IL-4 up-regulates epidermal chemotactic, angiogenic, and pro-inflammatory genes and down-regulates antimicrobial genes in vivo and in vitro: relevant in the pathogenesis of atopic dermatitis

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Abbreviations: AD, atopic dermatitis; AMPs, antimicrobial peptides; FBS, fetal bovine serum; HBD2, human β-defensin-2; NOS2, nitric oxide synthase 2; OSMR, oncostatin M receptor; Tg, transgenic; TLRs, Toll like receptors; for rest of the abbreviations, see table 2 and 3.

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Abstract

Atopic dermatitis (AD) is a common chronic inflammatory skin disease. Although the pathogenesis of AD is not fully understood, we and others have shown that IL-4 plays a key role. In this study we aimed to identify keratinocyte genes regulated by IL-4 that may play important roles in the pathophysiology of AD. HaCat cells were treated with IL-4 at various concentrations for 24 hours, and PCR gene array on inflammation/autoimmunity was performed three times for analysis of differential gene expression. Of all the 370 genes examined, 32 and 53 genes are up- and down-regulated, respectively. Specifically related to AD, chemokines CCL3L1, CCL8, CCL24, CCL25, CCL26, CXCL6 and CXCL16 are up-regulated by IL-4. Pro-inflammatory factors, such as IL-19, IL-20, IL-1α, IL-12Rβ2, IL-25, IL-31RA, OSMR and nitric oxide synthase 2, are also up-regulated. In addition, IL-4 up-regulates VEGFA, a pro-angiogenic factor. In contrast, antimicrobial peptides (AMPs) or factors involved in APM production, such as IFN-κ, S100s, Toll-like receptors, and several chemokines are down-regulated. Similarly IL-4 also down-regulates TNF-α, lymphotoxin-β, an IgE suppressor, TNFSF18, a T-cells function regulator, and the glucocorticoid receptor. On the in vivo level, real-time RT-PCR on the selected genes confirmed that IL-4 up-regulates chemokines, proinflammatory cytokines while it suppresses AMP production related genes in the skin obtained from IL-4 Tg mice. Detailed examination of these genes will delineate their specific roles in chemotaxis, inflammation, angiogenesis and AMP production, all of which may contribute to the development and progression of AD.

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Key Words: IL-4, PCR array, Atopic dermatitis, IL-4 transgenic mice

1. Introduction

Atopic dermatitis (AD), a chronic inflammatory skin disease, affects 10-20% of children and 1-3% of adults in developed countries [1]. In addition to skin manifestations, more than 50% of AD patients may have asthma or other atopic disorders [2]. However, the etiology and pathogenesis of AD have not been fully understood. We and others have shown that IL-4 might play an important role in the pathogenesis of AD [3-7]. Significant increase of IL-4-expressing T cells is detected in the early human AD skin lesions [8]. Through over-expressing IL-4 in the basal epidermis using a basal keratinocyte-specific keratin-14 promoter/enhancer, we have generated a mouse AD animal model, IL-4 transgenic (Tg) mice [9]. These Tg mice spontaneously develop skin lesions, which satisfy the clinical and histological diagnostic criteria for human AD, while the non-Tg littermates are lesion-free [9]. Using these Tg mice, we have shown in vivo that IL-4 over-expression in the epidermis leads to up-regulation of several proinflammatory cytokines [7], B cell activation molecules [10], angiogenic factors [5], and critical adhesion molecules [11].

Since keratinocytes are a dominant epidermal cell type that is known to participate in immune responses [8], and they are located adjacent to the IL-4 expression milieu in both human AD [8] and the IL-4 Tg mice [9], we aim to identify the keratinocyte genes that are directly regulated by IL-4. Compared to the traditional hybridization-based microarray technique, PCR array method combines quantitative real-time PCR with the multiple gene analysis in a single microarray, thus providing high levels of sensitivity, reproducibility and specificity. Different from ordinary microarray analysis, it does not

require RT-PCR confirmation. Our data showed that in the presence of IL-4, the expression of many chemotactic, pro-angiogenic and pro-inflammatory genes in cultured human keratinocytes is up-regulated, while many antimicrobial genes are down-regulated. Furthermore, selected real-time RT-PCR on IL-4 Tg mice confirmed our in vitro findings.

2. Materials and methods

2.1. Materials

IL-4, cell culture medium DMEM, fetal bovine serum (FBS), charcoal stripped FBS, Penicillin/Streptomycin, nonessential amino acids and sodium pyruvate were obtained from Invitrogen (Carlsbad, CA); RNA isolation kit was from Qiagen (Valencia, CA); First strand kit, qPCR master mix, and Inflammatory Response and Autoimmunity 384HT PCR Arrays were purchased from SABiosciences (catalogue number: PAHS-3803Z; Frederick, MD).

2.2. Animals

The IL-4 Tg mice were kept at 25 C with a 14-h light/10-h dark cycle. Skin tissues were collected before the onset of AD. Non-Tg littermates were used as controls. All experimental procedures were performed in accordance with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago.

2.3. Cell culture

Immortalized human keratinocytes, HaCat cells, were grown in DMEM medium supplemented with FBS (10%), nonessential amino acids, and antibiotic solution and incubated in a humidified atmosphere of 5% CO_2 at 37 C. Culture media were replaced every 48 hours. Cells were treated with various concentrations of IL-4 in DMEM medium supplemented with 1% charcoal-dextran-treated FBS for 24 hours. Cells were washed twice with ice cold PBS and were then frozen at –80 C until RNA extraction.

2.4. PCR array

Inflammatory Response and Autoimmunity 384HT PCR Array analysis was performed according to the manufacturer's instructions. Briefly, 1 μg of mRNA was extracted, DNase I digested, and reversely transcribed to cDNA. 10ul of master mix with cDNA was loaded into each well of the 384-well PCR array. The PCR reaction was run on a ABI 7900HT PCR machine. The cycling conditions were: 95ºC, 10 min; 40 cycles of (95ºC, 15 sec; 60ºC, 60 sec). The experiments were repeated three times. The raw data were analyzed by a web based Array Analysis Software provided by SABiosciences. Genes with at least 2-fold up-regulation or down-regulation in comparison to untreated cells and with a P-value of less than 0.05 were identified.

2.5. RNA isolation and real-time RT-PCR analysis

Total RNA from mouse skin tissues was isolated using Trizol reagent according to the manufacturer's instructions. The RT and real-time PCRs were performed as previously

described [12]. Briefly 1 μg of total RNA was reverse-transcribed and the final volume was 100 μl. The PCR were carried out in duplicate using a Stratagene Mx3000 real-time PCR machine (Santa Clara, CA). GAPDH was used as the internal reference. We used the standard –ddCT method to measure the relative mRNA expression. Primers synthesized by Integrated DNA Technologies (Coralville, IA) are listed in Table 1.

2.6. Statistical analysis

Data were examined by t-test (Figs. 1 and 2) using Prism software (GraphPad Software, Inc., San Diego, CA). Values were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. IL-4 regulates factors that are involved in chemotaxis in HaCat cells

As shown in table 2 and 3, IL-4 up-regulates several chemokines and related chemotactic factors, such as CCL8, CCL24, CCL25, CCL26, CCL3L1, CXCL6, CXCL16, CD74 and Stabilin 1, which attract different inflammatory cells to the skin. As eosinophil chemotactic factors, up-regulation of both CCL24 and CCL26 is associated with AD [13-14]. Furthermore, CD74, which works synergically with eotaxin to activate eosinophils [15], is up-regulated by IL-4. In addition, Stabilin 1, which is known for mediating migration of lymphocytes to the draining lymph nodes and granulocytes to the inflammatory site, is also up-regulated by IL-4 in HaCat cells. Conversely, TNF superfamily 9 (TNFSF9), which has a known function of inducing eosinophil apoptosis [16], is down-regulated by IL-4, thus it further prolongs the pro-inflammatory effect of

eosinophils. Consistent with our finding, TNFSF9 knockout mice displayed skin lesion, renal damage, lymphadenopathy, and high mortality, probably due to over-activation of $CD4^+$ T-cell and B-cell function [17]. Together, the up-regulation of the above group of keratinocyte-produced chemotactic factors by IL-4 might account in part for the prominent lymphocyte and eosinophil infiltration in skin lesions of human AD patients and our IL-4 Tg mice.

3.2. IL-4 regulates factors that are involved in inflammation in HaCat cells

Along with chemotactic factors, several proinflammatory factors, such as IL-1α, IL-19, IL-20, IL-25, IL-27, IL-12Rβ2, IL31RA, nitric oxide synthase 2 (NOS2), oncostatin M receptor (OSMR), and SOCS2 are up-regulated by IL-4 in HaCat cells. Consistent with our findings, Renne *et al.* demonstrated that IL-1 in the skin is essential for the development of chronic AD [18]. Interestingly, IL-4 also down-regulates the expression of IL-1R antagonist in our study, thus leaving the IL-1 pro-inflammatory function unopposed. Recently it was reported that certain IL-12Rβ2 polymorphisms are associated with AD [19] and this is in line with our data of its up-regulation by IL-4. More literature consistent with our data includes the report that keratinocytes derived from inflammatory skin (like AD and psoriasis) produce high amount of IL-19 and IL-20 [20]. IL-25, IL-33 and thymic stromal lymphopoetin produced by keratinocytes are known to work collectively to drive Th2 polarization [21]. This, together with our data of IL-4 up-regulating keratinocyte IL-25, supports that IL-4 from Th2 cells in AD skin lesions up-regulates IL-25, thus forming a positive feedback loop to further drive the Th2 pathway. IL-31 binding to its cognate receptors may induce skin inflammation and severe pruritus

in AD patients [22]. Two IL-31 receptors, IL-31RA and OSMR, are up-regulated in keratinocytes derived from AD patients [23-24]. Our findings that IL-4 up-regulates these two components of the IL-31 receptor are also in line with the current literature. SOCS2 is undetectable in normal skin, but is weakly expressed in AD [25], and this is consistent with our data that IL-4 up-regulated its expressions in HaCat cells. NOS2 has been suggested to play a role in the pathogenesis of AD in NC/Nga mice, an AD model [26], and our data of NOS2 up-regulated by IL-4 also support this notion.

3.3. IL-4 down-regulates antimicrobial factors in HaCat cells

While IL-4 up-regulated chemotactic and pro-inflammatory factors produced by keratinocytes, it showed strong down-regulatory effects on antimicrobial factors. Many chemokines have a substantial microbicidal activity against numerous microorganisms including Staphylococcus aureus, a common pathogen involved in AD [27]. We found that CCL17, CCL19, CCL20, CX3CL1, CXCL11, CXCL14, S100 proteins, and Toll like receptors (TLRs) were down-regulated by IL-4. S100 proteins, structurally similar to calmodulin, are involved in a variety of biological function, such as calcium homeostasis, inflammation and inhibition of microorganisms. Two of these proteins, S100A8 and S100A12, which have been reported to be deficient in AD skin [28], are down-regulated by IL-4 in keratinocytes in our study. TLRs and human β-defensin (HBD) are two important classes of epidermal biodefense molecules and HBD2 production is dependent on TLR2, which utilizes TLR1 and TLR6 as coreceptors [29]. As both TLR1 and TLR6 are down-regulated by IL-4 in our study, it supports a notion that the presence of IL-4 reduces the skin's ability to eliminate invading pathogens. Interestingly, TLR-2 missense

mutation is associated with AD [30]. In addition, IFN-κ, whose suppressed expression in AD is associated with low levels of antimicrobial peptides [31], and RIPK2, a signaling component in both the innate and adaptive immune pathways with ability to defend against pathogens [32], are all down-regulated by IL-4 in keratinocytes.

3.4. Other IL-4 regulated factors in HaCat cells

Other than regulating the keratinocyte chemotactic, pro-inflammatory, and antimicrobial factors, IL-4 is also found to influence other factors relating to immune responses. Previously we have shown that angiogenesis plays an important role in AD [5], and Zhang *et al*. have shown that VEGFA is up-regulated in AD lesions [33]. Our current data showed that IL-4 up-regulates VEGFA in keratinocytes, suggesting that the up-regulation of VEGF in AD is at least in part due to the effect of IL-4. Interestingly, TNF superfamily 15, an inhibitor of angiogenesis [34], is down-regulated by IL-4. In addition, the following genes are down-regulated by IL-4 in HaCat cells: IL-1F8 and 9, which play roles in skin immune defense [35], adenosine A1 receptor, involved in preventing tissue damage caused by harmful circumstances [36], lymphotoxin β (TNFSF3), an IgE suppressor [37], TNFSF18, a T-cells function regulator, and formyl peptide receptor 1, a G protein-coupled receptor involved in regulation of immune response and associated with AD development in cathepsin E null mice at lower levels [38]. For reasons yet to be determined, IL-18 is found to be reduced in young AD patients [39], and this is consistent with our findings that this gene is down-regulated by IL-4 in keratinocytes. In addition, we also found that IL-4 down-regulates the glucocorticoid receptor NR3C1, which may contribute to steroid resistance in some patients [40]. While IRF2 mutation has been

linked to AD [41], IRF4's role in AD remains to be investigated. Interestingly, conditional knockout of AP-2γ resulted in defects in skin barrier functions and abnormal expression of filaggrin, repetin and SLURP1 [42]. While IL-4 has been shown to regulate filaggrin production and low filaggrin expression is associated with AD, SLURP1's role in AD has not been reported. In AD patients, surfactant protein D and C3 are dysregulated [43-45], and our PCR array study suggests that this dysregulation may be caused by IL-4.

Interestingly, the regulation of IL-13Rα2 and TNFAIP6 by IL-4 appears to challenge the idea that IL-4 plays an essential role in the pathogenesis of AD. In keratinocytes, both IL-4 AND IL-13 bind to IL-4Rα and IL-13R1. IL-13Rα2, which binds to IL-13 with high affinity but lacks a significant cytoplasmic domain, has been reported as a dominant negative inhibitor for both IL-4 and IL-13 signal transduction pathways [46]. We found that IL-13Rα2 is up-regulated by IL-4 in HaCat cells. In AD patients this gene is similarly up-regulated [47]. This maybe is due to a negative feedback mechanism, by which the keratinocytes try to minimize overwhelming IL-4 effects. Along the same line, TNFAIP6, a member of the hyaluronan-binding protein family, which has anti-inflammation functions [48], is up-regulated dramatically in our PCR array study. Although CCL27 is over-expressed in AD lesions [6, 49], IL-4 appears to down-regulate it in keratinocytes. Since CCL27 is well known to be regulated by NF-κB [50], the up-regulation of CCL27 observed in AD lesions may be caused by proinflammatory cytokines other than IL-4. Similarly, although the expressions of CCL28 [51], IL-16 [52], IL-21R [53], and TNFα [7] are up-regulated in AD, it appears that IL-4 down-regulates them in HaCat cells.

3.5. In vivo studies of IL-4 regulated genes using IL-4 Tg mice

Through over-expressing IL-4 in the basal epidermis, we have generated a mouse AD animal model, IL-4 Tg mice [9]. Next we aimed to use the IL-4 Tg mice to verify whether the in vitro regulation of these genes by IL-4 also occurs in vivo. Towards that end, we collected skin tissues before the onset of AD to eliminate any possible effects of inflammation on gene regulation. Using real-time RT-PCR, we show that IL-4 up-regulates CCL8, CCL24, IL-12Rβ2, IL-13Rα2, IL-25 and TNFAIP6, while it down-regulates CCL17, FPR1, IL-1F9, TLR1 and S100A8 (Fig 1 and 2). Since our selected in vivo data are entirely consistent with the in vitro findings, our microarray data likely reflect the dysregulation of keratinocyte genes by high levels of IL-4 in AD.

AD is characterized by skin infiltration of IL-4-producing inflammatory cells and higher levels of IL-4 in the peripheral blood. High levels of IL-4, in turn, stimulate Th2 cell growth and synthesis of more IL-4, thus forming a positive feedback loop. We and others have shown that IL-4 plays an important role in the pathogenesis of AD. Using PCR array analysis, we for the first time extensively examined genes regulated by IL-4 in keratinocytes. Our data provide evidence that by up-regulating chemotactic, angiogenic, and pro-inflammatory genes in keratinocytes, IL-4 contributes directly to the AD inflammatory process. Similarly by down-regulating antimicrobial genes, IL-4 contributes indirectly to the AD inflammatory process in that it reduces skin's ability to eliminate invading pathogens, the source of persistent inflammation. Our data will hopefully pave pathways for future detailed examination of these genes, allowing

investigators to dissect the pathophysiology of AD and to develop more target-specific therapeutic options for the AD patients they care.

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Table 1. Mouse primers used for real-time RT-PCR.

Table 2. Genes up-regulated by IL-4 in HaCat cells.

HaCat cells were treated with various concentrations of IL-4 for 24 hours. PCR array studies were performed as described in Materials and methods. Gene names, corresponding accession numbers, abbreviations and fold change values are listed. All the fold change values are statistical significant except B, which means P>0.05.

Table 3. Genes down-regulated by IL-4 in HaCat cells.

HaCat cells were treated with various concentrations of IL-4 for 24 hours. PCR array studies were performed as described in Materials and methods. Gene names, corresponding accession numbers, abbreviations and fold change values are listed. All the fold change values are statistical significant except B, which means P>0.05.

Fig. 1. Up-regulated genes in the IL-4 Tg mice.

Tissue samples were collected from normal appearing skin of IL-4 Tg mice and non-Tg littermates. RNA extraction and real-time RT-PCR were performed as described in Materials and methods. Values are expressed as the mean ± SEM (n = 3). *, $P < 0.05$ *vs.* non-Tg mice.

Fig. 2. Down-regulated genes in the IL-4 Tg mice.

Tissue samples were collected from normal appearing skin of IL-4 Tg mice and non-Tg littermates. RNA extraction and real-time RT-PCR were performed as described in

Materials and methods. Values are expressed as the mean ± SEM (n = 3). *, $P < 0.05$ *vs.* non-Tg mice.