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IL-33 impacts on the skin barrier by downregulating the expression of filaggrin

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Key words: skin, skin barrier, atopic dermatitis, keratinocyte, IL-33, filaggrin.

Abbreviations used:

FLG: filaggrin

EDC: epidermal differentiation complex

LOF: loss-of-function

NMF: natural moisturing factor

ALI: air-liquid-interphase

HBD2: human beta defensin-2

AP1: activator protein 1

Capsule Summary:

IL-33 directly downregulates filaggrin expression in keratinocytes and in skin tissue affecting the filaggrin mediated barrier. This effect can promote skin inflammation as IL-33 also impairs human beta defensin 2 expression and amplifies Th2 related responses.

To the editor:

IL-33 is a member of the IL-1 family of cytokines that is constitutively expressed in healthy skin and was found to be increased the skin of atopic dermatitis (AD) patients. Since it can be released after tissue damage or physical stress including scratching of the skin[1], it has been classified as an alarmin concerned with alerting the immune system[2]. It enhances Th2 responses by inducing IL-5 and IL-13 as well as Th1 responses via upregulation of IFN-γ. Keratinocytes are known producer cells of IL-33 and do also express the receptor complex consisting of ST2 and IL-1RAcP on their surface. The aim of this study was to investigate the effect of IL-33 on keratinocytes, skin biopsies and living skin equivalents with regard to the regulation of the skin barrier molecule filaggrin (FLG). FLG is a structural protein promoting the outer epidermal barrier by aggregation of intermediate filaments[3] and influencing cell differentiation, what is considered to be a key step in establishing the structure and function of the stratum corneum[E1]. Furthermore, processed FLG contributes to natural moisturing factors (NMF) important for skin hydration[E2]. There is evidence that a lack of FLG breakdown products favours transepidermal water loss[E3], allergen penetration[4] and skin colonisation with Staphylococcus aureus[E4]. FLG-LOF may also play a role in the development of AD[5, 6] since it has been shown that up to 50% of AD patients carry such a mutation. Several cytokines like IL-4[7], IL-17[E5], IL-22[E5], IL-25[E6, E7] and IL-31[E8] have been described to negatively affect the FLG expression in keratinocytes. In this study we have analyzed the effect of IL-33 on FLG expression in sub-confluent and differentiated monolayer keratinocytes, skin biopsies and skin equivalents.

In a first approach, monolayer keratinocytes and skin samples were stimulated with IL-33 or IL-4 to analyse FLG mRNA expression (See additional information on methods in the Online Repository). We confirmed the effect of IL-4 on FLG in $Ca2^{+}$-differentiated human primary keratinocytes (Fig 1A). Investigating an impact of IL-33 on FLG transcription, we observed a tendency towards downregulation using the same experimental setup, whereas no effect of IL-

33 was on FLG expression was observable in undifferentiated keratinocytes cultured in the absence of calcium (Fig 1B). When we analyzed keratinocytes from patients with atopic dermatitis that were kept in cell culture in a semi-confluent manner with or without $Ca2^{+}$ we observed significant decreases of FLG mRNA upon stimulation with IL-33 (Fig 1B). The median effect on mRNA reduction was more pronounced compared to keratinocytes from healthy donors (median .54 vs .73, respectively). Subsequently, we investigated the expression of FLG mRNA in skin biopsies after incubation for 24h with IL-33 (Fig 1C). Here, we could also confirm the down- regulatory effect of IL-4. Although the effect of IL-33 was not significant, which may be explained by the time point of measurement after 24h incubation, a clear tendency could be observed (p=.08). An even shorter stimulation (4h) of skin samples with IL-33 or IL-4 did not show a clear effect in the regulation of FLG mRNA expression (data not shown).

Histological staining of FLG expression in the skin confirmed our results on the protein level. When compared to the non-stimulated skin, addition of IL-4 or IL-33 led to a decrease of FLG protein expression in the epidermis (Fig 2). We analysed the staining regarding intensity and area. Since existing FLG within the stratum corneum is not expected to be affected by 24h-stimulation, evaluation was restricted to keratinocytes with detectable nuclear counter-staining. By immunohistochemical staining and software-assisted analysis we measured the stained area within the epidermis and observed a significant difference between the non-stimulated and IL-33 stimulated samples (Fig 2A). Stimulation with IL-4 also led to FLG-loss as expected (non-stimulated: 17.72%±11.92, IL-4: 8.97%±7.06, % epidermis stained, n=6). In a next step we studied the expression of FLG with fluorescence antibodies, where the signal strength is not dependent on an enzymatic reaction. Here, the intensity of staining with anti-FLG was reduced in skin samples upon IL-33 incubation (Fig 2B). Under these conditions IL-4 stimulation did not lead to a distinct decrease of FLG expression (non-stimulated: 14.07±4.3, IL-4: 13.08±5.29, fluorescence intensity, n=9).

Finally, in vitro skin equivalents were continuously stimulated with IL-33 to investigate a putative effect on keratinocyte differentiation and we observed a disturbed barrier formation by trend. After 8 days of culture in the air-liquid (ALI)-phase, FLG was reduced by IL-33 stimulation (see Fig. E1 in this article´s Online Repository). This effect was still observable after 14 days. Software-assisted measurement of FLG-staining depicts a tendency towards reduced protein expression under the influence of IL-33. To investigate an effect on the penetrance of allergens, we applied biotinylated natural peanut allergen Ara h 2 onto the skin equivalent. After two hours of incubation, the allergen was detected in fixed paraffin sections by streptavidin-phycoerythrin. In control skin equivalents, we were not able to detect allergen penetrance, while IL-33-treatment led to a modest but visible effect on facilitated allergen penetrance into the epidermal part of the skin equivalent (see Fig. E2 in this article´s Online Repository).

Based on these results we hypothesize that IL-33 contributes to eczematous inflammation not only by acting as an alarmin but also by virtue of its barrier weakening function. We here observed a direct action of IL-33 on downregulation of FLG expression independent of cytokines from mast cells or Th2 cells. The observed stronger reduction in AD derived cells compared to healthy donors fits our findings of a higher expression of ST2 on the surface of AD keratinocytes in a previous study[8].

Filaggrin expression was shown to be controlled by the AP1 (activator protein 1) family of transcriptional regulators[E11], POU-domain transcription factors[E12], and p63[E13]. Signaling of IL-33 via ST-2 may impact these pathways, since it has been shown to result in the recruitment of MyD88, IRAK, IRAK4 and TRAF6, followed by phosphorylation of the downstream targets extracellular-signal-regulated kinase1/2, p38, IκBα, and JUN amino-terminal kinase, finally resulting in NFκB activation[E14]. While the mitogen-activated protein

kinases Erk1/2, p38, and JNK stimulate AP1 activity and transcription[E15], NFκB has been shown to counteract AP1 by downregulation of cFos via Elk-1[E16].

Under most experimental conditions the effect of IL-33 on FLG reduction was weaker than observed for IL-4. We recently described another IL-4-mimetic action of IL-33 regarding the regulation of human beta defensin-2 (HBD2)[9], supporting the idea of IL-33 being a dichotomous cytokine having IL-1 family-like and Th2-associated properties.

In conclusion, we propose that IL-33 has skin barrier modulating effects apart from its well described function as an innate alarmin mediating the activation of the adaptive immune system by promoting Th2 as well as Th1 responses. IL-33 bears the potential to directly or indirectly promote epidermal barrier impairment and thereby plays a crucial role in sustaining and enhancing the local inflammatory responses in diseases such as AD.

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Figure Legends

FIG 1. mRNA expression of FLG in monolayer keratinocytes and skin samples measured by qt-RT PCR. **A**, $Ca2^{+}$-differentiated keratinocytes from healthy donors stimulated for 4h with 50ng/ml IL-4 (n=7). **B**, $Ca2^{+}$-differentiated and undifferentiated keratinocytes from healthy donors and AD patients stimulated for 4h with 50ng/ml IL-33 as indicated (n=3-7). **C**, Whole skin samples stimulated with 50ng/ml of IL-4 or IL-33 for 24h (n=6). Means and SEM are depicted. ns: non-stimulated. t-test: *p<.05, **p<.005, ***p<.001.

FIG 2. Detection of FLG protein expression in the skin. Samples were stimulated with 50ng/ml of IL-33 or IL-4 for 24h. **A**, Immunohistochemistry staining. Stained area within the epidermis except the stratum corneum (dotted line) was measured using cellSensDimension software (Olympus). **B**, Immunofluorescence staining. Fluorescence intensity within the epidermis except the stratum corneum (dotted line) was measured using ImageJ (NIH). NS: non-stimulated. paired t-test, *p<.05, **p<.005.