Skin Wound Healing Is Accelerated by a Lipid Mixture Representing Major Lipid Components of Chamaecyparis obtusa Plant Extract

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In chronic nonhealing wounds, the healing process is disrupted and wounds are often infected with bacteria. About 85% of lower extremity amputations in diabetes are attributed to deep infection of foot ulcers. Therefore, infection control is critical for wound care. In this study, we analyzed lipid composition of Chamaecyparis obtusa extract, and we describe the wound-healing properties of its combination of 10 major lipid components. A 10-lipid mixture up-regulated HBD-3 and LL-37 through the olfactory receptor 2AT4 and induced phosphorylation of extracellular signal-regulated kinases and p38 mitogen-activated protein kinases in primary human keratinocytes. In addition, the 10-lipid mixture had direct bactericidal effects against Staphylococcus aureus and Streptococcus pyogenes and protected against staphylococcal α-toxin–induced keratinocyte cell death. In an animal model, the 10-lipid mixture accelerated skin wound healing and was also effective in healing wounds superinfected with S. aureus. We suggest that the 10-lipid mixture, because of its wound-healing and antimicrobial properties, can be beneficial for wound treatment.


INTRODUCTION
Chronic nonhealing wounds such as pressure ulcers, venous stasis ulcers, and diabetic wounds are a major health burden (Sen et al., 2009). The prevalence of chronic nonhealing wounds is about 2% in the general population (Sen et al., 2009), and over $25 billion is spent annually in the United States for the medical management of nonhealing wounds (Brem et al., 2007; Driver et al., 2010). In chronic wound infections with bacteria, Staphylococcus aureus often disrupts the wound-healing process (Apelqvist and Larsson, 2000; Creager et al., 2003; Robson, 1997; Vuorisalo et al., 2009). Therefore, infection control in chronic wounds, particularly those infected with antibiotic-resistant bacteria, is a major management concern (Apelqvist and Larsson, 2000; Falanga, 2004; Robson, 1997). To date, several medications have been developed for treatment of chronic pressure ulcers and diabetic wounds, but most of them have proven ineffective, except for PDGF (Shah et al., 2012; Tecilazich et al., 2013). However, it has been reported that PDGF increases the risk for cancer development and is ineffective for chronic wounds (Ma et al., 2015; Park et al., 2014; Tecilazich et al., 2013). Therefore, new medicines for chronic nonhealing wounds are needed.

Antimicrobial peptides (AMPs) produced in the epidermis, such as HBD-3 (Hirsch et al., 2009) and LL-37 (Nakatsuji and Gallo, 2012), play critical roles in the wound healing process. HBD-3 and LL-37 kill S. aureus, stimulate re-epithelialization, and induce angiogenesis (Heilborn et al., 2003; Hirsch et al., 2009; Kiatsurayanon et al., 2014; Koczulla et al., 2003; Nakatsuji and Gallo, 2012; Niyonsaba et al., 2007). Recently, it also has been reported that olfactory receptors (ORs) may support re-epithelialization and skin wound healing (Busse et al., 2014; Tsai et al., 2017). Stimulation of OR2AT4 with an odorant ligand positively affected keratinocyte proliferation, migration, and regeneration (Busse et al., 2014).

Beneficial effects of plant extracts on wound healing and skin barrier function in skin disorders have been documented (Mantle et al., 2001; Reuter et al., 2010). Researchers have shown that whole plant oils from Chamaecyparis obtusa have anti-inflammatory effects and are not cytotoxic (An et al., 2013; Chien et al., 2014). However, bactericidal activities and wound-healing properties of the C. obtusa extract have not been evaluated. Plant extracts may have lot-to-lot variations in their biological activity due to the differences in concentration of the bioactive components in these extracts. In a preliminary study, we examined the protein, carbohydrate, and lipid...
fractions of *C. obtusa* to establish which fraction(s) was/were the most effective in the induction of AMPs, had bactericidal effects against *S. aureus*, and promoted skin wound healing. We found that the lipid fraction of *C. obtusa* (see Supplementary Table S1 online) had all of these attributes. The 10 main lipids of the *C. obtusa* lipid extract were selected for this study (see Supplementary Table S2 online).

**RESULTS**

**A 10-lipid mixture, a mimic of the 10 major lipids of *C. obtusa* plant extract, induces AMPs and OR2AT4 in primary human keratinocytes**

The preparation of a 10-lipid mixture is detailed in the Methods section. We examined whether the mixture of 10 major lipids can regulate the expression of AMPs and ORs. HBD-3 mRNA expression was significantly induced in primary human keratinocytes treated with 0.01% and 0.02% solutions of the 10-lipid mixture compared with keratinocytes grown only in culture medium (Figure 1a). Similarly, the gene expressions of LL-37 (Figure 1b) and OR2AT4 (Figure 1c) were significantly induced in keratinocytes by the 10-lipid mixture. Immunodot-blot assay confirmed that the mixture of 10 lipids induces HBD-3 (Figure 1d) and LL-37 (Figure 1e) protein production by keratinocytes. Various concentrations of the 10-lipid mixture were nontoxic to primary human keratinocyte culture (see Supplementary Figure S1 online). Additionally, we found that the 10-lipid mixture accelerated cell migration (see Supplementary Figure S2a and b online).

HBD-3 and LL-37 are induced by the 10-lipid mixture through OR2AT4

Because some of the 10 lipids are aromatic, we investigated whether these lipids induced HBD-3 and LL-37 via OR2AT4 using small interfering RNA (siRNA) technique. β-pinene (0.02%) was used as a vehicle, because it did not induce OR2AT4, HBD-3, or LL-37 in human primary keratinocytes (data not shown). The specificity of OR2AT4 siRNA was confirmed because expressions of OR2AT4 mRNA
Figure 2. HBD-3 and LL-37 are induced by the 10-lipid mixture through OR2AT4. Gene expressions of (a) OR2AT4, (b) HBD-3, and (c) LL-37 were evaluated by real-time reverse transcriptase—PCR. Cells were fixed and stained with antibodies for (d) OR2AT4 (red), (e) HBD-3 (red), and (f) LL-37 (red). Nuclei were visualized with DAPI (blue), and wheat germ agglutinin-conjugated FITC stained the cytoskeleton (green). Original magnification ×250. Scale bar = 50 μm. The mean fluorescent intensities of (g) OR2AT4, (h) HBD-3, and (i) LL-37 are shown. (j) Western blot analysis for phosphorylated MAPK activation in human primary keratinocytes after stimulation with 0.02% of the 10-lipid mixture. The data are shown as the mean ± standard error. n = 3. siRNA, small interfering RNA.
and protein were significantly decreased in keratinocytes transfected with OR2AT4 siRNA compared with keratinocytes transfected with a control siRNA using real-time reverse transcriptase–PCR and Western blotting (see Supplementary Figure S3a and b online).

Gene expression of OR2AT4 was significantly decreased in keratinocytes transfected with OR2AT4 siRNA and induced by a 0.02% solution of the 10-lipid mixture (Figure 2a). The 10-lipid mixture did not induce OR2AT4 mRNA in keratinocytes transfected with OR2AT4 siRNA compared with cells transfected with control siRNA (Figure 2a). Interestingly, the induction of HBD-3 (Figure 2b) and LL-37 (Figure 2c) by the 0.02% solution of the 10-lipid mixture was significantly inhibited in keratinocytes transfected with OR2AT4 siRNA compared with cells transfected with control siRNA. Immunofluorescence staining confirmed these findings (Figure 2d–i). These data suggest that the 10-lipid mixture induced HBD-3 and LL-37 after engagement of OR2AT4.

Additionally, we showed that the 10-lipid mixture induced phosphorylation of ERK1 and 2 and p38 MAPKs in keratinocytes (Figure 2j).

The 10-lipid mixture has direct bactericidal effects and protects α-toxin–induced cell death

Elimination of bacteria is an important treatment strategy for wound care (Falanga, 2004; Robson, 1997; White and Cutting, 2006). We examined whether the 10-lipid mixture had bactericidal activity against methicillin-sensitive S. aureus (MSSA), methicillin-resistant S. aureus (MRSA), Streptococcus pyogenes, and Pseudomonas aeruginosa using a bactericidal assay. MSSA, MRSA, and S. pyogenes were killed by as low as 0.01% solution of the 10-lipid mixture (Figures 3a, b, and c, respectively). However, P. aeruginosa was resistant to the effects of the 10-lipid mixture (Figure 3d).

S. aureus α-toxin is the major S. aureus virulence factor involved in cytotoxicity (Brauweiler et al., 2014), and its effects are detrimental to skin wounds (Tkaczyk et al., 2013). We found that keratinocyte pretreatment with the mixture of 10 lipids blocks α-toxin–induced keratinocyte death using lactate dehydrogenase assay (see Supplementary Figure S4 online).

Wound healing is enhanced by a 10-lipid mixture in mice

To extend our findings in primary human keratinocytes that the 10-lipid mixture induces AMP production, we examined the effects of the 10-lipid mixture on AMP production in mouse skin. Mouse skin was treated with a vehicle or 0.02% of the 10-lipid mixture three times a day for 3 days. Significantly increased MBD-2, which is the mouse ortholog of HBD-3, and CRAMP production were observed in mouse skin treated with the 10-lipid mixture (see Supplementary Figure S5a–d).

These findings prompted us to investigate whether the 10-lipid mixture can enhance skin wound healing in an animal model. Wounds were inflicted using 4-mm skin punch biopsies on the back of hairless mice. Vehicle or 0.02% solution of the 10-lipid mixture were applied three times a day for 10 days. 0.02% solution of β-pinene was used as a vehicle in these experiments. Vehicle or the 10-lipid mixture did not cause any rash or edema in the skin. Wound sizes are shown in Figure 4a, and the composite data are presented in Figure 4b–d. Importantly, the wound sizes...
on days 5 (Figure 4c) and 10 (Figure 4d) were significantly reduced in wounds treated with the 10-lipid mixture compared with wounds treated with vehicle. The levels of MBD-2 expression in wounds were not significantly different between these treatments (Figure 4e and g). However, CRAMP expression was significantly higher in wounds treated with the 10-lipid mixture compared with wounds treated with vehicle (Figure 4f and h).

The mixture of 10 lipids regulates the expression of proinflammatory mediators in skin wounds of hairless mice

Several studies have reported that the expression of TNF-α, IL-1α, IL-6, and MMP-9 is increased in nonhealing wounds but is decreased in healing wounds (American Diabetes Association, 1999; Shah et al., 2012; Zoller et al., 2014). Moreover, IL-10 (Peranteau et al., 2008; Wise et al., 2014) and ORs (Busse et al., 2014; Tsai et al., 2017) are critical in wound healing. Therefore, we took skin biopsy samples from wounded areas treated with vehicle or the 10-lipid mixture on day 10 to examine the expression of these biomarkers. Gene expressions of TNF-α (Figure 5a), IL-1α (Figure 5b), and IL-6 (Figure 5c) were significantly decreased in the wounds treated with 0.02% solution of the 10-lipid mixture compared with wounds treated with vehicle. In contrast, mRNA levels of IL-10 and olfactory receptor 520 (Olfr520), which is the murine ortholog of OR2AT4, were significantly increased in wounds treated with the 10-lipid mixture (Figure 5d and e, respectively). No significant difference was noted in MMP-9 expression (Figure 5f).

In addition, it was reported that the expression of filaggrin is down-regulated in nonhealing wounds (Cheng et al., 2013) but is up-regulated in healing wounds (Jang et al., 2012). We found that FLG gene expression was significantly increased in wounds treated with 0.02% solution of the 10-lipid mixture compared with wounds treated with vehicle only (Figure 5g).

The 10-lipid mixture accelerated wound healing in wounds infected with S. aureus

We further tested whether the 10-lipid mixture can support healing of the wounds infected by S. aureus. Wounds were instilled on the back of hairless mice as described and inoculated with 1 × 10^6 MRSA on day 0. Vehicle or the 10-lipid mixture was then applied to the wounds three times a day for 10 days. Wound sizes are shown in Figure 6a, and the composite data for all samples is summarized in Figure 6b—d. Wound sizes on day 5 were not significantly different among wounds (Figure 6c). However, wound sizes on day 10 were significantly reduced by the 10-lipid mixture treatment compared with vehicle treatment (Figure 6d). These findings were confirmed by the hematoxylin and eosin staining of the wounded area biopsy samples. As shown in Figure 6e, wound sizes were decreased in wounds treated with 0.02% of the 10-lipid mixture compared with wounds treated with vehicle. Importantly, re-epithelialization rate (Figure 6f) was significantly increased, and wound width (Figure 6g) was significantly smaller in wounds treated with the 10-lipid mixture compared with wounds treated with vehicle.

S. aureus abundance was measured by immunostaining and PCR in skin biopsy samples on days 5 and 10 of the
The staining intensity of *S. aureus* in biopsy samples from day 5 was significantly lower in wounds treated with 0.02% solution of the 10-lipid mixture compared with wounds treated with vehicle (see Supplementary Figure S6a and b online). Additionally, protein expressions of MBD-2 (see Supplementary Figure S6c and d) and CRAMP (see Supplementary Figure S6e and f) on day 5 were significantly higher in wounds treated with 0.02% solution of the 10-lipid mixture compared with wounds treated with vehicle. Moreover, *S. aureus* in biopsy samples from day 10 was detected in three out of eight wounds treated with vehicle but were not detected in any of the wounds treated with the 10-lipid mixture (see Supplementary Figure S6g). We further examined protein levels of AMPs in wounds from day 10. Protein expression of MBD-2 on day 10 was not different in wounds treated with vehicle or the 10-lipid mixture (Figure 6h and j). In contrast, CRAMP expression on day 10 was significantly higher in wounds treated with 0.02% solution of 10-lipid mixture compared with wounds treated with vehicle (Figure 6i and k).

**DISCUSSION**

In this study, we showed that a mixture of 10 lipids, major components of lipids from *C. obtusa*, induced biomarkers
Figure 6. Wound healing is enhanced by the 10-lipid mixture in wounds with bacterial infection. (a) Representative photos and (b–d) the composite data are shown. Scale bars = 4 mm. Wounds from day 10 were stained with hematoxylin and eosin. (e) Representative hematoxylin and eosin-stained sections of wounds. The composite data for (f) re-epithelialization rate and (g) wound width in all wounds are shown. Black arrows indicate wound margins. Values are given as mean ± standard error. n = 8. Scale bars = 200 μm. Expressions of (h) MBD-2 (red) and (i) CRAMP (red) in wounds were examined. Scale bars = 50 μm. Wheat germ agglutinin-conjugated FITC (green) stained the cytoskeleton. Arrows point to MBD-2 or CRAMP. The mean fluorescence intensity of (j) MBD-2 and (k) CRAMP are shown. MRSA, methicillin-resistant *Staphylococcus aureus*. 
associated with wound healing and enhanced skin wound healing in mouse models. Earlier studies have documented that organic compounds from *C. obtusa* inhibit the development of atopic dermatitis-like skin lesions and suppress IL-1 and IL-6 in a mouse model (Yang et al., 2015a).

Lipids in the 10-lipid mixture used in this study belong to the following phytochemical families: terpenes (terpinyl acetate, guaiol, elemol, sabinene, thujoepene, totarol, β-pinene, and cembrene) and fatty acids: (palmitic acid and 9-octadecenamide). The components of *C. obtusa* lipid extract including terpinyl acetate (Devkota et al., 2008; Rudolf et al., 2017; Zhang et al., 2016), sabinene (Vimal et al., 2017), guaiol (Choudhary et al., 2007), cembrene (Chen et al., 2009), and totarol (Kubo et al., 1992) have been shown to have antibacterial activities. Guaiol (Liu et al., 2013) and β-pinene (da Silva et al., 2012) also have been shown to have antifungal activities. Additionally, other components of *C. obtusa* have been reported to have immunomodulatory activities. For example, elemol (Yang et al., 2015b) and thujoepene (Kim et al., 2013) decreased mast cell infiltration and IL-4 production. Palmitic acid has been shown to induce dendritic cell secretion of IL-1β (Nicholas et al., 2017) and activated production of IL-6, TNF-α (Zhou et al., 2013), and IL-8 (Ohtsu et al., 2017) by epithelial cells; 9-octadecenamide was suggested to have anti-inflammatory activities (Chen et al., 2011). It was also reported that terpenoid compounds may cause contact dermatitis (Mobacken and Fregert, 1975; Picman and Picman, 1990).

AMPs such as HBD-3 and LL-37 have polymorphic functions that not only kill microbes, such as *S. aureus*, but also regulate epidermal barrier, induce cell proliferation and migration, and stimulate re-epithelialization and angiogenesis (Hirsch et al., 2009; Mijalovic et al., 2010; Nakatsuji and Gallo, 2012). Additionally, HBD-3 (Gibson et al., 2012; Hirsch et al., 2009) and LL-37 (Carretero et al., 2008) have been tried as therapeutic agents for wound care. In our study, we showed that the 10-lipid mixture induced HBD-3 and LL-37 in both primary human keratinocytes and mouse skin and accelerated cell migration. Because the components of the 10-lipid mixture are aromatic and the engagement of one of the olfactory receptors, OR2AT4, by its odorant ligand was found to regulate skin wound healing (Busse et al., 2014), we examined whether the effects of the 10-lipid mixture in keratinocytes were mediated through OR2AT4. We found that the 10-lipid mixture increased OR2AT4 mRNA and protein expression. We also showed that the induction of HBD-3 and LL-37 by the 10-lipid mixture was inhibited by OR2AT4 siRNA. These data suggest that AMP production in response to the 10-lipid mixture is regulated by OR2AT4.

Of the approximately 400 intact human odorant receptors, only about 10% have known ligands. To date it is believed that some ORs respond to a large number of odorants, which are structurally diverse, whereas other ORs are more narrowly tuned, and some respond to only a small number of closely related odorants (Mainland et al., 2014, 2015; Malnic et al., 2004). It is possible that only certain lipids in the 10-lipid mixture engage OR2AT4, and other odorant lipids in this mixture interact with additional ORs expressed by keratinocytes. Our data point out that ORs may be involved in regulation of AMPs. Of interest, OR2AT4 is among very few dysregulated genes found to be suppressed in diabetic skin in comparative genomic analyses between human normal foot skin and intact diabetic foot skin (Ramirez et al., 2015). Regulation of OR2AT4 expression by the *C. obtusa* 10-lipid mixture and involvement of OR2AT4 in the production of AMPs in response to this lipid mixture shown in our study provide insights into potential therapeutic use of the *C. obtusa* lipid mixture.

*S. aureus* is commonly present in chronic wounds such as diabetic wounds (Apelqvist and Larsson, 2000; Lipsky et al., 2012; Roberts and Simon, 2012) and complicates wound healing (Apelqvist and Larsson, 2000; Creager et al., 2003; Vuorisalo et al., 2009). Additionally, α-toxin from *S. aureus* induces keratinocyte cell death (Brauweiler et al., 2014). Therefore, control of staphylococcal infection and the actions of its cytolysins are a critical strategy for wound management. Our current data show that the 10-lipid mixture effectively killed MSSA, MRSA, and *S. pyogenes* even at 0.01% solution of the 10-lipid mixture. This concentration of the 10-lipid mixture was not toxic to the keratinocytes. Furthermore, pretreatment with this lipid mixture protected primary human keratinocytes from the staphylococcal α-toxin—induced cell death. However, *P. aeruginosa* was not killed by 0.02% of the lipid mixture.

Given the induction of the AMPs and bactericidal effects of the 10-lipid mixture, we have examined its skin wound-healing effects. We have shown accelerated wound healing by the studied lipid mixture. In addition, this lipid mixture enhanced healing of the skin wounds that were infected with MRSA. MRSA was significantly inhibited in wounds treated with the 10-lipid mixture. The exact mechanisms of antibacterial activities by the 10-lipid mixture have not been elucidated in this study. However, given direct bactericidal effects of the 10-lipid mixture against *S. aureus* in vitro and induction of MBD-2 and CRAMP in infected wounds, we suggest that the 10-lipid mixture inhibited MRSA in wounds by direct killing and induction of AMPs.

In addition, we also showed that TNF-α, IL-1α, and IL-6 were down-regulated by the 10-lipid mixture in mouse skin. Inhibition of the expression of these cytokines was previously reported in association with wound healing (American Diabetes Association, 1999; Shah et al., 2012; Zoller et al., 2014). Overexpression of IL-10 (Peranteau et al., 2008; Wise et al., 2014), ORs (Busse et al., 2014; Tsai et al., 2017), and filaggrin (Jang et al., 2012) play important roles in wound healing. We found that IL-10, Olfr520, and filaggrin were up-regulated in wounds treated with the 10-lipid mixture.

The degree of contribution of the bactericidal effects of the 10-lipid mixture versus its effects on the expression of AMPs, skin barrier function, and inflammatory mediators in the process of accelerated wound healing requires further investigation and could be addressed using genetically modified mouse strains that are deficient in production of AMPs, skin barrier proteins, inflammatory mediators, and/or their receptors. The composition, concentration of lipids of the lipid mixture, and components responsible for bactericidal, antimicrobial, and anti-inflammatory
effects of this lipid mixture require further refinement and investigation to achieve the most efficient wound healing.

In conclusion, we show here that the 10-lipid mixture, a mimic of 10 major lipids from C. obtusa, induced biomarkers associated with wound healing and had bactericidal activity against MSSA, MRSA, and S. pyogenes. The 10-lipid mixture enhanced wound healing in vivo in wounds with bacterial infection. Therefore, the 10-lipid mixture may be a strong candidate for future treatment of chronic wounds with bacterial infections.

MATERIALS AND METHODS

Preparation of the C. obtusa 10-lipid mixture

C. obtusa protein, carbohydrate, and lipid extracts were prepared using a supercritical fluid extraction (Capuzzo et al., 2013; Yang et al., 2007). Lipid extract fraction was found to be the most potent inducer of the AMP production by primary human keratinocytes (data not shown). Therefore, all further experiments were focused on the C. obtusa lipid extract. C. obtusa lipid extract was analyzed using a gas chromatography-mass spectrometry as previously described (Yang et al., 2007). A total of 38 individual lipids were identified (see Supplementary Table S1), and 10 major lipids (see Supplementary Table S2) were selected among the 38 individual lipids based on the percentage abundance in the C. obtusa lipid extract, in order from the most to the least prevalent. Ten purified lipids from this list were purchased from Sigma-Aldrich (St. Louis, MO) or Chemos Chemicals (Regenstauf, Germany), and a 10-lipid mixture was generated based on natural percentages of the lipids in the C. obtusa lipid extract. Therefore, the 10-lipid mixture examined in this study represents the major lipid components of C. obtusa plant extract. The mixture of 10 lipids was dissolved in phosphate buffered saline with 0.1% DMSO (Fisher Scientific, Fair Lawn, NJ). Institutional approval or patient consent was not required because we did not use human tissue samples for this study.

Immunofluorescence staining

Human primary keratinocytes or mouse skin sections were fixed and blocked. Slides were then stained with antibodies against OR2AT4 (Abcam, Cambridge, MA), HBD-3 (Abcam), LL-37 (Abcam), MBBD-2 (Abcam), CRAMP (Abcam), and S. aureus (Abcam). Nuclei were visualized with DAPI, and wheat germ agglutinin-conjugated FITC stained the cytoskeleton. The slides were visualized with fluorescent microscopy (Leica, Wetzler, Germany). Images were collected at ×250 or ×400 magnification, and the levels of mean fluorescence intensity were measured with Slidebook 6.0 (Intelligent Imaging Innovations, Denver, CO).

Mice

Hairless mice (Crl: SKH1-Hhr, female, 13 weeks old, strain no. 477; Charles River Laboratories, Wilmington, MA) were used for experiments. Mice were anesthetized with isoflurane against OR2AT4 (Abcam, Cambridge, MA), HBD-3 (Abcam), LL-37 (Abcam), MBBD-2 (Abcam), CRAMP (Abcam), and S. aureus (Abcam). Nuclei were visualized with DAPI, and wheat germ agglutinin-conjugated FITC stained the cytoskeleton. The slides were visualized with fluorescent microscopy (Leica, Wetzler, Germany). Images were collected at ×250 or ×400 magnification, and the levels of mean fluorescence intensity were measured with Slidebook 6.0 (Intelligent Imaging Innovations, Denver, CO).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism, version 5.0 (GraphPad, San Diego, CA). Statistical differences between groups were determined by using one-way analysis of variance, and significant differences were determined by a Tukey-Kramer test. In cases in which two groups were compared, data were analyzed using a paired t test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.11.039.

REFERENCES


