Effect of Cationic Antimicrobial Protein CAP37 on Cytokine Profile during Corneal Wound Healing

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Abstract: The cationic antimicrobial protein of 37 kDa (CAP37) mediates proliferation, migration, and adhesion of human corneal epithelial cells and promotes corneal re-epithelialization in mouse. The purpose of this study was to investigate the cytokine profile following abrasion of the corneal epithelium, and to identify the cytokines modulated by topical treatment with CAP37 to determine the mechanism by which CAP37 contributes to the recruitment of inflammatory cells and healing of the cornea.

The corneal epithelium in mouse eyes was removed and wounds were treated with a saline vehicle or human recombinant CAP37. Wounds were visualized with fluoresce in staining at 0, 16, 24 and 48 h. Mouse corneas were excised at 0, 6, 16, 24 and 48 h post corneal abrasion. The excised corneas were analyzed by immunohistochemistry for re-epithelialization and infiltration of inflammatory cells while the expression profiles of thirty-two cytokines were investigated by multiplex analysis.

Results corroborating previous studies showed accelerated wound closure in corneas treated with CAP37 compared to those treated with the saline vehicle. Immunohistochemistry revealed less neutrophil infiltration in CAP37-treated corneas when compared to controls at 24 h. By 48 h post-wounding, histological analysis revealed more staining for neutrophils than the staining observed in the controls. Modulation of cytokine expression occurred for the majority of the cytokines tested at the time of corneal abrasion, during re-epithelialization, and/or by CAP37 treatment. Cytokines monocyte chemoattractant protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES) were induced during re-epithelialization, at the early 16 h time point. Interleukin 6 (IL-6), leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), IL-12p70, macrophage inflammatory protein 1 beta (MIP-1β), and interferon gamma-induced protein 10 (IP-10) were induced at 24 h and unchanged during CAP37 treatment. By contrast, IL-15, monokine induced by gamma interferon (MIG), keratinocyte-derived cytokine (KC), tumor necrosis factor alpha (TNF-α), MIP-1α, IL-1β, and macrophage colony-stimulating factor (M-CSF) were modulated by CAP37 treatment. In general, CAP37 appeared to decrease pro-inflammatory cytokines at 24 h and increase them at 48 h when compared to the control group.

These data demonstrate that CAP37 modulates the production of cytokines in the cornea and suggest that limiting the number of neutrophils recruited during the early inflammatory phase may support corneal re-epithelialization.

Keywords: Corneal abrasion, corneal re-epithelialization, neutrophil, monocyte, macrophage, natural killer cells, immunohistochemistry, multiplex analysis, interleukin, chemokine.

1. INTRODUCTION

The integrity of the cornea is important for optimal vision and for protection against invading pathogens. The cornea is a transparent, avascular structure composed of five distinct layers: the corneal epithelium, Bowman's layer, stroma, Descemet's membrane, and the endothelium from anterior to posterior respectively. The corneal epithelium serves as the external barrier and first line of defense against pathogens and foreign molecules. This layer rests on the epithelial basement membrane. The Bowman's layer is located directly underneath the epithelial basement membrane. This layer forms a strong anterior limit for the underlying stroma and consists of a dense accumulation of collagen fibrils that are tightly interwoven. The corneal stroma is the thickest layer of the cornea and consists of distinct lamellae composed of parallel arranged, tightly packed and very regularly spaced collagen fibrils. The stroma also has a few resident keratocytes and bone marrow-derived defense cells. The Bowman's layer and stroma are formed by highly ordered arrangement of collagen fibrils packed densely, ensuring the high transparency of the cornea. Beneath the stroma, the Descemet's membrane is the basement membrane that supports the innermost layer of the cornea, the corneal endothelium. The corneal endothelium seals the connective tissue stroma to the anterior chamber. It prevents the uncontrolled entrance of foreign substances including water from the anterior chamber and actively removes water from the stroma through the action of water pumps in the endothelial cells. Both the transparency and the refraction power of the cornea ensure optimal vision and its barrier function

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E-ISSN: 2309-6136/17

ensures protection against foreign material. Any breach in the epithelial barrier of the eye, such as corneal abrasion can lead to an infection, which can in turn lead to delayed, pathological healing, complications, and irreversible vision loss [1].

More than 2 million eye injuries and infections occur each year in the United States and corneal abrasion is one of the most common of these ocular injuries. It can be caused by extended wear of contact lenses, sand or particles in the air, eye rubbing, or anything else that accidentally enters in contact with the eyes. Immediate treatment of superficial ocular injuries is still rudimentary and involves the topical administration of prophylactic antibiotics and anesthetic eye drops. The rate of healing determines how long the eye remains vulnerable to bacterial colonization but unfortunately the current standard of care does not involve the use of pro-healing therapeutics [1].

Our lab has been studying the endogenous cationic antimicrobial protein of 37 kDa (CAP37) as a defense factor of the cornea [2] as well as synthetic CAP37-derived peptides with antimicrobial and wound healing therapeutic properties that could be used for ocular injuries and infections [1]. CAP37 is constitutively expressed in polymorphonuclear neutrophils where it was first discovered as part of a battery of antimicrobial proteins and peptides that act as the immune system's first line of defense against pathogens [3]. Following corneal abrasion, neutrophils migrate to the site of injury where they ingest and destroy microorganisms, clean up cellular debris of damaged cells, and release the content of pre-packaged granules containing antimicrobial proteins and peptides, including CAP37 [1]. In addition to being expressed in neutrophils, CAP37 has more recently been found to be induced in the corneal epithelium, stromal fibroblasts, ciliary epithelium, related limbus, ciliary vascular endothelium, and bulbar conjunctiva following intrastromal injection of *Staphylococcus aureus* in the rabbit eye [2]. CAP37 has antimicrobial activity, especially against Gram-negative organisms such as *Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella typhimurium* [4]. CAP37 also binds and neutralizes an outer membrane component of Gram-negative bacteria known as lipopolysaccharide (LPS) [1, 4]. In addition to being a pro-inflammatory protein with chemotactic activity towards monocytes/macrophages, and microglia [3, 5-7], CAP37 also promotes migration, proliferation, and adhesion of human corneal epithelial cells [8]. *In vivo*, CAP37 promotes faster corneal re-epithelialization in mouse [9]. Our lab is currently investigating the mechanisms by which CAP37 supports corneal wound healing, at the cellular and molecular levels. We hypothesize that CAP37 modulates cytokines in the wounded cornea, which in turn modulates the recruitment of inflammatory cells to promote faster healing. This study was designed to compare the cytokine profile in untreated and CAP37-treated corneas during the first 48 h following epithelial abrasion.

2. MATERIAL AND METHODS

2.1. Production of recombinant CAP37

Recombinant CAP37 was produced in human embryonic kidney (HEK) 293 cells using an RSV-PL4 expression vector [10]. The recombinant protein was purified on an HPC4 immunoaffinity column as previously described [7]. All preparations of CAP37 were dialyzed in 0.01% acetic acid and determined to be pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Functional activity was assessed using the modified Boyden chemotaxis chamber assay as previously published [8, 11]. The CAP37 preparation used in this study had < 0.05 endotoxin units per microgram of protein as determined by the limulus amebocyte lysate assay (QCL 1000, Lonza, Basel, Switzerland).

2.2. In Vivo Corneal Epithelial Abrasion Model

Mice (C57BL/6) were anesthetized using ketamine (100 mg/kg) and xylazine (10 ng/kg) and the right cornea was wounded in the following manner: a 2 mm trephine (Miltex, York, PA, USA) was first used to demarcate the mouse cornea. The corneal epithelium was then removed within the demarcated area using an Algerbrush II® (Alger Company, Inc., Lago Vista, TX, USA). Treatments, vehicle control saline (0.9% sodium chloride, pH 5.5; Baxter, Deerfield, IL, USA), and recombinant CAP37 (250 ng/ml in saline), were applied as 20 µl eye drops at 0 and 16 h. Wounds were visualized using sterile fluorescein sodium ophthalmic strips United States Pharmacopeia (Fluorets; Laboratoire Chauvin, Aubenas, France), dampened with sterile PBS at 0, 16, 24, and 48 h. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.3. Histology and Immunohistochemistry

Whole mouse eyes were collected for histology at 0, 6, 24, and 48 h post wounding and were placed in Prefer fixative (Aantech LTD., Battle Creek, MI, USA)

for 20 min before being transferred to 70% ethanol. Tissues were paraffin-embedded and cut at a thickness of 5 µm. Sections were incubated overnight at 4°C with rat monoclonal anti-Ly6G antibody (neutrophil marker, eBioscience™) or IgG control and incubated with anti-rat IgG coupled with horseradish peroxidase (HRP) for 30 min at room temperature. Sections were stained with 3', 3' diaminobenzidine tetrahydrochloride (DAB) chromogen and counterstained with hematoxylin as previously described [9].

2.4. Milliplex® MAP Mouse Cytokine Assay

Mouse corneas were excised at 0, 16, 24 and 48 h post-wounding and immediately flash frozen in liquid nitrogen. Corneas were thawed in 200 µl of buffer containing 20 mM Tris-HCl, 150 mM sodium chloride (NaCl), 1 mM phenylmethylsulfonylfluoride (PMSF), 0.05% Tween® 20, and a 1X cocktail of complete ULTRA Protease Inhibitors (Roche, Indianapolis, IN, USA). Homogenates were created by disrupting the corneas for 10 min at maximum speed in a Bullet Blender® (Next Advance, Inc., Averill Park, NY) using 0.9-2 mm stainless steel beads. Homogenates were centrifuged at 16,000 X *g* for 10 min and the pellet discarded. Protein concentrations of lysates were determined and equal amounts of proteins were assayed in duplicate for 32 cytokines using the Milliplex® MAP mouse cytokine assay (EMD Millipore Corporation, Billerica, MA). Results were obtained and analyzed using the Bio-Plex® 200 program (Bio-Rad, Hercules, CA). Data are expressed as mean ± SEM. The data are representative of 4 to 6 mice per group. The unwounded, wounded saline-treated, and wounded CAP37-treated were compared at each time point, using two-way ANOVA and Tukey's multiple comparisons test, $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.0001$.

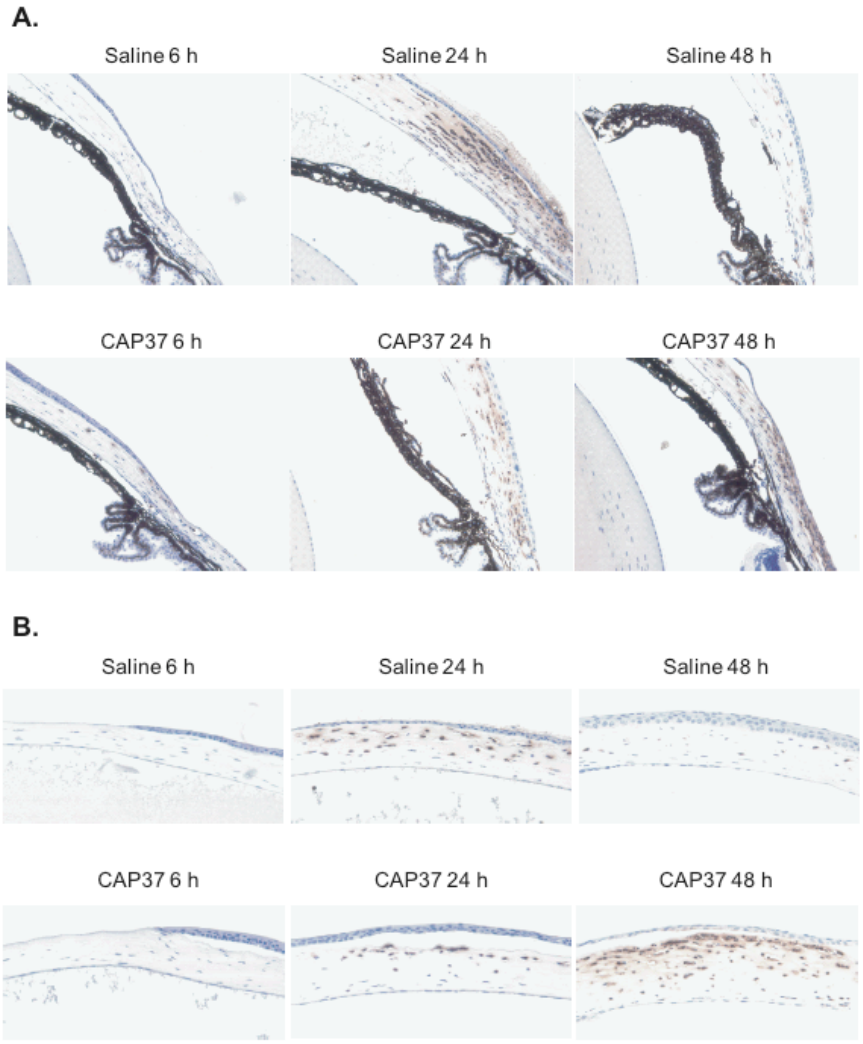

Figure 1: CAP37 induces delayed neutrophil infiltration at 48 h in the cornea during wound healing. Whole eye globes were enucleated at 6 h, 24 h, and 48 h post-wounding, and sections were stained for the neutrophil marker Ly6G (brown signal). (**A**) Representative images of the limbus area are shown. (**B**) Representative images of the corneal wounded areas are shown.

3. RESULTS

3.1. CAP37 Promotes Delayed Infiltration of Neutrophils During Corneal Re-Epithelialization

After corneal abrasion, neutrophils have been shown to migrate through the limbus area and into the injured cornea in two waves, one peaking at 18 h and one peaking around 30 h [12]. As shown in Figure **1**, in absence of CAP37 treatment, neutrophil infiltration in the limbus area (Figure **1A**) and in the cornea (Figure **1B**) was the highest at 24 h and was essentially resolved at 48 h. However, the number of neutrophils detected in the limbus area and in the injured area of the cornea was lower in CAP37-treated than in controls at 24 h, suggesting that the first wave of neutrophils immigrating in the cornea during the early stages of healing was decreased by CAP37 treatment. At 48 h, this trend was reversed, with more neutrophils found in the CAP37-treated eyes, suggesting that there is a delayed response in the CAP37 treatment group. Since re-epithelialization is complete in both groups before 48 h, it is unlikely that a difference in number and/or activation of neutrophils at this late time-point could influence the rate of re-epithelialization. Earlier events, such as the number of neutrophils migrating into corneal stroma during the first wave of neutrophil infiltration that peaks at 18 h after epithelial abrasion, are more likely to modulate the rate of re-epithelialization.

3.2. Changes in Cytokine Profile During Corneal Re-Epithelialization and CAP37 Treatment

To determine if CAP37's modulation of neutrophil recruitment was mediated by a change in cytokines profile, we quantified the expression of thirty-two cytokines during the first 48 h following corneal abrasion. Eleven out of the thirty-two cytokines tested had unchanged expression during corneal abrasion

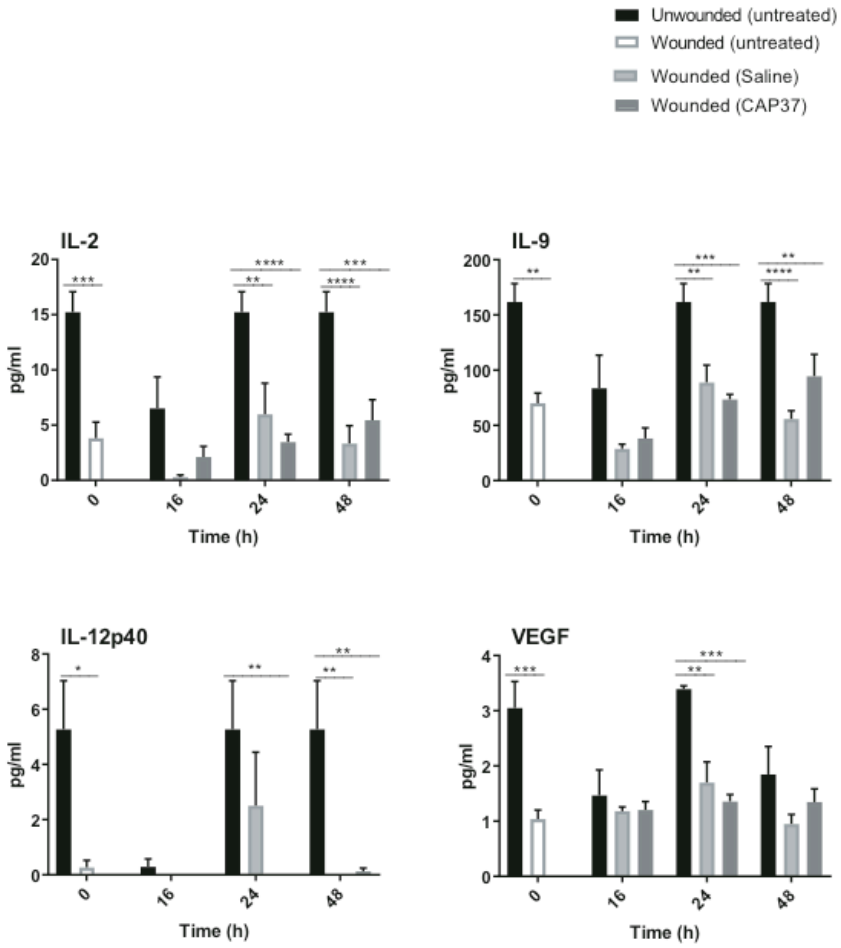

Figure 2: Corneal levels of IL-2, IL-9, IL-12p40 and VEGF are down regulated by corneal abrasion and also during re-epithelialization. A 2 mm circular epithelial abrasion was created on the mouse cornea. The abrasions were treated with vehicle (saline) or CAP37 in saline as an eye drop at 0 and 16 h. Corneas were collected and flash frozen at indicated times post corneal abrasion. Corneal lysates were analyzed for cytokines using the Milliplex® MAP mouse cytokine assay. The mean of 4 to 6 independent values obtained from 4 to 6 mice are shown ± SEM. The unwounded corneas were compared to wounded saline treatment and to wounded CAP37 treatment corneal samples for each time point using two-way ANOVA and Tukey's multiple comparisons test, **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.0001.

and re-epithelialization. Among them, four cytokines were not detected at all in the mouse cornea during the 48 h course of this experiment: interferon gamma (IFN-γ), interleukin 3 (IL-3), interleukin 5 (IL-5), interleukin 13 (IL-13). The following seven were detected but not significantly changed at any time during corneal re-epithelialization: Eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 1 alpha (IL-1α), interleukin 4 (IL-4), interleukin 7 (IL-7), interleukin 17 (IL-17), lipopolysaccharide-inducible CXC chemokine (LIX, also known as CXCL5).

Twenty-one out of the thirty-two cytokines tested had either decreased or increased expression during corneal abrasion and re-epithelialization. Among these twenty-one cytokines, eleven were not modulated by CAP37. The remaining ten were decreased by CAP37 at 24 h and/or increased by CAP37 at 48 h post-abrasion.

The data are categorized according to cytokines that are down regulated by corneal abrasion and during re-epithelialization in Figure **2**, cytokines that are induced during re-epithelialization in Figure **3**,

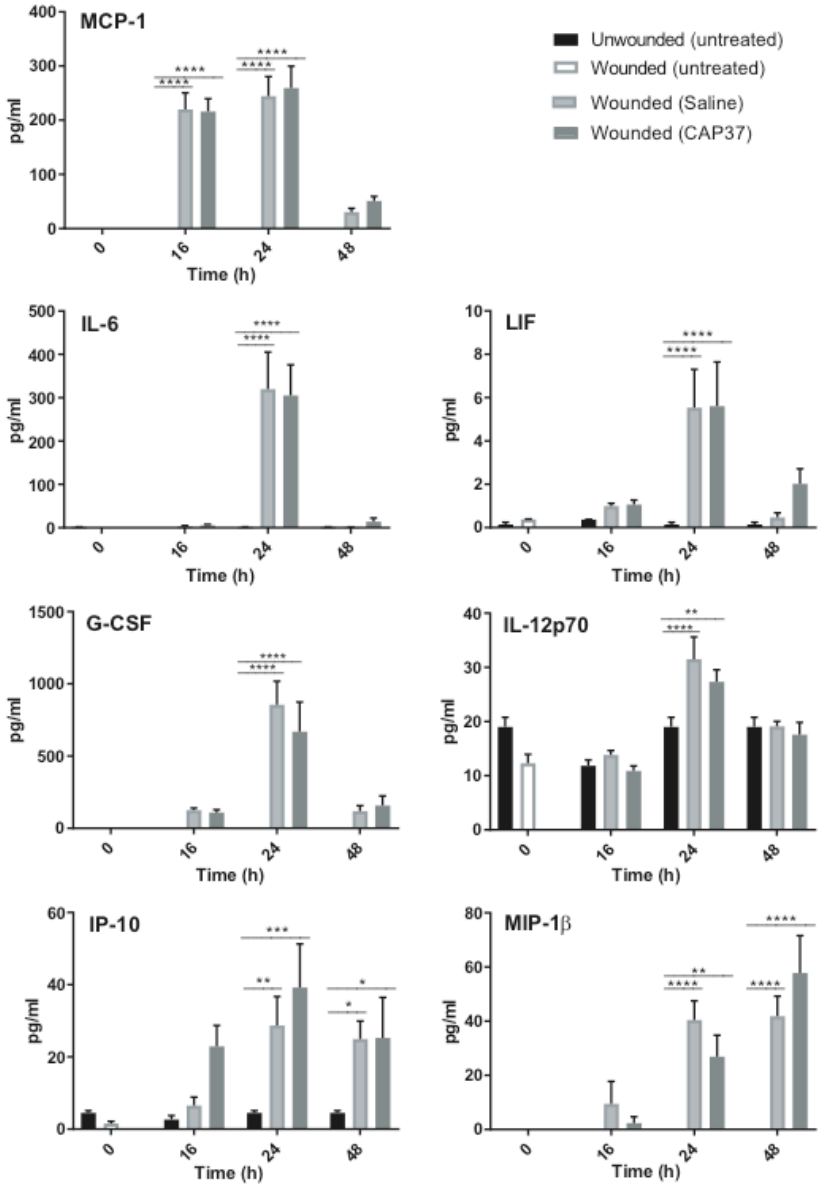

Figure 3: Corneal levels of MCP-1, IL-6, LIF, G-CSF, IL-12p70, IP-10 and MIP-1β are induced during re-epithelialization. A 2 mm circular epithelial abrasion was created on mouse cornea and treated with saline or CAP37 eye drop at 0 and 16 h. Corneas were collected and flash frozen at indicated times post corneal abrasion. Corneal lysates were analyzed for cytokines using the Milliplex® MAP mouse cytokine assay. The mean of 4 to 6 independent values obtained from 4 to 6 mice are shown ± SEM. The levels of cytokines in unwounded corneas were compared to wounded corneas treated with saline and to wounded corneas treated with CAP37 corneal samples for each time point using two-way ANOVA and Tukey's multiple comparisons test, **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.0001.

cytokines that are induced during corneal re-epithelialization and down regulated by CAP37 at 24 h in Figure **4**, and cytokines that are induced during corneal re-epithelialization and up regulated by CAP37 at 48 h in Figure **5**.

As shown in Figure **2**, interleukins IL-2, IL-9, IL-12p40, and vascular endothelial growth factor (VEGF) were significantly and immediately decreased by corneal abrasion, and remained low during the 48 h time-course of the experiment. These cytokines were not affected by CAP37 treatment. Surprisingly, these cytokines appeared to be down regulated at 16 h in unwounded retinas (Figure **2**). The reason for this down-regulation is not clear.

Among the seventeen cytokines that were induced during corneal re-epithelialization, only monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) and regulated on activation, normal T cell expressed and secreted (RANTES, also known as CCL5) were significantly induced at the early 16 h time point. They both remained elevated at 24 h, and then returned to a low expression level at 48 h. MCP-1 expression profile was unchanged by CAP37 treatment (Figure **3**). By contrast, RANTES expression profile

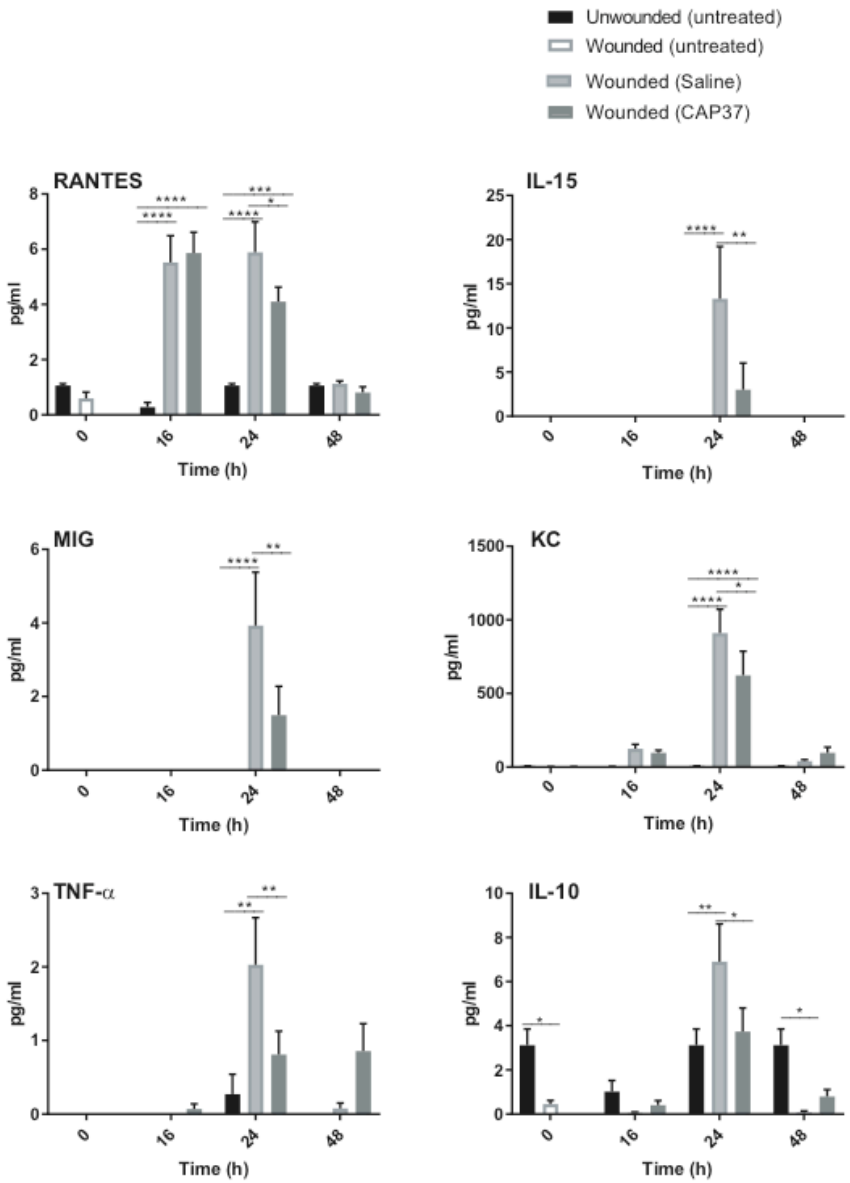

Figure 4: Corneal levels of RANTES, IL-15, MIG, KC, TNF-α and IL-10 are induced during corneal re-epithelialization and down regulated by CAP37 at 24h. A 2 mm circular epithelial abrasion was created on mouse cornea and treated with saline or CAP37 eye drop at 0 and 16 h. Corneas were collected and flash frozen at indicated times post corneal abrasion. Corneal lysates were analyzed for cytokines using the Milliplex® MAP mouse cytokine assay. The mean of 4 to 6 independent values obtained from 4 to 6 mice are shown ± SEM. The levels of cytokines in unwounded corneas were compared to wounded corneas treated with saline and to wounded corneas treated with CAP37 corneal samples for each time point using two-way ANOVA and Tukey's multiple comparisons test, **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.0001.

was modulated by CAP37. As shown in Figure **4**, RANTES returned to low expression level faster in CAP37-treated corneas and was already significantly lower than in untreated corneas at 24 h post corneal abrasion. MCP-1 and RANTES are chemokines that play an active role in attracting leukocytes at the site of injury [13]. As previously described, the first wave of neutrophil influx into corneal stroma after epithelial abrasion peaks at 18 h [12] so MCP-1 and RANTES are likely to participate in attracting this first wave of neutrophils.

Two subsets of macrophages were previously found to undergo dynamic changes during corneal re-epithelialization [14]. The $CCR2^+$ macrophages were shown to peak between 12 and 18 h post epithelial abrasion, overlapping the first wave of infiltrating neutrophils. Pro-inflammatory $CCR2^+$ macrophages express the canonical genes expressed by typical M1 activated macrophages. MCP-1 and RANTES are likely to participate in attracting this first subset of macrophages. A second subset of M2-like anti-inflammatory $CCR2^-$ macrophages was shown to peak at a later stage, around 36 h post epithelial abrasion [14]. Finally, MCP-1 was found to be importantly involved in attracting natural killer (NK) cells to the cornea, which number peaks at 24 h post corneal abrasion [15].

Most of the other fifteen cytokines induced during corneal re-epithelialization were still low at 16 h, peaked at 24 h, and returned to a low level at 48 h. As shown in Figure **3**, IL-6, leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and IL-12p70 are in this category and their profiles were unchanged by CAP37 treatment. These cytokines are produced by activated leukocytes (monocytes, macrophages and neutrophils) that infiltrate the site of injury [16]. Macrophage inflammatory protein 1 beta (MIP-1β, also known as CCL4) and interferon gamma-induced protein 10 (IP-10, also known as CXCL10) were induced at 24 but remained high at 48 h, and their profiles were unchanged by CAP37 (Figure **3**). MIP-1β is a chemoattractant of neutrophils, monocytes, and T cells and is also produced by these cells [17]. IP-10 can be produced by monocytes, endothelial cells and fibroblasts and has been attributed several roles, such as chemoattraction for monocytes/macrophages, T cells, NK cells, and dendritic cells [13]. Interestingly, although IP-10 is not statistically significantly changed in presence of CAP37, it is the only chemokine that shows a trend (P=0.06) for up regulation in response of CAP37 at the early time point 16 h (Figure **3**). IP-10 was shown to be essential for the migration of NK cells to the cornea, and NK cells were shown to support corneal re-epithelialization by decreasing the number of neutrophils emigrating to the cornea during the first wave of neutrophil infiltration [15]. It is thus possible that CAP37 treatment induces immigration of more NK cells in the cornea between 16 and 24 h, which in turn would limit the number of immigrating neutrophils during the same time frame.

Expression of IL-15, monokine induced by gamma interferon (MIG, also known as CXCL9), the murine IL-8 homolog keratinocyte chemoattractant (KC, also known as CXCL1), tumor necrosis factor alpha (TNF-α) and IL-10 were also transiently induced at 24 h, but their induction at 24 h was significantly lowered by CAP37 treatment (Figure **4**). TNF-α is a pro-inflammatory cytokine, produced by M1 type activated macrophages and can be produced by neutrophils [14]. It is a potent chemoattractant for neutrophils [14]. Monocytes and macrophages can also release the pro-inflammatory IL-15 and KC, which attracts neutrophils and monocytes [16]. IL-15 increases the expression of CD11b on neutrophils and is essential in wound healing [1]. Taken together, these results suggest that at 24 h, there might be less neutrophils and monocytes/macrophages infiltrating the cornea in the CAP37-treated group than in the control group. At 48 h, TNF-α is more elevated in the CAP37-treated group than in the vehicle control group, suggesting an increased number of neutrophils and activated macrophages at the site of injury in the CAP37-treated group. IL-10 is an anti-inflammatory cytokine released by $CCR2^-$ macrophages in the M2 alternative activation state. Less M2 type macrophages in the CAP37-treated group would result in less IL-10 release as shown in Figure **4** and more neutrophil recruitment at later time points.

As shown in Figure **5**, macrophage inflammatory protein 1 alpha (MIP-1α, also known as CCL3) and macrophage inflammatory protein 2 (MIP-2, also known as CXCL2), two pro-inflammatory chemokines that attract neutrophils [13] were elevated at 24 h and remained high at 48 h in the CAP37-treated corneas but not in the vehicle-treated group, suggesting again more infiltration of neutrophils in the CAP37-treated corneas at 48 h. Finally, IL-1β and macrophage colony-stimulating factor (M-CSF) were also higher in presence than in absence of CAP37 at 48 h. These are produced by activated M1 macrophages, suggesting more pro-inflammatory cells in the CAP37-treated corneas than in the controls at 48 h post-wounding.

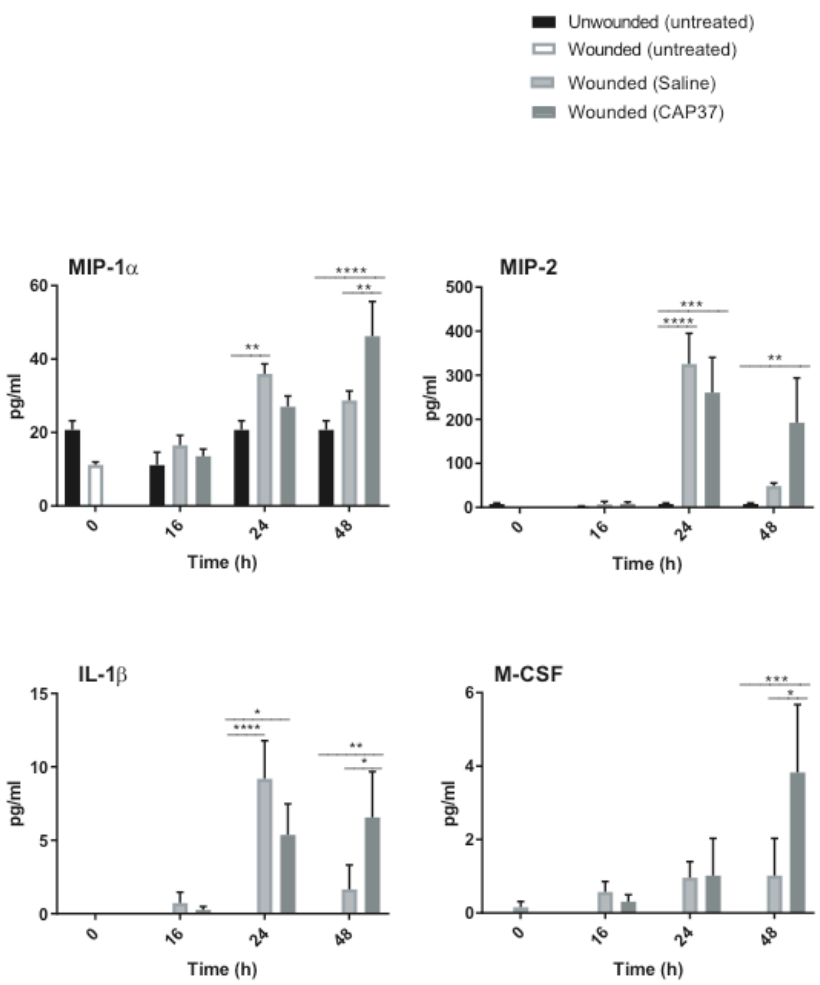

Figure 5: Corneal levels of MIP-1α, MIP-2, IL-1β and M-CSF are induced during corneal re-epithelialization and up regulated by CAP37 at 48 h. A 2 mm circular epithelial abrasion was created on mouse cornea and treated with saline or CAP37 eye drop at 0 and 16 h. Corneas were collected and flash frozen at indicated times post corneal abrasion. Corneal lysates were analyzed for cytokines using the Milliplex® MAP mouse cytokine assay. The mean of 4 to 6 independent values obtained from 4 to 6 mice are shown ± SEM. The levels of cytokines in unwounded corneas were compared to wounded corneas treated with saline and to wounded corneas treated with CAP37 corneal samples for each time point using two-way ANOVA and Tukey's multiple comparisons test, **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.0001.

4. DISCUSSION

Immune cells are recruited after epithelial abrasion by proinflammatory cytokines released from epithelial cells and keratocytes at the injured site [18]. Monocytes, macrophages, neutrophils, and NK cells have been shown to play a role in corneal re-epithelialization. MCP-1, IP-10, RANTES, IL-1, IL-6, and TNF-α have been shown to be important mediators in the recruitment of these cells. Our results demonstrated the release of these cytokines and a number of others, following corneal abrasion. In absence of CAP37 treatment, neutrophil infiltration is the highest at 24 h and is essentially resolved at 48h. By contrast, CAP37 treatment modulates the recruitment of neutrophils. In presence of CAP37, the number of neutrophils is lower than in controls at 24 h, and higher at 48 h. The lower neutrophil recruitment at 24 h in CAP37-treated eyes correlates with the following significant changes in the cytokine profiles: Pro-inflammatory cytokines RANTES, IL-15, MIG, KC, and TNF-α are significantly lower at 24 h in CAP37-treated than in control corneas. Additionally, IP-10 appears higher at 16 h in CAP37-treated corneas which may attract more NK cells, and in turn lower the number of neutrophils.

These results suggest that the number of neutrophils recruited during the early stages prior to 24 hours maybe critical for corneal re-epithelialization. After corneal epithelial injury, the ensuing inflammatory response is necessary for efficient wound healing [18]. While beneficial healing effects are attributed to recruited neutrophils and platelets, dysregulated inflammation (too little or too much) is associated with impaired wound healing. Therefore, the quantification of neutrophils and other inflammatory cells at the site of injury and the kinetics of influx of these cells in absence

and in presence of CAP37 treatment needs to be investigated more closely because this modulation of cellular events could be the mechanism by which CAP37 promotes corneal wound healing in the mouse.

ACKNOWLEDGEMENTS

We thank Craig Land for technical help with the figures, Stephen Neely for his help with statistical analysis of the results, and Jim Henthorn for the utilization of the Flow Cytometry and Imaging Core Facility at the University of Oklahoma Health Sciences Center (OUHSC). The National Eye Institute (NEI) of the National Institutes of Health (NIH) under Award Numbers R21EY026229 and R01EY015534 supported research reported in this publication. This work was also supported by the National Institute of General Medical Sciences (NIGMS) of the NIH under Award Number U54GM104938. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Received on 30-12-2017 Accepted on 05-01-2018 Published on 10-01-2018

DOI: https://doi.org:10.12974:2309-6136.2017.05.04