. Staphylococcal Resistance against AMPs

Most neutrophil AMPs are of cationic nature. Staphylococcal resistance against CAMPs is therefore achieved by partially neutralizing the negative charge of the cell surface that would attract cationic molecules. The genes of the *dltABCD* operon insert positive charge modifications to wall teichoic acid and lipoteichoic acid. The Dlt proteins result in D-alanine substitutions of teichoic acid (91). Similarly, MprF adds L-lysine residues to phosphatidylglycerol exposed on the outer surface of the staphylococcal cytoplasmic membrane (92, 93). In both cases, the modifications reduce the affinity of CAMPs released into the phagosome and repel them from the cytoplasmic membrane. Staphylococcal mutants in *dltA* and *mprF* are highly

susceptible to killing by CAMPs and neutrophils *in vitro*, and show reduced virulence in animal infection models (94-96). In addition to positive charge modifications, *S. aureus* has the ability to neutralize CAMPs. Secretion of staphylokinase protects *S. aureus* from defensins due to its potent defensin-binding activity and aureolysin can inactivate LL-37 by cleavage (97, 98).

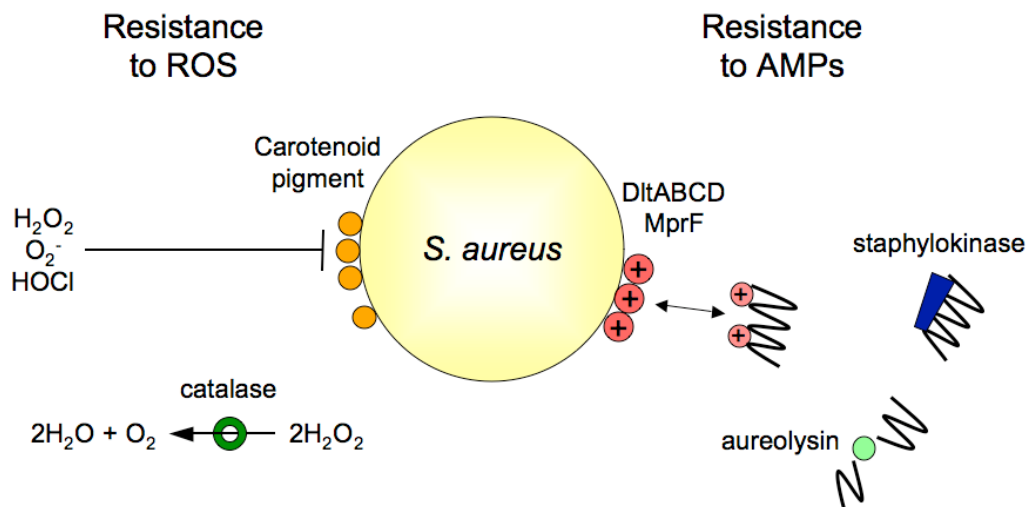


Figure 4. Resistance mechanisms of *S. aureus* against killing by reactive oxygen species and antimicrobial peptides. Carotenoid pigment provides an antioxidant shield whereas catalase detoxifies hydrogen peroxide protecting *S. aureus* against ROS. Resistance to CAMPs is given by positive charge modifications of the cell wall, aureolysin-mediated proteolysis, and inactivation by staphylokinase. Modified from Nizet *et al.* (99).

2.3.3. Staphylococcal Resistance against ROS

ROS produced during the oxidative burst include superoxide anion, which is subsequently converted to H₂O₂, hydroxyl radical, and HOCl. Exogenously generated superoxide does not kill bacteria directly. *S. aureus* expresses two superoxide dismutases enzymes (SodA, SodM) responsible for removal of endogenous superoxide. There are contradictory results about the role of Sod in staphylococcal

resistance against superoxide *in vivo*. Clement *et al.* found evidence that inactivation of the major superoxide dismutase SodA has no effect on virulence in a mouse abscess model (100). A more recent study by the same group showed that mutants in SodA and SodM are less virulent in the mouse abscess model (101), indicating that SodA and SodM are involved in ROS resistance of *S. aureus in vivo*. H₂O₂ is bactericidal only at high concentrations. *S. aureus* can further convert H₂O₂ to H₂O by the catalase KatA which accumulates extracellularly protecting *S. aureus* from external H₂O₂. In addition, *S. aureus* scavenges oxygen free radicals by expression of carotenoid pigment increasing resistance against oxidative neutrophil killing *in vitro* and *in vivo* (102).

2.3.4. Staphylococcal Escape from NETs

S. aureus can induce the formation of NETs and is itself entrapped in NETs pre-activated with soluble stimuli (72, 75). Partial killing of *S. aureus* by NETs has been described but the killing activity was most likely due to the antimicrobial activity of histones and not granular proteins (75). Therefore, evasion of *S. aureus* from NETs must either depend on degradation of DNA backbone and escape from NETs or the inactivation of histones. Since *S. aureus* secretes a DNase similar to those described in NETs degradation by *S. pneumoniae*, this seems so far the most probable mechanisms for *S. aureus* to evade NETs. This interesting question has not yet been addressed and might be a future topic of our research.

3. Aim of Study

Neutrophils are the first cells recruited to the site of infection providing immediate defense. Their relevance becomes obvious in persons who have neutrophils with defects in phagocytosis or killing and suffer from recurrent, often life-threatening infections. Neutrophils kill invading pathogens by a combination of non-oxidative and oxidative mechanisms involving antimicrobial peptides, reactive oxygen species and neutrophil extracellular traps. The microbicidal potential of many of these components alone is well established. First evidence of direct interaction of non-oxidative and oxidative killing was given by the finding that the NADPH oxidase is involved in the liberation of granule proteases. However, it is not fully understood how these processes cooperate or depend on each other to kill bacteria.

The first aim of this study was to examine the antimicrobial potential of neutrophil-derived CRAMP and its site of action in staphylococcal killing. Further, the cooperation of non-oxidative and oxidative killing mechanisms was targeted with particular attention to the contributing roles of NETs and NADPH oxidase to cathelicidin-mediated host defense (Part 1).

TLR2 contributes to efficient clearance of *S. aureus* by neutrophils and has been described to be involved in the regulation of antimicrobial peptide expression and in activation of the NADPH oxidase. Therefore, the second aim of the study was to investigate the role for TLR2 in cathelicidin-mediated staphylococcal killing and the potential regulation of CRAMP. Further, we intended to investigate the contribution of TLR2 in the oxidative killing of *S. aureus* and whether sensing of *S. aureus* by TLR2 induces the formation of NETs (Part 2).

Taken together, the aim of this thesis was to contribute to a better understanding of the complex network of non-oxidative and oxidative killing defense of neutrophils against *S. aureus* – on the one hand, by investigating the murine cathelicidin (CRAMP), its localization and site of action and on the other hand, by characterizing the TLR2-dependent regulation of non-oxidative and oxidative killing induced by *S. aureus*.

4. Results

Neutrophil antimicrobial defense against *Staphylococcus aureus* is mediated by phagolysosomal and extracellular trap-associated cathelicidin

Neutrophil antimicrobial defense against *Staphylococcus aureus* is mediated by phagolysosomal and extracellular trap-associated cathelicidin

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Abstract

Neutrophils kill invading pathogens by antimicrobial peptides (AMPs) including cathelicidins, reactive oxygen species and neutrophil extracellular traps (NETs). The human pathogen *Staphylococcus aureus* exhibits enhanced resistance to neutrophil AMPs in part due to alanylation of teichoic acids by the *dlt* operon. In this study, we took advantage of the CRAMP-susceptible phenotype of *S. aureus* $\Delta dltA$ to study the impact of the murine cathelicidin CRAMP on staphylococcal killing and to identify its key site of action in murine neutrophils.

We demonstrate that CRAMP remained intracellular during PMN exudation from blood and was secreted upon PMA stimulation. We show first evidence that CRAMP was recruited to phagolysosomes in infected neutrophils and exhibited intracellular activity against *S. aureus*. Later in infection, neutrophils produced NETs and immunofluorescence revealed association of CRAMP with *S. aureus* in NETs. NETs similarly killed both *S. aureus* wt and $\Delta dltA$ indicating that CRAMP activity was reduced when associated with NETs; CRAMP regained activity after DNase treatment. CRAMP localization in response to *S. aureus* was independent of the NADPH oxidase whereas killing was partially dependent. Our study unravels that neutrophils use CRAMP in a timed and locally coordinated manner in defense against *S. aureus*.

Introduction

Antimicrobial peptides (AMPs) are widely distributed in animals and plants and are among the most ancient innate host defense factors. Mammalian AMPs include α -defensins, β -defensins, and cathelicidins. Cathelicidins are found in several mammalian species and increasing evidence suggests a key role for cathelicidins in innate immune defense. Human and murine leukocytes each constitutively express a single cathelicidin: hCAP-18/LL-37 and CRAMP, respectively. hCAP-18/LL-37 and CRAMP are stored as pro-peptides in secondary granules of neutrophils. hCAP-18 is proteolytically cleaved to the antimicrobial form LL-37 by extracellular proteinase 3 (1), while the processing protease of CRAMP is unknown. Additionally to leukocytes, various epithelia including keratinocytes can induce the production of cathelicidins after injury or infection (2). However, significant production of cathelicidin in keratinocytes takes several hours; neutrophils entering the injured or infected skin immediately, therefore confer a rapid first defense.

In murine polymorphonuclear neutrophils (PMN), CRAMP is among the major cationic AMPs (CAMPs) due to a lack of α -defensins in mice (3). The human and murine cathelicidins are strongly induced in response to Gram-positive and Gram-negative species *in vitro* and *in vivo* (4-9). Lately, the generation of CRAMP-deficient mice made it possible to identify the importance of CRAMP against invading pathogens (10). Experimental infection studies in CRAMP-deficient mice have demonstrated a critical role of CRAMP in defense against *Streptococcus pyogenes* skin infections (11), *Escherichia coli* urinary tract infections (12), and *Neisseria meningitidis* bacteremia (4).

Recently, neutrophil extracellular traps (NETs) were described as a new phagocytosis-independent antimicrobial mechanism of PMN. NETs are composed of chromatin and specific enzymes and proteins from PMN granules. They are released in response to chemokines and microbial stimuli in a specialized form of cell death that depends upon the NADPH oxidase (13) providing a new linkage of oxidative burst and AMP function. Cathelicidins have been identified in similar extracellular traps released by mast cells (14) and D-alanyl modification of cell wall teichoic acid increased pneumococcal survival in NETs (15).

Non-oxidative killing and oxidative killing pathways in PMN have long been considered independent of each other. During oxidative burst, specific granules containing the transmembrane component of the NADPH oxidase fuse with the forming phagosome accompanied by the activation of the oxidase complex through recruitment of cytosolic subunits to the central transmembrane core. The assembled NADPH oxidase transfers electrons from the cytosol to the phagosomal lumen, where they are used to generate superoxide ions, which together with myeloperoxidase promote microbial killing (16). Recent studies propose an additional role of K^+ influx from the cytosol into the phagosomal lumen, promoting disaggregation of the granule proteoglycan matrix and activation of proteases including those responsible for processing of AMPs (17, 18).

The crucial importance of NADPH oxidase in pathogen killing is evidenced in persons with chronic granulomatous disease (CGD) that bear inactivating mutations in genes of subunits of the oxidase complex and suffer from repeated life-threatening bacterial and fungal infection (19, 20). PMN of these patients exhibit normal degranulation but the initial phagosomal alkalization, which normally accompanies initiation of superoxide production, does not occur (21, 22). Additionally, correction

of phagocytic pH to more physiological values restores the ability of CGD neutrophils to kill *S. aureus* (22). Therefore, phagosomal alkalization was proposed to be essential for liberating granule enzymes in the phagosome (16). These observations suggest that granule proteases act in concert with oxidases for effective bacterial killing; however, the functional role of NADPH oxidase in AMP processing has not been studied.

The major human pathogen *S. aureus* exhibits relative resistance to CAMPs due to positive-charge modifications to its cell wall such as peptidoglycan acetylation (23) and teichoic acid D-alanylation (24), the capacity to degrade CAMPs with specific proteases (25), and AMP-binding properties of staphylokinase (26). We had previously observed a reduced virulence of a *S. aureus* mutant with dealanylated teichoic acids (SA113 $\Delta dltA$) in septic and local infection models (27, 28). This phenotype was tentatively correlated to an enhanced susceptibility to cathelicidin AMPs, but only through *in vitro* susceptibility testing (28). In order to more fully understand the potential role of CRAMP in the response to *S. aureus* infection, we here probe the regulation and cellular location of CRAMP expression in murine blood and exudate granulocytes. We further investigated the role of CRAMP in staphylococcal killing and its key site of action with particular attention to the contributing roles of NETs and NADPH oxidase to cathelicidin-mediated host defense.

Our results demonstrated that PMN exert intracellular antimicrobial activity mediated by CRAMP against *S. aureus*. Additional extracellular entrapping and killing of *S. aureus* by NETs was partially mediated by CRAMP and may help to protect the host against further bacterial spreading and development of systemic disease.

Materials and Methods

Bacterial strains. *S. aureus* wt (ATCC 35556, SA113 wt) and its isogenic mutants $\Delta dltA$, Δspa , and $\Delta spa/\Delta dltA$ were grown overnight in tryptic soy broth (Difco) at 37°C. For stimulation experiments, a subculture was inoculated 1:100 (v/v) from overnight culture in fresh tryptic soy broth and grown to late-log phase. Bacteria were washed twice with 0.9% NaCl prior to use.

FITC-labeling of staphylococci. *S. aureus* subculture was grown to mid-log phase in fresh tryptic soy broth. Bacteria were washed twice with 0.9% NaCl and labeled in 0.1 mg/ml FITC (Sigma) in PBS for 1h at 37°C with shaking. Prior to use, bacteria were washed twice with 0.9% NaCl and resuspended in Dulbecco's PBS with 100 mg/L MgCl₂ and 100 mg/L CaCl₂ (DPBS⁺⁺, Invitrogen).

Mice and tissue cage model. C57BL/6, CRAMP^{-/-}, and gp91^{phox^{-/-}} mice were kept under specific pathogen-free conditions in the Animal House of the Department of Biomedicine, University Hospital Basel, and University of California, San Diego, according to the regulations of the Swiss veterinary law and the Veterans Administration of San Diego Committee on Animal Use, respectively. Mice were euthanized by CO₂ or i.p. injection of 500 mg/kg Thiopental® (Abbott Laboratories).

12-14-weeks-old female mice were anesthetized and sterile Teflon tissue cages were implanted subcutaneously, as described previously (28). Two weeks after surgery, the sterility of tissue cages was verified. To harvest tissue cage fluid (TCF) for isolation

of PMN, mice were anesthetized by isofluorane (MINRAD INC.) and TCF percutaneously collected with EDTA.

Antibody generation. Two New Zealand White rabbits were immunized by repetitive subcutaneous injections of 150 μ g synthetic CRAMP peptide (GL Shangai Biochem Ltd.) in adjuvant (MPL® + TDM + CWS Adjuvant System, Sigma) at monthly intervals. The titer of the antiserum was estimated by immunoblotting. The IgG fraction from the polyclonal anti-serum was isolated on a protein-G sepharose (Amersham) column and further affinity purified via affinity chromatography over a sepharose column (GE Healthcare) conjugated with synthetic CRAMP peptide used as immunogen. Bound antibody was eluted with 0.1 M glycine (0.002% sodium azide, pH 2.5) and dialyzed against PBS. A portion of the affinity-purified antibody was biotinylated as described previously (29), dialyzed against PBS and stored at -20°C. To demonstrate specificity, affinity-purified antibody was used to detect native 18 kDa CRAMP from murine blood PMN lysates. Affinity-purified anti-CRAMP Ab was specific for one band of ~18 kDa, corresponding to the predicted size of pro-CRAMP (Figure 2A).

PMN isolation. For peripheral blood PMN (bPMN), mouse blood was harvested by intracardiac puncture in EDTA. bPMN were isolated as described previously for human PMN (30), but using a modified density gradient centrifugation on a discontinuous Percoll gradient with 59% and 67% Percoll (Amersham Biotech) in PBS. bPMN were collected at the interface of the two Percoll layers.

For peritoneal PMN (pPMN), 1 ml of 3% thioglycollate (BD Biosciences) were injected i.p. After 6h, pPMN were collected by peritoneal lavage with 5 ml RPMI 1640 complete medium (5% FBS, 2 mM glutamate, 1 mM sodium pyruvate, 1.5 mM HEPES, non-essential amino acids) and pelleted by centrifugation.

For TCF-PMN, TCF was collected in EDTA and pelleted by centrifugation.

Where mentioned, bPMN, pPMN, and TCF-PMN were purified over a Percoll gradient as described above to a purity of > 97% (NIMP-R14 staining) and viability of PMN was 99% as assessed by trypan blue staining.

Stimulation of PMN for flow cytometry and immunofluorescence. After isolation from blood, peritoneum, or TCF, erythrocytes were lysed in water and PMN were resuspended in DPBS⁺⁺ at $1-2 \times 10^6$ cells/ml. PMN were incubated with 1 μ g/ml PMA, unlabeled (for flow cytometry) or FITC-labeled (for immunofluorescence) SA113 Δspa and SA113 $\Delta spa/\Delta dltA$ for 15 and 30 min at 37°C, 200 rpm. Stimulation was stopped on ice and PMN were collected by centrifugation for further use in flow cytometry or immunofluorescence. Supernatants were collected and released CRAMP was measured by ELISA.

Immunofluorescence. After stimulation, cells were spun onto glass coverslips and fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). After permeabilization with 0.2% saponin for 30 min at RT and 5 min in methanol, cells were blocked with 2% normal goat or donkey serum (NGS or NDS) for 30 min at RT. Cells were stained with affinity-purified rabbit anti-CRAMP (1 μ g/ml), biotinylated rabbit anti-CRAMP (5 μ g/ml), goat anti-cathepsin D (10 μ g/ml, Santa

Cruz Biotechnology), and rabbit anti-LAMP-1 (10 µg/ml, Abcam) Ab, followed by donkey anti-rabbit/goat IgG-Cy3 Ab (7.5 µg/ml, Jackson ImmunoResearch) or Streptavidin-Alexa647 (10 µg/ml, Molecular Probes). Isotype-matched Ab served as negative controls. To confirm specificity of antibody binding, parallel slides were treated identically with affinity-purified rabbit anti-CRAMP Ab that had been preincubated for 1h at RT with 20 µg/ml synthetic CRAMP peptide which abolished staining (Figure 2B). Specimens were analyzed with a Zeiss Axiovert 100M microscope (Carl Zeiss AG) using the confocal system LSM 510 META and LSM 510 v3.2 SP2 software (Zeiss).

Flow cytometry. Cells were blocked with 2% NGS, fixed with 4% PFA, and permeabilized for intracellular staining with 0.1% saponin. Rat anti-mouse CD16/CD32 (Pharmingen) has been used to block Fc-receptor binding of IgG. After Fc-blocking, cells were sequentially stained with biotinylated rabbit anti-CRAMP Ab (1 µg/ml) and Streptavidin-RPE (0.25 µg/ml) or the neutrophil marker rat- anti mouse NIMP-R14 (10 µg/ml, own hybridoma) and FITC-conjugated goat anti-rat Ab (7.5 µg/ml, Jackson ImmunoResearch). Biotinylated rabbit IgG and rat IgG2a (Pharmingen) were used as isotype controls.

PMN killing assay. TCF-PMN were resuspended in DPBS⁺⁺ (with 10% pooled mouse plasma) and 2×10^5 PMN were incubated with SA113 wt or $\Delta dltA$ at a multiplicity of infection (MOI) of 1 and incubated at 37°C, 200 rpm. After 2h, samples were diluted in H₂O (pH 11) to lyse PMN and serial dilutions were plated on Mueller-Hinton agar (MHA) to enumerate surviving intra- and extracellular bacteria.

Intracellular killing assay. pPMN were resuspended at 1×10^6 PMN in RPMI 1640 (10 mM HEPES, 2% pooled mouse plasma), seeded into 24-well plates and allowed to adhere for 1h. PMN were infected with SA113 wt and $\Delta dltA$ at a MOI of 1, centrifuged at $800 \times g$ for 5 min to synchronize phagocytosis and further incubated at 37°C and 5% CO_2 for 10 min. 50 U lysostaphin was added for 10 min to kill extracellular bacteria. Immediately (0 min) and 30 min after lysostaphin treatment, PMN were lysed with H_2O (pH 11) and serial dilutions were plated on MHA to assess surviving intracellular bacteria. Intracellular killing was calculated as the percentage of intracellular bacteria at 30 min versus 0 min.

NET-dependent killing assay. pPMN were resuspended at 2×10^6 PMN in RPMI 1640 (10 mM HEPES, 2% pooled mouse plasma), seeded into 24-well plates and activated with 50 nM PMA for 4h. The medium was carefully replaced with medium containing cytochalasin D (10 μM) with or without 50 U DNase1 to degrade NETs. Samples were infected with SA113 wt and $\Delta dltA$ at a MOI 0.01, centrifuged at $800 \times g$ for 10 min and incubated at 37°C and 5% CO_2 for 30 min. Wells were thoroughly scraped and serial dilutions were plated on MHA to assess surviving bacteria. NET-dependent killing was calculated as percentage of bacteria incubated without neutrophils.

Immunofluorescence of NETs. 2×10^5 bPMN or pPMN were seeded in poly-L-lysine-covered 16-well glass chamber slides, allowed to settle and either treated with PMA (50 nM), SA113 Δspa , SA113 $\Delta spa/\Delta dltA$ at a MOI of 10 or left unstimulated

for 4h. Cells were fixed with 4% PFA, blocked with 2% NGS, and stained with affinity-purified rabbit anti-CRAMP (1 µg/ml) and donkey anti-rabbit IgG-Cy3 (7.5 µg/ml) Ab. Controls were done with isotype-matched Ab. For labeling of DNA, SYTOX[®] Green (1 µM, Molecular Probes) was used. Specimens were analyzed as described above in immunofluorescence.

ELISA. An ELISA for CRAMP was developed using 96-well flat-bottom immunoplates (Nunc). Plates were coated overnight with 1 µg/ml affinity-purified rabbit anti-CRAMP Ab in 1 M carbonate buffer (pH 9.6) at 4°C. After blocking with 1% casein in PBS, samples and standards were added and incubated for 2h. Biotinylated anti-CRAMP Ab (400 ng/ml) was added for 1h and further incubated with Streptavidin-HRP (Zymed). After incubation for 30 min with TMB substrate (Pharmingen), reaction was stopped with 1 M H₂SO₄ and absorbance was measured at 450 nm. Between each step, plates were washed 4 times with PBS (0.05% Tween-20). All incubations were carried out at RT.

Immunoblot analysis. 1×10^6 pPMN were infected for 15 min with SA113 wt or left unstimulated. Cells were then lysed in 0.9% NaCl containing 1% Triton X-100, 1 mM PMSF and a protease inhibitor cocktail (Roche). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schüll). Membranes were blotted with affinity-purified rabbit anti-CRAMP Ab (1 µg/ml) followed by horseradish peroxidase conjugated donkey anti-rabbit IgG (0.16 µg/ml) and were visualized with enhanced chemiluminescence (Amersham Bioscience) on films (KODAK).

Statistical analysis. PMN killing assay was analyzed with Mann-Whitney. Flow cytometry data, intracellular killing and NET-dependent killing assays were analyzed with paired student's t-test. Statistical analysis was done with Prism 5.0a (GraphPad Software, Inc.). A p-value of $p < 0.05$ was considered statistically significant.

Results

PMN-derived CRAMP is active against *S. aureus*

S. aureus is resistant to CRAMP due to a variety of mechanisms including D-alanylation of teichoic acids (24). We sought to exploit the differential sensitivity of *S. aureus* (SA113) wt and its isogenic $\Delta dltA$ mutant to better understand the regulation, cellular localization and function of neutrophil-derived CRAMP. PMN from tissue cages (TCF-PMN) in C57BL/6 mice exhibited significantly increased bactericidal activity against SA113 $\Delta dltA$ compared to wt *in vitro* (Figure 1). In contrast, TCF-PMN from CRAMP^{-/-} mice showed similar bactericidal activity against SA113 wt and $\Delta dltA$. In addition, the bactericidal activity of CRAMP^{-/-} TCF-PMN against SA113 wt was significantly lower than of C57BL/6 PMN (Figure 1).

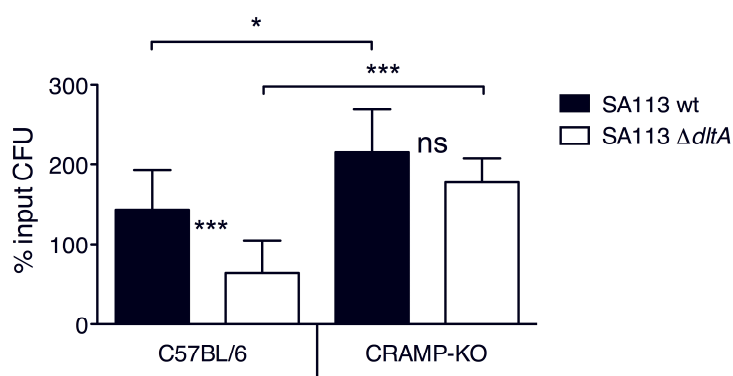


Figure 1. Bactericidal activity of TCF-PMN from C57BL/6 and CRAMP^{-/-} mice. The numbers of viable CFU of SA113 wt (closed) and SA113 $\Delta dltA$ (open) after 2h of incubation with TCF-PMN *in vitro* are expressed as percentage of the initial inoculum. Data are mean \pm SD of three independent experiments. Significant differences are indicated by *: $p < 0.05$ and ***: $p < 0.001$.

These data showed that the susceptibility of SA113 $\Delta dltA$ to PMN is predominantly mediated by CRAMP; allowing the $\Delta dltA$ mutant to serve as a powerful tool to study CRAMP function and activity. Furthermore, the decreased bactericidal activity of

CRAMP^{-/-} PMN implies that the expression of CRAMP in PMN is indeed important for the defense against *S. aureus in vivo*, despite the apparent resistance of the bacterium to the isolated AMP *in vitro*.

Degranulation of CRAMP does not occur during PMN migration

First, the specificity of affinity-purified rabbit anti-CRAMP Ab was tested by immunoblot analysis of blood PMN lysates. The antibody recognized a single band at ~18 kDa corresponding to the predicted size of pro-CRAMP (Figure 2A). The anti-CRAMP Ab was also specific for CRAMP in immunofluorescence as preincubation of anti-CRAMP Ab with excess synthetic CRAMP abolished staining on peritoneal PMN (Figure 2B). Next, we studied the location and the site of action of CRAMP by investigating intracellular CRAMP expression by flow cytometry in C57BL/6 PMN. To exclude that exudation from the bloodstream itself affects CRAMP expression, PMN purified from TCF and peritoneal exudates were compared to peripheral blood PMN. CRAMP was expressed intracellularly in PMN from all sites (Figure 2Ci-iii, upper row). Mean fluorescence intensity (MFI) was similar in all purified PMN indicating no loss of CRAMP during exudation from blood. Purified PMN were >97% positive for the granulocyte marker NIMP-R14 Ab as shown for TCF-PMN in Figure 2Aiv (upper row). Using fluorescence microscopy we showed that CRAMP is distributed in a granular pattern in PMN from blood, peritoneal cavity, and TCF (Figure 2Bi-iii, lower row).

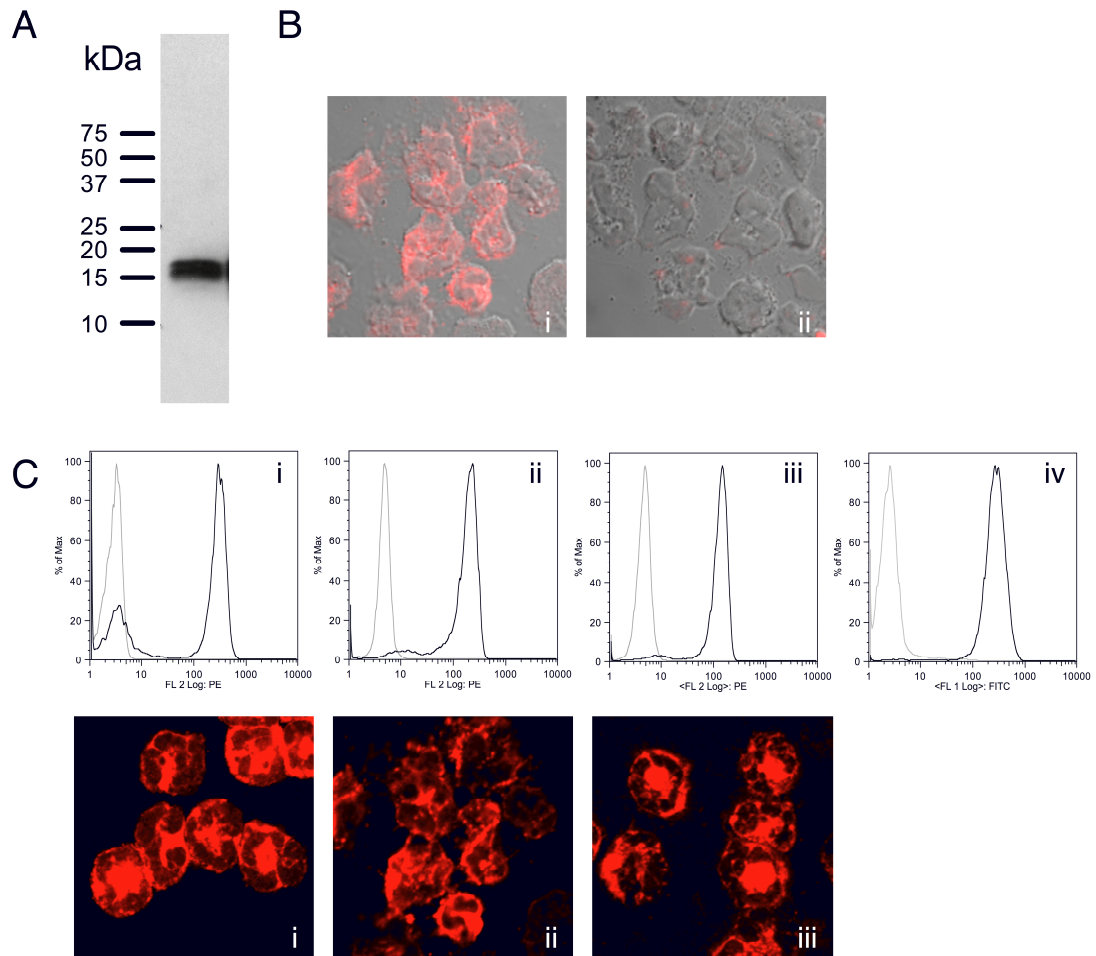


Figure 2. Intracellular CRAMP expression in blood and exudate PMN. (A) Western blot analysis of bPMN lysates using affinity-purified rabbit anti-CRAMP Ab. (B) Intracellular staining of pPMN with rabbit anti-CRAMP Ab followed by Cy3-conjugated donkey anti-rabbit Ab (i) and identical staining performed with antibody preincubated with excess of synthetic CRAMP peptide (ii). (C, upper row) PMN purified from blood (i), peritoneal exudate (ii), and TCF (iii) from C57BL/6 mice were intracellularly stained with biotinylated rabbit anti-CRAMP (black) or isotype control (grey) Ab followed by RPE-conjugated Streptavidin and analyzed by flow cytometry. (iv) Purified TCF-PMN stained with the neutrophil marker NIMP-R14 (black) or isotype control (grey) Ab followed by FITC-conjugated goat anti-rat Ab. Graphs are representative of two to five independent experiments. (C, lower row) bPMN (i), pPMN (ii), and TCF-PMN (iii) were immunolabeled with rabbit anti-CRAMP or isotype control (not shown) Ab followed by Cy3-conjugated donkey anti-rabbit Ab and examined by confocal microscopy. Isotype controls showed no detectable staining. Fluorescence micrographs (original magnification x100) are representatives of three independent experiments.

These results indicate that CRAMP-containing granules are not released during recruitment of PMN, such that full antimicrobial activity can be exerted at the site of infection.

PMN release CRAMP after PKC activation

Degranulation of secondary granules in human PMN was shown to be dependent on protein kinase C (PKC) (31). The signaling pathways promoting release of CRAMP after stimulation of murine PMN is unknown. We investigated the intrinsic ability of C57BL/6 pPMN to secrete CRAMP in response to the PKC activator PMA by flow cytometry. Unpurified pPMN were used to avoid preactivation of PMN by percoll purification. Therefore, two populations with bright and dim fluorescence are seen in the histograms, which were identified as PMN and monocytes by Wright's stain (data not shown). As shown in Figure 2Cii, PMN correspond to the bright CRAMP-expressing population. The fluorescence histogram of PMA-stimulated versus non-treated cells shows a reduction in intracellular MFI. Intracellular CRAMP was significantly decreased after PMA-stimulation as shown in the bar graph indicating release of CRAMP. During secondary granule release lactoferrin and LL-37 transiently locate to the cell surface (32, 33). Thus, we examined surface translocation of CRAMP as readout for secretion in response to PMA. Non-treated cells had no detectable CRAMP on their surface, whereas PMA-stimulated cells showed surface localization of CRAMP with significantly increased MFI compared to non-treated cells (Figure 3B). Using fluorescence microscopy we found that PMN stimulated for 15 min with PMA had less intracellular CRAMP and its distribution was more dispersed than in non-treated cells (Figure 3C).

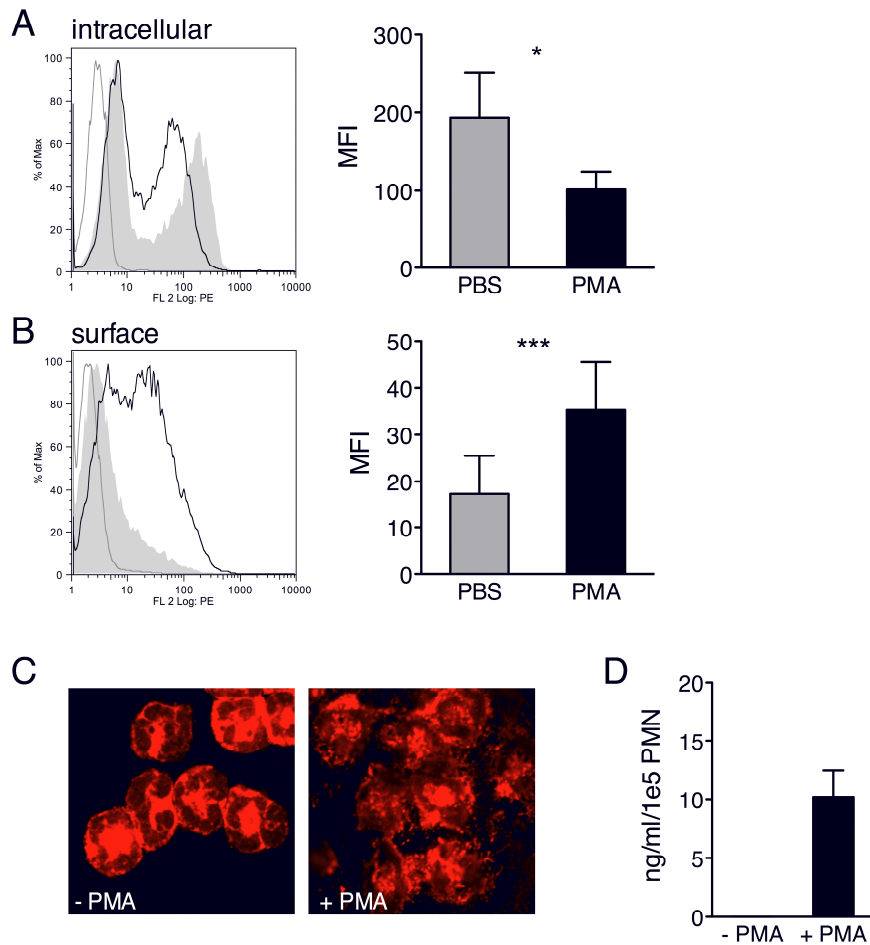


Figure 3. PMA-induced intracellular decrease and surface translocation of CRAMP. Flow cytometric analysis of unpurified pPMN from C57BL/6 mice stimulated for 15 min with PMA (black line) or left untreated (grey area) and subsequently stained with biotinylated rabbit anti-CRAMP or isotype control (grey line) Ab followed by RPE-conjugated Streptavidin. (A) Permeabilized cells stained for intracellular CRAMP and (B) non-permeabilized cells stained for surface-associated CRAMP. Bar graphs show MFI of non-treated (grey) and PMA-stimulated (black) pPMN. Representative histograms of two independent experiments are shown. Data are mean \pm SD of two independent experiments with two mice per group. (C) Immunofluorescence of non-treated and PMA-stimulated bPMN stained with rabbit anti-CRAMP Ab followed by Cy3-conjugated donkey anti-rabbit Ab. Fluorescence micrographs (original magnification $\times 100$) are representatives of three independent experiments. (D) Released CRAMP from bPMN non-stimulated or stimulated with PMA detected by ELISA. Significant differences are indicated by *: $p < 0.05$ and ***: $p < 0.001$.

Analysis of supernatants from PMA-stimulated PMN by ELISA confirmed that CRAMP was released after 30 min (Figure 3D). Other stimuli, such as fMLP, *S. aureus* lipoteichoic acid (LTA), the synthetic lipopeptide Pam3CSK4, or *Salmonella abortus equis* LPS, did not induce secretion of CRAMP (data not shown). These results give evidence that CRAMP is released from granules into the extracellular space after activation by PMA but not by a number of microbe-associated molecular patterns.

CRAMP is recruited to the phagosome and kills *S. aureus* intracellularly

The inability of PMN to secrete CRAMP after exposure to bacterial components raised the question whether viable *S. aureus* induce the release of CRAMP. In the following experiments we used the staphylococcal protein A (*spa*) deletion mutants SA113 Δspa and $\Delta spa/\Delta dltA$ instead of SA113 wt and $\Delta dltA$ to avoid the confounding factor of unspecific IgG binding to Protein A. pPMN were infected with either SA113 Δspa and $\Delta spa/\Delta dltA$ for 15 min and intracellular CRAMP expression and surface localization was studied by flow cytometry. In pPMN infected with either SA113 Δspa and $\Delta spa/\Delta dltA$ intracellular and surface localization of CRAMP remained unaltered compared to non-treated cells (Figure 4A-B).

Interestingly, confocal microscopy revealed that CRAMP localized with internalized *S. aureus* strains after infection (Figure 4C-D, arrows). We hypothesized that this localization of CRAMP is the result of granule fusion with *S. aureus*-containing phagosomes. Indeed, the phagosomal marker LAMP-1 colocalized with CRAMP at the site of *S. aureus*-containing phagosomes (Figure 4Civ,Div, arrows).

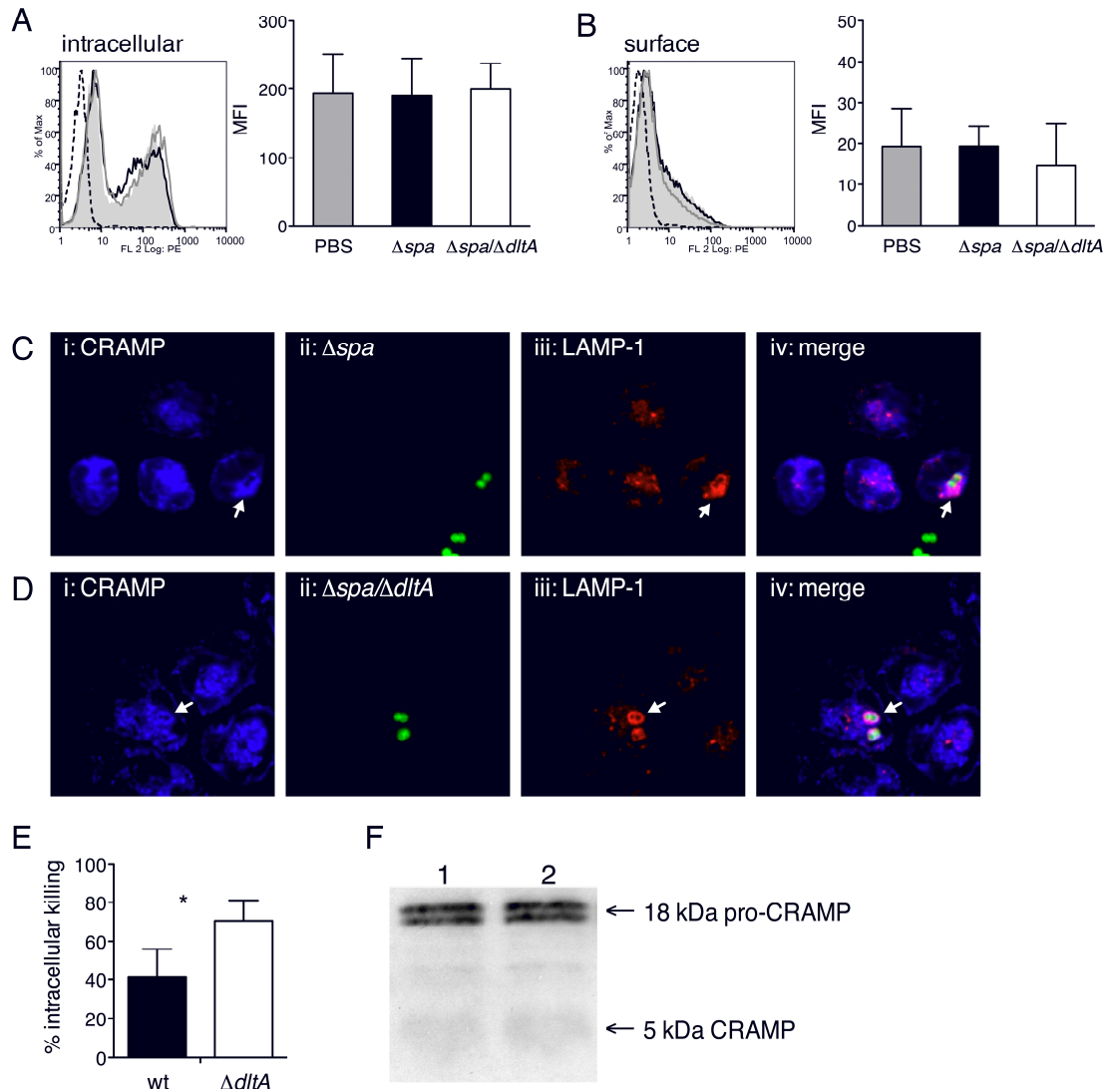


Figure 4. Intracellular localization and activity of CRAMP in *S. aureus* infection. Flow cytometric analysis of pPMN from C57BL/6 mice infected for 15 min with SA113 Δspa (black line) and SA113 $\Delta spa/\Delta dltA$ (grey line) or left untreated (grey area) stained with biotinylated rabbit anti-CRAMP or isotype control (dotted line) Ab followed by RPE-conjugated Streptavidin. (A) Permeabilized cells stained for intracellular CRAMP and (B) non-permeabilized cells stained for surface-associated CRAMP. Bar graphs show MFI of non-treated (grey), SA113 Δspa -infected (black), and SA113 $\Delta spa/\Delta dltA$ -infected (white) PMN. Representative histograms of three independent experiments are shown. Data are mean \pm SD of three independent experiments with two mice per group. (C, D) Immunofluorescence of bPMN infected for 30 min with FITC-labeled SA113 Δspa and $\Delta spa/\Delta dltA$ (MOI 1). Colocalization of CRAMP, FITC-labeled *S. aureus*, and LAMP-1: (i) immunostaining of CRAMP with biotinylated rabbit anti-CRAMP Ab followed by Streptavidin-Alexa647, (ii) FITC-labeled SA113 Δspa and $\Delta spa/\Delta dltA$, (iii) immunostaining of LAMP-1 with rabbit anti-LAMP-1 Ab followed by Cy3-conjugated donkey anti-rabbit Ab, and (iv) overlay of i-iii. Arrows indicate colocalization of markers with *S. aureus*. Fluorescence micrographs (original magnification x150) are

representatives of three independent experiments. (E) Intracellular killing of SA113 wt (closed) and $\Delta dltA$ (open) (MOI 1) by pPMN of C57BL/6 mice 30 min after infection. Data are mean \pm SEM of three independent experiments. Significant differences are indicated by *: $p < 0.05$. (F) Immunoblotting of PMN lysates. 1×10^6 pPMN of C57BL/6 mice were lysed, cell lysates were run on SDS-PAGE and analyzed by immunoblotting with rabbit anti-CRAMP or isotype control Abs. Lysates of untreated PMN (lane 1) and PMN infected with SA113 wt (lane 2). 10 μ g of total protein of lysates were loaded per lane. Immunoblots with isotype control antibody was negative.

Granule fusion to the phagosome was also confirmed by colocalization of CRAMP with cathepsin D (data not shown). No differences in localization of CRAMP toward the resistant SA113 Δspa and the CRAMP-susceptible $\Delta spa/\Delta dltA$ mutant were observed. Secretion of CRAMP into the extracellular space after 30 min of infection with both strains was not detectable by ELISA (data not shown). Although PMN are able to secrete CRAMP after soluble stimuli, these data point toward a preferential intracellular retention of CRAMP in infection to kill *S. aureus* in phagolysosomes. Consequently, we performed an intracellular killing assay using *S. aureus* infected pPMN to evaluate whether CRAMP is not only recruited but also active in phagolysosomes. SA113 wt and $\Delta dltA$ were killed intracellularly to 41.2% and 70.77%, respectively (Figure 4E). SA113 $\Delta dltA$ was significantly more susceptible to such intracellular killing. From this finding we conclude that the active form of CRAMP is present in phagolysosomes.

To show whether CRAMP is processed from its precursor to its active form in the phagosomes, pPMN were stimulated with SA113 wt and analyzed by SDS-PAGE and Western blot. In both untreated and infected cells, the pro-form of CRAMP appeared as two bands at 18 kDa and a faint band of the cleaved 5 kDa form of CRAMP was found in uninfected cells and after phagocytosis of SA113 wt (Figure 4F) indicating intracellular processing of CRAMP.

CRAMP is present in NETs but DNA-binding reduces CRAMP activity

Besides their phagocytic activity, human neutrophils were recently found to extracellularly entrap and kill *S. aureus* by forming NETs when phagocytic killing is exhausted (34). Proteases and antimicrobial peptides are associated with NETs comprising an extracellular site of bactericidal action of granule contents (35). Therefore, we first investigated NETs induction in murine PMN following stimulation with PMA or SA113 Δspa . Following both stimuli, NET formation was induced within 4h in bPMN (Figure 5Aii,Bii) and pPMN (not shown); unstimulated cells did not release DNA (Figure 5Av). In PMA-induced NETs, CRAMP was associated with extracellular DNA (Figure 5Aiv). In NETs induced by *S. aureus*, CRAMP colocalized with entrapped bacteria (Figure 5Biv, arrow). These results raised the possibility that association within NETs represents an extracellular site of action for CRAMP. To investigate the antibacterial activity of NETs, PMA-activated pPMN were incubated with SA113 wt and $\Delta dltA$ in the presence of cytochalasin D to inhibit phagocytic uptake. Both, SA113 wt and $\Delta dltA$ were killed with similar efficiency in the presence of NETs (Figure 5C). Interestingly, when NETs were degraded with DNase, the killing of SA113 wt was completely abolished while $\Delta dltA$ remained partially susceptible. From the similar susceptibility of SA113 $\Delta dltA$ and wt to NET-dependent killing we conclude that CRAMP, although present, is not the only important effector in the antimicrobial activity of NETs against *S. aureus*. Tight association of the cationic AMP with anionic DNA may reduce access of the peptide to the microbial cell surface.

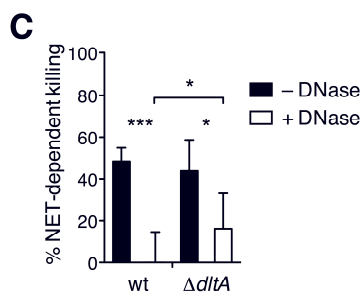
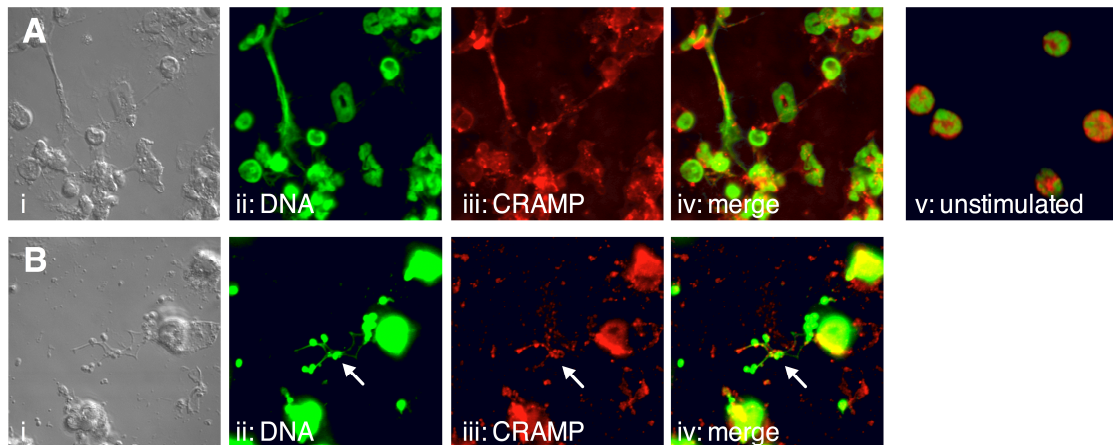


Figure 5. Induction of NETs and antimicrobial activity against *S. aureus*. (A, B) Immunofluorescence of bPMN activated for 4h with PMA (A i-iv) and SA113 Δspa (B). Colocalization of DNA, CRAMP, and *S. aureus*: (i) bright field, (ii) SYTOX[®] Green-labeled DNA and SA113 Δspa , (iii) immunostaining of CRAMP with rabbit anti-CRAMP Ab followed by Cy3-conjugated donkey anti-rabbit Ab, and (iv) merge of i-iii. (v) Immunofluorescence of unstimulated bPMN indicating no NETs formation after 4h. Arrows indicate colocalization of markers with *S. aureus*. Fluorescence micrographs (original magnification x150) are representatives of three independent experiments. (C) NET-dependent killing of SA113 wt and $\Delta dltA$ (MOI 0.01). NETs were treated with (open) or without (closed) DNase before infection to distinguish the contribution of DNA and the other NETs-components to the killing. Data are mean \pm SEM of three independent experiments. Significant differences are indicated by *: $p < 0.05$ and ***: $p < 0.001$.

NADPH oxidase is a weak contributor to CRAMP expression and response

Phagosomal alkalization following the NADPH oxidase activation was proposed to be essential for liberating granule enzymes in the phagosome (16). These granule enzymes are likely necessary for cleavage of CRAMP into its active form raising the question if NADPH oxidase-dependent liberation of granule enzymes might affect the processing and activity of CRAMP. We hence investigated function and phagosome translocation of CRAMP in $gp91^{phox-/-}$ mice lacking a functional NADPH oxidase. We assessed the bactericidal activity of $gp91^{phox-/-}$ TCF-PMN toward SA113 wt and $\Delta dltA$ *in vitro*. $gp91^{phox-/-}$ TCF-PMN were highly impaired in the killing of both strains compared to C57BL/6 TCF-PMN (Figure 6A compared to Figure 1). The persistent increased susceptibility of SA113 $\Delta dltA$ in $gp91^{phox-/-}$ TCF-PMN suggests that they still possess active CRAMP.

To exclude a defect in granule release we stimulated $gp91^{phox-/-}$ pPMN with PMA for 15 min and studied intracellular CRAMP expression and surface localization by flow cytometry. The MFI of non-treated $gp91^{phox-/-}$ pPMN was lower than in pPMN of C57BL/6 mice (Figure 6B versus 4A) indicating a reduced basal intracellular CRAMP level. However, intracellular CRAMP was significantly decreased after PMA stimulation as shown in the fluorescence histogram and bar graph in Figure 6B (left panels). PMA-stimulated cells showed more surface localization of CRAMP than non-treated cells with significantly increased MFI (Figure 6B, right panels). Regarding degranulation we found no defect in $gp91^{phox-/-}$ pPMN. Further we infected $gp91^{phox-/-}$ PMN with SA113 Δspa and $\Delta spa/\Delta dltA$ strains and observed that CRAMP was still recruited to internalized bacteria but LAMP-1 was only partially (Figure 6Civ,Div, arrows).

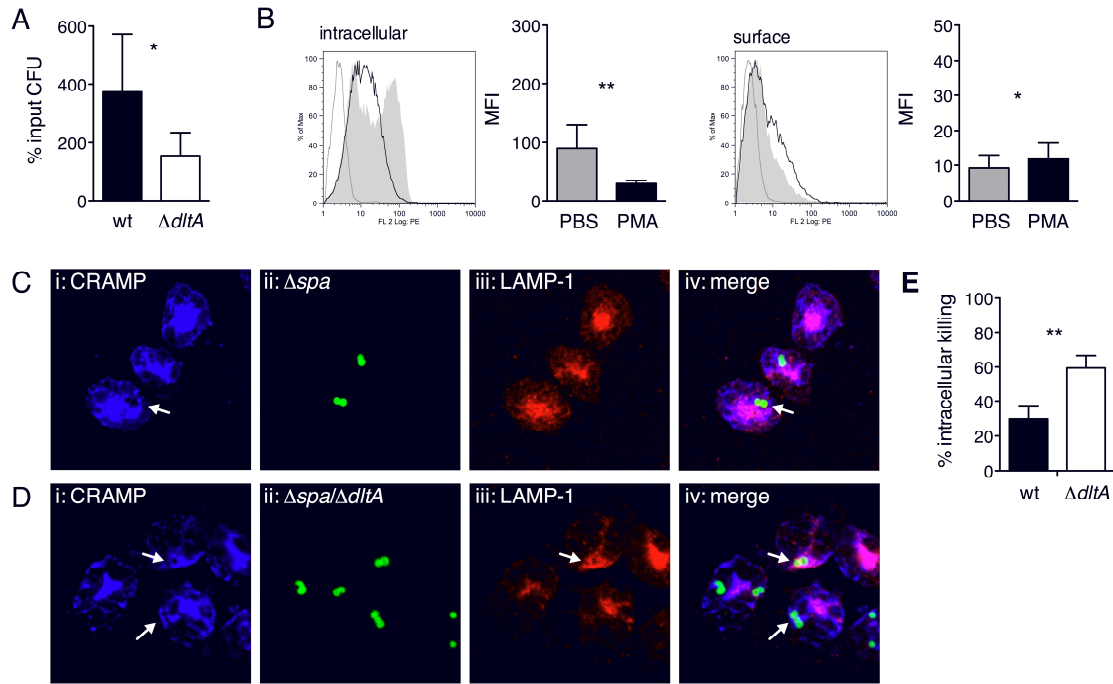


Figure 6. Contribution of NADPH oxidase to CRAMP function. (A) Bactericidal activity of TCF-PMN from $gp91^{phox-/-}$ mice. The numbers of viable CFU of SA113 wt (closed) and SA113 $\Delta dltA$ (open) after 2h of incubation with TCF-PMN are expressed as percentage of the initial inoculum. (B) Flow cytometric analysis of pPMN from $gp91^{phox-/-}$ mice stimulated for 15 min with PMA (black line) or left untreated (grey area) and stained with biontynlated rabbit anti-CRAMP or isotype control (grey line) Ab followed by RPE-conjugated Streptavidin. (left) Permeabilized cells stained for intracellular CRAMP and (right) non-permeabilized cells stained for surface-associated CRAMP. Bar graphs show MFI of non-treated (grey) and PMA-stimulated (black) pPMN. Representative histograms of three independent experiments are shown. Data are mean \pm SD of 3 independent experiments. (C, D) Immunofluorescence of bPMN of $gp91^{phox-/-}$ mice infected for 30 min with FITC-labeled SA113 Δspa (C) and $\Delta spa/\Delta dltA$ (D) (MOI 1). Colocalization of CRAMP, FITC-labeled *S. aureus*, and LAMP-1: (i) immunostaining of CRAMP with biotinylated anti-CRAMP Ab followed by Streptavidin-Alexa647, (ii) FITC-labeled SA113 Δspa and $\Delta spa/\Delta dltA$, (iii) immunostaining of LAMP-1 with rabbit anti-LAMP-1 Ab followed by Cy3-conjugated donkey anti-rabbit Ab, and (iv) merge of i-iii. Arrows indicate colocalization of markers with *S. aureus*. Fluorescence micrographs (original magnification x150) are representatives of two independent experiments. (E) Intracellular killing of SA113 wt (closed) and $\Delta dltA$ (open) (MOI 1) by pPMN of $gp91^{phox-/-}$ mice 30 min after infection. Data are mean \pm SEM of 3 independent experiments. Significant differences are indicated by *: $p < 0.05$ and **: $p < 0.01$.

Intracellular killing assays revealed that gp91^{phox-/-} PMN are significantly impaired in intracellular clearance of SA113 wt (p=0.0109) and $\Delta dltA$ (p=0.0392) compared to C57BL/6 PMN. SA113 $\Delta dltA$ remained more susceptible than wt (Figure 6E) suggesting the existence of active CRAMP in these PMN. The formation of NETs was described to be dependent on NADPH oxidase (13). Indeed, PMN from gp91^{phox-/-} mice were unable to induce NETs (data not shown). Taken together, the NADPH oxidase is only a weak contributor to the antimicrobial function of CRAMP.

Discussion

Since the original discovery of CRAMP in murine PMN (10), most studies have been performed on CRAMP expression and function in keratinocytes and other epithelial cells. In this study we provide evidence that PMN-derived CRAMP confers antimicrobial activity against the pre-eminent human pathogen *S. aureus*. Furthermore, we identified two different sites of action of CRAMP: Intracellularly in the phagolysosome and extracellularly associated in NETs, accentuated by DNA degradation.

To study CRAMP function, we made use of the CRAMP-susceptible *S. aureus* mutant $\Delta dltA$. We had previously shown the CAMP susceptible phenotype of this mutant *in vitro* and in a local murine infection model (28). In this study, we showed that the susceptibility of SA113 $\Delta dltA$ to murine PMN was selectively mediated by CRAMP by the use of CRAMP^{-/-} PMN. Thus, the dealanylated teichoic acids render *S. aureus* predominantly susceptible to CRAMP in PMN. The heightened susceptibility of SA113 $\Delta dltA$ to other PMN-derived AMPs (e.g. lactoferrin, lysozyme) was found to be less pronounced than for CRAMP (36, 37). Additionally, major contributions to resistance to other PMN-derived AMPs are provided by other staphylococcal virulence mechanisms, i.e. resistance to lysozyme is associated with acetylation of peptidoglycan (23) while resistance against lactoferrin (38) and lipocalin (39) are governed by multiple iron uptake systems in *S. aureus*, which might be not affected in the $\Delta dltA$ phenotype.

Our results show that TCF-PMN remain active with CRAMP against *S. aureus* despite its high resistance against AMPs *in vitro*. Similarly, whole blood PMN from C57BL/6, but not from CRAMP^{-/-} mice, blocked the proliferation of Group A

Streptococcus, another major Gram-positive pathogen with several well-defined cathelicidin resistance mechanism against CRAMP (11, 40).

Most studies on PMN activation and degranulation have been performed using PMN isolated from peripheral blood. However, PMN exert their function *in vivo* mainly after exudation from blood. Here we show that secondary granules wherein CRAMP is stored are not extensively released during PMN migration. This finding is in agreement with a study of granule mobilization during *in vivo* exudation of human PMN. The authors showed that control and exudate PMN have similar total content of lactoferrin and myeloperoxidase in secondary and primary granules, respectively (41). Nevertheless, our result for TCF-PMN contrasts with a previous study showing that TCF-PMN from guinea pigs have reduced myeloperoxidase and lysozyme content compared to peritoneal exudate PMN (42). The authors suggested that partial degranulation has occurred during contact with the implanted tissue cage. The retention of secondary and also primary granules, which harbor the most potent antimicrobial peptides including CRAMP, during extravasation may be crucial for retaining the antimicrobial activity of PMN for deployment at the site of infection.

We observed that secretion of CRAMP is induced after activation of PKC by PMA, but not by stimulation with fMLP, staphylococcal LTA, Pam₃CSK₄, *Salmonella*-LPS, or after phagocytosis of viable *S. aureus*. The mobilizable granules of PMN differ in availability for exocytosis and follow a strict hierarchy of exocytosis. Secretory vesicles have the highest propensity for release followed by gelatinase granules, secondary granules, and primary granules. This hierarchy could be the explanation why stimulation with fMLP did not lead to a significant release of CRAMP, but the more powerful agonist PMA induced secretion of CRAMP. Stie *et al.* showed that the human cathelicidin hCAP-18 accumulates at the PMN surface following stimulation

by 1 μ M fMLP (33). In our hands, 1 μ M fMLP did not lead to the surface translocation of CRAMP in pPMN, which might be due to different reactivity of human blood and murine peritoneal PMN. Nevertheless, surface-translocated CRAMP of strongly activated PMN might contribute to the killing and degradation of bacteria during their phagocytosis. The signaling pathways that link ligation of surface receptors or activation of PKC to degranulation of secondary granules include phospholipids, the MAP kinase p38, Ca^{2+} and the Src kinase Fgr, Rab GTPases and finally SNARE molecules (31). Their respective roles for CRAMP mobilization are subject of further investigation.

In infection, CRAMP was recruited to the phagosome where it exerted antimicrobial activity on *S. aureus*. This finding is confirmed by the work of Sorenson et al. showing hCAP-18 in the lumen of phagosomes after internalization of Latex beads by human PMN (1). By the increased susceptibility of SA113 $\Delta dltA$ to intracellular killing we showed that CRAMP was intraphagosomally active indicating that cleavage might have been occurred. Cathepsin D, a protease found in primary granules, was recruited to the phagosome as well. In hand with primary granule release into the phagosome, other cathepsins and neutrophil elastase accumulate in the lumen and might cleave CRAMP to the active form. By immunoblotting, we showed that intracellular CRAMP was mainly present as pro-form as it was shown for the human hCAP18/LL-37 (1). However, low amounts of the active form were detectable. This unexpected finding was contradictory to our high intracellular killing of SA113 $\Delta dltA$. Therefore, we think that active CRAMP might interact with negatively charged macromolecules (e.g. DNA) as shown for LL-37 (43, 44) or the bacterial membrane, which could explain low detectable amounts.

We demonstrated that murine PMN are able to form NETs after activation by PMA and viable *S. aureus* in a fashion similar to human PMN. In addition, CRAMP was localized in NETs and with entrapped *S. aureus*. CRAMP activity could not fully explain the NET-mediated killing of *S. aureus*, since SA113 wt and $\Delta dltA$ were killed to a similar extent. Extracellular killing of *S. aureus* therefore requires contributions from additional antimicrobial components of NETs, including histones. This is consistent with the finding that human NETs pretreated with antibodies against histones show reduced killing of *S. aureus* and *Shigella flexneri* (34). In contrast, D-alanylation of teichoic acids enhances resistance against NET-dependent killing in non-encapsulated but not encapsulated *S. pneumoniae* (35). Unlike *S. aureus* and other pathogens, pneumococci are not killed by the antimicrobial components in NETs (45). Therefore, the authors argued that NET-dependent killing was a cooperative effect of enhanced trapping and increased autolysis of non-encapsulated $\Delta dltA$ pneumococci. More importantly, we showed that the bactericidal activity of CRAMP is impaired when associated with NETs, while release of soluble CRAMP after degradation of the DNA backbone of NETs restored bactericidal activity of CRAMP against SA113 $\Delta dltA$. Our study provides first evidence that NET-associated AMP may be in a state of reduced activity; this idea is supported by the fact that the antimicrobial activity of LL-37 is inactivated by binding to DNA (44). We hypothesize that NETs may serve as a storage site of antimicrobial active peptides and enzymes to combat bacteria that are freed during NET breakdown or following DNase expression by certain pathogens.

We found reduced killing of *S. aureus* by PMN from gp91^{phox}^{-/-} mice and normal degranulation after PMA activation similar to human CGD PMN (22). The proposed dependence of granular proteases liberation on the NADPH oxidase (18) let us

hypothesize an impaired processing of CRAMP in $gp91^{phox-/-}$ mice. However, we could not find a link between the activity of the NADPH oxidase and CRAMP activity as SA113 $\Delta dltA$ remained significantly more susceptible to killing by $gp91^{phox-/-}$ PMN than the wt. The level of CRAMP was reduced in the absence of a functional NADPH oxidase. Both membrane subunits of the NADPH oxidase are absent in CGD patients with mutations in either the $gp91^{phox}$ or $p22^{phox}$ genes as well as in $gp91^{phox-/-}$ mice (46, 47). As CRAMP and the membrane subunits of NADPH oxidase are normally localized in the secondary granules (48), a lack of the NADPH oxidase membrane complex might alter membrane integrity and thereby leading to reduced granular content. Another explanation for the reduced intracellular CRAMP level might be augmented degranulation as previously described for primary granules in CGD neutrophils resulting in decreased levels of human α -defensins HNP 1-3 (49). The results of our study provide first evidence that PMN-derived CRAMP exhibits direct intracellular activity ensuring rapid initial protection from invading pathogens. NETs harboring CRAMP during prolonged infection may serve for storing AMPs, which might be freed during DNase expression by certain pathogens.

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Authorship

Contribution: N.J.J., M.S., K.A.R. and S.A.K. performed experiments; N.J.J., M.S., and S.A.K. analyzed the results and made figures; N.J.J., R.G., V.N., A.P., and R.L. designed the research and wrote the paper. This work was supported by SNF Nr. 3100A0-104259/1 and /2 and SNF Nr. 3100A0-120617.

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Role of TLR2 in non-oxidative and oxidative killing mechanisms of PMN
against *Staphylococcus aureus*

Role of TLR2 in non-oxidative and oxidative killing mechanisms of PMN against *Staphylococcus aureus*

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Abstract

We previously published that TLR2 contributes to efficient clearance of *Staphylococcus aureus* by polymorphonuclear leukocytes (PMN) in a local murine infection model. This study focused on the role of TLR2 in the activation of non-oxidative and oxidative killing mechanisms against *S. aureus*. We show that TLR2 clearly contributes to killing of *S. aureus*. The importance of antimicrobial peptide was shown in CRAMP-deficient PMN where killing of *S. aureus* was similarly reduced as in TLR2-deficient PMN. However, TLR2 was not involved in the regulation of CRAMP expression or function. PMN from gp91^{phox}^{-/-} were unable to eradicate *S. aureus* comparable to TLR2^{-/-} PMN and suggested that TLR2 may act via the NADPH oxidase. Indeed, induction of oxidative burst was delayed in TLR2-deficient PMN. We further investigated whether sensing of *S. aureus* by TLR2 is involved in the formation of neutrophil extracellular traps (NETs), which are induced when phagocytic killing is exhausted. Formation of NETs was independent of TLR2-MyD88 signaling. Summarized, TLR2 enhances oxidative killing of *S. aureus* possibly by accelerating NADPH oxidase assembly in the phagosome.

Introduction

Staphylococcus aureus (*S. aureus*) is a human commensal colonizing the skin and mucous membranes. When crossing the skin barrier, *S. aureus* causes abscess formation from which the bacteria can disseminate and cause severe systemic diseases. Once *S. aureus* breaches the skin barrier, polymorphonuclear leukocytes (PMN) are recruited to the site of infection to prevent further bacterial spread. Then, *S. aureus* is phagocytosed by PMN and eradicated by non-oxidative and oxidative mechanisms. The non-oxidative killing mechanisms are mediated by cationic antimicrobial peptides (CAMPs) such as defensins and cathelicidin stored in PMN granules. The oxidative mechanisms depend on reactive oxygen species (ROS) generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase (MPO). On one hand, the importance of non-oxidative killing comes clear in two rare inherited diseases, the Chediak-Higashi syndrome (1-3) and specific granule deficiency (4, 5). Both disorders are characterized by recurrent infections and shortened life expectancy underlining the fundamental role of granule proteins in host defense. On the other hand, the significance of ROS for antimicrobial activity is evidenced in patients with chronic granulomatous disease (CGD) lacking a functional NADPH oxidase complex and suffering from recurrent infections (6-9). All three diseases render patients susceptible to staphylococcal infection indicating that *S. aureus* has evolved the ability to resist both non-oxidative and oxidative PMN killing. Resistance to CAMPs is due to positive-charge modifications to the staphylococcal cell wall such as teichoic acid alanylation and peptidoglycan acetylation (10-12), whereas resistance to ROS is mediated by the expression of carotenoid pigment (13).

In addition to the intracellular killing mechanisms, PMN release neutrophil extracellular traps (NETs) consisting of chromosomal DNA and granule proteins (14). Formation of NETs presumably occurs after phagocytic killing mechanisms are exhausted. NETs then capture extracellular bacteria and are supposed to facilitate microbial killing by concentrating the antimicrobial arsenal to the site of infection and inhibiting further spreading. The formation of NETs was shown to be dependent on ROS and inhibitable by serum concentrations > 5% (15) but the precise mechanisms of NETs induction need further investigation. Traditionally, non-oxidative and oxidative killing mechanisms have been considered independent of each other. Recently, ROS have been implicated in the activation of granule proteases (16, 17). Activation of NADPH oxidase is followed by the influx of K⁺ and alkalization of the phagosome, which may be crucial to release the cationic granule proteases from the anionic proteoglycan matrix of the granules.

Sensing of *S. aureus* is mediated by staphylococcal lipoproteins and to a lesser extent by lipoteichoic acids (LTA), which are recognized by Toll-like receptor (TLR) 2 and its comolecules CD14 and CD36 (18-22). After ligand binding, TLR2 forms heterodimers with either TLR1 or TLR6 (23); dimerization causes conformational changes thereby triggering activation of myeloid differentiation factor 88 (MyD88). TLR2-MyD88-signaling pathway results in the activation of NF-κB and MAP kinases to induce pro-inflammatory genes and an antimicrobial response. The importance of TLR2 in inflammatory and antimicrobial response is evidenced in TLR2-deficient mice, which are highly susceptible to *S. aureus* septicemia (24), *Streptococcus pneumoniae* meningitis (25), and infections with *Mycobacterium tuberculosis* (26).

We previously showed that TLR2 contributes to efficient clearance of *S. aureus* in a local infection model and that alanylation of LTA protected *S. aureus* from this

TLR2-mediated defense (27). The protective effect of LTA alanylation was tentatively correlated to the resistance against cationic antimicrobial peptides. Recently, we could demonstrate that the increased susceptibility of a *S. aureus* mutant deficient in the gene *dltA* for alanylation of teichoic acids, SA113 $\Delta dltA$, to PMN-dependent killing is predominantly mediated by the murine cathelicidin CRAMP (28). Therefore, we here investigate whether TLR2 regulates the antimicrobial defense against *S. aureus* by activating non-oxidative and oxidative killing mechanisms.

We show that TLR2 on murine PMN enhances killing of *S. aureus in vitro*. However, TLR2 had no influence on expression level of the murine cathelicidin CRAMP and granule recruitment to phagosomes was TLR2-independent. Activation of TLR2 signaling accelerated the generation of superoxide induced by *S. aureus*. In contrast, formation of NETs was independent of TLR2-MyD88 signaling.

Materials and Methods

Bacterial strains. *S. aureus* wt (ATCC 35556, SA113 wt) and its isogenic mutants $\Delta dltA$, Δspa , and $\Delta spa/\Delta dltA$ were grown overnight in tryptic soy broth (Difco) at 37°C. For stimulation experiments, a subculture was inoculated 1:100 (v/v) from overnight culture in fresh tryptic soy broth and grown to late-log phase. Bacteria were washed twice with 0.9% NaCl prior to use.

FITC-labeling of staphylococci. *S. aureus* subculture was grown to mid-log phase in fresh tryptic soy broth. Bacteria were washed twice with 0.9% NaCl and labeled in 0.1 mg/ml FITC (Sigma) in PBS for 1h at 37°C with shaking. Prior to use, bacteria were washed twice with 0.9% NaCl and resuspended in Dulbecco's PBS with 100 mg/L MgCl₂ and 100 mg/L CaCl₂ (DPBS⁺⁺, Invitrogen).

Mice and tissue cage model. C57BL/6, TLR2^{-/-}, CRAMP^{-/-}, and gp91^{phox^{-/-}} mice were kept under specific pathogen-free conditions in the Animal House of the Department of Biomedicine, University Hospital Basel, and University of California, San Diego, according to the regulations of the Swiss veterinary law and the Veterans Administration of San Diego Committee on Animal Use, respectively. Mice were euthanized by CO₂ or i.p. injection of 500 mg/kg Thiopental® (Abbott Laboratories).

12-14-weeks-old female mice were anesthetized and sterile Teflon tissue cages were implanted subcutaneously, as described previously (27). Two weeks after surgery, the sterility of tissue cages was verified. To harvest tissue cage fluid (TCF) for isolation

of PMN, mice were anesthetized by isofluorane (MINRAD INC.) and TCF percutaneously collected with EDTA.

Antibody generation. Two New Zealand White rabbits were immunized by repetitive subcutaneous injections of 150 μ g synthetic CRAMP (GL Shangai Biochem Ltd.) in adjuvant (MPL® + TDM + CWS Adjuvant System, Sigma) at monthly intervals. The titer of the antiserum was estimated by immunoblotting. The IgG fraction from the polyclonal anti-serum was isolated on a protein-G sepharose (Amersham) column and further affinity purified on CRAMP-coupled NHS-activated sepharose (GE Healthcare). Bound antibody was eluted with 0.1 M glycine (0.002% sodium azide, pH 2.5) and dialyzed against PBS. A portion of the antibody preparation was biotinylated as described previously (29), dialyzed against PBS and stored at -20°C.

PMN isolation. For peripheral blood PMN (bPMN), mouse blood was harvested by intracardiac puncture in EDTA. bPMN were isolated as described previously for human PMN (30), but using a modified density gradient centrifugation on a discontinuous Percoll gradient with 59% and 67% Percoll (Amersham Biotech) in PBS. bPMN were collected at the interface of the two Percoll layers.

For peritoneal PMN (pPMN), 1 ml of 3% thioglycollate (BD Biosciences) were injected i.p. After 6h, pPMN were collected by peritoneal lavage with 5 ml RPMI 1640 complete medium (5% FBS, 2 mM glutamate, 1 mM sodium pyruvate, 1.5 mM HEPES, non-essential amino acids) and pelleted by centrifugation.

For TCF-PMN, TCF was collected in EDTA and pelleted by centrifugation.

Stimulation of PMN for flow cytometry and immunofluorescence. After isolation from blood, peritoneum, or TCF, erythrocytes were lysed in water and PMN were resuspended in DPBS⁺⁺ at $1-2 \times 10^6$ cells/ml. PMN were incubated with 1 μ g/ml PMA, unlabeled (for flow cytometry) or FITC-labeled (for immunofluorescence) SA113 Δ *spa* and SA113 Δ *spa/\Delta**dltA* for 15 and 30 min at 37°C, 200 rpm. Stimulation was stopped on ice and PMN were collected by centrifugation for further use in flow cytometry or immunofluorescence

Immunofluorescence. After stimulation, cells were spun onto glass coverslips and fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). After permeabilization with 0.2% saponin for 30 min at RT and 5 min in methanol, cells were blocked with 2% normal goat or donkey serum (NGS or NDS) for 30 min at RT. Cells were stained with affinity-purified polyclonal rabbit anti-CRAMP (1 μ g/ml), biotinylated rabbit anti-CRAMP (5 μ g/ml), goat anti-cathepsin D (10 μ g/ml, Santa Cruz Biotechnology), and rabbit anti-LAMP-1 (10 μ g/ml, Abcam) Ab, followed by donkey anti-rabbit/goat IgG-Cy3 Ab (7.5 μ g/ml, Jackson ImmunoResearch) or Streptavidin-Alexa647 (10 μ g/ml, Molecular Probes). Isotype-matched Ab served as negative controls. Specimens were analyzed with a Zeiss Axiovert 100M microscope (Carl Zeiss AG) using the confocal system LSM 510 META and LSM 510 v3.2 SP2 software (Zeiss).

Flow cytometry. Cells were blocked with 2% NGS, fixed with 4% PFA, and permeabilized for intracellular staining with 0.1% saponin. They were sequentially

stained with biotinylated rabbit anti-CRAMP Ab (1 $\mu\text{g/ml}$) and Streptavidin-RPE (0.25 $\mu\text{g/ml}$). Biotinylated rabbit IgG was used as isotype control.

PMN killing assay. TCF-PMN were resuspended in DPBS⁺⁺ (with 10% pooled mouse plasma) and 2×10^5 PMN were incubated with SA113 wt or $\Delta dltA$ at a multiplicity of infection (MOI) of 1h and incubated at 37°C, 200 rpm. After 2h, samples were diluted in H₂O (pH 11) to lyse PMN and serial dilutions were plated on Mueller-Hinton agar (MHA) to enumerate surviving intra- and extracellular bacteria.

Quantification of DNA release from activated neutrophils. TCF-PMN were resuspended in RPMI 1640 complete (2% FCS), seeded into 96-well plates (OptiPlate-96F, Perkin Elmer) at 2×10^5 /well and stimulated with 50 nM PMA, SA113 wt, and SA113 $\Delta dltA$ at MOI 10. After indicated time points, Sytox Green (MolecularProbes) was added to the cells at a final concentration of 10 μM to detect extracellular DNA. Non-stimulated TCF-PMN were used as control. The plates were read in a fluorescence microplate reader (Spectramax GeminiXS, Molecular Devices) with a filter setting of 485(excitation)/538 (emission).

Superoxide production. Intracellular superoxide anion production of 2×10^5 TCF-PMN was measured using luminol-enhanced chemiluminescence (31). TCF-PMN in DPBS⁺⁺ were stimulated with 1 $\mu\text{g/mL}$ PMA, SA113 wt, and SA113 $\Delta dltA$ at MOI 10 in the presence of 50 μM luminol and 2000 Units catalase. Chemiluminescence was measured every 5 min throughout the stimulation with a luminometer (Microlumat Plus, Berthold Technologies).

Statistical analysis. PMN killing assay and *in vivo* experiments were analyzed with Mann-Whitney. Flow cytometry data, intracellular killing and NET-dependent killing assays were analyzed with paired student's t-test. Generation of superoxide was analyzed with 2way-ANOVA. Statistical analysis was done with Prism 5.0a (GraphPad Software, Inc.). A p-value of $p < 0.05$ was considered statistically significant.

Results

TLR2^{-/-} PMN have impaired bactericidal activity against *S. aureus*

We previously showed that TLR2 contributes to efficient clearance of *S. aureus* in a local infection model and that alanylation of LTA protected *S. aureus* from this TLR2-mediated defense (27). The protective effect of LTA alanylation was tentatively correlated to the enhanced susceptibility of the $\Delta dltA$ mutant to CAMPs. Indeed, we could recently demonstrate that the increased susceptibility of SA113 $\Delta dltA$ to PMN-dependent killing is predominantly mediated by the murine cathelicidin CRAMP (28). Together, these results raised the hypothesis that TLR2 might regulate the expression or function of CRAMP in murine PMN.

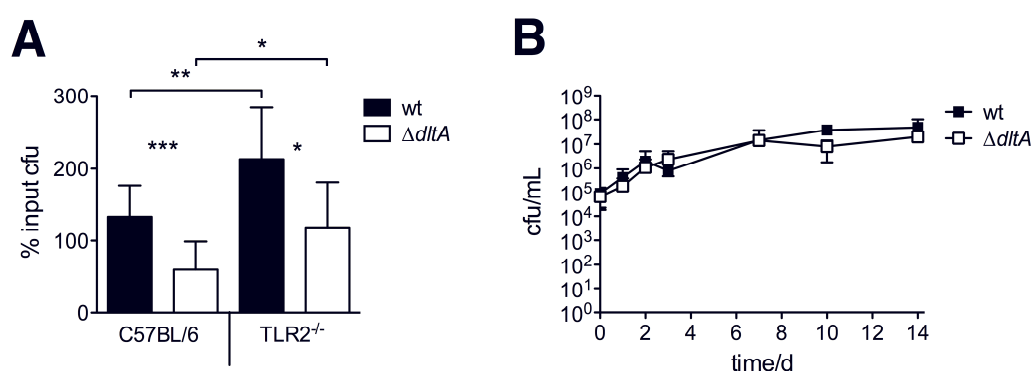


Figure 1. Bactericidal activity of TCF-PMN and *in vivo* growth of *S. aureus* in CRAMP^{-/-} mice. (A) The numbers of viable CFU of SA113 wt (closed) and SA113 $\Delta dltA$ (open) after 2h of incubation with TCF-PMN of C57BL/6 and TLR2^{-/-} mice are expressed as percentage of the initial inoculum. (B) *In vivo* bacterial growth for 14 days after infection with 10⁵ cfu of SA113 wt (closed) and $\Delta dltA$ (open) in CRAMP^{-/-} mice. Data are mean \pm SD of three independent experiments. Significant differences are indicated by *: p<0.05, **: p<0.01 and ***: p<0.001.

We investigated whether TLR2 enhances killing of *S. aureus* by comparing the bactericidal activity of TLR2^{-/-} and C57BL/6 PMN towards SA113 wt and $\Delta dltA$.

Indeed, PMN isolated from tissue cage fluid (TCF-PMN) of TLR2^{-/-} mice exhibited significantly decreased bactericidal activity against SA113 wt and $\Delta dltA$ compared to C57BL/6 TCF-PMN (Figure 1A) confirming the results found *in vivo* (27). SA113 wt and $\Delta dltA$ survived much better in the presence of TLR2^{-/-} than C57BL/6 TCF-PMN, but the enhanced susceptibility of $\Delta dltA$ to killing persisted in TLR2^{-/-} TCF-PMN, indicating the presence of active cathelicidin in TLR2^{-/-} TCF-PMN. Interestingly, the percentage of surviving SA113 wt (213.0% \pm 70.77) and $\Delta dltA$ (118.3% \pm 62.46) in presence of TLR2^{-/-} TCF-PMN was similar to the percentages of SA113 wt (214.9% \pm 53.5) and $\Delta dltA$ (177.4% \pm 30.01) in the presence of CRAMP^{-/-} TCF-PMN published earlier (28). Additionally, *in vivo* experiments revealed similar growth of SA113 wt and $\Delta dltA$ in CRAMP^{-/-} mice (Figure 1B), which was comparable to the results previously found in TLR2^{-/-} mice. These data give first evidence that TLR2 might mediate staphylococcal killing by regulating the expression or function of CRAMP in PMN.

TLR2-deficient PMN have normal levels of CRAMP

To investigate whether CRAMP expression is affected by TLR2-deficiency we compared intracellular CRAMP levels of TLR2^{-/-} and C57BL/6 PMN by flow cytometry. Cells from murine peripheral blood, TCF, and peritoneal exudates were used to cover the different populations of PMN encountered by *S. aureus in vivo*. Intracellular expression of CRAMP shown as mean fluorescence intensity (MFI) was similar in PMN from TLR2^{-/-} and C57BL/6 mice (Figure 2A). Additionally, MFI of PMN from different sites were comparable indicating minor degranulation of CRAMP during PMN exudation from blood. Immunofluorescence revealed that cellular distribution of CRAMP was similar in TCF-PMN of TLR2^{-/-} and C57BL/6

mice (Figure 2B). In addition, the experiment identified PMN as the major CRAMP-expressing cells in TCF, as monocytes showed no positive staining for CRAMP (arrows). These results indicate that TLR2-deficiency does not affect expression of CRAMP in PMN granules.

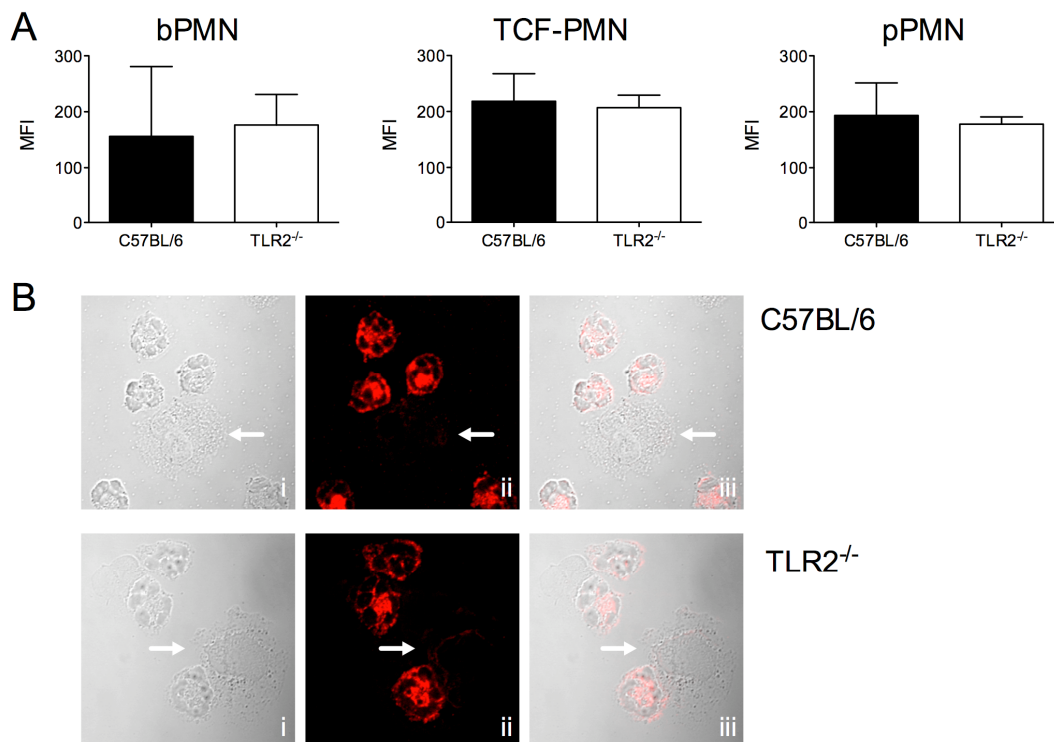


Figure 2. Intracellular CRAMP expression in blood and exudate PMN. (A) MFI of purified blood PMN, peritoneal exudate cells, and TCF cells from C57BL/6 and TLR2^{-/-} mice intracellularly stained with biotinylated rabbit anti-CRAMP Ab followed by RPE-conjugated Streptavidin and analyzed by flow cytometry. (B) Immunostaining of CRAMP in TCF cells examined by confocal microscopy. (i) Bright field, (ii) immunostaining of CRAMP with rabbit anti-CRAMP Ab followed by Cy3-conjugated donkey anti-rabbit Ab, and (iii) merge. Fluorescence micrographs (original magnification x64) are representatives of three independent experiments. Arrows indicate unstained monocytes.

Granule recruitment to *S. aureus*-containing phagosomes is TLR2-independent

The reduced antimicrobial activity of TLR2^{-/-} PMN against *S. aureus* could further base on a defect in degranulation or impaired recruitment of granules to phagocytosed *S. aureus*. In TLR2-deficient macrophages, a defect in phagosome maturation was described (32). We recently observed that release of cathelicidin of peritoneal PMN takes neither place after stimulation with staphylococcal LTA and synthetic lipopeptide nor after phagocytosis of *S. aureus* and can therefore rule out a defect in degranulation (28). To address recruitment of granules to phagosomes we used the staphylococcal protein A (*spa*) deletion mutants SA113 Δspa and $\Delta spa/\Delta dltA$ instead of SA113 wt and $\Delta dltA$ to avoid the confounding factor of unspecific IgG binding to Protein A. pPMN were infected with FITC-labeled SA113 Δspa and $\Delta spa/\Delta dltA$ for 30 min and CRAMP was stained to localize secondary granules after infection. CRAMP colocalized with internalized *S. aureus* strains in TLR2^{-/-} PMN similar as in C57BL/6 PMN (Figure 3); no differences in localization of CRAMP toward the resistant SA113 Δspa and the CRAMP-susceptible $\Delta spa/\Delta dltA$ mutant were observable. Summarized, the normal expression level and recruitment of granules to phagosomes in PMN point toward a TLR2-mediated bactericidal mechanism independent of cathelicidin.

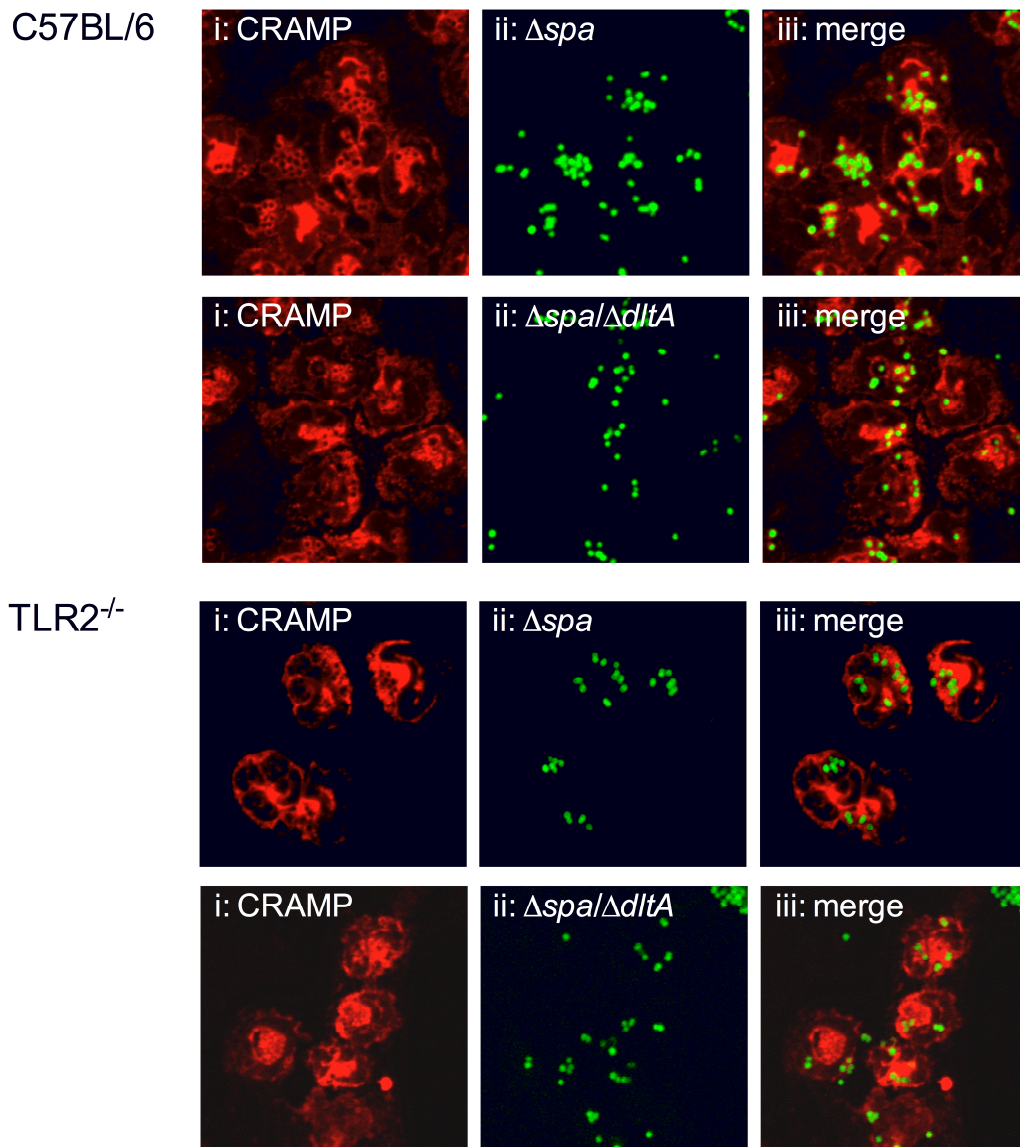


Figure 3. Intracellular localization of CRAMP in *S. aureus* infection. Immunofluorescence of bPMN of C57BL/6 and TLR2^{-/-} mice infected for 30 min with FITC-labeled SA113 Δspa and $\Delta spa/\Delta dltA$ (MOI 10). Colocalization of CRAMP and FITC-labeled *S. aureus*: (i) immunostaining of CRAMP with rabbit anti-CRAMP Ab followed by Cy3-conjugated donkey anti-rabbit Ab, (ii) FITC-labeled SA113 Δspa and $\Delta spa/\Delta dltA$, and (iii) overlay. Fluorescence micrographs (original magnification x100) are representatives of three independent experiments.

TLR2 signaling accelerates oxidative burst

The findings so far excluded an involvement of TLR2 in non-oxidative killing mechanisms of PMN. A recent study showed that phosphorylation of the NADPH oxidase subunit p47^{phox} is dependent on TLR2 and macrophages of TLR2^{-/-} mice showed no superoxide and H₂O₂ production (33). Additionally, Laroux *et al.* showed that MyD88 controls assembly of the NADPH oxidase and killing of gram-negative bacteria (34). Based on these findings, the TLR2-mediated bactericidal activity against *S. aureus* might be attributed to oxidative killing mechanisms. We thus first determined the bactericidal activity of gp91^{phox}^{-/-} TCF-PMN against *S. aureus*. gp91^{phox}^{-/-} TCF-PMN were highly impaired in staphylococcal killing (Figure 4). This implies that the NADPH oxidase is involved in staphylococcal defense.

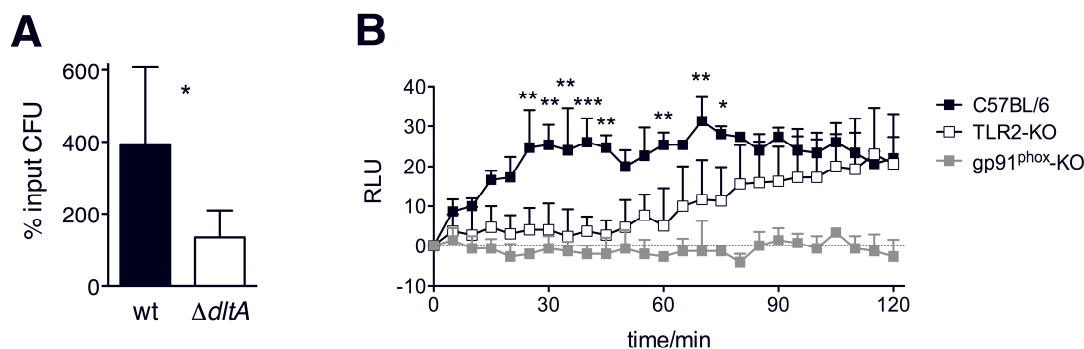


Figure 4. Impact of TLR2 on oxidative burst in PMN. (A) The numbers of viable CFU of SA113 wt (closed) and SA113 $\Delta dltA$ (open) after 2h of incubation with TCF-PMN from gp91^{phox}^{-/-} mice are expressed as percentage of the initial inoculum. (B) *S. aureus*-induced production of intracellular superoxide in TCF-PMN from C57BL/6, TLR2^{-/-}, and gp91^{phox}^{-/-} mice measured by luminol-enhanced chemiluminescence. Data are mean \pm SD of two independent experiments. Significant differences are indicated by *: p<0.05, **: p<0.01, and ***: p<0.001.

The involvement of TLR2 in the signaling process leading to activation of NADPH oxidase and generation of ROS was tested in PMN of TLR2^{-/-} and C57BL/6 mice. PMA served as control stimulus and induced a rapid production of superoxide

measured by luminol-enhanced chemiluminescence (Figure 4B). Interestingly, production of superoxide induced by *S. aureus* was delayed in TLR2^{-/-} compared to C57BL/6 PMN. This result indicates acceleration of oxidative burst by TLR2 signaling. Further investigation will reveal if a delay in assembly of the NADPH oxidase or reduced phosphorylation of subunits causes this defect in TLR2^{-/-} PMN.

Induction of NETs is independent of TLR2-MyD88 signaling

NETs are made by neutrophils activated with an increasing number of stimuli. However, not much is known about exact induction pathways. Urban *et al.* assumed that Fc γ receptors or pattern-recognition receptors such as TLRs might be involved (35). The formation of NETs is dependent on the activation of the NADPH oxidase (15). With the observed delay of oxidative burst in TLR2^{-/-} PMN formation of NETs might be affected as well. Thus, we analyzed whether TLR2 and MyD88 are involved in the induction of NETs by *S. aureus*. One to 3 hours post-infection with *S. aureus*, PMN from TLR2^{-/-} and MyD88^{-/-} mice released NETs comparable to C57BL/6 mice (Figure 5). Control PMN from gp91^{phox-/-} mice were unable to produce NETs. Taken together, these data show that TLR2-MyD88 signaling is not involved in NET formation indicating that phagocytic or complement receptors might be of importance in this process.

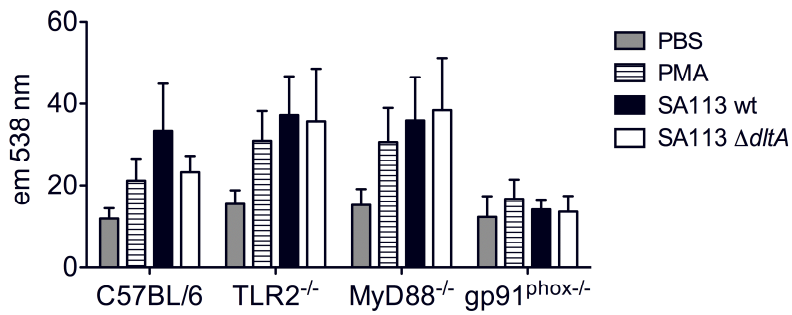
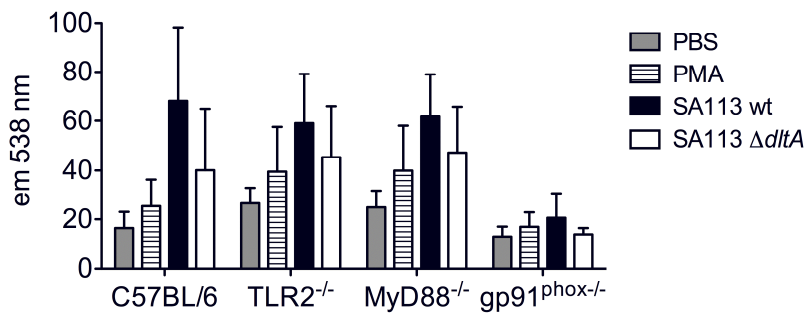
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Figure 5. Role of TLR2 signaling in formation of NETs. Quantification of NETs formation by TCF-PMN of C57BL/6, TLR2^{-/-}, MyD88^{-/-}, and gp91^{phox-/-} mice activated for 1 hour (A) and 3 hours (B) with PMA (stipped), SA113 wt (filled), and $\Delta dltA$ (open). Untreated PMN (grey) were used as negative control.

Discussion

The role of TLRs in the regulation of antimicrobial peptide expression was first described in *Drosophila*. Expression of human beta-defensin hBD-2 was induced by activation of TLR2 by bacterial lipoprotein in lung epithelial cells (36). TLR2 activation by soluble agonists enhanced cathelicidin protein expression in human macrophages (37) and CRAMP mRNA in murine fibroblasts (38). Recently, staphylococcal lipoproteins were evidenced to up-regulate human cathelicidin and hBD-2 in a TLR2-dependent manner in corneal epithelial cells (39). Altogether previous studies supported the hypothesis of a possible interaction between TLR2 and CAMP-mediated host defense.

We confirmed the importance of TLR2 in efficient killing of *S. aureus* by PMN. In line with our previous findings *in vivo*, alanylation of teichoic acid protected *S. aureus* from killing. Additionally, *S. aureus* infection in CRAMP-deficient mice mirrored the situation published for TLR2-deficient mice where *S. aureus* wt and $\Delta dltA$ showed similar growth strengthening our hypothesis. However, our study revealed a negligible role of TLR2 in the expression of CRAMP as well as in secondary granule recruitment to phagosomes in PMN. PMN, which have a life span of only a few hours, do store cathelicidin in the secondary granules as inactive form and processing after activation of the cell confers rapid antimicrobial defense. Since PMN are the first cells recruited to the site of infection we consider that they use their antimicrobial arsenal to eradicate bacteria after phagocytosis independently of TLR2. In epithelial cells and macrophages, cathelicidin is constitutively not or only weakly expressed and its expression is up-regulated after infection. Therefore, sensing pathogens by TLR2 and resulting up-regulation of cathelicidin in long-lived epithelial cells and

macrophages seems necessary to provide subsequent protection after the first intervention of PMN.

Nevertheless, TLR2 contributed to oxidative killing by accelerating NADPH oxidase activation. In agreement with this, we have previously shown that TLR2 enhanced oxidative killing of pneumococci in murine PMN (40). Additionally, p47^{phox} mRNA induction and phosphorylation were found TLR2-dependent in infected macrophages (33, 41). The role of TLRs in oxidative burst generation is further documented as mice, which lack MyD88, a downstream adaptor of TLR2, failed to produce reactive oxygen upon stimulation with group B streptococci (42). Furthermore, MyD88 enhanced NADPH oxidase assembly and controlled killing of gram-negative bacteria (34). Future investigation will reveal the molecular mechanism of TLR2-mediated oxidative killing and in particular whether TLR2 activation affects translocation and assembly of the NADPH oxidase.

PMN are able to form NETs that trap and degrade bacteria. The presence of bacteria activates PMN and leads to the formation of NETs. Urban *et al.* proposed that Fcγ receptors and pattern-recognition receptors might be involved in the initiation of NETs formation by opsonized and non-opsonized microbes (35). Here, we showed that induction of NETs formation by non-opsonized *S. aureus* is independent of TLR2-MyD88-signaling. It is now known that induction of NETs depends on ROS-signaling, as inhibition of H₂O₂ formation blocked NET formation and application of H₂O₂ to PMN from CGD patients led to formation of NETs (15). Additionally, NET formation is inhibited in a concentration-dependent fashion by serum due to its antioxidant activity (15, 43). On the other hand, ROS production can be induced by stimulation of PMN with specific TLR ligands. LPS-induced TLR4-MyD88-IRAK-4 signaling was shown to regulate NADPH oxidase activation (44-47). However, only a

few reports have clearly identified the interplay between TLRs and NADPH oxidases and needs further investigation. Therefore, signaling mechanisms involved in NETs formation by different stimuli might be complicated but future studies on ROS and TLR signaling will help to fully understand the process of NET formation.

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Authorship

Contribution: N.J.J., M.S., K.A.R. and S.A.K. performed experiments; N.J.J., M.S., and S.A.K. analyzed the results and made figures; N.J.J., R.G., V.N., A.P., and R.L. designed the research and wrote the paper.

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5. Overall Discussion

In the first part of this thesis we aimed to investigate the antimicrobial potential of neutrophil-derived CRAMP in staphylococcal killing. The focus was on the identification of its site of action, since little is known so far about extra- or intracellular activity of neutrophil-derived cathelicidins. Although the human cathelicidin hCAP-18 is secreted to the extracellular space by neutrophils after soluble stimuli (14), it is plausible that a particle stimulus such as live *S. aureus* leads to release of cathelicidin into the phagolysosome. Evidence for this assumption is given by the fact that hCAP-18 is found in latex bead-containing phagosomes. Interestingly, cleavage of hCAP-18 was only observed extracellularly but not yet in the phagolysosome (14). Therefore, we intended to investigate extra- and intracellular activity of CRAMP against *S. aureus* and the presence of active CRAMP in phagolysosomes.

We found that neutrophils retain CRAMP during the process of extravasation. This documents the importance of CRAMP to exert full antimicrobial activity at the site of infection. Although we found degranulation of CRAMP after strong activation of neutrophils, soluble MAMPs and importantly live *S. aureus* did not induce secretion of CRAMP to the extracellular space. In contrast, CRAMP was recruited to *S. aureus*-containing phagolysosomes. Most interestingly, by using the CRAMP-susceptible *S. aureus* $\Delta dltA$ mutant we could demonstrate intracellular activity of CRAMP that was not reported before. The main function of CRAMP, therefore, seems to be intracellular. This finding is novel and contrasts with the known mainly extracellular activity of human cathelicidin.

A further approach in this study was to target the interaction of non-oxidative and oxidative killing mechanisms of neutrophils.

A novel link between non-oxidative and oxidative mechanisms was found in the formation of NETs, which is dependent on ROS (72). Antimicrobial activity of NETs against several pathogens including *S. aureus* has been established but was not correlated to the AMP content. Since AMP activity in NET-association has not yet been addressed and might be of importance, we focused on the activity of CRAMP in NET-mediated defense. We demonstrated that CRAMP is in a state of reduced activity when associated with NETs and does not contribute to staphylococcal killing. This new finding contradicts the current opinion that NETs kill bacteria with locally concentrated AMPs. Whether this inactivation is common to all NET-associated AMPs remains unclear. Approaches addressing this question will be necessary for our detailed understanding how NETs exert microbial killing. We hypothesize that NETs serve as a storage site of antimicrobial active peptides and enzymes to combat bacteria that are freed during NET breakdown or following DNase secretion by certain pathogens including *S. aureus*.

The ingenious model of synergistic interaction between the NADPH oxidase and granule-associated proteases proposed by Segal and co-workers did not consider that the subsequent activation of antimicrobial peptides might be affected as well (13). Therefore, we further addressed the relationship between a functional NADPH oxidase and the efficiency of CRAMP-mediated staphylococcal defense. Using *gp91^{phox-/-}* mice, lacking a functional NADPH oxidase, we found normal CRAMP recruitment to the phagolysosome; however, intracellular CRAMP level and killing of *S. aureus* were reduced. Since intracellular killing of *S. aureus* Δ *lta* remained enhanced, activation of CRAMP by granule proteases could not be correlated to the

NADPH oxidase in this study. While total killing of *S. aureus* by gp91^{phox-/-} PMN was markedly reduced, intracellular killing was only slightly impaired. This might reflect an importance of ROS for extracellular but negligibility for intracellular killing. An additional finding during our observations was that LAMP-1, which we used as phagosomal marker, did only partially co-localize with *S. aureus*-containing phagosomes in gp91^{phox-/-} neutrophils. Few studies are available localizing LAMP-1 in the neutrophil; however, LAMP-1 was identified in the membrane of multivesicular bodies (MVB) and multilaminar compartments (MLC) (103, 104). Little is known about the role of MVB and MLC in neutrophil phagosome formation but they are speculated to be the true lysosomes in neutrophils (105). Characterization of neutrophil MVB and MLC might broaden our knowledge of phagosome maturation in neutrophils and identify a possible role of the NADPH oxidase in this process.

In the second part of this thesis we intended to investigate whether TLR2 mediates the activation of non-oxidative or oxidative killing mechanisms in response to *S. aureus*.

TLR2 activation has been shown to up-regulate cathelicidin protein and mRNA expression in fibroblasts and epithelial cells (88, 89). Evidence for TLR2 in mediating a cathelicidin-dependent defense in neutrophils was proposed by our group before (96). First, we investigated the role of TLR2 in cathelicidin-mediated staphylococcal killing and the potential regulation of CRAMP. We showed that TLR2 contributes to efficient staphylococcal killing in neutrophils. A correlation of TLR2-mediated killing and CRAMP expression or phagosome recruitment was not found. In accordance with the neutrophil's role in rapid defense these results underscore the concept of storing large amounts of inactive cathelicidin for immediate antimicrobial activity upon neutrophil activation. In contrast, cathelicidin expression is low in epithelial cells but

strongly up-regulated in infection. Accordingly, sensing pathogens by TLR2 and resulting up-regulation of cathelicidin is necessary to provide long-term protection by epithelial cells whereas the antimicrobial arsenal stored in neutrophil granules is sufficient for the first intervention by neutrophils.

Since TLR2 clearly contributed to efficient staphylococcal killing in neutrophils, independent of CRAMP, we assumed that TLR2 might promote oxidative killing in response to *S. aureus*. Our hypothesis was supported by the fact that TLR2 enhances oxidative killing of pneumococci in murine PMN (106). In addition, a recent study showed that TLR2 activation induced the phosphorylation of the NADPH oxidase subunit p47^{phox} in macrophages (107).

Our results revealed that TLR2 activation by *S. aureus* accelerates the induction of an oxidative burst in neutrophils. Future experiments addressing the phosphorylation and kinetics of assembly of NADPH oxidase subunits will specify the position of TLR2 in promoting oxidative killing. Under assumption of Segal's theory it may be argued that a delayed production of toxic ROS might additionally affect bacterial killing by hindering protease liberation and AMP activation. From this perspective, detailed examination of the relationship between TLR2 and NADPH oxidase activation will reveal further insights in the complex network of microbial sensing and killing.

Recent work suggested that TLRs might be involved in the initiation of signaling cascades leading to the formation of NETs (108). Although proposed, this hypothesis has not been subject of recent studies and there is no clear evidence for it. Therefore, we investigated the induction of NETs under the focus of TLR2 signaling. Our results revealed that TLR2-MyD88 signaling is not involved in the induction of NETs by non-opsonized *S. aureus*. This result suggests that induction of NETs might rather depend on phagocytosis-mediated signaling events than pattern recognition receptors.

Clearly, future research is necessary to elucidate which signaling pathways lead to the induction of NETs and whether opsonization might be of influence.

In conclusion, the direct interplay of non-oxidative and oxidative killing in neutrophils remains complicated and elusive. Overall, we believe that the neutrophil's ability to target many essential processes of microbial pathogens and to synergize non-oxidative and oxidative mechanisms with one another results in broad antimicrobial activity that is difficult for microorganisms to completely resist or circumvent. This needs temporal and spatial coordination of the distinct defense mechanisms, which makes it difficult to separate these processes. The remaining uncertainties and controversies leave many fascinating questions in the interactions between neutrophils and microorganisms to answer. Nevertheless, a deeper understanding of this area would be of crucial importance in developing methods to interfere with both old and newly emerging pathogens.

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Publication List

Schroeder GN, Jann NJ, Hillbi H

„Intracellular type III secretion by cytoplasmic *Shigella flexneri* promotes caspase-1-dependent macrophage cell death“

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