CLASSIFICATION MODELS FOR IDENTIFYING SKIN SENSITIZERS USING *IN VITRO* ALTERNATIVES TO ANIMAL TESTING

by

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A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Biomedical Engineering

Written under the direction of

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And approved by

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New Brunswick, New Jersey

October, 2014

ABSTRACT OF THE DISSERTATION

CLASSIFICATION MODELS FOR IDENTIFYING SKIN SENSITIZERS USING *IN VITRO* ALTERNATIVES TO ANIMAL TESTING

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Allergic contact dermatitis (ACD) is an inflammatory disease that occurs when chemicals known as sensitizers come in contact with the skin. Recent European legislation prohibits animal based screens of cosmetic ingredients. Current alternatives to animal testing are limited by their poor ability to identify a subset of non-innate contact sensitizers known as pre-/pro-haptens which require transformation in the skin. Furthermore, these approaches only evaluate a single cell type with 1 or 2 biomarkers.

To address this, we performed an initial study using RealSkin, a full thickness skin equivalent, in co-culture with MUTZ-3 derived Langerhan's cells (MUTZ-LCs). This co-culture was treated with model pro-/pro-haptens from an irritant control and multiple cellular metrics were evaluated. A novel feature selection method was developed using a support vector machine (SVM) to rank the margin distances of each metric and identify biomarkers of sensitization. A panel (IL-12, IL-9, VEGF, IFN-γ) was identified by SVM and predicted sensitizers with over 90% accuracy. Although promising, this method is costly and resource

intensive. Thus, we designed a more economic, high throughput screening approach to metabolize pro-hapten sensitizers.

MUTZ-LCs were cultured alone and in parallel with a co-culture of HaCaT keratinocytes, dermal fibroblasts, and MUTZ-LCs. Both cultures were treated with a panel of pre- and pro-hapten sensitizers and non-sensitizers. The secretome of both cultures were evaluated for 27 cytokines, chemokines, and growth factors. Feature selection by SVM identified predictive signatures of sensitization for each culture type. These cellular metrics was used to develop a classification model of sensitization. The MUTZ-LCs classification model was 83.3% accurate at identifying pro-hapten sensitizers using MIP-1β, MIP-1α, RANTES, IL-8, and IL-9. The co-culture classification model was 89.6% accurate at identifying pro-hapten sensitizers using a panel of IL-8, GM-CSF, and RANTES. The presence of the keratinocytes and fibroblasts enhanced the identification of pre- and pro-haptens to sensitize the MUTZ-LCs. This approach also preserves the cross-talk signals between all three skin cell types. Thus, the co-culture of HaCaT keratinocytes, dermal fibroblasts, and MUTZ-LCs is an attractive, high throughput *in vitro* alternative to animal testing for the identification of pre- and pro-hapten skin sensitizers.

DEDICATION

For my dear mother, your endless love and encouragement inspires me daily.

ACKNOWLEDGEMENTS

"If I have seen further than anyone, it is because I have stood on the shoulder of giants."– Isaac Newton.

I would like to thank members of my thesis committee, Dr. Martin Yarmush, Dr. Rene Schloss, Dr. Michniak-Kohn, Dr. Timothy Maguire, and Dr. Miri Seiberg. Their individual expertise, guidance, and support helped shape this project from conception to completion. I would especially like to thank my advisor, Dr. Yarmush, for providing me with the opportunity to continue my education through the Biotechnology Training Program. This training and support gave me the strength to continuously challenge myself to become a stronger scientist and communicator. I would also like to thank Dr. Rene Schloss for our weekly meetings in which her immunology background was instrumental to the development of this project. Her weekly words of encouragement and positive outlook were a source of light that carried me through the troubleshooting aspects of this project and gave me the courage to push through. I would like to thank Dr. Michniak- Kohn for her expertise in dermal research and the gift of the HaCaT keratinocyte line. She instilled in me a passion for dermal research ever since my senior design project as an undergraduate student in her laboratory. The Center of Dermal Research that she founded continues to be an excellent source of education. Dr. Tim Maguire played a seminal role in establishing the computational framework of developing the *in silico* aspects of this project. I would also like to thank Dr. Miri Seiberg for her unique industry perspective on developing this project.

I would like to extend my appreciation to my fellow lab members from past and present, Andrea Gray, Mehdi Ghodbane, Ileana Merrero-Berrios, Agnes Yeboah, Alvin Chen, Max Balter, Liz Stucky, Dr. Jeffery Barminco, Dr. Nir Nativ, Dr. Lulu Li, Dr. Rohit Jindal, Dr. Bhaskar Mitra, and Dr. Nripen Sharma. I am very grateful to have had such a wonderful support team over the years. I would especially like to thank Andrea Gray for being an incredible friend, bridesmaid, and source of light. Renea Faulknor and Melissa Olekson from Dr. Berthiaume's lab were also a great source of encouragement and support.

I would like to thank my dear friends outside of academia, Dr. Bhumika Desai and Krishna Purohit. Their daily words of encouragement and unwavering belief in me instilled a stronger sense of confidence to pursue my passions. Also, planning a wedding during the final year of my dissertation could not have been done without such an incredible maid of honor and wonderful team of bridesmaids at the helm.

Last but not least, I would like to thank my husband, parents, grandmother, and brother for their continued love, support, and encouragement. My loving husband, Peter, you inspire me to be a better person every day. My parents' incredible work ethic is a constant source of inspiration to me. I would especially like to thank my mother who instilled in me a thirst for knowledge and love for learning as a child with all those trips to the library and science museums.

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ABBREVIATIONS

2AP – 2-Aminophenol

ACD - Allergic Contact Dermatitis

APC – Antigen-presenting Cell

ARE – Antioxidant Response Element

CA – Cinnamic Alcohol

CD – Cluster of Differentiation

CLD - Cinnamaldehyde

CYS - Cysteine

DC – Dendritic Cell

DMSO - Dimethylsulfoxide

DNCB - Dinitrochlorobenzene

EU - Eugenol

FB – Fibroblast

HQ - Hydroquinone

IL – Interleukin

IE - Isoeugenol

IFN – Interferon

KC - Keratinocyte

LC - Langerhan's Cell

MHC – Major Histocompatability Complex

MUTZ-LC – Mutz-3 differentiated Langerhan's cell

NS – Non-sensitizer

PPD – *p*-phenylenediamine

QSAR – Quantitative Structure Activity Relationship

S – Sensitizer

SA – Salicylic Acid

SDS – Sodium Dodecyl Sulfate

SVM – Support Vector Machine

CHAPTER 1: INTRODUCTION

1.1 CONTACT DERMATITIS

Skin serves as the primary barrier between our external environment and our body. Thus, it is unsurprising that there are relentless insults on the skin due to exposure of ultraviolet rays, micro-organisms, viral pathogens, and xenobiotics. Contact dermatitis (CD) is an inflammatory skin disease that can commonly occur as a consequence of exposure to harmful xenobiotics such as irritants or sensitizers. The symptoms of contact dermatitis can range from mild itching to more serious health complications such as the formation of blisters with long-lasting effects. Contact dermatitis is the most common skin disease with an estimated 20% of people in the general population that are sensitive to at least one allergen in their environment [1]. Furthermore, CD is one of the most commonly reported occupational diseases, with a significant economic burden that is estimated at nearly $1 billion in medical costs, worker's compensation, and lost work days [2].

Irritant Contact Dermatitis

Irritant contact dermatitis (ICD) is the more prevalent form of contact dermatitis that constitutes nearly 70% of all diagnoses. ICD is an acute response to an irritant that triggers the innate immune system and does not lead to the formation of memory T-cells or antigen-specific antibodies. Irritants are commonly surfactants, detergents, and weak acids that compromise the barrier properties of the stratum corneum in the epidermis. Though the precise mechanism that leads to ICD is still not fully understood, this non-specific

inflammation is thought to occur due to cytotoxic effect of irritants on skin cells. This triggers an inflammatory cascade of events where mediators such as cytokines (e.g., IL-1α and TNF-α) and prostaglandins are released and immune cells such as neutrophils, macrophages, and eosinophils are recruited. This ultimately results in the clinical symptoms characteristic of ICD such as oedema and erythema.

Allergic Contact Dermatitis

Although Allergic Contact dermatitis (ACD) occurs with less frequency than ICD, it is the more serious of the two disorders in terms of duration and clinical expression. ACD is a delayed type IV hypersensitivity response that is mediated by antigen-specific T lymphocytes and leads to the development of a memory response. ACD can often occur as a complication of ICD and there is evidence that frequent exposure to irritants can condition the development and severity of an ACD response [3]. Additional risk factors of ACD include gender, age, occupation, genetic predisposition, and underlying pathologies such as atopic eczema where the skin's barrier is compromised. These statistics represent a growing public health concern since the incidence rate of ACD is on the rise (especially in the youth demographic) due to the increased prevalence of contact allergens in the environment [4]. Clinical symptoms can manifest as early as 24-72 hours after re-exposure and is characterized by erthryma that can lead to the formation of hives, blisters or scaly plaques pending on severity [5]. These effects can be enduring with up to 50% of cases lasting nearly 6 months

[6]. Thus, it is imperative to minimize the greater public's exposure to these harmful agents that can potentially cause great duress.

1.2 *IN VIVO MECHANISM*

The *in vivo* mechanism of ACD can be generally broken down into two distinct phases: 1) sensitization phase and 2) elicitation phase.

During the sensitization phase, contact allergens penetrate the stratum corneum barrier and bind to nucleophilic proteins or peptides in the skin to form the macromolecular immunogen. Keratinocytes in the epidermis play an integral role in allergic contact dermatitis in initiating the inflammatory cascade of events. Keratinocytes sense contact allergens via their toll house receptors (e.g., TLR-2 and TLR-4), which leads to the secretion of inflammatory cytokines such as IL1-α, TNF-α, IL-8 and GM-CSF [7, 8]. These factors promote the antigen presentation process of resident Langerhan's cells and dermal dendritic cells and recruitment of inflammatory cells. In addition to providing necessary danger cues in the microenvironment, they are also the major source of xenobiotic metabolism in the skin. Keratinocytes possess oxidizing phase I enzymes such as members of the Cytochrome P450 (CYP) family, alcohol dehydrogenases, aldehyde dehydrogenases, monoamine oxidases, flavin containing monooxygenases, and hydrolytic enzymes [9-12]. Phase II enzymes such as acyltransferases, glutathione S-transferases, uridine 5′-diphospho-glucuronosyltransferases, and sulfotransferases are also present in the skin and may play a role in metabolizing pro-haptens [11-13]. Langerhan's cells (LCs) reside in the epidermis and are a subset of dendritic cells with antigen presenting capability. The immunogen is

taken up and processed by LCs. Dendritic cells (DCs) that reside in the dermis are also capable of internalizing the immunogen and processing it as well. While the antigen is being processed, a series of cellular changes occur where LCs and/or DCs down-regulate adhesion molecules and up-regulate chemokine receptors such as CXCR4 and CCR7 on their cell surface [14][15, 16]. This ultimately leads to a migration response of LCs/DCs out of the skin and towards their corresponding local lymph node. During this migratory maturation process, co-stimulatory molecules such as CD86, CD83, CD80, and adhesion molecules such as CD54 will also be expressed on the surface of LCs/DCs[17]. These surface molecules help facilitate the presentation of the antigen to naïve T-cells in the lymph node via the MHC-I and MHC-II receptors [18, 19]. Once presented with an antigen, activated T-cells undergo a proliferation and differentiation process into Th1 or Th2 cells depending on the cytokine environment. Memory T-cells specific to the contact allergen are also produced. The sensitization phase can take anywhere from 10–15 days in humans and is typically asymptomatic [20]. However, an acute inflammatory response that is hapten specific could occur 5-15 days after initial contact with the allergen [21]. This response is mediated by CD8+ T-cells that subsequently enter circulation after activation and mount an antigen-specific inflammatory response at the site where the allergen was in contact with the skin [21].

The elicitation phase of ACD occurs when one is re-exposed to the sensitized contact allergen. Memory T-cells are recruited to the site of contact and mediate the antigen-specific inflammatory response. This response appears

within 24-72 hours after re-exposure to the contact allergen [20]. CD8+ T-cells are the main effector cells that are recruited early after elicitation and are responsible for KC apoptosis. They also recruit additional leukocytes that participate in the development of the clinical lesions characteristics of ACD [22]. The accessory lymphocytes that are recruited include natural killer cells, mast cells, and neutrophils [20].

1.3 SKIN SENSITIZERS

It is estimated that up to 17% of 60,000 chemicals currently in commercial use could be contact sensitizers with nearly 3000 confirmed allergens determined by patching testing [23, 24]. The most common allergens are nickel, fragrances, dyes, and preservatives [25]. Contact sensitizers have molecular properties that allow them to penetrate through the stratum corneum barrier such as a low molecular weight (<1000Da) and lipophilicity (logp ~ 2) [26]. There are three broad classes of contact allergens that are grouped according to their mechanistic pathways to form macro-molecular immunogens that initiate the allergic response. The first class of contact allergens was discovered in 1935 by Landsteiner and Jacobs when they observed that small organic molecules can readily bind to skin proteins to form a sensitizing entity [27]. These small organic molecules are known as haptens and the process by which they bind to skin proteins is known as haptenization. Haptens are directly reactive to proteins due to the presence of electrophilic functional groups such as alkyl halides, aldehydes, ketones, amides, and esters [28]. The skin is a nucleophilic hotspot that is filled with many proteins that contain nucleophilic amino acids such as

cysteine, lysine, histidine methionine, and tyrosine [26]. Contact sensitizers preferentially bind to cellular proteins, rather than serum proteins [29]. One specific example of a cellular protein is the sensor protein Keap1 that contains highly reactive Cys residues [30]. Several pathways theorized in these electrophilic-nucleophilic binding reactions include S_N2 reactions, S_NAr reactions, Schiff base formations, Michael type additions, and acylation reaction [26]. Once bound together, this conjugated complex serves as the antigen that is further processed by antigen-presenting cells and presented to T-cells that ultimately mediate the resulting immune response. Non-classical haptens such as metal cations, Ni^{2+}, and Cr^{3+} can directly bind to HLA-DR52c, an MHC-II molecule and an unknown HC-bound peptide via histidine or acidic residues on the side chain [31]. Ni^{2+} may also bind to TLR-4/MD-2 and death ligands on keratinocytes to mediate an innate immune response that leads to the conditioning of ACD [32].

Unlike haptens, the following two classes of sensitizers known as pre- and pro- haptens are not inherently reactive due to a lack of binding site susceptible to nucleophilic attack. However, they can undergo chemical reactions to form reactive intermediates or products that readily bind to proteins in the skin. Pre-haptens undergo abiotic mechanisms such as auto-oxidation to form reactive hydroperoxide products[33]. Common pre-haptens include dyes such as *p*-phenylenediamine and fragrances such as linalool [25]. Pro-haptens are sensitizers that undergo chemical activation by metabolic conversion that is mediated by the host's enzymes. These enzymatic reactions likely take place in skin cells such as keratinocytes, fibroblasts, melanocytes, and antigen-

presenting cells native to the skin such as Langerhan's cells in the epidermis and dermal dendritic cells based on mRNA expression studies of CYP enzymes [34] [9]. The primary group of enzymes implicated in bio-transforming pro-haptens includes members of the cytochrome P450 family. However, additional enzymes such as alcohol dehydrogenases, aldehyde dehydrogenases, monoamine oxidases, flavin-containing monooxygenases, hydrolytic enzymes, acyltransferases, glutathione S-transferases, uridine 5′-diphospho-glucuronosyltransferases, and sulfotransferases are also present in the skin and may play a role in metabolizing pro-haptens [12]. The reactive intermediates and products formed during these enzymatic mechanisms are often sensitizing haptens such as aldehydes and epoxides [26, 35]. These reactive products can be actively pumped out through multi-drug resistant transporters[36] where neighboring Langerhan's cells or dendritic cells may internalize it.

Pre- and pro-hapten sensitizers constitute an estimated 30-60% of all known contact sensitizers [37, 38]. There are also several examples of chemicals that could act as both pre- and pro-haptens and are capable of undergoing both auto-oxidative and enzymatic reactions such isoegeunol, eugenol, and *p*-phenylenediamine [39, 40].

1.4 CURRENT SCREENING APPROACHES

Traditional aspects of the multi-step *in vivo* pathway of allergic contact dermatitis and predict screening tools for skin sensitizers include animal tests such as the guinea pig maximization assay (GPMA) and the local lymph node assay (LLNA) in the murine model. Compared to human data, these animal

models are 72% accurate at predicting the sensitization potential of chemicals [2]. This discrepancy is due the misclassification of irritants such as sodium dodecyl sulfate that induce non-specific proliferation of T-lymphocytes in the LLNA[2]. There are also several examples of common false negatives such as pro-haptens geraniol, eugenol, and abietic acid[41]. Additional limitations with these animal models include their low throughput nature, high cost, variability, and ethical concerns. For these reasons, the European Union has placed a ban on animal testing of cosmetic ingredients that has been in effect since March 2013[42]. In lieu of these recent legislative events, many alternatives to *in vivo* screening of chemicals have been developed *in chemico* and *in vitro.* These novel alternatives to animal assays in development seek to model various events that occur during the ACD mechanism to predict sensitization potential of chemicals. However, all of these assays have yet to be formally approved by the European Union as a suitable alternative for animal testing.

In silico Tools

In silico tools are based on principles of QSAR (quantitative structure activity relationship) that relates molecular structure with the biology activity of the compound. The first QSAR model in context of sensitization was founded by Roberts and Williams in 1982 where they discovered correlations between physico-chemical properties and sensitizing abilities of alkylating agents [43]. Since then, many classification models based on machine learning tools and computer-based expert systems (**Table 1.1**) are available for the prediction of contact allergens.

A summary of *in silico* tools with their corresponding metrics used to determine the sensitization potential of chemicals, and their accuracy rates. The OECD toolbox accuracy rate was determined with respect to a panel of 54 chemicals. The DEREK and TOPKAT accuracy rate was determined with a panel of 178 chemicals.

Table 1. 1 In Silico Tools for Predicting Sensitization. A summary of *in silico* tools with their corresponding metrics used to determine the sensitization potential of chemicals, and their accuracy rates. The OECD toolbox accuracy rate was determined with respect to a panel of 54 chemicals. The DEREK and TOPKAT accuracy rate was determined with a panel of 178 chemicals.

In Silico	Metrics	Accuracy
OECD ToolBox	QSAR	80%
DEREK	QSAR + Metabolism + Skin Penetration	73%
TOPKAT	QSAR + GPMT data base	83%

The OECD QSAR toolbox is a free software tool that uses structural alerts of molecules for predicting direct covalent protein binding to identify possible mechanisms involved. When 54 chemicals were evaluated *in silico* using this toolbox, an accuracy of 80% was achieved [44]. Although non-sensitizers that do not have any electrophilic binding potential were all identified correctly, there were several false-negatives due to the complete misclassification of all pre- and pro-haptens evaluated. Thus, electrophilic structural alerts alone are only sufficient for the proper prediction of hapten sensitizers.

Derek for Windows (Deductive Estimation of Risk from Existing Knowledge) is a rule-based system that uses a set of molecular sub-structures

as alerts that are correlated with skin sensitization. These structural alerts include potential acylating or alkylate/arylating agents, Michael electrophiles, aldehydes, free radical generators, and thiol-exchange units [33]. DEREK is also capable of being updated with new rules that can allow additional QSAR data such as skin penetration and metabolic predictions to be implemented [33]. When using a test set of 178 chemicals from the GMPT and LLNA databases, an accuracy score of 73% was achieved[45]. This score is due to erroneous predictive mechanisms which led to the misclasssification of several known pre- and pro- haptens such as geraniol, vinyl pyridine, benzo-pryene, abietic acid, oximes, and dienes [46, 47].

TOPKAT (Toxicity Prediction by Komputer Assisted Technology) is another available rule-based system where electrotopical descriptors of atoms in a given molecule are used in conjunction with statistical regression analysis to predict the sensitizing potential of chemicals and their potencies. Additionally, TOPKAT checks whether query structures are part of an internal database of known sensitizers used to build the model. When testing a panel of 178 chemicals from a GPMT database, a 73% accuracy score was obtained [45]. A major limitation of the TOPKAT model is that it was developed based solely on the guinea pig model. Hence, many chemicals that are falsely classified in the GPMT were also falsely classified using TOPKAT [33].

Although *in silico* tools are an extremely efficient method of screening potential skin allergens based on their structural characteristics, the correct identification of pre- and pro-haptens is a major issue. This is especially clear for

in silico tools that do not account for metabolism such as the OECD tool box where 0% accuracy is reported for 8 pre- and pro-haptens [44]. Even when a metabolic component is introduced, the fact that predictions are made based on speculative chemistries and metabolic reactions that have yet to be empirically validated show the limitations of *in silico* tools. With the development of *in chemico* assays and tools, sensitizer mechanisms can be elucidated and these *in silico* models can be improved.

***In chemico* Assays**

An *in chemico* peptide binding assay was developed to empirically assess the formation of the macromolecular immunogen that develops during the initial step of sensitization. This assay measures the depletion of free peptides or the formation of adducts due to covalent binding of electrophilic sensitizers to nucleophilic regions on peptides. The latter method of measuring haptenization may provide more structural and mechanistic insights on how macromolecular immunogens form. The synthetic peptides utilized for these studies were 7 amino acids long with an exposed glutathione, cysteine, lysine, or histidine residue (**Table 1.2**) [48]. When this assay was evaluated with a panel of 38 sensitizers, peptides with cystiene showed the greatest degree of accuracy with 83% accuracy. A major caveat of this assay platform is that metabolism and oxidation reactions are not considered. This leads to an assay system that is poorly predictive of pre- and pro-hapten sensitizers without a source of metabolism or oxidation [38]. Although peptide binding is an integral step during ACD and this *in chemico* assay is an insightful tool for evaluating the

sensitization potential of chemicals, cellular responses to contact allergens should be considered.

Table 1.2 In Chemico Peptide Binding Assay for Predicting Sensitization. Peptide binding assay accuracy results when 38 sensitizers were evaluated for their ability to covalently bind to nucleophilic amino acids: cysteine, lysine, glutathione, or histidine. Peptide depletion was measured using HPLC. Most contact sensitizers in this study preferentially binded to cysteine residues on peptides.

In Chemico	Metrics	Accuracy
Binding	Cysteine - Binding	83%
	Lysine - Binding	67%
	Glutathione - Binding	66%
	Histidine - Binding	36%

***In vitro* Assays**

In vitro assays encompass the vast majority of current screening tools in development. In the interest of brevity, only *in vitro* assays that were submitted to the European Union and are currently undergoing multi-laboratory validation studies will be discussed in this section (**Table 1.3**). Prominent *in vitro* assays branch into two general categories that assess the cellular response of sensitizers on either keratinocytes or dendritic cells.

Table 1.3 A summary of *in vitro* assays for predicting sensitization. These cell-based *in vitro* alternatives are currently undergoing multi-laboratory validation studies in Europe. The h-CLAT and MUUST are dendritic cell based assays that evaluated the surface expression of CD54 and/or CD86 using flow cytometry methods. The GARD assay is a dendritic

cell based assay that utilized a 200 gene signature of sensitization. The KeratinoSens assay used a gene reporter cell line derived from HaCat keratinocytes with an antioxidant response element (ARE) promoter. The NCTC2544 keratinocyte cell line is used to measure IL-18 production by ELISA.

In Vitro	Cell Line	Cell Type	Metrics	Accuracy
h-CLAT	THP-1	Dendritic Cell	CD54, CD86	85 - 93%
MUSST	U-937	Dendritic Cell	CD86	77 - 85%
GARD	MUTZ-3	Dendritic Cell	200 Gene Signature	89 - 98%
KeratinoSens	HaCaT	Keratinocyte	ARE	83 - 94%
NCTC IL-18	NCTC2544	Keratinocyte	IL-18	78 - 97%

The KeratinoSens assay is a keratinocyte (KC) based test that utilizes a reporter gene cell line derived from HaCaT cells. This assay carries a luciferase reporter gene for the antioxidant response element (ARE) promoter [49]. This approach was based on the finding that the Nrf2-Keap1-ARE regulatory pathway was activated by skin sensitizers due to haptens binding to cysteine residues on Keap1 as part of the innate inflammatory response mediated by keratinocytes [50]. Genes that are activated via this regulatory pathway include IL-8, quinone reductase, aldo-keto reductase, thioredoxin, and thioredoxin-reductase [50]. Pending on the test set of chemicals used, the KeratinoSens assays report accuracies ranging from 77-83% [51, 52]. A limitation of this assay is that Nrf2-Keap1-ARE regulatory pathway is activated by cysteine-reactive skin sensitizers, thus leaving out a group of skin sensitizers that preferentially bind to other amino acids or the amino-terminus such as aldehydes with no α-β-unsaturation, pthalic anhydride, and oxazolone [52].

Another keratinocyte based assay that is currently undergoing validation studies is the NCTC2544 IL-18 assay. This assay utilizes the NCTC2544 keratinocyte cell line and measures IL-18 production as a biomarker of skin sensitization [53, 54]. IL-18 is produced during allergic contact dermatitis and is released when cytotoxic concentrations of chemicals are used. IL-18 is a potent inducer of IFN-γ production and promotes a Th-1 response. This assay appears to be highly predictive (78-97%) with the current test set of chemicals that consist primarily of hapten [53, 55]. Although both keratinocyte-based assays show promising results, dendritic cells that play an integral role in the presentation of the allergens to naïve T-cells during sensitization should also be investigated as a complementary approach.

Current *in vitro* dendritic cell (DC) assays commonly use myeloid leukemia-derived cell lines such as the THP-1 and U937. The THP-1 cell line is used in the human cell line activation test (h-CLAT) and the U937 cell line is used in the Myeloid U937 skin sensitization test (MUSST). Both of these assays predict the sensitization potential of chemicals using viability and dendritic cell maturation metrics such as surface molecules CD54 and CD86. Pending on the test set of chemicals evaluated, the MUSST assay reported accuracy scores that range from 71-86% [44, 51, 56]. When both CD54 and CD86 expression are evaluated in the h-CLAT assay, accuracy scores that range from 76 – 93% were reported. [44, 57].

Another potential source of LCs/DCs for *in vitro* studies includes the MUTZ-3 cell line. The Mutz-3 cell line is derived from myeloid leukemia and is

established as a viable cell source for differentiation into Langerhan's cells or dendritic cells upon exposure to different growth factors and cytokines [58-60]. Mutz-3 derived cells physiologically resemble human DCs more than THP-1 and KG1 cell lines based on gene expression studies [61]. The Genomic Allergen Rapid Detection (GARD) assay utilizes the MUTZ-3 cell line with a highly predictive genomic signature for sensitization comprising of 200 genes that was initially reported to be 98% accurate [62]. The genomic signature includes genes that regulate xenobiotic metabolism, cell proliferation, cell death, lipid metabolism, hematopoietic development, cell cycle, molecular transport, and carbohydrate metabolism. This screening approach was combined with a support vector machine classifier to predict the sensitization potential of chemicals. A recent in-house validation study using the GARD assay found it to be 89% accurate [63].

1.5 CURRENT LIMITATIONS AND CHALLENGES

Despite the promising numbers initially reported for overall assay performance in current alternatives to animal testing, it is important to evaluate the specifics of these numbers. After all, many assays are nearly 100% successful for some classes of chemicals and unsuccessful for others. Thus, the performance of a given assay will depend on the diversity of chemicals used in the study. This is reflected in current literature reports where reported assay accuracies vary pending on the chemical panel (**Table 1.3**). When each assay's ability to identify pre- and pro-hapten sensitizers was specifically assessed, significantly lower accuracies were observed and highlight a major limitation with

several of the current methods (**Table 1.4**). This was especially apparent for the peptide binding assay that measures the covalent binding of haptens that are innately electrophilic to nucleophilic peptide residues.

Table 1. 4 Assay Accuracies Detecting Pre-/Pro-Hapten Sensitizers. Assay performance of *in chemico* and *in vitro* assays when a panel of only pre- and pro-haptens were evaluated. Reduced accuracy rates were observed compared to assay performances determined in Table 2 and Table 3.

In Chemico			**Metrics**	**Accuracy**
Peptide Binding			Cysteine - Binding	58%
Peptide Binding			Lysine - Binding	37%
In Vitro	**Cell Line**	**Cell Type**	**Metrics**	**Accuracy**
h-CLAT	THP-1	DC	CD54, CD86	63%
MUSST	U-937	DC	CD86	66%
GARD	MUTZ-3	DC	200 Genes	75%
KeratinoSens	KeratinoSens	KC	ARE	77%
NCTC2544 IL-18	NCTC2544	KC	IL-18	83%

Dendritic cell based assays, h-CLAT, and MUSST also performed very poorly when detecting pre- and pro-hapten sensitizers with low accuracies in the range of 63 - 66% [56, 57]. Several known pre- and pro-haptens (e.g., 2-aminophenol, potassium dichromate, *p*-phenylenediamine, propyl gallate, hydroquinone, isoeugenol, ethylene diamine, anilin, eugenol, and geraniol) are commonly misclassified as non-sensitizers in DC-based assays [64]. This could be due to insufficient metabolic activity in dendritic cells as compared to keratinocytes where THP-1 cells used in the h-CLAT assay were shown to exhibit mRNA expression and protein content of CYP1A1 and CYP3A5 at lower levels compared to the normal human skin [65]. THP-1 cells also lack the aryl hydrocarbon receptor that regulates the induction of CYP1A1 expression [65].

Thus, dendritic cell based assays that utilize the THP-1 cell line may be insufficient tools to accurately predict potential sensitizing agents that undergo metabolic reactions in the skin. Interestingly, the GARD assay that utilized the MUTZ-3 cell line appears less impaired in accurately predicting 3 out of 4 four test pre/pro-hapten sensitizers. This is likely due to the fact that the GARD signature includes activation of genes that are involved in xenobiotic metabolism. Furthermore, several pro-hapten sensitizers were utilized as part of the training set. Thus, the MUTZ-3 cell line may possess greater metabolic capacity than THP-1 and U937 sources of DCs. Since keratinocytes are considered the major cell source of xenobiotic metabolism in the epidermis, it is not surprising that the KeratinoSens assay and the NCTC2544 keratinocyte IL-18 assay generally performed better at identifying pre- and pro-haptens than DC-based assays with accuracies ranging from 77% to 83% respectively [52, 66].

Another limitation of current methods is that they only investigate one facet of a multi-step ACD pathway or a single cell type. Thus, an assay based on the use of one to two metrics or “biomarkers” is insufficient to predict all sensitizers. This observation is true across a variety of DC sources and cell lines such as CD34+ hematopoetic progenitor cells, CD14+ monocytes, THP-1, U-937, Mutz-3, and KG-1[64]. As more chemicals are being evaluated using current alternatives, it is evident that the use of CD54 and CD86 alone is insufficient. Thus, the use of a molecular signature that takes into account several cellular metrics will be more sensitive than the use of a single biomarker. This effect is likely why the GARD assay is successful at predicting sensitization since it

utilizes 200 genes to classify chemicals. Furthermore, it appears that multi-metric approaches that incorporate *in vitro* data from various assays are more predictive than the use of data from one metric alone [44, 51, 66]. However, these methods still incorporate the combination of less predictive metrics and could benefit from a combination of superior markers. To aid in the development of predictive models of sensitization that incorporate various cellular metrics of skin sensitization, computational approaches that include machine learning can be utilized.

1.6 MACHINE LEARNING

Machine learning is the process of learning from data where predictions are made based on a set of metrics or features (e.g., physico-chemical properties). In supervised machine learning, there is a training set (e.g., physico-chemical properties of known sensitizers) of data where the outcome (e.g., Sensitizer vs. Non-sensitizer) is known based on the feature. Using this data, a prediction model or “learner” will be able to determine the outcome of new test set (e.g., chemicals with unknown sensitization potential) of data. A variety of machine learning methods such as linear discriminate analysis, decision trees, neural nets, and support vector machine have been used as classification tools to perform feature selection and build classification models to predict the sensitization potential of chemicals [37] . These computational methods initially utilized QSAR knowledge of known sensitizers from animal model data banks to ”train” their classification model and a subset of ”test” chemicals were used to evaluate the classifier performance [67].

Due to a large panel of potential cellular metrics currently being explored in literature and in our studies to predict sensitization responses, an *in silico* analysis method based on machine learning principles can provide valuable insights in a high throughput manner. Feature selection is the process where a subset of metrics is selected and uninformative metrics are discarded without altering the original data set. This is in contrast to other dimensionality reduction techniques such as principal component analysis or compression[68]. Supervised classification models can be used to perform feature selection on cellular secretion data that we evaluated. This will allow us to identify molecular signatures of sensitization at the secreted protein level that are most predictive. This information can be used to build a classification model that is capable of predicting the sensitization potential of unknown chemicals. An additional benefit of coupling an *in vitro* culture alternative with an *in silico* analysis method is that the classification model can be improved as more *in vitro* information becomes available when additional cellular metrics and chemicals are evaluated. Thus, there is an iterative feedback loop set in place to improve the model's performance with empirical data.

1.7 DISSERTATION SUMMARY

The primary goal of this thesis work is to develop a non-animal alternative for screening skin sensitizers. Current *in vitro* assays generally utilize a single cell type and/or biomarker that are involved in the ACD pathway to predict sensitization. This has led to the accurate prediction of many known hapten-sensitizers. However, correct identification of pre-/pro-haptens is limited due to

the lack of a sufficient metabolic component. We propose to address these issues by 1) establishing an *in vitro* culture system that preserves the dynamic signaling environment and metabolic machinery found *in vivo* during allergic contact dermatitis and 2) using computational methods to identify the most predictive cellular metrics and build a classification model of sensitization using these selected biomarkers.

In chapter 2, we initially developed an *in vitro* system where a full thickness skin model with keratinocytes and fibroblasts was co-cultured with MUTZ-3 derived Langerhans' cells. With this co-culture assay, we were able to topically apply a variety of treatments and induce metabolism of model pro-haptens to sensitize the Mutz-LCs placed below the skin. This culture system set up the platform to evaluate many different sensitization metrics based on the *in vivo* pathway with ease and led to the development of a novel feature selection method. Post-sensitized Langerhan's cells were subsequently isolated and characterized for the presence of maturation surface molecules, cytokine secretion, and chemotaxis towards CCL19. We developed a novel method of performing feature selection by ranking cellular metrics using the margin distances calculated from the support vector machine (SVM). This work highlighted the limitations of CD54 and CD86 as biomarkers and the use of the trans-well migration assay using CCL19. However, we found that the combination of IL-12, IL-9, IFN-γ, and VEGF together can be used to build a predictive classification model using SVM. Although promising, this co-culture assay is potentially costly and resource intensive. Thus, an alternative means to

metabolize pro-hapten sensitizers in a manner that is more amenable for cost efficient, high throughput screening was established. Furthermore, with our feature selection method validated, we can apply our *in silico* tools to other culture systems where multiple secreted metrics are evaluated.

In chapter 3, we developed a co-culture system that combined HaCaT keratinocytes, dermal fibroblasts, and MUTZ-LCs in a single well and compared this to MUTZ-LCs alone. The co-culture method enables the keratinocytes and fibroblasts to carry out the necessary metabolism to activate pro-hapten sensitizers and to provide additional cross-talk signals to promote LC maturation. A panel of secreted cytokines, chemokines, and growth factors were evaluated in the supernatants generated after sensitizer treatment for the two culture systems. Support vector machine analysis was utilized to perform feature selection to identify promising cellular metrics and to develop a predictive classification model of skin sensitization for both culture systems.

In chapter 4, a summary of the key dissertation findings and implications will be provided. Furthermore, the limitations of our assays will be discussed in greater detail and potential future studies will be outlined.

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CHAPTER 2: PREDICTING FULL THICKNESS SKIN SENSITZATION USING A SUPPORT VECTOR MACHINE

Note: This chapter is reproduced from the following publication:

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Predicting Full Thickness Skin Sensitization Using a Support Vector Machine, Toxicology *In vitro* (Accepted July 2014).

2.1 INTRODUCTION

Allergic contact dermatitis (ACD) is a common inflammatory skin disease that is mediated by adaptive immunity. It is reported that up to 19.5% of the general population is sensitive to at least 1 allergen [1]. Traditional *in vivo* screening assays for sensitizers include the guinea pig maximization assay and a more quantitative assay known as the murine local lymph node assay (LLNA). However, there are still several limitations with these animal models that include their low throughput nature, high cost, variability, and ethical concerns. Furthermore, compared to human clinical data, these *in vivo* assays are only 73% accurate at predicting the sensitization potential of chemicals [2]. For these reasons, the European Union has placed a ban on animal testing of cosmetic ingredients [3]. Alternatives to animal testing should allow for high throughput screening of chemicals using sensitive metrics that can reliably predict human immunotoxicity.

There are many novel alternatives to animal assays in development that seek to model aspects of the *in vivo* pathway of allergic contact dermatitis and predict the sensitization potential of chemicals [4]. Sensitizers are commonly haptens, which are molecules that can easily penetrate through the epidermal barrier and bind to nucleophilic regions of proteins in the skin. This conjugated complex serves as the immunogen that initiates the allergic contact dermatitis response [5]. A high through-put *in chemico* assay was developed that measures this peptide binding with a high degree of accuracy [6]. Low through-put *in vitro* assays that utilize dendritic cells have also been explored to predict the sensitization potential of chemicals using maturation metrics such as surface molecules, cytokine production, chemotaxis, and ability to stimulate a T-cell proliferation response.

A major limitation of many of these systems is that they do not contain a metabolic component to accurately identify a class of haptens known as pro-haptens [7] [6]. Pro-haptens are innately non-electrophilic and require chemical activation or biotransformation to form reactive intermediates or products that subsequently bind to peptides to form the immunogen [8]. A metabolic component integrated with dendritic cells would be a valuable addition for the identification of pro-hapten sensitizers. Furthermore, due to the complexity of the *in vivo* mechanisms that trigger allergic contact dermatitis, a tiered approach that tests for a more comprehensive panel of cellular metrics may be needed. To aid in the analysis of these *in vitro* metrics, high throughput computational tools can

be utilized to identify the best predictors of sensitization and streamline the low through-put collection of cellular data.

To develop a culture platform that is physiologically relevant to humans and capable of metabolizing pro-haptens, a re-constructed full thickness skin model known as RealSkin was used either as a stand-alone assay or co-cultured with Langerhan's cells derived from the MUTZ-3 cell line. This allows for key *in vivo* events such as permeation through the skin barrier, metabolic activation of pro-haptens, and a dynamic signaling environment from a variety of cell types *in vitro*. Post-sensitized Langerhan's cells were subsequently isolated and characterized for the presence of maturation surface molecules, cytokine secretion, and chemotaxis towards CCL19. The secretome from the post-sensitized cultures was evaluated using hierarchical cluster analysis and support vector machine (SVM) classification tools.

SVM is a powerful machine learning tool that is commonly used in pattern recognition and was previously used to classify skin sensitizers based on molecular structures [9]. We utilized the support vector machine as a feature selection tool to compare different cytokine secretion profiles as potential predictors and using a small sensitizer panel identified the best molecular signature for sensitization, as a proof of concept for the utility of our approach. This analysis tool can also potentially aid in the understanding of key events in allergic contact dermatitis associated with sensitizer potency differences.

2.2 MATERIALS AND METHODS

RealSkin and Cell Lines

RealSkin, a full thickness skin model from EpiSkin^{TM} consists of a dermal equivalent with a lattice of acido-soluble collagen and normal human adult fibroblasts overlaid by a stratified, well differentiated epidermis layer derived from normal human adult. The RealSkin kit including the tissue model and its respective culture medium was provided by EPISKIN^{TM} (Lyon, France) as a donation from L'Oreal (Paris, France). MUTZ-3 cells were a donation from L'Oreal (Paris, France) and are available for purchase from DSMZ (Braunschweig, Germany). 5637 urinary bladder carcinoma cell line was purchased from ATCC (Manassas, Va). RealSkin was cultured for up to a two week period and the medium was changed every other day. A 1 cm^2 biopsy punch was applied to the RealSkin prior to experimental use and the excised piece was placed on top of sterile inserts supplied by L'Oreal (Paris, France). The 5637 cell line was maintained at 37^{o}C and 5% CO2 in RPMI medium supplemented with 10% FBS, 2% L-glutamine, and 1% penicillin-streptomycin. Media was changed every other day and conditioned medium was collected when the cells were 90% confluent. This conditioned medium was supplemented into the MUTZ-3 culture medium as per the guidelines from DSMZ. MUTZ-3 cell line was maintained at 37^{o}C and 5% CO2 in alpha-MEM medium with Glutamax, ribonucleosides, and deoxyribonucleosides (Invitrogen) supplemented with 20% heat inactivated FBS, 10% 5637 conditioned medium, 1% Penicillin-streptomycin, and 50uM2-mercaptoethanol. Media was changed every other day and the cells were split on day five of culture.

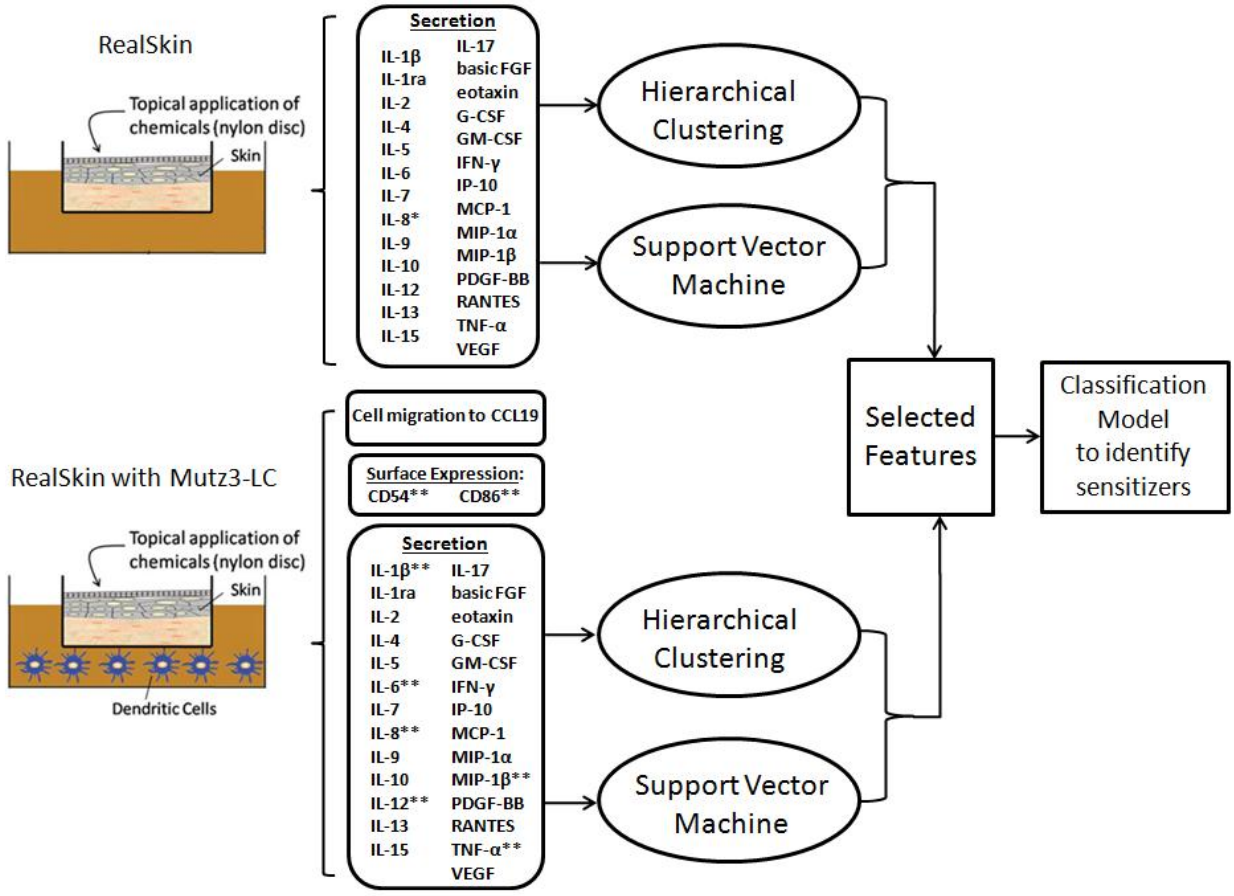

Figure 2. 1 Schematic of our experimental system, comparing sensitized Realskin with Realskin+Mutz co-cultures. RealSkin was cultured at the air-liquid interface (ALI) and topically dosed with either non-sensitizers (vehicle and salicylic acid (SA)) or sensitizers (isoeugenol(IE) or p-phenylenediamene (PPD)) for 48 hours. For the co-culture configuration, the RealSkin was also cultured at the ALI with the MUTZ-3 derived Langerhan's cells, cultured in the medium below. The supernatant from each treatment condition was collected and analyzed using the Bioplex assay to screen up to 27 cytokines. Of these cytokines, the * indicates factors that have been previously explored by other groups in literature for skin equivalent sensitization studies and ** indicates factors that have been

commonly utilized in dendritic cell based assays[10] [4]. The secretome data was further analyzed *in silico* using feature selection tools such as hierarchical cluster analysis and support vector machine to identify potential biomarkers for sensitization. The MUTZ-3 derived LC's from the co-culture was also analyzed *in vitro* for surface marker expression changes and chemotaxis towards CCL19.

Chemicals

The chemical panel selected to perform the initial proof-of-concept studies were irritant, Salicylic acid (SA), a moderate pro-hapten sensitizer, isoeugenol (IE), and a strong pre-hapten, *p*-phenylenediamine (PPD). These chemicals were selected as representative pre- and pro-haptens that are currently on the list provided by Sens-It-Iv initiative *(Newsletter number 02-2007, January 26. http://www.sens-it-iv.eu/files/newsletter/Sens-it-iv_Newsletter_0.8.html).* All chemicals were purchased from Sigma-Aldrich. The stock solutions were prepared in dimethylsulfoxide (DMSO) and then subsequently diluted to their final working concentrations using RPMI cell culture medium (Invitrogen). The final concentration of DMSO was 0.4% across all conditions. These working concentrations were determined from dose-response studies where at least 90% of the skin cells were viable as determined by MTT conversion assay (data not shown). These concentrations are 180 µM and 360 µM for SA, 112 µM and 224 µM for IE, and 185 µM and 92.5 µM for PPD. The chemicals were topically applied on either RealSkin alone or on RealSkin as a co-culture with MUTZ-3 derived Langerhan's cells for 48 hours (**Figure 2.1**).

Flow Cytometry

The phenotype of sensitized MUTZ-LC was determined by flow cytometry. Cells were stained using mouse anti-human monoclonal antibodies to CD54 (IgG1-FITC, R&D Systems) and CD86 (IgG1-FITC, R&D Systems). IgG1-FITC (R&D Systems) was used as an isotype control to assess non-specific binding. Cells were incubated with antibodies for 30 minutes on ice with 1% mouse serum for blocking, washed three times with PBS, fixed in 4% paraformaldeyhyde for 15 minutes, and then re-suspended in the PBS for FACS analysis with a FACScan flow cytometer (Beckton Dickinson, San Jose, Ca.). The data was analyzed using CellQuest Software. Comparisons between different treatment conditions were performed by measuring the stimulation index (SI) which was determined by taking into account the fluorescent intensity and % positive population of cells from the treatment conditions relative to the fluorescent intensity and % positive population of cells treated with the vehicle.

Trans-well Migration Assay with CCL19

Sensitized MUTZ-3 LCs co-cultured with RealSkin were harvested and placed into the upper chamber of a 24-well transwell insert (8 um pore size) at a density of 5×10^4 cells in the presence and absence of 250 ng/mL of chemokine, CCL19. (R&D Systems) Four hours after the cells were exposed to the chemokine, the MUTZ-LCs that migrated were centrifuged and re-suspended in 100uL of fresh media prior to counting by hemocytometer. The net migration of cells was determined by the following equation using conditions where cells were in the presence and absence of CCL19.

[Net # of Cell Migration] = [# Cells migrated in presence of CCL19] – [# Cells migrated in absence of CCL19]

The data was further processed to find the fold difference between all the sensitizer treated conditions with respect to the irritant treated condition. The net migration of LCs for SA treated conditions across a concentration range was averaged since the values were approximately the same. This average net migration for irritant treated cells was then used to determine the fold increase in migration for different sensitizers using the formula below.

$$\text{Fold Increase in Migration} = \frac{[\text{Net Migration for Sensitizer Treated Cells}]}{[\text{Average Net Migration for Irritant Treated Cells}]}$$

Cytokine Multiplex Analysis

Supernatant was collected after treating RealSkin alone and RealSkin co-cultured with MUTZ-LCs with salicylic acid, isoeugenol, and p-paraphenylenediamine for 48 hours. Basal alpha-MEM media fortified with 20% heat inactivated FBS and 1% P/S was used as control. The supernatants were then analyzed for 27 human cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCAF, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF) using a Bioplex Assay following the manufacturer's instructions (Bio-Plex Human Cytokine 27-plex panel; Bio-Rad Laboratories, Hercules, CA, USA).

Hierarchical Cluster Analysis

Unsupervised agglomerative hierarchical clustering was performed as a feature selection method in Matlab on secretome data from the Bioplex that was normalized to the vehicle treated cultures.

Support Vector Machine

The supervised classification approach for distinguishing chemical potency utilizes support vector machine (SVM) as a feature selection method, which determines the best discriminate metrics between the two classes of chemicals (sensitizing vs. non-sensitizing). For each cytokine, the training data is given by $\{x_i, y_i\}, i = 1 \dots l, y_i \in \{-1, 1\}$, where x denotes the cytokine concentration and y corresponds to the label of the chemical (-1 for non-sensitizers and 1 for sensitizers). Using a linear kernel, the points that lie on the hyperplane that separates the positives and negatives is $w \cdot x + b = 0$, which leads to the following constraints to the training data:

$$x_i \cdot w + b \geq +1 \; for \; y_i = +1 \qquad (1)$$

$$x_i \cdot w + b \leq -1 \; for \; y_i = -1 \qquad (2)$$

where w is the norm to the hyperplane and $|b|/\|w\|$ is the perpendicular distance from the hyperplane to the origin, and $\|w\|$ is the Eucledian norm of w. From inequality (1) the perpendicular distance of the hyperplane to the origin is found to be $|1 - b|/\|w\|$. Similarly, the distance of the hyperplane in (2) to the origin is $|-1 - b|/\|w\|$. Therefore the margin distance between these two hyperplanes is $2/\|w\|$. Thus we can find a pair of hyperplanes that best separates the two classes by minimizing $\|w\|$ subject to the constraints (1) and (2). This problem is solved using quadratic programming provided by the bioinformatics toolbox in

Matlab. We used the aforementioned method to compute the margin distance 2/||w| for each cytokine. These margin distances and classification accuracy values were subsequently ranked to identify metrics that had the greatest distance of separation between the non-sensitizer (untreated, vehicle, and salicylic acid) and sensitizer treated classes (isoeugenol and paraphenylenediamine).

The support vector machine was also used as a classification model to predict sensitization. Model performance (accuracy, sensitivity, specificity) was assessed using 10-fold cross validation for each individual cytokine metric and molecular signatures identified through various feature selection methods described above.

Statistical Analysis

All data is presented as mean ± standard error. All data in the paper was based on N≥3 independent biological replicates. Specifically, the data for the CCL19 migration assay was N=4 independent biological replicates and the data for all other evaluated metrics (IL-8, CD54, CD86, 27-cytokines) were N=3 independent biological replicates. To compare the data from the different chemical treatments, we used ANOVA followed by Fisher's least significant difference post-hoc analysis. Statistical significance was determined at $p \leq 0.05$ and these values were ranked to perform feature selection on the multi-plex data.

2.3 RESULTS

IL-8 Secretion

Secretion of IL-8 is a commonly utilized metric for sensitization of several dendritic cell lines and skin cultures [4]. Thus, we assessed IL-8 levels in our co-culture system to see if elevated levels were present in sensitized conditions when normalized to vehicle. IL-8 secretion was evaluated in the supernatant collected from the co-culture configuration with RealSkin and MUTZ-LC 48 hr. post-chemical treatment. IL-8 secretion from RealSkin alone indicated that chemical treatments with salicylic acid (SA), isoeugenol (IE), and *p*-phenylenediamine (PPD) did not significantly alter relative to vehicle treatment (**Figure 2.2A**). Differential secretion between non-sensitized groups (untreated and vehicle) compared to sensitized groups (IE and PPD) were observed (**Figure 2.2B**). However, only PPD induced a significant elevation in secretion levels as compared to the vehicle.

Evaluation of CD54 and CD86 on MUTZ-LC

Studies were also designed to evaluate the RealSkin full thickness skin co-culture sensitization system using commonly evaluated surface expression metrics for dendritic cell maturation, CD54 and CD86. Sensitizers, isoeugenol (IE) and *p*-phenylenediamine (PPD) or controls, vehicle (0.4% DMSO in RPMI medium) and irritant salicylic acid (SA) were topically applied to the RealSkin and DC sensitization was evaluated via immunofluorescence labeling after 48 hours. The flow cytometric results (**Figure 2.3**) of IE and PPD sensitization indicated that exposure to a strong pre-hapten 185 μM PPD, yielded the greatest stimulation for CD86 and CD54. However, isoeugenol, a moderate sensitizer, induced a very mild increase in CD86 and CD54 that was not significant.

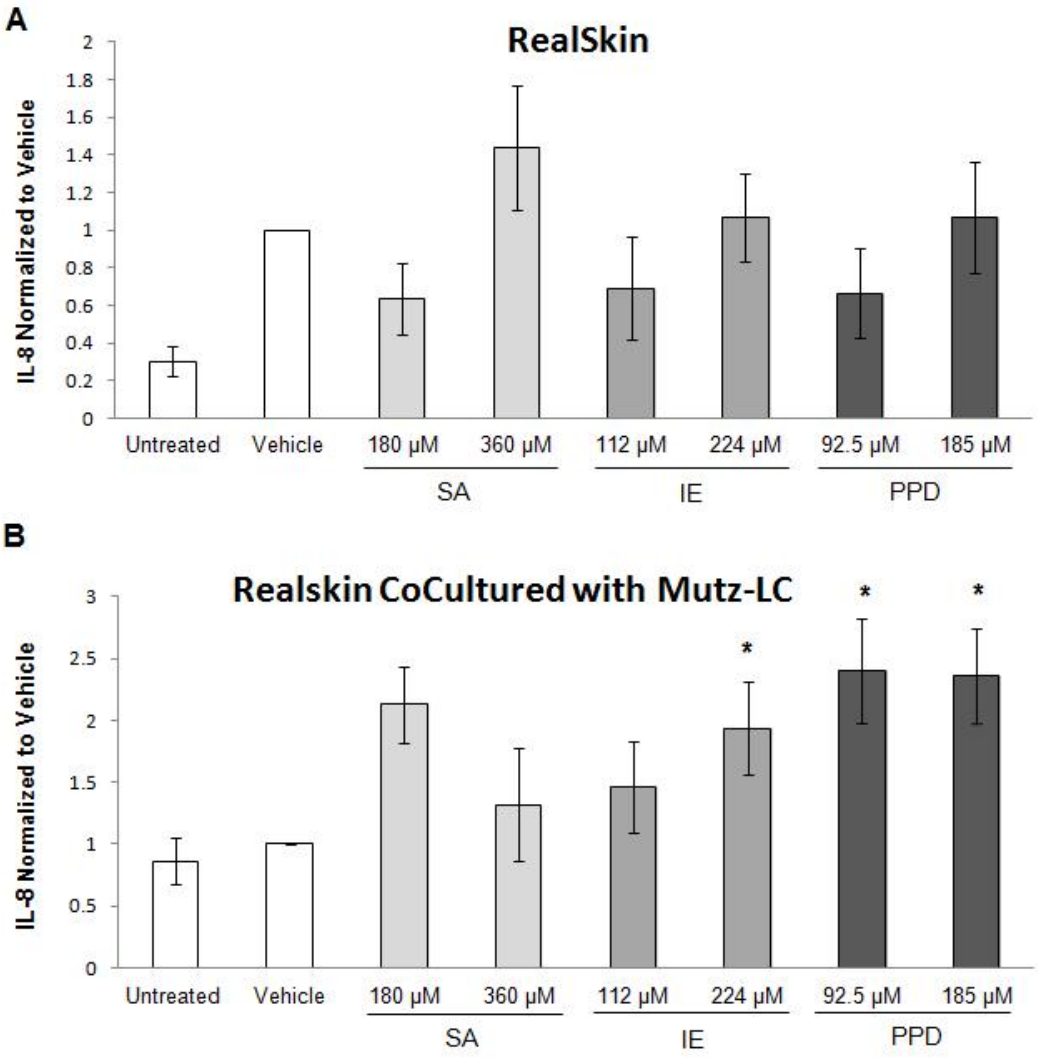

Figure 2. 2 Secretion of IL-8 from A) RealSkin and B) RealSkin co-cultured with Mutz-3 LCs. After 48-hours of chemical treatment the secretion level of IL-8 was normalized to the baseline vehicle level. Data is represented as Means ± S.E. for N=3 independent replicates. (A) RealSkin alone did not induce any significant changes in IL-8 secretion following treatment with the chemicals salicylic acid (SA), isoeugenol (IE) or *p*-phenylenediamine (PPD). (B) RealSkin Co-Cultured with MUTZ-LCs showed significant IL-8

increases with 224 µM IE and PPD treatments with respect to the untreated and vehicle where p≤0.05. However, these conditions were not significant when compared with the 360 µM dose of SA.

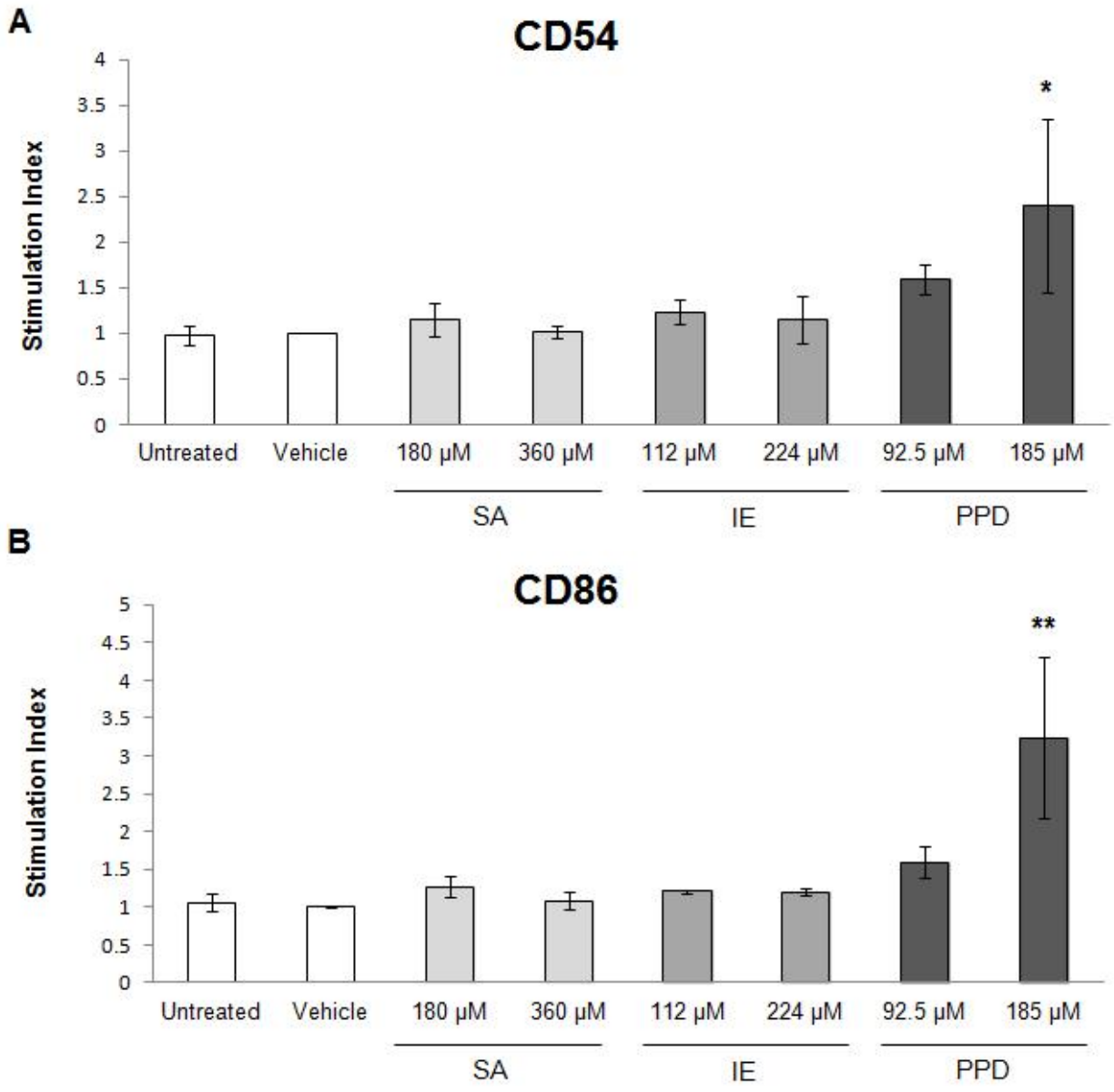

Figure 2. 3 Expression of CD54 and CD86 of MUTZ-LCs following sensitization. The relative percent positive in the sensitized MUTZ-3 LC's populations was compared to vehicle treatment for A) CD54 and B) CD86 expression. The expression level of each corresponding surface molecule

treated with vehicle is indicated by the black dotted line. CD54 surface expression did not significantly change with treatments salicylic acid (SA), isoeugenol (IE), or *p*-phenylenediamine (PPD). CD86 expression increased significantly following treatment with the strong sensitizer PPD. * indicates significance at p≤0.05. Although there is an increased CD86 trend following moderate sensitizer IE treatment, the difference was not significant. Data is represented as Means ± S.E. for N=3 independent replicates.

Evaluation of MUTZ-LC Migration to CCL19

To functionally assess the MUTZ-3 derived LC sensitization mediated chemotaxis towards the chemokine CCL19, a 4 hour trans-well migration assay was performed. After 48 hour sensitizer exposure, an enhanced dose responsive migration towards CCL19 was observed relative to SA for IE and PPD, respectively (**Figure 2.4**).

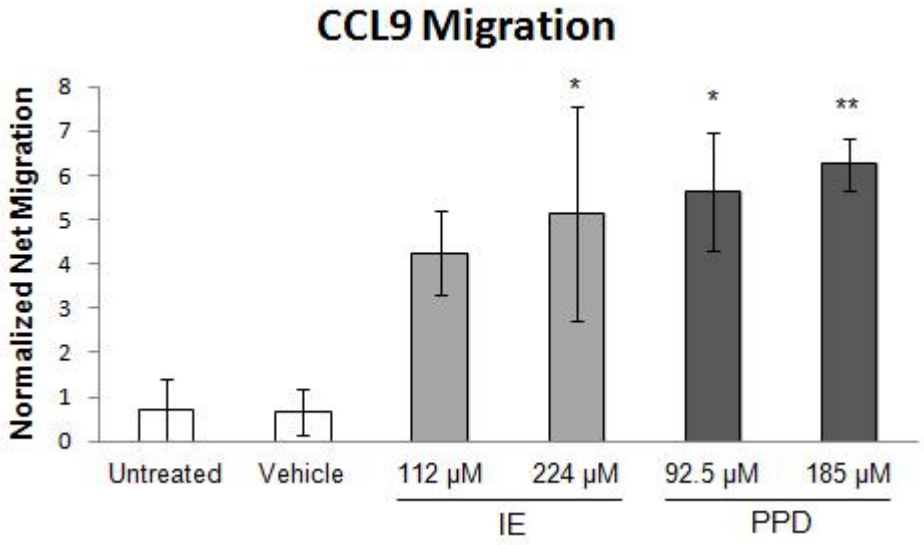

Figure 2. 4 Migration of MUTZ-LCs co-cultured with RealSkin toward CCL19. Cultures were established and sensitized as described above. Migration was quantified post-exposure to chemical treatments. The data was not normalized to vehicle for the migration assay due to zero net migration when treated with vehicle alone. Instead, the data was normalized to irritant salicylic acid (SA) instead as represented by the dashed line to indicate mean level of migration for SA treated MUTZ-LCs. Chemotaxis in response to CCL19 exposure was greatest for the strong sensitizer treatment *p*-phenylenediamine (PPD) followed by the moderate sensitizer isoeugenol (IE). This difference was significant for both sensitizer treatments, where ** indicates p≤0.01. The non-sensitizer treatments (untreated, vehicle, and salicylic acid (SA)) showed minimal cell migration. Data is represented as Means ± S.E. for N=4 independent replicates.

Hierarchical Cluster Analysis of Secretome Data

In order to expand our quantitative analysis of RealSkin mediated sensitization, we examined the secreted molecular patterns post-chemical treatment using a 27-cytokine Bioplex screen. The data from this secretome screen was analyzed using high throughput classification tools to identify molecular patterns related to sensitization. An unsupervised agglomerative hierarchical clustering analysis was performed to identify potential cytokines that are elevated post-sensitization and for the two culture conditions (RealSkin treated alone or RealSkin co-cultured with MUTZ-LCs).

The heat map representation of the data shows increased relative levels of secretion in shades of red and decreased levels of secretion in shades of green. The cluster analysis for the RealSkin alone treated with chemicals shows a dendrogram that clusters a node with sensitizer PPD and irritant SA. This demonstrates that these two treatment conditions share similar secretion patterns (**Figure 2.5A**). In contrast, the cluster analysis for the co-culture secretome from RealSkin with MUTZ-LC shows a dendrogram that clusters non-sensitizer treatments (untreated, vehicle, and irritant SA) together. However, the moderate sensitizer, isoeugenol, branches out from this node, indicating a change in the baseline secretion. Moreover, the strong sensitizer PPD, branches off from the isoeugenol treatment conditions and farthest away from the non-sensitizer treatment conditions. Therefore, the non-sensitizer treatment conditions share similar patterns in secretion post-treatment with low levels of inflammatory cytokine secretion and sensitizer treatment conditions show increased levels of inflammatory cytokine secretion. A panel of secreted metrics that is clustered together to reflect these patterns include VEGF, IL-4, IL-9, and IL-12 (**Figure 2.5B**) and are selected as potential candidates to predict skin sensitization.

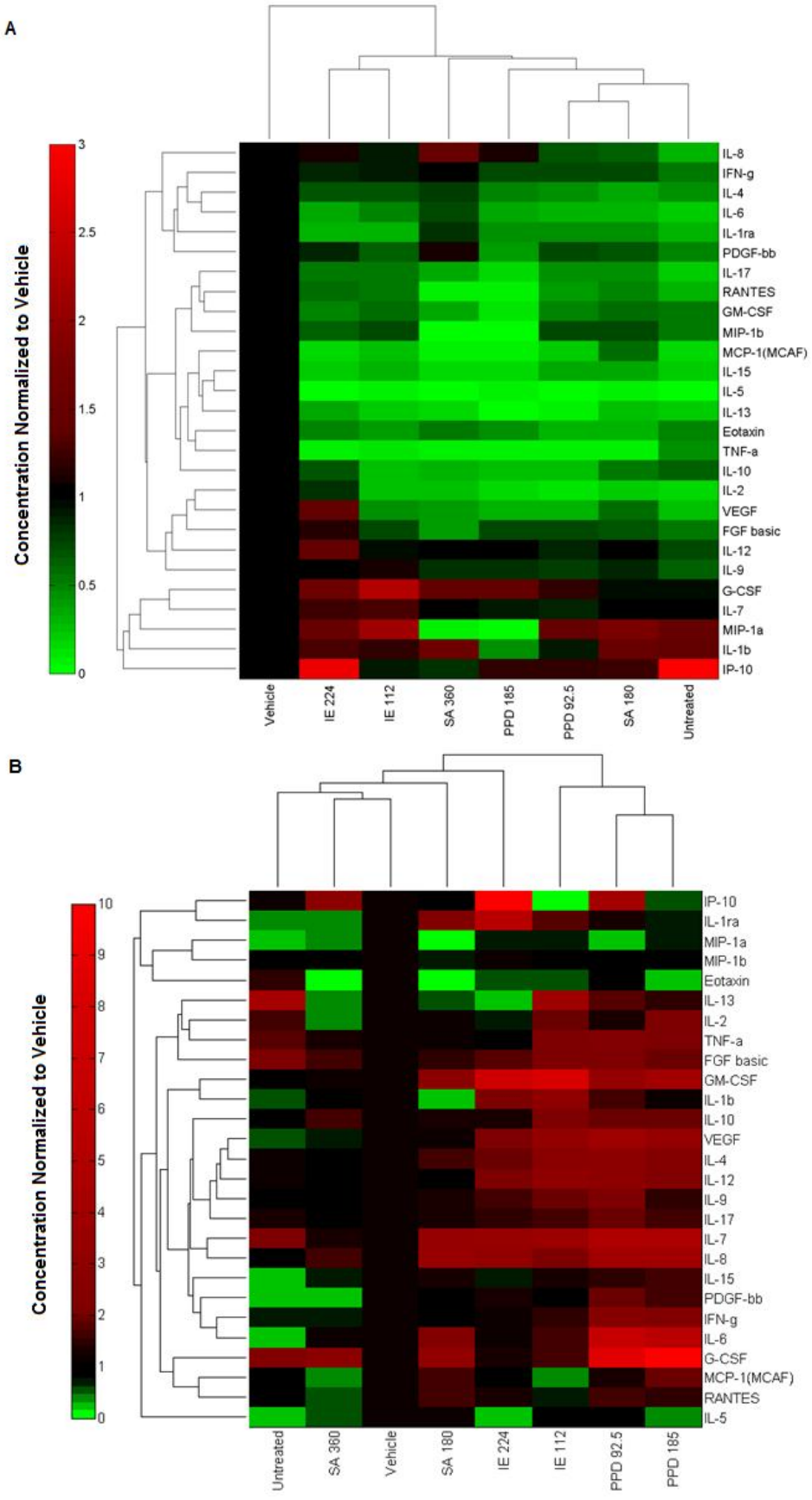
A
Concentration Normalized to Vehicle
3
2.5
2
1.5
1
0.5
0
IL-8
IFN-g
IL-4
IL-6
IL-1ra
PDGF-bb
IL-17
RANTES
GM-CSF
MIP-1b
MCP-1(MCAF)
IL-15
IL-5
IL-13
Eotaxin
TNF-a
IL-10
IL-2
VEGF
FGF basic
IL-12
IL-9
G-CSF
IL-7
MIP-1a
IL-1b
IP-10
Vehicle
IE 224
IE 112
SA 360
PPD 185
PPD 92.5
SA 180
Untreated
B
Concentration Normalized to Vehicle
10
9
8
7
6
5
4
3
2
1
0
IP-10
IL-1ra
MIP-1a
MIP-1b
Eotaxin
IL-13
IL-2
TNF-a
FGF basic
GM-CSF
IL-1b
IL-10
VEGF
IL-4
IL-12
IL-9
IL-17
IL-7
IL-8
IL-15
PDGF-bb
IFN-g
IL-6
G-CSF
MCP-1(MCAF)
RANTES
IL-5
Untreated
SA 360
Vehicle
SA 180
IE 224
IE 112
PPD 92.5
PPD 185

Figure 2. 5 Hierarchical cluster analysis of A) RealSkin and B) RealSkin co-cultures with MUTZ-LCs secretome. A) RealSkin secretome normalized relative to vehicle and B) RealSkin co-cultured with MUTZ-LCs secretome normalized relative to vehicle after treatment with sensitizers isoeugenol (IE), *p*-phenylenediamine (PPD), and irritant, salicylic acid (SA) for 48-hours. No discriminant patterns emerged in the RealSkin mono-culture with sensitizer treatment and therefore the cluster analysis does not arrange the treatments according to their actual potency. However, for the co-culture condition, a panel of cytokines (VEGF, IL-4, IL-12, IL-9) show elevated levels of secretion relative to both irritant and vehicle for sensitizer treatments of moderate and strong potency. Heat map data is represented as mean for N=3 independent replicates.

Support Vector Machine Analysis on Secretome Data

Although the hierarchical cluster analysis gave some insight in parsing the potencies of each chemical and identifying potential biomarkers for sensitization based on the multi-plex data, it does not systematically rank the metrics to identify the best possible biomarker(s) quantitatively. Thus, alternative feature selection methods were explored such as the utility of p-values from ANOVA and a support vector machine (SVM) classifier. The SVM calculated the margin distance of separation between two classes of chemicals: non-sensitizer control treatments and sensitizer treatments (IE and PPD). The greater margin distances indicated a greater degree of separation between the two classes for any given metric. Based on this information, we were able to rank each cytokine based on

its ability to distinguish between the controls and the sensitizer treatments and select the key features necessary to produce an accurate prediction. The margin distances for the cytokine data from the co-culture assay are greater than the margin distances from the skin equivalent secretome indicating a greater degree of separation from non-sensitized treatment groups and sensitized treatment groups (data not shown). Furthermore, for RealSkin alone, no cytokines were found to be statistically significant and all cytokine metrics showed low accuracies (<75%) for correctly classifying sensitized treatments.

The margin distances of the top ten secretome cytokines collected through the co-culture system with RealSkin and MUTZ-LCs in ranked order are IL-12, IL-9, VEGF, IFN-γ, PDGF, IL-7, IL-8, GM-CSF and IL-6 **(Table 2.1)**. A representative scatter plot of the top metric, IL-12 shows the separation of the sensitizer (IE and PPD) data points in contrast to the non-sensitizer (Vehicle, SA) data points that are separated by the margin distance **(Figure 2.6)**. The top 3 cytokines, IL-12, IL-9, and VEGF all have accuracy, sensitivity, and specificity values that exceed 80% as individual biomarkers. Using the support vector machine to rank the cytokines by their accuracy instead of by margin distances yields the same panel of cytokines. However, the ranking order is slightly altered to reflect the following hierarchy in descending order of IL-12, IL-9, VEGF, PDGF, IL-4, GM-CSF, IL-6, IL-8, IFN-γ, and IL-7. Ranking the cytokines by p-values determined through ANOVA results in the same cytokines listed in the top 10 as the panel identified by ranking margin distances. However, the order of the top

ten cytokine ranking is slightly shuffled to reflect the following order of IL-12, VEGF, IL-9, IFN-γ, IL-4, PDGF, IL-8, IL-7, IL-6, and GM-CSF.

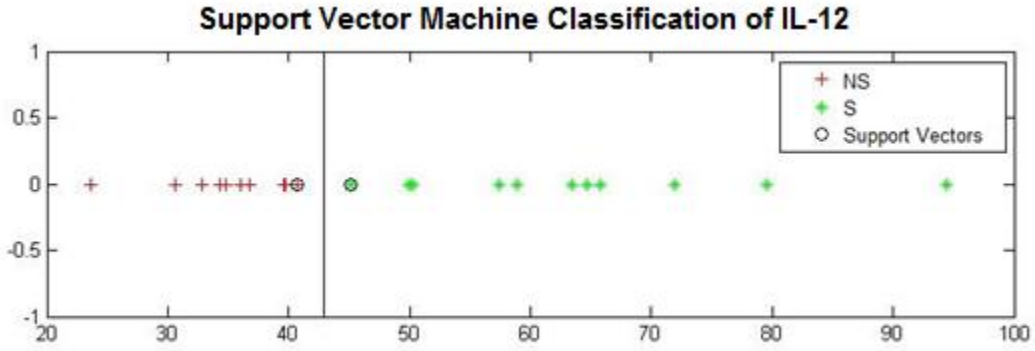

Figure 2. 6 Representative scatter plot of IL-12 using support vector machine. Red + indicates non-sensitizer (NS) treatments (untreated, vehicle, and salicylic acid (SA)) and green + indicates sensitizer (isoeugenol (IE) and *p*-phenylenediamine (PPD)) treated conditions. The line separating the two classes in this plot indicates the center of the margin distance boundary. The two encircled points near the margin distance center boundary indicates the critical points that are used as support vectors. IL-12 data analyzed by the SVM classifier included all treatment conditions (untreated, vehicle, SA, IE, and PPD) and all of their respective concentrations for N=3 independent replicates.

Table 2. 1 Margin distances of Cellular Metrics from Support Vector Machine Analysis. The margin distances quantified from the support vector machine ranked, from greatest to least distance of separation, between non-sensitizer treatment groups and sensitized groups for metrics from a Bioplex screen using supernatant from co-culture of RealSkin with MUTZ-3 derived Langerhan's cells (RSLC). The accuracy, sensitivity, and

specificity for each cytokine metric were determined using a support vector machine classification model with 10-fold cross. The top ten secretion metrics (IL-12, IL-9, VEGF, IFN-γ, IL-4, PDGF, IL-8, IL-7, GM-CSF, and IL-6) that can accurately classify non-sensitizers (vehicle and salicylic acid (SA)) from sensitizers (isoeugenol (IE) and *p*-phenylenediamine(PPD)) identified by SVM all have p values ≤0.05 as determined by ANOVA. IL-12 is the only metric that has an accuracy, sensitivity, and specificity value that exceeds 90%. Data analyzed by the SVM included all treatment conditions (untreated, vehicle, SA, IE, and PPD) and all of their respective concentrations for N=3 independent replicates.

Rank	Metrics	Margin Distance	Accuracy (%)	Sensitivity (%)	Specificity (%)	P-value
1	IL-12	0.5564	91.67	91.67	91.67	< .0001
2	IL-9	0.339	83.33	83.33	83.33	0.00011
3	VEGF	0.3176	83.33	83.33	83.33	0.00022
4	IFN-γ	0.2645	66.67	66.67	66.67	0.00146
5	IL-4	0.2564	75	66.67	83.33	0.00287
6	PDGF-bb	0.2473	79.17	83.33	75	0.00732
7	IL-8	0.2438	70.83	66.67	75	0.01131
8	IL-7	0.2403	66.67	50	83.33	0.01559
9	GM-CSF	0.2391	75	58.33	91.67	0.02965
10	IL-6	0.2336	70.83	58.33	83.33	0.02993
11	IL-17	0.2278	66.67	75	58.33	0.05489
12	IL-15	0.2233	58.33	66.67	0.5	0.083
13	IL-10	0.2219	66.67	58.33	75	0.09419
14	IL-1β	0.2196	62.5	50	75	0.09501
15	FGF basic	0.2189	66.67	66.67	66.67	0.13044
16	TNF-α	0.2181	66.67	75	58.33	0.15035
17	IL-1ra	0.216	50	25	75	0.16244
18	G-CSF	0.2147	50	25	75	0.22958
19	IL-2	0.2137	54	50	58.33	0.2565
20	IP-10	0.2117	50	16.67	83.33	0.2815
21	Rantes	0.2104	50	50	50	0.42441
22	Eotaxin	0.209	33.33	33.33	33.33	0.53499
23	MIP-1α	0.2089	33.33	25	41.67	0.79776
24	MIP-1β	0.2087	25	33.36	16.67	0.80659
25	IL-13	0.2087	45.83	8.33	83.33	0.86399
26	MCP-1	0.2087	33.33	16.67	50	0.89127
27	IL-5	0.2085	45.83	50	41.67	0.90373

Table 2. 2 Performance of classification models using metrics identified through feature selection methods. Classification performance in terms of accuracy, sensitivity, and specificity was determined by 10-fold cross validation. By utilizing all of the 27 cytokines from the Bioplex without performing any feature selection to build a predictive model, the accuracy, sensitivity and specificity of the classifier was very poor. Performing

feature selection by ranking the margin distances from the SVM and p-values determined from ANOVA identified IL-12, IL-9, VEGF, IFN-γ as a molecular signature to build the classification model. This classification model performed superiorly as compared to a model built using features selected using hierarchical cluster analysis or by ranking the accuracies computed from the SVM. Data analyzed by all feature selection methods included all treatment conditions (untreated, vehicle, SA, IE, and PPD) and all of their respective concentrations for N=3 independent replicates.

Feature Selection Method	Metrics	Accuracy	Sensitivity	Specificity
No Feature Selection	All 27 Cytokines from Bioplex	75.0%	67.0%	83.0%
P-Values	IL-12, IL-9, VEGF, IFN-γ	92.0%	92.0%	92.0%
Hierarchical Cluster Analysis	IL-4, IL-9, IL-12, VEGF	91.7%	91.7%	91.7%
SVM Margin Distance	IL-12, IL-9, VEGF, IFN-γ	92.0%	92.0%	92.0%
SVM Accuracy	IL-12, IL-9, VEGF, PDGF	91.3%	90.9%	91.7%

When hierarchical cluster analysis was used to perform feature selection, a panel of cytokines (IL-12, VEGF, IL-4, IL-9) was identified and used to build the SVM classification model. This feature selection method resulted in a model that performed with accuracy of 91.67% and with a sensitivity and specificity score of 91.67%. When feature selection was performed by ranking the margin distances and p-values, the top 4 metrics (IL-12. IL-9, VEGF, and IFN-γ) were identified and utilized to build a SVM classification model. Both feature selection methods led to the best classification performance where the accuracy, sensitivity, and specificity were all 92% (**Table 2.2**). The accuracy value for sensitizer classification was greater when a panel of metrics was used instead of individual cytokine metrics alone (**Table 2.1**). Interestingly, the cytokine panel identified using feature selection also performed better than all other cellular metrics

including CD54, CD86, and chemotaxis to CCL19 which had respective accuracy scores of 72.73%, 81.83%, and 87.5%. Thus, feature selection methods are useful tools to identify potential molecular signatures to build predictive classification models of skin sensitization.

2.4 DISCUSSION

Predictive *in vitro* assays that can accurately identify skin sensitizers and fully replace animal testing are in demand. Although high through-put alternatives such as the peptide binding assays accurately predicted 89% of the 82 sensitizers tested, it failed to accurately identify a class of haptens known as pro-haptens [6]. Pro-haptens require auxiliary chemical reactions to transform the innately inert molecule into a hapten that will bind to peptides in the skin and serve as the antigen that triggers the allergy response. Furthermore, there is evidence that pro-hapten transformation involves Cytochrome p450 enzymes present in the epidermis and dermis [11] [12]. Although peptide binding provides a helpful initial tool for evaluating the sensitization potential of chemicals, other cellular factors such as skin permeation, metabolism, cytokine signaling environment, and dendritic cell activation should also be considered.

To address the need for a metabolic component that is more *in vivo*-like, we developed a co-culture assay system where a full thickness skin model with keratinocytes and fibroblasts known as RealSkin was cultured together with MUTZ-3 derived Langerhan's cells. RealSkin's™' mRNA levels for phase I and phase II enzymes were found to be more similar to excised skin than the epidermal skin model, EpiSkin™. This is thought to occur due to fibroblasts in

RealSkin which modulate the enzymes' expression levels [13]. Dermal fibroblasts also have immunomodulatory properties that can facilitate dendritic cell maturation though soluble signals such as TNF-alpha and direct cell-cell contact [14]. Thus, a full thickness skin model that contains both epidermal keratinocytes and dermal fibroblasts co-cultured with Langerhan's cells can ensure that the dynamic signaling environment across different cell types during sensitization is preserved *in vitro*. The MUTZ-3 cell line is derived from myeloid leukemia and is established as a viable cell source for differentiation into Langerhan's cells or dendritic cells upon exposure to different growth factors and cytokines [15] [16].

In a previous study by Oewehand *et al*, a full thickness skin equivalent model with MUTZ-3 derived Langerhan's included within the epidermis was developed and showed dendritic cell maturation responses upon exposure to skin sensitizers. Here, the LCs were stained for CD83 via immunohistochemistry, IL-1β and CCR7 mRNA were measured, and migration into the dermis of the skin equivalent was observed [17]. This system is uniquely poised to evaluate the induction of LC migration out of sensitized skin and may also be useful in identifying important sensitization metrics which can be ranked in importance using our analysis tools. Thus far secretome analysis was not performed so we are unable to compare the results of their and our systems in identifying important sensitization metrics. In our co-culture assay the LC are not incorporated directly into the skin equivalent. Nevertheless, we are able to take advantage of the many benefits of skin equivalents. These advantages include the ability to use commercially available products to topically apply chemicals,

preservation of the cellular cross talk between keratinocytes, fibroblasts, and LCs, and a source of xenobiotic metabolism for pre/pro-haptens to sensitize the MUTZ-LCs placed below the skin. Thus, with the MUTZ-LCs cultured below the skin compartment, the two components are easily separated and we were able to assess multiple dendritic cell maturation metrics. A small panel of chemicals that include representative pre-/pro-haptens isoeugenol and *p*-phenylenediamine and irritant salicylic acid were selected in this initial study to show proof-of-concept. Others have also reported small chemical panels for initial system characterization [4, 16, 18, 19]. Future studies that incorporate additional chemicals will be necessary to further validate our approach.

Based on the results from our co-culture sensitization platform, we were able to distinguish PPD as a sensitizer relative to vehicle and irritant salicylic acid controls, through CD54 and CD86 surface expression. However, both surface molecules were not elevated for the moderately potent isoeugenol. CD86 and CD54 have been reported as promising surface molecule biomarkers for sensitization with several cell lines including THP-1, U-937, KG-1 and MUTZ-3 [4]. However, an evaluation of CD86 on MUTZ-3 cells using a panel of 20 sensitizers (where PPD and IE were included) and 20 non-sensitizers (where DMSO and SA were included) corroborated our findings where CD86 was upregulated for PPD verses SA and DMSO, but IE was not [7]. Thus, CD86 and CD54 may not be sufficient to accurately predict sensitization as standalone metrics.

In addition to surface molecule expression, the level of IL-8 mRNA or secretion is also a commonly evaluated metric of sensitization among several different cell-based assays. Secreted IL-8 was measured from RealSkin alone and from the co-culture of RealSkin with the MUTZ-LCs. RealSkin alone did not secrete IL-8 in a discriminate fashion for sensitizers as compared to the control treatments. IL-8 secretion from the co-culture of RealSkin and MUTZ-LC was able to correctly identify only PPD as a sensitizer. While reports in literature are generally supportive of IL-8 as a distinguisher of sensitization, there are still several reports with false negatives where a contact sensitizer was mis-classified [20]. Similar to CD54 and CD86, IL-8 expression may be better suited as a metric that is evaluated within a panel of additional cellular markers. In addition to molecular metrics, a trans-well migration assay was used to functionally assess the MUTZ-LC's post-sensitization. A dose response with respect to concentrations of the sensitizer was observed for both IE and PPD. These results indicate that a functional assessment of the sensitized LCs to migrate in response to chemokine CCL19 can be used to distinguish pro-hapten IE and pre-hapten PPD from irritant SA and vehicle control (0.4% DMSO in RPMI medium) in our co-culture system.

Several prominent cellular metrics such as CD54, CD86, and IL-8 performed poorly on correctly identifying the less potent sensitizer, isoeugenol. Although these metrics are useful as an initial screening tool, the need to find more sensitive metrics and assays still persist. Thus, a 27-cytokine screen was performed to assess the secretome from the topical application of sensitizers on

RealSkin alone and on RealSkin co-cultured with MUTZ-LCs. An unsupervised classification tool known as hierarchical cluster analysis was used to analyze the secretome data and identify molecular patterns of sensitization. Based on the results of hierarchical cluster analysis, RealSkin secretome did not show any distinguishing cytokine metrics that could be used to cluster sensitizer IE and PPD relative to non-sensitizers SA and vehicle. However, hierarchical clustering of the co-culture system grouped the vehicle and SA treatments with moderate sensitizer IE branching off and with strong PPD treatments clustering adjacent to the IE treatments. Thus, the cluster pattern for this culture configuration also separates treatments by the potency of sensitizers. This suggests that cytokines secreted through co-culture can discriminate between non-sensitizers and sensitizers more accurately than cytokines secreted from the skin alone. Secreted metrics that were identified as prospective predictors in the co-culture setup for the identification of sensitizers include VEGF, IL-4, IL-9, and IL-12. Interestingly, several of these metrics include cytokines currently implicated during skin sensitization and are involved in the immune regulation of T-cells [21-23].

To further aid in the discovery of sensitization molecular signatures, a supervised classifier known as a support vector machine was used to classify and identify the best secreted metrics that were capable of distinguishing between sensitizers and non-sensitizers. A support vector machine is a powerful machine learning tool that has been extensively applied in the area of pattern recognition and classification. SVM analysis was compared with linear

discriminate analysis (LVA) in screening 130 organic compounds based on 6 molecular descriptors associated with quantitative structure activity relationships (QSAR) based on electrophile-nucleophile reactions that occur during skin sensitization [9]. Based on this study, SVM methods proved to be superior in classifying compounds as non-strong/moderate or strong/moderate sensitizers according to their QSAR properties when these *in silico* results were compared to the Gerberick LLNA database. Additional physicochemical property descriptors were utilized such as hydrophobicity and polarity of the test molecules. However, limited descriptors derived from *in vitro* biological experiments were applied in this study. Since then, genomic data collected post-sensitization from MUTZ-3 cells has also been used to built a classification model with a support vector machine where 89% accuracy was achieved using a 200 gene signature (GARD assay) [7, 24]. Here, we report the novel use of a SVM for sensitizer classification applied to biological activity at the protein level to build a classification model and develop a feature selection tool to compare different biomarkers. Furthermore, the molecular signature that we've identified utilizes four secreted soluble proteins that are easily detectable by ELISA or multi-plex and do not require the more labor intensive nucleic acid based protocols.

By comparing the computed margin distances from the secreted metrics of the RealSkin alone and the co-culture of RealSkin with MUTZ-LCs, the co-culture system showed superior assay performance in the correct prediction of both the moderate pro-hapten isoeugenol and strong pre-hapten PPD. This is likely due to the more *in vivo*-like nature of the co-culture system where the dynamic cellular

interactions between keratinocytes, fibroblasts and Langerhan's cells are preserved. By systematically ranking the margin distances and accuracy values, a panel of cytokines capable of distinguishing sensitizers was identified. This panel includes IL-12, IL-9, VEGF, IFN-γ, IL-4, PDGF, IL-8, IL-7, GM-CSF, IL-6, and IL-17 with the top three metrics with accuracies, sensitivities, and specificities all exceeding 83.33%. By ranking the statistical p-values from ANOVA, we found the same panel of potential cytokine markers.

After identifying cytokines of interest, we explored whether using them as a panel led to greater accuracies in identifying sensitizers than using each secreted metric alone. Using 10-fold cross-validation, it was determined that using a panel of metrics identified independently through cluster analysis, SVM, and ANOVA improved the accuracy, specificity, and sensitivity in correctly identifying PPD and IE as sensitizers better than using a single cellular metric alone. The results indicate that traditional feature selection methods such as ANOVA and hierarchical cluster analysis validate our support vector machine feature selection approach. However, additional benefits of using SVM as a feature selection tool over traditional statistical methods based on filtering significant p-values or cluster analysis are that SVM accounts for dependencies amongst the features, enables quantitative ranking of individual metrics, interacts with the classifier, and affords superior computational complexity [25]. The ability to interact with the classifier is especially important as the classification model is progressively trained with more biological information provided by expanding the panel of chemicals and cellular metrics.

The classifier accuracy, sensitivity, and specificity were all greater than 90% when the best secreted features were selected based on our small panel of sensitizers. This supports the recent notion that a molecular signature or a battery of assays needs to be implemented together to correctly identify potential contact allergens [26, 27]. The true predictive power of our specific molecular signature will need to undergo validation studies with an expanded panel of chemicals. However, the accuracy of our classification model suggests that this identified signature validates our feature selection method by SVM and the need to use a panel of several metrics. Thus, as more chemicals are evaluated, it is feasible that the number of predictive metrics may expand as was the case in the GARD study where initial studies utilized 10 genes and was later expanded to 200 genes [7]. Furthermore, additional sensitizers or varying potencies will need to be evaluated to determine whether the system can accurately predict potencies of chemicals as well as their sensitizing potential.

Our molecular panel includes secreted products that are implicated during antigen presentation such as cytokines IL-12, IFN-γ, and IL-9. VEGF is a potent vasodilator that facilitates lymphocyte infiltration into the skin during an allergy response [28, 29]. Thus, all proteins identified are physiologically relevant to allergic contact dermatitis. Recent literature also indicates IL-18 as an effective predictor of skin sensitization using epidermal equivalents and the NCTC2544 keratinocyte cell line [30, 31]. Although IL-18 was not measured as part of our 27-cytokine Bioplex screen, we did identify IFN-γ as a predictive marker of skin sensitization. Since IL-18 is a potent inducer of IFN-γ production and promoter of

a Th-1 response, it is feasible that there could be some IL-18 release in our co-culture model [32]. However, previous studies with skin equivalents indicate that the accuracy of IL-18 as a biomarker depends on cytotoxicity levels that yield <50% [31]. Nonetheless, future studies could benefit from characterizing IL-18 release and this cellular metric could be compared to the others evaluated in this paper using our SVM selection approach. Also, these studies should include more cytotoxic concentrations as part of the dose response studies to allow for the full predictive potential of IL-18 release as a biomarker.

Although the use of skin equivalents may be more time and resource intensive than submerged cell cultures if they are developed in-house, these inconveniences could be significantly reduced if the skin equivalents are purchased directly through a vendor. Although RealSkin is currently not available for purchase through EpiSkin, there are several full thickness skin equivalents available on market such as MatTek's EpidermFT, and CellSystems' AST2000 [33, 34]. Commercially available epidermal equivalents such as MatTek EpiDerm™, SkinEthic™ EpiSkin, and SkinEthic™ RHE may also be used in a co-culture model [31]. There is evidence that assays that use of epidermal equivalents may be more amenable to inter-laboratory transfers to generate reproducible results than submerged cell lines that are more sensitive to specific culture conditions [31, 35]. Ultimately, it is clear that a single assay and cellular metric of sensitization is insufficient as a stand-alone predictor and tiered strategies using a battery of assays improved the overall accuracy of identifying known skin sensitizers [26, 27]. Thus, we envision this type of co-culture system

to be utilized as a second tier assay following *in silico* or peptide binding screening methods. Unlike studies where a battery of assays with different cell sources, metrics, and specifications are evaluated separately, we envision a more streamlined approach where several key *in vivo* sensitization steps such as permeation, metabolism, keratinocyte activation, and dendritic cell maturation can all be measured within a single assay system.

In conclusion, we established an assay system that utilizes an organotypic skin model co-cultured with differentiated Langerhan's cells from the MUTZ-3 cell line as a potential alternative to animal testing. Furthermore, we describe a novel feature selection method to identify key biomarkers of sensitization using a support vector machine to rank the margin distances from a panel of 27 secreted cellular metrics. Unlike standard dendritic cell based assays, chemicals can be topically applied to the skin model and permeate through the skin to activate the MUTZ-LCs below. Additional benefits of a co-culture system include the metabolic capabilities of the skin model to convert pro- and pre-hapten sensitizers into electrophilic products and the preserved cellular interactions between keratinocytes, fibroblasts, and Langerhan's cells. Based on our comprehensive analysis of multiple cellular metrics, we found that CD54, CD86, and IL-8 may be unreliable markers to identify potential skin sensitizers in our system. However, using a multi-plex screen combined with unsupervised and supervised classification tools, we identified a molecular pattern of cytokines that were expressed after sensitization. Furthermore, the support vector machine was utilized as a tool to systematically rank these cytokines of interest and select for

the best panel of sensitization biomarkers in our system. This signature that includes IL-12, IL-9, IFN-γ, and VEGF showed greater accuracy, sensitivity, and specificity than using a single secreted metric. Future studies will focus on evaluating an expanded panel of sensitizers with wider ranges of dosages to further optimize and validate our culture approach and molecular signature. The support vector machine feature selection method and classification model we have developed can be continually expanded to compare cellular metrics obtained by our laboratory and by others in training the classification model to predict sensitizers and their respective sensitizer potencies.

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CHAPTER 3: CO-CULTURE OF KERATINOCYTES, FIBROBLASTS, AND LANGERHAN'S CELLS IMPROVES SENSITIZATION PREDICTION

3.1 INTRODUCTION

Allergic contact dermatitis is an inflammatory skin disease that is a rising public health concern due to its growing prevalence. To assess the public's propensity for contact dermatitis, many alternatives to *in vivo* screening of chemicals have been developed. However, these systems are limited by their poor ability to accurately identify a subset of contact sensitizers known as pro-haptens that require metabolic activation to serve as the antigen. Pro-hapten sensitizers are innately un-reactive until they are bio-transformed by enzymes in the skin to form sensitizing intermediates and products. Thus, for the accurate identification of pro-hapten sensitizers, a metabolic component must be incorporated with the screening tool.

Several strategies to introduce a metabolic component to transform pre– and pro-hapten sensitizers were previously investigated in literature using Cytochrome p450 enzyme cocktails, liver microsomes, and keratinocyte cell lines [1-3]. A skin-like cocktail of CYP enzymes (CYP1A1, CYP1B1, CYP2B6, CYP2E1, and CYP3A5) was used to metabolize pro-hapten dienes, and metabolism of these substrates was confirmed using LC-MS [3]. The products of this reaction were added to peripheral blood mononuclear derived dendritic cells, and elevated IL-8 mRNA was measured to confirm sensitization. While this method shows great promise, the CYP enzymes in this study were enriched a

1000-fold and may not be fully representative of the *in vivo* content and activity of these enzymes [4].

Another alternative source of metabolism previously explored utilized human liver microsomes co-incubated with THP-1 cells to facilitate the metabolism of pro-haptens benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, carvone oxime, cinnamic alcohol, and isoeugenol [1]. THP-1 cells that were co-incubated with the microsomal enzymes had greater CD54 and CD86 expression as compared with control cells that were not incubated with microsomes. Thus, liver microsomes derived from hepatocyte's endoplasmic reticulum where CYP enzymes are concentrated could be a viable source of metabolism that is both cost effective and amenable for high throughput screens based on their extensive use by the pharmaceutical industry to perform drug metabolism and pharmacokinetic studies [5]. However, there are tissue specific discrepancies in the panel of Cytochrome P450 enzymes and their respective activity levels between skin and liver derived microsomes [6, 7]. Thus, a more physiologically relevant source of enzymes that are involved during sensitization such as keratinocytes and fibroblast cell cultures may be a suitable, cost effective alternative. In a previous study, co-culture of HaCaT keratinocytes and THP-1 cells was able to improve the detection of pro-haptens using CD86 expression as an end point metric [2].

Keratinocytes (KCs) in the epidermis are primarily responsible for carrying out xenobiotic metabolism in the skin. An immortalized KC line, HaCaT cells express several key phase I enzymes such as CYP1A1, CYP1 B1, CYP3A, and

COX [6, 7]. CYP1A1 is particularly important in generating reactive intermediates from pro-haptens such as eugenol, isoeugenol, and geraniol [8-11]. KCs also produce several inflammatory mediators such as members of the IL-1 family, TNF-α, IL-6, IL-18 and GM-CSF during ACD. These factors orchestrate various events during the sensitization phase such as the migration and maturation LCs, the recruitment of T-lymphocytes, and accessory lymphocytes such as natural killer cells, macrophages, and neutrophils [12-14]. Fibroblasts in the dermis also play an important role by secreting chemokines such as CXCL12 and cytokines such as TNF-α that further direct LC migration and maturation during sensitization [15, 16]. Fibroblasts may also regulate the expression of Cytochrome P450 enzymes in keratinocytes by secreted factors [17]. Thus, a co-culture approach that includes both of skin cell types may confer additional benefits as compared with the use of keratinocytes alone.

In a previous study described in Chapter 2, a full thickness skin model co-cultured with Mutz-3 derived Langerhan's cells was assessed as an *in vitro* model to identify pro-hapten isoeugenol and pre-hapten *p*-phenylenediamine. Although the preliminary results were promising, there are several limitations to this approach. The use of a full thickness skin equivalent can be resource intensive from a labor and cost perspective. This can potentially limit its ability to perform high-throughput screening that is necessary to evaluate the sensitizer potential of numerous compounds. At this present time, there are no skin models that have been validated for sensitization testing. However, there are several models that have been deemed acceptable for irritant screening by the European

Commision. These models include three epidermal equivalent models from RHE: SkinEthic, EpiDerm, and Episkin. The Episkin model is available for purchase in a 12-well format at an estimated cost of ~$450/test kit/chemical [18]. It is estimated that nearly 20,000 new compounds will require sensitization information by 2018, according to the guidelines placed by REACH, the European community regulation on chemicals and their safe use [19]. A basic cost analysis for this class of new chemicals entering the market yields 20,000 x ~$450 = ~$9 million for just the cost of skin alone. This high cost and limited availability of reconstructed skin models are largely due to lengthy protocols that are labor intensive and performed by trained personnel. Thus, an alternative method that preserves the benefits of keratinocyte and fibroblast's presence in co-culture with MUTZ-LCs was explored in this chapter. In parallel, MUTZ-LCs were cultured alone to assess if these cells were capable of identifying an expanded panel of pre/pro-haptens. Previous studies with MUTZ-3 cells in the GARD assay and the CXCL12 migration assay indicated that this cell line can be used for identifying pre/pro-haptens [20-23].

3.2 MATERIALS AND METHODS

Cell culture

The HaCaT keratinocyte (KC) cell line was a donation from Dr. Bozena Michniak-Kohn. Primary dermal fibroblasts (FB) isolated from foreskin were a donation from Dr. Francois Berthiaume. Both HaCaT KCs and FBs were maintained in DMEM (Gibco) with 10% FBS and 100 U/mL Penicillin, 100ug/mL streptomycin supplementation.

The 5637 urinary bladder carcinoma cell line was purchased from ATCC (Manassas, Va.). The 5637 cell line was maintained at 37^{o}C and 5% CO2 in RPMI medium supplemented with 10% FBS, 2% L-glutamine, and 1% penicillin-streptomycin. Media was changed every other day and conditioned medium was collected when the cells were 90% confluent. This conditioned medium was supplemented into the MUTZ-3 culture medium as per the guidelines from DSMZ.

The MUTZ-3 cells were a donation from L'Oreal (Paris, France) and are available for purchase from DSMZ (Braunschweig, Germany). The MUTZ-3 cell line was maintained at 37^{o}C and 5% CO2 in alpha-MEM medium with Glutamax, ribonucleosides, and deoxyribonucleosides (Invitrogen) supplemented with 20% heat inactivated FBS, 10% 5637 conditioned medium, 1% Penicillin-streptomycin, and 50uM2-mercaptoethanol. Media was changed every other day and the cells were split on day five of culture. To differentiate the MUTZ-3 to LCs, cells were cultured for 7 days using 2.5 ng/mL TNF-α, 10ng/mL TGF-B1, and 100ng/mL GM-CSF. On day 2 and day 5, fresh cytokines were added at half those concentrations. Differentiated LCs utilized in experiments were maintained in complete MUTZ-3 media described above without the 5637 conditioned medium. This medium will be described as the Maturation Medium in future sections.

Chemicals and Reagents

Test chemicals included both non-sensitizers and known skin sensitizers that are pre-/pro-haptens (**Table 3.1**). Non-sensitizing chemicals included the

vehicle (0.1% Dimethylsulfoxide) and 2 skin irritants, salicylic acid (SA) and sodium dodecyl sulfate (SDS). A panel of 9 sensitizers of varying potencies and classes outlined in Table 3.1 were evaluated. This panel includes eugenol (EU), gerionol (GER), cinnamic alcohol (CA), isoeugenol (IE), Cinnamaldehyde (CLD), 2-Aminophenol (2AP), Hydroquinone (HQ), p-phenylenediamine (PPD), and dinitrochlorobenzene (DNCB). All chemicals are purchased through Sigma-Aldrich and prepared in dimethylsulfoxide (DMSO) where the final concentration of DMSO in cell culture during treatment was 0.1%. Concentration ranges were based on values commonly reported in a literature review of these chemicals.

Table 3. 1Panel of chemicals evaluated.

Chemical	Abbreviation	Chemical Type	Potency	Concentration (µM)
Sensitizers				
Dinitrochlorobenzene	DNCB	Hapten	Extreme	6.25
2-Aminophenol	2AP	Pre-/pro-hapten	Strong	100
Hydroquinone	HQ	Pre-/pro-hapten	Strong	25
p-Phenylenediamine	PPD	Pre-/pro-hapten	Strong	62.5
Cinnamaldehyde	CLD	Hapten	Moderate	125
Isoeugenol	IE	Pre-/pro-hapten	Moderate	250
Cinnamic Alcohol	CA	Pre-/pro-hapten	Weak	500
Eugenol	EU	Pre-/pro-hapten	Weak	250
Gerianol	GER	Pre-/pro-hapten	Weak	500
Non-Sensitizers				
Dimethylsulfoxide	DMSO			0.10%
Salicylic Acid	SA	Irritant	-	500
Sodium Dodecyl Sulfate	SDS	Irritant	-	250

Co-Culture of HaCaT Keratinocytes, Dermal Fibroblasts, and MUTZ-3 Langerhan's Cells

HaCaT keratinocyte cells (1.25×10^5) and human dermal foreskin fibroblasts (1.25×10^5) were plated 24-hours into 96-well plates before the start of the experiment in the complete DMEM medium described above. After the

cells attached to the bottom of the plate, the wells were washed with the Maturation culture medium. Fully differentiated (day 7) MUTZ-LCs ($2.5x10^5$) was added to the wells in 220 µL of Maturation culture medium with the chemical treatment at the listed concentration for 48 hours to induce sensitization. After this incubation period, the supernatant was collected, stored at -20C, and thawed prior to performing the Multi-plex analysis.

Cytokine Multi-plex Analysis

Supernatant was collected after treating MUTZ-LCs alone and HaCaT KCs and FBs co-cultured with MUTZ-LCs with a panel of non-sensitizers and sensitizers. The supernatants were then analyzed for 27 human cytokines, chemokines, and growth factors (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCAF, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF) using a Bioplex Assay following the manufacturer's instructions. (Bio-Plex Human Cytokine 27-plex panel; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

A total of N=4 independent, biological replicates were analyzed by support vector machine to determine the margin distances and develop the classification models. Statistical significance was determined at $p \leq 0.05$ by Student's T-test in Kaleidagraph.

Support Vector Machine as a Feature Selection Method

In the previous chapter, we described a novel method of performing feature selection by support vector machine using the calculated margin

distances between 2 classes of chemicals: non-sensitizer control treatments and sensitizer treatments. For each cytokine, the training data is given by $\{x_i, y_i\}$, $i = 1 \ldots l$, $y_i \in \{-1,1\}$, where x denotes the cytokine concentration normalized to vehicle and y corresponds to the label of the chemical (-1 for non-sensitizers and 1 for sensitizers). For potency evaluation, the labels were changed to indicate -1 for non-sensitizers and 1 for weak/moderate sensitizer or strong sensitizer. Factors that were unable to be normalized to the vehicle or below the detection threshold of the Bioplex assay were excluded. Using a linear kernel, the points that lie on the hyperplane that separates the positives and negatives are $w \cdot x + b = 0$, which leads to the following constraints to the training data:

$$x_i \cdot w + b \geq +1 \; for \; y_i = +1 \qquad (1)$$

$$x_i \cdot w + b \leq -1 \; for \; y_i = -1 \qquad (2)$$

where w is the norm to the hyperplane and $|b|/||w||$ is the perpendicular distance from the hyperplane to the origin, and $||w||$ is the Eucledian norm of w. From inequality (1), the perpendicular distance of the hyperplane to the origin is found to be $|1 - b|/||w||$. Similarly, the distance of the hyperplane in (2) to the origin is $|-1 - b|/||w||$. Therefore the margin distance between these two hyperplanes is $2/||w||$. Thus we can find a pair of hyperplanes that best separates the two classes by minimizing $||w||$ subject to the constraints (1) and (2). This problem is solved using quadratic programming provided by the bioinformatics toolbox in Matlab. We used the aforementioned method to compute the margin distance 2/||w| for each cytokine. These margin distances and classification accuracy values were subsequently ranked to identify metrics that had the greatest

distance of separation between the non-sensitizer (vehicle, SA, SDS) and sensitizer treated classes (EU, GER, CA, CLD, IE, 2AP, HQ,PPD, DNCB).

Development of a Classification Model

A support vector machine was used to develop classification models in MATLAB based on the identified secreted metrics from ranking margin distances to predict sensitization for each culture configuration (co-culture model and MUTZ-LC model). The classifier's performance (accuracy, sensitivity, and specificity) was assessed using 5-fold cross validation for each individual cytokine metric and molecular signature identified through various feature selection methods described above. This method trains the data with 80% of randomly selected data and tests the data with the remaining 20% in an iterative fashion 5 times.

Principal Component Analysis

Principal component analysis (PCA) is a multi-variate statistical tool that reduces dimensionality of datasets in a manner that preserves the information of the original data. Dimensions are reduced down to their principal components that are calculated from an orthogonal transformation that converts correlated variables into a new set of uncorrelated variables. These principal components are ordered in terms of the variance such that the first principal component contains the greatest amount of variance from the original data set. Since we utilized the SVM as a feature selection method to reduce the dimensionality of our data, PCA was used primarily as a visualization tool using the identified

molecular signatures to generate scatter plots along the data's principal components. PCA was performed using the statistical toolbox in MATLAB.

3.3 RESULTS

Feature Selection

The support vector machine was utilized as a feature selection method. A greater margin distance computed between the two classes of chemicals indicated a greater degree of separation for any given secreted factor. Based on this information, we were able to rank each secreted metric based on its ability to distinguish between the non-sensitizing controls and the sensitizer treatments and select the key features necessary to produce an accurate prediction. The margin distances of the top ranked secretome cytokines collected through the MUTZ-LCs alone in ranked order were MIP-1β, MIP-1α, IL-8, RANTES, and IL-9. **(Table 3.2)**. Each metric alone in this panel had accuracies that did not exceed 75%.

The margin distances of the top ranked secretome cytokines collected through the co-culture system with HaCaT KCs, FBs, and MUTZ-LCs in ranked order are IL-8, GM-CSF, RANTES, MIP-1β, and MIP-1α **(Table 3.3)**. The greatest accuracy score out of all the metrics evaluated in the co-culture was for IL-8 with ~80%. The margin distance and accuracy for the top ranked cytokine from the co-culture assay was greater than any of the margin distances from the MUTZ-LC secretome indicating a greater degree of separation of the two chemical classes in the co-culture configuration.

Classification Model for Distinguishing Sensitizers from Non-Sensitizers

Classification models for identifying sensitizers was developed using both individual metrics and using a panel of metrics that was identified by ranking the margin distances. The identified top metrics identified in the MUTZ-LC's secretome showed accuracies that did not exceed 75% when a single biomarker was assessed. However, IL-8 secretion in the co-culture setting was identified as the best individual metric overall with the greatest margin distance, accuracy (80%), sensitivity (91.7%), and specificity (77.8%) based on 5-fold cross validation (**Table 3.4**). Although IL-8 secretion in the co-culture showed an accuracy of 80%, we were interested in evaluating whether the use of a panel of biomarkers can improve the classifier performance as we observed in Chapter 2. Based on the results of the feature selection method, we compared different combinations of the identified features to build a predictive classification model. When a predictive model was developed using a signature of the top 5 metrics (MIP-1β, MIP-1α, IL-8, RANTES, and IL-9) identified in the MUTZ-LC secretome, the classifier performance increased to 83.3% (**Table 3.3**). Including additional metrics beyond the top 5 ranked cytokines did not improve the classifier performance. When a classification model was built using the selected features from the co-culture system, the combination of the top three cytokines (IL-8, MIP-1β, RANTES) provided the best prediction with accuracy of 89.6% (**Table 3.5**).

Table 3. 2 The performance of classification models predicting skin sensitization using secreted metrics from MUTZ-LCS identified through SVM feature selection. The margin distances quantified from the support vector machine ranked, from greatest to least distance of separation from a

Bioplex screen using supernatant from Mutz-3 derived Langerhan's cells. The accuracy, sensitivity, and specificity of each metric were determined by 5-fold cross validation. Data analyzed by the SVM included non-sensitizers conditions (DMSO, SA, SDS) and sensitizers (EU, GER, CA, IE, CLD, 2AP, PPD, HQ, DNCB) for N=4 independent replicates.

Rank	Metric	Margin Distance	Accuracy	Sensitivity	Specificity
1	MIP-1β	0.18273	65.0%	91.7%	58.3%
2	MIP-1α	0.15715	61.0%	83.3%	52.8%
3	IL8	0.15665	71.0%	83.3%	66.7%
4	RANTES	0.15438	65.0%	100.0%	47.2%
5	IL-9	0.15128	60.0%	83.3%	55.6%
6	IL-17	0.15071	75.0%	91.7%	69.4%
7	MCP-1(MCAF)	0.14815	48.0%	32.4%	92.9%
8	IL-15	0.14185	53.0%	83.3%	44.4%
9	IL-1b	0.14	70.0%	83.3%	58.3%
10	IL-6	0.13386	48.0%	83.3%	47.2%
11	VEGF	0.13183	69.0%	75.0%	63.9%
12	IL-13	0.13063	54.0%	83.3%	50.0%
13	IL-1ra	0.12957	65.0%	66.7%	61.1%
14	IL-4	0.12811	54.0%	66.7%	52.8%
15	IL-7	0.12733	53.0%	75.0%	50.0%
16	IL-12(p70)	0.12731	59.0%	66.7%	50.0%
17	TNF-a	0.12723	40.0%	25.0%	27.8%
18	PDGF-bb	0.12709	39.0%	50.0%	44.4%
19	IL-10	0.12682	46.0%	58.3%	41.7%
20	IFN-γ	0.12667	42.0%	41.7%	38.9%
21	IP-10	0.12651	55.0%	33.3%	33.3%
22	FGF basic	0.1265	47.0%	25.0%	36.1%
23	G-CSF	0.12638	65.0%	75.0%	52.8%
24	Eotaxin	0.12634	36.0%	41.7%	38.9%

Table 3. 3 The performance of classification models predicting skin sensitization using secreted metrics from MUTZ-LCS identified through SVM feature selection. Classification performance in terms of accuracy, sensitivity, and specificity was determined by 5-fold cross validation.

Performing feature selection by ranking the margin distances from the SVM identified MIP-1β, MIP-1α, IL-8, RANTES, and IL-9 as a predictive molecular signature to build the classification model. This classification model performed superiorly as compared to a model built using the MIP-1β alone or in combination with the top 4 ranked metrics. Using these 5 metrics, an overall accuracy of 83.3% was observed. Data analyzed by the SVM to develop these classification models included non-sensitizers conditions (DMSO, SA, SDS) and sensitizers (EU, GER, CA, IE, CLD, 2AP, PPD, HQ, DNCB) for N=4 independent replicates.

Metrics	Accuracy	Sensitivity	Specificity
MIP-1B	66.7%	100.0%	50.0%
MIP-1B, MIP-1α	75.0%	100.0%	66.7%
MIP-1B, MIP-1α, IL-8	75.0%	100.0%	66.7%
MIP-1B, MIP-1α, IL-8, RANTES	80.0%	100.0%	75.0%
MIP-1B, MIP-1α, IL-8, RANTES, IL-9	**83.3%**	**100.0%**	**75.0%**

Table 3. 4 Margin distances of Cellular Metrics from HaCaT KC, dermal FB, and MUTZ-LC co-culture secretome. The margin distances quantified from the support vector machine ranked from greatest to least distance of separation from the co-culture are shown. The accuracy, sensitivity, and specificity of each metric were determined by 5-fold cross validation. Data analyzed by the SVM included non-sensitizers conditions (DMSO, SA, SDS) and sensitizers (EU, GER, CA, IE, CLD, 2AP, PPD, HQ, DNCB) for N=4 independent replicates.

Rank	Metric	Margin Distance	Accuracy	Sensitivity	Specificity
1	IL8	0.18698	79.0%	91.7%	77.8%
2	GM-CSF	0.16002	67.0%	91.7%	58.3%
3	RANTES	0.14471	63.0%	91.7%	50.0%
4	MIP-1β	0.14302	58.0%	91.7%	47.2%
5	MIP-1α	0.13638	58.0%	83.3%	50.0%
6	MCP-1	0.13581	41.0%	91.7%	19.4%
7	IL-4	0.12976	61.0%	66.7%	55.6%
8	TNF-a	0.12971	75.0%	75.0%	63.9%
9	G-CSF	0.12912	40.0%	83.3%	36.1%
10	IL-6	0.12891	37.0%	83.3%	22.2%
11	IL-1ra	0.12873	67.0%	66.7%	61.1%
12	IL-15	0.12867	61.0%	66.7%	52.8%
13	IL-17	0.12836	43.0%	75.0%	38.9%
14	IP-10	0.12727	49.0%	91.7%	22.2%
15	IL-13	0.12721	52.0%	91.7%	33.3%
16	VEGF	0.12706	52.0%	91.7%	33.3%
17	IL-1b	0.12684	50.0%	41.7%	38.9%
18	Eotaxin	0.12679	67.0%	33.3%	66.7%
19	IFN-γ	0.12644	67.0%	50.0%	47.2%
20	PDGF-bb	0.12622	14.0%	60.0%	40.0%

Table 3. 5 The performance of classification models predicting skin sensitization using secreted metrics from HaCaT KC, dermal FB, and MUTZ-LC co-culture identified through SVM feature selection. Classification performance in terms of accuracy, sensitivity, and specificity was determined by 5-fold cross validation. Feature selection identified IL-8, GM-CSF, RANTES, and MIP-1β as a predictive molecular signature for the classification model. This classification model performed superiorly as compared to a model built using each of the metrics alone. Using these 3 or 4 metrics in combination, an overall accuracy of 89.6% was observed.

Data analyzed build these classification models included all treatment conditions for N=4 independent replicates.

Metrics	Accuracy	Sensitivity	Specificity
IL-8	79.2%	91.7%	75.0%
IL-8, GM-CSF	83.3%	83.3%	83.3%
IL-8, GM-CSF, RANTES	**89.6%**	**83.3%**	**91.7%**
IL-8, GM-CSF, RANTES, MIP-1β	**89.6%**	**83.3%**	**91.7%**
IL-8, GM-CSF, RANTES, MIP-1β, MIP-1α	83.3%	83.3%	83.3%

Potency Analysis

To identify secretome metrics that are sensitive to weak and moderate sensitizers, SVM analysis was performed using only non-sensitizers (DMSO, SA, SDS) and weak to moderate sensitizers (EU, GER, CA, CLD, IE) from both the co-culture and MUTZ-LC's secretome. SVM analysis of the MUTZ-LCs treated with weak to moderate strength potency showed that MIP-1β was the most predictive metric. However, the accuracy of this metric was very poor (59%) due to the low specificity rate (**Table 3.6**). Using a panel of the subsequently ranked metrics did not improve the performance of this classification model based on MUTZ-LC data. SVM analysis of the MUTZ-LCs treated with strong sensitizers, however, showed the best separation between non-sensitizers and strong sensitizers when using a panel of 4 top ranked metrics (MIP-1β, MIP-1α, IL-17, and IL-8). In this classification model, the accuracy was 92.9% (**Table 3.6**). Thus, MUTZ-LCs alone treated with strong sensitizers is best suited for identifying strongly sensitizing pro-hapten chemicals.

Table 3. 6 Classification models using metrics that are predictive of weak to moderate sensitizers in the MUTZ-LC secretome. Classification performance in terms of accuracy, sensitivity, and specificity was

determined by 5-fold cross validation. Performing feature selection by ranking the margin distances from the SVM identified MIP-1β, RANTES, IL-8, MCP-1, and IL-9. However, all classification models generated showed poor performance rates and increasing the panel size did not improve accuracy. Data utilized to build these classification models included all non-sensitizer treatment conditions (DMSO, SA, SDS), weak sensitizers (EU, GER, CA), and moderate sensitizers (CLD and IE) for N=4 independent replicates.

Metrics	Accuracy	Sensitivity	Specificity
MIP-1β	**59.4%**	**91.7%**	**40.0%**
MIP-1β, RANTES	53.1%	83.3%	35.0%
MIP-1β, RANTES, IL-8	**59.4%**	**83.3%**	**45.0%**
MIP-1β, RANTES, IL-8, MCP-1	56.3%	83.3%	40.0%
MIP-1β, RANTES, IL-8, MCP-1, IL-9	59.4%	83.3%	45.0%

Table 3. 7 Classification models using metrics that are predictive of strong sensitizers in the MUTZ-LC secretome. Classification performance in terms of accuracy, sensitivity, and specificity was determined by 5-fold cross validation. Performing feature selection by ranking the margin distances from the SVM identified MIP-1β, MIP-1α, IL-17, IL-8, and IL-1β. Using the top 4 or 5 metrics yield the same classification performance with high degree of accuracy, sensitivity, and specificity. Data utilized to build these classification models included all non-sensitizer treatment conditions (DMSO, SA, SDS) and strong sensitizers (2AP, HQ, PPD, DNCB) for N=4 independent replicates.

Metrics	Accuracy	Sensitivity	Specificity
MIP-1β	82.1%	83.3%	81.3%
MIP-1β, MIP-1α	85.7%	83.3%	87.5%
MIP-1β, MIP-1α, IL-17	85.7%	83.3%	87.5%
MIP-1β, MIP-1α, IL-17, IL-8	**92.9%**	**91.7%**	**93.8%**
MIP-1β, MIP-1α, IL-17, IL-8, IL-1b	**92.9%**	**91.7%**	**93.8%**

To identify secretome metrics that are prominent in identifying weak sensitizers in the co-culture secretome, SVM analysis was performed to rank the margin distances and develop predictive models based on the identified metrics. In table 3.8, using a panel of IL-8, RANTES, and GM-CSF led to an accuracy of 84.4% in identifying weak to moderate strength sensitizers from non-sensitizers. This demonstrates that the co-culture assay is more predictive at identifying pro-hapten chemicals that are weakly sensitizing than the use of MUTZ-LCs alone. When SVM analysis was performed to develop a classification model to distinguish strong sensitizers from non-sensitizers using co-culture data, IL-8, GM-CSF, and RANTES used together were found to be 92.9% accurate (T**able 3.9**).

Table 3. 8 Classification models that are predictive of weak and moderate sensitizers in the co-culture secretome. **Classification performance in terms of accuracy, sensitivity, and specificity was determined by 5-fold cross validation. Performing feature selection by ranking the margin distances from the SVM identified IL-8, RANTES, GM-CSF, MIP-1β, and MIP-1α. The use of the top 3 (IL-8, RANTES, and GM-CSF) developed the highest performing classification model with an accuracy of 84.4%. Data utilized to build these classification models included all non-sensitizer treatment**

conditions (DMSO, SA, SDS), weak sensitizers (EU, GER, CA), and moderate sensitizers (CLD and IE) for N=4 independent replicates.

Metrics	Accuracy	Sensitivity	Specificity
IL-8	68.8%	83.3%	60.0%
IL-8, RANTES	78.1%	91.7%	70.0%
IL-8, RANTES, GM-CSF	**84.4%**	**91.7%**	**80.0%**
IL-8, RANTES, GM-CSF, MIP-1β	75.0%	75.0%	75.0%
L-8, GM-CSF, MIP-1β, MIP-1α, RANTES	78.1%	75.0%	80.0%

Table 3. 9 Classification models that are predictive of strong sensitizers in the co-culture secretome. Classification performance in terms of accuracy, sensitivity, and specificity was determined by 5-fold cross validation. Performing feature selection by ranking the margin distances from the SVM identified IL-8, GM-CSF, MIP-1β, MIP-1α, and RANTES. The use of the top 2 (IL-8 and GM-CSF) or the top 4 (IL-8, GM-CSF, MIP-1β, and MIP-1α) developed the highest performing classification model with an accuracy of 92.9%. Data utilized to build these classification models included all non-sensitizer treatment conditions (DMSO, SA, SDS) and strong sensitizers (2AP, HQ, PPD, DNCB) for N=4 independent replicates.

Metrics	Accuracy	Sensitivity	Specificity
IL-8	89.3%	91.7%	87.5%
IL-8, GM-CSF	**92.9%**	**83.3%**	**100.0%**
IL-8, GM-CSF, MIP-1β	89.3%	83.3%	93.8%
IL-8, GM-CSF, MIP-1β, MIP-1α	92.9%	83.3%	100.0%
IL-8, GM-CSF, MIP-1β, MIP-1α, RANTES	89.3%	83.3%	93.8%

Principal Component Analysis

PCA was utilized as a visualization tool of the data using the identified features that were used to build a classification model and to demonstrate separation of the chemicals classes by plotting them against their principal

components. In **Figure 3.1** a 3-Dimensional scatter plot is shown for the top 5 metrics found in the MUTZ-LC secretome using the first three principal components. The separation of the non-sensitizers (in circles) and sensitizers (in squares) can be best seen along the first principal component where the greatest variation in the data is present, as confirmed by the associated Scree plot. This scatter plot shows poor separation between non-sensitizers and weakly sensitizing chemicals. However, strong sensitizers (indicated by yellow, orange, and red) separate away from this cluster of non-sensitizers and weak sensitizers.

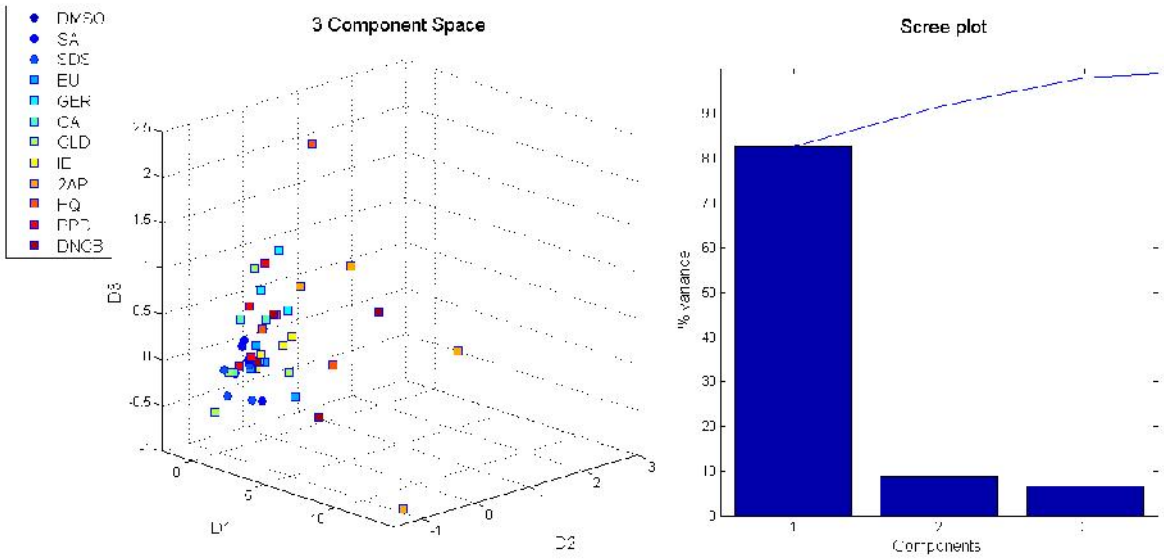

Figure 3. 1 A 3-dimensional visualization of PCA on data from the MUTZ-LC secretome using the top 5 ranked metrics (MIP-1β, MIP-1α, IL-8, RANTES, IL-9) for distinguishing sensitizers from non-sensitizers. The non-sensitizers (DMSO, SA, SDS) are shown as blue circles and the sensitizers are marked as squares. Sensitizers with weaker potencies are shown in light blues, green, and yellow (EU, GER, CA, CLD, IE). Strong sensitizers

(2AP, HQ, PPD, DNCB) are represented in orange and red hues. The scree plot on the right shows that most of the separation of the 2 chemical classes (the variance) is contained along the axis of the first principal component. Using Mutz-LCs alone with MIP-1β, MIP-1α, IL-8, RANTES, and IL-9, there is separation between strongly sensitizing chemicals and weakly sensitizing chemicals. However, non-sensitizers did not separate clearly from weak sensitizers as our classification model indicated.

In **Figure 3.2**, PCA visualization of the data for the top three cytokines identified in the co-culture can be observed. The greatest degree of separation of non-sensitizers and sensitizers can be seen along the first and second principal components. Here, the non-sensitizers appear grouped together tightly with most of the weak sensitizers shown just at the periphery of this cluster. Strong sensitizers are shown distal to the non-sensitizers and weak sensitizers along principal component 1 and 2.

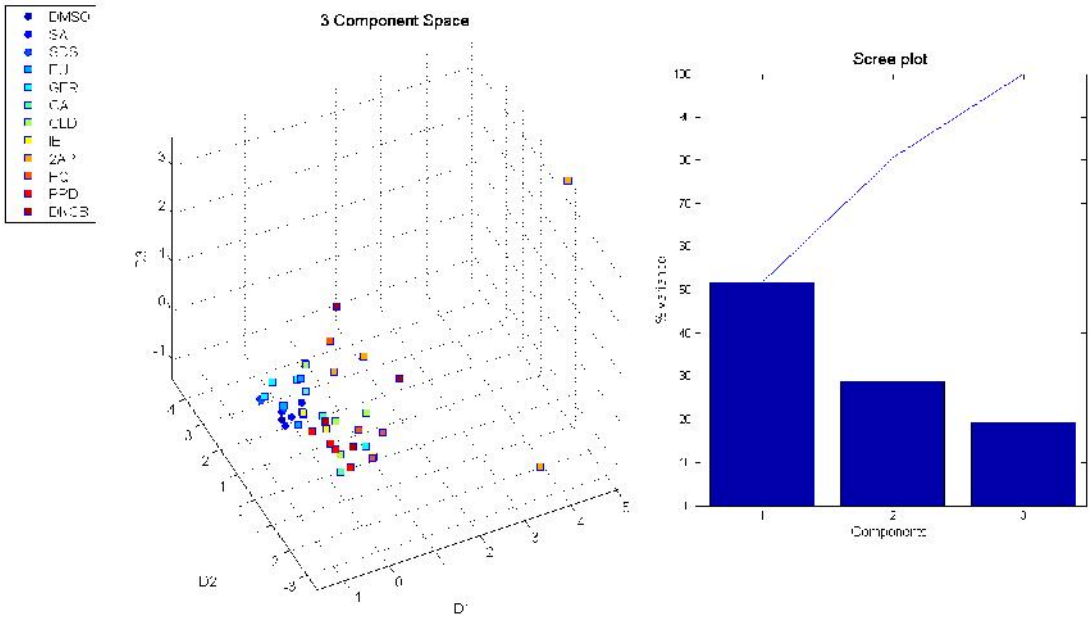

Figure 3. 2 A 3-dimensional visualization of PCA on data from the KC/FB-LC secretome using the top 3 ranked metrics (IL-8, GM-CSF, and RANTES). The non-sensitizers are shown as circles and the sensitizers are marked as squares. Sensitizers with weaker potencies (EU, GER, CA, CLD, IE) are shown in blues, greens, and yellow. Stronger sensitizers (2AP, HQ, PPD, DNCB) are shown in the orange to red color range. The scree plot shows that most of the separation of the two chemical classes (the variance) is along the first and second principal component.

In **Figure 3.3A**, PCA visualization of the data for the top 3 cytokines (MIP-1β, RANTES, IL-8) that best separate non-sensitizers from weak/moderate sensitizers with MUTZ-LCs can be observed. Here, the non-sensitizers appear to separate poorly from the weak sensitizers. This visually confirms that the MUTZ-LCs alone is potentially a poor predictor of weak pro-hapten sensitizers using this panel of identified metrics. In **Figure 3.3B**, PCA visualization of the data for the top 3 cytokines that best separate non-sensitizers from weak/moderate sensitizers with the co-culture can be observed. Unlike the MUTZ-LCs, there appears to be better separation between the two classes that confirms the classification performance data. However, there are still some overlapping regions between the two classes.

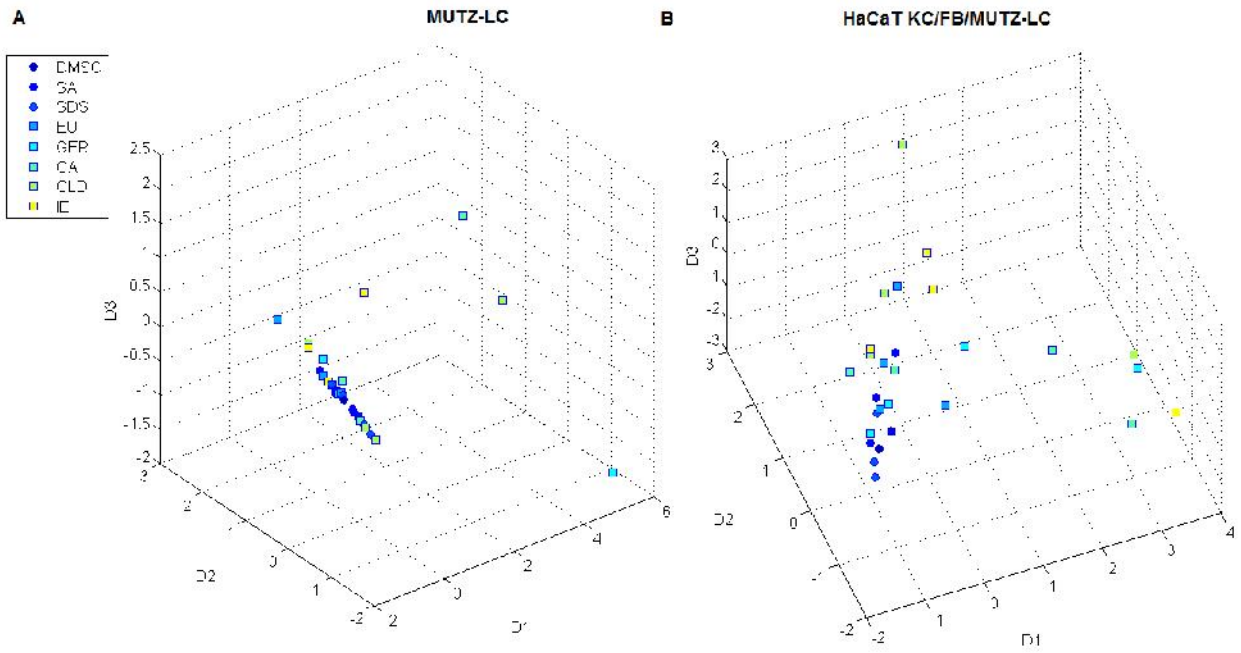

Figure 3. 3 PCA visualization of PCA of non-sensitizers vs. weak/moderate sensitizers for both A) MUTZ-LCs and B) HaCaT keratinocyte, fibroblasts, and MUTZ-LC Co-culture. The non-sensitizers (DMSO, SA, SDS) are indicated by blue circles and the weak/moderate sensitizers (EU, GER, CA, CLD, IE) are indicated by light blue, green, and yellow. The metrics used to generate the MUTZ-LC figure were MIP-1β, RANTES, and IL-8 (A). The signature used to generate the co-culture figure includes IL-8, GM-CSF, and RANTES (B). Both plots were generated using data from N=4 independent replicates.

Feature selection was also performed for both culture conditions to identify metrics that best distinguish between non-sensitizers and strong sensitizers as well. **Figure 3.4A** represents the use of the top 5 metrics identified from the MUTZ-LC cultures with 3 principal components. There is a separation of non-sensitizers (blue circles) from strong sensitizers (orange and red squares) that is

in agreement with the classification performance data where 92.9% accuracy was achieved. **Figure 3.4B** represents the use of the top 3 metrics identified from the MUTZ-LC cultures with 3 principal components. Non-sensitizing treatments (blue circles) cluster closely together away from strongly sensitizing treatment groups (orange and red squares).

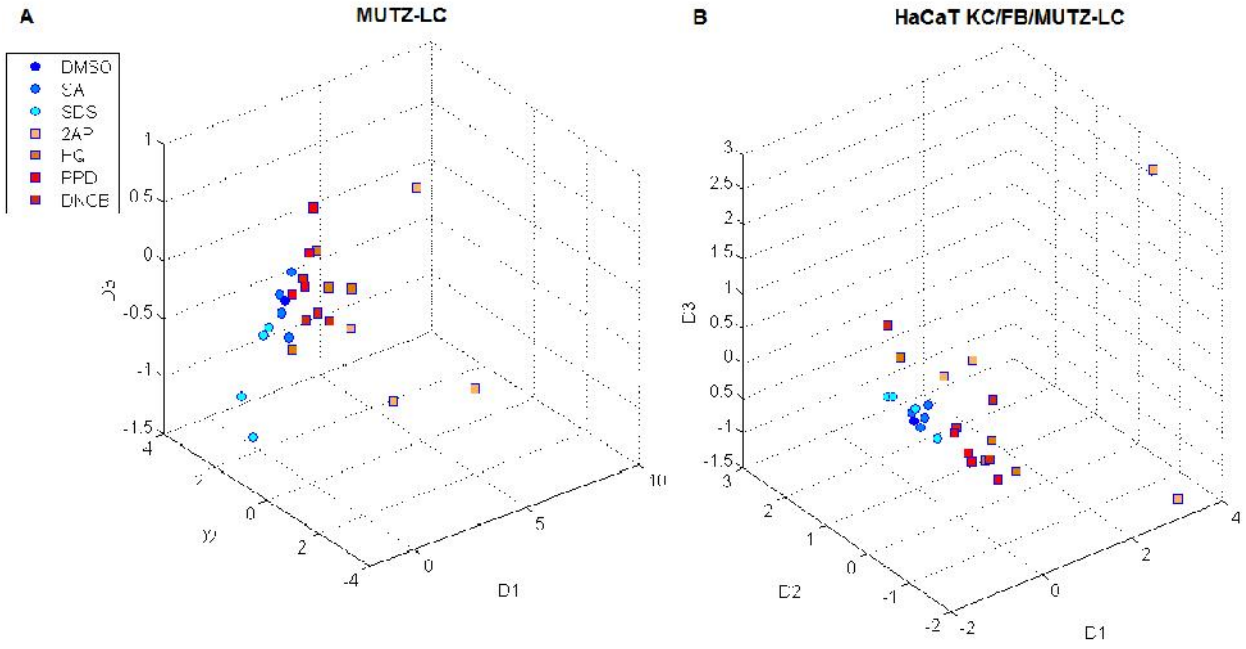

Figure 3. 4 PCA visualization of non-sensitizers vs. strong sensitizers for both A) MUTZ-LCs and B) HaCaT keratinocyte, fibroblasts, and MUTZ-LC co-cultures. The non-sensitizers (DMSO, SA, SDS) are indicated by blue circles and the strong sensitizers (2AP, HQ,PPD, DNCB) are indicated by orange and red. The metrics used to generate the MUTZ-LC figure were MIP-1β, MIP-1α, IL-17, and IL-8 (A). The signature used to generate the co-culture figure includes IL-8, GM-CSF, MIP-1β, and MIP-1α (B). Both plots were generated using data from N=4 independent replicates.

3.4 DISCUSSION

Current screening methods for predicting skin sensitizers show variable accuracies pending on the test panel of chemicals. These assays appear best suited for detecting hapten sensitizers that are innately electrophilic and bind to nucleophilic peptides or proteins in the skin to form the antigen-complex that triggers the ACD response. However, sensitizing chemicals that are not directly reactive such as pre- and pro-haptens do not see the same success rate as their hapten counterparts. This is problematic as pre- and pro-hapten chemicals encompass at least 30% of all known skin allergens [24] [25]. Pre-hapten sensitizers require abiotic means of activation, such as auto-oxidation, to form immunogenic products. Pro-haptens require enzymatic conversion to from reactive intermediates or products that are capable of binding to nucleophilic peptides in the skin. Keratinocytes in the skin play a major role in metabolizing xenobiotics and detoxification through phase I and phase II enzymes. Fibroblasts in the dermis also possess several Cytochrome p450 enzymes and may participate in xenobiotic metabolism [17, 26].

In this study, we investigated whether HaCaT keratinocytes and dermal fibroblasts can also be used as a source of metabolism for improving the sensitization response of MUTZ-LCs cells treated with a panel of pre-/pro-hapten chemicals. A co-culture of KCs, FBs, and LCs were sensitized in parallel with MUTZ-LC cultures that were treated alone. After 48 hours of incubation, the supernatant was collected and the secretome from both cultures were evaluated. The Bioplex screen includes a panel of 27 cytokines, chemokines, and growth

factors. The secretome data was analyzed using support vector machine to perform feature selection and to build a predictive classification model of sensitization.

Based on our results, we identified a panel of markers consisting of MIP-1β, IL-8, MIP-1α, RANTES, and IL-9 for MUTZ-LCs that was found to be 83% accurate when used together to build a classification model. SVM analysis was also used to identify metrics that are potentially more sensitive to less potent allergens. Building a classification model of these metrics highlighted the limitations of utilizing the MUTZ-LCs alone in identifying weak sensitizers using secreted metrics as an end point predictor. Previous studies utilizing the MUTZ-LCs alone in the GARD assay showed that they are capable of distinguishing a panel of pro-hapten sensitizers from non-sensitizers with changes in gene expression using a 200 gene signature. Furthermore, MUTZ-LCs treated with weak pro-haptens eugenol, cinnamic alcohol, and moderate pro-hapten isoeugenol were able to migrate in response to chemokine CXCL12 [22]. This indicates that MUTZ-LCs were able to undergo functional changes in response to weak, pro-hapten sensitizers. Although this is in contrast with our findings, future dose response studies of these weaker sensitizers could increase classification performance.

Based on our results, we see that MUTZ-LC secretome can be used to discriminate between non-sensitizers and pro-hapten sensitizers that are more potent. The identification of 2AP, PPD, HQ, and DNCB from non-sensitizers was 89.6% accurate with a classification model developed using MIP-1β, MIP-1α, IL-

17, and IL-8. Thus, we conclude that the MUTZ-LC's can identify a panel of sensitizers with 83.3% accuracy from non-sensitizers when using a panel of 5 secreted metrics. However, this approach may not be as sensitive to weak, pro-hapten sensitizers.

To enhance the metabolism of pro-hapten sensitizers, we established a co-culture method using HaCaT keratinocytes, dermal fibroblasts, and Mutz-LCs and observed that the use of IL-8, GM-CSF, and RANTES can accurately identify sensitizers with 89.6% accuracy. When compared to the MUTZ-LCs treated alone, a higher accuracy was achieved using fewer cytokine metrics. We also performed SVM analysis to identify metrics that are potentially more sensitive to less potent allergens in this co-culture. Using IL-8, RANTES, and GM-CSF together in the co-culture system led to 84.4% accuracy in correctly distinguishing weak sensitizers from non-sensitizers. This demonstrates that the presence of keratinocytes and fibroblasts significantly enhances detection of weakly sensitizing chemicals as compared with the use of metrics from MUTZ-LC secretome. Furthermore, we observed that this effect also extended to the identification of strong sensitizers with the co-culture where the classification model using IL-8 and GM-CSF together was 92% accurate. These trends comparing the two culture methods were visually confirmed by PCA. In the MUTZ-LC scatter plot (**Figure 3.1**), there was poor separation of non-sensitizers and sensitizers. However, when PCA was used to visualize the secretome data from the co-culture, the non-sensitizers grouped together with the weak sensitizers surrounding this cluster area. Further along the axis of all principal

components, the strong sensitizers evaluated scattered distally away from the non-sensitizer/weak sensitizer cluster region.

Factors of interest that were identified through the SVM feature selection method include IL-8, GM-CSF, MIP-1β, MIP-1α, RANTES, IL-9, and IL-17. All of these factors were found to play a role during the clinical manifestation of ACD and related inflammatory skin disorders such as psoriasis and atopic contact dermatitis [23, 27-31]. IL-8, MIP-1β, MIP-1α, and RANTES are all chemokines that recruit both adaptive and innate immune cells. GM-CSF and IL-17 are 2 factors that are commonly found on ACD lesions and are thought to play a potential role in remodeling of the skin [14, 32]. IL-8 was identified as one of the top ranking metrics in both the MUTZ-LC and co-culture conditions. IL-8 is a potent chemokine that recruits inflammatory cells during ACD such as neutrophils, natural killer cells, and CD8+ T-cells. IL-8 secretion was previously identified as a potential biomarker for skin sensitizers in monocyte derived dendritic cells, THP-1, and MUTZ-3 cell line [22, 23, 33]. Our work continues to support this metric as a viable distinguisher of skin sensitizers from non-sensitizers. However, its performance can be vastly improved when it is coupled with additional secreted metrics that we've identified. MIP-1α and MIP-1β are chemokines that recruits inflammatory cells involved during ACD such as T-lymphocytes, natural killer cells, macrophages, and monocytes. This chemokine is also important in T-cell trafficking in lymph nodes [34]. MIP-1β and MIP-1α were previously identified in the THP-1 cell line, MUTZ-3 cell line, and MoDCs as potential biomarkers of sensitization [23, 29]. Here, we report similar findings for

MUTZ-LCs treated alone and in co-culture with keratinocytes and fibroblasts with an expanded panel of pro-hapten sensitizers.

Although several of these factors were previously investigated as a potential screening metrics, there is a consensus that a single biomarker is unlikely to be predictive of all skin sensitizers. Thus a tiered strategy that evaluates several different metrics of sensitization together to make an informed prediction is necessary. As we expand our chemical panel to validate our *in vitro* approach and identified molecular signatures, it is feasible that these metrics will no longer be predictive. This was the case for the GARD assay that initially utilized a 10 gene signature for predicting skin sensitizers [20]. However, when this method was adopted by industry and more chemicals were evaluated, the 10 gene signature was expanded to 200 genes. It is feasible that a similar expansion of secreted proteins or other cellular metrics will be necessary as more chemicals are evaluated using our co-culture approach.

In this chapter, we continued to show proof of concept that our feature selection method using the support vector machine enables us to rank potential sensitization metrics and refine our classification model. Thus, new *in vitro* data that evaluated additional cellular metrics can be analyzed by SVM and incorporated into our final prediction model with ease. We also identified that the MUTZ-LCs in culture alone can be used to distinguish sensitizing pro-haptens with 83.3% accuracy when using a combination of MIP-1β, MIP-1α, IL-8, RANTES, and IL-9. The co-culture of HaCaT, fibroblasts, and MUTZ-LCs can also be utilized to identify sensitizers with nearly 90% accuracy when IL-8, GM-

CSF, and RANTES are used. However, the detection of weakly sensitizing pro-haptens was greater with the co-culture system. Co-culturing keratinocytes, fibroblasts, and MUTZ-LCs together may be utilized for enhancing the identification of potential pro-hapten allergens that may not be distinguished using current dendritic cell based approaches.

This approach is also cost efficient and amenable for high-throughput screening as compared to the use of skin equivalents. The HaCaT cell line that was utilized in this study is not formally available for purchase. However, it can be obtained through DKFZ (Heidelberg, Germany) where the cell line was originally established [35]. Since HaCaT's are an immortalized cell line, it can be rapidly expanded indefinitely and frozen down in liquid nitrogen. If this cell line is unavailable for use, primary keratinocytes may also be purchased through several vendors such as the ATCC ($430), Lonza ($424), and LifeTechnologies ($387) [36-38] . These cells can undergo a minimum of 15 population doublings when properly maintained with the use of growth factors and supplementation. However, the HaCaT line does not require additional medium supplementation with a cocktail of growth factors. Thus, the use of cell lines is an attractive economic approach as compared to the use of skin equivalents or primary keratinocytes. Primary dermal fibroblasts can also be purchased through several vendors such as ATCC ($234), Lonza ($229), and LifeTechnologies ($365) [36-38]. These cells can also undergo a minimum of 10-15 population doublings. For example, a single vial may contain approximately 1 x 10^6 cells that can be expanded 10 fold. This generates 10 vials each with 1 x 10^6 cells that can be

stored in liquid nitrogen. Each vial can be thawed and subsequently expanded and passaged up to 10-15 times. Currently, the cell seeding density used in this study was 1.25 x 10^5/well in a 96-well plate. Thus, 1.2 x 10^6 cells are required to screen up to 8 chemicals where each chemical is evaluated for 4 concentrations in triplicate on a single 96-well plate. This ultimately can lead to ~80 chemicals per vial. With 10 frozen vials, this is further expanded to 800 chemicals that can be screened with an initial purchase of one vial. Combining the two primary cell types can cost as much as ~$800 initially. However, once a cell bank is established, up to 800 chemicals can be screened which result in approximately ~$1/per chemical in terms of skin. If only primary dermal cells need to be purchased due to the use of HaCaT cells that are obtained through donation, this cost can be further reduced to $300/800 chemicals or $0.38. This is in stark contrast to the purchase of a single 12-well plate of skin equivalents that can cost up to ~$450 to screen 1 chemical. Thus, initial screening studies *in vitro* can benefit from the use of submerged co-cultures as a viable source of xenobiotic metabolism with low additional cost.

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CHAPTER 4: DISSERTATION CONCLUSIONS

4.1 SUMMARY OF FINDINGS

There are two general issues that lead to the false prediction of known skin sensitizers in current *in vitro* alternatives to animal testing. The first relates to the poor detection of pre- and pro-hapten sensitizers due to a lack of chemical activation. The second relates to the use of 1 or 2 cellular metrics that are may not be sensitive enough to identify a variety of chemicals. To address these issues, we initially developed a co-culture assay with a full thickness skin model and MUTZ-3 derived Langerhan' cells. We also investigated the use of a submerged co-culture system with keratinocytes, fibroblasts, and MUTZ-LCs as an approach that is more amenable to high throughput screening with reduced cost. A variety of cellular metrics such as the secretion of cytokines, chemokines, and growth factors were evaluated and predictive molecular signatures were identified using a support vector machine. A predictive classification model of skin sensitization was developed based on these *in vitro* results. Thus, a potential alternative to animal testing is proposed using secreted metrics from a co-culture system with keratinocytes, fibroblasts, and MUTZ-LCs.

Co-Culture with Full Thickness Skin Equivalent and MUTZ-3 Langerhans' Cells

To screen potential skin sensitizers in cosmetics and consumer care products, many alternatives to *in vivo* chemical screening have been developed which generally incorporate a small panel of cellular metrics with only one cell type involved during sensitization. However, given the underlying complexity of

ACD, these limited approaches may be insufficient to predict contact sensitizers accurately. To identify a molecular signature that can further characterize sensitization, we developed a novel system using RealSkin, a full thickness skin equivalent, in co-culture with Mutz-3 derived Langerhan's cells. This system was used to distinguish a model moderate pro-hapten isoeugenol (IE) and a model strong pre-hapten *p*-phenylenediamine (PPD) from irritant, salicylic acid (SA). Commonly evaluated metrics such as CD86, CD54, and IL-8 secretion were assessed, in concert with a 27-cytokine multi-plex screen and a functional chemotaxis assay. Data were analyzed with feature selection methods using ANOVA, hierarchical cluster analysis, and a support vector machine to identify the best molecular signature for sensitization. A panel consisting of IL-12, IL-9, VEGF, and IFN-γ predicted sensitization with over 92% accuracy using this co-culture system analysis was identified. Thus, a multi-metric approach that utilizes molecular signature of secreted may be more predictive of contact sensitization than single biomarkers such as CD86 and CD54 expression.

Co-Culture with HaCaT keratinocytes, dermal fibroblasts and MUTZ-3 Langerhan's Cells

In an effort to develop an *in vitro* assay that is more amenable to high throughput screening while preserving the cellular interactions that occur during ACD, a co-culture assay that utilizes HaCaT keratinocytes, dermal fibroblasts, and MUTZ-3 Langerhan's cells was developed and compared to the treatment of MUTZ-3 LCs alone. This co-culture approach provides a source of xenobiotic metabolism to facilitate the conversion of pre-/pro-hapten sensitizers and

provides inflammatory mediators to activate MUTZ-LCs. A panel of chemicals consisting of 3 non-sensitizers (DMSO, SA, SDS) and 9 sensitizers (EU, CA, GER, IE, CLD, 2-AP, HQ, PPD, DNCB) were dosed to both culture types for 48 hours. After this incubation time, the supernatant was collected and evaluated with a Bioplex screen that includes up to 27 cytokines, chemokines, and growth factors. These secreted metrics were evaluated using the SVM feature selection method and the top ranked factors were used to develop classification models. We identified a panel of markers consisting of MIP-1β, IL-8, MIP-1α , RANTES, and IL-9 for MUTZ-LCs that was found to be 83% accurate when used together to build a classification model. To enhance the metabolism of pro-hapten sensitizers, we established a co-culture method using HaCaT keratinocytes, dermal fibroblasts, and Mutz-LCs and observed that the use of IL-8, GM-CSF, and RANTES can accurately identify sensitizers with 89.6% accuracy. The co-culture of KC, FB, and MUTZ-LCs showed greater accuracy than the use of MUTZ-LCs alone. Potency analysis showed that this was due to the poor classification of weak sensitizers from non-sensitizers for MUTZ-LCs alone. The presence of KC and FBs in the culture promotes the proper identification of weak pro-hapten sensitizers. This is likely due to the additional source of xenobiotic metabolism to convert pro-haptens and the cross-talk of inflammatory cytokines amongst KCs, FBs, and MUTZ-LCs.

4.2 LIMITATIONS

Skin Equivalents

In chapter 2, we evaluated the use of a full thickness skin equivalent together with MUTZ-LCs. There are several key benefits of using tissue engineered skin equivalents over traditional, submerged cell cultures. Skin equivalents provide a more physiologically relevant platform with a differentiated epidermis and dermis that resembles the 3-D architecture of *in vivo* tissue. Furthermore, culturing these constructs at the air-liquid interface enables topical formulations of chemicals to be evaluated. This is especially important for chemicals that may be poorly soluble in water. Despite these benefits, compared to other cell based *in vitro* assays, a co-culture assay that utilizes a full thickness skin equivalent is undoubtedly more time, labor, and resource intensive if the skin equivalents are developed in house. Skin equivalents developed in house often require protocols that utilize a large quantity of cell culture reagents and may take weeks to form a fully differentiated stratum corneum barrier[1]. These inconveniences could be significantly reduced if the skin equivalents are purchased directly through a vendor.

Although RealSkin is currently not available for purchase through Episkin, there are full thickness skin equivalents available on market such as MatTek's EpidermFT and AST2000 that may be utilized in a similar fashion[2] [3]. Epidermal equivalents may also be used in a co-culture model where epidermal equivalents such as MatTek EpiDerm™, SkinEthic™ EpiSkin, and SkinEthic™ RHE [4] The Episkin model is available for purchase in a 12-well format at an estimated cost of ~$450/test kit/chemical [5]. It is estimated that nearly 20,000 new compounds will require sensitization information by 2018, according to the

guidelines placed by REACH, the European community regulation on chemicals and their safe use [6]. A basic cost analysis for this class of new chemicals entering the market yields 20,000 x ~$450 = ~$9 million for just the cost of skin alone. This high cost and limited availability of reconstructed skin models are largely due to lengthy protocols that are labor intensive and performed by trained personnel.

Limitations of Cell Lines

The use of cell lines *in vitro* provides many advantages necessary for performing high throughput screening studies. This includes the ability to store them in liquid nitrogen, to rapidly expand them when needed, to produce reproducible results due to the use of a single donor source, and to maintain them at relatively low cost. However, a major caveat of using traditional submerged cell cultures is the inability to screen chemicals that are poorly soluble in aqueous medium. Furthermore, with a single donor source, it is difficult to assess how the results will translate to a heterogeneous population. In our studies, the MUTZ-3 cell line was used as a source of dendiritc cells and the HaCaT cell line was used as a source of keratinocytes. The dermal fibroblasts utilized in Chapter 3 are primary cells isolated from foreskin. The full thickness skin equivalent evaluated in Chapter 2 used both primary keratinocytes and primary fibrboblasts.

The HaCaT line is an immortalized keratinocyte cell line and it is currently utilized in the KeratinoSens assay with a reporter gene for the antioxidant response element [7]. HaCaT KCs express several key phase I enzymes

involved in xenobiotics such as CYP1A1, CYP1 B1, CYP3A, and COX[8]. CYP1A1 is particularly important in generating reactive intermediates from pro-haptens such as eugenol, isoeugenol, and geraniol [9, 10]. HaCaT KCs also possess phase II enzymes involved in detoxification such as glutathione-S-transferases, n-acetyltransferase, and UDP-glucuronosyltransfers [11]. When comparing the phase I metabolic enzyme activities of HaCaT KCs and tissue engineered epidermal equivalent with excised skin, and epidermal equivalent and excised skin showed superior activity levels of enzymes such as CYP enzymes [8]. 2014). Additionally, when comparing the phase II enzyme activities between cell lines and *ex vivo* skin, cell lines exhibit greater phase II enzyme activity [11]. Thus, it is feasible that the higher activity of phase II detoxifying enzymes can lead to de-activation of pre-haptens and haptens with the use of HaCaT KCs. This detoxification effect was previously observed in a study where we evaluated the use of sensitizer treated HaCaT KC conditioned medium to activate the MUTZ-LCs (data not shown). Chemicals that undergo rapid detoxification such as CA, PPD, and DNCB were unable to stimulate IL-8 secretion from conditioned medium treated MUTZ-LCs. However, directly co-culturing the HaCaT KCs, dermal FBs, and MUTZ-LCs together addressed this delicate balance of providing the reactive hapten intermediates to MUTZ-LCs in a timely fashion before the chemical is detoxified.

The MUTZ-3 cell line was used as a source of dendritic cells in our present study. Although the MUTZ-3 cell line appears to be closer to monocyte derived DCs than THP-1 cells in gene expression studies, there are still some

potential limitations to note[12]. For example, MUTZ-3 cells show impaired ability to react to classic immunogens such as LPS, Poly(I:C), and Pam3SCK. This is due to the lower gene expression of Toll-like receptors 2, 3, and 4 (TLR2, TLR3, and TLR4) in MUTZ-3 cells as compared to monocyte derived DCs[13]. Interestingly in the GARD assay, other members of the TLR family (TLR6 and TLR9) were included in the panel of 200 genes for predicting sensitization [14]. There is evidence that metal contact allergens such as nickel, cobalt, and palladium bind to TLR-4 and induce activation of dendritic cells [15]. Although nickel sulfate was correctly identified in the GARD assay and in a CXCL12 migration assay that utilize the MUTZ-3 cell line, it is feasible that additional metal sensitizers that ligate with TLR-4 may not be correctly identified as skin sensitizers using the MUTZ-3 cell line[16].

Limitations of Selected Metrics

In our studies evaluating several different *in vitro* cultures configurations, we found that each culture method had a unique molecular signature that was most predictive. In the co-culture system with the full thickness skin equivalent and MUTZ-LCs, IL-12, IFN-γ, IL-9, and VEGF were identified as a panel of interest using a panel of 4 chemicals. In the submerged co-culture assay, IL-8, GM-CSF, and RANTES were identified. When MUTZ-LCs were treated alone, MIP-1β, MIP-1α , IL-8, and IL-17 were the most predictive combination of factors.

All of these factors that were identified are implicated in ACD *in vivo* in both animal models and humans[17-23]. Factors such as IL-12, IFN-γ, IL-9, and GM-CSF influence T-cell recruitment, stimulation, proliferation, and

differentiation. Other factors such as IL-8, RANTES, MCP-1, MIP-1α , and MIP-1β are all potent chemokines that recruit both specific and non-specific leukocytes such as T-cells, neutrophils, macrophages, and natural killer cells to mediate the inflammatory response during ACD. Several of these metrics such as IL-8, MIP-1β, and MIP-1α were previously explored in literature as single read out metrics of sensitization across various DC cell sources such as peripheral blood monocytes, THP-1, and MUTZ-3 [16, 21, 24]. Although additional chemicals will need to be screened to validate whether these secreted metrics are truly predictive of skin sensitization, we have developed a computational method to continuously compare these metrics with new ones that can be evaluated. As we expand our chemical panel, it is feasible that these metrics we've identified will face similar problems that the developers of the GARD assay encountered. The GARD study initially utilized a 10 gene signature for predicting skin sensitizers [14]. However, when this method was adopted by industry and more chemicals were evaluated, the 10 gene signature was expanded to 200 genes. It is feasible that a similar expansion of secreted proteins or other cellular metrics will be necessary as more chemicals are evaluated using our co-culture approach. However, if we are able to identify a smaller panel of secreted soluble proteins that are easily detectable by ELISA and is predictive of skin sensitization and their respective potencies using our feature selection approach to compare different metrics, it may be less labor and resource intensive than nucleic acid based protocols.

It is widely established that a single biomarker is unable to accurate identify all known skin allergens due to the diverse nature of these chemical. Skin sensitizers are diverse in the manner at which they form the immunogen. Hapten sensitizers are directly reactive due to their electrophilicity. Other sensitizers such as pre-haptens and pro-haptens require either abiotic or enzymatic conversion to form reactive intermediates or products that are able to bind to nucleophilic peptides. Not only are skin sensitizers diverse in how they form the antigen complex, but they also are diverse in terms of the differential intra-cellular pathways that are activated such as nrf2, p38 MAPK, JAK/STAT, and NF-kB [25]. Thus, the downstream products of these activated pathways can vary and this is likely a contributing factor why a single metric is insufficient. This is especially clear in the KeratinoSens assay that only measures nrf2 activation that occurs when sensitizers preferentially bind to cysteine on sensor protein, Keap1. Therefore, chemicals that do not activate this particular pathway were not properly identified [26]. This is also likely the reason why the GARD assay that utilizes a 200 gene signature to predict sensitization shows promising predictive power. Current measures to approach this issue of chemical diversity also include combining different established assays together to improve the accuracy of correctly identifying sensitizers. By combining *in silico* screening, peptide binding, KeratinoSens, mMUSST, and hCLAT, the overall performance of the classification model was improved to 94%[27]. However, this process could be further streamlined and optimized if biomarkers that are now known to be poorly predictive such as CD86 and CD54 are discarded and newer, more sensitive

metrics are evaluated instead in combination with *in silico* and *in chemico* approaches.

4.3 FUTURE DIRECTION

Assay Optimization and Validation

Three *in vitro* culture methods were assessed and the best performing systems were the 2 co-culture methods. Although both co-culture methods are able to predict sensitizers with high accuracy (≥90%), there are still additional studies that can be done to further optimize these assays before validating it with an extended panel of chemicals. In a previous study optimizing the IL-18 assay with the NCTC2544 cell line, several culture parameters were identified that could influence the proper identification of sensitizers [28]. Key factors that were identified include the cell density of keratinocytes, adhesion time before chemical treatment, and duration of chemical treatment. These are all culture parameters that we can optimize in the future as well as the seeding ratio between the keratinocytes, dermal fibroblasts, and the MUTZ-LCs.

For the full thickness skin equivalent co-culture method, the optimal number of Mutz-LCs to skin equivalent should be evaluated. The duration of treatment that is topically applied to the full thickness skin equivalent may also be an important factor that can be influenced by the chemical's permeation rate. Further studies can utilize HPLC methods to assess transcutaneous permeation of the compounds as another metric of importance. Once these cultures are fully optimized, we can validate our system by screening more chemicals of different potencies and sensitizer class. A reference list of chemicals available from

ECVAM and the LLNA data bases could be utilized [29, 30]. We have already evaluated all of the recommended pre-/pro-haptens from ECVAM on validating *in vitro* methods for detecting skin sensitizers. Once our chemical panel is further expanded, we can perform in-house validation studies to train and test our classification model with different subsets of chemicals. We envision that both co-culture methods can be used together as a tiered strategy where high throughput studies are initially performed using the submerged cell cultures. Furthermore, we can continue to refine our classification model as more *in vitro* data is generated. Ultimately, it is clear that a single assay and cellular metric of sensitization is insufficient as a stand-alone predictor, and that tiered strategies using a battery of assays improve the overall accuracy of identify known skin sensitizers [27, 31]. Thus, we envision our type of co-culture system to be utilized as a second tier assay following *in silico* or peptide binding screening methods. Unlike studies where a battery of assays with different cell sources, metrics, and cell specifications are evaluated separately, we envision a more streamlined system where several key *in vivo* sensitization steps such as metabolism, keratinocyte and fibroblast activation, and dendritic cell maturation can all be measured within a single assay system.

Functional Assays

In our study, we found that a panel of secreted factors was predictive of skin sensitization for the MUTZ-LCs and co-culture assays with either full thickness skin equivalents or submerged keratinocytes and fibroblasts. However, a readout of biomarker expression may not be a true test of functional

activity of the MUTZ-LCs. In chapter 2, we evaluated the MUTZ-LCs chemotactic response towards CCL19 in a trans-well migration assay. Although chemotaxis towards CCL19 may be predictive for IE and PPD, this assay is cumbersome and difficult to scale up, and the chemokine gradient is unstable over time. Despite these limitations with trans-well chemotaxis, the CXCL12/CCL5 migration assay that utilizes MUTZ-LCs shows promising results at accurately distinguishing a small panel of sensitizers in an inter-laboratory validation study [32]. Thus, this migration assay can also be performed in the future to confirm the functionality of the MUTZ-LCs sensitized in our co-culture system. An alternative functional test that may be even more indicative of LC maturation is to co-culture sensitized MUTZ-LCs with T-cells and evaluate T-cell stimulation and proliferation [33]. Since allergic contact dermatitis is ultimately a T-cell mediated disease, functional assays that measure T-cell stimulation *in vitro* as an alternative to the Local Lymph Node Assay should be incorporated in future studies.

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