Harnessing system-level modelling to effectively alleviate the needs for animal testing.

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Abstract.

Therapeutic and cosmetic products containing novel ingredients must first pass thorough safety evaluation testing before they are allowed for use in human populations. These toxicology studies have classically been carried out using laboratory animals (in vivo methods). However, the needs to evaluate the safety of an increasingly large number of chemicals and their mixtures, has prompted a fundamental paradigm shift in toxicology testing, calling for a rapid transition from animal to in vitro, virtual tissues methods. Yet, while a significant number of in vitro methods allowing reliable assessment of the sensitising potency of individually tested chemicals already do exist, they have so far met with scant industrial utilisation where in vivo testing remains the preferred approach.
In this article, the authors investigate the reasons behind this situation and demonstrate how the utilisation of a heuristic modelling approach (CADI) can significantly improve the robustness, efficacy and flexibility of existing in vitro safety testing methods, thereby fostering their industrial utilisation in preference to in vivo tests for the development of innovative cosmetic/skin-care products, as well as for that of novel therapeutic approaches addressing neurodegenerative disorders.

Background

When developing a novel product (i.e.: containing novel ingredients), be it for pharmaceutical or cosmetic purposes, the problem of safety for use in human populations invariably arises and safety/pre-clinical testing is a strictly enforced regulatory requirement addressing any candidate investigational product (new chemical entities and biologics, known as test articles) ultimately intended for therapeutic or cosmetic utilisation by humans.
Safety/pre-clinical testing is carried-out to evaluate pivotal toxicology & safety properties of a test article to either determine its pharmacological characteristics or to evaluate the effect that it has upon a physiological test system. This involves recourse to in vitro systems and, ultimately, to laboratory animals since a wide range of toxicology studies, such as pharmacokinetic, repeat dose toxicity, eye irritation and developmental or reproductive alterations (teratogenicity) classically requires in vivo testing.
Yet, while a full ban on animal testing for cosmetics came into force on the 11th of March 2013 in the European Union, the regulatory safety requirements addressing any novel candidate product do remain.
In vitro testing, using cell/tissue-cultures, could, at least theoretically, uncover a wide range of toxicological issues that may be attached to a novel product and considerable progress has been achieved in addressing the necessity to ensure safety without animal testing (see http://www.alttox.org/ttrc/overarching-challenges/way-forward/fentem-westmoreland/; http://www.alttox.org/spotlight/086.html). However, while in vitro methods allowing reliable assessment of the sensitising potency of individually tested chemicals do exist (see http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations/file-kerati/JRC_SPR_Keratinosens_Rec_17_02_2014.pdf; http://www.alttox.org/ttrc/existing-alternatives/skin-irritation.html), there is a paucity of methods allowing reliable safety assessment of complex natural products, such as plant extracts (see http://www.efsa.europa.eu/en/efsajournal/pub/1249.htm). Hence, production of validated, cost-effective, highly reliable and flexible non-animal, in vitro methods that can be applied industry-wide

for safety evaluation purposes remains an unmet challenge. Indeed, the range of in vitro test systems that must be utilised for safety assessment of complex products is extensive (see http://www.altex.ch/resources/rISC_008_Pereira1.pdf) and these procedures are not only industrially cumbersome and costly to operate, they also require that the safety issues be pre-defined since to each safety parameter corresponds a specific range of in vitro testing systems. Furthermore, in order to approach what could correspond to a physiological response in an individual, the results generated by in vitro testing systems require extensive data integration procedures that are yet to be implemented.
Thus, in vitro approaches which, currently, do not really address what could correspond to physiological responses in an individual, are only partly capable of answering regulatory requirements (1, 2). As a result, safety/pre-clinical in vivo testing remains, of necessity, practised on a large scale by many research institutions, by all pharmaceutical companies, by many cosmetic companies and by some chemical companies.
Although legislation is slowly changing world-wide in favour of various forms of bans on animal testing for a variety of products, the situation could rapidly reverse. Indeed, should the lack of cost-effective, highly reliable in vitro methods capable of addressing safety requirements for complex preparations result in increased frequency of adverse events, not only could regulatory authorities consider revising legislations, but companies themselves might be induced to ignore the bans and ensure the safety of novel products rather than obeying the bans and face escalating frequencies of product withdrawals from the market and/or damaging law suits.
However, before asking "How could in vitro safety testing methods be improved so as to foster their industrial utilisation in preference to in vivo tests?" it might be appropriate to ascertain the reasons behind the current situation.

The types and extent of current adverse events attributable to cosmetics.

According to recent studies addressing the frequency of dermatoses caused by the use of cosmetics in patients with specific complaints, 52% of patients had no skin condition related to cosmetics, 45% had dermatoses associated with cosmetics, 14% of which were caused by inappropriate cosmetic use, and 3% had inconclusive results (1). Allergens most often responsible for contact dermatitis in cosmetics users are methylisothiazolinone (19 %), paraphenylenediamine (15.2 %), and fragrance mixtures (7.8 %). Acrylates are the most common allergens in cases of occupational disease. The products most often implicated among cosmetics users are hair dyes (18.5 %), gels/soaps (15.7 %), and moisturizers (12.7 %) (2, 3). Women become sensitized at a younger age than men, and the frequency of adverse reactions to cosmetics increases with age, reaching a maximum at between 60 and 69 years of age, when the greatest rate of sensitization occurs (4).
Cumulatively, these studies suggest that

1) Safety assessment of a cosmetic product clearly depends upon how it is used, as this determines the amount of substance which may be ingested, inhaled, or absorbed through the skin or mucous membranes; and
2) Besides inappropriate cosmetic use, repeated exposure to weak allergens/irritants is the primary cause of cosmetics-associated sensitisation which worsens with age and may develop into full-fledged skin pathologies.

It therefore follows that, to meet both industrial and regulatory requirements, any non-animal, in vitro method designed to assess a cosmetic preparation's potential to cause skin sensitisation or skin damage, must be capable of reliably addressing weak allergens/sensitizers that may be simultaneously present in a preparation and predict the physiological effects these will have upon the targeted human epidermal regions.
Thus, to promote their industrial utilisation in preference to animal tests, not only the robustness, efficacy and flexibility of existing in vitro safety testing methods must be improved but means to integrate in vitro test results into reliably defined in vivo physiological responses must be devised.

Bridging the gap between in vitro tests results and in vivo physiological responses.

As stated above, one of the main impediments to a wider utilisation of non-animal testing methods resides with the facts that in vitro approaches rely upon subsequent extensive data integration in order to approach what could correspond to a physiological response in an individual. This indispensable task calls upon the implementation of analytical processes collectively known as "systems biology".

Systems biology addresses the need to shift from a component-based reductionist view of biology to a system-wide perspective. Systems biology explores the dynamic interactions between components of a living system, as well as their interactions with the environment, to elucidate how they determine its phenotype. These procedures can be characterised as interdisciplinary, iterative, computationally intensive and information greedy (5). However, there are no inherent limits to the levels at which "a system" may be defined as an entity. In fact, there is no such thing as "a system" because structures that are parts of one system (a transport vesicle in a cell) may form systems in their own right at a different level of integration (in the context of cytokines secretion in an inflammatory response). Thus, systems biology interprets biological phenomena as dynamic processes, the mechanisms and consequences of which depend upon the behaviour of the living entity studied. The time-scales addressed range from sub-microseconds for molecular-level interactions to days, months, and even years for the development of a disease in humans such as, for example, cosmetics-associated dermatoses.

Two broad approaches to systems biology currently exist: the frequently followed mathematical procedures (6) and the more rarely encountered heuristic approaches (7-9), both of which are largely regarded as mutually incompatible (10, 11).

Mathematical modelling starts from quantitative data to produce models capable of reiterating these data and predict the outcome of a different experimental paradigm. Focusing mainly on chemical and physical cellular processes, with the expectation that living systems can be fully explained from this perspective, these formal models, often based on ordinary differential equations, also includes the widespread analogy that presents functions within an organism as resulting from "modular organisation" (12, 13).

However, physiological systems are integrative and non-linear by nature and functional behaviours, which can be easily observed but not analytically approached through chemical and physical processes only, do emerge from indirectly linked organisational hierarchies which constitute physiological systems. The pacemaker rhythm in the heart constitutes a prime example of this. The site of the pacemaker rhythm cannot be located at the sub-cellular and molecular levels. Yet, there is no difficulty in locating it anatomically at the level of certain cells within the whole organ (10). Thus, if a particular biological process or response does not exist at one level, this does not mean that it doesn't exist at all. Functional principles on a higher level obviously include phenomena which are not reducible to the molecular level. Hence, attempting to productively apply mathematical systems biology principles to translate in vitro cellular responses into in vivo physiological responses is fraught with considerable difficulties.

In contrast, the alternative heuristic model-building approach, known as CADI™ (computer-assisted deductive integration), has repeatedly proven its efficacy in bridging the gap between in vitro cellular responses and in vivo physiological responses (14, 15, 46-53).

Heuristics can be characterised as a problem solving approach evaluating each step in a process, searching for satisfactory solutions rather than for optimal solutions, using all available qualitative information. Thus, heuristic modelling starts from accumulated knowledge to produce a model capable of describing the biological events and the mechanisms that generated the observed experimental data and predict their modifications associated with a different outcome.

Associating algorithmics and heuristics, the CADI™ process does not assume functional linearity and the components of a model do not incorporate solely what is known. Indeed, since this approach relies upon strict and systematic implementation of negative selection of hypotheses, models arising

from this procedure contain elements that have never been described but cannot be refuted by current knowledge and/or available biological data (see also http://www.bmsystems.net/).
Here, heuristic modelling plays the role of an architect (defines the nature, the structure, the functionalities and the contextual constraints of the system under study) while mathematical modelling, to be implemented at a later stage, plays the role of an engineer (reveals the dynamics and robustness of the structures while defining the set of parameters sufficient to give rise to similar or very different phenotypes).
Although the models arising from this analytical approach cannot, by any means, be regarded as biologically true in the absolute, they do represent a "least biased" and detailed view of the mechanisms potentially associated with a given physiological state and/or governed by the biological components under consideration, together with precise indications of the means whereby these could be manipulated. The new data arising from subsequent experimental verifications can then be re-injected into the model, rapidly leading to a clear and factual understanding of the biological processes under investigation.
This approach is currently being implemented to by-pass the recourse to animal testing by considerably improving the industrial usefulness of current in vitro reconstituted skin models, with particular emphasis on the efficient detection of weak allergens/sensitizers within complex preparations, including natural plant extracts.
In order to achieve this, it is first necessary to distinguish the structural and functional characteristics that differentiate in-vitro reconstituted skin systems from actual, in situ human skin.

In vitro skin models versus in situ human skin.

Human skin consists of two principal layers: the epidermis which is the outer epithelial tissue layer of the skin and the dermis which is the connective tissue layer of the skin. Embedded within the dermis are hair follicles, sebaceous glands and sweat glands, all of which are epithelial invaginations from the epidermis, as well as blood vessels and sensory nerve endings which penetrate into the epidermis. Blood vessels and nerves in the dermis are capable of active response to injury, yielding the defensive reaction of inflammation. These two compartments are separated by a complex basement membrane, the dermal-epidermal junction (DEJ). The epidermis displays a multilayers organisation. These layers are not distinctly different tissues but rather reflect visible stages along the continuous process of keratinocyte maturation, or keratinization. This organisation can easily be replicated in reconstructed skin models (Fig.1.).

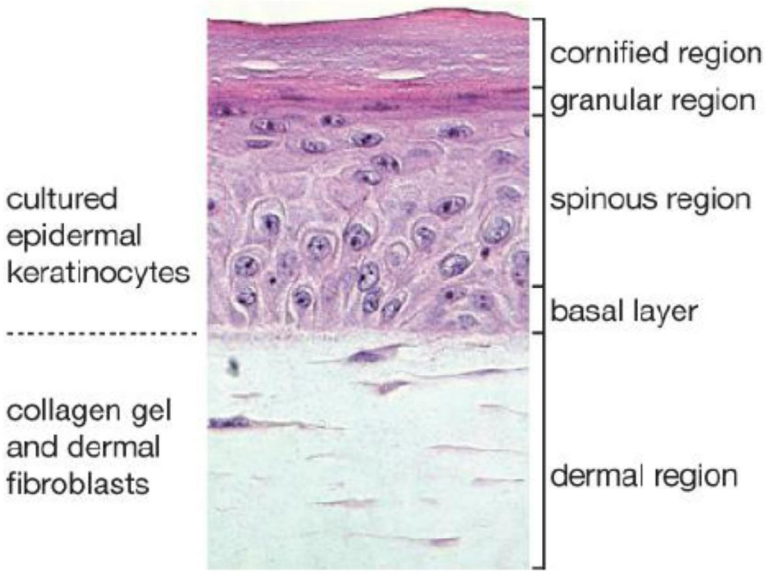

Fig.1. In vitro reconstituted skin model: three-dimensional (3D) cultures of skin epithelial cells simulate the multilayer tissue architecture present in vivo.

The numerous proteoglycans (negatively charged hepran sulphates) present in the lower layer of the DEJ act as effective traps for signalling peptide (growth factors, cytokines, etc.) and it is the controlled turnover of these components which, by regulating the segregation, release and exchange of peptidic factors (16), is largely responsible for the homeostatic control of continuous skin tissue renewal, keratinocytes proliferation and differentiation mechanisms as well as interferon (IFN)-γ and tumor necrosis factor (TNF)-α-driven cell death which must be tightly regulated to prevent inappropriate and excessive epidermal growth (17).
However, while all 3D skin models possess a stratified epidermis with a differentiated stratum corneum, they also lack most of the key components which mediate skin responses to the presence of potential allergens/sensitizers. It is in this sense that in vitro reconstituted skin models simulate but do not replicate actual human skin.
In vivo, skin sensitization is a two-step process by which exposure to a chemical first potentiates the immune system and subsequent exposure elicits an allergic response. The mechanism, well understood and described elsewhere (http://www.alttox.org/spotlight/086.html), relies upon cross-talk between innate and adaptive immune responses driven by innate effectors (dendritic [Langerhans] cells and CD8+ T cells) that reside within the epidermis (Fig.2).
The Langerhans cells are dendritic cells normally present in the stratum spinosum and the basal cell layers in the epidermis. They are similar in morphology and function to macrophages and are termed the "outposts" of the cutaneous immune system. They have a double function in the skin's immune system: 1) to capture harmful substances which have penetrated and report the incident to the immune system, and 2) in their phagocytes capacity, to take part in the destruction of pathogens.

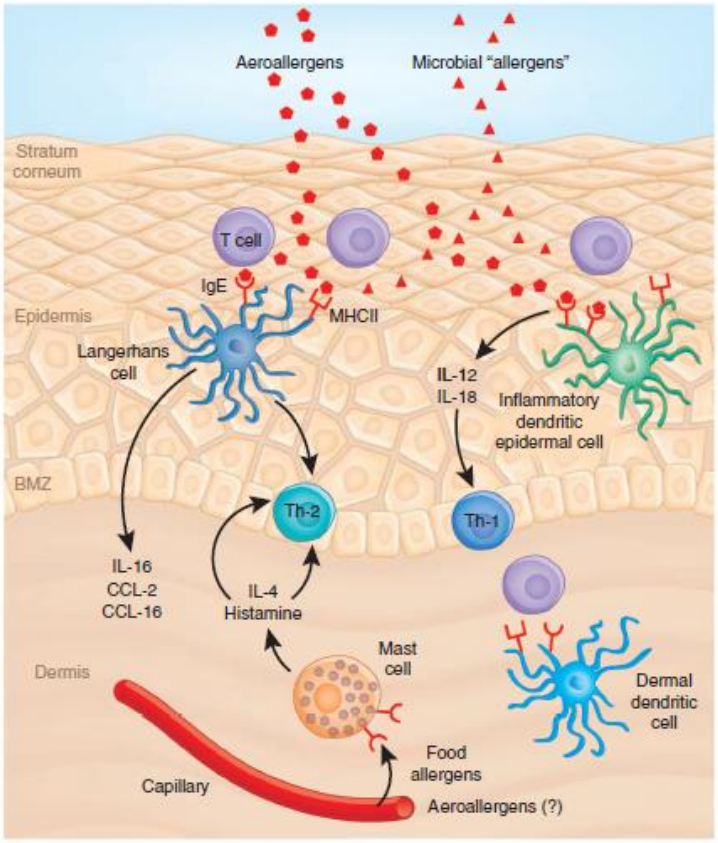

Fig.2. Effects of allergens in cutaneous inflammation and atopic dermatitis. Allergens can enter the skin both through the bloodstream (for example, food) or through the stratum corneum (for example, cosmetics). In the skin, receptors on epidermal dendritic cells and T cells, which are components of the innate immune system, and on dermal mast cells contribute, together with monocytes, to the early inflammatory response (3-12h) and to the recruitment and activation of adaptive immune components (specific T and B cells), leading to prolonged inflammatory responses (3-5 days) (adapted from 18).

Whether or not an inflammatory response will be evoked in response to a potential allergen thus depends upon 1) the qualitative and quantitative forms of cross talk between immune components resident in the epidermal and dermal compartments of the skin 2) the presence of functional microcirculation (dermal capillaries and lymphatic drains) to harness adaptive immune components, and 3) the forms of immune tolerance (regulatory T cells [Treg] repertoire) established within the adaptive immune system. Most components of the adaptive immune system activated by the induction of innate immune responses secrete a variety of peptides which participate in maintaining immune tolerance through two distinct mechanisms: by regulating the balance between pro-inflammatory and anti-inflammatory factors, and by inducing the emergence of Treg cells with suppressive activity against auto-reactive T cell effectors (19, 20).
None of these key components are present within in vitro reconstituted skin models.
Nerve endings, through the release of neuropeptides, also play a key role in response to potential irritants by modulating vascular function (vasodilatation, plasma extravasation, endothelial cell activation, etc.), immune cell function (T-cell proliferation, mast cell degranulation, B-cell development, etc.), and cell activation (keratinocyte migration, antigen presentation by dendritic cells, etc.) (21). Furthermore, chemicals capable of inducing itching sensations without directly affecting resident immune effectors can nevertheless lead to scratching which damages skin barrier integrity, opening the way to acute or chronic inflammation in response to usually innocuous skincare products. None of these mechanisms can be addressed by in vitro reconstituted skin models which are bereft of nerve endings.
Finally, in normal skin, the protease inhibitor cystatin A is secreted in sweat and flows out onto the surface of the skin forming a protective layer. Exogenous proteases from, for example, house dust mites (DerP1) are inhibited by the protective layer of cystatin A and cannot break down the corneodesmosomes that lock corneocytes together and give rise to a layer of water resistant lipid lamellae, preventing water loss while providing a resilient permeability barrier that impedes the penetration of allergens. Furthermore, the balance between the expression and activity of proteases, such as KLK7 (SCCE), and protease inhibitors, such as LEKTI and cystatin A, determines the rate of desquamation (corneocytes shedding) and thereby the thickness of the barrier (22).
Since functional sweat glands are entirely absent in reconstituted in vitro skin models, it follows that the barrier properties of their cornified and granular layers stands to present significant differences with respect to healthy human skin in situ (23).
As a consequence of all these functional differences, keratinocytes are, in reconstituted skin models, the only cells capable of initiating a pro-inflammatory response to the presence of potentially allergenic/sensitizing chemicals or preparations. From an immunological standpoint, these keratinocyte-mediated mechanisms are probably the only responses common to both in vitro skin models and in situ human skin.
Therefore, any improvements one may attempt bringing to the efficacy, flexibility and relevance of in vitro reconstituted skin models in terms of safety testing shall require focusing upon keratinocyte-mediated pro-inflammatory mechanisms.

Keratinocyte-mediated pro-inflammatory mechanisms.

In keratinocytes, there are 5 mechanisms that can be triggered by allergens/sensitizers through innate pattern recognition receptors. These mechanisms can be subdivided into canonical danger signalling pathways (Toll-like receptors [TLRs], NOD-like receptors [NLRs], and NLRP inflammasomes) and non-canonical danger signalling pathways where additional functions of some of these receptor classes may affect outcomes (Fig.3).

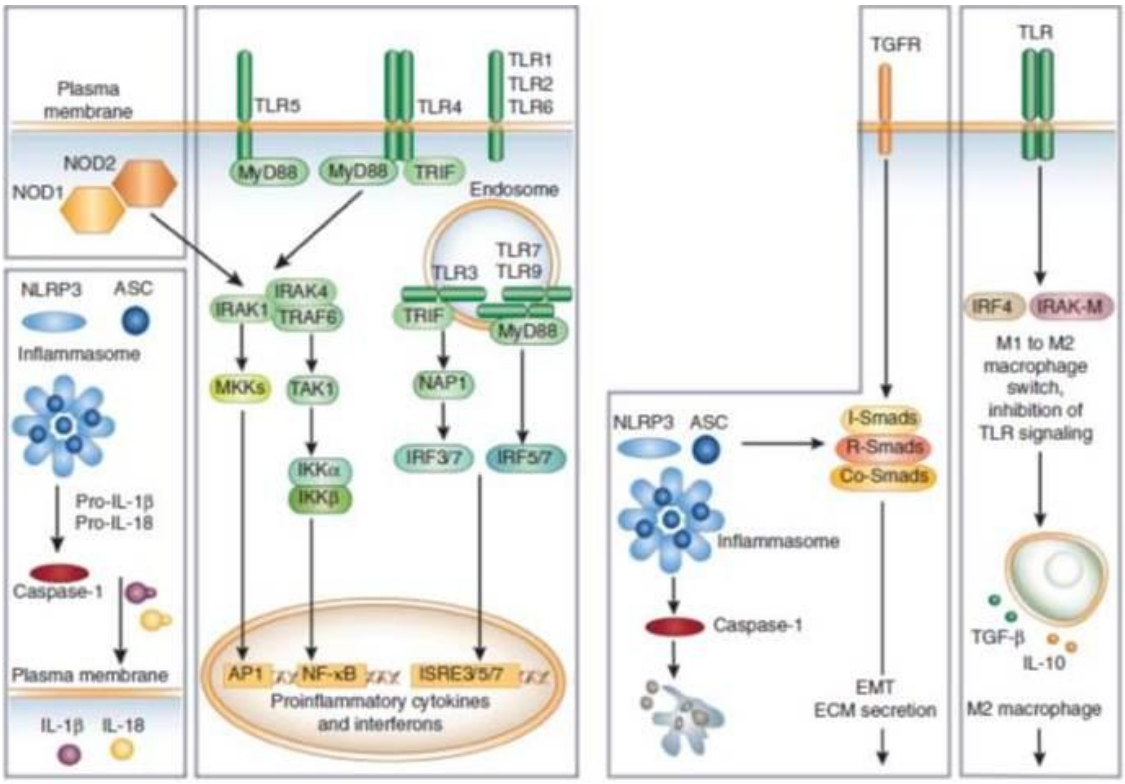

Fig.3. Biological effects of innate pattern recognition receptors activation. Left: Canonical danger signaling pathways. Toll-like receptors (TLRs), NOD-like receptors (NLRs), and NLRP inflammasomes are germ line–encoded pattern recognition receptors that translate danger recognition into the activation of proinflammatory signaling pathways and transcription factors, such as nuclear factor-κB (NF-κB). This induces the secretion of multiple proinflammatory cytokines and chemokines, which sets up inflammation for danger control, usually involving some immunopathology. Right: Non-canonical danger signalling pathways. In chronic sensitization, additional non-canonical functions of these receptor classes may affect outcomes, such as TLR-mediated shifts in the activation of macrophage phenotypes, and NLRP3-mediated pyroptosis or effects on transforming growth factor-β (TGF-β) signalling. AP1: activator protein 1; ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain; Co-Smads: common mediator Smads; ECM: extracellular matrix; EMT: epithelial–mesenchymal transition; IKK: IκB kinase complexes; IL: interleukin; IRAK: interleukin-1-receptor-associated kinase; IRF: interferon regulatory factor; I-Smads: inhibitory Smads; ISRE: interferon-stimulated response element; MKK: mitogen-activated protein kinase kinase; MyD88, myeloid differentiation primary response gene 88; NAP1, nucleosome assembly protein 1; NOD: nucleotide-binding oligomerization domain; R-Smads: receptor-regulated Smads; TAK1: transforming growth factor beta-activated kinase 1; TGFR: transforming growth factor receptor; TRAF: tumor necrosis factor receptor–associated factor; TRIF: TIR-domain-containing adaptor-inducing interferon-β.

While the TLRs are classical transmembrane receptor proteins, the NLRs consists of intracellular soluble proteins that sense cytosolic pathogen-associated molecular patterns (PAMPs) as well as a range of environmental and host-derived stress signals, known as danger-associated molecular patterns (DAMPs).

All three inflammasome systems are in cross-talks and contribute to complex responses (Fig.4).

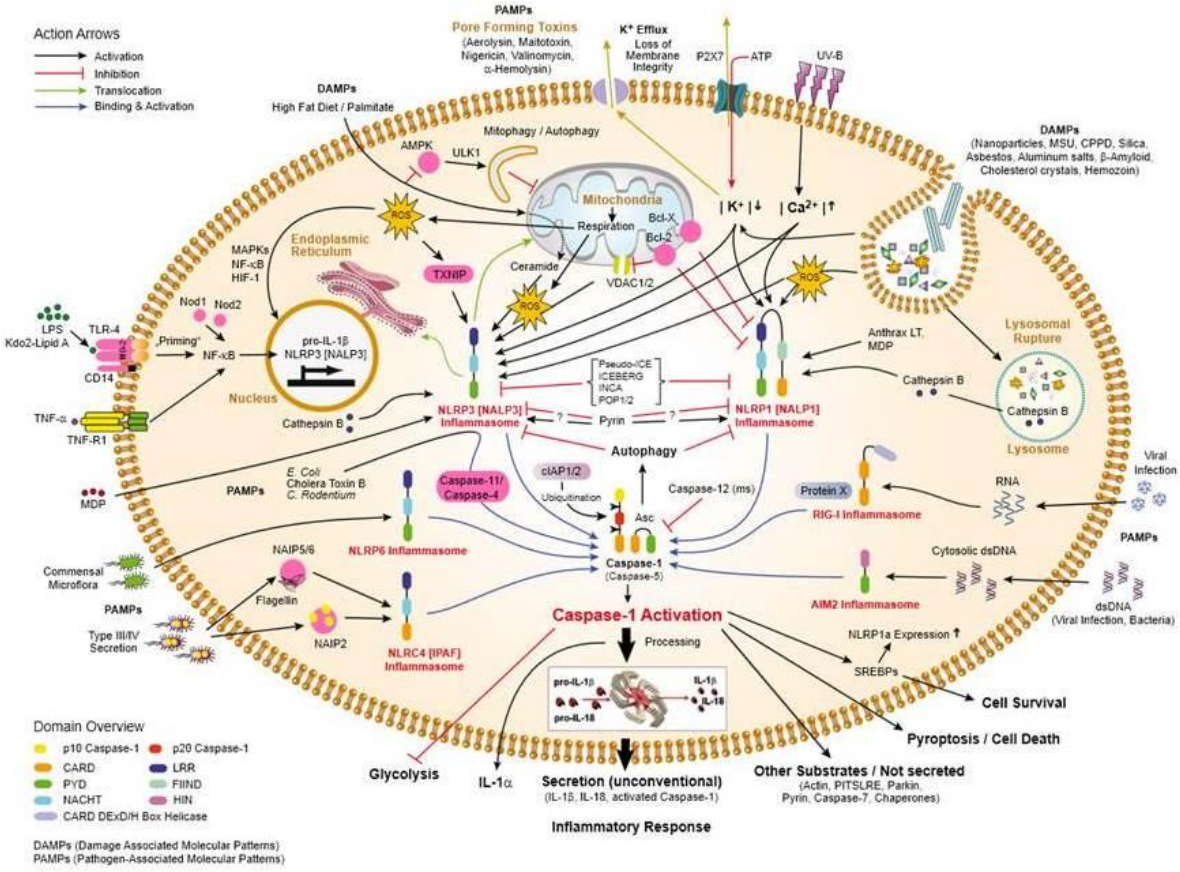

Fig.4. Inflammasomes activation and signalling. The inflammasomes activate mainly caspase-1, leading to the processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18. Assembly of inflammasomes depends on the NOD-like receptor family members, such as the NLRP/NALP proteins. Inflammasomes link pathogenic triggers and metabolic or genotoxic stress signals to the proteolytic processing of interleukins, which promote the inflammatory response.

In keratinocytes, the activation of inflammasomes signalling leads to the secretion of numerous cytokines, the nature, levels and time-course of which depends upon the inflammasome components activated, the strength of inflammasome stimulation and the forms of cross-talks elicited (24).
It is this complex interplay that can be harnessed to considerably improve the efficacy, flexibility and relevance of in vitro reconstituted skin models for robust safety assessment of complex natural products and cosmetic preparations while reliably addressing weak allergens/sensitizers that may be simultaneously present in a preparation and predict their probable effects upon the targeted human epidermal regions.

Harnessing keratinocyte inflammasome signalling to significantly improve the robustness, efficacy and flexibility of in vitro reconstituted skin models.

Whatever potential sensitizer may be applied onto a “skin model” (be it reconstituted skin or biopsies), it will be first in contact with the cornified layer (stratum corneum, Fig. 1.) of the epidermis and must therefore penetrate this layer in order to encounter keratinocytes capable of responding to its presence.
The layer immediately below this stack of horny cells embedded in a dense matrix of proteins and lipids which cement the cells together into a continuous membrane, is the stratum lucidum, consisting of several layers of flattened dead keratinocytes incapable of any biological response.
Below this layer is the stratum granulosum, consisting, in thick skin, of a few layers of flattened keratinocytes containing numerous keratohyalin granules forming "free" accumulations in the

cytoplasm. Only one layer of granulous keratinocytes may be visible in thin skin. These dying keratinocytes, which begin to release the contents of their lamellar bodies, the lipid contents filling the entire interstitial space, also are incapable of biological response.
This thin layer is followed by the thicker stratum spinosum, composed of keratinocytes becoming irregularly polygonal. The cells are often separated by narrow, translucent clefts spanned by spine-like extensions of the keratinocyte's outer membrane (hence the name of the layer), which interconnect the cells of this layer. This is the first layer in which keratinocytes are capable of mounting a response to sensitising agents.
The deepest layer of the epidermis (closest to the dermis) is the stratum basale. It consists of a single layer of columnar or cuboidal keratinocytes which rest on the basement membrane (DEJ). Basal keratinocytes are the stem cells of the epidermis. Their mitotic activity replenishes the cells in more superficial layers as these are eventually shed from the epidermis. The renewal of the human epidermis takes about 3 to 4 weeks. Stratum basale keratinocytes are the most responsive to sensitizing agents.

In many signalling systems, and particularly so in inflammasome-mediated mechanisms, a strong stimulus results in strong and rapid induction of negative feedback pathways that quickly suppress the signalling response. This is in contrasts to a weaker stimulus that reaches a lower peak of signalling but in which the response is sustained because of slower and less-robust induction of the negative feedback mechanisms.
Because of the way immediate-early and early gene-transcription programs are kinetically linked to signalling inputs, these two different outcomes of inflammasome stimulation can lead to very different cytokine-mediated downstream effects ranging from acute to chronic inflammation.
For instance, IL-1β production by keratinocytes, bioavailability following secretion and paracrine as well as autocrine signalling (25) are all negatively regulated (inhibited) by at least six interrelated mechanisms. These include the initial counter-regulation of TLR signalling (26), sustained TTP activity via DUSP1 (27, 28), mRNA processing by TTP and related mRNA-binding proteins (regulated by uridine-rich element in 3' untranslated regions) down-regulating IL17A and IL22 production (29) both of which enhance the secretion of IL-1β by keratinocytes via the ROS-NLRP3-caspase-1 pathway (30), type I interferon-mediated inhibition of the NLRP3 inflammasome (31) and autophagy-mediated destruction of inflammasomes (32).
Therefore, in response to a test preparation, a given spectrum of secreted keratinocyte cytokines together with their secretion time-course and relative levels actually reflects a defined pattern of inflammasome components activation, stimulation strengths, signalling levels and feedback implementations that are entirely pertinent to in vivo situations.
Furthermore, while allowing fine distinctions to be made between different forms and levels of irritation potentials, this approach also allows detection of weak allergens/sensitizers together with the type of response pathways they prime. This is particularly important since it leads to identification of components that should not be simultaneously present in a complex preparation because of their potential to suddenly provoke adverse events in vivo through synergistic interactions between primed inflammasome-mediated pathways.
However, in order to be actually harnessed, this approach requires extensive systems modelling in order to define in detail

- The patterns of cytokines that constitute mechanism-associated signatures,
- Their secretion time-course characteristics,
- The differential effects of modulatory/regulatory feedback mechanisms, and
- The forms and time-course of pattern alterations indicative of delayed exacerbation/extinction.

This is what we have undertaken. The main modelling and compilation tasks have now been completed. We are now looking for complementary partners (mathematical modelling companies,

cosmetics companies, and academic teams to conduct the validation phases. Internal coherence cross-checks and refinements prior to experimental validations need to be conducted.

But this approach, which uses extensive systems modelling to by-pass the recourse to animal testing, does not solely apply to cosmetics. It can also be utilised for novel therapeutic developments.

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