

# Identification of Gene Expression Changes Induced by Chemical Allergens in Dendritic Cells: Opportunities for Skin Sensitization Testing

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Cellular changes within resident skin dendritic cells (DCs) after allergen uptake and processing are critical events in the acquisition of skin sensitization. Here we describe the development of a set of selection criteria to derive a list of potential target genes from previous microarray analyses of human peripheral blood-derived (peripheral blood mononuclear cells (PBMCs)-DCs) treated with dinitrobenzene sulfonic acid for predicting skin-sensitizing chemicals. Based on those criteria, a probing evaluation of the target genes has been conducted using an extended chemical data set, comprising five skin irritants and 11 contact allergens. PBMCs-DCs were treated for 24 hours with various concentrations of chemicals and in each instance the expression of up to 60 genes was examined by real-time PCR analysis. Consistent allergen-induced changes in the expression of many genes were observed and further prioritization of the targets was conducted by analysis of the same genes in DCs treated with non-sensitizing chemicals to determine their specificity for skin sensitization. Real-time PCR analyses of multiple chemical allergens, irritants, and non-sensitizers have identified 10 genes that demonstrate reproducibly high levels of selectivity, specificity, and dynamic range consistent with providing the basis for robust and sensitive alternative approaches for the identification of skin-sensitizing chemicals.

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## INTRODUCTION

Langerhans cells (LCs) are considered to be the principle antigen-presenting cell in the skin and they typify the sentinel role of immature dendritic cells (DCs) as they are critical to the development of allergic contact sensitization (Steinman and Banchereau, 1998). Following exposure to a chemical allergen, LCs effect antigen internalization, processing, and presentation; processes that require their migration from the epidermis to regional lymph nodes and maturation into immunostimulatory DCs capable of presenting antigen effectively to responsive T lymphocytes (reviewed by Cumberbatch *et al.*, 2000). Many biological changes occur

in LCs throughout this process including the alteration of surface markers (Aiba and Katz, 1990; Schwarzenberger and Udey, 1996; Verrier *et al.*, 1999), the production of cytokines (Enk and Katz, 1992; Wang *et al.*, 2002), and the induction of signal transduction pathways (Kühn *et al.*, 1998).

Expression profiling provides an opportunity to investigate genome-wide changes induced in LCs or DCs following encounter with allergen. This approach has been used by others to study various biological responses in DCs and/or LCs such as activation and differentiation (Richards *et al.*, 2002), tolerogenicity (Suciu-Foca Cortesini *et al.*, 2001), modulation by steroids (Griffin *et al.*, 2004), and antimicrobial responses (Messmer *et al.*, 2003; Semnani *et al.*, 2003; Ju and Zenke, 2004). Since few reports have focused on contact allergy, we used transcript profiling to characterize responses to chemical allergens in a type of LCs surrogate, human peripheral blood-derived DCs (peripheral blood mononuclear cells (PBMCs)-DCs). One important objective was to identify significant gene changes that could be exploited for the development of *in vitro* methods for identifying skin sensitizing chemicals (Ryan *et al.*, 2004). A number of genes were examined and evaluated in detail for their reproducibility using real-time PCR analysis. These genes have been analyzed further in PBMCs-DCs after treatment with multiple chemical allergens and irritants. Genes identified through this approach that display measurable discriminatory capability will provide new and exciting opportunities to develop novel

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Abbreviations: BA, benzoic acid; BsA, benzene sulfonic acid; DC, dendritic cell; DCP, diphenylcyclopropanone; DNBS, dinitrobenzenesulfonic acid; Eug, eugenol; HC, hydroxycitronellal; HCA, hexylcinnamic aldehyde; LC, Langerhans cell; NiSO<sub>4</sub>, nickel sulfate; PBMC, peripheral blood mononuclear cell; PenG, penicillin G sodium salt, Benzylpenicillin; PLG, propyl gallate; SA, salicylic acid; SADE, 3,4-diethoxy-3-cyclobutene-1,2-dione; SDS, sodium lauryl sulfate; SLAM, signaling lymphocytic activation molecule

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alternative methods for the identification and characterization of skin-sensitizing chemicals.

## RESULTS

### Selection of candidate genes

Microarray experiments conducted previously on dinitrobenzenesulfonic acid (DNBS)-treated PBMCs-DCs-identified genes that appeared related to the activation of DCs by a contact allergen (Ryan *et al.*, 2004). Although approximately 2,880 significant ( $P \leq 0.01$ ) gene changes were observed, we chose to consider for further analysis by PCR only those genes that were regulated highly significantly ( $P \leq 0.001$ ) by both concentrations of DNBS tested (1 and 5 mM) to reduce the potential selection of genes that were regulated as a result of cellular cytotoxicity. Thus, we selected 60 genes from the 118 significant at  $P \leq 0.001$  to validate further in another DNBS experiment with PBMCs-DCs derived from a different donor. Genes were originally selected from the microarray data and later categorized by either their magnitude of altered expression (largest fold change), presumed or confirmed mechanistic association with skin sensitization or DCs biology, immunologic function, cellular function, or baseline constitutive expression (detectable by other methods).

The 60 selected genes were characterized by real-time PCR in two additional DNBS experiments for further evaluation as potential targets important to the regulation of DCs activation by an allergen. These experiments were performed with PBMCs-DCs derived from different donors to address donor-to-donor variability and included multiple concentrations of DNBS to allow definition of dose-response relationships and to determine the dynamic range of gene expression. Additionally, expression levels of at least 50 of the 60 genes were examined in a larger experiment with elutriated monocyte-derived immature DCs from a single donor that were treated with three contact allergens, one skin irritant, and one non-sensitizer. From these experiments the potential gene list was reduced to 29 that could be analyzed more quickly with a larger number of chemicals (data not shown). Genes that showed altered expression following treatment with the skin irritant (and therefore did not permit discrimination between allergens and irritants), or failed to display sufficiently substantial changes in expression following exposure to chemical allergens were eliminated.

### Evaluation by real-time PCR gene expression changes in chemical-treated DCs

Expression of the 29 selected genes was analyzed by real-time PCR using additional allergens and non-allergens. It is well known that differences between contact allergens with respect to their relative skin-sensitizing potency can span four or more orders of magnitude (Basketter *et al.*, 2000; Kimber *et al.*, 2003). Therefore, we evaluated chemicals that display a range of sensitizing potencies (weak to extreme). The potency categories used are those outlined by the European Centre for Ecotoxicology and Toxicology of Chemicals that are based on the results of the Local Lymph Node Assay in the form of their EC3 values (ECETOC, 2003). Initially, DNBS (the water soluble analog of the extreme contact allergen

2,4-dinitrochlorobenzene), a strong allergen (propyl gallate (PLG)), a non-sensitizing skin irritant (benzoic acid (BA)), and a non-sensitizer (benzene sulfonic acid (BsA)) were tested in independent experiments. Extreme and strong allergens were included to gauge the degree of altered expression that was observed with these genes, and to compare their expression levels with two different allergens with similar potencies, while the irritant and non-sensitizer were included also to determine selectivity of the genes. Multiple doses of each chemical were tested to determine the dynamic range of each gene, but the doses used maintained a cell viability of 85% or greater as measured by propidium iodide staining (data not shown). However, the highest dose used in each experiment was intended to induce 10–15% cell death in order to assess the impact of viability on expression levels.

Owing to the limited number of PBMCs-DCs generated from each leukoprep and the number of cells required to obtain sufficient quantities of RNA to test in multiple reactions, Table 1 depicts representative mean fold change data for the 29 genes from independent experiments using different donor cells for each experiment. Although the data presented here illustrate results from one individual experiment for each of the four chemicals, at least one additional experiment using a different donor was conducted for each chemical. DNBS treatment induced measurable changes in expression compared with control for all genes analyzed, and in most cases for all doses used, except for IL3RAX where only the high dose was found to be effective. As expected, the magnitude of alterations in expression varied between genes as there was a 43.6-fold decrease in ABCA6 gene expression, whereas only a 2.1-fold increase in the expression of TTRAP, although which genes would be regulated more robustly was not predicted. In all cases the direction of regulation measured was the same as that observed in the microarray study and confirms our initial findings with DNBS. The magnitude of expression changes was generally slightly greater by real-time PCR analysis compared with microarray analysis. However, the fold-changes observed were comparable with changes identified in subsequent real-time experiments using DNBS with some variation in the magnitude of regulation between PCR reactions with particular genes as mentioned above.

The strong allergen, PLG, also induced measurable changes in a majority of the genes analyzed (Table 1) and was analogous to DNBS treatment, although with some exceptions. Unlike DNBS treatment, the level of expression of CCRL2, RIT1, and IL3RAX in PLG-treated cells was unaffected at all doses examined and only modest changes were observed for TXN and TTRAP. Although the irritant BA did not generate many changes in gene expression, the non-sensitizer BsA, that is structurally similar to DNBS but lacks the reactive moiety associated with skin sensitization, did cause changes in the expression of a few genes such as G1P2, IL3RAX, AK1RC2, S100A4, CD1E, and SPN but not to the same degree as that induced by the allergens, DNBS and PLG. Moreover, the fold changes observed with BsA treatment for the CTSH and CCL2 genes (>2-fold increase)

Table 1. Mean fold change of chemical-treated DCs versus control-treated DCs<sup>1</sup>

Gene symbol	DNBS			PLG			BsA		BA		
	1.0 mM	2.5 mM	5.0 mM	100 $\mu$ M	250 $\mu$ M	500 $\mu$ M	1.0 mM	5.0 mM	200 $\mu$ M	400 $\mu$ M	800 $\mu$ M
ABCA6	-5.2	-18.6	-43.6	ND	-11.5	-39.2	1.2	1.4	-1.5	-1.3	-1.4
AKR1C2	15.9	42.9	229.2	8.0	10.1	15.5	1.7	2.1	-1.3	-1.3	-1.7
ARHGDIB	-2.2	-5.1	-7.0	-2.0	-3.4	-19.6	1.0	1.2	-2.2	-1.4	-2.0
BLNK	-4.7	-10.9	-39.8	-5.3	-2.8	-23.6	-1.3	-1.3	-1.3	-1.3	-1.2
CCL2				-1.5	-1.8	-53.8	-1.7	2.0	-1.2	-2.8	-1.4
CCL23	-3.5	-7.4	-9.8	-2.5	-3.0	-11.2	-1.3	-1.6	-1.3	-1.9	-1.1
CCL4	-3.4	-2.8	-17.5	-2.2	-2.8	-3.9	-2.3	1.1	-1.5	-1.2	-1.2
CCRL2	4.6	7.2	5.2	1.4	1.5	1.5	-1.1	1.1	-1.3	-1.1	-1.1
CD1E	-2.3	-3.6	-25.3	-2.2	-4.5	-30.8	-2.9	-4.1	1.2	-1.2	-1.5
CTSH	-4.8	-5.0	-5.1	-1.6	-2.1	-4.2	2.2	2.5	-2.4	-1.4	-1.6
CYP27A1	2.8	15.1	6.2	2.83	4.3	7.1	1.1	1.5	-1.2	1.2	-1.2
EPB41L2	-6.2	-6.3	-8.3	-2.6	-6.6	-15.3	-1.1	1.3	1.2	1.1	-1.1
FCER1A	-3.4	-4.5	-10.7	-3.7	-4.7	-23.9	1.1	1.1	1.4	1.1	1.1
FGL2	-4.5	-5.1	-22.5	-1.9	-3.1	-20.1	1.0	1.2	-1.4	-1.3	-1.1
G1P2	3.5	6.9	11.9	1.2	-1.1	-4.7	3.0	2.8	-1.2	1.4	-1.4
HML2	-12.7	-36.3	-42.2	-2.7	-3.2	-18.1	1.0	-1.3	-1.6	-1.2	-1.6
IER3	-7.3	-4.3	-2.5	-1.87	-2.5	-5.5	-1.5	-1.8	-1.2	-1.2	-1.1
IL3RAX	1.0	1.1	3.4	-1.4	1.3	-1.2	3.0	4.7	-1.4	-1.4	-1.5
MRC1	-1.4	-2.3	-6.5	-1.1	-1.8	-3.8	-1.4	-1.4	1.1	1.4	-1.3
NOTCH3	3.7	7.6	18.7	5.0	8.0	8.5	1.0	1.5	1.5	1.4	-1.2
QPCT	-2.2	-2.1	-3.3	-1.6	-2.3	-4.8	-1.2	1.2	-1.0	1.2	-1.1
RIT1	1.2	2.4	5.8	1.1	-1.0	-1.5	-1.0	1.1	-1.0	1.4	-1.0
S100A4	-12.7	-36.3	-42.2	-1.9	-3.9	-97.5	-1.8	-2.6	-1.6	-1.7	-2.0
SH3BP5	4.1	13.2	12.3	1.5	1.3	3.4	-1.9	-2.0	1.0	-2.2	-1.9
SLAM	-9.6	-7.3	-15.6	ND	-6.1	-3.3	1.2	1.9	-1.4	-1.2	1.2
SPN	-1.4	-3.1	-5.5	-2.7	-4.0	-20.0	-2.1	-2.2	1.0	-1.2	-1.1
TRIM16	1.6	5.7	15.5	2.6	2.3	7.1	-1.3	-1.7	-1.3	-1.1	1.0
TTRAP	-2.5	2.1	2.1	2.4	2.4	2.0	1.1	1.2	-1.3	-1.9	-2.0
TXN	3.8	2.9	12.8	2.3	2.2	2.5	-1.5	-1.6	-1.1	-1.1	-1.1

<sup>1</sup>Mean fold change was derived from real-time PCR reactions run on either replicate wells of a single sample or single wells of biological replicates for each experiment. Each chemical was analyzed in separate experiments using different donor cells and the data are from a single experiment per chemical and are representative of results observed in additional experiments.

were in a direction opposite of that seen with the allergenic chemicals PLG and DNBS.

#### Scoring of regulated genes

To determine that a gene is associated strongly with DCs activation by an allergen, we decided that it must be regulated by multiple allergens (robust) and must be specific for allergens and not irritants (selective). Therefore, to prioritize the 29 genes of interest with respect to their robustness and selectivity as well as to reduce the number of candidate genes, the genes were scored according to their expression level induced by additional allergens and irritants bringing the total number of allergens evaluated to 11 and

irritants to four. The allergens used represented multiple chemical classes (e.g., aldehydes, quinones) estimated to follow different reaction mechanisms and varying potency categories ranging from weak to extreme (ECETOC, 2003). The weak allergens (eugenol (Eug), hydroxycitronellal (HC), nickel sulfate (NiSO<sub>4</sub>), and penicillin g sodium salt, Benzylpenicillin (PenG)), moderate allergens (hexylcinnamic aldehyde (HCA) and, IsoEug), strong allergens (PLG, 3,4-diethoxy-3-cyclobutene-1,2-dione (SADE), and HQ), and extreme allergen diphenylcyclopropanone (DCP) and DNBS (the water soluble analog of the extreme allergen DNCB) were among those tested. The irritants tested were sodium lauryl sulfate (SDS), BA, methyl salicylate, and salicylic acid

(SA). Several of these chemicals were tested in more than one experiment and therefore have multiple data points for each gene. However, due to limited number of RNA samples obtained from certain experiments, not all genes were tested with each chemical. Nevertheless, the information in Table 2 indicates how many chemicals a particular gene was tested against, which chemical tested negative for the allergens and positive for the irritants, and which chemicals were not tested at all for that particular gene.

Scoring of fold changes listed in Table 2 was made by determining a positive response for a gene in all chemicals tested and the number of chemicals tested per gene. A positive response was defined as being greater than a 2-fold change in the direction identified originally in the initial DNBS experiment at one of the doses tested. Since the purpose of the analysis was to identify genes that were capable of discriminating between allergens and irritants, separation of allergens into specific potency categories was

**Table 2. Scoring of candidate genes by positive and negative responses in real-time PCR reactions upon chemical treatment<sup>1</sup>**

Gene symbol ( <sup>2</sup> *)	Allergen scoring <sup>2</sup> (no. positive/no. tested)	Allergen that tested negative <sup>3</sup> (no. negative/no. tested)	Allergen not tested <sup>4</sup>	Irritant scoring <sup>5</sup> (no. positive/no. tested)	Irritant that tested positive <sup>6</sup> (no. positive/no. tested)	Irritant not tested <sup>7</sup>
ABCA6*	11/11			1/4	SDS (1/3)	
AKR1C2*	11/11			1/4	SDS (3/5)	
ARHGDIB*	10/10		NiSO <sub>4</sub>	2/4	BA (2/2) SA (1/2)	
BLNK*	11/11			1/4	SA (1/1)	
CCL2	8/10	DPC Eug	HQ	1/3	BA (1/2)	MS
CCL23*	11/11			1/4	SA (1/1)	
CCL4*	10/11	HQ		0/4		
CCRL2	4/9	DCP HQ PLG HCA Pen G	IsoEug Eug	0/3		SA
CD1E	11/11			2/4	SDS (2/4) BA (1/3)	
CTSH	8/11	HQ HCA HC		0/4		
CYP27A1*	11/11			1/4	SA (1/1)	
EPB41L2	9/11	HC Eug		1/4	SDS (1/4)	
FCER1A	7/8	SADE	NiSO <sub>4</sub> Eug IsoEug	0/3		SA
FGL2	10/11	DCP		0/4		
G1P2	2/9	PLG HC SADE NiSO <sub>4</sub> Pen G HCA DCP	IsoEug Eug	1/3	SDS (1/4)	SA
HML2*	11/11			0/4		
IER3	4/8	DCP HQ HCA Pen G	IsoEug Eug NiSO <sub>4</sub>	1/3	BA (1/3)	SA

Table 2 Continued on the following page

Table 2. continued

Gene symbol ( <sup>7</sup> *)	Allergen scoring <sup>2</sup> (no. positive/no. tested)	Allergen that tested negative <sup>3</sup> (no. negative/no. tested)	Allergen not tested <sup>4</sup>	Irritant scoring <sup>5</sup> (no. positive/no. tested)	Irritant that tested positive <sup>6</sup> (no. positive/no. tested)	Irritant not tested <sup>7</sup>
IL3RAX	5/8	DPC PLG SADE	IsoEug Eug PenG	1/4	SDS (1/2)	
MRC1	5/11	HQ HC NiSO <sub>4</sub> Eug HCA DCP		1/4	SDS (1/3)	
NOTCH3*	11/11			1/4	SDS (1/5)	
QPCT	6/10	DPC HCA HQ NiSO <sub>4</sub>	IsoEug	1/3	SDS (1/2)	SA
RIT1	5/9	NiSO <sub>4</sub> HCA DCP PLG	IsoEug Eug	0/4		
S100A4*	6/6		HC PenG IsoEug Eug DCP	0/4		
SH3BP5	7/9	Pen G DCP	IsoEug Eug	0/4		
SLAM*	11/11			1/4	BA (1/2)	
SPN	8/9	DCP	IsoEug Eug	1/3	BA (1/3)	SA
TRIM16	9/11	NiSO <sub>4</sub> Eug		1/4	SDS (1/4)	
TTRAP	5/8	NiSO <sub>4</sub> HCA PenG	IsoEug Eug DCP	0/4		
TXN	10/11	SADE		2/4	BA (1/3) SDS (1/4)	

<sup>1</sup>The expression of candidate genes in allergen-treated DC were analyzed in real-time PCR reactions and the mean fold change levels of expression for each gene was measured and compared to the levels induced by irritant treatment.

<sup>2</sup>Allergen scoring was performed by recording the number of allergens in which a positive response was observed out of the number of allergens that particular gene was tested. A positive response represents a fold change level that was larger than 2 for upregulated genes and -2 for downregulated genes. A positive response was recorded regardless of the dose at which it registered positive or the number of times a positive response was observed.

<sup>3</sup>Lists the allergen to which no positive response was observed at any dose in any experiment.

<sup>4</sup>Some genes were not tested in all allergens mentioned in the table due to limited quantity of RNA thus the allergens not tested are listed.

<sup>5</sup>Irritant scoring describes the number of irritants in which a positive response was observed out of the number of allergens tested and a positive response is the same as that described for allergen response.

<sup>6</sup>The irritant that tested positive at any dose analyzed is described. Parenthetic numbers indicate the number of experiments for which a positive response was observed out of the number of times that particular gene was tested with that specific irritant.

<sup>7</sup>Genes that initially appear to have the most predictive potential for an alternative method (\*).

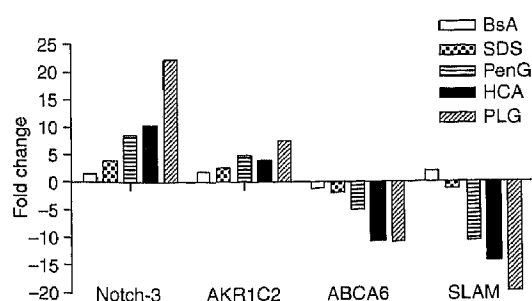
not performed at this time and no associations with fold change and a chemical's potency were made at this time. In addition, a gene was scored positive regardless of the number of times it registered a positive in any experiment tested with that particular chemical. For further clarity, Table 2 also lists the number of times that a particular gene was tested and registered as positive or negative for a chemical.

As shown in Table 2, several genes were affected by all of the allergens tested such as AK1RC2, ARHGDIB, CCL23, CD1E, CYP27A1, HML2, NOTCH3, S100A4, and signaling lymphocytic activation molecule (SLAM). Other genes (ABCA6, BLNK, CCL4, EPB41L2, TRIM16, and TTRAP) showed an association with the majority of allergens tested. Interestingly, it was usually the weak allergens that did not

meet the 2-fold change criterion. The results of the scoring also show that some genes are not strongly associated with allergen activation such as CCRL2, G1P2, MRC1, and QPCT. The specificity of the genes can be assessed by determining if they are regulated by irritants. For example, CTSH, FCER1A, HML2, or RIT1 were induced in only one irritant tested and in only one out five independent experiments with that particular chemical. It is important to note that even if genes were regulated up or down by an irritant, the degree of change could be considerably more robust with an allergen as was the case with AKR1C2, for example the highest concentration of SDS induced only a 2.6-fold change in AKR1C2 whereas the allergen isoeugenol (IsoEug) induced a 17.2-fold change in expression (data not shown). As a result, the data presented in Table 2 allows determination of which genes are associated strongly with allergen activation of DCs and which also show specificity for allergens versus irritants.

#### Comparison of gene expression for use as potency predictor

To determine whether any of 29 genes display potential for categorization of relative sensitization potency as is currently derived using the local lymph node assay (Basketter et al., 1999; Kimber et al., 2003), the fold change levels of four candidate genes were analyzed in separate experiments with five chemicals from different potency categories ranging from non-sensitizer to strong allergen. The data are from individual PCR experiments for each chemical but are representative of results obtained from multiple runs of a particular sample set or from single PCR runs of multiple experiments per chemical. As shown in Figure 1 all genes show differences in expression upon treatment with chemicals from different potency categories, but to a different degree. NOTCH3 and SLAM display measurable differences between all potency groups analyzed, whereas ABCA6 showed expression differences between the irritant and the weak and moderate allergen but did not further separate the moderate and strong allergen. Distinguishable levels of AK1RC2 expression were observed between the irritant and the weak allergen but were



**Figure 1. Expression of candidate genes in chemical-treated DCs.** PBMCs-DCs were exposed to vehicle alone (either medium only or medium containing 0.1% DMSO), 5 mM BsA, 175  $\mu$ M SDS, 12.5 mM PenG, 100  $\mu$ M HCA, and 500  $\mu$ M PLG for 24 hours. Real-time PCR analysis after treatment expressed as fold change of expression induced upon treatment compared to control treated DCs. Each chemical represents an independent experiment performed on separate occasions using different donor cells for each experiment. Data are from an individual experiment for each chemical and are representative of additional experiments.

not noticeably different among the chemical allergens of higher potency classification. Therefore, this gene might be capable of separating the irritants from allergens without potency classification.

#### DISCUSSION

The aim of this study was to identify genes in DCs that are associated strongly with activation by contact allergens and do so in a selective manner such that they might serve as end points for an *in vitro* method to predict the skin sensitization potential of an unknown chemical. Therefore, we focused on transcriptional analysis by real-time PCR of genes identified previously by the Affymetrix Genechip<sup>®</sup> platform (Ryan et al., 2004) to elucidate pathways in DCs that are involved in "immune recognition" or antigen uptake, and subsequent cell activation, as well as to identify additional genes that are associated with allergen treatment that might have not been considered otherwise.

The evaluation of surface marker expression, cytokine production, and other markers of DCs activation is not new to the field of alternative *in vitro* approaches. Early work identified IL-1 $\beta$ , CD86, major histocompatibility complex class II, and CD54 as candidate molecules (Enk and Katz, 1992; Degwert et al., 1997; Reutter et al., 1997; Pichowski et al., 2000; Rougier et al., 2000; Staquet et al., 2004). While these studies have provided possible new approaches, many of those published have been confronted with some degree of restriction (Kimber et al., 2001; Ryan et al., 2001). Recently, Aebly et al. (2004) described results using IL-1 $\beta$  and aquaporin P3 gene expression combined with flow cytometric analysis of CD86-positive cells to characterize the sensitizing potential of chemicals. While their data are promising, additional allergens and irritants will need to be evaluated to determine the ability of these markers to predict the skin sensitization potential of unknown chemicals.

Owing to the variability reported in many of the surface markers studied to date, it is appropriate to consider alternative experimental strategies, including microarray transcript profiling, that permit a more holistic interrogation of genes and pathways that influence the ability of DCs to initiate adaptive immune responses to chemical allergens. Recently, we reported the phenotypic and functional changes at the genomic scale in DCs treated with contact allergens (Ryan et al., 2004). From this original data set we have sought to identify novel markers to investigate for their mechanistic relevance for contact allergy and their potential as effective markers that could be exploited in the development of alternative approaches for skin-sensitization testing.

We evaluated by real-time PCR analysis 60 out of 118 genes that were statistically significant in our original gene profile at  $P \leq 0.001$  (Ryan et al., 2004). The genes were selected using several criteria such as expression level, biological function and reproducibility as they were significantly regulated similarly in subsequent experiments using DNBS-treated PBMCs-DCs derived from different donors (data not shown). Furthermore, experiments conducted with cells from a single donor treated with six chemicals demonstrated genes which appeared to be associated with

allergen-induced DCs activation. From those experiments, 29 candidate genes were analyzed further in subsequent experiments that included a strong allergen, PLG, as well as an irritant, BA, and a non-sensitizer, BsA. For the most part similar responses were observed between the two allergens, DNBS and PLG, for most of the genes examined with fewer similarities being observed between the allergens and the irritant or the non-sensitizer.

To investigate further the association of these 29 genes with DCs activation by allergen, additional allergens, and irritants were tested. In order to categorize these genes initially, we separated the chemicals into two groups, allergens and irritants, without consideration for potency. As the amount of data generated at this point was sizeable, this process allowed us to determine whether some genes demonstrated little specificity with allergen activation of the PBMCs-DCs. For this evaluation, objective parameters such as presence or absence in a allergen/non-allergen, the dose to which a response was registered, and the nature of the chemical to which a response did not qualify were considered and thus resulted in a group of 11 genes that initially appear to be strongly associated with DCs activation by allergens and thus serve as potential predictors of skin sensitization (Table 2).

While we are hesitant to designate this as a priority ranking at this time, many of these genes have been the most robust and selective markers to date and therefore are the most likely candidates. Notch receptors play a key role in cellular processes including proliferation, differentiation, and apoptosis. Mammals have four known Notch receptors and Langerhans cells have been reported to constitutively express Notch-3 and -4 (B. Nickloff, unpublished observations reported in Weijzen *et al.*, 2002). In addition, Jagged-1, a Notch ligand that is constitutively expressed at high levels in keratinocytes, is reported to induce maturation of human monocyte-derived DCs via Notch-1 signaling (Weijzen *et al.*, 2002). We have observed consistent upregulation in message for Notch-3 in allergen-treated DCs versus control.

SLAM was first identified on activated T and B cells. Of late, SLAM signaling appears to be associated with the induction of maturation (Bleharski *et al.*, 2001; Kruse *et al.*, 2001), however, in a direction opposite of our observations. We consistently observe SLAM downregulation after 24 hours of treatment by both microarray and real-time PCR analysis (Ryan *et al.*, 2004) and further exploration may reveal that these differences are due to the kinetics of DCs activation.

BLNK or B-cell linker protein represents a central linker protein that bridges the B-cell receptor-associated kinases with a multitude of signaling pathways (Fu *et al.*, 1998). Linker or adapter proteins provide mechanisms by which receptors can amplify and regulate downstream effector proteins and BLNK has been shown to be critical in the integration of signaling cascades downstream of immunoreceptor tyrosine-based activation motif-bearing receptors. While the specific function of BLNK in DCs has not been elucidated, DCs do express immunoreceptor tyrosine-based

activation-containing receptors such as the Fc receptors (Bonnerot *et al.*, 1997; Kanazawa *et al.*, 2003).

The membrane-associated protein encoded by ABCA6 is a member of the superfamily of ATP-binding cassette transporters which transport various molecules across extra- and intracellular membranes. Although the exact function of this gene is unknown it may play a role in macrophage lipid homeostasis (Dean *et al.*, 2001; Kaminski *et al.*, 2001) and conceivably function in either receptor-specific or passive transport of molecules across DCs surfaces.

AKR1C1/C2 genes encode members of the aldo-keto reductase family of proteins. These enzymes convert aldehydes and ketones to their corresponding alcohols using nicotinamide adenine dinucleotide (reduced form) and/or nicotinamide adenine dinucleotide as cofactors. Additionally, they work to control ligand access to nuclear receptors as part of a switch mechanism with short-chain dehydrogenases/reductases (Bauman *et al.*, 2004). Although these proteins are 97.8% homologous and only differ in seven amino acids (Shiraishi *et al.*, 1998) their activities and affinities for their steroid substrates are different (Couture *et al.*, 2003) and since the Affymetrix target sequence that we used to design primer pairs contains the region of homology between the two genes, we cannot speculate at this time as to a role of either or both of these genes in DCs biology. However, at this time only two reports have been published on the expression of AKR1C1 in skin, a mouse study (Pelletier *et al.*, 2003) and a human study reporting expression in abdominal subcutaneous and adipose tissue (Blouin *et al.*, 2005). No studies reporting DCs, LCs, or keratinocyte expression of either AKR1C1 or AKR1C2 have been published.

CCL4, also known as macrophage inflammatory protein-1 beta, is one of four members of the macrophage inflammatory protein-1 CC chemokine subfamily. Chemokines are low molecular weight cytokines that are produced by many cell types and stimulate/regulate the movement of leukocytes between blood and tissues by acting via G-protein-coupled cell surface receptors (Maurer and von Stebut, 2004) and thus make excellent candidates to study. In addition, Verheyen *et al.* (2005) studied the response of CCL4, as well as CCL2, CCL3, and CCL3L1, in CD34+ derived DCs following treatment with contact allergens and irritants and observed clear increases in expression with nickel, DNCB, oxazalone, and Eug treatment.

Several of the analyzed genes have emerged as better predictors of DCs activation and thus are more likely candidates for a predictive model for skin sensitization. The top genes that displayed the most promise based on our selection criteria of fold-change levels induced in individual experiments, the scoring results from Table 2 such as the dose at which a positive response was recorded and the number of times it tested positive is denoted with an asterick in Table 2. However, this list is not intended to diminish the potential for other genes of interest that are not listed in this table to be used in future assay development or to conclusively associate the genes listed in Table 2 with predictive power.

Data from four genes listed in Table 2 are shown in Figure 1 and they all display measurable differences between allergens and irritants/non-sensitizers. Although it is a bit premature to determine which, if any genes are capable of classifying allergens into specific potency categories, preliminary data on Notch3, AKR1C2, ABCA6, and SLAM, as well as other genes listed in Table 2, suggest that this is feasible in addition to designating a compound as an allergen or irritant. It also appears that some genes show good potential to differentiate between an irritant and a non-sensitizer.

It is difficult, but important, to incorporate all of the components involved in the induction of skin allergy into the development of an alternative method. It is also important to correctly choose the genes that should be investigated more thoroughly and the number and nature of the chemicals to which these genes should be validated against. In addition, there is no standard chemical test set identified to evaluate the utility of any new gene that shows predictive potential or any fixed cellular criteria, such as viability and activation state, determined. Therefore, it was the goal of this study to identify a panel of candidate genes and evaluate them for their potential against a large number of chemicals using cytotoxicity restrictions. As any gene identified in this study has been chosen for its robustness or specificity, it must be examined in a larger-scale study with even more unique chemicals. We envision the design of a model that incorporates both a mechanistic understanding of the immunobiology of skin sensitization and the results from these gene expression profile studies. We do not anticipate that any one gene will be capable of predicting skin sensitization alone, but rather foresee a select panel of genes to be used to phenotype the activity of a test chemical.

## MATERIALS AND METHODS

### Materials

DNBS (CAS-no. 885-62-1), PLG (CAS-no 121-79-9), SADE (CAS-no 2892-51-5), HCA (85% purity; CAS-no 101-86-0), Eug (CAS-no 97-53-0), NiSO<sub>4</sub> (CAS-no 10101-97-0), SA (CAS-no 69-72-7), BsA (CAS-no 98-11-3), BA (CAS-no 65-85-0), IsoEug (CAS-no 97-54-1) and DCP (CAS-no 886-38-4) were purchased from Aldrich, Milwaukee, WI. HC (CAS-no 107-75-5), HQ (CAS-no 123-31-9), PenG (CAS-no 61-33-6), and methyl salicylate (CAS-no 119-36-8) were purchased from Sigma, St Louis, MO. SDS (10% solution; CAS-no 151-21-3) was purchased from Invitrogen Inc. (Carlsbad, CA). All chemicals used, except for HCA, were at least 95% pure and concentration was calculated using w:v. HCA (85% pure) dose solutions were prepared taking purity into account.

### Culture medium

Complete culture medium consists of RPMI-1640 containing 1X L-glutamine supplemented with 1X penicillin-streptomycin antibiotic mixture (GIBCO, Rockville, MD), 30  $\mu$ M 2-mercaptoethanol (GIBCO), and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT). For DCs generation, complete culture medium was supplemented with 10 ng/ml granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) and 10 ng/ml IL-4 (R&D Systems) referred below as cytokine-containing medium.

### Generation of peripheral blood-derived DCs

Enriched human leukocyte preparations were purchased from Sera-Tec Biologicals (North Brunswick, NJ) and were received as numerically coded units with no identifiable information. As such this study was conducted in accordance with the Declaration of Helsinki Principles. Following dilution with an equal part of complete medium the leukocytes were separated over Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). The PBMCs fraction was collected, washed, and counted using a Coulter Counter (Beckman-Coulter Inc., Miami, FL). The PBMCs concentration was adjusted to  $5 \times 10^6$  cells/ml with complete medium and 30 ml of the cell suspension was plated in T75 flasks. Following 2 hours incubation at 37°C/5% CO<sub>2</sub>, the non-adherent cells were removed. Of cytokine-containing medium, 10 ml was added to the remaining adherent cells in the flask. The cultures were incubated at 37°C/5% CO<sub>2</sub> for 48 hours during which the adherent cells became loosely or non-adherent. On day 2, the cells were collected, centrifuged, resuspended to a concentration of  $1 \times 10^6$  cells/ml in fresh cytokine-containing medium and re-plated. On day 5, residual T cells and B cells were removed from the cultures using two passages over CD2 (pan T cell) and CD19 (pan B cell) immunomagnetic beads (Dynal, Oslo, Norway) according to the manufacturer's directions. Following depletion of T- and B cells, the remaining DCs were resuspended to concentration of  $1 \times 10^6$  cells/ml in fresh cytokine-containing medium and re-plated in a T75 culture flask. The cultures were incubated for another 48 hours before experimental use. As previously described (Hulette *et al.*, 2002), DCs cultured for a total of 7 days in this manner express an immature DCs phenotype: HLA-DR<sup>+</sup>, CD1a<sup>+</sup>, CD83<sup>-</sup>, CD80<sup>lo</sup>, and CD86<sup>lo</sup> (data not shown). Alternatively, DCs were derived from elutriated human monocytes (Advanced Biotechnology Inc., Columbia, MD) that were cultured in the same manner as the PBMCs. The phenotype of these elutriated monocyte-derived immature DCs at day 7 was found to be identical to that of the DCs cultured from the enriched human leukocyte preparations (data not shown).

### Chemical treatment of DCs

Day 7 PBMCs-DCs or elutriated monocyte-derived immature DCs were collected, washed in complete medium and resuspended in cytokine-containing medium. In all,  $2 \times 10^6$  cells were plated in each well of a six-well culture plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). The test chemical dosing solution or vehicle control was added to the cells (final volume 3 ml/well) and the cultures were incubated for 24 hours at 37°C/5% CO<sub>2</sub>. Chemicals were initially dissolved in either complete medium or 100% DMSO and subsequent dilutions were performed in cytokine-containing media. Vehicle-treated PBMCs-DCs (either cytokine-containing medium or 0.1% DMSO in cytokine-containing medium) were used as controls. Either single or replicate wells were plated for each control and chemical treatment cultures. Final in-well concentrations of chemicals were chosen by the amount of cytotoxicity that it induced in DCs. PBMCs-DCs viability following chemical treatment was assessed by propidium iodide dye exclusion using a Coulter Epics<sup>®</sup> XL flow cytometer (Beckman Coulter, Miami, FL). The viability of the control PBMCs-DCs ranged from 95 to 99% while exposure to higher chemical concentrations were slightly cytotoxic, resulting in DCs viability ranging from 85 to 95%. Any



chemical concentration that induced more than 15% cytotoxicity was excluded from further analysis.

### RNA isolation and cDNA generation

Total RNA was isolated from after 24 hours of incubation using TRIzol<sup>®</sup> Reagent (GIBCO). Pellet Paint<sup>™</sup> NF (Novagen, Madison, WI) was used as a co-precipitant to aid in the recovery of the RNA. The resulting total RNA was purified further using RNeasy Mini Kit (Qiagen, Valencia, CA) and the RNA content was determined spectrophotometrically. Reverse transcription of RNA was performed using the Omniscript RT kit (Qiagen). In all reactions, cDNA was synthesized in 20  $\mu$ l using 1.5  $\mu$ g of total human RNA from DCs and 10  $\mu$ M Oligo-dt primers (Ambion Inc., Austin, TX). All reactions contained 10 U of RNase Out RNase inhibitor (Invitrogen) and the resulting cDNA was diluted to 10 ng/ $\mu$ l with sterile PCR-grade water and stored frozen ( $-20^{\circ}\text{C}$ ) until assayed.

### Real-time PCR analysis

Quantitative PCR amplification of gene-specific cDNA's was performed in 96-well PCR plates with the iCycler thermal cycler and the iCycler iQ<sup>™</sup> Real-Time PCR Detection System (Bio-Rad, Hercules, CA). An equivalent of 50 ng of total RNA was used in 25  $\mu$ l of QuantiTect SYBR Green PCR Master Mix (Qiagen). The real-time PCR reaction mixture included 5  $\mu$ l of sample, forward and reverse primers specific for each gene (0.3  $\mu$ M each), and the PCR master mix containing probe (2  $\times$  stock). The specific primer pair for each gene was designed using either the Genbank sequence or the Affymetric target sequence used for the U95A chips. The 96-well real-time PCR format included seven, 10-fold dilutions of a PCR-purified DNA standard (2  $\times 10^{-2}$  to 2  $\times 10^{-8}$  ng), a PCR negative control (minus template), and up to 12 genes independently analyzed per plate. The positive control and PCR standard was a rat intestinal calcium binding protein PCR product that was gel purified away from the primers. Test samples analyzed were either biological triplicates performed individually within a given PCR run or single biological samples performed in duplicate within a PCR run. In all experiments donor-specific vehicle-treated controls were included for comparison.

### Analysis of real-time PCR data

Optical real-time PCR data was analyzed using the default and variable parameters available in the software provided with the iCycler iQ<sup>™</sup> Real-Time PCR Detection System. The PCR threshold cycle number ( $C_T$ ) and starting quantity of test RNA samples was calculated after PCR baseline subtraction and  $C_T$  determination had been carried out on the standards. Standard curve equations were calculated by regression analysis of the log of the copy number (starting quantity) versus threshold cycle. The standard curve equations ( $R^2$  usually  $>0.96$ ) were used to calculate quantities of test RNA. The mean relative fluorescence units were calculated using individual well readings within a given PCR run on biological samples and then converted to mean fold change comparing mean relative fluorescence units of control samples versus mean relative fluorescence units of treated samples. For each gene described in Table 1, the data for each chemical presented represent either the combined mean fold change from multiple PCR reactions (2–4) within a single run or are the mean of fold changes calculated from single real-time reverse transcriptase-PCR reactions run on each of three replicate cultures from a single donor for any given chemical

within an experiment. Although each chemical represents a single donor, each chemical was tested individually in independent experiments.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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