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REVIEW ARTICLE

Dendritic cells and the assessment *in vitro* of skin sensitizing potential

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Abstract

It is now well established that dendritic cells (DC) play pivotal roles in the initiation and orchestration of adaptive immune responses, including cutaneous immune responses to chemical allergens that drive the acquisition of skin sensitization. It is not unexpected, therefore, that a large number, and wide variety, of proposed approaches for the identification of skin sensitizing chemicals *in vitro* are based upon the use of cultured DC or DC-like cells. The use of DC in this context is legitimate. However, with our rapidly increasing understanding of the diversity of cutaneous DC with respect to both phenotype and function, it is timely now to review briefly the potential limitations and interpretive difficulties that are associated with the use of DC-based assays. Among the important considerations are the fact that chemical-induced changes in the characteristics and function of cultured DC will not necessarily reflect accurately the events that support the development of skin sensitization *in vivo*. In addition, most DC-based assays are predicated on a view that cutaneous DC have as their primary function the initiation of adaptive immune responses. However, it is now appreciated that cutaneous DC, and in particular epidermal Langerhans cells (LC), may also play important immunoregulatory roles that serve to limit and contain skin immune responses. Notwithstanding these considerations there is reason to believe that at least some *in vitro* DC-based assays are of value, and indeed some are currently the subject of a formal validation process. However, it is appropriate that such assays are configured and interpreted carefully, and with an appreciation of the complexity of DC biology.

Keywords: Skin sensitization, hazard identification, Langerhans cells, dendritic cells

Introduction

For a variety of reasons, there is currently considerable interest in, and commitment to, the development of alternative methods for the identification of skin sensitizing chemicals that avoid the use of experimental animals. A variety of experimental strategies are being explored that, to a greater or lesser extent, reflect what is known of the key events and processes that are required for the successful acquisition of skin sensitization (1,2). Among the most popular approaches are those based upon the use of cultured dendritic cells (DC) or DC-like cells (3–7).

DC are known to play pivotal roles in the development of sensitization (8,9), and there is no doubt therefore that, in principle at least, they represent legitimate target or indicator cells for *in vitro* assays. Nevertheless, in recent years our understanding of the diversity, phenotype and function of DC has increased significantly, and it is

appropriate therefore to consider progress that has been made with DC based assays, and future opportunities, in the light of that newer information about the complex biology of these cells (3,4,10–12).

The purpose of this short commentary is to review briefly the main strategies that have been developed for alternative skin sensitization tests based on the use of DC or DC-like cells, and to explore the advantages and limitations of such approaches in the light of our evolving appreciation of the biology of cutaneous DC.

DC and the characterization of skin sensitizing chemicals *in vitro*

The (relatively recent) history of the use of DC and DC-like lines has its roots in the concept that the Langerhans cells (LC) of the epidermis were the “master

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key" to the development of skin sensitization (13). As will be seen later in this review, that fundamental basis may be somewhat flawed, or at least over-simplistic. Nevertheless, it remains the case that chemical sensitizers, in one way or another, do interact with, and produce changes in, DC, and consequently this remains a legitimate target for those attempting the development of *in vitro* alternatives (4,14).

Following the original observations of the rapid elevation of interleukin (IL)1 β in response to topical exposure to a skin sensitizer (15,16), efforts were made to reproduce this observation in DC (17–21). This work involved the use of CD34⁺ monocyte-derived DC that were obtained from peripheral blood of human volunteers, the isolation of usable numbers of viable DC directly from skin representing an insuperable technical hurdle. Typically the monocyte fraction required several days of culture in the presence of cytokines, including granulocyte/macrophage colony stimulating factor (GM-CSF), to yield a cell population with DC characteristics. The presence/absence of transforming growth factor (TGF) β was employed to achieve a switch between an immature and mature phenotype, the logic being that the primary interaction of DC with a skin sensitizing chemical occurred with immature cells.

Work continues to optimize the above approach, but the limitations of the constant need for freshly obtained human peripheral blood from multiple donors (to overcome the limitations of interindividual variation) remains a significant barrier to progress. In addition, there is some evidence that although DC characteristics are displayed by the monocyte-derived population, perhaps not surprisingly, there may also be functional differences compared with skin DC (22,23). Other investigators have focused instead on the use of cord blood derived DC (24,25). Perhaps to an even greater extent than with peripheral blood, the use of cord blood, although an excellent research tool, represents an obvious and significant challenge in terms of translation into routine predictive toxicology.

An alternative approach to the above has been adopted by a number of groups, who have preferred dendritic-like cell lines to the freshly isolated article. This topic has been the subject of a relatively recent detailed review (14) and therefore will not be considered exhaustively here. Instead, emphasis will be focused upon those assays based on dendritic-like cell lines that are the subject of relatively advanced formal validation under the auspices of the European Union (EU). In reality, several of these cell lines exist, but those that have come into prominence in terms of predictive test methods are THP-1 (a human monocytic leukemia cell line), U-937 (a human histiocytic lymphoma cell line), and most recently MUTZ-3 (a human acute myeloid leukemia cell line). For each of these cell lines, chemical sensitizers have been shown to induce changes in either cytokine production and/or the expression of membrane markers associated with DC activation.

The best described of the methods involving DC-like cell lines is the human cell line activation test (h-CLAT) that was developed in Japan (26–28). This method employs 48-h culture of THP-1 cells with exposure to a range of concentrations of the test chemical either side of the dose which causes a 25% reduction in cell viability. The endpoint measures increases in membrane markers associated with increasing DC maturity (CD54 and CD86) using flow cytometry. Recent publications have detailed investigations on the dependence of the outcome of the method on cytotoxicity (29) and also report the reproducibility and interlaboratory transferability of the h-CLAT (30). At present, the method is undergoing a blinded evaluation of within and between laboratory variability, involving the assessment of 24 chemicals in each of 4 laboratories (31).

A second DC-like cell line assay, the myeloid U-937 skin sensitization test (MUSST) has been developed by scientists at L'Oreal and is mentioned in recent publications from the European cosmetics industry (32,33). In this method, cells are cultured for 24 h in the presence of test chemical, followed by flow cytometric analysis of changes in expression of CD86. The MUSST is currently being assessed in the European Centre for the Validation of Alternative Methods (ECVAM) validation process alongside the h-CLAT.

The outcome of this validation exercise is not expected to be known until well into 2013. Even then, it has to be recognized that these efforts are directed solely towards the predictive identification of skin sensitization hazards (e.g. (34,35)), notably as characterized in the latest version of the United Nations Globally Harmonized System (36). As a consequence, there remains the challenge regarding assessment of the relative potency of identified skin sensitizing chemicals, which of course is the real key to meaningful risk assessment and risk management (31).

An alternative endpoint measurement for DC relates to their tendency to migrate towards draining lymph nodes in response to allergen exposure. This property was explored in skin explants some years ago, but found not to be specific to sensitizing chemicals, perhaps reflecting instead a more generic response to noxious stimuli (37,38). However, the latter authors also argued that by careful selection of concentrations, sensitizers could be distinguished from irritating chemicals (39). It is important to be aware that this work raises two important issues – the practical utility in a routine toxicology assay of freshly obtained skin explants and the frequent need to use cytotoxic concentrations of test chemicals to obtain results. A similar set of issues applies to the use of freshly derived blood cells. These and other aspects are considered in the subsequent section.

Advantages and limitations of DC-based assays

By definition, allergic sensitization, including skin sensitization, is dependent upon elicitation of an adaptive immune response and immunological priming. The acquisition of skin sensitization following topical

encounter with a contact allergen requires engagement of the innate and adaptive immune systems through complex cellular and molecular interactions that are tightly orchestrated (9,40–45). Many of the early events induced during sensitization culminate in and support the activation of DC in the skin and their deployment for presentation of antigen to responsive T lymphocytes. The paradigm until relatively recently focused on the role played by epidermal LC. The view was that LC are activated by skin sensitizing chemicals, that they recognize, internalize and process antigen, and that they migrate from the epidermis, via afferent lymphatics, to regional lymph nodes. The evidence is that the mobilization and migration of LC is induced and regulated by cytokines and chemokines, and that while in transit from the skin they are subject to phenotypic changes that equip them to present antigen effectively to T lymphocytes (46–49).

Although the above summary is essentially correct, there have been two relatively recent developments that impact significantly on our view of DC and skin sensitization. These are first that LC are not the exclusive DC within skin that are able to support the development of skin sensitization, and the second is that LC have a more complex impact on the skin immune system than was appreciated previously because it is now known that they possess immunoregulatory, as well as immunostimulatory, properties.

Thus, it is now firmly established that there are DC other than epidermal LC that can support the acquisition of skin sensitization. In mice, epidermal LC express Langerin, a determinant that was at one time believed to be specific for these cells. However, it is now known that there are (at least) two discrete populations of dermal DC (dDC), one of which also expresses Langerin (50). Characterization of the activity of these cells has been facilitated by the availability of engineered mice that lack discrete populations of skin DC. Both LC and Langerin⁺ dDC are able to migrate to regional lymph nodes following mobilization, and the available evidence suggests that both populations have the potential to induce sensitization. In practice, however, the relative requirement for LC and dDC may be dependent upon the availability in the skin of the inducing chemical allergen, and possibly other as yet undefined variables (51–55). The important observation is, therefore, that in mice there exist DC other than epidermal LC that may contribute to the development of skin sensitization, and that may in some circumstances play a predominant or even exclusive role in driving responses to contact allergens. There is also heterogeneity among cutaneous DC in human skin, although the exact speciation appears to be somewhat different (56).

The second important observation has been that LC, and possibly other skin DC, may display immunoregulatory activity that serves to inhibit, or at least constrain, cutaneous immune responses including the acquisition of skin sensitization (54,55,57–59). It has even been suggested that LC are precommitted to an immunoregulatory

function in the skin; the purpose being to maintain local tolerance and provide a mechanism to prevent the expression of excessive or damaging cutaneous immune responses (60). The mechanisms through which LC mediate these regulatory constraints have not yet been characterized in detail, although there is evidence that the induction of regulatory T (Treg) cells and the production of IL-10 may both be important (54,61,62).

One suggestion is that, in some circumstances at least, it is antigen-bearing dDC that arrive first in draining lymph nodes during skin sensitization, and this drives the activation and expansion of allergen reactive T lymphocytes. It is further argued that somewhat later LC with acquired antigen reach the same lymph nodes where they interact with T lymphocytes, become fully activated, secrete IL-10 and dampen the T cell response (54,55).

Setting aside for one moment this added complexity, it remains the case that the basic premise that skin sensitization is dependent upon the activation, mobilization, migration and maturation of cutaneous DC remains valid. On that basis it is not unreasonable to regard cultured DC as relevant indicator cells for *in vitro* assays of skin sensitizing potential. However, what the emerging information about the phenotypic diversity and functional characteristics of skin DC should alert us to is that the results of such assays need to be interpreted with caution.

In this context, there are a number of relevant points to consider. Not least among these is the fact that, although it is well established that skin sensitization is associated with the migration and maturation of cutaneous DC, that does not imply that the phenotypic changes associated with the activation of these cells are caused by the direct interaction of the chemical allergen with DC. Thus, for example, as indicated above, there are DC-based *in vitro* assays that seek to measure skin sensitizing potential as a function of chemical-induced increases in the expression of membrane CD86 (a cofactor required for effective activation of T lymphocytes). This is a perfectly legitimate marker of DC activation, and there is every reason to believe that *in vivo* those DC that provoke T lymphocyte responses express membrane CD86. However, there is no reason to suppose that changes in the expression of this costimulatory molecule are triggered by a direct contact of the DC with chemical allergen.

Another important consideration is that the activation of some DC during skin sensitization, and in particular the activation of LC, may have an inhibitory, rather than a stimulatory, impact on the development of hapten-specific immune responses. The implication is that not all DC activation induced by exposure to a chemical allergen is necessarily reflective of immune stimulation and the development of skin sensitization. An associated issue is the fact that activation of different DC populations, or the activation of the same DC population in different ways or in different contexts, may result in the selective development of qualitatively distinct immune responses. There is, for instance, a view that epidermal LC favor the

development of T helper (Th)2, rather than Th1-type immune responses; the latter being more commonly associated with skin sensitization (56). It may be relevant also that topical exposure of mice to Th1-inducing and Th2-inducing chemical allergens is associated with different tempos of LC migration from the skin; the hypothesis being that the kinetics of LC migration to regional lymph nodes will impact on the quality of immune response that is provoked (63).

Key considerations for the design and interpretation of DC/DC-like based assays for skin sensitization

The most important considerations are listed below. Some of these, such as the problem of delivering lipophilic chemicals to aqueous culture systems, are of course not restricted to DC-based assays. Other issues are, however, related to our evolving understanding of the biology of DC.

- There remains a real challenge in ensuring the effective delivery of lipophilic chemicals to aqueous cell cultures, and having confidence that there has been opportunity for the test material to interact with target cells.
- DC are present in tissues and blood in only very small numbers and it is difficult to use isolated primary cells in *in vitro* assays. Moreover, the act of isolation itself will cause the damage to and/or activation of DC.
- Due to the difficulties in using primary cells for *in vitro* assay systems, attention has focused on employing either DC derived from peripheral blood, cord blood or bone marrow precursors, or DC-like cell lines. In neither case is there necessarily direct phenotypic or functional comparability between the cells used in test systems and native DC.
- With regard to the previous point, and in the light of the fact that more than one type of DC is able to support the development of skin sensitization, it is difficult to define what characteristics an ideal DC-like cell should have for the purposes of an *in vitro* test system.
- It is possible that for effective assessment of the impact of chemicals on cultured DC there is a requirement for a certain level of cell trauma or cell death. Changes in cell viability also provide some reassurance that the test chemical has indeed had opportunity to interact with the cultured cells. However, it is not easy to judge what level of cell death is acceptable/desirable, and the degree of cell death that will render the assay system uninterpretable.
- Although it may be the case that the interaction of DC with antigen, including chemical allergen or adducted proteins, provides partial activation, it is possible – and indeed likely – that other factors or signals (danger signals) deriving from the surrounding tissue microenvironment are required for full activation.
- It is possible that chemicals may cause changes in cultured DC that are more reflective of acquired

immunoregulatory function than they are of immunostimulatory activity.

- Even if a test system based on DC or DC-like cell lines can be established that provides a sound basis for hazard identification, assessment of relative skin sensitizing potency will remain a challenge. Currently there is no information available about whether or in what ways chemical induced changes in DC phenotype or function *in vitro* reflect sensitizing potency *in vivo*.

Finally, it is worth addressing one other feature of DC-based assay methods. As described previously, and in spite of the considerations listed above, some of the DC-based assays appear to perform well and to display impressive levels of specificity and selectivity. The question this poses is as follows: if the introduction of a chemical allergen to a monoculture of DC-like cells is able to cause changes in the phenotype of those cells that differ from effects that may be associated with skin irritants/nonsensitizing chemicals, how is it that DC are able to draw that distinction? It is easier to understand how within the complex cell and molecular matrix of whole skin decisions are reached about the need to launch an immune response based upon interpretation of multiple signals. But, how are DC in isolation, and in the absence of that tissue matrix, able to determine which chemicals are allergens and to distinguish those from nonallergenic materials?

Concluding comments

DC are now widely acknowledged to be the orchestrators of adaptive immune function, and there is no doubt that they play a pivotal role in driving cutaneous immune responses, and in the acquisition of skin sensitization. It is, therefore, entirely legitimate to view DC as a potentially useful and informative cellular basis for alternative methods for the identification of skin sensitizing chemicals. However, with a growing awareness of the diversity of DC, including DC resident in the skin, and of their of their potential immunoregulatory function, it is appropriate that DC-based assays are configured appropriately and interpreted with caution.

Declaration of interest

Two authors declare that there are no conflicts of interest. DAB is a chairman of the European Centre for Validation of Alternative Methods Validation Management Group which is conducting a validation trial on the h-CLAT and the MUSST.

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