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Cathelicidin-Deficient (*Cnlp*^{-/-}) Mice Show Increased Susceptibility to *Pseudomonas aeruginosa* Keratitis

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Abstract

Purpose—To examine the clinical progression and innate immune responses during *Pseudomonas aeruginosa* (PA) keratitis in cathelicidin-deficient (KO) mice.

Methods—PA (ATCC 19660) keratitis was induced in KO mice and wild-type (WT) littermates generated on a 129/SVJ background. Clinical score and histopathology were used to monitor the progression of infection at postinfection (PI) days 1, 3, 7, 14, and 21. Mouse corneas were harvested for viable bacteria quantitation, and myeloperoxidase (MPO) assays were performed to determine the number of infiltrating neutrophils. ELISA was used to quantitate interleukin (IL)-1 β , IL-6, macrophage inflammatory peptide (MIP)-2, keratinocyte-derived chemokine (KC), tumor necrosis factor (TNF)- α , and vascular endothelial growth factor (VEGF) levels in the corneas.

Results—WT mice were resistant (cornea healed), whereas KO mice showed increased susceptibility (corneas failed to recover by 21 days or perforated) to PA infection. Clinical scores were significantly elevated in the infected corneas of KO mice versus WT mice at 7, 14, and 21 days PI. Absence of cathelicidin resulted in significantly delayed clearance of PA in the cornea and an increased number of infiltrating neutrophils at 1, 3, 7, and 14 days PI. KO mice also exhibited differential expression of protein levels for IL-1 β , IL-6, MIP-2, KC, TNF- α , and VEGF up to day 21 PI compared with the WT mice.

Conclusions—Cathelicidin-deficient mice showed considerable susceptibility to PA keratitis. The present study demonstrates direct in vivo evidence that endogenous expression of cathelicidin provides defense against corneal PA infection indicating its importance in host innate immunity at the ocular surface.

The bacterium *Pseudomonas aeruginosa* (PA) is one of the leading virulent corneal pathogens associated with contact lens-related keratitis.^{1,2} Although an opportunistic organism, PA is notorious for its resistance to antibiotics and is therefore considered a particularly dangerous and dreaded ocular pathogen. PA induced ulcerative keratitis is a rapidly developing and devastating corneal disease that typically presents with severe inflammation, neovascularization, and liquefactive necrosis of the cornea. Apart from the

suppurative stromal ulceration and mucopurulent exudate, the clinical picture of advanced PA keratitis may also reveal descemetocoele (keratocoele) formation resulting from corneal melting. In the absence of timely and appropriate treatments, the infected cornea undergoes progressive degradation leading to perforation and therefore permanent vision loss.^{3–5}

The host immune response to PA corneal infection, which is critical in determining the outcome of the disease, consists primarily of an influx of neutrophils from the tear film and the limbal vasculature into the cornea. Previous studies suggest that both tissue-destructive bacterial proteases and stromal-degrading enzymes liberated by activated neutrophils and other stimulated inflammatory cells (e.g., macrophages) account for the corneal perforation observed in patients with severe PA keratitis.^{6,7} Angiogenesis associated with neovascularization observed in PA-induced corneal infection is a complicated and tightly regulated process and is known to be mediated by both proangiogenic and antiangiogenic growth factors and certain cytokines and chemokines.^{8,9} During an active PA corneal infection, bacterial toxins can stimulate cytokine, such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α , production in the cornea which synergize to elicit further inflammatory events including the induction of chemokine (IL-8) expression, which in turn facilitate recruitment of additional immune cells to defend the infected cornea.^{5,10} Thus, these chemokines and cytokines play an important role in determining the resolution or exacerbation of the inflammatory response induced by PA.

Cathelicidins are a family of mammalian cationic antimicrobial peptides with broad activity against a wide range of pathogens including Gram-positive and -negative bacteria, fungi, and some viruses.¹¹ In keeping with their role in host innate immunity providing the first line of defense against microbial infection, cathelicidins have been identified in leukocytes such as neutrophils and macrophages,^{12,13} skin epidermis,^{14–16} and epithelial surfaces of various tissues including that of the ocular surface.^{17–21} The mechanism of action by which cathelicidins exert their antimicrobial effects is through disruption of the anionic microbial membrane by electrostatic interaction resulting in cell permeabilization and death.²² In addition to being antimicrobial, cathelicidins are increasingly known to be multifunctional, regulating processes unrelated to microbial killing, such as the ability to chemoattract cells of the innate and adaptive immune systems including neutrophils, mast cells, monocytes and T-lymphocytes.^{23–26}

The mature human and mouse cathelicidins, each representing the sole cathelicidin in their respective species, share a highly conserved structure, identical regulatory functions, analogous tissue distribution/cellular expression and comparable spectra of antimicrobial activity against various microorganisms including PA.^{27–31} The murine cathelicidin gene, *Cnlp*, gives rise to the peptide CRAMP (cathelin-related antimicrobial peptide), a homologue to the human cathelicidin LL-37.³² Evidence supporting cathelicidin's being actively involved in innate immune defense comes from in vivo studies documenting that CRAMP-deficient mice are more susceptible to group A *Streptococcus* skin infection^{33,34} and show increased pox formation after inoculation with vaccinia virus in the skin.³⁵ In a separate study, Howell et al.³⁶ have reported that cathelicidin deficiency predisposes mice and humans with atopic dermatitis to eczema herpeticum. Rosenberger et al.¹³ have also shown enhanced *Salmonella typhimurium* survival within macrophages derived from

CRAMP-deficient mice. Conversely, Bals et al. have demonstrated that mice overexpressing LL-37 had a lower bacterial load and reduced inflammatory response in the lung after a challenge with PA, and in a different study, showed that transfer of the LL-37/hCAP18 gene restores bacterial (PA and *Staphylococcus aureus*) killing in a human cystic fibrosis bronchial xenograft model.^{37,38} Furthermore, Koczulla et al.³⁹ have shown that exogenous application of LL-37 resulted in angiogenesis and arteriogenesis in a rabbit model of hind-limb ischemia. In the same study, mice deficient for CRAMP also showed decreased neovascularization during cutaneous wound repair in vivo.³⁹

We have reported that LL-37 is a potent antimicrobial effective against various ocular pathogens.¹⁸ Furthermore, LL-37 is capable of stimulating cell migration and modulating chemokine and cytokine production by corneal epithelial cells¹⁹ which suggest that LL-37 may act as a multifunctional mediator and play an important part in stimulating the innate immune response in the cornea. Although our in vitro studies support a physiological role for cathelicidin (LL-37) in ocular innate immunity, there have been no studies addressing this directly in vivo. Therefore, the purpose of this study was to investigate the role of CRAMP in vivo by examining the clinical progression and innate immune responses during PA keratitis in CRAMP-deficient mice.

Materials and Methods

Animals

Homozygous wild-type (*Cnlp*^{+/+}, WT) and CRAMP knockout (*Cnlp*^{-/-}, KO) mice were generated from breeding pairs of heterozygous (*Cnlp*^{+/-}, HZ) 129/SVJ mice deficient in CRAMP expression.³³ All protocols were approved by the Institutional Animal Care and Use Committee of the University of Houston and conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Polymerase Chain Reaction

Mouse genotypes, *Cnlp*^{-/-} (KO) and age-matched wild-type (WT) mice, were verified by PCR performed on tail genomic DNA, as described by Nizet et al.³³ Genomic DNA was isolated from the tails of 3-to 4-week-old mice using reagents (DirectPCR Lysis Reagents; Viagen, Los Angeles, CA) and a genomic DNA isolation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. PCR was performed with reagents obtained from Invitrogen on the genomic DNA isolated from the WT and KO mice. The specific primers used were CRAMP³³: forward 5'-TGTTTTCTCAGATCCTTGGGAGC-3' and reverse 5'-AATTTTCTTGAACCGAAAGGGC-3', 241 bp; *Neo*³³ (the knockout gene): forward 5'-CCAGGGACTTCCATCCAGTAGAC-3' and reverse 5'-AGACTGCCTTGGGAAAAGCG-3', 88 bp. The PCR reaction included an initial 5-minute denaturation at 94°C. Amplification of the cDNA was performed for 35 cycles: denaturation, 95°C for 30 seconds; annealing, 60°C for 30 seconds; and extension, 72°C for 1 minute. A DNA ladder (HyperLadder I; Midwest Scientific, St. Louis, MO) was used as a marker (M). PCR products were visualized on 2% agarose gels by using a gel documentation system (Alpha Imager; Alpha Innotec, San Leandro, CA).

Experimental Keratitis

PA ATCC 19660 (American Type Culture Collection, Manassas, VA) were prepared as previously described.¹⁹ This ATCC strain is known to produce severe ocular infection in experimentally infected animal models of bacterial keratitis.^{40,41} PA cultures were suspended in phosphate buffer (PB) to a concentration of 2.0×10^8 cfu/mL. The bacterial concentration was adjusted turbidimetrically, and the dose was confirmed retrospectively by counting viable cells. Inbred 6- to 8-week-old mice (CRAMP KO and age-/sex-matched WT mice) were anesthetized by intraperitoneal injection (ketamine, 60 mg/kg; xylazine 6 mg/kg; Vedco, Inc., St. Joseph, MO). With the aid of a surgical stereomicroscope, three parallel 1-mm scratches penetrating the epithelial basal lamina into the superficial corneal stroma were made in the left eye of anesthetized mice using a sterile 27-gauge needle. Immediately after the corneal scarification, 5 μ L of the bacterial suspension (1.0×10^6 CFU) of PA was pipetted directly onto the surface of the wounded cornea. The clinical progression of the induced infection was monitored, and the severity of corneal damage determined at 1, 3, 7, 14 and 21 days PI with a photo slit lamp biomicroscope. At each time point, mice were killed and the corneas were removed. Six mice per group (WT and KO) per time point were used. The entire experiment was repeated at least twice to ensure reproducibility.

Clinical Examination of Corneal Infection

The mice were examined before bacterial challenge, immediately subsequent to bacterial challenge, and at intervals during the experiment. The animals were anesthetized for examination at 1, 3, 7, 14, and 21 days PI, and the corneas were examined at 40 $\times$ magnification under white light with a photo slit lamp biomicroscope. Digital photographs were taken under normal slit lamp illumination. The severity of corneal damage from PA infection was determined based on a grading system: 0, no disease; +1, isolated small opacity partially covering the cornea (focal infiltrate); +2, large opacity partially covering the cornea or paracentral stromal ring infiltrate; +3, large and dense opacity with neovascularization partially covering the cornea; +4, large and dense opacity with neovascularization fully covering the cornea +5, descemetocele/keratocele or corneal melting; +6, perforation or phthisis. The mean clinical scores were calculated from six mice per group (WT and KO) per time point and analyzed statistically using an unpaired Student's *t*-test, with $P < 0.05$ being considered significant.

Histology

Corneal sections from the infected eyes of WT mice were compared qualitatively with those from KO mice. The histology of the infected corneas was assessed at 1, 14, and 21 days PI. Assessment at other days was not possible, as the corneas perforated on enucleation. The eyes ($n = 1$ to 2 per group at each time point) were immediately enucleated and fixed in 2% glutaraldehyde in 80 mM sodium cacodylate (pH 7.4) at 4°C for at least 24 hours. After fixation, the corneas were isolated and washed three times with 0.1 M sodium cacodylate buffer then dehydrated in graded xylene and ethanol solutions before being embedded in paraffin. Five-micrometer-thick sections were cut and stained with Harris' hematoxylin and eosin and viewed by light microscopy for the presence of infiltrating leukocytes, epithelial defects, and stromal melting.

Viable Bacterial Counts

Corneas of control and challenged eyes of five mice per group (WT or KO mice) were harvested at 1, 3, 7, 14, and 21 days PI and homogenized on ice in 1 mL of sterile PBS at a pH of 7.4, as previously described by Cole et al.⁴² For the quantitation of viable bacteria, a 100- μ L aliquot from the homogenate was serially diluted (10 fold dilutions) in sterile PBS. Duplicate aliquots (20 μ L) of each dilution, including the original homogenate, were plated onto nutrient broth agar. Plates were incubated for 14 to 16 hours at 37°C and the number of colonies counted. The mean cfu is expressed as log₁₀ number per cornea $\pm$ SEM. Data were examined statistically by using an unpaired Student's *t*-test, with *P* < 0.05 being considered significant.

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity was determined by a method previously reported by Cole et al.,⁴² using the homogenate prepared as just described. Hexadecyltrimethylammonium bromide (final concentration of 0.5% wt/vol in 50 mM phosphate buffer [pH 6.0]) was added to 90 μ L homogenate. Samples were sonicated for 10 seconds in an ice bath and then subjected to three freeze-thaw cycles before centrifugation at 8000g for 20 minutes at 4°C. Ten-microliter aliquots of the resulting supernatants were pipetted in triplicate into a flat-bottomed microtiter plate, and the enzymatic reaction was started by the addition of 90 μ L 0.0167% (wt/vol) *o*-dianisidine dihydrochloride and 0.002% (vol/vol) H₂O₂ in PBS. The change in absorbance was continuously monitored for 30 minutes at 450 nm with a plate reader and compared to a standard curve on the same plate. The standard curve was prepared from purified MPO (Calbiochem, San Diego, CA). Results are expressed as relative units of MPO activity $\pm$ SEM (1 unit is proportional to 2×10^5 infiltrating neutrophils)⁴³ per cornea. Data were compared statistically by unpaired Student's *t*-test with values of *P* < 0.05 being considered significant.

Enzyme-Linked Immunosorbent Assay

Homogenates prepared as described earlier were centrifuged at 4000g for 20 minutes at 4°C, and the resultant supernatants were immediately frozen at -80°C until required for assay. IL-1 β , IL-6, TNF- α , MIP-2, KC, and VEGF levels in supernatant samples were measured in triplicate for each sample using specific ELISA kits (R&D Systems) as per the manufacturer's instructions. The data were analyzed by Student's *t*-test with values of *P* < 0.05 being considered significant.

Results

PCR Genotyping

PCR was performed on genomic DNA isolated from mouse tails to detect the expression of the *Cnlp* gene (WT) or the *Neo* gene (KO). For comparison, PCR products were also generated using genomic DNA from HZ to show the expression of both the CRAMP and Neo genes. Figure 1 demonstrates that the genomic PCR products were amplified as expected in the appropriate groups of mice.

Clinical Examination

Mice ($n = 6$ per group) were examined at 1, 3, 7, 14, and 21 days PI with a slit lamp biomicroscope to grade the severity of PA infection. The typical appearance of the corneal disease response in two cathelicidin-deficient KO mice and WT mice is shown in Figure 2. The mean clinical scores of both WT and KO mice are shown in Figure 3. Corneas of both WT and KO mice inoculated with PA (ATCC 19660) at day 1 postinfection (PI) showed isolated focal infiltrates in the central cornea, a paracentral stromal ring infiltrate or a dense opacity partially covering the cornea. Eyes from the WT group showed mild to moderate anterior chamber inflammatory reaction, whereas those in the KO exhibited a severe inflammatory response. The mean clinical score for the WT mice at this time was 2.33 ± 0.27 and for the KO mice was 2.83 ± 0.25 . Mean clinical scores for the infected corneas of WT and KO mice were not significantly different at 1 day PI. At 3 days PI, the corneal response appeared more severe in the KO mice (mean clinical score, 3.83 ± 0.25) than in the WT mice (mean clinical score, 3.00 ± 0.21) although this did not reach statistical significance. Dense cellular infiltration and edema of the cornea in KO mice became more generalized and extended to the periphery with mild conjunctival injection. Moderate-to-severe anterior chamber responses were observed in the infected eyes of both strains of mice, as indicated by the presence of cells, flare, and hypopyon. At day 7 PI, the responses of the corneas of the KO mice (mean clinical score, 4.33 ± 0.17) were significantly more severe than those observed in the WT mice (mean clinical score, 2.83 ± 0.25). Whereas the corneal disease appeared slightly improved in the WT mice from that observed at day 3 PI, the infected corneas in KO mice worsened and became markedly more edematous, with large and central leukocytic infiltrates covering nearly the entire cornea. Peripheral corneal neovascularization and severe conjunctival injection were also observed in the infected eyes of the KO mice. At 14 days PI the responses of the corneas in the WT and KO mice remained significantly different. KO (mean clinical score, 4.50 ± 0.28) animals showed increased thinning/melting of the central cornea leading to descemetocoele or keratocoele formation, and one of the six KO mice examined had progressed to perforation. In contrast, the infected WT corneas showed progression toward resolution of the infection with mean clinical score of 2.00 ± 0.30 . No anterior chamber response was noted in the WT mice by day 14. At 21 days PI the KO mice (mean clinical score, 4.50 ± 0.28) continued to show significantly more severe corneal response than that in the WT animals. A characteristic feature of the response of KO corneas (four of six mice) at this time was an extensive neovascularization that extended over approximately 90% of the diameter of the cornea. By day 21 PI, infected corneas of 80% of the WT mice (five of six mice) had recovered from the PA infection whereas five of the six infected corneas in the KO animals showed persistent infection and one progressed to perforation. Residual stromal scarring and a very mild amount of corneal neovascularization (notably markedly less than that in the KO animals) in the central cornea was observed in the WT mice, but no inflammation was observed throughout the corneas by day 21 PI. In the KO animals, dramatic corneal inflammation was observed during the course of PA infection and became progressively severe at the later time points. Mean clinical scores for the infected corneas of WT and KO mice were significantly different at days 7, 14, and 21 ($P < 0.05$) PI. Comparable results were seen in a separate round of infection experiments.

Histopathology

Histologic sections from the normal and PA-infected eyes of KO mice were examined and compared qualitatively to those from WT mice. Noninfected corneas from KO mice were structurally normal and comparable to those in the WT mice (Figs. 4G, 4H). Histologic examination of the infected corneas of WT and KO mice at 1 day PI showed localized neutrophilic infiltrate near the scratch site, and abundant infiltrating cells streaming through the limbus and conjunctiva into the central stroma (Figs. 5A, 5B). Although full-thickness epithelial defects were observed in both strains of mice, the epithelial loss in the KO mice appeared considerably more extensive in the central cornea. Infected KO animals also displayed mucopurulent exudates in the corneas and a markedly more pronounced inflammatory response in the anterior chamber (hypopyon, predominantly neutrophilic) than that in the WT mice. Massive inflammatory infiltration (composed predominantly of neutrophils) was observed throughout the infected cornea in the KO mice and to a much lesser extent in the WT mice (Figs. 4A, 4B). At 14 days PI, re-epithelialization of the cornea was evident, and the anterior chamber showed no sign of inflammatory cells in either strain of mice (Figs. 5C, 5D). The corneas of the WT mice exhibited recovery from the PA infection as shown by reduced edema and significant reduction (compared with that at 1 day PI) in the density of infiltrating neutrophils which were localized to the anterior stroma (Fig. 4C). Acellular spaces were observed in some parts of the corneal stroma as a result of extracellular matrix remodeling. Remnants of regressed blood vessels known as ghost vessels were also evident in the stromal tissue. In contrast at day 14 PI, KO mice showed dense and diffuse infiltration of neutrophils, severe stromal edema, destruction extending to the peripheral cornea, and neovascularization throughout the cornea (Fig. 4D). By day 21, the infected eyes in KO mice exhibited a very swollen and neovascularized cornea (Fig. 4F) progressing toward perforation, with infiltrating cells (mostly polymorphonuclear neutrophils [PMNs]; the MPO activity at this time point, as shown in Fig. 6, is approximately equal to 8×10^4 PMNs/cornea, and some macrophages) in the corneal stroma (Fig. 5F). On the contrary, eyes of the WT mice have recovered from PA infection and showed no sign of infiltrating cells in the cornea or anterior chamber, as demonstrated in Figure 4E and 5E. These histologic observations were consistent with the macroscopic clinical presentation and progression of PA corneal infection (Fig. 2).

Viable Bacteria Count

Viable bacterial counts (in cfu) per cornea were determined in the infected corneas of both WT and KO mice at 1, 3, 7, 14, and 21 days after PA challenge ($n = 5$ per group at each time point). As shown in Figure 7, no bacteria were observed in the noninfected control eyes, whereas the infected corneas in both WT and KO mice showed peak bacterial load at 3 days PI, which remained high up to 14 days and declined markedly at 21 days compared with 1 day PI. Approximately 1 to 1.5 log units cfu increase at days 1, 3, 7, and 14 PI, and almost 2 log units cfu increase at day 21 PI in bacterial load was detected in the infected eyes of KO mice when compared with those in the WT mice. The mean number of viable bacteria recovered from the infected corneas of KO mice was significantly higher than that of WT mice at all time points ($P < 0.05$). Identical results were obtained from a separate round of infection experiments.

Neutrophil Infiltration

The number of neutrophils was quantitated by measuring the relative MPO activity of the infected eyes in KO mice and compared with those in the WT mice at 1, 3, 7, 14, and 21 days after PA challenge ($n = 5$ per group at each time point). As shown in Figure 6, no MPO activity was found in homogenates of noninfected control corneas from CRAMP-deficient KO mice or WT mice, whereas high levels of MPO activity were observed in the corneas inoculated with PA in both WT and KO as early as 1 day PI (approximately 7.9×10^5 and 1.3×10^6 neutrophils, respectively). Infected corneas in both WT and KO mice showed peak MPO activity at 7 days PI and declined considerably at 21 days, compared with 1 day PI. Approximately a 2-unit increase in relative MPO activity at days 1, 3, and 7 PI and a 1-unit increase at day 14 PI was detected in the infected eyes of KO mice when compared with that in the WT mice. MPO activity was significantly elevated in the KO mice compared with WT mice at 1, 3, 7, and 14 days PI ($P < 0.05$). Histopathology (Figs. 4, 5) correlated qualitatively with MPO activity at 1 and 14 days PI. At day 21, very low MPO activity was detected in the WT mice (reaching a baseline level) compared with the noninfected control eyes, and there was no significant difference in the number of infiltrating neutrophils per infected cornea between KO and WT mice. Identical findings were observed in a separate round of infection experiments.

Corneal Chemokine and Cytokine Levels

ELISAs were performed to determine the levels of chemokines KC and MIP-2, the proinflammatory cytokines IL-1 β , IL-6, TNF- α , and VEGF in the infected corneas of WT and KO in response to PA challenge. As shown in Figure 8, IL-1 β and MIP-2 were not expressed, whereas very low constitutive levels of KC, TNF- α , IL-6, and VEGF were present in the normal noninfected corneas of WT and KO mice. As early as 1 day PI, high levels of all chemokines and cytokines examined were detected in the infected corneas of both groups of mice (Fig. 8). PA-inoculated corneas showed increase production in IL-1 β (Fig. 8A) in the KO and WT mice which reached peak levels at 1 and 3 days after the challenge, respectively, then began to decline thereafter. Significant elevation was observed in the production of IL-1 β ($P < 0.05$) in the infected corneas of the KO mice as compared with those of the WT mice throughout the course of the PA infection (up to 21 days). As shown in Figure 8B, MIP-2 protein expression in PA-inoculated corneas reached peak levels at 1 day PI. This expression returned to baseline level by 14 days in the WT mice and decreased to a very low level by 21 days PI in the KO mice. At days 1, 3, 7, and 14 PI, levels of MIP-2 in the corneas of KO mice were significantly higher than those in WT mice ($P < 0.05$). The levels of KC (Fig. 8C) reached a peak at 3 and 7 days PI in the WT and KO mice, respectively. During the course of PA infection, levels of KC in corneas of KO mice were significantly higher than those in WT mice ($P < 0.05$). Although the level of expression of KC declined drastically in the infected corneas of WT mice by day 14 PI, it remained high in those of the KO mice up to day 21 PI. KC expression did not return to near baseline levels until 21 day PI in the WT mice. The expression patterns of IL-1 β , MIP-2, and KC observed in the WT and KO mice paralleled the relative MPO activity (Fig. 6). The expression pattern of TNF- α (Fig. 8D) was similar to that observed for KC, reaching its peak at 3 and 7 days PI in the WT and KO mice, respectively. The level of expression of

TNF- α , however, did not return to near baseline levels by 21 day PI in the WT mice and was sustained at high levels in the KO mice up to day 21 PI. IL-6 and VEGF production patterns (Figs. 8E, 8F) observed in response to the PA infection in the WT and KO mouse corneas were different from those of the other four cytokines initially (1 and 3 days PI). The level of IL-6 was significantly lower in the infected corneas of the KO mice compared with those of the WT mice at 1 and 3 days PI ($P < 0.05$). At the later stage of infection, the level of IL-6 in the infected corneas of the WT mice declined markedly by day 14 and was reduced to baseline level by 21 days PI. On the contrary, significantly higher levels of IL-6 ($P < 0.05$) were detected in the infected corneas of the KO mice compared with those of the WT mice at 7, 14, and 21 days PI. The expression of VEGF (Fig. 8F) in PA-inoculated corneas of both WT and KO mice reached peak levels at 1 day. The upregulated expression of VEGF in the infected corneas of WT mice declined considerably by day 7 and approached baseline level by day 21 PI. On the contrary, significantly higher concentrations of VEGF were measured in the infected corneas of KO mice than in those of the WT mice at 7, 14, and 21 days PI. For all chemokines and cytokines tested, comparable results were observed in a separate round of infection experiments.

Discussion

In the present study, the inbred WT mouse strain (129/SvJ) was resistant to PA corneal infection, as these mice are able to resolve the infection and restore corneal integrity within 21 days PI. Similarly, resolution of PA keratitis has been observed by Hobden et al.⁴⁴ in a strain of mice created on a similar background (129/Sv) within 21 days. On the contrary, mutant mice deficient in CRAMP showed markedly greater severity of the corneal infection as reflected by failure to resolve by 21 days and considerable increase in viable bacterial counts when compared with the WT mice, suggesting that CRAMP may play a part in controlling PA replication in the infected cornea. This finding is in line with recently published data for CRAMP-deficient mice showing increased susceptibility in other microbial infection models.^{13,33–36,45} The present study is the first to demonstrate direct in vivo evidence that cathelicidin serves important roles in defending the ocular surface from infection, as the experimental PA keratitis in cathelicidin-deficient mice failed to resolve and progressed more rapidly to severe ulceration and, in some cases, perforation within 21 days.

Nizet et al.³³ have shown that, although leukocytes derived from CRAMP-deficient mice are functionally competent and similar to those obtained from the WT mice in terms of oxidative burst activity and recruitment or response to chemoattractants, they are deficient in direct bacteria-killing, indicating the importance of CRAMP in neutrophil action. Furthermore, Chromek et al.⁴⁵ have recently reported that epithelium-derived CRAMP contributed substantially to the antimicrobial properties of the urinary tract against infection in neutrophil-depleted mice. In the present study, KO mice had enhanced recruitment of neutrophils, but the absence of the peptide in neutrophils, macrophages, and epithelial cells clearly leads to defects in controlling PA infection. Indeed, higher levels of bacterial burden and delayed clearance of pathogens have also been reported in several studies in CRAMP-deficient murine models.^{33,34,36} In some cases, the KO animals showed very serious tissue damage secondary to uncontrolled infections, which was similarly observed during PA keratitis in the present study.

The clinical and pathologic responses to PA keratitis induced with the cytotoxic PA strain in our WT animals is comparable to that published previously by others showing an initial ring infiltrative response (at day 1 PI) and a dense stromal infiltrate underlying a full-thickness epithelial ulceration at the later stages of the disease.^{3,46,47} Histologic assessment at days 3 and 7 was not possible, as the corneas perforated on enucleation. In addition to the clinical signs observed in the WT animals, PA keratitis in the KO mice presented with a grossly edematous and ectatic central cornea with ensuing descemetocoele (keratocoele) formation at the end stage of the disease. It has been shown that corneal infection with PA can trigger an extensive host inflammatory response causing devastating tissue damage. With regard to initiation of these events, the release of the proinflammatory cytokines IL-1 β , IL-6, TNF- α and the chemokines MIP-2 and KC by corneal and infiltrating immune cells at the site of infection appears to be key in the regulation of the processes of inflammation, and in leukocyte recruitment, and such release contributes to tissue pathology and outcome of the disease.^{7,10} Therefore, we investigated the corneal expression patterns of these cytokines and chemokines in response to PA infection. The relative early and rapid upregulated expression of all cytokines and chemokines examined at the initial stage of the disease and the timely down-regulation of their expression at the later time points in the infected corneas of the WT mice found in the present study have also been noted by others and are comparable to previous findings in other known PA-resistant strains of mice, the BALB/c and 129/Sv.^{7,44,47-54} Whereas the responses in the infected corneas of WT animals returned to or approached baseline levels as measured in the fellow noninfected control corneas, the majority of the responses in the KO animals remained significantly elevated or at levels greater than the control corneas by 21 days after ocular challenge. Data from the present study suggest that the prolonged and elevated expression patterns of proinflammatory cytokines and chemokines observed in the KO mice, possibly due to increased bacterial load as a result of CRAMP deficiency, may have contributed to a more vigorous immune response than that necessary for containing the PA infection and maintenance of corneal integrity as seen in the WT mice, and therefore resulted in substantial corneal destruction.

The proinflammatory cytokine IL-1 β is known to be involved in cytokine/chemokine production, activation and regulation of leukocytes in the innate and adaptive systems, and induction of neovascularization.⁵ Rudner et al.⁵⁵ have reported detection of significantly greater amounts of IL-1 β in the infected corneas of susceptible (C57BL/6) vs. resistant (BALB/c) mice and concluded that excess amounts of this cytokine can result in poor disease outcome. In the present study, a significant upregulation in IL-1 β expression was observed in the infected corneas of KO mice compared with those of WT mice, and this increase may have contributed to irreversible tissue damage. The chemokines MIP-2 and KC have been implicated as functional mouse homologues of the human chemokine IL-8, a potent chemoattractant and activator of neutrophils.^{10,56-58} Although both MIP-2 and KC are known to facilitate recruitment of neutrophils into the cornea, previous studies suggest that MIP-2 has the predominant role.^{52,55,59} In the present study, both MIP-2 and KC were found in greater amounts in the infected corneas of KO mice than in those of WT mice, and the expression pattern of IL-1 β , MIP-2, and KC paralleled the relative number of neutrophils found. These findings suggest that the higher number of neutrophils present in the infected

corneas of the KO mice throughout the infection probably resulted from the elevated levels of these cytokines and chemokines.

Previously, it has been reported that corneal epithelium, keratocytes, neutrophils and macrophages are the major sources of TNF- α in PA keratitis.^{10,60} The kinetics of TNF- α expression in the infected corneas of WT mice found in the present study were analogous to those reported by Hobden et al.⁴⁴ TNF- α has been demonstrated to induce corneal angiogenesis,⁶¹ and, notably, in our study significantly upregulated levels of this cytokine were seen in the infected corneas of KO mice compared with those of WT mice, particularly toward the end stage of the infection, a time when prominent corneal neovascularization was observed in this group of mice. IL-6 is known to be produced by activated macrophages, lymphocytes, and resident cells in the cornea and has been shown to share overlapping activities with IL-1 β and TNF- α .^{53,62} Cole et al.⁶³ have demonstrated that the presence of IL-6 is required for neutrophil recruitment into the central cornea. In the present study, the kinetics of IL-6 production observed in the PA-infected corneas of the WT mice were comparable to those observed in other resistant mouse strains.^{7,53} A recent study reported that IL-6 produced during herpes simplex virus keratitis can stimulate resident corneal cells and inflammatory cells, including neutrophils and macrophages to secrete VEGF, a proangiogenic mediator, in a paracrine manner.⁶⁴ In keeping with this, we have found that elevated levels of IL-6 expression in the infected corneas of the KO mice correlate with the increased production in VEGF during the course of PA keratitis.

Angiogenesis or neovascularization is a prominent early feature of PA infection in the cornea. It has been noted that expression of VEGF is upregulated in inflamed, infected, and vascularized corneas in humans and in animal models.⁸ The temporal pattern of VEGF expression in the infected corneas in the WT animals seen in the present study appeared similar to that reported by Xue et al.⁵¹ for the BALB/c resistant strain. The fact that the deficiency of CRAMP did not inhibit, but rather enhanced neovascularization during PA keratitis in vivo, indicates that mediators other than CRAMP are involved in stimulating angiogenic activity in the cornea and that CRAMP may play inhibitory roles in corneal angiogenesis during PA infection. Koczulla et al.³⁹ have recently documented that the human cathelicidin LL-37 can act as a potent inducer of angiogenesis and arteriogenesis; however, the angiogenic effect of the peptide is VEGF independent. Although Koczulla et al. have also reported that CRAMP is necessary for angiogenesis and arteriogenesis, the prerequisite processes for normal wound vascularization in the skin, data from the present study indicated that CRAMP does not play a role in stimulating neovascularization in an avascular and transparent tissue such as the cornea. Further, the vessel growth observed in the KO mice could have been initiated by the higher number of infiltrating neutrophils and possibly macrophages, as these cells contain significant amounts of angiogenic mediators that can be released in response to cellular activation.^{65–67} Notably, the ability of cytokines to induce other cytokines and pro- and/or antiangiogenic factors secondarily and function as chemotactic factors for inflammatory cells makes it difficult to sort out the final and critical mediators of angiogenesis, infiltration, and inflammation conclusively in vivo.

In the cornea, epithelial cells are a source of various antimicrobial peptides (including CRAMP, data not shown), particularly after injury or in response to inflammatory

stimuli.^{19,68–70} Furthermore, infiltrating neutrophils and macrophages present in the cornea as a result of microbial challenge also play an important role in limiting bacterial invasion by the production of certain peptides including the cathelicidins. Findings from the present study showed that functional activities of cathelicidin are necessary for bacterial clearance and innate corneal immunity at the ocular surface. This observation, combined with previous studies^{13,33–36,45} showing the effects of cathelicidin deficiency in other tissues have better defined the function of this family of antimicrobial peptides in infectious disease.

We have shown that, in addition to being antimicrobial, the human cathelicidin LL-37 stimulates corneal epithelial cell migration and cytokine and chemokine production *in vitro*; thus, we have speculated that cathelicidin may have multifunctional roles at the ocular surface.¹⁹ Data from the present study indicate that cathelicidin is unlikely to make a direct contribution to stimulating cytokine and chemokine secretion in experimental keratitis as the levels of these inflammatory mediators were generally equivalent or higher in KO mice than in WT mice. Rather, the data suggest that the major contributory role of cathelicidin in this model is as an antimicrobial peptide. It should be noted that in addition to a direct ability to kill bacteria, cathelicidin (LL-37) has also been shown to neutralize endotoxin and block activation of toll-like receptors,^{71–75}; thus, all these factors probably contribute to the overall antimicrobial effect of the peptide. It remains to be determined whether the *in vitro* effects of LL-37 we have seen¹⁹ are meaningful in terms of corneal epithelial wound healing *in vivo*.

Previous studies have established that mice can be categorized as susceptible (cornea perforates) to PA infection, which is mediated by a Th1 response, or resistant (cornea heals), which is mediated by a Th2 response.⁷ As the WT mice in our study showed recovery from PA infection, we presume (but did not confirm this experimentally) them to fall in to the category of the Th2 responders. A recent study by Huang et al.⁷⁶ showed that deficiency of toll-like receptor 4 resulted in resistant BALB/c mice becoming susceptible to PA keratitis. Notably, this was accompanied by an increase in type-1-associated cytokines. These observations raise the possibility that, in our experiments, CRAMP deficiency resulted in a switch from a Th2 to a Th1 response. Cathelicidin (LL-37) has been shown to exert a variety of influences on immune cells^{71,72,77–79}; however, current evidence suggests that it drives a Th1 response. Thus, future studies are needed, to address the effect of CRAMP deficiency on the immune response and particularly cytokine production in PA keratitis.

In summary, mice deficient in CRAMP are phenotypically different from WT mice in disease progression in PA keratitis, showing delayed bacterial clearance due to lack of CRAMP, persistent elevation in the number of circulating neutrophils, and differential expression patterns of infection/inflammation-induced cytokines and chemokines released by cells in the cornea, which include the resident corneal epithelial and stromal cells as well as the infiltrating leukocytes. These contributing factors augment or prolong the inflammatory response that ultimately results in permanent corneal tissue damage and in some cases perforation as the net effect in the experimental PA keratitis model of our study. Collectively, findings from the present study highlight the importance of cathelicidin as an integral element in the host innate immune defense protecting against corneal infection caused by PA at the ocular surface.

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Figure 1.

PCR genotyping of tail genomic DNA from CRAMP WT and KO mice. Genomic PCR products were amplified in the wild-type (WT, *Cnlp*^{+/+}), heterozygous (HZ, *Cnlp*^{+/-}), and CRAMP knockout (KO, *Cnlp*^{-/-}) mice. *Cnlp*, CRAMP gene; *Neo*, KO gene.

Figure 2.

Clinical examination of mouse corneas inoculated with PA (ATCC 19660). Digital photographs of the noninfected (control) and infected corneas in the wild-type (WT) and knockout (KO) mice at days 1, 3, 7, 14, and 21 PI. Shown are representative images of corneas from two different mice per group at each time point from one representative experiment. Magnification, $\times 40$.

Figure 3.

Clinical scores of corneal disease responses. Clinical scores were obtained from the infected corneas of the CRAMP-deficient KO mice and WT mice at 1, 3, 7, 14 and 21 days PI. Data are expressed as mean clinical score $\pm$ SEM of six mice per group at each time point. The graph shows representative data from one experiment. (* $P < 0.05$, KO versus WT mice).

Figure 4.

Histopathology of PA-infected mouse corneas. The figure shows corneal sections from WT (A, 1 day PI; C, 14 days PI; E, 21 days PI), KO (B, 1 day PI; D, 14 days PI; F, 21 days PI) mice, and noninfected eyes (control) of WT (G) and KO (H) mice. Ep, epithelium; S, stroma; En, endothelium; NV, new blood vessels; GV, ghost vessels; Sp, space. Magnification, $\times 400$.

Figure 5.

Histologic examination of mouse corneas inoculated with PA (ATCC 19660). Light microscopic histopathology of PA-infected corneas from WT (A, 1 day PI; C, 14 day PI; E, 21 day PI) and KO (B, 1 day PI; D, 14 day PI; F, 21 day PI) mice. (F) The only one of six corneas at day 21 PI that did not proceed to perforation and hence could be processed for histologic analysis. Magnification, $\times 40$.

Figure 6.

Relative MPO activity in the infected corneas. Relative MPO activity determined from the infected corneas of the CRAMP-deficient KO mice and WT mice at 1, 3, 7, 14, and 21 days PI. Data shown are expressed as relative MPO activity units per cornea $\pm$ SEM of five mice per group at each time point. At 1, 3, 7, and 14 days PI, significantly greater MPO activity was present in the corneas of the KO than the WT mice. At 21 days PI, very low MPO activity was detected in homogenates of corneas from WT mice. No significant differences were observed at 21 days PI in the relative MPO activity per infected cornea between KO and WT mice. Data are representative of results in one experiment. C, noninfected control corneas. * $P < 0.05$, KO versus WT mice.

Figure 7.

The amount of viable PA in the infected corneas. Viable bacterial counts from the infected corneas of the CRAMP-deficient KO mice and WT mice at 1, 3, 7, 14 and 21 days PI. The number of cfus per cornea at various times PI is expressed as $\log_{10} \pm \text{SEM}$. At 1, 3, 7, 14, and 21 days PI, significantly more viable bacteria were recovered from the corneas of KO than WT mice ($n = 5$ per group per time point). Data are representative of results from one experiment. C, noninfected control corneas. * $P < 0.05$, KO versus WT mice.

Figure 8.

Cytokine and chemokine protein expression in the infected corneas. ELISA was performed to measure the level of IL-1 β (A), MIP-2 (B), KC (C), TNF- α (D), IL-6 (E), and VEGF (F) in the infected corneas of WT and KO mice. Results are expressed as picograms of cytokine per milligram of tissue protein $\pm$ SEM of five mice per group at each time point. Data are representative results from one experiment repeated two times, each performed in triplicate. C, noninfected control corneas. * $P < 0.05$, KO versus WT mice.