Psoriasis is a chronic skin disease characterized by the activation of various T cell subsets secreting IFNγ, IL-17, and IL-22, dendritic cells producing TNFα and IFNα, and other immune cells including neutrophils and mast cells. Keratinocytes respond to different cytokine signals orchestrating innate and adaptive immune responses. In vitro studies sought to clarify the cytokine effects on keratinocytes in order to evaluate the centrality of these mediators in psoriasis pathogenesis. The aim of this review is to highlight the relevance of this peculiar in vitro approach in investigating cytokine effects on skin or multilayered epidermis. Particularly, we reported keyfindings supporting the cytokine role in psoriasis pathogenesis.

Key words: Psoriasis, RHE, Skin equivalent, IL-17, IL-22, TNFα, IFNγ
act in a paracrine way in amplifying the cytokines cascade production. Overall, each cytokine modulates distinct keratinocyte-response pathways with a certain grade of overlap in their gene expression induction. For instance, IL-17 and TNFα strongly induce the synthesis of pro-inflammatory mediators (Chiricozzi et al., 2011; Prignano et al., 2015), while IL-22 and IL-20 mainly stimulate keratinocyte hyperplasia (Wolk et al., 2006, 2009a; Donetti et al., 2016). Once activated, keratinocytes participate in the immune response secreting pro-inflammatory mediators and releasing chemokines and other chemoattractants (i.e., CCL20, CXCL1, CXCL8-11, antimicrobial proteins), which are crucial for the recruitment of T cells, neutrophils, and inflammatory myeloid dendritic cells to skin.

In order to investigate the cytokine contribution to the psoriasis phenotype and to specifically determine their effects on keratinocytes, in vitro models have been developed. The simplest model is represented by cultured monolayer keratinocytes, either pooled normal human or HaCat cells, which are able to define cytokine effects and cytokine interactions (Nograles et al., 2008; Chiricozzi et al., 2011). Nevertheless, this approach shows some limitations as keratinocytes appear undifferentiated, similar to basal layer keratinocytes, lacking the physiological stratified keratinocyte differentiation process occurring in vivo and, moreover, characterized by lack of junctional proteins which play a unique role in epidermal barrier. Thereby, various three-dimensional (3-D) skin equivalents reproducing in vivo conditions have been developed in order to elucidate peculiar pathogenic aspects in psoriasis, particularly the contribution of key-cytokines to the psoriatic pathogenic mechanism. In this review we will attempt to describe some of these in vitro approaches testing cytokine effects on skin, assessing both morphological and gene expression changes.

**Full thickness human skin equivalent**

Among the commercially available skin equivalents, a full thickness 3-D human skin model consisting of an epidermis and a dermal compartment that contains viable normal human dermal fibroblasts embedded into a collagen matrix, has been widely used. The dermis supports a multilayered human epidermis, cultivated at the air-liquid interface consisting of differentiating keratinocytes, which are distributed into basal, spinous, and granular layers (8-12 layers in total), whose terminal differentiation results in the presence of the stratum corneum, analogously to the epidermal in vivo structure.

This skin equivalent has been used to test the effects of certain psoriasis-signature cytokines, namely IL-22, IL-17, IFN-γ (Nograles et al., 2008, Chiricozzi et al., 2014). Treatment with IL-22 increased epidermal thickness, induced parakeratosis and downward epidermal projections, altering the keratinocyte differentiation process, conversely to the other conditions (Nograles et al., 2008). Similarly to the induction in cultured monolayer keratinocytes, IL-22 and IL-17 were found to increase the expression of S100A7, while CCL20, β-defensin 2 (DEFB4) and CXCL8 were induced by IL-17 alone (Nograles et al., 2008). Another study investigated the transcriptomes induced by the same panel of psoriasis-signature cytokines in skin equivalents, though it was mainly focused on IL-17 spectrum-of-action (Chiricozzi et al., 2014). Notwithstanding the centrality of IL-17 signaling in the psoriasis pathogenic mechanism, IL-17 was found to regulate the expression of a limited number of inducible genes (60 genes) in monolayer keratinocytes. On the contrary, in the 3-D skin model IL-17 altered the expression of a wider set of genes, 490 genes (322 upregulated and 168 downregulated), as compared to monolayer keratinocytes (Chiricozzi et al., 2014). This gene induction did not represent the mere sum of induced gene sets detected in monolayer fibroblasts and keratinocytes, cultivated separately. Notably, the presence of fully differentiated keratinocytes within the full thickness skin model seemed to allow an enhanced response to IL-17 stimulation as they uniquely expressed CEBP-β, one of the key-transcription factors involved in the IL-17 signaling (Chiricozzi et al., 2014). The gene expression response was specific as demonstrated by the small overlap with the other transcriptomes obtained in IL-22- and IFNγ-treated skin equivalents, and was confirmed by the upregulation of IL-17-signature genes such as IL-19, DEFB4, LCN2, IL-1F9, IL-1β, S100A7A, and S100A12, in the IL-17-treated skin equivalent. Gene set enrichment analysis revealed the enhancement of the IL-17-treated skin equivalent gene profile in the psoriasis transcriptome (Chiricozzi et al., 2014), with half of the genes induced by IL-17 in skin equivalent that were also included in the psoriasis transcriptome, suggesting that this IL-17 response may represent a reasonable model for the in vivo role of IL-17 in psoriasis, and may reflect gene activation commonly observed in psoriasis lesion. Furthermore, the specificity of this model was also determined by the positive correlation between the gene set induced by IL17 in vitro and the gene set downregulated in vivo by ixekizumab, an anti-IL-17 antibody (Chiricozzi et al., 2014).

**Reconstituted human epidermis**

Similarly to full-thickness skin equivalents, reconstituted human epidermis (RHE) models have been used to evaluate cytokine contribution to psoriasis pathogenesis.

In particular, they helped to clarify the pathogenic role of IL-20 cytokine subfamily members, which contribute to the development of epidermal acanthosis and parakeratosis in psoriasis (Kunz et al., 2006; Wolk et al., 2006, 2009b; Sa et al., 2007). The commercially available RHE model consists of a multistratified epidermis that exhibits morphological and growth characteristics similar to human epidermis (Rosdy et al., 1997), displaying basal, spinous, granulous with
numerous keratohyalin granules within the upper portion, and cornified cell layers. This 3-D tissue model consisting of normal, human-derived epidermal keratinocytes (NHEK) is cultured on a specially prepared tissue culture supplement, at the air-liquid interface. Some studies using this model confirmed the IL-22 effects in altering epidermal differentiation and triggering epidermal hyperplasia. Indeed, IL-22 was found to downregulate keratinocyte differentiation-related genes, such as involucrin, loricrin, filaggrin, desmocollin (DSC)1, keratin (K)10, 27-kDa heat shock protein (Hsp), calmodulin like (CALML)5, K1, and late cornified envelope (LCE)1B. IL-22 also caused the loss of keratohyalin granules in the granular layer and the presence of picnotic nuclei. Notably, the epidermal acanthosis did not result from an increased basal keratinocyte proliferation as the number of Ki67-positive cells was not significantly different between control and IL-22-treated RHE (Boniface et al., 2005; Wolk et al., 2009b). Beside the inhibition of keratinocyte differentiation, IL-22 also activates keratinocytes in producing CXCL5, platelet-derived growth factor (PDGF)-A, S100A7, S100A8, and S100A9 gene (Boniface et al., 2005; Wolk et al., 2009b).

This model was strikingly useful to distinguish the effects of IL-22 from other members of the same cytokine family, including IL-20, IL-19, IL-24, and IL-26, and other psoriasis-signature cytokines (i.e., IL-17, TNF-α and IFN-γ). RHE response to IL-20 was found to be similar, though less marked compared to IL-22, showing a slight acanthosis that was associated with reduced expression of LCE1B, DSC1, CALML5, K1, and K10. Limited IL-20 effects were also detected in psoriasis-related chemokine expression (namely CXCL1, CXCL8, CXCL5, CXCL2) and STAT3 activation (Wolk et al., 2009a). Notably, the combined treatment with both IL-22 and IL-20 did not show a marked synergistic effect in downregulating key-genes involved in the keratinocyte terminal differentiation.

Along these lines, all members of the IL-20 cytokine subfamily were able to increase epidermal thickness in RHE, with the exception of IL-26. The effects on RHE induced by each cytokine were distinct, in particular IL-22 showed a unique capability in causing (i) hypogranulosis; (ii) a reduced granular cell layer; (iii) parakeratosis; (iv) a more prominent downregulation of keratinocyte terminal differentiation-related genes; (v) an increased STAT3 activation; and (vi) an increased expression of AMPs, chemokines, notwithstanding the similarity of the gene profiles induced by IL-20, IL-22, and IL-24 and their common usage of the IL-22 receptor (Boniface et al., 2005; Wolk et al., 2006, 2009a; Sa et al., 2007).

Conversely to the IL-20 cytokine subfamily, particularly to IL-22, other pathogenic cytokines, namely IL-17, IFN-γ, and TNF-α were not able to impair keratinocyte differentiation or to induce morphological alterations of the epidermis (Wolk et al., 2006, 2009a). Though TNF-α alone did not increase epidermal thickness, it synergized with IL-22 in inducing epidermal acanthosis, which was more pronounced than what was observed with IL-22 alone. This synergism between IL-22 and TNF-α was also observed in the enhanced expression of immune-related genes, S100A7 and CXCL8, but it did not occur in increasing IL-22-induced morphological changes. A synergism between IL-22 and IL-17 was also observed in stimulating IL-20 expression in RHE, and it could be potentially extended to RHE chemokine production as both cytokines stimulated the expression of chemoattractants for dendritic cells (CCL20) and granulocytes (CXCL1, CXCL2, CXCL5, CXCL8), whereas IL-22 did not strengthen chemokine production induced by IFN-γ (CXCL9, CXCL10) or IL-1β (CCL27) (Wolk et al., 2009a,b).

Three dimensional organotypic culture obtained by normal human skin biopsies

To investigate the cellular mechanism(s) involved in the early epidermal response to different exogenous stimuli an effective experimental setting is represented by a three dimensional organotypic culture of normal human skin biopsies. Three-dimensional models occupy an intermediate position between cell cultures and animal-based models and allow to overcome the multiple limitations existing in each proposed in vivo model. In the last decade, we have standardized a three-dimensional model of organotypic cultures of normal human skin obtained from aesthetic surgery, in our model mammal reconstructive surgery of young healthy women after written informed consent (Bedoni et al., 2007). The main advantage of this model is to reproduce the physiological condition since the skin is cultured in a Transwell system at the air-liquid interface with the epidermis exposed to the air and the dermis immersed in the culture medium. As no growth factors were normally added to the medium, the focus was to investigate the direct and prompt response of the main epidermal cytotypes, i.e. keratinocytes and Langerhans cells, to exogenous stimuli at early time points (5, 24, 48, and 72 hours after surgery). In these experimental conditions, both lymphatic/blood vessels and innervation are lacking in the dermis and, consequently, the potential outcomes derived from soluble factors by the many cells of dermal origin can be easily excluded. The epidermal response was firstly evaluated after exposure to physical agents as a standard dose of gamma rays used for radiotherapy of breast tumors (Donetti et al., 2005). More recently, the effects of UVA and UVB rays were compared and the potential protective activity of a natural antioxidant such as thymol was evaluated (Cornaghi et al., 2016). In the last few years, another field of use with a relevant clinical impact is represented by the epidermal effects induced after the exposure of normal human skin biopsies to pro-inflammatory psoriatic cytokines alone.
or in combination (Donetti et al., 2014, 2016; Prignano et al., 2015). This is a key issue as genetically modified mice cannot reproduce the exact clinical features of psoriasis because of the differences in the skin and in the immune systems between humans and mice (Gudjonsson et al., 2007). The role of the main psoriatic cytokines (IL-17, IL-22, TNF-α) on different processes typical of plaque growth in a micro-environment mimicking the psoriatic lesion can be specifically defined. Initially, IL-17 and TNF-alpha were added to the culture medium and induced a strong and immediate decrease of epidermal proliferation starting from 24 hours of incubation. This phenomenon can be, at least partially, responsible for a later “response-to-injury” leading to the epidermal hyperplasia distinctive of psoriatic plaques. The inhibition of cell proliferation was accompanied by an altered occludin expression in the granular layer, i.e. one of the main psoriatic specific changes in the epidermal barrier, without influencing K10 expression throughout the epidermal layers (Donetti et al., 2014). On the other hand, each cytokine affected at a different degree LC activation features, while the combination of both of them had no effect on their ultrastructure (as shown by the many organelles, but more specific by rough endothelium reticulum, mitochondria and Birbeck granules). TNF-α, but not IL-17, exerted a chemoattractant activity, trapping LCs in the epidermal compartment without inducing any evident morphological activation. IL-17, conversely, is able to induce only LC maturation which then migrate leaving the epidermal compartment. Interestingly, when both cytokines were added to the medium, a remarkable and statistically significative increase of epidermal LCs occurred, with a possible synergic effect between IL-17 and TNF-α (Prignano et al., 2015). Conversely to IL-17 and TNF-α, IL-22, itself, was shown to alter keratinocyte terminal differentiation, but not proliferation. Only when associated with IL-17 and TNF-alpha, IL-22 inhibited keratinocytes proliferation confirming the homeostatic role of IL-22 upon epidermal cells. On the other hand, IL-17 and IL-22, but not TNF-alpha, stimulated the expression of a well known psoriatic biomarker such as keratin K17.

The specific and intrinsic activity of each cytokine on different steps of the psoriatic lesion formation/progression strongly support the hypothesis that multiple cytokine inhibition may be a valuable strategy for psoriasis therapy.

Concluding remarks

The use of skin equivalents represented an important step in the in vitro investigation of cytokine effects. Conversely to monolayer keratinocytes cultures with clear limits in terms of viability and differentiation, skin equivalents show the advantage of a structure similar to in vivo condition. In particular, the presence of differentiating multilayered keratinocytes constitutes the key-feature of this approach. These 3-D skin models differ because of their complexity and their technical advances and overall they represent a useful tool for testing cytokine effects, cytokine antagonists, or topical therapeutics. Primarily they helped to dissect the spectrum of action of psoriasis-signature cytokines, providing relevant insights about their specific role in mediating key pathogenic steps in psoriasis. For instance, the in vitro approach clarified the capability of IL-22, to induce psoriasis-like alterations such as epidermal acanthosis, parakeratosis, and STAT3 activation, while IL-17 was found to increase the production of AMPs, chemokines, and other pro-inflammatory mediators.

The three dimensional organotypic culture obtained by normal human skin biopsies has proved to be very effective in experimenting the direct effects of the many tested cytokines, without the influence of growth factors and cells from vascular and lymphatic origin. Moreover, this model has shown the relevance of the epidermal barrier in psoriasis, specifically, of the tight junction proteins, an aspect that had been underestimated so far.

All together these experimental models prove to be effective investigative tools avoiding the use of animal models, with the advantage of being able to experiment the effects of cytokines and soluble factors on differentiated keratinocytes. These experimental models have added new evidence concerning the role of many tight junction proteins in the epidermal barrier modification as a new pathogenetic clue in psoriasis.

Nevertheless, transcriptomic and translational studies are crucial to accurately evaluate the cytokine contribution to psoriasis pathogenesis, as in vitro findings may not necessarily mirror the in vivo observations.

References

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3-D skin models and cytokines in psoriasis


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