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Cyr61/CCN1 induces CCL20 production by keratinocyte *via* activating p38 and JNK/AP-1 pathway in psoriasis

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ABSTRACT

Background: Psoriasis is a common chronic skin disease characterized by epidermal hyperplasia and inflammation. Cysteine-rich angiogenic inducer 61 (Cyr61/CCN1) has recently been implicated in psoriasis pathogenesis by promoting keratinocyte activation. However, the mechanisms by which CCN1 enhances cutaneous inflammation are not fully understood.

Objective: In this study, we investigated the role of CCN1 on the expression of CCL20 in human keratinocyte.

Methods and results: By double-label immunofluorescence staining, we first identified that the expression of CCN1 colocalized well with CCL20 production in the epidermis of psoriasis skin lesion. Furthermore, *in vivo*, blocking or knockdown CCN1 expression ameliorated skin inflammation and reduced the expression of CCL20 in both imiquimod and IL-23-induced psoriasis-like mouse models, which indicated that CCN1 might be involved in the regulation of CCL20 production in psoriasis. Next, *in vitro*, we stimulated primary normal human epidermal keratinocyte (NHEK) with exogenous protein CCN1 and found that CCN1 directly upregulated CCL20 production independent of TNF-α, IL-22 and IL-17 pathway. Lastly, the signaling pathway study showed that CCN1 enhanced the binding of AP-1 to the CCL20 promoter *via* crosstalk with p38 and JNK.

Conclusions: Our study demonstrates that CCN1 stimulates CCL20 production *in vitro* and *in vivo*, and thus supports the notion that overexpressed CCN1 in hyperproliferating keratinocyte is functionally involved in the recruitment of inflammatory cells to skin lesions affected by psoriasis.

1. Introduction

Psoriasis is a common, potentially disfiguring immune-mediated skin disease characterized by hyperproliferation, aberrant differentiation of keratinocytes, chronic inflammation, and high relapse rate [1–3]. The overall prevalence of this condition is 0.2%–5% worldwide, depending on the population of origin [4–7]. Most scientific research refers to the common clinical variant termed psoriasis vulgaris, which affects approximately 85% to 90% of all patients with the disease [8,9]. Physical manifestations of psoriasis include the presence of raised, well-demarcated, erythematous oval plaques with adherent silvery scales [3,10]. The precise pathogenesis of psoriasis is complex with interplay between genetics, external environmental triggers, skin barrier disruption, and immune dysfunction [11–13]. Substantial studies have illustrated the importance of both keratinocytes and the immune system in the pathophysiology of psoriasis [14–16]. An early cellular event in the development of psoriatic lesions is the infiltration of target sites by activated DCs, T cells and γdT cells, which, in turn, produce inflammatory mediators, such as IL-17, induce epidermal hyperplasia, and may act with keratinocytes and dermal macrophages to sustain a cycle of inflammation that finally leads to the psoriatic phenotype [17,18]. In this regard, knowing the molecular mechanisms involved in the formation of dermal aggregates of DCs and T cells in psoriasis may be important for further understanding of chronic inflammation.

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Chemokine, CC chemokine ligand (CCL) 20 is a key mediator of leukocytes migration during pathogenic insult and also play a prominent role in homeostasis [19]. Most, but not all, chemokine receptors can interact with more than one chemokine; CCL20 is the only chemokine known to interact with CC chemokine receptor 6 (CCR6), a property shared with the antimicrobial β-defensins [19]. In psoriasis, CCL20 is produced predominantly by keratinocyte and this chemotactic molecule is a key stimulus for chemoattracting both CCR6-positive immature DCs and T cells (especially IL-17-producing ones) from blood into inflamed cutaneous tissue [20–22]. Moreover, Yihua Cai et al. have revealed that CCR6 is constitutively expressed in dermal γδ T cell subset which displays IL-17-producing capability [23]. Summarily, overexpression of CCL20 results in enhanced dendritic cell trafficking, subsequent T-cell priming, and pathologic inflammation. Previous study has showed that Th17 cytokines (IL-22, IL-17 and TNF-α) could stimulate CCL20 production which suggest a mechanism of Th17 cells promoting their continued presence in psoriatic tissue [24]. However, little is known about whether there are other stimuli affecting CCL20 production by keratinocyte in psoriasis.

Cysteine-rich angiogenic inducer 61 (Cyr61/CCN1) is a matricellular protein of the CCN family, comprising six secreted proteins [25]. Matricellular proteins are secreted extracellular matrix (ECM) proteins that regulate diverse cell functions *via* its interaction with, and integration of, integrins, proteoglycans and growth factor signaling [26]. Initially, CCN1 was identified as an immediate–early gene that plays major roles in embryonic development, tissue injury repair, fibrosis, angiogenesis and tumorgenesis [25]. For example, CCN1-null mice suffer embryonic death due to loss of vascular integrity and impaired placental development [27]. However, accumulating evidence has indicated that CCN1 is involved in inflammatory responses as a new pro-inflammatory factor. In the lung, CCN1 overexpression exacerbates lung injury and causes neutrophilic alveolitis and obstructive bronchiolitis in mice [28]. In rheumatoid arthritis, CCN1 up-regulates the cytokines production (IL-6/IL-8/IL-1β) of synovium cells, which in turn promotes Th17 differentiation, activates macrophages and recruites neutrophils respectively [29–31]. Moreover, we reported previously that the expression of CCN1 is greatly enhanced in lesion and non-lesion skin in psoriasis patients, and increased expression of CCN1 aggravates epidermal hyperplasia and inflammation *via* promoting keratinocyte activation in psoriasis [32]. Though CCN1 has such profound effects on keratinocyte physiology, the effects of CCN1 on the expression of CCL20 in keratinocyte remain unknown yet.

In this report, we first identified that CCN1 colocalized well with the expression of CCL20 in the epidermis of psoriasis lesion by confocal colocalization studies. Furthermore *in vivo*, blocking or knockdown CCN1 expression ameliorates skin inflammation and reduces the expression of CCL20 in imiquimod (IMQ)/IL-23-induced psoriasis mouse models, which indicated CCN1 might be involved in the regulation of CCL20 production in psoriasis. *In vitro*, we stimulated primary normal human epidermal keratinocyte (NHEK) with exogenous protein CCN1 and found that CCN1 directly upregulated CCL20 production in an IL-22, IL-17 and TNF-α independent pathway. Moreover, CCN1 enhanced the binding of AP-1 complexes to the CCL20 promoter *via* crosstalk with p38 and JNK. Taken together with the results of our existing and previous work, these findings provide new evidence that CCN1 participates in psoriasis pathogenesis not only through promoting keratinocyte activation but also triggering the recruitment of inflammatory cells (Th17, γδT and DC).

2. Materials and methods

2.1. Human skin specimens

A total of 4 formalin-fixed, paraffin-embedded tissue samples were obtained from patients with a dermatologist-confirmed diagnosis of psoriasis vulgaris. Exclusion criteria included previous treatment received, psoriasis with local complications such as ulceration, bleeding or local infection. Psoriatic skin samples were obtained by punch biopsy under local anesthesia (1% Lidocaine). The fresh tissue samples were snap-frozen and stored in liquid nitrogen or fixed in 4% paraformaldehyde solution and embedded in paraffin. The study was performed in accordance with the declaration of Helsinki Principles and approved by the Institutional Medical Ethics Review Board of the Shanghai Ninth People's Hospital. All the patients provided written informed consent to allow the collection and use of their tissues for the present study.

2.2. Mice

Female, BABL/c mice, six- to eight-weeks old, were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science. Mice were housed under pathogen-free and climate-controlled conditions with free access to water and food. All animal procedures were performed in accordance with guidelines and approved by the Animal Care and Use Committee of Shanghai Jiao tong University School of Medicine (2013028). To ameliorate any suffering of mice observed throughout these experimental studies, mice were euthanized by CO_2 inhalation.

2.3. Induced psoriasis-like mouse models and treatment

The establishment and treatment of psoriasis-like mouse models induced by imiquimod (IMQ)/IL-23 were as same as previously description[32]. Briefly, for the IMQ-induced mouse model, about 62.5 mg and 20.8 mg IMQ cream (Aldara; 3 M Pharmaceutical, St Paul, MN) was applied daily on the shaved back and ear skin respectively for 16 days. Treatment with mAb 093G9 was carried on 200 μg/mouse ip twice a week. For the IL-23-induced mouse model, 2×10^6 lentivirus particles targeting CCN1 were injected intradermally into the mouse ears 3 days before the IL-23 (eBioscience, USA) injection. And then the mice received 1 μg/mouse IL-23 every day for 7 days.

2.4. Primary human epidermal keratinocyte culture

Primary normal human epidermal keratinocytes were purchased from Lifeline Cell Technology (Walkersville, MD USA). Cells were cultured in Dermalife basal Medium supplemented with DermaLife K LifeFactors® Kit (Lifeline Cell Technology), which contained 6 mM L-Glutamine, 0.5 ng/ml rh TGF-a, 100 ng/ml hydrocortisone hemisuccinate, 0.4% Extract P, 1.0 μM Epinephrine, 5 μg/ml rh-insulin and 5 μg/ml apo-transferrin in supplemented medium. The media was changed at least every 24 h. When 80%-90% confluence was reached, cells were propagated with a detach kit from Lifeline Cell Technology (Walkersville, MD USA) and used within 6 passages for our study. Keratinocyte, pretreated with neutralizing antibody anti-CCN1, anti-IL-22/TNF-α/α6/β1 (R&D, Minneapolis, MN) or not, was stimulated with exogenous CCN1, and incubated at a humidified 5% CO_2 incubator. At indicated time points, conditioned culture medium and cell lysate were collected for protein and mRNA quantification.