

## Keratinocyte Proliferation and Differentiation on IL-9 Stimulation: An Explorative *In vitro* Study

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Recent studies have indicated the significance of Th9 cells in a variety of inflammatory and allergy-related diseases (see Kaplan et al. (1)). Th9 cells are a distinct subset of CD4<sup>+</sup> T cells present in the skin, which secrete interleukin (IL)-9 (2–4). IL-9 is transiently expressed by skin-tropic and skin-resident Th9 cells to regulate the production of inflammatory cytokines (5). IL-9, like IL-4, is associated with predominantly type 2 immune responses, and aberrant IL-9 expression or signalling in skin is implicated in allergic contact dermatitis (ACD) (6), atopic dermatitis (AD) (7–9), and psoriasis (5, 10). Targeting of IL-9 or its receptor may therefore be an interesting new therapeutic avenue to be explored. Although the IL-9 receptor (IL9R) is expressed by immune cells as well as epithelial cells (11), the majority of research focuses on the Th9/IL-9 axis in immune cells. Recent *in vitro* studies, however, have shown the regulation of *IL9R* expression in keratinocytes by IL-4 (12) and increased IL9R expression in basal keratinocytes of psoriatic lesions (10). Furthermore, IL-9 increased IL-8 (CXCL8) and vascular endothelial growth factor (VEGF) secretion by keratinocytes *in vitro* (6, 7). The effects of IL-9 on human keratinocytes with regard to epidermal proliferation, differentiation and host defence are still poorly understood. This study examined the effects of IL-9 on epidermal morphology, proliferation, differentiation and host defence, and studied cytokine-mediated regulation of *IL9R* expression on keratinocytes, and investigated the potential additive or synergistic effects by IL-9 in Th2-cytokine mediated epidermal responses.

### MATERIALS AND METHODS

Human epidermal equivalents (HEEs) and monolayer cultures generated from adult primary human keratinocytes were exposed to human recombinant IL-9 or other pro-inflammatory cytokines, as indicated. Epidermal morphology was studied by histology, and gene and protein expression was analysed by quantitative PCR analysis and immunohistochemistry, respectively. For detailed description, see Appendix S1<sup>1</sup>.

### RESULTS

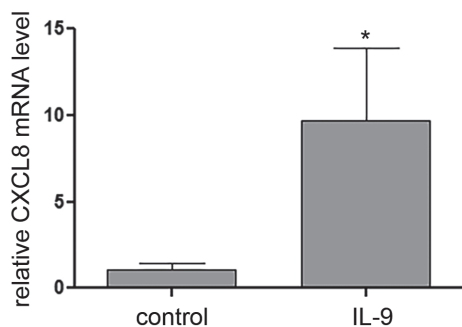
First, a dose range of IL-9 was tested on differentiating submerged keratinocyte monolayer cultures and no effect on cell morphology and viability was observed,

even at the highest concentration of 500 ng/ml (data not shown). For further in depth analysis of keratinocyte proliferation and differentiation, HEEs were exposed to 50 ng/ml human recombinant IL-9, being a biologically relevant concentration as shown by the induction of *CXCL8* expression (Fig. 1, (10)). After 72 h of IL-9 stimulation, HEEs showed normal epidermal morphology with a fully stratified epidermis and a well-developed stratum corneum (Fig. S1a<sup>1</sup>). The number of proliferating cells, measured with Ki67 staining, and the epidermal thickness was similar to that of control HEEs (Figs S1a and S2<sup>1</sup>). Next, we analysed the expression of the major epidermal differentiation proteins, keratin 10 (K10), involucrin (IVL), filaggrin (FLG) and loricrin (LOR) (Fig. S1b<sup>1</sup>). For all markers, IL-9 did not alter protein localization or expression levels, nor did it change the expression of epidermal differentiation genes (Fig. S3<sup>1</sup>). Also, marker expression for keratinocyte activation or host defence, namely keratin 16 (K16) and SKALP remained unaffected. Human beta defensin 2 (hBD2) is absent in unstimulated HEEs and is also not induced by IL-9 (Fig. S1c<sup>1</sup>). This in contrast to the classical Th2 cytokines, IL-4 and IL-13, which downregulated FLG, LOR and IVL expression (Fig. S4a<sup>1</sup>) and induced K16 and SKALP expression (Fig. S4b<sup>1</sup>).

The suggested role of IL-9 in atopic diseases, which are largely Th2 driven, led us to investigate the interaction of IL-9 with IL-4 and/or IL-13. Co-stimulation with IL-9 did not alter the effect of Th2 cytokines on downregulation of epidermal differentiation proteins (Fig. S5a<sup>1</sup>) or the induction of inflammatory epidermal markers, K16 and SKALP (Figs S5b and S6<sup>1</sup>).

We hypothesized that the absence of significant effects by IL-9 in our study could be due to low *IL9R* expression (mean Ct value 34) under the conditions described above. *IL9R* expression appears to be induced in inflammatory processes, and we therefore stimulated keratinocyte monolayers and HEEs with various cytokine combinations and found interferon gamma (IFN- $\gamma$ ) to be the main inducer of *IL9R* expression (Fig. 2). We determined the minimal, but effective, IFN- $\gamma$  exposure time and observed significantly induced *IL9R* expression after 6 h of IFN- $\gamma$  stimulation (Fig. S7<sup>1</sup>). Thereafter, HEEs were exposed to IL-9 for 72 h. IFN- $\gamma$  alone induced the mRNA expression of *IVL*, and the chemokines *CCL5* and *CXCL10*, yet no additional effect of IL-9 stimulation was observed on these or other genes analysed (Fig. S8a<sup>1</sup>). Similar to previous

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**Fig. 1. Interleukin-9 (IL-9) induces epidermal CXCL8 expression.** CXCL8 mRNA expression of human epidermal equivalents stimulated with IL-9 (50 ng/ml) for 72 h. Bars represent mean  $\pm$  standard deviation (SD),  $n = 3$  keratinocyte donors,  $*p < 0.05$ .

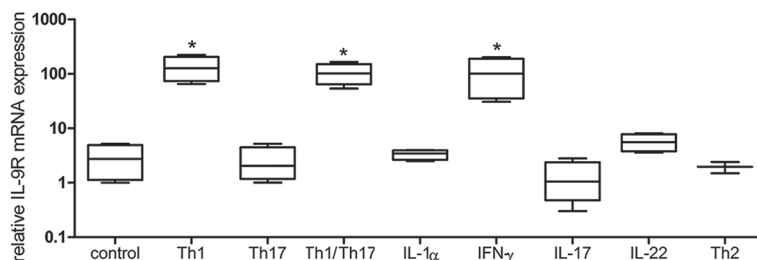
experiments, IL-9 did not alter epidermal morphology or differentiation protein expression (K10, IVL, LOR and FLG) even under conditions of high *IL9R* expression due to 6 h IFN- $\gamma$  pre-stimulation (Fig. S8B<sup>1</sup>).

## DISCUSSION

This study explored the potential effects of IL-9 on keratinocytes and investigated its potential role in multiple biological processes involved in epidermal homeostasis, such as proliferation, differentiation, host defence and inflammatory responses. Even after the induction of *IL9R* in keratinocytes we did not detect any effect of IL-9 on any of the aforementioned processes, besides the induction of *CXCL8* mRNA expression, which has been reported previously (12). Induced expression of this chemokine may potentially aid in the inflammatory process mediated by Th9 cells and cytokines.

Our data indicate that *IL9R* expression in human keratinocytes is constitutively low, but highly inducible upon inflammatory conditions, and that this regulation is mainly due to IFN- $\gamma$ . This finding is in line with the upregulated expression of this receptor as found in psoriasis and allergic contact dermatitis (2, 3, 5), where IFN- $\gamma$  levels prevail and contribute to disease pathogenesis. In contrast, Hong et al. (12) found *IL9R* to be upregulated by IL-4. We did not detect Th2-cytokine mediated induction of *IL9R* mRNA expression in HEEs (Fig. 1A) or monolayer cultures (data not shown). The differences in cell source or post-transcriptional regulation may explain this discrepancy, since Hong et al. studied IL9R protein expression in foreskin keratinocytes.

The limitation of the current study is that we examined a selection of genes. Genome-wide transcriptomic analysis may reveal additional IL-9 responsive genes in epidermal keratinocytes, other than *CXCL8* reported here, but this is outside the scope of the current study. Based on our data we conclude that IL-9 does not affect important keratinocyte functions, such as proliferation



**Fig. 2. IL-9R expression after pro-inflammatory cytokine stimulation.** IL9R mRNA expression of submerged keratinocytes stimulated for 48 h with single cytokines or mixes thereof: Th1: IL-1a (30 ng/ml), TNF- $\alpha$  (30 ng/ml), IFN- $\gamma$  (500 U/ml), Th2: IL-4 (50 ng/ml), IL-13 (50 ng/ml), Th17: IL-17 (30 ng/ml), IL-22 (30 ng/ml). Boxplots represent mean  $\pm$  standard deviation (SD),  $n = 3$  keratinocyte donors,  $*p < 0.05$ .

or transcription of major differentiation-related genes involved in skin barrier function.

*The authors declare no conflicts of interest.*

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