

Review

Dendritic cells and skin sensitization: Biological roles and uses in hazard identification

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Abstract

Recent advances have been made in our understanding of the roles played by cutaneous dendritic cells (DCs) in the induction of contact allergy. A number of associated changes in epidermal Langerhans cell phenotype and function required for effective skin sensitization are providing the foundations for the development of cellular assays (using DC and DC-like cells) for skin sensitization hazard identification. These alternative approaches to the identification and characterization of skin sensitizing chemicals were the focus of a Workshop entitled “Dendritic Cells and Skin Sensitization: Biological Roles and Uses in Hazard Identification” that was given at the annual Society of Toxicology meeting held March 6–9, 2006 in San Diego, California. This paper reports information that was presented during the Workshop.

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Introduction and background

It is now well established that via the activation, regulation and modulation of T lymphocyte function, bone marrow-derived dendritic cells (DCs) play pivotal and essential roles in the orchestration of adaptive immune responses and maintenance of

effective host resistance (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2001; Pulendran et al., 2001; Smits et al., 2005). Generically these cells have responsibility for the processing of antigen and its presentation to T lymphocytes, and the qualitative alignment of induced immune responses.

Among the wider family of DCs are Langerhans cells (LCs) that reside within the epidermis and which play important and probably decisive roles in the development of cutaneous immune function. In the skin LC are believed to serve as sentinels of the immune system, recognizing and responding to

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changes in the microenvironment and encounter with foreign antigen. Specifically, the roles of LC are to recognize, internalize and process antigen and to transport it from the skin, via afferent lymphatics, to regional lymph nodes. The LC that bear antigen and which become mobilized for transit from the skin undergo a functional maturation such that by the time they reach the paracortical areas of draining lymph nodes they have lost the characteristics of antigen processing cells and acquired instead the properties of immunostimulatory DC. These cells present antigen effectively to responsive T lymphocytes and thereby initiate cutaneous immune responses. These same events are involved in the processing and presentation by LC of chemical antigens encountered at skin surfaces, and the subsequent acquisition of skin sensitization (Kimber et al., 1998, 2000; Cumberbatch et al., 2000, 2003; Griffiths et al., 2005). In recent years understanding of LC, their life history, and the mechanisms through which they provoke and modulate immune responses has increased (Wang et al., 2002; Merad et al., 2002; Hunger et al., 2004; Fujita et al., 2005), although there remains a debate about the exact contribution of these cells to contact hypersensitivity (Kaplan et al., 2005; Bennett et al., 2005; Kissenpfennig and Malissen, 2006; Yu et al., 2006). Nevertheless, it is the case that, under normal circumstances, LC affect the earliest cellular responses to contact allergens, and therefore represents a legitimate focus of interest for the development of novel approaches to the identification of skin sensitization hazards.

One seminal observation was reported by Enk and Katz (1992) who found that topical exposure of mice to contact allergens was associated with a rapid increase in the expression by LC of mRNA for IL-1 β . This discovery, combined with the increasing availability of cultured DC with LC-like properties (Inaba et al., 1992), provided impetus for exploration of new cell-based assays for skin sensitization hazard identification (De Silva et al., 1996). Since then experience has grown, new cell systems have been described, and additional allergen-selective markers have been proposed (Casati et al., 2005). However, it is clear that there remain important challenges and technical hurdles to be negotiated if DC/LC-based assay systems are to be applied widely in safety assessment. Here we review recent progress that has been made in exploiting an appreciation of the roles played by LC as the basis for the development of new approaches to skin sensitization testing.

Langerhans cells, cytokines and skin sensitization

As indicated above, there has been considerable interest during the last two decades in defining the roles played by epidermal LC in the acquisition of skin sensitization. In simplistic terms it is the responsibility of LC to translate antigenic signals at skin surfaces, and in particular antigenic signals associated with cell or tissue trauma, into adaptive immune responses. To fulfil these responsibilities it is necessary that LC are able to recognize, internalize and process chemical allergens (usually associated with a protein carrier), and to transport processed antigen from the skin to regional lymph nodes. Here, either the mature DC into which migratory LC

develop, and/or DC already resident within the paracortex of draining lymph nodes, present antigen to responsive T lymphocytes and initiate a cutaneous immune response. If this primary immune response is of sufficient magnitude and vigor then skin sensitization will be acquired (Cumberbatch et al., 2000, 2003, 2004; Kimber et al., 1998, 2000, 2002a, 2002b; Griffiths et al., 2005).

There is no doubt that cytokines and chemokines available locally play decisive roles in the migration and maturation of LC following skin sensitization. Of particular importance in the mobilization of LC are interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) and IL-18 (Antonopoulos et al., 2001; Cumberbatch and Kimber, 1992; Cumberbatch et al., 1997, 2001; Wang et al., 1996), with IL-10 possibly playing a counter-regulatory role (Wang et al., 1999). In addition, it is apparent also that in addition to cutaneous cytokines providing a stimulus for LC to transit from the epidermis, chemokines, and in particular, *CCL21* and *CCL19*, ligands for the CCR7 receptor, are responsible for guiding migrant cells into, and positioning them within, peripheral lymph nodes (Caux et al., 2000; Cyster, 1999; Ohl et al., 2004).

Collectively, the changes to which LC are subject following skin sensitization, and which are induced and regulated largely by cytokines and chemokines, equip these cells for their journey from the skin to regional lymph nodes, and for the effective presentation of antigen to T lymphocytes (Fig. 1). Included among these changes are: (a) the increased production by LC of a variety of cytokines, including IL-1 β , (b) the reduced expression of E-cadherin that allows LC to disassociate from surrounding keratinocytes in the epidermis, (c) the elevated expression of adhesion molecules and other factors that facilitate migration within and through tissue matrices, (d) the induced expression of CCR7 for homing to draining lymph nodes, and (e) acquisition of the phenotypic characteristics of mature antigen presenting cells (increased levels of MHC class II determinants and costimulatory molecule expression) that facilitate activation of responsive T lymphocytes (Caux et al., 2000; Cumberbatch et al., 1996; Enk and Katz, 1992; Ohl et al., 2004; Price et al., 1997; Ratzinger et al., 2002; Schuler and Steinman, 1985; Schwarzenberger and Udey, 1996). It is these, and other associated changes in LC phenotype and function required for effective skin sensitization, that have provided the foundations for the development of cellular assays (using DC and DC-like cells) for skin sensitization hazard identification.

Approaches to skin sensitization hazard identification using cultured dendritic cells

T cell immunity against tumor and bacterial or viral infections relies essentially on the recognition of antigenic peptides processed and presented to T cells by antigen presenting cells (APCs). During *in vivo* immune responses, the role of APC is played primarily by dendritic cells (DCs) forming a sentinel network able to detect, capture, and process antigens (Aiba et al., 1997; Cella et al., 1997). In peripheral tissues DC can be found in epithelial tissues in an immature state with a high capacity for antigen uptake and processing, but

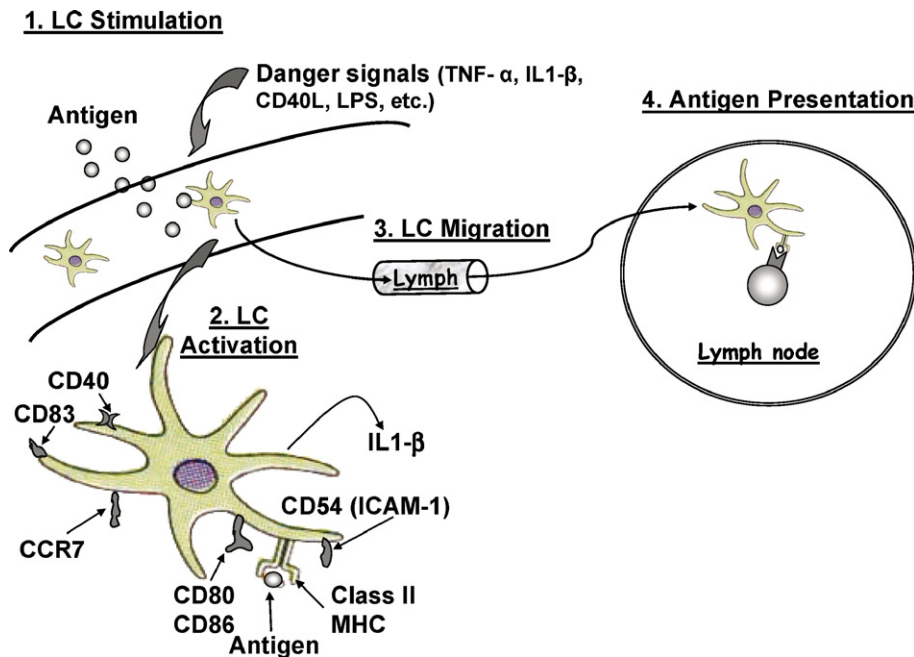


Fig. 1. Langerhans cell activation, migration to the draining lymph node and antigen presentation.

unable to stimulate primary T lymphocyte responses (Banchereau and Steinman, 1998; Cella et al., 1997). Upon antigen capture, stimulation by microbial products through Toll-like receptors (TLR) but also in response to inflammatory cytokines (TNF- α , IL-1 β) local DC undergo a maturation process leading to the up-regulation of certain co-stimulatory molecules (including, for instance, CD86, CD80, CD40), MHC class II molecules and the CD83 protein. Thereafter, DCs migrate to the T cell areas of lymphoid organs during which time they lose antigen-processing activity and become potent immunostimulatory cells. These maturing DCs acquire the ability to migrate in response to gradients of chemokines (*CCL21* and then *CCL19*) through expression of chemokine receptors such as CCR7 and down-regulation of molecules such as E-cadherin (Gunn et al., 1999). Expression of CCR7 is particularly important in skin sensitization, as CCR7-deficient mice show an impaired migration of activated LCs into draining lymph nodes after skin painting with FITC and consequently, lack any contact hypersensitivity (Forster et al., 1999).

Dendritic cells were first identified in the epidermis in 1868 and were named Langerhans cells after their discovery. Their presence in other tissues was identified in 1973 (Steinman and Cohn, 1973). Currently, it seems that DCs comprise at least three distinct subsets, including two within the myeloid lineage, LC and interstitial DC (termed dermal DC in the skin), and one within the lymphoid lineage, the so-called lymphoid DC subset, also named plasmacytoid DC. LC appear to represent a sublineage of DC that are distinct from both interstitial and plasmacytoid DC. Dependence on transforming growth factor-beta (TGF- β) for the development and derivation from slowly cycling local precursors are properties that differentiate LC from other DC including interstitial DC in the dermis (Valladeau and Saeland, 2005). The skin harbors two DC subsets, LC and interstitial/dermal DC (IDDC).

Dendritic cells are a minor population within epithelial cells in organs such as epidermal cells in the skin; isolation of this population in sufficient numbers is a difficult task and often results in unspecific activation of DC. Generating DC from murine bone marrow CD34⁺ hematopoietic cell precursors (HPC) has been used as an alternative, but this procedure is time-consuming and requires a significant number of animals. In the early 1990s, the discovery of culture techniques supporting the *in vitro* generation of large numbers of human DC from hematopoietic progenitors has allowed the development of human DC models (Caux et al., 1992). Two main protocols to generate DC, from either monocytes or CD34⁺ hematopoietic cell precursors, have been described. CD34⁺ HPC, isolated from cord blood, or bone marrow mononuclear cells using immunomagnetic beads, can be induced to proliferate *in vitro* in response to several cytokines in combination. TNF- α enhances significantly the proliferation of CD34⁺ HPC induced by either IL-3 or granulocyte/macrophage colony-stimulating factor (GM-CSF). Addition of SCF (stem cell factor) or Flt-3L (fms-like tyrosine kinase-3 ligand) increases the yield of DC, but does not affect DC differentiation status. Many candidate DCs are CD1⁺CD14[−] during the later stages of culture (days 12–14) from CD34⁺ HPCs in FCS-containing medium. However, when examined earlier two DC subsets emerge independently by days 5–7, as defined by the mutually exclusive expression of CD1a and CD14. When cultured with GM-CSF and TNF- α , CD14⁺CD1a[−] intermediates generated E-cadherin[−], langerin[−]CD1a⁺ interstitial DC that lack Birbeck granules, and CD14[−]CD1a⁺ intermediates generated E-cadherin⁺langerin⁺CD1a⁺ LC-like DC possessing Birbeck granules by a TGF- β -independent pathway. However, adding TGF- β to the media induced a considerable increase of LCs type cells. Peripheral blood monocytes cultured with GM-CSF and IL-4 or IL-13 generate immature DC that can be driven into a mature state by TNF- α . In

Table 1
Phenotypic markers of immature human Langerhans DCs and human dermal/interstitial DCs (IDDC) generated *in vitro*

Marker	LC	IDDC
CD1a	+	+
Birbeck granule	+	–
E-cadherin	+	–
CLA	+	–
CCR6	+	–
Langerin/CD207	+	–
DC-SIGN/CD209	–	+
CD86	+ low	+ low
CD83	–	–
CCR7	–	–

Human LC are generated either from CD34⁺ hematopoietic cell precursors using GM-CSF and TNF- α with or without SCF or Flt-3L (in some protocols TGF- β is added to increase the number of LC) or from human monocytes using GM-CSF, IL-4 and TGF- β . IDDC can be obtained from human monocytes using GM-CSF and IL-4.

this case, the DC population obtained resembles interstitial/dermal DC characterized by the expression of DC-SIGN/CD209 (Table 1). Addition of TGF- β to culture of monocytes with GM-CSF and IL-4 has been shown to induce the differentiation of peripheral blood monocytes in LC (Geissmann et al., 1998). Recently, using a new culture system with monocytes and cytokines related to the T helper 2 (Th₂)-type environment (GM-CSF/TNF- α /IL-13/TGF- β), it has been possible to generate both LC and IDDC DC. This new model provides a mean of studying cross-talks between two skin DC outside of their respective compartment (Bechetoille et al., 2006).

Considering similarities between immunity to simple chemicals and that to infectious agents, it is reasonable to speculate that a hapten itself stimulates DC maturation. The establishment of human *in vitro* models of DC offered the possibility to demonstrate that haptens were able to directly activate cultured DC derived from peripheral blood monocytes or from CD34⁺ HPC (Aiba et al., 1997; Degwert et al., 1997; De Smedt et al., 2001). Several studies confirmed these observations showing the upregulation of maturation markers (CD83, CD80, CD86, CD40) on human DC (Arrighi et al., 2001; Aiba et al., 2000; Coutant et al., 1999; Tuschl et al., 2000). Cytokine production such as IL-12p40, TNF- α and IL-1 β has also been reported upon hapten stimulation (Aiba et al., 2003; De Smedt et al., 2001). Recently, the expression of *ccr7* mRNA in human DC stimulated by haptens and the migration of these cells in response to *CCL19* has been observed (Boislève et al., 2004; Aiba et al., 2000). Boislève et al. have also documented the presence of CD83⁺CCR7⁺ DC in response to nickel and DNCB (Boislève et al., 2004). From these observations, it can be concluded that some haptens in common with danger signals are able to trigger DC maturation associated with changes in marker expression (Table 2).

Mitogen-activated protein kinase (MAPK) have been described to play a major role in DC maturation induced by Toll-like receptors (Ardeshna et al., 2000) or by inflammatory cytokines through binding to specific membrane receptors. Recently, it has been possible to show that haptens such as

nickel (NiCl₂ and NiSO₄) and DNCB induce the phosphorylation of p38MAPK, c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK) in DCs derived from CD34⁺ HPC (Boislève et al., 2005). It has been reported also that inhibition of MAPKs is correlated with alteration in phenotypic indicators of maturation such as CD86, HLA-DR, CD40, CCR7 or CD83 in DC derived from monocytes or from CD34⁺ HPCs stimulated with nickel (Aiba et al., 2003; Arrighi et al., 2001; Boislève et al., 2004). Same types of observations were also found for IL-12p40 and IL-6 with p38MAPK playing a major role (Antonios, unpublished observations).

In conclusion, the recent development of culture techniques has allowed the development of two main protocols for generating *in vitro* models of human DC. Human DCs are generated either from CD34⁺ HPC or from monocytes. Addition of TGF- β during the process of differentiation allows the emergence of LC-like cells. In both models, some haptens are able to alter DC phenotype and activation of MAPK suggests that sensitizers can be perceived as danger signals by DC. Similar types of results have been obtained within several research groups suggesting that it may prove feasible in the future to develop *in vitro* models based on analysis of DC function for the purposes of hazard identification.

Gene expression changes induced in cultured dendritic cells by chemicals allergens

To evaluate the ability of chemical sensitizers to interact with and activate immune cells, gene expression changes in cultured human peripheral blood-derived DC (PBMC-DC) induced by exposure to contact allergens have been examined. Initial studies focused on holistic transcript profiling. By taking a more global approach, the entire mRNA profile could be examined and thereby provide an opportunity to investigate genome-wide changes that could identify unique or novel biomarkers of interaction of allergens with DC. One approach taken has been

Table 2
Phenotypic markers modulated by Nickel and DNCB in human CD34-derived DC (from Boislève et al., 2004, 2005; Antonios and Ade, unpublished results)

Marker	NiSO ₄	DNCB
CD1a	–	=
E-cadherin	–	=
Langerin/CD207	–	=
CD86	+	+
CD83	+	+
CCR7	+	+
HLA-DR	+	+/-
CD40	+	+/-
IL-12p40	+	=
IL-6	+	=
IL-8	+	+

CD34-DC were obtained from CD34⁺ hematopoietic cell precursors using GM-CSF and TNF- α with Flt-3L for 7 days. Cells were treated for 24 h with the chemicals. Expression of membrane markers was then measured using specific antibodies and cytofluorometry. Cytokines were measured using ELISA assays. The concentrations of NiSO₄ and DNCB were 500 μ M and 25 μ M, respectively. –: down-regulated; +: up-regulated; =: not modified; +/-: slightly up-regulated.

to conduct transcript profiling using the Affymetrix Genechip platform (Affymetrix Inc., Santa Clara, CA) (Ryan et al., 2004). Briefly, DC generated from the adherent fraction of PBMC by 7 days of culture in the presence of GM-CSF and IL-4 were exposed for 24 h to two different concentrations of dinitrobenzenesulfonic acid (DNBS), the water-soluble analog of the contact allergen DNCB. Total RNA was obtained from the allergen-treated and vehicle control cells and cRNA targets were prepared for hybridization to the Affymetrix Human Genome U95Av2 GeneChip microarrays. Many changes in specific transcripts were observed in both the 1 mM and 5 mM DNBS treatments. Although approximately 2880 genes were differentially regulated upon DNBS treatment versus controls, at either 1 mM, 5 mM, or both ($p \leq 0.01$), 118 genes, 60 up-regulated and 58 down-regulated, were significant in both 1 mM and 5 mM DNBS treatment groups ($p \leq 0.001$).

Real-time PCR analyses were performed on 60 of the 118 significantly regulated genes to determine whether they would exhibit the same pattern and degree of regulation upon DNBS treatment as observed in the initial Genechip experiments. The 60 genes were selected based on criteria that included the magnitude of change over control observed following hapten treatment and a known association with immune mechanisms or DC. Dose response analyses were conducted with PBMC-DC derived from two different donors to address donor-to-donor variability and included multiple concentrations of DNBS to determine the dynamic range of the genes. Additionally, the expression levels of at least 50 of the 60 genes chosen were examined in a larger experiment using elutriated monocyte-derived DC from a single donor that were treated with 3 allergens, 1 irritant, and 1 non-sensitizer. From those data the gene candidate list was narrowed to 29 genes (Table 3) that were analyzed further for their predictive potential using an expanded chemical test set, which included 4 irritants and 11 allergens (ranging in allergenic potency from weak to extreme) (Gildea et al., 2006). This list includes many genes with known immune functions, such as the chemokines and chemokine receptors and immune cell surface molecules (e.g. *CD1E*, *FCER1A*, *MRC1*, *SLAM*) and some genes that currently are not associated specifically with immune function or DCs (e.g. *ABCA6*, *AKRIC2*, *NOTCH3*). In addition, the levels of constitutive expression of these genes in controls cells spans a wide range, with some being highly expressed, like *HML2*, some moderately expressed like *AKRIC2*, and others normally expressed at only low levels, like *NOTCH3*.

As mentioned previously, an initial evaluation of the sensitivity, specificity, dynamic range, and reproducibility of these genes has been conducted to determine their suitability as endpoint measures to predict skin sensitization potential. Fold change comparisons were made for each dose of chemical that was tested and those data were used to generate a scoring table of candidate genes to identify those that appear to have the highest predictive potentials and to distinguish these from those that appear to be of lower predictive value (Gildea et al., 2006). Ultimately this work will lead to the development of a select panel of genes that can be used to characterize the sensitizing activity of chemicals. However, it is important to emphasize

that in advance of that further evaluation with an expanded panel test chemicals is required. Since the desire is to have a test system that incorporates an understanding of the biological mechanisms of the induction of skin sensitization with a defined gene expression profile, it will be necessary and appropriate to consider the biological relevance of selected candidate genes.

Some of the gene expression changes observed reflect DC maturation following exposure to contact allergens and the acquisition of antigen-presenting activity at the expense of the ability to process antigen. Among the list of candidate genes, a down-regulation in the expression of some genes associated with antigen capture such is observed. One such gene is *FCER1A*, the high-affinity receptor for IgE. This receptor is expressed on the surface of human epidermal LC and it allows the uptake of IgE-bound antigens (Mudde et al., 1990). As the LC differentiate or mature into a lymphoid DC both surface and

Table 3
Twenty-nine candidate genes that were analyzed for their predictive potential

GenBank accession no.	Gene symbol	Gene name
NM080284	<i>ABCA6</i> ↓ ^a	ATP-binding cassette, sub-family A (ABC1), member 6
NM001354	<i>AKRIC2</i> ↑	Aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3- α hydroxysteroid)
NM001175	<i>ARHGDIB</i> ↓	Rho GDP dissociation inhibitor (GDI) beta
NM013314	<i>BLNK</i> ↓	B-cell linker
NM002982	<i>CCL2</i> ↓	Chemokine (C–C motif) ligand 2
NM145898	<i>CCL23</i> ↓	Chemokine (C–C) motif ligand 23
NM002984	<i>CCL4</i> ↓	Chemokine (C–C motif) ligand 4
NM003965	<i>CCRL2</i> ↑	Chemokine (C–C motif) receptor-like 2
NM030893	<i>CD1E</i> ↓	CD1E antigen
NM004390	<i>CTSH</i> ↓	Cathepsin H
NM000784	<i>CYP27A1</i> ↑	Cytochrome P450, family 27, subfamily A, polypeptide 1
NM001431	<i>EPB41L2</i> ↓	Erythrocyte membrane protein band 4.1-like 2
NM002001	<i>FCER1A</i> ↓	Fc fragment of IgE, high affinity I receptor
NM006682	<i>FGL2</i> ↓	Fibrinogen-like 2
NM005101	<i>ISG15</i> ↑	Interferon, alpha-inducible protein
NM006344	<i>CLEC10A</i> ↓	C-type lectin domain family 10, member A
NM003897	<i>IER3</i> ↓	Immediate early response 3
NM002183	<i>IL3RA</i> ↑	Interleukin-3 receptor, alpha (low affinity)
NM002438	<i>MRC1</i> ↓	Mannose receptor, C type 1
NM000435	<i>NOTCH3</i> ↑	Notch homolog 3 (<i>Drosophila</i>)
NM012413	<i>QPCT</i> ↓	Glutamyl-peptide cyclotransferase (glutamyl cyclase)
NM006912	<i>RIT1</i> ↑	Ras-like without CAAX 1
NM019554	<i>S100A4</i> ↓	S100 calcium binding protein A4
NM004844	<i>SH3BP5</i> ↑	SH3-domain binding protein 5 (BTK-associated)
NM003037	<i>SLAMF1</i> ↓	Signaling lymphocytic activation molecule family member 1
NM003123	<i>SPN</i> ↓	Sialophorin (CD43)
NM006470	<i>TRIM16</i> ↑	Tripartite motif-containing 16
NM016614	<i>TTRAP</i> ↑	TRAF and TNF receptor-associated protein
NM003329	<i>TXNRD1</i> ↑	Thioredoxin reductase 1

^a Reported direction of regulation following exposure to DNBS in Gildea et al. (2006); ↑=up-regulated; ↓=down-regulated.

intracellular expression of this receptor is lost (Kraft et al., 1998). *CLEC10A* (*HML-2*) is a C-type lectin receptor that has been proposed to be an endocytic receptor for glycosylated antigens, and it has been shown to be expressed on immature monocyte-derived DC, but not on DC following maturation with LPS (Higashi et al., 2002). *MRC1*, also known as CD206, is a mannose receptor that allows DC to take up mannoseylated protein antigens like the mannoseylated glycoproteins derived from many bacteria and fungi. Mature DC do not express CD206 (Sallusto et al., 1995; Mellman et al., 1998).

A down-regulation in the *cathepsin H* and *CD1e* genes have been observed following exposure to contact allergens. Cathepsin H is an endosomal cysteine protease that is reported to be involved in MHC class II antigen processing. It is also secreted from DC and is thought to facilitate DC migration to lymph nodes (Zavašnik-Bergant et al., 2005). The CD1 molecules are associated with the presentation of antigenic glycopeptides or lipopeptides. Unlike the other surface expressed CD1 molecules (a, b, c and d), *CD1e* cannot be detected on the plasma membrane by flow cytometry and displays an unusual cellular distribution which, in DC, is dependent upon their stage of maturation. It has been shown that as DC mature, *CD1e* synthesis progressively declines (Angénieux et al., 2005). To date, the exact role of *CD1e* is not fully understood.

As DC mature, they undergo changes which permit them to interact more effectively with T lymphocytes. *SPN*, sialophorin, or CD43 is a DC cell surface molecule which can regulate cell adhesion and has the potential to mediate cell activation signals. Both LC and monocyte-derived DC express high levels of CD43 (Fanales-Belasio et al., 1997). A down-regulation in CD43 gene expression was recorded after exposure to allergen. Removal of CD43 expression enhances cell interactions (Ostberg et al., 1998), therefore it would be reasonable to suggest that as the DC mature and begin to prepare for interactions with T lymphocytes, CD43 expression will decrease. Following allergen exposure there has been observed an up-regulation in the gene expression of thioredoxin (Trx). It's been shown that during antigen presentation DC release cysteine and thioredoxin which creates a reducing microenvironment that facilitates immune response as T cells require exogenous thiols for activation and function (Angelini et al., 2002).

Changes in the expression of several of chemokine, chemokine receptor, cytokine and cytokine receptor genes were noted following chemical sensitizer treatment. When activated by contact with foreign invaders, immature DC, like LC, begin to produce inflammatory chemokines such as MCP-1 (*CCL2*) and MIP1- β (*CCL4*) and as the DC mature the capacity to produce them is lost (Sallusto et al., 1999). As a result of allergen exposure down regulation in gene expression for both *CCL2* and *CCL4* has been found. *CCL4* is reported to be produced rapidly and at high levels for a short period of time by DC following exposure to maturation stimuli like LPS and TNF- α (Sallusto et al., 1999). Since the examination of gene expression changes are made at 24-h post-allergen exposure, it may be that by this time the DCs have reached a maturation state where they cease to produce message for *CCL2* and *CCL4*. In

addition, Türeci et al. (2003) reported a decrease in gene expression for *CCL2* and *CCL4* in DC after 40 h of maturation with CD40L. *CCL23*, also known as myeloid progenitor inhibitory factor-1, is a ligand for CCR1 (Berahovich et al., 2005) which induces DC chemotaxis. On maturation DC lose CCR1. Monocyte-derived DC constitutively produce *CCL23* and treatment of the DC with anti-CD40 or IFN- γ results in a decrease in *CCL23* transcripts and protein production (Nardelli et al., 1999). CD40L is often used as a means of inducing DC maturation. The speculation is that the observed decrease in *CCL23* gene expression is due to DC maturation resulting from hapten exposure. The *CCRL2* gene encodes a chemokine receptor like protein. The function of this gene is currently unknown but its expression has been shown to increase on monocyte-derived DC following stimulation by LPS, IFN- γ and CD40L (Migeotte et al., 2002). Exposure to chemical allergen also induces an increase in *CCRL2* gene expression and in the expression of the IL-3 receptor alpha subunit gene (*IL3RAX*). The alpha subunit is specific for IL-3 and has been reported to be up-regulated in DC following exposure to maturation-inducing stimuli such as CD40L and TNF- α (Moschella et al., 2001; Dietz et al., 2000) and bacterial antigens (Huang et al., 2001).

Another sensitizer-induced gene expression change of interest is the up-regulation in *NOTCH3*. Notch receptors play a role in many cellular processes. Of the four mammalian Notch receptors, LC are reported to express Notch-3 (B. Nickloff, unpublished observations reported in Weijzen et al., 2002). Jagged-1, a Notch ligand, is constitutively expressed at high levels in keratinocytes and has been reported to induce maturation in monocyte-derived DC (Weijzen et al., 2002). Interestingly, an increase in Notch-3 expression has been reported on DC matured by treatment with LPS (Chen et al., 2002).

In the course of the investigations summarized above a list of 29 candidate genes has been derived. It is unlikely that any single gene will alone be capable of predicting skin sensitization. However, it is hoped that a small battery of carefully selected genes may provide a viable approach to hazard characterization.

Ring trials of dendritic cell surrogate cell lines

There is interest in the use of cell lines as surrogates for DC. This is because the culture procedures used to generate DC from human peripheral or cord blood are lengthy, typically taking 5 to 7 days and require the use of cytokines to induce DC differentiation which add costs to the procedure (Bender et al., 1996; Caux et al., 1996; Romani et al., 1996; Sallusto and Lanzavecchia, 1994). In addition, the resulting DCs do not proliferate so only a limited number of cells can be obtained from any one donor, thus restricting experimental design. From a performance perspective, there are many reports of donor-to-donor variability in the responses to allergen exposure including differences in cell surface marker expression (Aiba et al., 1997), IL-1 β mRNA expression (Pichowski et al., 2000, 2001), and cytokine production (Aiba et al., 1997). As a result,

standardization of test methods that rely on primary cultures of DC from human peripheral blood may be difficult.

Various cell lines have been, or currently are being, explored as potential DC surrogates and include the THP1 (Ashikaga et al., 2002; Yoshida et al., 2003), U937 (Python et al., 2007), KG-1 (Hulette et al., 2002; Yoshida et al., 2003), and MUTZ-3 (Azam et al., 2006) lines. As *in vitro* methods for skin sensitization which use these monocytic cell lines are developed, some types of pre-validation work are needed to evaluate the protocols and prediction models among several laboratories. These small inter-laboratory trials provide an opportunity for optimization and standardization of the protocol, data analysis procedures and refinement of the prediction model prior to submitting the method for a large-scale formal validation study.

The European Cosmetic, Toiletry and Perfumery Association, COLIPA, via its Skin Tolerance Task Force (STTF) has an interest in the development of alternative, non-animal test methods for skin sensitization and irritation. COLIPA has provided funding for a number of projects in these areas and is currently coordinating ring trials of cell line-based *in vitro* assays for skin sensitization. The overall objectives of these trials are to evaluate protocol transfer and performance and, if indicated, to identify areas for protocol and/or prediction model refinement. Two such test systems are currently being evaluated, one which uses the THP-1 cell line and another with the U937 cell line. Human monocyte-derived DC have been used in the development of *in vitro* methods for identifying skin sensitizing chemicals. These two cell lines were selected for evaluation as DC surrogates because of their monocytic lineage. The THP-1 cell line, derived from an acute monocytic leukemia patient, has monocyte characteristics (Tsuchiya et al., 1980) and can be induced with phorbol ester to have macrophage-like function (Tsuchiya et al., 1982). U937 cells, derived from a histiocytic lymphoma patient (Sundstrom and Nilsson, 1976), can be induced to terminal monocytic differentiation with appropriate stimulation (Larrick et al., 1980; Olsson et al., 1983).

The THP-1 cell line is available through the American Type Tissue Collection, Manassas, VA (ATTC culture #TIB-202). Initial work was conducted by researchers from Kao Corporation and Shiseido Co., Ltd. to examine this cell line for its potential as a replacement for peripheral blood mononuclear cell-derived DC in the development of an *in vitro* test for contact sensitizers. They reported that THP-1 cells up-regulate cell surface expression of CD86 (Ashikaga et al., 2002; Yoshida et al., 2003) and CD54 (Yoshida et al., 2003) in response to chemical allergen exposure. There is mechanistic support for the examination of these two markers as indicators of sensitization potential from studies conducted using epidermal LC (Ozawa et al., 1996).

Based on the methods used in those initial studies, the two laboratories jointly developed and evaluated a standardized protocol and named the test the human Cell Line Activation Test (h-CLAT) (Ashikaga et al., 2006; Sakaguchi et al., 2006). The h-CLAT uses flow cytometry to measure changes in cell surface marker expression of CD86 and CD54, as mean fluorescence intensity (MFI), in THP-1 cells following 24-h chemical

exposure. After subtracting the MFI of the isotype control, the relative fluorescence intensity (RFI) of each surface marker on the treated cells as compared with the vehicle control cells is calculated. Three independent studies are conducted for each chemical and with the original prediction model, a mean $\text{RFI} \geq 150\%$ for either CD86 or CD54, with a cell viability $>50\%$ as determined by propidium iodide (PI) exclusion is considered to be a positive response.

To date, the h-CLAT has been evaluated by five independent laboratories in a series of three ring trials coordinated by COLIPA. The results of the first ring trial which examined ten chemicals demonstrated that the protocol was transferable and several areas for protocol and prediction model improvement were identified. As a result, the method originators modified the dose selection procedure. In the original protocol, four concentrations of each test chemical were set by the originating labs based on the concentration which induced 50% cytotoxicity (IC_{50}) as determined by the MTT assay. In the modified protocol, the number of test concentrations was increased to eight in a 1.2-fold dilution series based on the concentration that resulted in a cell viability of 75% (CV_{75}) determined by PI exclusion and a 5 $\mu\text{g/mL}$ 1-chloro-2,4-dinitrobenzene positive control was included. The prediction model was refined to reduce the number of false positive results by increasing the criteria of a CD54 positive response to a mean $\text{RFI} \geq 200\%$. A second ring trial using the modified protocol and prediction model tested twelve chemicals that included eight from the first ring trial in order to evaluate protocol and prediction model changes relative to the first ring trial results. The outcome of the second ring trial indicated that there were still areas for protocol and prediction model improvement. The reproducibility of the data for some chemicals across the labs was not good and was thought to be related to the variations in the solubility and/or stability of the test materials. To address these concerns, dose solution preparation procedures were modified to include sonication of dosing solutions of chemicals with limited solubility and treatment of cells within 30 min after dose solution preparation. Additional criteria were developed to assess data acceptability and further refinements were made to the prediction model; not only the mean, but also two of three independent data should exceed the cut-off RFI values for CD86 and/or CD54 for any dose to be considered 'positive'. A third ring trial to evaluate seven chemicals has been initiated. Pending the outcome of this trial more work may be required to finalize the protocol and prediction model. At that time it will be necessary to determine the value of h-CLAT for skin sensitization hazard identification.

The other DC surrogate cell line test system being evaluated currently in a collaborative study coordinated by COLIPA is the U937/CD86 Test which employs the U937 cell line (ATTC culture #CRL-1593.2). U937 test systems were being developed by two independent laboratories; one method examined the % CD86 positive cells after 48 h incubation with test chemical (L'Oréal's U937/CD86 test) and the other model examined IL-1 β and IL-8 gene expression in addition to % CD86 positive cells at 24, 48 and 72 h following treatment (Python et al.,

2007). Initial investigations indicated that the U-937 cell line showed some promise as the basis for an alternative approach to hazard identification.

The first COLIPA collaborative U937 studies involved the development of a harmonized protocol and assessment of method transferability. The starting protocol, the L'Oréal U937/CD86 test, examines five test chemical concentrations, cell viability determined by PI exclusion must be >70% for any dose to be considered and a CD86 stimulation index (SI) of % CD86 positive cells in the treated versus vehicle control cells is calculated. Three independent studies are conducted. A chemical is considered to have sensitizing potential if the SI is ≥ 120 with two of the three studies in agreement and there is evidence of a dose response.

Three laboratories participated in the first round of collaborative studies in which three chemicals were tested with each lab using different lots of cells and different monoclonal CD86 antibodies. Despite the non-standardized test conditions, the results were broadly comparable and a second round of testing was initiated to re-evaluate the existing protocol under more standardized experimental conditions. However, issues of low cell viability and inconsistent assay results both within and between laboratories developed and the study was stopped. Follow-up studies were conducted to examine the effect of various test conditions on cell lot growth and performance and, as a result, the protocol was modified. No changes were made to the prediction model.

Three allergens and two irritants were tested with the modified protocol. Data from the three labs were consistent; the sensitizing potential of four test chemicals was predicted correctly and sodium lauryl sulfate was a false positive in all labs. The COLIPA coordinated work on the U937/CD86 test continues with further protocol optimizations possible. A ring trial is planned with the addition of two more laboratories.

In conclusion, it is clear that the use of cell lines as surrogate DC in the development of *in vitro* test methods offers advantages compared with primary cultures of DC from peripheral blood. Cell lines are easy to obtain and grow, thus providing sufficient numbers of cells so test design is not limited. It is important that test protocols using cell lines should be robust enough so that the impact of any lot-to-lot variability on the performance of the assay is minimized. It is also necessary to consider using methods to assess different batches of cells (with respect to both the growth characteristics and performance within the assay) prior to use. Based on the outcome of the COLIPA coordinated work conducted to date, the most judicious view at present is that with further refinement some of these cell lines may show some promise as surrogates for DC in development of cell-based assays that can be used as part of an *in vitro* test battery.

The way forward

Our focus here has been upon how knowledge of DC immunobiology might be exploited for the predictive identification of chemicals with the potential to cause skin sensitization. In this type of approach, greatest weight is given to the

discrimination of significant skin sensitizers from those chemicals which are either non-sensitizing, or are sufficiently weakly sensitizing not to merit formal classification (for example, in relation to EU criteria [EEC, 1992]). However, as has been shown in recent sensitization datasets (Aptula et al., 2005; Gerberick et al., 2005), skin sensitizing chemicals cover not only a wide spectrum in terms of their chemistry, but also present a very wide range of potencies. Furthermore, a significant minority of chemicals possess substantial skin sensitization hazard; many of these chemicals also possess valuable properties (topical medicaments, preservatives, epoxies, acrylates and many more). The consequence is that there is a need to determine not only whether a chemical is a skin sensitizer, but also to understand its potency and thereby permit appropriate risk assessment and risk management to be undertaken.

When the guinea pig was the primary skin sensitization model, "potency" was typically characterized by the proportion of animals which were sensitized, independently of the concentration(s) deployed in the test procedure (e.g. Magnusson and Kligman, 1970). Consequently, risk assessment/management was accomplished largely by a comparative approach, in which attempts were made to judge a new sensitizer by its similarity to existing materials whose safety profile was well understood (see Basketter and Kimber, 2006 for more details). In recent years, the preferred approach to the characterization of sensitization potency has become the mouse local lymph node assay (LLNA) (Basketter et al., 2000; Gerberick et al., 2001; Kimber et al., 2002a, 2002b; Basketter et al., 2005). In this assay, estimation of the concentration of a chemical required to cause a 3-fold stimulation in lymph nodes draining the site of application, the EC3 value, has been shown to correlate well with what is known of potency in predictive human testing (Schneider and Akkan, 2004; Griem et al., 2003; Basketter et al., 2005). EC3 potency values can then inform risk management and can also be used as a basis for quantitative risk assessment (QRA) (Felter et al., 2002, 2003; Basketter et al., 2005). QRAs for skin sensitization are increasingly being used to identify safe levels for many types of ingredients in consumer products (Zachariae et al., 2003; Basketter et al., *in press*).

In light of the above, it is very clear therefore that *in vitro* (and *in silico*) alternatives in skin sensitization will need to provide more than a yes/no answer to the question "Is my chemical a potential skin sensitizer?". *In vitro* assays based on an analysis of DC responses to chemical exposure may well be expected to form an integral component of a non-animal testing strategy in this area of toxicology. However, the challenge will be not only to analyze their response in relationship to sensitization, but in particular to develop an appreciation of how this response contributes to the characterization of sensitization potency. This matter has been addressed recently by Jowsey et al. (2006). In essence the approach represents a hypothetical view of how, once the assays are developed, data from a variety of sources might be integrated (in a transparent way) to provide an index of sensitizing potency. All that remains therefore is to do the groundwork to develop and validate the individual elements, including a DC assay. It is our

view that output from a DC will only properly inform toxicologists regarding potential skin sensitization, and particularly potency, if it is integrated in the type of scheme indicated below. With so many variables being combined, it will be essential that in the development of the methods, results are compared with a considerable number of chemicals of known sensitizing potency. Such data are increasingly available and should help to ensure there is a real opportunity to fully validate *in vitro* models (Gerberick et al., 2004, 2005).

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