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Mesenchymal stromal cell-secreted CCL2 promotes antibacterial defense mechanisms through increased antimicrobial peptide expression in keratinocytes

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Abstract

Mesenchymal stromal cells (MSCs) from both humans and horses, which represent a clinically relevant translation animal model for human cutaneous wound healing, were recently found to possess antimicrobial properties against planktonic bacteria, and in the case of equine MSCs, also against biofilms. This, together with previous findings that human and equine MSCs promote angiogenesis and wound healing, makes these cells an attractive approach to treat infected cutaneous wounds in both species. The anti-biofilm activities of equine MSC, via secretion of cysteine proteases, have only been demonstrated in vitro, thus lacking information about in vivo relevance. Moreover, the effects of the equine MSC secretome on resident skin cells have not yet been explored. The goals of this study were to (a) test the efficacy of the MSC secretome in a physiologically relevant ex vivo equine skin biofilm explant model and (b) explore the impact of the MSC secretome on the antimicrobial defense mechanisms of resident skin cells. Our salient findings were that secreted factors from equine MSCs significantly decreased viability of methicillin-resistant *Staphylococcus aureus* bacteria in mature biofilms in this novel skin biofilm explant model. Moreover, we demonstrated that equine MSCs secrete CCL2 that increases the antimicrobial activity of equine keratinocytes by stimulating expression of antimicrobial peptides. Collectively, these data contribute to our understanding of the MSC secretome's antimicrobial properties, both directly by killing bacteria and indirectly by stimulating immune responses of surrounding resident skin cells, thus further supporting the value of MSC secretome-based treatments for infected wounds.

KEYWORDS
antimicrobial peptides, biofilm, cutaneous wounds, keratinocytes, mesenchymal stromal cells, *MRSA*, secretome

Significance statement

This study demonstrates that the mesenchymal stromal cell (MSC) secretome effectively reduced viability of the methicillin-resistant *Staphylococcus aureus* (*MRSA*) biofilms in an ex vivo cutaneous wound explant model. Moreover, MSC secretome could actively modulate immune responses of keratinocyte via a CCL2-mediated mechanism. For these studies, the equine model was used, which is a recognized translational and clinically relevant animal model for human wound healing. Collectively, these data further support the value of MSC secretome-based treatments for infected wounds.

1 | INTRODUCTION

Mesenchymal stromal cells (MSCs) are adult multipotent progenitor cells that participate in the inflammatory, proliferative, and remodeling phases of tissue repair.[1,2] The primary mechanism contributing to tissue repair occurs via paracrine signaling and, therefore, MSC-secreted factors are considered a promising therapeutic approach in regenerative medicine.[1-3] Previously, our group demonstrated that the MSC secretome, containing all secreted bioactive factors, stimulates dermal fibroblast migration and promotes angiogenesis in the equine model.[3-5] We use the horse as a physiologically relevant and translational animal model to evaluate novel therapeutic approaches for cutaneous wounds.[6] In both horses and humans, particular types of chronic wounds are often therapy resistant and cause various complications, leading to high morbidity and mortality with significant economic impact.[6-8]

An important factor associated with compromised healing is wound colonization with pathogenic bacteria. In acute wounds, the main complication is infections, which, if not cleared, lead to wound chronicity.[9,10] Infections can trigger an excessive inflammatory host response that suppresses the healing process. Subsequently, the skin barrier remains open, creating a vicious cycle by allowing continuous bacterial infestation.[11] Bacterial wound colonization is a hallmark of all types of chronic wounds, regardless of the underlying pathology or species.[12,13]

Beside promoting skin tissue repair, we and others have found that MSC-secreted factors also possess antimicrobial properties, both against planktonic bacteria and bacteria in biofilms.[14-17] In light of increasing therapy-resistant bacterial infections, alternative approaches to conventional antimicrobial chemotherapy are of great interest, especially those for wounds infected with the biofilm forming methicillin-resistant *Staphylococcus aureus* (*MRSA*).[18,19] For the latter, we were the first to report that equine MSCs reduce *MRSA* biofilms by secreting cysteine proteases that degrade the biofilm's extracellular matrix (ECM), and moreover, that this mode-of-action significantly increased the efficacy of prior noneffective antibiotics.[17] This study was performed using an in vitro culture biofilm model, and although exceptionally well suited to screen for possible anti-biofilm treatments, this model lacks clinical significance as biofilm composition is highly dependent on the microenvironment.[20] Indeed, the milieu in cutaneous wounds is different from the sterile milieu of a culture dish, and biofilms that form in skin wounds are likely to have distinguished architecture and ECM patterns from in vitro biofilms.[21] Another question that needs to be addressed when evaluating the therapeutic value of the MSC secretome for infected wound management is whether the MSC secretome also affects the inherent host immune responses in the skin, in addition to directly affecting bacteria.

The skin, as the most exposed organ of the body, needs to guarantee a protective barrier against environmental insults such as microbes. The antimicrobial defense of the skin consists of complex interactions of resident skin and immune cells, as well as the skin microbiota. A healthy skin microbiota prevents invasion of the skin or wounds with pathogenic bacteria. The microbiome also interacts closely with the outer layer of the skin, the epidermis, and stimulates the host defense mechanism by inducing upregulation of antimicrobial peptide (AMP) expression.[22] The skin is organized in four main anatomical structures: epidermis, dermis, adipose tissue, and skin appendages. The outermost layer is the epidermis, where keratinocytes are the predominant cell type and play a key role in initial defense against microbial invasion.[23,24] In the following layer, the dermis, fibroblasts are the dominating cell type .[25] Specialized keratinocyte cell types are represented in the skin appendages, such as sweat and sebaceous glands that produce secretes, which are released to the skin surface where they contribute to the barrier function of the skin by regulating skin moisture, pH, temperature, and by release of AMPs,[24] which is the most important defense mechanism of resident skin cells against invasive microorganisms. Both human and equine keratinocytes have been found to express the AMPs ß-defensin, cathelicidin, elafin, and lipocalin.[26-29] These AMPs exert antimicrobial activity, but are also associated with a variety of other processes ranging from barrier homeostasis to inflammation and wound healing.[30]

Since the anti-biofilm activities of the MSC secretome have not yet been studied in a physiologically relevant skin environment and information on the effects of MSC-secreted factors on the antimicrobial response of resident skin cells is lacking, the goals of this study were to (a) test the efficacy of the MSC secretome on biofilms in a physiologically relevant ex vivo equine skin explant model and (b) explore the impact of the MSC secretome on the antimicrobial defense mechanisms of resident skin cells. Our salient findings were that secreted factors from equine MSC significantly decreased the viability of *MRSA* in mature biofilms in a clinically relevant ex vivo skin explant model. Moreover, we demonstrated that equine MSCs secrete the cytokine CCL2 that increases the antimicrobial activity of equine keratinocytes by stimulating their expression of the AMPs cathelicidin and ß-defensin.

2 | METHODS

2.1 | Cells

Equine peripheral blood-derived MSCs were isolated and characterized, exactly as described previously,[3,4,31] and cultured in low glucose Dulbecco's modified Eagle medium (DMEM; Corning, Acton, Massachusetts), supplemented with 30% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, Georgia), 1% penicillin/streptomycin (P/S; Invitrogen, Grand Island, New York), and 2 mM L-glutamine (Invitrogen). Blood collection was approved by the Cornell Institutional Animal Care and Use Committee (IACUC # 2014-0038). The equine dermal fibroblast cell line NBL6 (ATCC, Manassas, Virginia) was cultured in DMEM supplemented with 10% FBS and 1% P/S.

Primary keratinocytes were isolated from equine skin harvested from the inner thighs of horses that were euthanized for reasons unrelated to the study. Skin was washed three times with phosphate-buffered saline (PBS) (Corning, New York), quickly rinsed with 70% ethanol, followed by three washes with PBS supplemented with 3% Antibiotic/Antimycotic (Ab/Am) (Corning). Skin was cut in 1 cm^2 pieces and incubated overnight at 37°C in 2.4 U/mL Dispase II (Sigma Aldrich, St Louis, Missouri) to dissociate the epidermis from the dermis. Epidermis was removed using forceps and digested with 0.05% trypsin-0.02% EDTA at 37°C. Mixtures were substituted every 30 minutes until no more cells were recovered. Cell-containing supernatants were sequentially filtered through a 100 and 40 μm cell strainer, and then washed in 10 mL PBS at 300*g* for 5 minutes. Keratinocytes were plated in 6-well plates and keratinocyte growth medium, consisting of 50% DMEM and 50% sterile filtered NBL6 conditioned medium (CM) (see below), supplemented with 10% FBS, 10 μM Rho-kinase inhibitor (Sigma Aldrich), and 1% P/S.[32] Medium was changed the next day and then every other day. Colonies of keratinocytes typically appeared after 3 to 7 days.

2.2 | Isolation and characterization of skin explants

Skin collected from horses (see above) was washed three times with PBS, quickly rinsed with 70% ethanol, followed by three washes with PBS supplemented with 3% Ab/Am. Skin was placed on a solid agar consisting of 5 g/L agar (Sigma Aldrich) in DMEM, supplemented with 10% FBS and 1% P/S. Semisolid agar consisting of 2.5 g/L agar in DMEM, supplemented with 10% FBS and 1% P/S, was added around skin explant, so that the epidermis of the skin was lifted to an air-agar interface.

Skin explants were harvested at day 0, 1, 2, and 3, for further characterization. To determine cell survival of the skin explants, skin was stained with a whole mount live and dead staining, as described previously.[33,34] Briefly, skin explants were washed for 30 minutes in PBS with 0.01% Tween 20. A mixture of 15 μM propidium iodide (Thermo Fisher Scientific, Waltham, Massachusetts) and 1.4 μM 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich) in PBS[35] was added to skin explants and incubated for 24 hours at 4°C on a rotating shaker. Staining solution was removed and skin was washed three times for 10 minutes in PBS with 0.01% Tween 20, embedded in optimal cutting tissue (OCT) compound (Sakura, Torrance, California), snap frozen, and stored at −80°C. Skin was sectioned in 5 μm sections, fixed with 4% paraformaldehyde (PFA) (Thermo Fisher Scientific), mounted with Dako Glycergel mounting medium (Agilent Dako, Santa Clara, California), and cover-slipped. Images were acquired using an Eclipse TE2000-U inverted fluorescence microscope (Nikon Inc., Mellville, New York) with a ×4 magnification objective. To determine epidermal thickness, 5 μm sections of untreated snap-frozen skin explants were fixed with a 4% PFA solution and stained with hematoxylin-eosin (H&E). Sections were mounted with Dako Glycergel mounting medium and cover-slipped. Images were acquired using a ×20 magnification objective on a bright-field microscope (Olympus, Waltham, Massachusetts). Four different optic fields from four different sections of each skin explant were imaged for further analysis. Images were analyzed using the Fiji ImageJ Software (https://imagej.net/Fiji).

2.3 | Collection of CM and identification of MSC-secreted factors

CMs were collected from MSCs from three different horses, exactly as described earlier.[14,17] Briefly, 9×10^5 MSC were seeded in a T_{75} flask in 10 mL antibiotic-free medium, consisting of DMEM and 10% FBS. After 2 days of culture, medium was removed, cells were washed thoroughly with PBS three times, and refed with 6 mL of DMEM. Supernatants, named CM, were collected 24 hours later. A similar approach was used to collect CM from NBL6 cells (control CM or as supplement for keratinocyte growth medium) and keratinocytes.

To evaluate the presence of certain equine cytokines in the MSC CM, CM from MSC and NBL6 were submitted to the Animal Health Diagnostic Center at Cornell University, for detection using equine-specific bead-based multiplex assays, exactly as previously described.[36,37]

2.4 | Stimulation of keratinocytes with MSC CM

1×10^4 keratinocytes were seeded in wells of six-well plates in Abx-free keratinocyte growth medium. After 2 days of culture, medium was removed, cells were washed three times with PBS, and 300 μL of MSC CM, NBL6 CM, or DMEM, was added in triplicate. In parallel, 300 μL of each condition was also added to wells without cells to control for the inherent antimicrobial activity of the CM used (control). Supernatants were collected 7 hours later and after centrifugation twice for 5 minutes at 300*g*, stored at −80°C until further use in antibacterial assays.

For experiments evaluating the contribution of MSC-secreted cytokines, MSC CM was collected, supplemented with 0.2 μg/mL of mouse-anti-horse CCL-2 antibodies (clone 104-2, 49), mouse-anti-horse CCL-5 antibodies (clone 96, 91-1, 46), mouse-anti-horse

CCL11 antibodies (clone 24, 25) or a mouse IgG1 isotype control,[37] and incubated overnight at 4°C on a rocking shaker. This antibody-incubated MSC CM was then use to stimulate keratinocytes and supernatants were collected for antibacterial assays, as described above.

2.5 | Double immunofluorescence stainings

To investigate AMP expression in (un)stimulated keratinocytes, cells were seeded on microscopic Nunc Thermanox coverslips (Thermo Fisher Scientific), fixed with 4% PFA for 5 minutes, and washed twice with staining buffer consisting of PBS, 0.5% bovine serum albumin (BSA, Sigma Aldrich), 1% Tween 20 (Thermo Fisher Scientific), and 0.01% saponin (Sigma Aldrich). After blocking unspecific binding-sites for 30 minutes with 10% normal goat serum in staining buffer, cells were incubated overnight at 4°C with rabbit-anti-cathelicidin antibodies, rabbit-anti-lipocalin 2 antibodies, rabbit-anti-elafin antibodies, rabbit-anti-ß-defensin antibodies, rabbit-anti-CCR3 antibody, rabbit-anti-CCR4 antibodies, or rabbit-IgG isotype control antibodies (all at a 1:500 dilution and from Abcam, Cambridge, Massachusetts). For CCR3 and CCR4 staining, staining buffer contained no saponin. After three washes with staining buffer, keratinocytes were incubated for 1 hour at room temperature with Alexa Fluor 488-labeled goat-anti-rabbit secondary antibodies (1:500, Jackson Immunoresearch, West Grove, Pennsylvania). After three washes with staining buffer, mouse-anti-pan-cytokeratin antibodies (clone EA1/EA3, 1:1000, Abcam), used as a keratinocyte marker, were added for 1 hour, followed by three washes, and a second incubation for 1 hour with Alexa Fluor 647-labeled goat-anti-mouse secondary antibodies (1:500, Jackson Immunoresearch). Before the final three washes, DAPI was added for 3 minutes, slides were covered with Dako Glycergel mounting medium and cover-slipped.

To investigate AMP expression in skin explants, skin was embedded in OCT compound, snap-frozen, and 5 μm sections were cut using a cryotome. Skin sections were fixed with 4% PFA for 5 minutes and then washed three times with staining buffer. The staining procedure for the AMPs cathelicidin, lipocalin2, elafin, and ß-defensin was executed exactly as described above. AMP staining was combined with either a keratinocyte maker, that is, anti-pan-cytokeratin as described above, or a marker for cells of mesenchymal origin. For the latter, skin sections were incubated for 1 hour with mouse-anti-vimentin antibodies (1:500, Abcam), and after three washes, a secondary Alexa Fluor 647-labeled goat-anti-mouse antibody was added for 1 hour. Before the final three washes, 1.4 μM DAPI was added for 3 minutes, slides were covered with Dako Glycergel mounting medium, and cover-slipped.

Images were acquired using a ×40 magnification water immersion objective on the confocal laser scanning microscope FV3000 (Olympus) with the cellSens Dimension software (Olympus). Images were analyzed automatically using the Fiji ImageJ Software and AMP expression was presented as pixel intensity relative to pixel intensity of the DMEM control that was set to a 100%.

2.6 | Bacterial cultures

The *MRSA strain USA300* was a kind gift from Dr Whittaker, Cornell University. Methicillin-susceptible *S aureus* (*MSSA*) 25923 (ATCC) and *MRSA* were maintained on Tryptic Soy (TS) agar (Sigma Aldrich) at 4°C for up to 2 weeks. For each experiment, a single colony of the appropriate strain was incubated in 4 mL of TS broth (Hardy Diagnostics, Santa Maria, California) on an orbital shaker at 200 rpm, overnight at 37°C in a warm room. Overnight cultures were then used for further experimentation, as described below.

2.7 | CM-planktonic bacteria cocultures

To test the antibacterial effect of keratinocyte CM on planktonic bacteria, bacterial concentrations in TS broth were determined, exactly as described previously.[17] 100 μL of following conditions were added to triplicate wells of a 96-well plate: (a) CM from MSC CM-stimulated keratinocytes and MSC CM from cell-free wells as background control; (b) CM from NBL6 CM-stimulated keratinocytes and NBL6 CM from cell-free wells as background control; (c) CM from DMEM-stimulated keratinocytes (negative control) and DMEM from cell-free wells as background control; and (d) P/S (1% in DMEM) (positive control). Next, 50 μL of TS broth containing 1×10^3 colony forming units (CFU)/mL of bacteria were added to each well and plates were incubated on an orbital shaker at 200 rpm, at 37°C in a warm room for 8 hours. The optical density (OD) of the cultures was read using a 96-well Multiskan EX plate reader (Thermo Fisher Scientific) at 600 nm. Growth in the different conditions was normalized to growth in their respective background controls, and bacterial growth was presented as the OD. To determine the correlation of CFU/mL to OD, *MSSA* and *MRSA* were plated in serial dilutions in triplicate in wells of a 96-well plate and OD was read, as described above. After OD reading, bacterial cultures were plated in serial dilutions on TS agar and incubated for 18 hours at 37°C, to determine CFU/mL.

2.8 | Equine skin biofilm explant model

Skin explants were prepared, as described above. Epidermal wounds with a diameter of 4 mm were created using a biopsy punch (Integra LifeSciences, Princeton, New Jersey). Wounds were inoculated with 10 μL of overnight cultures of *MSSA* or *MRSA*, diluted 1:10 in TS broth to a concentration of 10^8 CFU/mL, and biofilms were allowed to form for 24 hours at 37°C.

To confirm biofilm formation, skin was snap-frozen, cut in 5 μm sections, fixed and stained in a 1% Alcian blue (Sigma) solution (pH 2.5) for 30 minutes, as previously described.[38] Additional sections were stained with 5 μg/mL of a rabbit anti-*S aureus* polyclonal antibody (Thermo Fisher) or a rabbit isotype control (Abcam), followed by a secondary horseradish peroxidase-labeled goat anti-rabbit antibody. After adding the substrate 3-amino-9-ethylcarbazole (*AEC*), sections were

counterstained with Gill's hematoxylin. Mounted and cover-slipped sections were imaged using a bright-field microscope (Olympus).

To test the effect of MSC CM on biofilms, established biofilms were treated in triplicate with 10 μL of MSC CM from three different horses, DMEM alone (negative control), DMEM with 1% P/S (positive control), or a combination of MSC CM with P/S, and incubated another 24 hours before explants were harvested, weighed, and homogenized in PBS. Serial dilutions of homogenates were plated on TS agar and incubated for 18 hours at 37°C, after which CFUs were counted to determine CFU/g tissue.[39] Whole mount Live/Dead staining was performed on distinct sets of explants, as described above.

Images of biofilms were taken using a ×100 oil immersion objective on the Eclipse TE2000-U inverted fluorescence microscope (Nikon). Four different optic fields from four different sections were imaged and analyzed automatically using the Fiji ImageJ Software.

2.9 | Statistical analysis

A two-way analysis of variance (ANOVA), followed by a Tukey's multiple comparison test, unless where indicated otherwise, was performed to determine statistically significant differences at $P < .05$. GraphPad

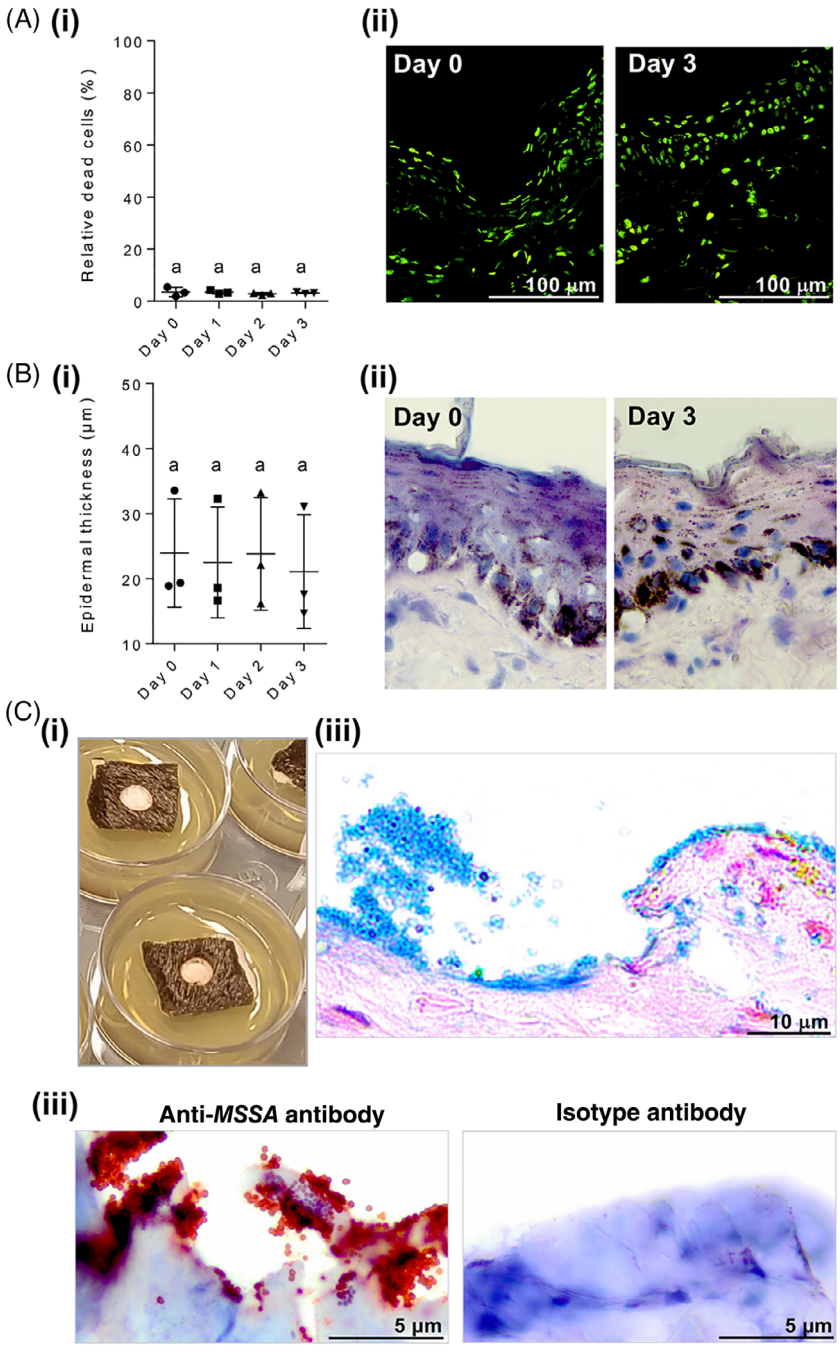

FIGURE 1 Equine ex vivo skin biofilm explant model. A,B, Ex vivo skin explants show no signs of decay over a 3-day culture period. Freshly harvested skin was either immediately processed (day 0, control) or cultured and collected daily to assess the ratio of live and dead cells (A) and epidermal thickness (B) over time. Different letters indicate statistically significant difference. n = 3 (i). Representative immunofluorescence (IF) images at day 0 and day 3 of green (live) and red (dead) cells are shown (A[ii]), as well as images of H&E stainings (B[ii]). C, Biofilms can be successfully established in an ex vivo skin explant wound model. A representative image of skin explants in culture (i) is shown. Bacterial methicillin-susceptible *Staphylococcus aureus* (*MSSA*) biofilms in skin explants were confirmed with an Alcian Blue staining to show presence of extracellular matrix (ii) and with an immunohistochemistry staining using a rabbit anti-*S aureus* antibody to show presence of *MSSA*. A rabbit isotype control staining was also performed (iii). H&E, hematoxylin-eosin

Software was used for the analysis (GraphPad Software, La Jolla, California). Data given are the mean of three replicates with SDs.

3 | RESULTS

3.1 | The MSC secretome reduces live *MRSA* in biofilms in an ex vivo cutaneous wound model

To investigate the impact of the MSC secretome, collected as CM, on survival of bacteria in a clinically relevant model, we first established an ex vivo equine skin biofilm explant model. In order to evaluate the suitability of this model, we started by culturing freshly harvested equine skin over a period of 3 days and investigated cellular survival and epidermal thickness using microscopy. Neither significant increase in cell death nor decrease in epidermal thickness was observed during this culturing period (Figure 1A,B). Next, we created an infected wound model by making uniform wounds in freshly harvested skin and inoculating these wounds with *MSSA* or *MRSA* for 24 hours (Figure 1C[i]). The establishment of biofilms in this explant model was confirmed by staining the extracellular polymeric substances with Alcian Blue (Figure 1C[ii]) and the bacteria with a specific anti-*S aureus* antibody (Figure 1C[iii]).

Biofilms in this novel equine explant model were then treated with DMEM (negative control), MSC CM, or antibiotics (Abx, positive control) for 24 hours and bacterial load was measured by evaluating CFU per gram (CFU/g) of tissue. We observed that treatments with MSC CM as the well as the antibiotic control reduced *MRSA*, but not *MSSA*, biofilms when compared to DMEM control, although this difference did not reach statistical significance (Figure 2A). In order to gather more information about biofilm composition after MSC CM treatments, we also determined live and dead bacteria using fluorescent staining. The area of live bacteria was not statistically significantly decreased when *MSSA* biofilms were treated with MSC CM or Abx when compared to treatment with DMEM control (Figure S1A). In contrast, MSC CM treatment did result in a statistically significant reduction of the area of live bacteria in *MRSA* biofilms when compared to DMEM and this reduction was comparable to the reduction observed with Abx treatment (Figure S1A). However, no differences of dead bacteria relative to live bacteria were found in *MSSA* or *MRSA* biofilms after treatments with either MSC CM or Abx (Figure S1B). These findings indicate that MSC CM not necessarily leads to bacterial killing, but still reduces the content of live bacteria in biofilms, likely due to biofilm destruction by protease activity or growth inhibition, as we previously reported.[14,17] Additionally, a treatment with a combination of MSC CM with Abx reduced CFU/g tissue of mature biofilms in the skin explants when compared to Abx alone, and reached significance for *MRSA* but not *MSSA* (Figure 2B). Collectively, our data show that the reduction of biofilms upon treatment with the equine MSC secretome, as we previously determined using in vitro assays,[14,17] remains valid in a physiological relevant ex vivo skin explant model, thus providing stronger support that the MSC secretome will most likely impact biofilm growth in vivo as well.

3.2 | MSC-secreted factors increase the antimicrobial properties of equine keratinocytes via stimulation of AMP expression

In addition to the direct effects of the MSC secretome on biofilms, we also explored its effects on resident skin cells, as this might affect bacterial infection indirectly via stimulation of inherent immune responses of these cells. First, we investigated the effect of the MSC CM on resident skin cell viability in our ex vivo skin explant model and did not find a negative impact after a 24 hours incubation (Figure S2), indicating therapeutic safety. CM from equine dermal fibroblasts (NBL6) also did not affect resident skin cell viability (Figure S2). The latter was

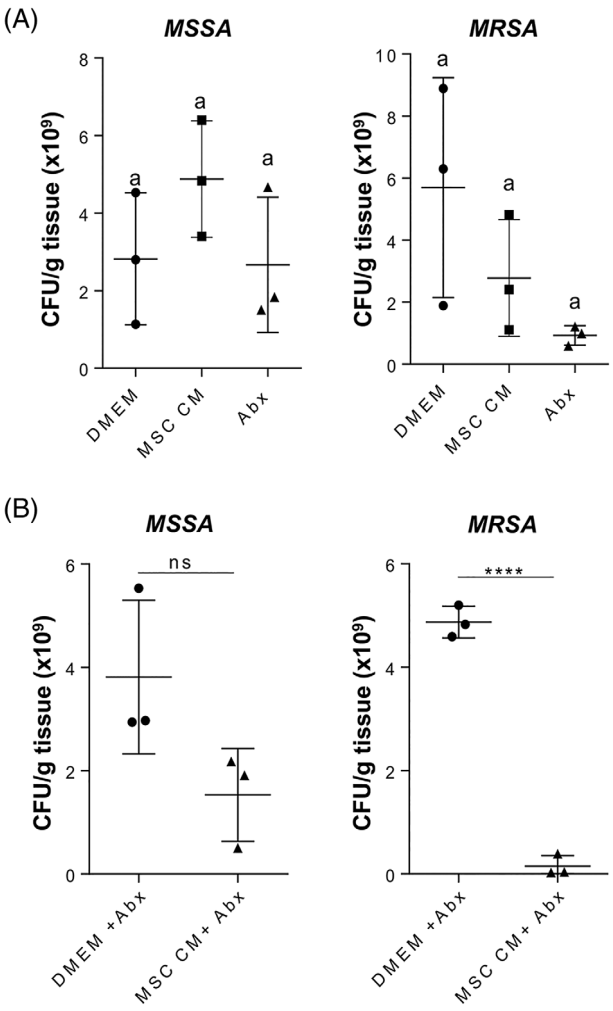

FIGURE 2 Effect of mesenchymal stromal cell (MSC) conditioned medium (CM) on mature biofilms in an ex vivo equine skin explant model. Methicillin-susceptible *Staphylococcus aureus* (*MSSA*) and methicillin-resistant *Staphylococcus aureus* (*MRSA*) biofilms grew for 24 hours before treatment with Dulbecco's modified Eagle medium (DMEM, negative control), equine MSC CM, or penicillin/streptomycin (Abx, positive control) (A) or combinations of Abx with DMEM or MSC CM (B). CFU/g tissues were assessed 24 hours later. Different letters indicate statistically significant differences. n = 3

included as control to distinguish between general cell secretome-mediated effects and specific MSC secretome-mediated effects, similar to previous work from our group when studying MSC secretome effects.[14,17]

Although MSCs have been shown to enhance inflammatory pathways, as well as reduce inflammation in multiple studies,[40] no studies have been conducted yet to our knowledge investigating the effects of the MSC secretome on the antimicrobial defense mechanisms of resident skin cells. AMPs are key molecules in the cutaneous innate immune response and altered expression is associated with skin pathologies.[24,41] To evaluate the presence of AMPs in the equine skin explants, immunofluorescence (IF) stainings were performed for the AMPs cathelicidin, elafin, lipocalin, and ß-defensin. Cathelicidin and elafin were found to be highly expressed, followed by lipocalin (Figure S3). Equine ß-defensin was only detected in low levels (Figure S3). To further localize AMP expression in the equine skin, AMP stainings were repeated in combination with a staining for keratinocytes, using a pan-cytokeratin marker, or a staining for skin cells of mesenchymal origin like fibroblasts, using a vimentin marker. Equine AMP expression was found in the epidermis as well as in the dermis, with the majority of AMP expression localized in keratinocytes (80.6%-99.9%) (Figure 3A) and between 0.8% and 7.6% localized in vimentin-positive cells, depending on the AMP (Figure 3B). Similar to studies on AMP expression in human skin,[42] our results show that keratinocytes are the main source for AMP expression in the equine skin as well.

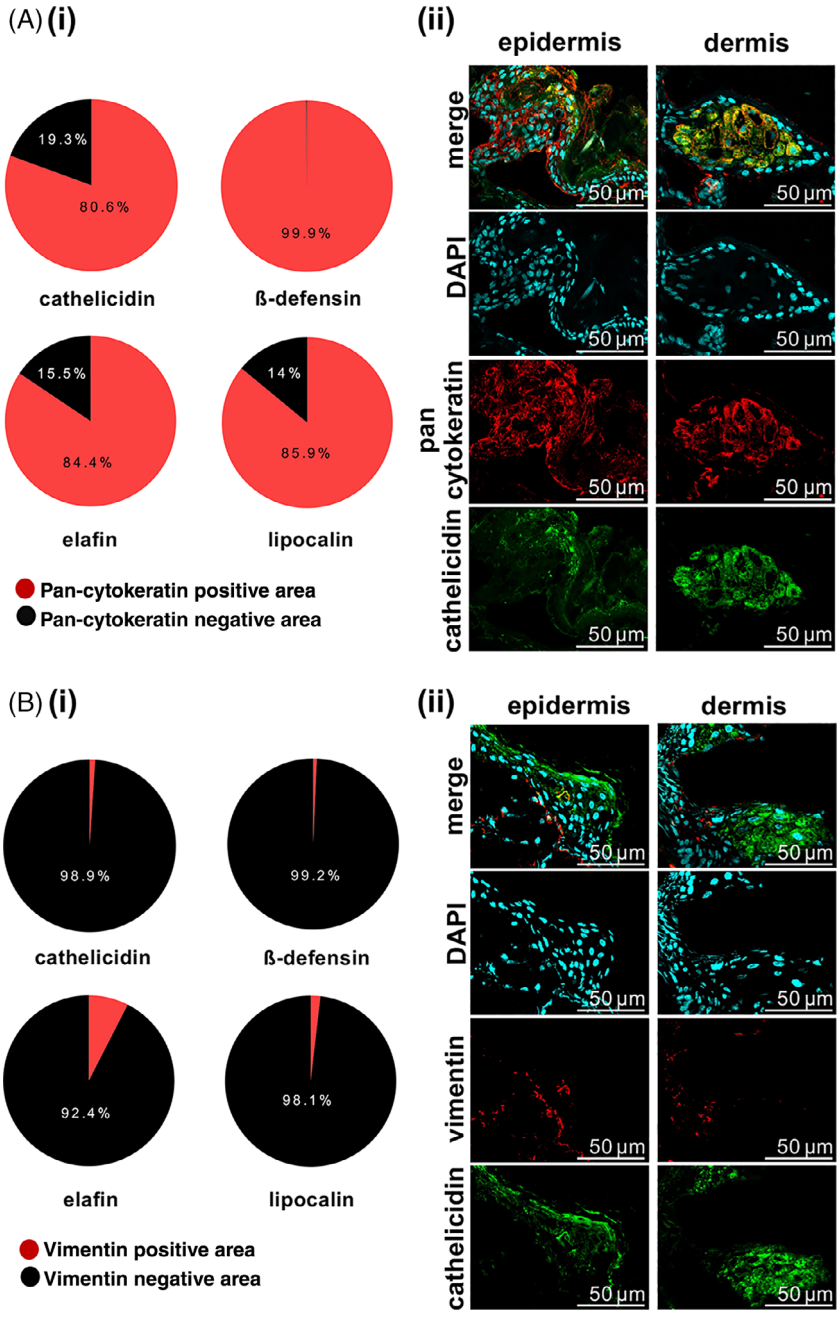

FIGURE 3 Antimicrobial peptide (AMP) colocalization in resident skin cells in an ex vivo equine skin explant model. The percentage of expression of the AMPs cathelicidin, ß-defensin, elafin, and lipocalin, in either pan-cytokeratin-positive cells (A) or vimentin-positive cells (B) in an ex vivo equine skin explant model was analyzed using confocal microscopy. AMP-positive areas generally overlapped with areas positive for a pan-cytokeratin marker, identifying keratinocytes (between 80.6% and 99.9%, depending on the AMP) and generally did not overlap with vimentin-positive areas (between 0.8% and 7.6%, depending on the AMP), indicative of cells of mesenchymal origin such as fibroblasts (i). Representative images are shown, with red cells positive for either pan-cytokeratin or vimentin marker and green cells positive for the AMP cathelicidin (as example) in equine epidermis and equine dermis. DAPI was used for nuclear staining (blue) (ii). DAPI, 4′,6-diamidino-2-phenylindole

In order to mechanistically study the effects of MSC CM on these skin keratinocytes, we decided to isolate and characterize equine keratinocytes and study their antimicrobial properties in a two-dimensional culture system (Figure S4A,B). After confirming that MSC CM, as well as control NBL6 CM, did not negatively affect keratinocyte viability (Figure S4C), keratinocytes were stimulated with MSC CM, NBL6 CM, or DMEM for 7 hours. After stimulation, CM was collected from these keratinocytes and added to *MSSA* and *MRSA* bacterial cultures for 8 hours. We observed that CM from MSC-stimulated keratinocytes could inhibit growth of *MRSA* when compared to DMEM- and NBL6 CM-stimulated keratinocytes (Figure 4A). A standard curve presenting the correlation between CFU/mL to the measured OD is shown in Figure 4B. These results indicate that stimulation of equine keratinocytes with factors secreted by equine MSCs increased the antimicrobial efficacy of these cells against antibiotic resistant *S aureus* strains.

Combining our findings that keratinocytes in our ex vivo equine skin explant model express various AMPs (Figure 3A) with the observation that the secretome of MSC CM-stimulated keratinocytes has an increased efficacy to inhibit bacterial growth (Figure 4), we sought to determine whether an increased AMP expression might be responsible for the increased antimicrobial efficacy of equine keratinocytes upon stimulation with MSC CM. To this end, we performed IF stainings for cathelicidin, ß-defensin, elafin, and lipocalin, in primary equine keratinocyte cultures that were stimulated with MSC CM, or NBL6 CM and DMEM as controls, and determined fluorescent pixel intensity/cell by running an unbiased automatic script created with Fiji ImageJ. We found that MSC CM-stimulated keratinocytes had significantly increased levels of the AMPs cathelicidin and ß-defensin, but not elafin and lipocalin, when compared to keratinocytes that were stimulated with either DMEM or NBL6 CM (Figure 5). These findings were corroborated by repeating these experiments in our ex vivo equine skin explant model, using equine skin from three different horses. Here, we found increased levels of all AMPs in skin treated with MSC CM when compared to DMEM treated skin and this increased expression reached statistical significance for the AMP cathelicidin (Figure S5).

3.3 | MSC secrete chemokines with pro-inflammatory effect on keratinocytes

Since various cytokines, such as IL-17, IL-1ß, IL-22, IFN-γ, and TNF-α, have been reported to mediate AMP expression in human keratinocytes,[43-49] we aimed to evaluate the MSC cytokine profile in search for potential candidates with stimulating activity toward AMP expression in equine keratinocytes. First, we mined through our recently acquired single-cell RNA sequencing data set of equine MSCs[50] to determine cytokine expression on a RNA level. Surprisingly, none of the cytokines described above were found in this data set (data not shown). RNA single-cell sequencing is known to provide data about the expression of a great range of genes at the individual cell level, but at the cost of a reduced sequencing depth resulting in identifying only highly expressed genes. Therefore, we decided to evaluate the expression of cytokines on a protein level. To this end, we analyzed MSC CM and NBL6 CM (control) samples with the equine cytokine 5-plex assay, detecting equine IL-4, IL-10, IL-17, IFN-γ and IFN-α,[36] and the chemokine 6-plex assay, detecting IL-1ß, CCL2, CCL3, CCL5, CCL11, and TNF-α.[37] The immunoassays did not detect IL-1ß, IL-17, IFN-γ, and TNF-α corroborating the sequencing data that these cytokines are most likely not produced by equine MSCs (Figure 6A[i]). In contrast, the equine chemokines CCL2, CCL5/Rantes, and CCL11/Eotaxin-1 were detected in the MSC CM, and moreover, in much higher concentrations compared to NBL6 CM, where these chemokines were either not detected or only at very low levels (Figure 6A[ii]). Interestingly, these chemokines play an important role in skin immune defense and their chemotactic activity toward immune cells, like macrophages and eosinophils, is well established.[51-53] More recent studies found that human keratinocytes express receptors for these chemokines, and that receptor-ligand interactions stimulated wound closure by keratinocytes in an in vitro

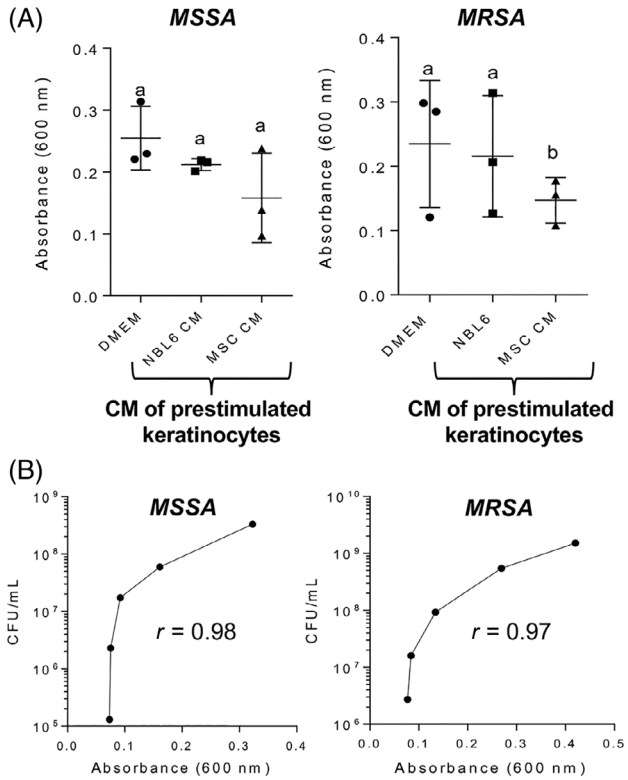

FIGURE 4 Effect of mesenchymal stromal cell (MSC) condition medium (CM) on antimicrobial properties of primary equine keratinocytes. Keratinocytes were prestimulated with Dulbecco's modified Eagle medium (DMEM), CM from MSCs, or dermal fibroblast (NBL6) CM for 24 hours. CM from these stimulated keratinocytes was collected and added to methicillin-susceptible *Staphylococcus aureus* (*MSSA*) or methicillin-resistant *Staphylococcus aureus* (*MRSA*) bacterial cultures for 8 hours, after which relative bacterial growth was assessed by optical density (OD). Different letters indicate statistically significant difference. n = 3. (A) Standard curve showing the correlation between CFU/mL and OD readings for both *MSSA* and *MRSA* (B)

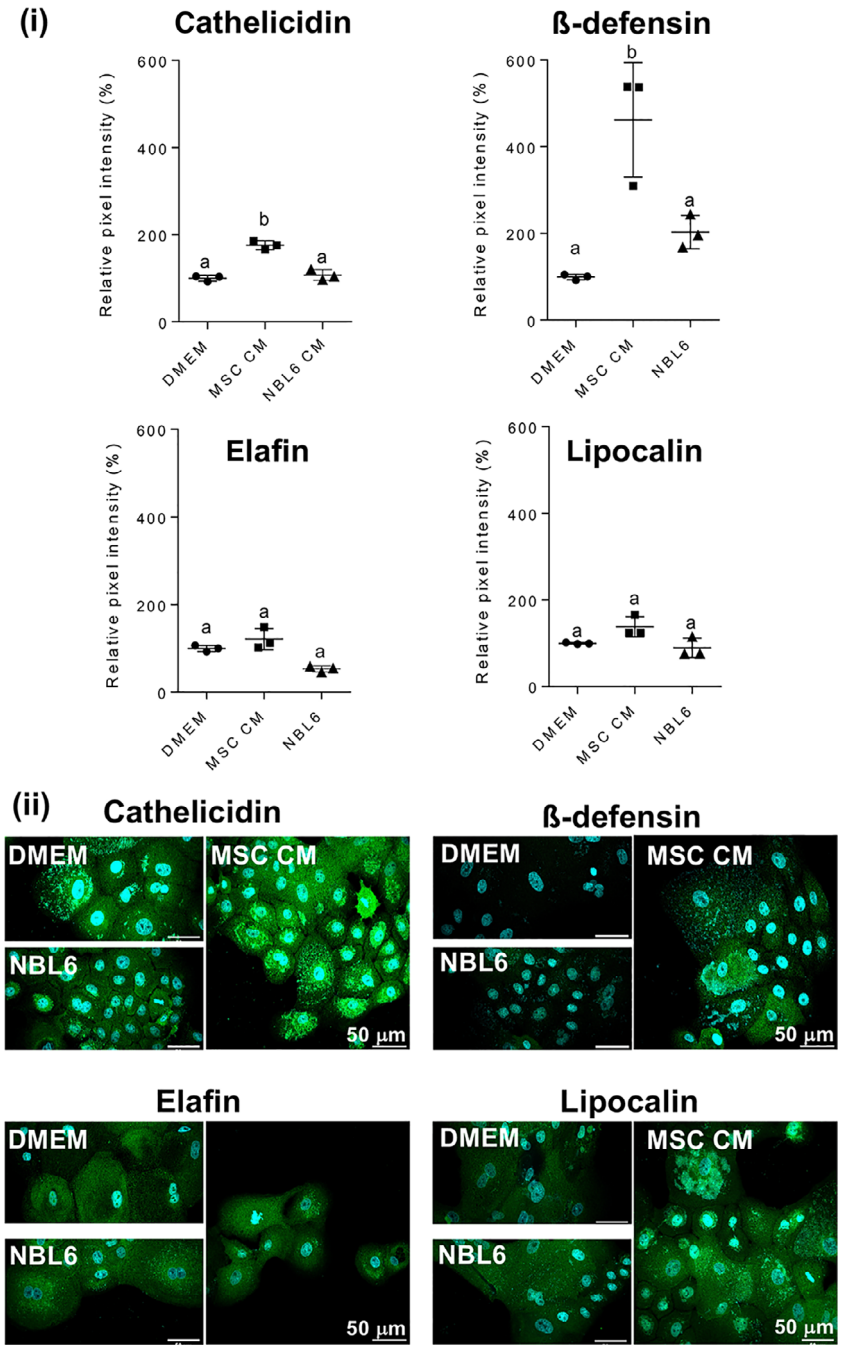

FIGURE 5 Effect of MSC CM on AMP expression in primary equine keratinocytes. Keratinocytes were incubated with Dulbecco's modified Eagle medium (DMEM), MSC CM, or dermal fibroblasts (NBL6) CM for 24 hours and AMP expression was visualized using IF stainings. Images were collected via confocal microscopy, analyzed, and normalized to cell count using Fiji ImageJ. Different letters indicate statistically significant differences. n = 3 (i). Representative images of AMP expression (green) in DMEM, MSC CM, or NBL6 CM-stimulated keratinocytes. DAPI was used for nuclear staining (blue) to determine cell number (ii). AMP, antimicrobial peptide; CM, condition medium; DAPI, 4′,6-diamidino-2-phenylindole; MSC, mesenchymal stromal cell

scratch assay.[54] However, and to our knowledge, no studies have been performed to evaluate whether these effects are mediated via an increased AMP expression in keratinocytes. In order to study this, we first confirmed CC chemokine receptor expression on equine keratinocytes of the receptors CCR3, which binds the chemokines ligands CCL11 and CCL5, and CCR4, which binds CCL2 and CCL5 (Figure 6B). Next, we inhibited these three chemokines in the MSC CM using equine specific monoclonal antibodies against CLL2, CCL5, or CCL11. MSC CM was also incubated with an isotype antibody control to exclude any effects due to steric hindrance by the physical presence of antibodies in the CM.

We first used these antibody-treated MSC CM to evaluate ß-defensin expression in keratinocytes, based on the knowledge that wound healing in humans is associated with increased ß-defensin expression in resident human skin cells,[55,56] combined with our finding that ß-defensin expression was significantly increased when equine keratinocytes were stimulated with MSC CM (Figure 5). Keratinocytes incubated with anti-CCL2 Ab-treated MC CM showed significantly reduced ß-defensin expression when compared to incubation with untreated MSC CM as determined by Dunnet's post hoc test (Figure 6C). No statistically significant differences in ß-defensin expression were observed when

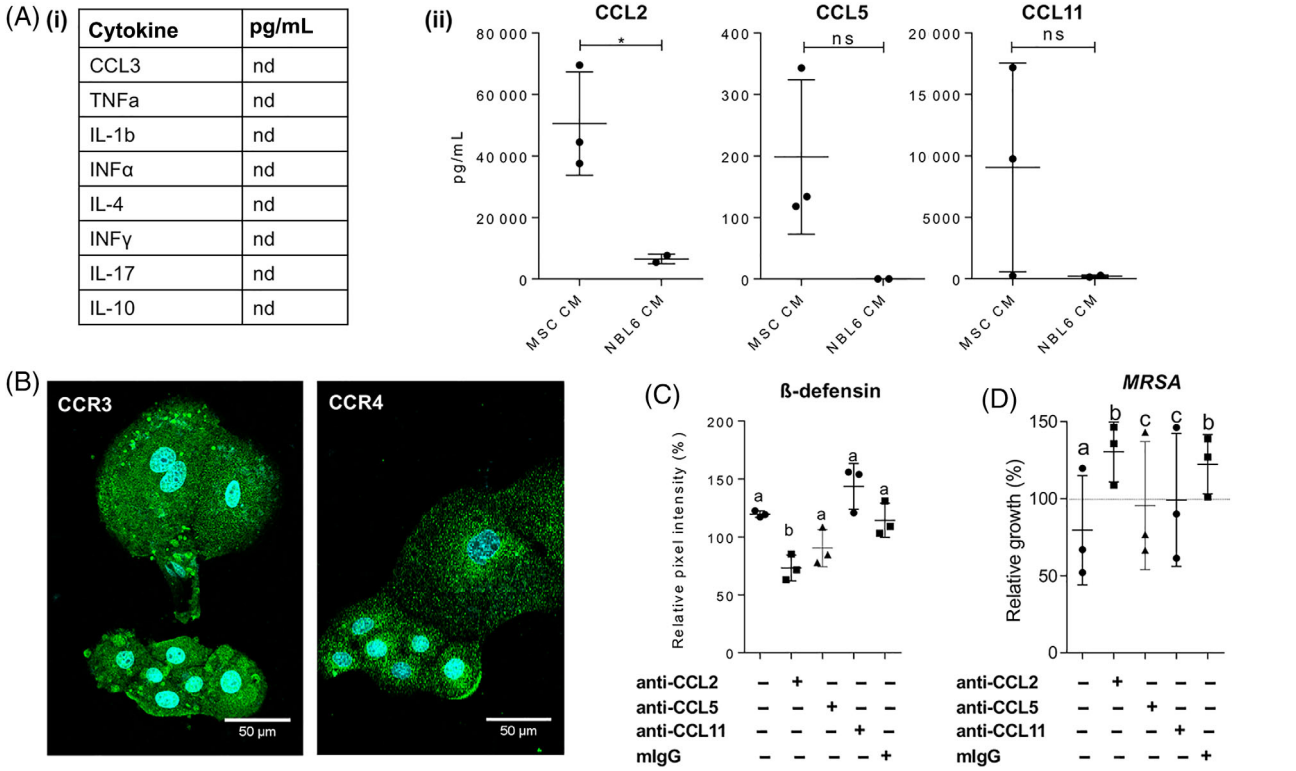

FIGURE 6 Mesenchymal stromal cells (MSCs) secrete chemokines with pro-inflammatory effect on keratinocytes. A, Conditioned medium (CM) from MSCs and dermal fibroblasts (NBL6) were submitted for analysis of secreted cytokines and chemokines using equine-specific immunoassays. Whereas several cytokines were not detected (i), the chemokines CCL2, CCL5, and CCL11 were found to be present in higher concentrations in MSC CM compared to NBL6 CM (ii). Asterisks indicate statistical significance ($P < .05$), nd = not detectable, ns = not statistically significant. B, Equine keratinocytes express CCR3 and CCR4 receptors as visualized using immunofluorescence (IF) staining, with CCR expression in green and DAPI used as nuclear staining (blue). C,D, Chemokines CCL2, CCL5, and CCL11 present in MSC CMs were blocked by preincubation with specific equine anticytokine antibodies for 24 hours. Untreated MSC CM and CM treated with an equine IgG1 isotype control were included as controls. MSC CMs were then used to stimulate equine primary keratinocytes and ß-defensin expression was determined in these keratinocytes (C) or CM was collected from these cells and used in a *MRSA* growth assay (D). Dotted lines indicate ß-defensin expression in equine primary keratinocytes stimulated with DMEM (C) and *MRSA* growth in keratinocyte CM from keratinocytes stimulated with DMEM (D). + and − indicate presence and absence of antibody in MSC CM, respectively. Different letters indicate statistically significant differences. n = 3. DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle medium; *MRSA*, methicillin-resistant *Staphylococcus aureus*

keratinocytes were incubated with anti-CCL5, anti-CCL11, or isotype control Ab-treated MC CM (Figure 6C), the latter indicating that reduced ß-defensin expression was not due to steric hindrance from the antibodies. Next, we used this antibody-treated MSC CM to stimulate keratinocytes for 7 hours, after which keratinocyte CMs were collected and used in a bacterial *MRSA* growth assay. Interestingly, the inhibition of bacterial growth we observed when using CM from keratinocyte that were stimulated with untreated MSC CM was abolished when using CM from keratinocytes that were stimulated with anti-cytokine Ab-treated MSC CM (Figure 6D). However, since a similar effect was also observed when stimulating keratinocytes with isotype control-treated MSC CM (Figure 6C), we cannot exclude that the reduced effect in inhibition of bacterial growth is due to steric hindrance.

Collectively, these results show that equine MSCs secrete various cytokines, most notably CCL2, that can stimulate ß-defensin expression in keratinocytes, as well as enhance the antimicrobial properties of these cells, thus providing a mechanism for the indirect antimicrobial properties of the MSC secretome by stimulating immune responses of surrounding resident skin cells.

4 | DISCUSSION

In the present study, we showed that the MSC secretome effectively reduces live bacteria in *MRSA* biofilms in an ex vivo wound explant model. For this, we used the equine model, which is a recognized translational and clinically relevant animal model for human wound healing.[6] Furthermore, we could demonstrate for the first time that MSCs secrete factors, such as the chemokine CCL2, that stimulate keratinocytes to (a) increase their expression of AMPs, such as ß-defensin and cathelicidin, and/or (b) enhance their efficacy of inhibiting bacterial growth, thus providing an indirect mechanism of the MSC secretome to reduce bacterial growth by stimulating innate antibacterial defense mechanisms in the skin.

We and others previously showed in vitro that MSCs secrete AMP and proteases that can kill bacteria on a single cell level and destroy biofilms by degrading proteins in the ECM, respectively.[14,17] Since the ECM composition of biofilms is highly dependent on the biofilm's microenvironment,[20,21] it was important to evaluate whether these effects of the MSC secretome could also be observed in a more physiologically relevant system, such as an ex vivo biofilm skin explant model. Although the differences between the treatments did not always reach statistical significance, likely because of individual donor variations and/or small sample size, our results did show that MSC CM is similarly effective in biofilm prevention as antibiotics. Interestingly, we found that *MRSA* biofilms in general are more prone to the antimicrobial properties of the MSC CM compared to *MSSA* biofilms. Since the ECM of *MRSA* biofilms contains on average more proteins compared to ECM of *MSSA* biofilms,[57] it could be hypothesized that the proteases present in the MSC CM, as we have previously shown,[17] more effectively destroy *MRSA* biofilms. Another interesting question is whether biofilms grown for a longer time period, for example, 48 or 72 hours instead of the 24 hours used in this study and many other studies, would undergo similar effects after MSC CM treatment. In addition to our ex vivo results showing that the MSC secretome is effective in biofilm control, we also obtained some preliminary findings in vivo further promoting the value of MSC secretome for treatment of infected wounds. Although these data are preliminary because the experimental setup was not optimized and the sample size was too small to perform statistical analysis, we deem them important to report as they form the basis for future in vivo studies. To this end, naturally occurring skin wounds of two horses were treated daily for 6 days with either MSC CM or DMEM (control) that was mixed with hydroxyethyl cellulose. We found that the MSC CM not only reduced the bacterial loads in these naturally occurring equine skin wounds when compared to DMEM treatment (Figure S6A), but also stimulated a shift of the bacterial colonization toward a Gram-negative bacterial spectrum (Figure S6B), indicating that the MSC secretome effectively reduces Gram-positive bacteria, such as *S aureus*. Additional in vivo experiments are planned in the near future, using an equine model of experimentally created wounds on torso and lower legs and infection with biofilm forming *MRSA*, to evaluate the efficacy of the MSC secretome in a complete skin wound environment in vivo.

In addition to evaluating the direct antimicrobial effects of the MSC secretome, this study also assessed potential indirect antimicrobial effects by stimulating innate immune responses of resident skin cells. We found that the MSC secretome stimulated equine keratinocytes to increase their ß-defensin and cathelicidin expression, AMPs that both have been shown to be effective against common skin pathogens like *S aureus* and *Pseudomonas (P) aeruginosa*.[58,59] Interestingly, human keratinocytes have been reported to upregulate ß-defensin and cathelicidin production after recognizing *S aureus* skin infections through pattern recognition receptors in vitro.[60,61] In vivo, ß-defensin and cathelicidin levels in the epidermis of human patients with atopic dermatitis (AD), an allergic skin disorder that is also often seen in horses, dogs, and cats,[62] were significantly decreased compared to ß-defensin and cathelicidin levels in skin from patients with psoriatic lesions.[63] In AD, but not psoriatic lesions, colonization with *S aureus* strains is a hallmark of lesioned skin in patients.[64] Although cathelicidin as well as the three main defensins identified in human skin, ß-defenin-1,-2, and -3, are all expressed in human keratinocytes upon contact with *S aureus*, only ß-defensin-3 is mobilized to the cell surface of keratinocytes in sufficient concentrations to kill *S aureus*, and therefore, is recognized as a key AMP in skin antimicrobial defense.[65] Since AD is a T helper type 2 (T_h2) immune-mediated inflammatory skin disorder, high levels of the T_h2 cytokines IL-4 and IL-13 prevent mobilization of functional ß-defensin-3, and thus, explain why AD patients are prone to *S aureus* skin infections.[66] Building on the results from these human AD studies, our finding that the MSC secretome directly stimulates increased expression of ß-defensin and cathelicidin in keratinocytes provides the rationale that such a treatment approach could be beneficial to treat AD and other types of T_h2-mediated skin infections. Moreover, we found that the increased ß-defensin expression is mediated through CCL2 secreted by MSCs, and interestingly, human CCL2 in combination with CCL7 was shown to limit IL-4 generation, which can result in a better mobilization of AMPs in T_h2 driven cutaneous disorders such as AD.[67]

To our surprise, none of the usual suspects known to drive AMP expression in human keratinocytes, like IFN-γ, TNF-α, IL-17, IL-1ß, or IL-22, were found to be expressed on a mRNA level in equine MSCs or on a protein level in their CM.[46-49] Instead, we did find high levels of the chemokines CCL2, CCL5, and CCL11, which were also part of the multiplex assays, in the MSC CM. Interestingly, activation of the CCL2/CCR4 axis and CCL11/CCR3 axis has been associated with better and faster wound healing in vitro.[68] When we inhibited the function of these cytokines in the MSC CM, using equine-specific antibodies, we found that CM treated with anti-CCL2 antibodies no longer stimulated expression of ß-defensin in keratinocytes. Combined with the notion that wound healing in humans is associated with increased ß-defensin expression in resident human skin cells,[55,56] our results present an intriguing and novel mechanism based on an MSC-secreted CCL2-mediated stimulation of ß-defensin expression in keratinocytes that can promote wound healing, and studies are planned to further investigate this pathway in more detail.

4.1 | Summary

In summary, we have demonstrated that the MSC secretome reduces biofilms of wound pathogens in an ex vivo skin explant model and actively modulates immune responses of keratinocyte via a CCL2-mediated mechanism, thus further supporting the value of MSC secretome-based treatments for infected wounds.

4.2 | Limitations

The evaluation of planktonic bacterial growth inhibition was assessed by measuring the OD in each condition. However, measuring CFU/mL to assess bacterial killing is more standard in the field is, and therefore, no conclusions were made as to whether the MSC CM is bactericidal or bacteriostatic.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

C.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. S.G., R.M.H., B.W.: collection and/or assembly of data. G.R.V.d.W.: conception and design, data analysis and interpretation, manuscript writing, administrative support.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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