Journal of Dermatological Science 78 (2015) 206–214

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

IL-9 induces IL-8 production via STIM1 activation and ERK phosphorylation in epidermal keratinocytes: A plausible mechanism of IL-9R in atopic dermatitis

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ARTICLE INFO

Article history:
Received 2 December 2014
Received in revised form 2 March 2015
Accepted 3 March 2015

Keywords:
IL-9
IL-9R
IL-8
STIM1
Keratinocytes
ERK

ABSTRACT

Background: IL-9 and its receptor play important roles in the pathogenesis of asthma. Its role in atopic dermatitis (AD) was examined in just a few studies, including nucleotide polymorphisms, increased transcriptional levels of IL-9 and IL-9R in diseased skin, and an association of blood IL-9 levels with clinical severity.
Objective: Little was known about the pathophysiological regulation of IL-9/IL-9R in AD skin. We asked whether IL-9R was expressed in epidermal keratinocytes; if so, what the functional outcome, cytokine production, and signaling pathway of IL-9/IL-9R in keratinocytes are.
Methods: We measured and compared the expression of IL-9R in skin from AD patients and controls by immunofluorescence. We also performed in vitro studies on the IL-9-treated primary keratinocytes, including flow cytometry for IL-9R expressions, Western blotting for mTOR, S6K, ERK, p38, and STAT3 activations, ELISA for cytokine levels, and immunofluorescence for STIM1.
Results: We found that IL-9R was indeed expressed in keratinocytes but not in fibroblasts. Its expression in keratinocytes was enhanced by IL-4 but not by TGF-beta1. IL-9 induced a moderate production of IL-8 but not CXCL16, CCL22, TSLP, nor IL-33. IL-9 induced formation of STIM1-puncta. IL-9 induced ERK phosphorylation both dose- and time-dependently, but not mTOR, S6K, p38, or STAT3. Pretreatment with U0126 (ERK inhibitor) but not rapamycin (mTOR inhibitor) abrogated the IL-9-mediated IL-8 production. Blockage of STIM1 with BTP2 or SKF96265 abrogated ERK phosphorylation and IL-8 production induced by IL-9.
Conclusion: This study represents the first to show the regulation of the IL-9-STIM1-ERK-IL-8 axis in keratinocyte, and how the axis might play an important role in the pathophysiology of AD.

1. Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease usually associated with personal or family history of allergic diseases, including AD, asthma, and allergic rhinitis [1,2]. Clinically, it occurs with eczematous changes and intense itching [3]. AD skin is characterized microscopically by spongiotic dermatitis with moderate dermal immune cell infiltrates. The immune abnormalities of AD have been found

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http://dx.doi.org/10.1016/j.jdermsci.2015.03.004
0923-1811/

to involve aberrant T helper cell polarization, imbalances in cytokines and chemokines, and dysregulation of innate immunity. Filaggrin mutations [4] and thymus stromal lymphopoietin (TSLP) [5] from epidermal keratinocytes are reported to mediate the development of asthma, suggesting that epidermal keratinocytes play a major role in the pathogenesis of AD and bronchial asthma. In fact, one of our previous studies found that IL-31, a pruritus-related cytokine, activated IL-31R, induced calcium propagation, activated STAT3, and released beta-endorphin in keratinocytes [6].

In AD skin, a significant portion of T cells is polarized to Th2 subsets. IL-9 was previously thought to be a Th2 cytokine, but it is now known that TGF-beta1 is able to reprogram Th2 cells to produce IL-9 instead of Th2 cytokines [7]. The transcriptional factors of IL-9, which is produced by mast cells, T cells, and NKT cells [8], include PU.1 [9] and IRF4 [10]. Its receptor, IL-9R, is expressed in T cells as well as bronchial epithelial cells [11], neutrophils [12], mast cells [13], B cells [14], and human airway smooth muscle cells [15]. The activation of IL-9R causes different physiological functions in different cells. One recent mouse study has described the biological role of IL-9 secreting cells in the mediation of tumor immunity to melanoma [16]. Only few studies examined the role of IL-9R of keratinocytes in AD. One recent study showed that K5.hTGF-β1 transgenic mice exhibiting a psoriasis-like phenotype with increased expressions of IL-9 and IL-9R in skin [17]. In the same study, they found IL-9R is increased in the basal layer of epidermis in psoriatic skin [17]. It is not known, however, what the functional outcome and the regulatory mechanisms of IL-9R in AD skin are.

Previous translational studies of IL-9 have mainly focused on asthma. In mice, transgenic expression of IL-9 causes allergic inflammation [18,19] while antibodies against IL-9 ameliorates its development [20]. In humans, IL-9 induces mucus production by lung epithelial cells [21]. The expression of IL-9 and IL-9R has also been found to be higher in the bronchial epithelium of asthmatic patients compared to controls [22,23]. In asthmatic families, IL-9 gene polymorphism and environmental exposures synergistically affect the clinical aggravation of asthma [24]. Compared with the large number studies addressing the role of IL-9/IL-9R in asthma, there are only a limited number of studies investigating its role in AD. One of these studies, reported that transcriptional level of IL-9 and IL-9 receptor is significantly increased in lesional skin areas of AD patients as compared to normal control skin [13], and another has reported a correlation between IL-9 level in the blood and severity of AD [25]. In addition, IL-9 single nucleotide polymorphisms have been associated with the susceptibility of AD [26]. Since keratinocytes make up the majority of cells in the skin, we sought to investigate whether IL-9R is expressed in keratinocytes and, if so, what functional outcomes and pathophysiological regulations are in AD.

2. Materials and methods

2.1. Reagents and ELISAs

IL-9 was purchased from R&D (Minneapolis, MN, USA). ELISA kits for IL-8, CXCL16, IL-33, CXCL1, and CCL22 were also purchased from R&D to measure their levels in conditioned media from IL-9-treated keratinocytes. ELISA kit for TSLP was obtained from BioLegend (San Diego, CA). Multiplex cytokine array kits, including Human cytokine array panel A (ARY005) and Human chemokine array kit (ARY017), were purchased from R&D (Minneapolis, MN, USA). The mTOR inhibitor rapamycin was obtained from Sigma–Aldrich (St. Louis, MO) and ERK inhibitor (U0126) from Promega (Madison, WI, USA).

2.2. Primary culture of epidermal keratinocytes and dermal fibroblasts

Normal human keratinocytes were obtained from adult foreskins through routine circumcision. The keratinocytes were harvested and cultured as described previously [27]. Briefly, skin specimens were washed with PBS (pH 7.2), cut into small pieces, and harvested in a medium containing 0.25% trypsin (Gibco, Grand Island, NY) overnight at 4 °C. The epidermal sheet was lifted from the dermis by a fine forceps. The epidermal cells were spun down by centrifugation (500 × *g*, 10 min) and then were dispersed into individual cells by repeated aspiration. The keratinocytes were gently resuspended in 5 ml of keratinocyte–serum-free medium (Gibco), which contained 25 μg/ml bovine pituitary extract and 5 ng/ml recombinant human epidermal growth factor. Keratinocytes at the third passage were then grown in a keratinocyte–serum-free medium without bovine pituitary extract and recombinant human epidermal growth factor for 24 h before experimentation.

For fibroblasts, the primary fibroblasts were harvested as described previously [28]. Briefly, dermal parts after lifting of epidermis were cut to 1–2 mm^3 and incubated with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.1 mM 2-mercaptoethanol (Sigma–Aldrich), along with 50 units/ml of penicillin and 50 g/ml of streptomycin (Invitrogen). The 3rd passage of human fibroblasts were used for the experiments.

2.3. Cytokine stimulations and blocking experiments

In preparation for the measurement of IL-9R, we treated cells with IL-4 (up to 10 ng/ml) and/or TGF-beta1 (up to 50 ng/ml) for 24 h and stained cells with 1:100 mouse anti-IL-9R (BioLegend, San Diego, CA) overnight at 4 °C. We then measured the expressions of IL-9R by flow cytometry (BD Biosciences, San Jose, CA, USA). To measure what cytokines/chemokines would be induced by IL-9 treatment, we treated keratinocytes with IL-9 at 0, 5, 10, and 20 ng/ml for 12 or 24 h and then measured the expressions of individual cytokines by ELISA. To determine the intracellular signaling pathways in keratinocytes by IL-9, we treated cells with IL-9 at 10 ng/ml for 0, 5, 10, 15, 20, and 30 min or IL-9 at 0, 1, 5, 10, and 20 ng/ml for 10 min and then evaluated the signaling pathways by Western blot. To determine whether IL-9 might induce the production of IL-8 through ERK or mTOR pathways, we pretreated keratinocytes with U0126 (an ERK inhibitor) or rapamycin (an mTOR inhibitor) at indicated concentrations for 2 h prior to their treatment with IL-9. Finally, for STIM1 blocking, we pretreated keratinocytes with STIM1 inhibitors (BTP-2 up to 0.1 nM or SKF96365 up to 50 nM, both from Sigma–Aldrich) for 24 h before IL-9 treatments.

2.4. Immunoflorescent study for IL-9R in skin

Immunofluorescent studies for IL-9R were performed on 5-μm serial tissue sections obtained from skin of AD patients (active and stable), patients with psoriasis, and controls (n = 3, 3, 2, and 5, respectively). The protocol for these studies were approved by IRB from the affiliated hospital. All participants provided written informed consent. To perform these studies, all sections were blocked with 3% bovine serum albumin at room temperature for 2 h. After the blocking, the sections were incubated with rabbit polyclonal antihuman IL-9R (1:200; GeneTex, GTX87356, Irvine, CA) at 4 °C overnight. Image analysis was performed using NIH IMAGEJ (http://rsbweb.nih.gov/ij/). Fluorescent intensity index (0–255) was calculated in five random mid-power fields above the dermoepidermal junction.