RESEARCH ARTICLE

Bovine *Staphylococcus aureus* Secretes the Leukocidin LukMF′ To Kill Migrating Neutrophils through CCR1

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ABSTRACT **Although *Staphylococcus aureus* is best known for infecting humans, bovine-specific strains are a major cause of mastitis in dairy cattle. The bicomponent leukocidin LukMF′, exclusively harbored by *S. aureus* of ruminant origin, is a virulence factor associated with bovine infections. In this study, the molecular basis of the host specificity of LukMF′ is elucidated by identification of chemokine receptor CCR1 as its target. Bovine neutrophils, the major effector cells in the defense against staphylococci, express significant cell surface levels of CCR1, whereas human neutrophils do not. This causes the particular susceptibility of bovine neutrophils to pore formation induced by LukMF′. Bovine *S. aureus* strains produce high levels of LukMF′ *in vitro*. In culture supernatant of the mastitis field isolate S1444, LukMF′ was the most important cytotoxic agent for bovine neutrophils. In a fibrin gel matrix, the effects of the *in situ* secreted toxins on neutrophils migrating toward *S. aureus* were visualized. Under these physiological *ex vivo* conditions, bovine *S. aureus* S1444 efficiently killed approaching neutrophils at a distance through secretion of LukMF′. Altogether, our findings illustrate the coevolution of pathogen and host, provide new targets for therapeutic and vaccine approaches to treat staphylococcal diseases in the cow, and emphasize the importance of staphylococcal toxins in general.**

IMPORTANCE **This study explains the mechanism of action of LukMF′, a bicomponent toxin found in bovine lineages of *S. aureus* that is associated with mastitis in cattle. At a molecular level, we describe how LukMF′ can specifically kill bovine neutrophils. Here, we demonstrate the contribution of toxins in the determination of host specificity and contribute to the understanding of mechanisms of coevolution of pathogen and host. Our study provides new targets that can be used in therapeutic and vaccine approaches to treat staphylococcal diseases in the cow. We also demonstrate the importance of toxins in specific elimination of immune cells, which has broader implications, especially in human infections.**

Received 27 February 2015 **Accepted** 15 April 2015 **Published** 4 June 2015

Citation Vrieling M, Koymans KJ, Heesterbeek DAC, Aerts PC, Rutten VPMG, de Haas CJC, van Kessel KPM, Koets AP, Nijland R, van Strijp JAG. 2015. Bovine *Staphylococcus aureus* secretes the leukocidin LukMF′ to kill migrating neutrophils through CCR1. mBio 6(3):e00335-15. doi:10.1128/mBio.00335-15.

Editor Steven J. Projan, MedImmune

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Staphylococcus aureus is a human and animal commensal but also a highly successful pathogen. In most mammalian species, *S. aureus* can cause bacterial infections ranging in severity from superficial skin and soft tissue infections (SSTI) to systemic infection and septicemia (1). *S. aureus* disease in animals is best documented in livestock, including rabbits, poultry, small ruminants, and cattle. In dairy cattle, *S. aureus* is a major cause of mastitis (2), a leading cause of economic losses in the dairy industry, and may have detrimental effects on the wellbeing and life span of cattle (3). Upon entry of bacteria into the lumen of the mammary gland, neutrophils are recruited in attempt to protect the gland from the establishment of infection and subsequent clinical mastitis (4, 5). A delayed influx of neutrophils in the mammary gland has a negative impact on the outcome of infection and is associated with invasive disease (6).

S. aureus has evolved a broad range of virulence factors, many of which are species specific, which contributes to the ability of this pathogen to infect various hosts (7, 8). Bicomponent pore-forming toxins are secreted virulence factors of *S. aureus* capable of killing a broad range of leukocytes. They consist of two distinct subunits, S and F components, of which the S component binds to a specific proteinaceous receptor on the cell surface (9, 10). Subsequent association of the F component results in pore formation, loss of cell membrane integrity, and cell lysis (11). The bicomponent leukocidins efficiently kill phagocytes, key players in the host defense against *S. aureus* (12). *S. aureus* strains of human origin can produce up to five different bicomponent toxins, including two types of γ-hemolysin (HlgAB and HlgCB) (13), LukAB (14) LukED (10), and Panton-Valentine leukocidin (PVL) (7, 15). A unique leukocidin exclusive to nonhuman *S. aureus* strains is LukMF′ (16). LukMF′ is predominantly found in strains isolated from bovine *S. aureus* mastitis (17). LukMF′ is located within the

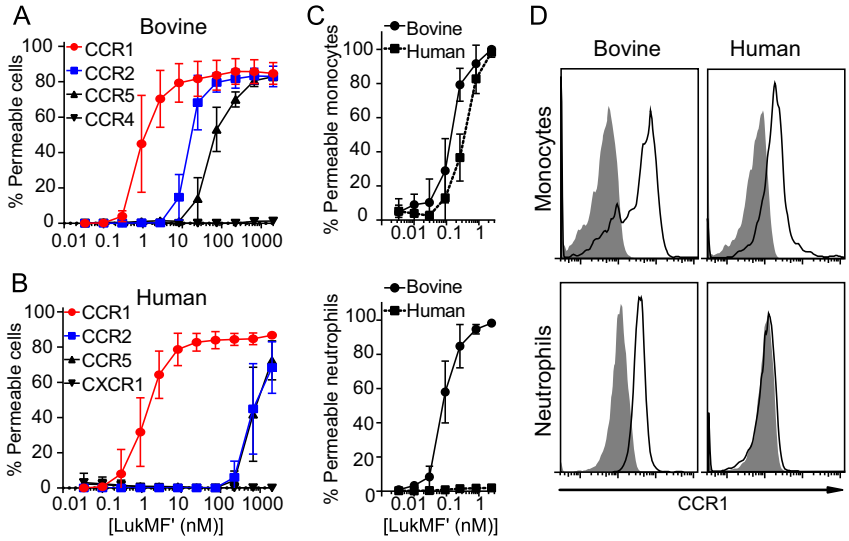

FIG 1 LukMF′ targets bovine phagocytes through CCR1, CCR2, and CCR5. HEK293T cells stably transfected with plasmids encoding bovine (A) or human (B) chemokine receptors were analyzed for pore formation upon incubation with LukMF′. Mean percentages of permeable cells ± standard deviations (SD) are shown ($n = 3$). (C) Bovine and human monocytes are efficiently permeabilized when treated with LukMF′. Bovine neutrophils are also efficiently targeted by LukMF′, whereas human neutrophils are resistant to pore formation. Mean percentages of permeable cells ± SD are shown ($n = 3$). (D) Surface expression of CCR1 on bovine and human phagocytes showing substantial expression of CCR1 on bovine but not human neutrophils. CCR1 was expressed abundantly on monocytes of both species. The results of one representative experiment out of three independently performed experiments are shown.

genome of temperate phage φSa1 and can be horizontally transmitted between strains (18, 19). The frequency of distribution of LukMF′ among bovine isolates ranges between 10 and 86% and differs according to geographic region and site of isolation (18, 20–23). In cattle, the presence of LukMF′ genes is associated with mastitis-causing strains (18, 20–23). LukMF′ is thought to be a virulence factor involved in the pathogenesis of mastitis because of its lytic effect on neutrophils, the most important effector cells in preventing intramammary infections (6).

LukMF′ is toxic to bovine neutrophils, monocytes, and macrophages but not to bovine B cells (24), suggesting the involvement of one or multiple specific receptors, not identified as yet. The receptors employed by LukMF′, however, have not yet been identified. In recent years, the host molecular counterparts of other leukocidins have been reported, explaining the cell selectivity and species specificity of the toxins (9, 10, 14, 25, 26). Members of the family of chemokine receptors are broadly used as target receptors by the closely related bicomponent toxins PVL, LukED, HlgAB, and HlgCB (15).

In this study, we identified bovine CCR1, CCR2, and CCR5 as target receptors for LukMF′ and showed that not only receptor compatibility but, to an even greater extent, differential expression of chemokine receptors are responsible for the species-specific efficiency of leukocidins. We demonstrated that LukMF′ is highly expressed and the most potent toxin killing bovine neutrophils. An *ex vivo* fibrin gel model showed that *S. aureus* kills migrating neutrophils at a distance by secretion of LukMF′. Altogether, this report shows that LukMF′ is the most potent and highly expressed leukocidin of bovine *S. aureus*, eliminating bovine neutrophils at distance in a CCR1-dependent manner.

RESULTS

LukMF′ targets bovine CCR1, CCR2, and CCR5. The cellular targets of LukMF′ have not been identified so far. In recent years, the host counterparts for PVL, LukED, HlgAB, and HlgCB were shown to be chemokine receptors (9, 10, 25, 26). Based on the close homology of these leukocidins to LukMF′, we hypothesized that LukMF′ targets bovine chemokine receptors. Therefore, we cloned and expressed, in human embryonic kidney (HEK293T) cells, all known bovine chemokine receptors that are present on LukMF′-sensitive cells. These cells were screened for permeabilization upon incubation with high concentrations of LukMF′ (see Table S1 in the supplemental material). Of these 27 receptors, only HEK293T cells expressing bovine CCR1, CCR2, and CCR5 were susceptible to pore formation induced by the toxin. Untransfected cells were resistant to permeabilization by LukMF′. HEK293T cells stably expressing bovine CCR1, CCR2 and CCR5 (see Fig. S1) were analyzed more closely for LukMF′-induced pore formation. LukMF′ was shown to permeabilize these cells in a dose-dependent manner (Fig. 1A). Calculation of half-maximal lytic concentrations (50% effective concentration [EC_{50}]) identified bovine CCR1 as the major target for LukMF′ toxicity, with a mean EC_{50} of 1.09 nM (±0.47 nM standard deviation) that was significantly lower than those of CCR2 (14.74 ± 5.19 nM) and CCR5 (57.12 ± 22.02 nM) ($P < 0.05$). LukMF′ was able to target the human counterpart of CCR1 with a similar EC_{50} (1.35 ± 0.67 nM), while pore formation through human CCR2 and CCR5 was inefficient (Fig. 1B).

To further specify the cellular tropism of LukMF′, we analyzed freshly isolated human and bovine neutrophils and monocytes for cell permeabilization upon incubation with LukMF′. In accordance with earlier observations (17), bovine neutrophils are highly susceptible to LukMF′-induced pore formation, with an EC_{50} of 0.08 nM (±0.03 nM), while human neutrophils are resistant (Fig. 1C). The susceptibilities of monocytes, on the other hand, are very similar between the two species, with an EC_{50} of 0.15 nM (±0.05 nM) for bovine versus 0.36 nM (±0.06 nM) for human monocytes. The surface expression of the target receptor

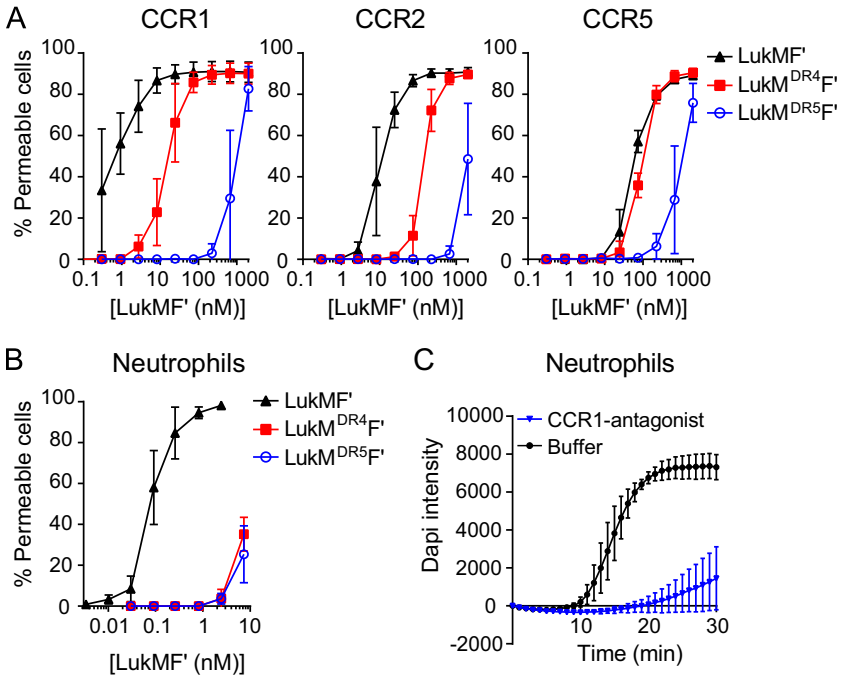

FIG 2 CCR1 defines the sensitivity of bovine neutrophils to LukMF′. (A) The pore-forming capacities of LukM^{DR4}F′ and LukM^{DR5}F′ were compared to that of LukMF′ in HEK293T cells expressing bovine CCR1, CCR2, or CCR5. Impairment of pore formation was receptor dependent for LukM^{DR4}F′ only. Mean results ± SD are shown ($n = 3$). (B) Bovine neutrophils were treated with either LukM^{DR4}F′ or LukM^{DR5}F′, and pore formation induced by the different toxin hybrids was compared to that induced by LukMF′. Decreased pore-forming capacity was found for both LukM^{DR4}F′ and LukM^{DR5}F′. Mean results ± SD are shown ($n = 3$). (C) Bovine neutrophils were treated with buffer or 0.1 μM CCR1 antagonist J113863, and pore formation upon the addition of 0.1 nM LukMF′ was measured over time in a fluorescence plate reader. Mean results ± SD are shown ($n = 3$).

defines the cell tropism of the leukocidin, as has been described previously for other members of the leukocidin family (25, 27). Therefore, we determined the expression levels of CCR1 on bovine and human leukocytes. Both species express CCR1 on monocytes (28), while CCR1 expression is restricted to bovine neutrophils (Fig. 1D). The absence of CCR1 on human neutrophils explains the observed resistance toward LukMF′ cytotoxicity. In summary, these data suggest that LukMF′ can efficiently target bovine phagocytes through a specific interaction with CCR1. Differential expression of CCR1 can explain the species-specific killing of neutrophils by LukMF′.

LukMF′-mediated killing of bovine neutrophils is CCR1 dependent. We set out to assess the relative importance of CCR1, CCR2, and CCR5 in mediating pore formation in bovine neutrophils upon incubation with LukMF′. Unfortunately, monoclonal antibodies recognizing bovine CCR2 and CCR5 are not available and the expression levels of these receptors on neutrophils are unknown. Therefore, we generated LukM/LukS-PV (S component of PVL) divergent region (DR) hybrids (LukM^{DR4} and LukM^{DR5}) with potentially restricted activities toward CCR1, CCR2, or CCR5 (26). LukM^{DR4} and LukM^{DR5} were tested for their ability to permeabilize HEK293T cells expressing CCR1, CCR2, and CCR5 upon the addition of LukF′ (Fig. 2A). LukM^{DR5}F′ was unable to permeabilize receptor-expressing cells efficiently, in accordance with earlier observations (26). LukM^{DR4}F′ showed a receptor-specific impairment of cytotoxicity. Toxicity toward CCR5-expressing cells was unaltered, while toxicity toward CCR1- and CCR2-expressing cells was decreased. Therefore, we used LukM^{DR4} to assess the relative importance of CCR1 and CCR2 for the induction of pore formation in bovine neutrophils. LukM^{DR4}F′ lost almost all cytotoxic activity on bovine neutrophils (Fig. 2B), suggesting that LukMF′ employs either CCR1 or CCR2 to kill these cells. In order to differentiate between these two receptors, we used a potent CCR1 antagonist (J113863) (29) that competed with LukM for binding to bovine CCR1 (see Fig. S2 in the supplemental material). Pretreatment of bovine neutrophils with J113863 protected these cells from LukMF′-induced pore formation (Fig. 2C). These data indicate that the cytotoxicity of LukMF′ toward bovine neutrophils is CCR1 mediated.

ECL2 and ECL3 define the sensitivity of CCR1 to LukMF′. LukM binds to CCR1 and is highly specific. In order to better understand this receptor specificity, we set out to identify the binding site for LukM on CCR1. We generated chimeras of bovine CCR1 and the highly similar bovine CCR1L, a receptor that is not recognized by LukMF′ (see Table S1 in the supplemental material) (28). The N terminus and extracellular loop 1 (ECL1) of bovine CCR1L are 100% identical to those of bovine CCR1, while the ECL2 and ECL3 of CCR1 and CCR1L differ slightly in their amino acid sequences (see Fig. S3A). Replacement of the ECL2 or ECL3 sequence in CCR1L by the corresponding sequences of CCR1 was sufficient to make CCR1L a target for LukMF′ (Fig. 3A). Substitution of ECL3 had a more pronounced effect on the recognition of the CCR1L/CCR1 chimeras than substitution of ECL2. Replacement of both ECLs together resulted in a chimera of CCR1L that was targeted by LukMF′ with the same efficiency as for bovine CCR1 (Fig. 3B). In ECL2, the residues interacting with LukMF′ were narrowed down to E177, F178, and H181 (Fig. 3B, EFH) by subsequently mutating individual amino acids. For ECL3, how-

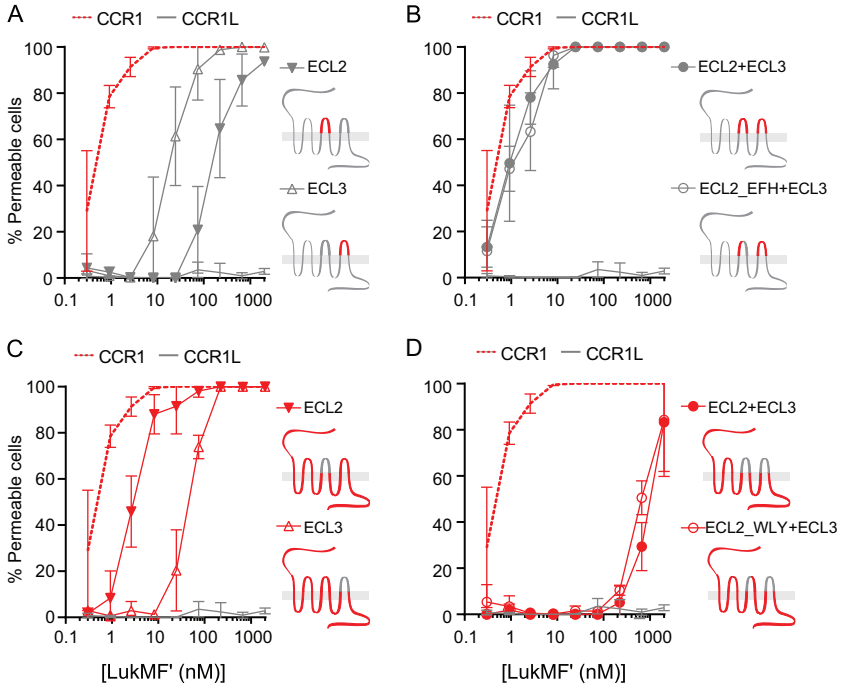

FIG 3 ECL2 and ECL3 of CCR1 are the target regions of LukMF′. (A and B) HEK293T cells were transfected with plasmids encoding CCR1L/CCR1 chimeras where ECL2 or ECL3 regions of CCR1L were replaced by nucleotide sequences of CCR1 separately (A) or together (B). (B) ECL2 was either replaced entirely or only by the ECL2_EFH region (E177, F178, and H181) of CCR1. Transfected cells were treated with LukMF′, and pore formation was measured to determine gain of function compared to that of WT CCR1L. Supporting GPCR schematics depict CCR1 regions of the chimeras in red and CCR1L regions in grey. Mean percentages of permeable cells ± SD are shown ($n = 2$ to 3). (C and D) HEK293T cells were transfected with plasmids encoding CCR1/CCR1L chimeras where ECL2 or ECL3 regions of CCR1 were replaced by nucleotide sequences of CCR1L separately (C) or together (D). (D) ECL2 was either replaced entirely or only by the ECL2_WLY region (W177, L178, and Y181) of CCR1L. Transfected cells were treated with LukMF′, and pore formation was measured to determine loss of function compared to that of WT CCR1. Supporting GPCR schematics depict CCR1 regions of the chimeras in red and CCR1L regions in grey. Mean results ± SD are shown ($n = 2$ to 3).

ever, the whole region appeared to be of importance (see Fig. S3B). These data were confirmed by the generation of CCR1/CCR1L chimeras, in which recognition of CCR1 by LukMF′ was gradually decreased by the replacement of ECL2 or ECL3 with CCR1L sequences (Fig. 3C). The effect was enhanced by simultaneous mutation of ECL2 and ECL3 (Fig. 3D). Again, the most important amino acids within ECL2 were E177, F178, and H181, because mutation into their CCR1L counterparts W177, L178, and Y181 (Fig. 3D, WLY) resulted in a loss of recognition similar to that caused by replacement of the entire ECL2. Thus, we identified the regions in bovine CCR1 that are involved in the interaction with LukMF′ and defined its receptor specificity.

LukMF′ is the major secreted toxin of *S. aureus* S1444 targeting bovine neutrophils. The genomes of bovine *S. aureus* strains can encode up to five leukocidins. A representative German bovine mastitis field isolate (S1444) was sequenced and shown to harbor the genes encoding LukMF′, LukAB (LukGH), LukED, HlgAB, and HlgCB. We investigated the relative contributions of these toxins to bovine neutrophil cytotoxicity. The relative promoter activities of the individual leukocidins were measured using each specific promoter region fused to a green fluorescent protein (GFP) sequence. In all culture media, the LukMF′ promoter was more active ($P < 0.005$ and $P < 0.0001$) than the other leukocidin promoters (Fig. 4A). The levels of fluorescence of the LukMF′ promoter in casein hydrolysate and yeast extract (CCY) medium (25), Todd-Hewitt broth (THB), and tryptic soy broth (TSB) were >27-, >30-, and >5-fold higher, respectively, than the levels of fluorescence of all other leukocidin promoters. In milk, an environment *S. aureus* would encounter when invading the mammary gland, the activity of the LukMF′ promoter was also >5-fold higher than those of all other leukocidin promoters. In order to confirm the high LukMF′ expression on the protein level, nine bovine *S. aureus* isolates harboring the LukMF′ operon (see Table S2 in the supplemental material) were analyzed for the presence of LukM in overnight culture supernatants by enzyme-linked immunosorbent assay (ELISA) (Fig. 4B). In accordance with the high promoter activity of LukMF′, the protein levels of LukM in culture supernatant were also observed to be high compared to the concentrations of other immune evasion proteins (30). Most strains produced 0.3 to 1 μg/ml of LukM, while two strains, including S1444, secreted significantly ($P < 0.05$ and $P < 0.005$) higher levels, 21 to 27 μg/ml, of LukM in THB (Fig. 4B). Overall, the LukM concentrations measured in culture supernatants were in range with the levels described for PVL (25) and at least 100-fold higher than needed to reach the EC_{50} of LukMF′ for bovine neutrophils.

To study the contribution of high LukMF′ production to cytotoxicity of *S. aureus*, we generated an isogenic LukMF′ deletion mutant strain (Δ*lukMF′*) in the S1444 background (see Fig. S4 in the supplemental material). The ability of secreted proteins from *S. aureus* S1444 wild type (WT) and Δ*lukMF′* to induce pore formation in neutrophils was analyzed. The crude THB medium-based bacterial supernatant of *S. aureus* S1444 WT was highly toxic to bovine neutrophils (Fig. 4C). In contrast, the supernatant

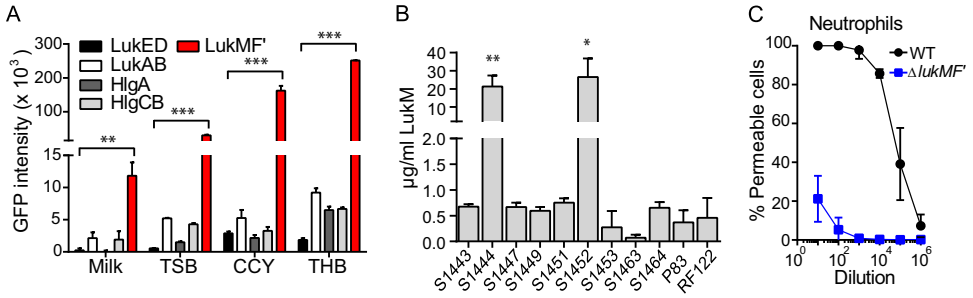

FIG 4 LukMF′ is the most highly expressed leukocidin of *S. aureus* S1444 and the major secreted toxin interacting with bovine neutrophils. (A) Promoter activities of HlgA, HlgCB, LukAB, LukED, and LukMF′ operons in S1444 GFP reporter strains cultured in CCY, THB, TSB, and milk. GFP fluorescence at 8 h postinoculation is shown. Mean results ± SD from three independent cultures are shown. Statistical significance was determined using Student's *t* test: **, $P < 0.005$; ***, $P < 0.0001$. (B) Protein levels of LukM in overnight culture supernatants of nine bovine *S. aureus* mastitis isolates in THB as measured by capture ELISA. Mean results ± SD from experiments performed with supernatants from three or four independent cultures are shown. Statistical significance was determined using Student's *t* test: *, $P < 0.05$; **, $P < 0.005$. (C) Bovine neutrophils were treated with overnight culture supernatants of S1444 WT and Δ*lukMF′* in THB, and pore formation was measured. Mean results ± SD are shown ($n = 3$).

of *S. aureus* S1444 Δ*lukMF′* showed very little residual cytotoxicity. This indicates that the other leukocidins of the bovine *S. aureus* S1444, of which recombinant HlgAB, HlgCB, and LukED are highly cytotoxic for bovine neutrophils (17), are not produced in sufficient concentrations in overnight culture supernatant to permeabilize bovine neutrophils. Functional quantification of HlgAB-, HlgCB-, and LukED-induced pore formation in HEK293T cells stably expressing their specific receptors (9) confirmed low activity of these leukocidins in the culture supernatants of both WT and Δ*lukMF′* S1444 (see Table S4). In summary, these data suggest that LukMF′ is the most important cytotoxic agent of bovine *S. aureus* S1444 toward bovine neutrophils.

***S. aureus* eliminates neutrophils at a distance by secreting LukMF′.** *S. aureus* uses secreted toxins to kill specific subsets of leukocytes and impair the host immune response (10). Among susceptible leukocytes, targeting of phagocytes has the most detrimental effect on the survival of the host (26). However, it was never shown directly how secretion of leukocidins protects *S. aureus* from phagocytes. We hypothesized that leukocidins can readily diffuse through tissue or biofilm to target phagocytes at a distance from the bacterial colonies. We designed a fibrin gel matrix that provided a culture environment amenable to both long-term survival (>12 h) of the bovine neutrophils and growth of *S. aureus* and expression of LukMF′. We studied the efficiency of neutrophil permeabilization by secreted factors of *S. aureus* S1444 WT and Δ*lukMF′* in the same fibrin gel using the methods described in the text and depicted in Fig. S5 in the supplemental material. A WT S1444 microcolony was captured at the 1 h 30 min time point and seen to expand while attracting neutrophils, as depicted in still images of a time lapse video (Fig. 5A). At 4 h 30 min, the neutrophils that moved into the near vicinity of the expanding microcolony started to lose their shape and become propidium iodide (PI) positive, indicative of compromised membrane integrity (Fig. 5A). The microcolony continued to grow and eventually (8 h 00 min) killed all adjacent neutrophils. In an overview image of the fibrin gel at 6 h 30 min, it became apparent that all *S. aureus* S1444 WT microcolonies were surrounded by PI-positive neutrophils (Fig. 5B and C). In contrast, the neutrophils in close proximity to the *S. aureus* Δ*lukMF′* microcolonies remained viable (Fig. 5D and E). The average PI intensity measured in cross sections of the gel was highest in the proximity of the WT microcolonies and still elevated at a distance of 2.0 mm from these colonies. In contrast, the PI intensity in the cross sections near the Δ*lukMF′* colonies remained low (Fig. 5F). These findings clearly demonstrate that the deletion of LukMF′ is sufficient to abolish killing of bovine neutrophils by *S. aureus* in an *ex vivo* situation. They also demonstrate that *S. aureus* S1444 secretes sufficient LukMF′ to kill neutrophils up to 2.0 mm away from the microcolonies and, thus, can form a protective zone around them.

DISCUSSION

The presence and production of the *S. aureus* leukocidin LukMF′ is associated with bovine mastitis in several epidemiological studies (18, 20–23). However, the mechanism of LukMF′ activity and its role in the pathophysiology of mastitis are not well understood. We identified CCR1 as the main molecular target for LukMF′ and showed that CCR1 is expressed abundantly on bovine neutrophils. This explains both the species specificity of LukMF′ and the susceptibility of bovine neutrophils to pore formation induced by this leukocidin. Since neutrophils are the major effector cells in the defense against staphylococci (6, 12), LukMF′ is expected to be an important virulence factor involved in bovine *S. aureus* disease.

Receptor recognition is defined by specific regions in the S component of the leukocidin and by specific motifs in the target chemokine receptor (25, 26). Recently, a domain involved in receptor selectivity has been identified in S component LukE (26). LukM shares ~77.5% homology with LukE and also employs CCR5 as one of its targets (10, 15). We found that the receptor binding DR4 domain identified in LukE is also important for receptor recognition by LukMF′. Because of the close homology with HlgAB, HlgCB, and PVL (31), we predict that for these leukocidins also, DR4 is the specific receptor-targeting region. Furthermore, we identified the binding site for LukM on its major receptor CCR1. We show that variation in a limited number of amino acids in ECL2 and ECL3 of this G protein-coupled receptor (GPCR) can impair receptor recognition by LukMF′. This finding emphasizes the selectivity of the leukocidins and helps in understanding the frequently observed lack of toxin-receptor compatibility between homologous receptors of different species (9, 25, 27).

Apart from CCR1, a unique receptor in relation to other staphylococcal leukocidins (9, 10, 14, 25, 26), LukMF′ also recognizes

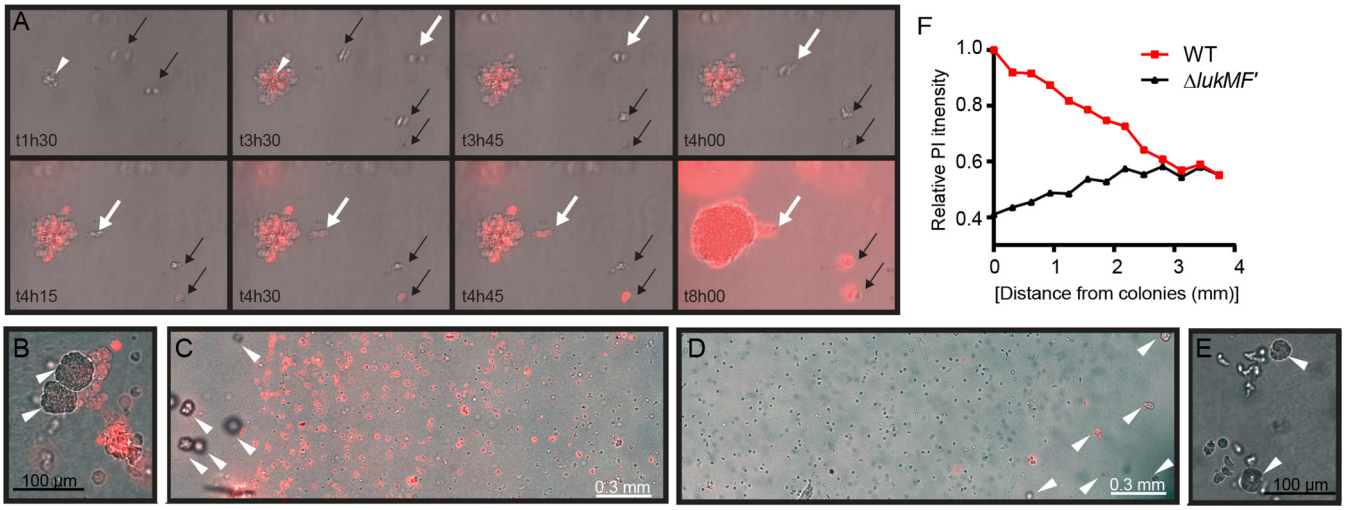

FIG 5 *S. aureus* S1444 eliminates distant neutrophils through secretion of LukMF′. (A) Still images obtained from time lapse video microscopy of a WT S1444 microcolony (white arrowheads) cultured in a fibrin gel according to the method described in the text (depicted in Fig. S5 in the supplemental material) in the presence of PI. The images shown are representative of at least 3 independent experiments. Neutrophils (black arrows) from the adjacent fibrin gel compartment had entered the compartment of the WT S1444 microcolony. One neutrophil (white arrows) is observed actively migrating toward the microcolony. Some out-of-focus neutrophils are seen as double images due to optical aberrations. Still images captured at 1 h 30 min, 3 h 30 min, 3 h 45 min, 4 h 00 min, 4 h 15 min, 4 h 30 min, 4 h 45 min, and 8 h 00 min after initiation of the experiment are shown. (B) Single image of expanded WT S1444 microcolonies located adjacent to the bovine neutrophils in the fibrin gel in the presence of PI. Images were captured 6 h 30 min after initiation of the experiment (time zero) as depicted in Fig. S5B. (C) Stitched overview image of the fibrin gel at the site where the WT S1444 microcolonies (arrowheads) are growing adjacent to the bovine neutrophils in the presence of PI. Images were captured 6 h 30 min after initiation of the experiment (time zero) as depicted in Fig. S5B. (D) Stitched overview image of the fibrin gel at the site where the Δ*lukMF′* S1444 microcolonies (arrowheads) are growing adjacent to the bovine neutrophils in the presence of PI. Images were captured 6 h 30 min after initiation of the experiment (time zero) as depicted in Fig. S5B. (E) Single image of expanded Δ*lukMF′* S1444 microcolonies located adjacent to the bovine neutrophils in the fibrin gel in the presence of PI. Images were captured 6 h 30 min after initiation of the experiment (time zero) depicted in Fig. S5B. (F) Average PI intensities in cross sections at fixed distances from WT and Δ*lukMF′* S1444 microcolonies in the fibrin gel from the experiment whose results are presented in panels C and D at 6 h 30 min. PI intensities were measured in a single well with WT colonies on one side and Δ*lukMF′* colonies on the other as depicted in Fig. S5. For clarity, the absolute distance toward the colonies is shown. PI intensities were plotted relative to the maximal PI intensity measured in the experiment.

CCR2 and CCR5. Although CCR1 is the most prominent target for LukMF′, the quantity of LukMF′ in culture supernatant is also sufficient to kill CCR2$^+$ and CCR5$^+$ cells. As a consequence, the range of susceptible cells is extended to subsets of T cells, inflammatory macrophages, and dendritic cells (9, 10). As described earlier, CCR5$^+$ T cells involved in the recruitment and activation of neutrophils through secretion of interleukin 17 (IL-17) and gamma interferon (IFN-γ) are important in the defense against *S. aureus* (10, 32). Furthermore, CCR2$^+$ inflammatory macrophages also play a role in *S. aureus* infections (9). Therefore, LukMF′-mediated killing of CCR5$^+$ and CCR2$^+$ leukocytes potentially promotes survival of *S. aureus*, in synergy with efficient killing of CCR1$^+$ neutrophils.

The leukocidins of *S. aureus* are species-specific virulence factors, highly active in humans but not in mice (15). Similar to the situation in humans (7, 12), *S. aureus* has adapted very well to infecting the bovine host (1, 33). Cattle have been reported to be highly susceptible to LukED, HlgAB, HlgCB, and LukMF′ (17) and are often naturally infected by *S. aureus* strains encoding these leukocidins. LukMF′ is a virulence factor that is likely to be involved in *S. aureus* adaptation to this nonhuman host (7, 12). So far, the mobile genetic element encoding LukMF′ has only been found in *S. aureus* strains of nonhuman origin (22). The insensitivity of human neutrophils to LukMF′-induced pore formation coincides with the observed absence of the gene in human-specific strains (15). Our report now directly links the LukMF′ sensitivity of neutrophils to surface expression of CCR1. This illustrates that differential expression of chemokine receptors, in addition to receptor compatibility (27), also defines the species specificity of leukocidins. Some rodent species are known to express CCR1 on neutrophils (34), which might be an explanation for their recently observed susceptibility to LukMF′-associated *S. aureus* disease (35).

All bicomponent leukocidins (PVL, HlgAB, HlgCB, LukED, LukAB, and LukMF′) kill neutrophils *in vitro*. However, the cytotoxic potencies of purified bicomponent toxins toward neutrophils differ. We identified LukMF′ as the most potent killer of bovine neutrophils. Compared to the cytotoxicity of LukED, a leukocidin that has been shown to contribute to mortality in mice *in vivo* (26), the cytotoxicity of recombinant LukMF′ toward bovine neutrophils is >500-fold higher than the activity of LukED toward murine neutrophils (26). The cytotoxicity of LukMF′ toward bovine neutrophils even exceeds that of PVL for human neutrophils (25). However, the relevance of a leukocidin is determined not only by its potency but also by its expression level. Although a single *S. aureus* strain can encode up to five pairs of bicomponent leukocidins, their expression has been shown to be subject to specific regulatory conditions. For example, the growth conditions and the presence of immune cells are known to influence the relative expression levels of several leukocidins (14, 36).

We show that LukMF′ is the most highly expressed leukocidin of bovine *S. aureus* and functionally the most active toward bovine neutrophils. Even though bovine neutrophils are highly susceptible to pore formation by HlgAB, HlgCB, and LukED, we observed

that their killing is largely dependent on the presence of LukMF′. This implies that, both during culture *in vitro* and during *ex vivo* growth of bovine *S. aureus* in a fibrin gel matrix, the other leukocidins are not produced at levels sufficient to kill bovine neutrophils. The expression of the staphylococcal leukocidins might be differentially regulated in bovine and human *S. aureus* isolates.

LukAB has been described as the main contributor to leukotoxin-mediated killing of human neutrophils in an *ex vivo* environment (14), consistent with its high expression in the presence of these cells (37). Since the susceptibility of bovine leukocytes to LukAB is unknown, it might be that the incompatibility of bovine CD11b prevents this leukocidin from exerting its effects in our experiments (14). In the human situation, LukAB contributes to bacterial escape from neutrophils after phagocytosis, resulting in survival of *S. aureus* colonies (14). Culture of LukMF′-secreting bovine *S. aureus* ex vivo in a fibrin gel matrix enabled us to be the first to visualize how a staphylococcal leukocidin functionally kills neutrophils a at distance, before physical contact between neutrophils and bacteria occurs. LukMF′ readily diffused through the fibrin gel matrix, which resulted in the formation of a gradient around *S. aureus* microcolonies that was toxic for approaching bovine neutrophils. Hence, *S. aureus* can form a protective zone around its colonies that safeguards the bacteria from harm by phagocytosis or the release of neutrophil extracellular traps (NETs), major defense mechanisms of neutrophils against *S. aureus* (12). In this way, it renders LukMF′ into a virulence factor that is beneficial for successful infection of the bovine host. HlgAB, HlgCB, LukED, and LukAB did not functionally contribute to the killing of bovine neutrophils in our experiments. Future research should point out whether these leukocidins have a complementary function in *S. aureus* infections with strains harboring LukMF′. Altogether, our data highlight the host-specific features of the staphylococcal leukocidins and further elucidate the mechanisms they employ to target and kill phagocytes.

MATERIALS AND METHODS

Ethics statement. For isolation of human leukocytes, written informed consent was obtained from all donors in accordance with the Declaration of Helsinki. The medical ethics committee of the UMC Utrecht (the Netherlands) approved the use of human material for this study. The use of animals was approved by the Ethical Committee for Animal Experiments of the Utrecht University (permit no. DEC2012.II.10.152) and conducted according to national regulations.

Leukocyte isolation. Bovine blood from three healthy Holstein Friesian donor cows was collected from the coccygeal vein using a sterile blood collection system with EDTA anticoagulant (BD Vacutainer). Neutrophils were isolated by using Percoll (1.09176 g/liter) centrifugation (38). Bovine peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat by centrifugation. Human blood from healthy volunteers was collected in heparin tubes, and PBMC and neutrophils were isolated by Ficoll/Histopaque centrifugation (39).

Quantification of human and bovine CCR1 expression levels. Freshly isolated human and bovine leukocytes (3×10^6 cells/ml) were incubated with CCR1 antibody (clone 53504) or an IgG2B isotype control (Dako) for 30 min on ice in a volume of 50 μl in the presence of 10% normal mouse serum. Cells were subsequently washed, and fluorescence levels were measured by flow cytometry. Neutrophils and monocytes were gated according to forward- and side-scatter properties using FlowJo software (Tree Star).

Cell permeability assays. Cell lines and leukocytes (3×10^6 cells/ml) were incubated with crude bacterial supernatant or recombinant LukMF′ in a volume of 50 μl in RPMI containing 0.05% human serum albumin (Sanquin) for 30 min at 37°C, 5% CO_2. Cells were analyzed by flow cytometry or in duplicate in a fluorescence plate reader, and pore formation was defined as intracellular staining by 4′,6-diamidino-2-phenylindole (DAPI). Equimolar concentrations of S and F components were used in all assays. The effect of nonspecifically cytotoxic phenol-soluble modulins (PSMs) was minimized by performing all experiments with culture supernatant in the presence of 10% fetal calf serum (40). For the analysis of pore formation in monocytes, human and bovine PBMC were prestained with mouse anti-human CD14 antibody clone MφP9 (BD Pharmingen) or mouse anti-bovine CD14 antibody clone CC-G33 (AbD Serotec), respectively. $CD14^+$ cells were analyzed for acquisition of DAPI and disappearance from the $CD14^+$ gate. Analysis of pore formation in HEK293T cells was restricted to receptor-positive cells. Maximal pore formation of HEK293T cells transiently transfected with plasmids encoding CCR1/CCR1L chimeras was defined by the percentage of DAPI-positive cells within the GFP-positive cell population as determined by flow cytometry. For analysis, the percentage of spontaneously lysed cells was subtracted from the percentage of permeable cells that were incubated with toxin. Half-maximal lytic concentrations (EC_{50}s) of LukMF′ were calculated using nonlinear regression analyses in Prism 6 (GraphPad).

Binding assays of polyhistidine-tagged LukM were performed as described in detail elsewhere (25).

Generation of fluorescent promoter reporters in *S. aureus* S1444. The promoter regions of the operons encoding LukMF′, LukED, LukAB, HlgA, and HlgCB were amplified by PCR from genomic DNA of strain S1444 and cloned into the pCM29 plasmid (41). The following primer pairs were used: 5′-GCTCTAGAAAACGCGCAGTTAATAAAAAG-3′ and 5′-GGGGTACCTTTATATTTTTAATACAAATTTATAC-3′ for LukMF′; 5′-AACTGCAGTTCAGTTATAACAGTATACG-3′ and 5′-GGGGTACCTATAAATTTTTAATACAAATTTATTCTAG-3′ for LukED; 5′-GCTCTAGATAAGCTCACACCTTTTCAAAGTAGT-3′ and 5′-GGGGTACCATGATTGATATTTGTTGATATG-3′ for LukAB; 5′-GCTCTAGACCACTTTTTTACCTGCAACTTG-3′ and 5′-GGGGTACCTTTAATTTTAAGTTCATAT-3′ for HlgA; and 5′-GCTCTAGAAATAATTAACTTTTAGATGTATTC-3′ and 5′-GGGGTACCTAATTTTATTTAACTTAATT-3′ for HlgCB. Restriction enzyme recognition sites are underlined. All plasmids were transformed into competent *Escherichia coli* DC10B cells and analyzed by sequencing. Plasmids were introduced into *S. aureus* S1444 by electroporation.

***Ex vivo* interaction of bovine neutrophils and secreted LukMF′.** To investigate the interaction of bovine neutrophils with secreted LukMF′ under physiological conditions, we set up an experiment where a diffusion gradient of secreted proteins could be established. Passive movement of *S. aureus* and bovine neutrophils was eliminated by embedding them in a fibrin gel. The fibrin gel was composed of components necessary for polymerization of the gel, as well as mimicking the *ex vivo* environment amenable to both long-term survival of the bovine neutrophils and growth of *S. aureus*. The gel was composed of two separate solutions: a fibrinogen solution containing 4 μg/ml fibrinogen, 25% TSB, 5% THB dissolved in HEPES buffer with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (wt/vol) human serum albumin (HSA) and a thrombin solution containing 2 U/ml thrombin, 20% precolostral bovine serum, and 12% THB dissolved in Iscove's modified Dulbecco's medium (IMDM). The resulting fibrinogen and thrombin solutions were combined 1:1 and mixed with either *S. aureus* or bovine neutrophils. To prevent direct phagocytosis and killing of single bacteria by the neutrophils, separate fibrin gels were made, containing either *S. aureus* or bovine neutrophils.

S. aureus S1444 WT or S1444 Δ*lukMF′* cells were grown overnight in THB. The culture was diluted 1:50 in fresh THB, grown for 3 h, diluted again in THB to an optical density at 660 nm (OD_{660}) of 0.0015, and vortexed vigorously, resulting in a suspension containing mostly single bacteria. This suspension was diluted in the freshly prepared liquid fibrin gel mixture, resulting in 4×10^4 bacteria/ml fibrin gel. Bovine neutrophils were diluted into the freshly prepared liquid fibrin gel, resulting in 2.5×10^6 neutrophils/ml fibrin gel. The suspensions containing either WT or

Δ*lukMF′* bacteria were carefully placed in the opposite corners in one well of an 8-well Lab-Tek II chambered coverglass (Thermo Scientific) (see Fig. S5A in the supplemental material). Subsequently, the neutrophil suspension was pipetted in between the two strains and the gel was allowed to polymerize for 5 min (see Fig. S5B). The solidified fibrin matrix was overlaid with 200 μl IMDM to prevent drying out of the gel matrix. At the initiation of the experiment (time zero), the slide was incubated at 37°C for 1.5 h to allow the single *S. aureus* bacteria to grow into small microcolonies and adjacent neutrophils to migrate toward these colonies (see Fig. S5C). Subsequently, propidium iodide (PI) (Invitrogen) was added at a final concentration of 4 μM to the IMDM overlay to allow it to diffuse into the fibrin gel to visualize membrane disruption of both neutrophils and bacteria. The chambered coverslip was transferred to the stage of a Leica TSC SP5 inverted microscope and imaged with an HC PL APO 20×/0.70 IMM objective (Leica Microsystems, The Netherlands). The microscope was encased in a dark environment chamber maintained at 37°C. The cells and bacteria were imaged using the N21 filter cube (red fluorescence from PI-positive cells) and bright-field microscopy every 15 min. When required to obtain a field of view covering the whole well while maintaining high resolution, a tile scan consisting of a maximum of 80 frames (4 × 20) was made, covering a rectangle of 11.00 × 1.88 mm. Individual bright-field and PI images were merged and exported using Leica LAS-AF software. These individual images were combined using Adobe Photoshop CS 6 "auto-align layers" followed by "auto-blend layers" to make one composite red-green-blue (RGB) image. To measure PI staining intensity, the composite RGB images mentioned above were split into their individual channels using ImageJ, and the red channel was divided into 28 identical sections along the width of the image, each being 0.312 mm wide and the height of the total image. The average pixel intensity for each section of this grayscale image representing only the red channel was measured using ImageJ and used to represent pore formation. The distribution of neutrophils throughout the fibrin gel was considered equal in each box. The signal at the last time point in the box adjacent to the WT colonies was assumed to represent total pore formation, and all other measurements were scaled to this by dividing their values by the total pore formation value, resulting in a pore formation scale where 1 represents full pore formation.

Statistical analysis. Flow cytometric data were analyzed with FlowJo (Tree Star Software). Statistical analysis was performed using Prism (GraphPad Software). Statistical significance was calculated using Student's *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00335-15/-/DCSupplemental.

Text S1, DOC file, 0.1 MB.
Figure S1, EPS file, 0.8 MB.
Figure S2, EPS file, 0.7 MB.
Figure S3, EPS file, 1 MB.
Figure S4, EPS file, 1.1 MB.
Figure S5, EPS file, 0.9 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0.04 MB.
Table S3, DOCX file, 0.02 MB.
Table S4, DOC file, 0.03 MB.

ACKNOWLEDGMENTS

This work was supported by a grant from ZonMw AntiMicrobial Resistance program (grant no. 205200004) and by the ALTANT program of the Dutch Government.

We thank Ross Fitzgerald Pascal, Rainard, and MSD Animal Health for donating *S. aureus* strains, Pieter Leliefeld for sharing expertise, and András Spaan for reviewing the manuscript.

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