University of Alberta

Effects of Contact Allergens on Innate Immune Cells

by

Christopher D. Green

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of

Master of Science

in

Immunology

Department of Medical Microbiology and Immunology

Edmonton, Alberta

ABSTRACT

The characteristics which cause a particular chemical to be a contact irritant, a contact allergen, or have no apparent interaction with our skin are not well understood, but undoubtedly complex. We propose that contact allergens, which are those chemicals that have the capacity to induce sensitization and the subsequent development of allergic contact dermatitis, possess two fundamental, but independent, properties: (1) the ability to create a non-self T-cell epitope by reacting with self-proteins within the skin, and (2) the ability to simultaneously activate the innate immune system to provide the necessary co-stimulatory signals required for sensitization. The latter property is the focus of this thesis. We first developed and characterized a proinflammatory cytokine-based assay, using the human monocytic cell line THP-1 as a model innate immune cell. Release of TNF-α, IL-8, IL-6 and IL-1β were measured following exposure of THP-1 cells to an array of contact allergens. We found that contact allergens, but not contact irritants, elicited robust innate immune cell activation. We then utilized parental and human Toll-like receptor 4 (hTLR4) transfected HEK293 cells to screen selected contact allergens to determine if they, like Nickel, also signal through hTLR4. We independently found that two Nickel-related contact allergens, Cobalt and Palladium, also elicit immune responses through hTLR4, while the eleven other contact allergens tested do not. Lastly, we attempted to find, or create, a THP-1 based reporter assay which would be suitable for high-throughput investigations to discover novel signaling pathways used by other contact allergens. While these efforts ultimately proved unsuccessful, direct measurement of inflammatory cytokines from THP-1 cells should prove to be a useful tool for the purposes of siRNA screens or related approaches.

CONTENTS

List of Tables
List of Figures
Abbreviations

Page

I. General Introduction

Contact Dermatitis 1
Irritant Contact Dermatitis 1
Allergic Contact Dermatitis 4
Sensitization Phase 4
Elicitation Phase 7
Allergen Recognition
Self-Haptenation 8
MHC Modification 9
Roles by Cell Type
Keratinocytes 10
Neutrophils 12
Monocytes/Macrophage 12
T-Cells 13
B-Cells 15
Mast Cells 16
NK Cells 17
Activation of Innate Immunity
Toll-like Receptors (TLRs) 18
NOD-like Receptors (NLRs) 19
Hypothesis 20
Research Objectives 20

II. Materials and Methods

2.1 Cell lines, Bacterial Strains & Plasmids 21

2.2 Growth Media 21
2.2.1 Antibiotics & Additives 23
2.3 Contact Allergens & Contact Irritants 23
2.4 DNA/RNA Manipulation
2.4.1 Plasmid Isolation 23
2.4.2 Ethanol-Sodium Acetate Purification 24
2.4.3 HPRM15772-PG02 Restriction Enzyme Digest 24
2.4.4 Chloroform Extraction 26
2.4.5 Plasmid Electroporation (THP-1) 26
2.4.6 Plasmid Electroporation (*E. coli*) 27
2.4.7 RNA Extraction 27
2.4.8 Quality/Quantity Assessment of Total RNA 27
2.4.9 Reverse Transcription (RT-PCR) 28
2.5 Assays
2.5.1 Chemical Treatment of THP-1 28
2.5.2 Chemical Treatment of Null & hTLR4 HEK-293 29
2.5.3 Assessing Cellular Viability 29
2.5.4 Inflammatory Cytokine Assays 30
2.5.5 IL-8 Luciferase Reporter Assays 31
2.5.6 SEAP Inhibition Assays 31
2.5.7 Quantitative Real Time-PCR (qRT-PCR) 32

III. Contact Allergens Trigger Cytokine Release from THP-1 cells, whereas Contact Irritants do not

INTRODUCTION 33
RESULTS 35
DISCUSSION 40

IV. Cobalt and Palladium also Signal Through hTLR4

INTRODUCTION 54

RESULTS 55

DISCUSSION 57

V. Characterization of Two THP-1 Derived Cell Lines Containing NFkB or IL-8 Reporters

INTRODUCTION 68

RESULTS AND DISCUSSION 70

VI. General Conclusions and Future Directions 86

References 92

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
2.1	Cell Lines, Bacterial Strains & Plasmids	22
2.2	Contact Allergens & Contact Irritants Solubility	25
3.1	Prevalence and common uses of selected Contact Allergens	43
3.2	THP-1 LD_{50} and peak TNF-α & IL-8 secretions for selected contact allergens	45
3.3	THP-1 peak IL-6 & IL-1β secretions for selected contact allergens	46
3.4	THP-1 LD_{50} and peak TNF-α & IL-8 secretions for selected contact irritants	48
3.5	THP-1 peak IL-6 & IL-1β secretions for selected contact irritants	49
4.1	HEK-hTLR4 LD_{50} and peak IL-8 secretion profiles following exposure to selected contact allergens	63
4.2	Parental HEK293 LD_{50} and peak IL-8 secretion profiles following exposure to selected contact allergens	64
4.3	HEK-hTLR4 TNF-α secretion when treated with metal contact allergens Nickel, Cobalt and Palladium	66
5.1	THP-1 XBlue $LD_{50\ and}$ NF-κβ reporter expression for selected contact allergens	77
5.2	THP-1 XBlue LD_{50} and NF-κβ reporter expression for selected contact irritants and one oxidative stressor	79
5.3	Inhibition of the secreted embryonic alkaline phosphatase (SEAP) reporter	81

5.4	THP-1 IL-8 luciferase reporter induction for selected contact allergens	83
5.5	THP-1 IL-8 luciferase reporter induction for selected contact irritants and one oxidative stressor	85

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
1.1	Anatomy and key cell types mediating allergic contact dermatitis within the skin	3
3.1	Example contact allergen viability, IL-8 release & TNF-α release characteristics	44
3.2	Example contact irritant viability, IL-8 release & TNF-α release characteristics	47
3.3	Comparison of TNF-α and IL-8 cytokine secretion profiles from THP-1 following exposure to selected contact allergens	50
3.4	Comparison of IL-6 and IL-1β cytokine secretion profiles from THP-1 following exposure to selected contact allergens	51
3.5	Comparison of TNF-α and IL-8 cytokine secretion profiles from THP-1 following exposure to selected contact irritants	52
3.6	Comparison of IL-6 and IL-1β cytokine secretion profiles from THP-1 following exposure to selected contact irritants	53
4.1	LPS triggers release of cytokine from hTLR4 transfected, but not parental, HEK293 cells	61
4.2	Nickel triggers upregulation of IL-8 mRNA transcript from hTLR4 transfected, but not parental, HEK293 cells	62
4.3	Cobalt and Palladium also result in increased cytokine expression from hTLR4 transfected HEK293 cells	65
4.4	HEK-hTLR4 TNF-α cytokine secretions when treated with metal contact allergens Nickel, Cobalt and Palladium	67
5.1	Viability and NF-κβ reporter characterization for three representative contact allergens	76
5.2	Viability and NF-κβ reporter characterization for two representative contact irritants and one oxidative stressor	78

5.3	THP-1 XBlue NF-κβ reporter characterization following exposure to selected contact allergens and irritants	80
5.4	Annotation of the IL-8 luciferase reporter plasmid HPRM15772-PG02	82
5.5	THP-1 IL-8 luciferase reporter characterization following exposure to selected contact allergens and irritants	84

ABBREVIATIONS

ACD	Allergic contact dermatitis
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BB	Bandrowski's base
bp	Base pair
BSO	Buthionine sulfoximine
CD	Contact dermatitis or Cluster of differentiation
CHS	Contact hypersensitivity
cps	Counters per second
Da	Dalton
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DCP	Diphenylcyclopropenone
DMEM	Dulbecco's modified eagles medium
DMF	Dimethyl fumarate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	2,4-dinitrochlorobenzene
DNFB	1-fluoro-2,4-dinitrobenzene
dNTP	Deoxynucleotide triphosphates
DTH	Delayed type hypersentivity
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein-5-isothiocyanate
GM-CSF	Granulocyte macrophage colony stimulating factor
GPMT	Guinea pig maximization test
h-CLAT	Human cell line activation test
HEV	High endothelial venule
HEK	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICD	Irritant contact dermatitis
IFN	Interferon
IL	Interleukin

kb	Kilobases
LC	Langerhans cell
LD	Lethal dose
LLNA	Local lymph node assay
LPS	Lipopolysaccharide
MCI/MI	Methylchloroisothiazolinone/Methylisothiazolinone
MI	Methylisothiazolinone
MHC	Major histocompatibility complex
NAT	N-acetyltransferase
NF-κβ	Nuclear factor kappa-β
NK	Natural killer
NLR	NOD-like receptor
PBS	Phosphate buffered saline
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
pPD	para-Phenylenediamine
qRT-PCR	Quantitative real time polymerase chain reaction
QSAR	Qualitative Structure-Activity Relationship
RAG	Recombination activating gene
RFI	Relative fluorescence intensity
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
rt-PCR	Reverse transcription polymerase chain reaction
SEAP	Secreted embryonic alkaline phosphatase
SDS	Sodium dodecyl sulfate
siRNA	Small interfering ribonucleic acid
TCR	T cell receptor
TEWL	Transepidermal water loss
TGF	Transforming growth factor
TIMES	Tissue metabolism simulator
TLR	Toll-like receptor
TNCB	2,4,6-trinitrochlorobenzene
TNF	Tumor necrosis factor
TSS	Transcription start site
UV	Ultraviolet

CHAPTER I

GENERAL INTRODUCTION

Contact Dermatitis

Throughout our daily lives, we encounter thousands of different chemical species, most of which have no apparent interaction with our skin. However, a relatively small proportion of chemicals can cause an inflammatory reaction upon contact, termed Contact Dermatitis (CD). Contact Dermatitis can be divided into two subtypes, dependent upon the type of chemical which has caused the inflammatory skin reaction: Irritant Contact Dermatitis (ICD) or Allergic Contact Dermatitis (ACD). Clinically, ICD and ACD both provoke similar inflammatory symptoms; however the non-immunological reactions in ICD and the T-cell mediated delayed type hypersensitivity responses from ACD are hallmark in differentiating the two reactions, as discussed in detail below.

Irritant Contact Dermatitis

The skin is our first line of defense against a variety of insults, both providing a barrier and acting as a sensory organ to physical trauma, temperature, infection, harmful radiation, dehydration and other environmental hazards including chemical species (Elias, 2005; Elias, 2007). The skin can be separated into three distinct regions, the Hypodermis, the Dermis and the Epidermis, which is most superficial of the three. The Epidermis can then be further divided into various strata which primarily contain keratinocytes in various stages of progressive differentiation except for the outermost

strata, or stratum corneum (Figure 1.1). The stratum corneum is comprised of a lipid component and dead or terminally differentiated keratinocytes, sometimes termed corneocytes, which are key in providing the physical and innate barrier described above. Therefore, before an inflammatory reaction is generated, both contact allergens and contact irritants must first penetrate the stratum corneum by one of the following paths: diffusion through the extracellular lipid component (intercellular), through the corneocytes (transcellular), across sweat glands or hair follicles, or at sites of injury where the extracellular lipid and corneocyte barrier has already been disrupted (Schaefer, 2001).

Typically, contact irritants cause inflammation within the viable layers of the epidermis and dermis through the latter route of barrier disruption. As contact irritants are those chemicals which have acido-basic, surfactant or solvent properties, they therefore excel at degradation of the cornified cells through extreme pH or amphipathic properties.

Irritant Contact Dermatitis is often referred to an as non-immunological inflammatory response as it arises from proinflammatory mediators produced and released due to simple toxicity of contact irritants upon the skin (McFadden, 2000). These non-antigen specific ‘danger signals’ cause keratinocytes to initiate the release of IL-1α; which has been shown to be stored in large quantities for immediate release following tissue insult (De Johng, 2007; Wood, 1996). Following a positive feedback mechanism, IL-1α then stimulates resting keratinocytes to further produce IL-1α, other inflammatory cytokines (TNF-α, IL-8) and tissue repair mediators such as GM-CSF and TGF-β (Spiekstra, 2005; Effendy, 2000) until resolved.

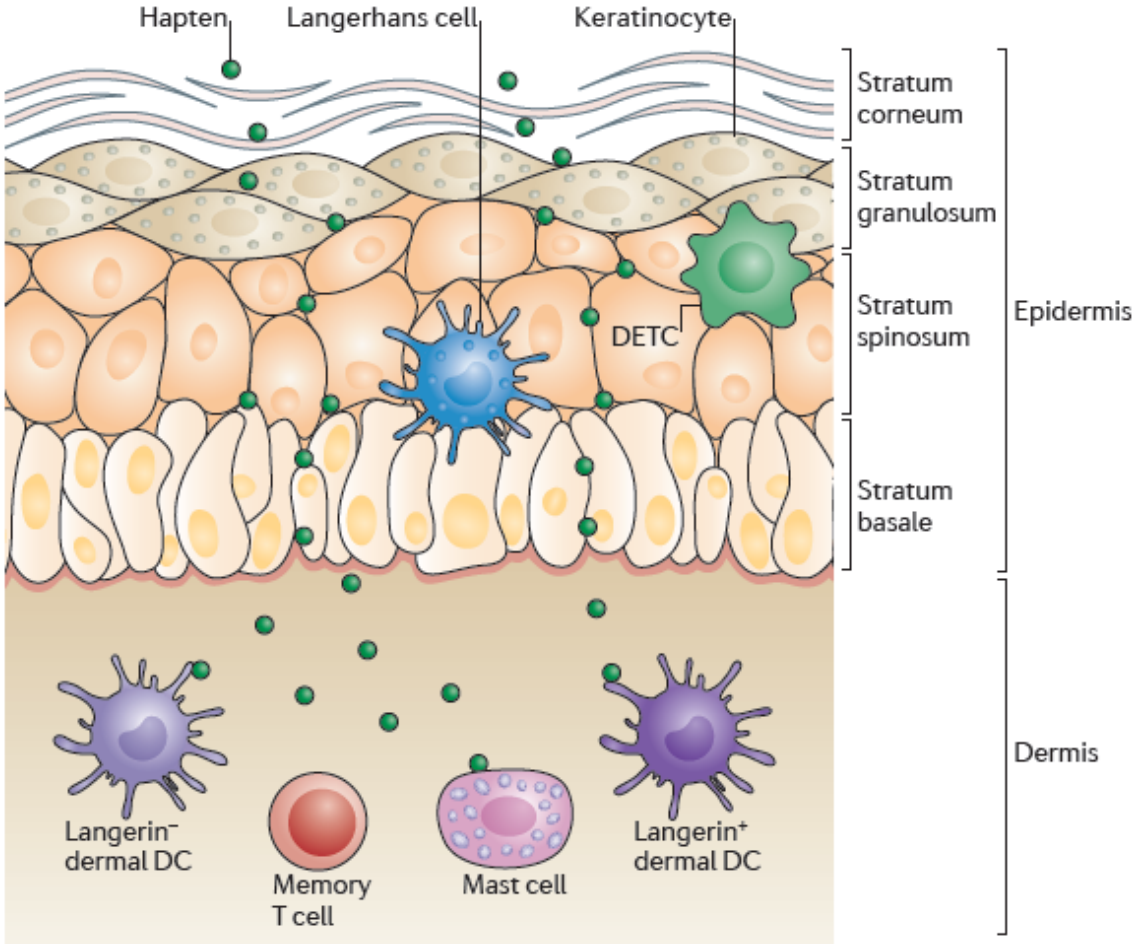

Figure 1.1. Anatomy and key cell types mediating allergic contact dermatitis within the skin. The epidermis consists of various strata of differentiated keratinocytes including the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Langerhans cells, thought to be the primary mediators during sensitization following hapten penetration, are found here. Below the Epidermis lies the Dermis, which is innervated by both nervous and circulatory networks and houses dermal dendritic cells and recruited memory T-cells found within the skin. Below the Dermis is the Hypodermis, which is not pictured here. Figure adopted from Kaplan *et al*. (2012).

Allergic Contact Dermatitis

There are over 3700 chemicals identified as being able to cause Allergic Contact Dermatitis, however only a relatively small subset of these are regularly encountered and thus identified by clinicians. These chemicals are typically found in jewelry, alloys, pigmentations or act as preservatives in a variety of every-day cosmetic products like deodorant or shampoo and conditioners. While both contact allergens and contact irritants share irritating properties in the sense that they are able to produce an inflammatory reaction due to the cytotoxic nature of the chemicals, the hallmark difference between ACD and ICD is the involvement of allergen specific T-cells; which is why ACD is classified as a delayed-type hypersensitivity reaction. The involvement of T-cells augment the non-antigen specific characteristics of ICD with both chemical memory and more potent responses with respect to the proinflammatory cytokines released and the amount of chemical required to generate a productive immune response. Due to the involvement of memory T-cells, the responses to contact allergens can be thought to occur in two phases: Sensitization and Elicitation.

Sensitization Phase (Primary Exposure)

During the sensitization phase, the first immunological contact has been made following a breach of the stratum corneum. Since most contact allergens have a relatively small molecular weight (less than 500 Da), these are generally regarded as non-antigenic by themselves, but rather chemical haptens (Bos, 2000). Due to their reactive nature, allergenic haptens are able to readily associate with components of the skin and create a modified-self hapten complex through covalent bonding, or coordination bonding as in

the case of some metal ions, such as Nickel (Budinger, 2000; Schmidt, 2010). However, not all contact allergens are initially reactive and instead require some sort of modification prior to interacting with our immune system; these are termed pre- and pro-haptens, depending upon the site of modification. Pre-haptens are those which require pre-modification outside of the body whether through exposure to oxygen or UV-radiation (Lepoittevin, 2006). An example of this is the contact allergen tetrachlorosalicylanilide, a bacteriostatic agent that becomes dechlorinated under UV-exposure to produce a reactive product which can then form complexes to proteins within the skin (Epling, 1988). Contact allergens can also be converted to a chemically reactive state within in the body and skin and are termed pro-haptens in their native state. Typically, this conversion is accomplished through metabolic enzymatic processing, as in the case of para-phenylenediamine (pPD) which is oxidized by N-acetyltransferases, and the reactive intermediates can then polymerize to the suspected agent of hair-dye allergens, Bandrowski's Base (BB). In fact, several studies looking for genetic markers that predispose an individual toward ACD have suggested that those carrying at least one allele of NAT2*4 or NAT2*12A results in a 'rapid acetylation' phenotype and was found to be overrepresented in ACD patients compared to a control population (Schnuch, 1998; Westphal, 2000).

Following formation of a self-hapten complex, Langerhans cells (LCs), which are specialized antigen presenting cells found within the epidermis and account for up to 5% of the total cell population, become stimulated (Hoath, 2003). This stimulation, whether directly from the haptenated product and/or indirectly through the toxicity of the contact allergen, results in the production of IL-1β and IL-18 from LCs which in turn mediate the

production of IL-1α, TNF-α and GM-CSF from keratinocytes (Weinlich, 1998; Jakob, 2001; Jakob, 1998; Schwarzenberger, 1996). This results in both the upregulation of CD54, α6 integrin and CD44, as well as the downregulation of E-cadherin, which results in LC disassociation from neighboring keratinocytes and migration away from the epidermis (Tang, 1993; Ma, 1994; Price, 1997; Weiss, 1997). Further, LC migration is honed to the local draining lymph node by upregulation of chemokine receptor CCR7, which like in immature T-cells, responds to ligands CCL19 and CCL21 that are produced in large quantities within high endothelial venules (HEV's) and lymphatic tissues (Randolph, 2001; Saeki, 1999; Gunn, 1998).

What haptenated self protein a TCR actually recognizes will be discussed at length further below, however both CD8+ and CD4+ T-cells can be activated based upon whether the modified-self product is processed via the endogenous or exogenous pathways, respectively. Upon engagement of a hapten-MHC:TCR complex, costimulation is provided by the LCs via further cytokine expression such as IL-1β and expression of costimulatory molecules CD86 (B7.2), CD80 (B7.1) and CD40 (Acuto, 2003; Quezada, 2004). These interactions result in a stronger engagement of the MHC:TCR complex, and through intracellular signaling cascades, activated naïve T-cells produce IL-2 which then promotes survival, proliferation and maturation. IL-12 production from LCs and dendritic cells within the skin-draining lymph nodes, as well as proinflammatory IFN-γ from activated T-cells, promotes differentiation toward a type-1 effector T-cell lineage that is believed to be the primary T-cell response in ACD (Nakamura, 1997; Kang, 1996). However, it is noteworthy to mention that studies also exist showing a role of T_H2 and T_H17 responses in ACD, and will be discussed further

when detailing the roles of various cell types involved in ACD (Probst, 1995; Larsen, 2009).

Lastly, imprinting of the activated T-cells within the skin draining lymph nodes seems to be attributed to the expression of the cutaneous lymphocyte antigen (CLA) for homing to the cutaneous endothelium, and chemokine receptors CCR4 and CCR10 which complex with ligands CCL17 and CCL27 produced by keratinocytes in the epidermis, respectively (Fuhlbrigge, 1997; Woodland, 2009).

Elicitation Phase (Secondary/Subsequent Exposure)

Once an individual has become sensitized, the clinical manifestation of Allergic Contact Dermatitis develops upon re-exposure to the specific contact allergen. Secondary exposure will again cause proinflammatory cytokine and chemokine signaling within the epidermis and subsequent migration of allergen carrying LCs and dermal dendritic cells from the epidermis to the skin draining lymph. However, proinflammatory cytokine directed upregulation of adhesion molecules within the dermal endothelial capillaries also aid in the recruitment of leukocytes to the site of contact, including the allergen-specific T-cells which were generated upon sensitization (Waldorf, 1991; Shimizu, 1991). Further, it has been suggested that preferential recruitment of allergen specific T-cells may also result from interactions with allergen presenting endothelial cells, whereby hapten penetration into the dermal tissue can be detected within minutes (Scheper, 1985; Goebeler, 1993; Macatonia, 1987). Indeed, both application of DNCB using a guinea-pig model and urushiol patch testing studies found significant recruitment of allergen-specific T-cells to the site of inflammation when compared to treatment with unrelated

compounds or when comparing the concentration of allergen-specific T-cells within the blood (Scheper, 1985; Kalish, 1990). Therefore, due to the presence of both T_{EM} and T_{CM} T-cell populations that were generated previously, both the speed and magnitude of the resulting elicitation inflammatory response will be augmented and this response is the basis of clinical patch testing to diagnose contact allergen sensitivity. Unlike immediate type allergic reactions, which occur within minutes due to humoral IgE mediated degranulation of mast cells, the cell-mediated delayed type hypersensitivity (DTH) reaction of ACD typically reaches a peak maximal inflammatory reaction within 2-3 days of challenge.

Allergen Recognition

Self-Haptenation

It is believed that some of the key features distinguishing contact allergens from irritants or benign chemicals are the abilities to penetrate the skin and to form an adduct with self-proteins in the skin. This haptenation reaction ultimately generates a T-cell response. The exact protein(s) being modified or what peptide-hapten product is presented is unknown, but is likely highly dependent upon the contact allergen. However, studies do exist that have looked at the reactivity of various amino-acid groups that provide some insight into how contact allergens may interact with our self-proteins and form T-cell epitopes. Specifically, methyl alkanesulfonates have been shown to preferentially modify methionine and histidine residues (Lepoittevin, 1992). Meanwhile, another class of contact allergens which are found in plants, α-methylene-γ-butyrolactones, were shown to be able to modify lysine residues (Franot, 1993;

Lepoittevin, 2009). The importance of lysine residues as a reactive site for haptenation was also highlighted by binding studies using methylchloroisothiazolinone (MCI) and methylisothiazolinone (MI), which are preservatives commonly found in rinse-off consumer products such as shampoos and conditioners. Here, MI was found to primarily react with cysteine residues, while MCI was found to preferentially react with cysteine, histidine and lysine (Alvarez-Sanchez, 2003; Alvarez-Sanchez, 2004a; Alvarez-Sanchez, 2004b).

However, not all contact allergens covalently bond to proteins. A 1991 study that looked at the coordination bonding sites of Nickel proposed that Nickel interacted with histidine residues of peptides bound to MHC (Romagnoli, 1991). The clinical relevance of these bonding assays is highlighted by the recent study showing Nickel activates the innate immune system through the dimerization of human toll-like receptor 4 (hTLR4) via histidine residues 431, 456 and 458 (Schmidt, 2010). Furthermore, mice lacking these histidine molecules within their TLR4 structures could only become sensitized to Nickel by the transgenic expression of H456 and H458 containing human TLR4, showing the absolutely essential role of these histidine residues in development of ACD to Nickel (Schmidt, 2010).

MHC Modification

Rather than forming a hapten-protein complex that is then recognized as a T-cell epitope, another model by which contact allergens may elicit a T-cell response is by direct modification of HLA complexes. One such report on the forefront of this hypothesis centers on the drug abacavir, which is a reverse transcriptase inhibitor used to

control HIV infection, but can also cause serious drug hypersensitivity reactions (Illing, 2012). Like contact allergens, abacavir is a relatively small molecule and by itself isn't believed to be antigenic, yet delayed type hypersensitivity reactions can be detected through patch testing. Until recently, how exposure to the drug could result in generating a T-cell response was not well understood. The report showed that abacavir can noncovalently bind to HLA-B*57:01, which results in an alteration of the antigen binding pocket. This alteration activates polyclonal abacavir-specific CD8+ T-cells, ultimately leading to drug-induced hypersensitivity (Illing, 2012). A similar report further showed that abacavir accomplishes these phenomena by binding the F pocket of the peptide-binding groove, resulting in a shift of affinity for tryptophan and phenylalanine residues toward peptides containing valine and isoleucine (Ostrov, 2012). Lastly, a third paper identified that some of the endogenous valine and isoleucine containing peptides that abacavir mediates HLA-B*57:01 affinity toward included those expressed in the skin (BPAG1 and MX1) and liver (ACOD4), and thus may explain the multi-organ hypersensitivity reactions mediated by CD8+ T-cells in response to abacavir treatment (Norcross, 2012). Together, these findings put together the hypothesis that hypersensitivity reactions may, at least in part, be mediated via drug induced HLA modification which directs non-traditional self-peptide presentation and the generation of chemical-specific T-cell responses toward a repertoire of self peptides.

Roles by Cell-Type

Keratinocytes

As mentioned previously, keratinocytes are an essential cell type in both our protection from contact allergens, and also in mediating the sensitization phase of

Allergic Contact Dermatitis. As a protecting factor, keratinocytes are indispensable to barrier function by limiting hapten penetration into the viable dermal layers, and thus to the epidermal Langerhans cells or dermal dendritic APCs. In fact, recent studies have shown that patients with a mutation in filaggrin, a protein that binds keratin and is an essential part of the cornified cell layer, correlated to greater transepidermal water loss (TEWL) and an increased sensitivity to haptens (Fallon, 2009; Oyoshi, 2009; Scharschmidt, 2009).

Following hapten penetration, keratinocytes are also responsible for producing a battery of cytokines, again including IL-1α and TNF-α, which mediate the upregulation of adhesion molecules, further recruitment of leukocytes, as well as maturation and migration of APC's from the skin into local draining lymph nodes (Nishibu, 2007; Cumberbatch, 1999; Cumberbatch, 1997). Further, keratinocytes also appear to play a role in the resolution, or late phase, of ACD. Keratinocytes can indirectly regulate inflammation by increasing expression of Receptor Activator of NF-κβ Ligand (RANKL) which is thought to promote recruitment of regulatory T-cells through activation of dendritic cells (Loser, 2006). Further, keratinocytes can also directly suppress responses during resolution through the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Ferguson, 1994; Effendy, 2000). Lastly, it has also been proposed that once activated, keratinocytes may be able to induce anergy in effector T-cell populations by acting as non-professional APC's. This is accomplished through the expression of high levels of Class I and II MHC, but lacking sufficient expression of costimulatory molecules such as CD80 (B7.1), which are required for full activation of responding T-cells (Nasir, 1994; Williams, 1994).

Neutrophils

Following treatment of the skin with contact allergen, tissue biopsies show neutrophil infiltration to the site of inflammation (Larsen, 2009). Neutrophil chemotaxis within ACD is presumed to be due to the recruitment by the adaptive and innate branches of the immune system. The adaptive immune system functions to recruit neutrophils via T_H17 T-cells that produce IL-17 upon activation, which then mediates the expression of several proinflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, among others. Further, IL-17 receptors expressed within the vascular endothelium respond to IL-17 by producing CXCL1, which is a chemokine that serves as a neutrophil chemoattractant (Kish, 2009). Direct neutrophil recruitment is also accomplished via an innate immune response with IL-8 (CXCL8), which has been shown to be produced in large quantities by antigen presenting cells when stimulated with a variety of contact allergens (Toebak, 2006). These two pathways are thought to be the principal factors facilitating neutrophil recruitment to the site of allergen contact, which aids in the overall inflammation and edema noted in contact allergen elicitation reactions. However, the exact role neutrophils play once recruited to the site of inflammation remains to be investigated.

Monocytes/Macrophage

Although the uptake and presentation of hapten-modified self proteins are thought to be primarily mediated by resident APCs in the epidermis, such as Langerhans cells, or from within the dermis (e.g. dermal dendritic cells), infiltrating monocytes are also thought to play a role in ACD reactions. Although the literature regarding the role of infiltrating monocytes/macrophage at the site of contact allergen challenge is sparse, one

recent report showed that Ly6C+CD11b+ infiltrating monocytes were recruited via IL-17 expressing CD8+ T-cells in a CCR2 dependant mechanism (Chong, 2013). Here, depletion of this Ly6C+CD11b+ monocyte subset at the site of contact through CCR2 knockout mice or ablation using clodronate treatment resulted in a decreased expression of TNF-α and induced nitric oxide synthase (iNOS). The authors also showed that these cells expressed costimulatory molecules such as CD80 and CD86 and were further activated to increase expression of class II MHC via IFN-γ (Chong, 2013). This data would then suggest that infiltrating monocytes/macrophage may play a role in ACD pathogenesis primarily through the production of TNF-α, a key inflammatory cytokine as discussed previously. In addition, through the increased expression of MHCII and costimulatory molecule expression, monocyte infiltrates may also aid in the presentation of haptenated protein complexes. However, whether these infiltrating cells also migrate from the skin to the draining local lymph once bearing haptenated protein, remains to be investigated. Interestingly, in the absence of these monocyte infiltrates, the CCR2 knockout and clodronate treated mice were still able to present CHS responses (Chong, 2013), which shows the functionally redundant nature of infiltrating monocytes in conjunction with the resident antigen presenting Langerhans cells and dermal dendritic cells.

T-cells

Whether CD4+ or CD8+ T-cells are the primary effector cell populations within Allergic Contact Dermatitis remains in question, although it seems likely that both play a crucial role in both the elicitation phase of this inflammatory skin disease. Due to their

ability to destroy antigen bearing cells, CD8+ T-cells are thought to significantly contribute to the overall skin inflammation when challenged by contact allergen. Indeed, within the murine model of contact hypersensitivity (CHS), it has been shown that CD8+ T-cells mediate sensitization to DNFB, a potent laboratory contact allergen related to DNCB (Kish, 2009). Interestingly, one report found that in humans it is primarily CD4+ T-cells that hone to the skin following treatment by Nickel (Moed, 2004). However, another clinical study found that both sensitized and non-allergic patients have Nickel specific CD4+ T-cells, and thus was believed that CD8+ T-cell populations only in the former group, are the primary effector T-cells (Cavani, 1998). Additionally, one study found that mice were unable to be sensitized to weak contact allergens in the presence of functional CD4+ T-cells, and thus hypersensitivity could only be established following depletion of these cells (Vocanson, 2006). This result then raises the question regarding a potential regulatory role of CD4+ T-cells in ACD patients, which may also be dependent upon the type (T_H1, T_H2 or T_H17) of cytokine response and whether it is an early or late phase response to allergen exposure.

Indeed, it is becoming clear that the development of ACD is not restricted to a single T-cell subset, but rather employs the use of at least T_H1, T_H2 and $T_H17$subsets. IFN-γ, a well characterized type-1 cytokine, has long been shown to be a common denominator across a panel of clinical ACD reactions (Flier, 1999), and even enhanced expression within epidermal tissues will alone cause a significantly increased ACD sensitivity, albeit among other complications (Carroll, 1997). IL-17 is also known to be produced in response to DNFB via CD8+ T-cells in mice (He, 2006) as well as by the CD4 T-cells that infiltrate to the site of patch testing in humans and within *in vitro* assays

(Albanesi, 1999; Larsen, 2009). T_H2 cytokine responses have also been noted, especially in later phases of ACD. Here, type-1 T-cells can become more susceptible to activation induced cell-death (AICD) than their type-2 counterparts, due to an increased expression of Fas-ligand and lower amounts of apoptosis protecting factor, FAP-1 (Orteu, 1998; Zhang, 1997). Type-2 cytokines produced by T-cells in ACD reactions include IL-4 and IL-10, the latter of which has also been shown to be produced from keratinocytes and macrophage infiltrates, an important anti-inflammatory cytokine (Enk, 1992; Schwarz, 1994; Berg, 1995). Due to the production of IL-10 and the apparent absence of a humoral response in ACD (as discussed below), T_H2 responses likely mediate ACD severity in part through immune suppression, at least during the late or resolution stages of ACD.

B-cells

Studies to find allergen specific antibody within humans have failed, suggesting that humoral responses may not play a major role in Allergic Contact Dermatitis. However, it has also been shown that blockade of IL-4, a principal cytokine in T_H2 responses, also inhibits ACD pathogenesis. Therefore, humoral responses may instead indirectly mediate ACD symptoms (Asherson, 1996). Indeed, production of IgM by B cells binds antigen and can activate complement. C5a, resulting from the cleavage of C5 in the complement cascade, in turn results in the upregulation of adhesion molecules within the endothelium, stimulating TNF-α production from locally activated mast cells and the recruitment of both neutrophils and monocytes. Further, C5a found within the endothelium has also been shown to recruit T-cells to the site of inflammation via expression of the C5a receptor (Nataf, 1999). Therefore, antibody responses within ACD

may play a role in mediating leukocyte recruitment to the site of contact by increasing expression of adhesion molecules, cytokines and chemotactic factors. However B-cells are not thought to be a major component of contact allergen pathogenesis, at least in humans.

Mast Cells

Whether mast cells have an essential or redundant role in ACD is controversial and clinical relevance to humans can be questioned since many studies have been done within mouse models. However, some recent studies have suggested that mast cells may play a role in both promoting and suppressing contact hypersensitivity, depending upon whether their role is evaluated during early or late phase ACD responses. These studies have been accomplished by utilizing mast cell deficient mice due to a functional knockout of the *kit* gene. Further, this model can be reconstituted with mast cells harvested from various cytokine knockout mice, essentially creating a mast cell-specific cytokine knockout phenotype. Here, contact hypersensitivity toward FITC was abrogated in the absence of mast cells, which would argue toward an essential role of mast cells in the development of CHS within mice. Additionally, while the development of contact hypersensitivity was able to rescued by the reintroduction of mast cells, transfer of mast cells that lacked the ability to produce TNF-α were again unable to mount a productive hypersensitivity response (Suto, 2006). Apparently, mast cell derived TNF-α is not the only requirement of CHS responses, as it has been further shown that mast cell knockout, IgE-deficient and FceRI-deficient mice fail to sensitize to oxazolone (Bryce, 2004). While these reports argue an essential role for mast cell activation in the development of

CHS, other reports show that mast cell deficient CHS responses to urushiol (the causative agent of poison ivy/oak reactions) were not abolished. In fact, the response was augmented as the CHS failed to resolve as compared to wild-type mice (Grimbaldeston, 2007). Here it was suggested that mast cells actually play a suppressive role during late-phase CHS responses through the production of IL-10, as resolution could be restored by reconstitution of wild-type mast cells, but not following addition of IL-10 deficient mast cells. Together, these experiments show that the role of mast cells is at least dependent upon timing, the allergen used to induce contact hypersensitivity, and potentially highlights a difference in responses between mice and humans.

NK Cells

Natural killer (NK) cell populations have also been shown to play a role in ACD pathogenesis, through their ability to produce IFN-γ upon activation. In RAG-/- mice, which lack both functional B & T cells, it was shown that activation through the NK cell receptor, NKG2D, was able to substitute for the lack of adaptive immunity and these mice were still able to generate ACD responses upon allergen challenge (O'Leary, 2006).

Additionally, NKT cells, which express both markers of NK cells (NK1.1) and express a limited αβ TCR that recognizes non-classical MHC CD1d molecules on APCs, have also been shown to be involved in Allergic Contact Dermatitis. Within mice, blockcade of CD1d inhibited both the sensitization and elicitation challenges of allergen, showing the importance for NKT cells in both early and late stages of ACD responses (Nieuwenhuis, 2005). Further, this result becomes significant as it has subsequently been shown that NKT infiltration in humans at the site allergen challenge results in 10-100

fold higher NKT population than compared in the blood (Gober, 2008).

Activation of Innate Immunity

Toll-like Receptors (TLRs)

Toll-like receptors (TLRs) are a well characterized class of innate immune receptors that recognize a wide variety of pathogen associated molecular patterns (PAMPs) such as proteins, lipoproteins, nucleic acids and carbohydrates. Recently Toll-like receptors have become an area of growing interest with several reports showing these innate immune receptors play an essential role in the development of contact hypersensitivity. One such report showed that mice deficient for both TLR2 and TLR4 resulted in a failure to develop sensitivity toward the common experimental contact allergens trinitrochlorobenzene (TNCB), fluorescein isothiocyanate (FITC) and oxazolone (Martin, 2008). Further, as these contact allergens are not known to activate any of these receptors, CHS responses were also observed in germ-free mice, which developed normally. This suggested that TLR2 and TLR4 were both required for sensitization, and are likely detecting endogenous ligands. A likely candidate for this activation was proposed to be low molecular weight hyaluronic acid, which is known to activate DC's via TLR2 and TLR4 (Scheibner, 2006; Termeer, 2002). Further, blockade of hyaluronic acid function in germ-free mice resulted in a reduced CHS severity as measured by ear swelling, again indicated the role of hyaluronic acid products in CHS responses, likely through signaling via TLR2 and TLR4 (Martin, 2008).

As discussed previously, TLRs have also been implicated in a direct response to contact allergens, as is the case with Nickel binding with histidine residues within human

Toll-like receptor 4 (Schmidt, 2010).

NOD-like Receptors (NLRs)

Another class of innate immune receptors is the nucleotide-binding oligomerization domain-like receptor (NLR) family which recognizes both PAMPs and DAMPs, and results in the formation of an intracellular complex known as the inflammasome. Here, upon receptor activation, caspase 1 becomes activated via the adaptor protein ASC, which then leads to the cleavage and activation of proinflammatory cytokines IL-1α and IL-18. The relevance of this pathway in ACD was recently highlighted by showing that mice deficient for Asc, Nlrp3 and Casp1 had reduced sensitivity to trinitrochlorobenzene (TNCB) and dinitroflourobenzene (DNFB) (Watanabe, 2007). Interestingly, dinitrothiocyanobenzene (DNTB), a weak sensitizer that is unable to stimulate inflammasome-formation, can become a stronger sensitizing allergen by the addition of inflammasome inducing SDS or by direct addition of IL-1β. Together, these studies highlight the crucial role of the innate immune system in providing a proinflammatory signal to initiate ACD, whether by detecting hapten induced cell damage or direct receptor activation. Therefore, one may hypothesize that in addition to being able to generate a T-cell epitope, a contact allergen must also be able signal the innate immune system to overcome a threshold of activation, which otherwise may induce T-cell anergy or even tolerance. However, the specific nature of how the innate immune system interacts with the vast majority of contact allergen remains to be investigated.

Hypotheses

This thesis will explore the following hypotheses:

1. Contact allergens have the intrinsic capacity to directly and potently activate innate immune cells, whereas contact irritants do not.
2. Other contact allergens, besides Nickel, will signal through human Toll-like receptor 4 (hTLR4).
3. For contact allergens that do not signal through hTLR4, reporter cell lines will facilitate the discovery of new signaling pathways (e.g. using siRNA screens, etc.).

Research Objectives

1. Using the human monocytic cell line THP-1 as a model innate immune cell, to characterize survival and acute phase proinflammatory cytokine production (TNF-α, IL-8, IL-6 & IL-1β) following exposure to an array of contact allergens and contact irritants.
2. Using untransfected and hTLR4 transfected HEK293 cells, to screen an array of other contact allergens to determine if they, like nickel, also signal via hTLR4.
3. To test an existing NF-κβ reporter cell line (THP-1 XBlue) for the capacity to detect the immune-activating potential of an array of contact allergens. If necessary, to create a new reporter cell line using an appropriate cytokine:promoter luciferase construct.

CHAPTER II

MATERIALS AND METHODS

2.1 Cells, Bacterial Strains & Plasmids

All cell lines, bacterial strains and plasmids used in this investigation are listed in Table 2.1. THP-1 and HEK293 cell lines were cyropreserved in RMPI 1640 media supplemented with 15% (v/v) fetal bovine serum and 7.5% (v/v) DMSO and stored in liquid nitrogen. Plasmid harboring DH5-α E. coli stocks were maintained in sterile 10% (w/v) skim milk-water solution and frozen at -80°C.

2.2 Growth Media

Parental THP-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and routinely cultured in RPMI-1640 medium (Gibco, Burlington, ON, CAN) supplemented with 10% ultra low endotoxin fetal bovine serum (FBS; NorthBio Inc., Toronto, ON, CA), 1% antibiotic–antimycotic (Gibco, Burlington, ON, CAN) and 100 ug/ml Noromocin (Invivogen, San Diego, CA, USA). THP-1 IL-8 transfectants were cultured as described above, however with the addition of 1 ug/mL puromycin (Sigma Adrich, St. Louis, MO, USA) as a selective marker for the integrated HPRM15772-PG02. Human TLR4 (hTLR4) transfected and parental (null2) HEK293 cell lines were obtained from Invivogen (San Diego, CA, USA) and cultured in high glucose DMEM medium with L-glutamine (Gibco, Burlington, ON, CAN), further

Table 2.1. Cell Lines, Bacterial Strains & Plasmids

Strain or Plasmid	Description	Selective Antibiotic Resistance Marker
THP-1		
THP-1	Parental cell-line (ATCC)	
THP-1 XBlue	NF-κβ reporter cell line	Zeocin^{R}
IL-8 THP-1	IL-8 luciferase reporter cell line	Puromycin^{R}
HEK-293		
HEK-null2 XBlue	Parental cell line	Zeocin^{R}
HEK-hTLR4 XBlue	hTLR4 transfected cell line	Zeocin^{R} HEK-Blue^{R*}
E. coli		
DH5-α	Transformation efficient *E. coli*	
Plasmids		
HPRM15772-PG02	IL-8 promoter:luciferase reporter construct	Kanamycin ^{R} Puromycin^{R}

*HEK-Blue^{R} is a combination of antibiotics for the selection and maintenance of various transgenes expressed by the hTLR4-transfected HEK293 cell line

supplemented with 10% ultra low endotoxin fetal bovine serum (FBS; NorthBio Inc., Toronto, ON, CA), 1% antibiotic–antimycotic (Gibco, Burlington, ON, CAN), 100 ug/ml Noromocin (Invivogen, San Diego, CA, USA) and 100 µg/ml Zeocin (Invivogen, San Diego, CA, USA). Additionally, hTLR4-HEK293 cells were cultured with HEK-Blue selection (Invivogen, San Diego, CA, USA) antibiotics for marker selection. *E. coli* were routinely cultured at 37°C in Luria-Bertani (LB) broth (10 g NaCl, 5 g yeast extract and 10 g typtone) or on LB solid-media, supplemented by 1.5% agar.

2.2.1 Antibiotics & Additives

Antibiotics used in this study were prepared in sterile dH_2O and filter sterilized prior to storage at -20°C. Antibiotic concentration for plasmid HPRM15772-PG02 maintenance in *E. coli* was 50 µg/mL kanamycin.

2.3 Contact Allergens & Contact Irritants

All contact allergens and contact irritants used within this investigation are listed in Table 2.2. The final concentration of DMSO as a vehicle was always ≤ 0.5%.

2.4 DNA/RNA Manipulation

2.4.1 Plasmid Isolation

Escherichia coli DH5-α strains harboring the HPRM15772-PG02 plasmid were cultured with appropriate antibiotic, and plasmid isolation was carried out as suggested by the kit manufacturer (Qiagen), including the optional wash step. Purified plasmids were eluted in 50 µl buffer EB and stored at -20°C.

2.4.2 Ethanol-Sodium Acetate DNA Purification

10% (v/v) of sodium acetate (3M, pH 5.2) was added to the DNA sample, followed by the addition of 2.5x (v/v) of ethanol (95%). The mixture was then incubated at 4°C for at least two hours. The DNA was pelleted via centrifugation (13,000 rpm; thirty minutes) at 4°C, with the supernatant being discarded. Residual salt was then removed by the addition of 800 µl ethanol (70%), followed by gentle inversion prior to another round of centriguation (13,000 rpm; thirty minutes) at 4°C, again discarding any supernatant. The pellet was then centrifuged again for one minute at 13,000 rpm and any remaining supernatant carefully removed. The pellet was then air dried for ten minutes to completely removal any residual ethanol. The purified DNA pellet was then resuspended in sterile, distilled water or EB Buffer (10mM Tris, pH 8.5) and the quantity and quality of DNA assessed via nanodrop.

2.4.3 HPRM15772-PG02 Restriction Enzyme Digest

HPRM15772-PG02 plasmid was linearized by restriction enzyme digestion prior to electroporation via the following protocol: 160 µg of plasmid was diluted in 900 µl of sterile distilled water, followed by the addition of 100 µl NEB Buffer #2 (10x) and 80 µl of NheI (10U/µl). Digestion was then carried out at 37˚C for four hours. Immediately, a second round of digestion was performed following the addition of another 8 µl NheI (10U/µl) and incubated at 37˚C for two hours. Complete digestion was verified via electrophoresis on a 1% (w/v) agarose gel (60 minutes at 100V) and the digested plasmid was then purified via chloroform extraction.

Table 2.2. Contact Allergens & Contact Irritants Solubility

Chemical	Supplier	Solubility/Vehicle
Contact Allergens		
2,4-Dinitrochlorobenzene	Sigma Aldrich	DMSO
Cinnamic Aldehyde	Chemotechnique Diagnostic	DMSO
Citral	Chemotechnique Diagnostic	DMSO
Cobalt chloride hexahydrate	BDH Chemicals	Culture Media
Diazolidinyl urea	Chemotechnique Diagnostic	Culture Media
Diphenylcyclopropenone	Sigma Aldrich	DMSO
DMDM Hydantoin	Chemotechnique Diagnostic	Culture Media
Methylchloroisothiazolinone/ Methylisothiazolinone	Chemotechnique Diagnostic	Culture Media
Methylisothiazolinone	Sigma Aldrich	Culture Media
Nickel chloride hexahydrate	Sigma Aldrich	Culture Media
Palladium chloride	Sigma Aldrich	Culture Media
Potassium dichromate	Sigma Aldrich	Culture Media
p-Phenylenediamine	MP Biomedicals	DMSO
Thimerosal	Sigma Aldrich	DMSO
Contact Irritants		
Dimethyl sulfoxide	Fisher Scientific	Culture Media
Isopropanol	Sigma Aldrich	Culture Media
Sodium dodecyl sulfate	Bio-Rad	Culture Media
Buthionine sulfoximine	Sigma Aldrich	Culture Media

2.4.4 Chloroform Extraction

Remaining endonuclease was then removed from the linearized plasmid via the following protocol: NheI was first heat-inactivated following heating at 65˚C for twenty minutes, followed by the addition of 1x volume chloroform, mixing well via multiple inversions. The mixture was then centrifuged (13,000 rpm; 3 minutes) and the DNA-containing aqueous phase (top) was carefully removed, avoiding the protein aggregate at the interface. The linearized plasmid DNA was then concentrated and purified once again via ethanol-sodium acetate purification prior to electroporation**.**

2.4.5 Plasmid Electroporations (THP-1)

THP-1 stable transfectants with HPRM15772-PG02 were transformed using electroporation as described: Freshly cultured THP-1 cells were collected and resuspended to a concentration of $1x10^7$ cells/ml. A 400 µl aliquot of these cells was then added to a sterile 4mm gap cuvette, followed by the addition of 10 µg of linearized HPRM15772-PG02 plasmid. Electroporation was then performed using 960µF, 250V and infinite resistance using a Gene Pulser (BioRad). Immediately following the pulse, the cells were incubated on ice until being transferred to warmed RPMI-1640 medium (Gibco, Burlington, ON, CAN), supplemented with 10% ultra low endotoxin fetal bovine serum (FBS; NorthBio Inc., Toronto, ON, CA) and 1% antibiotic–antimycotic (Gibco, Burlington, ON, CAN) and incubated at 37°C for forty-eight hours. Cells were then washed and selected following the addition of 1 µg/mL puromycin into the culturing medium.

2.4.6 Plasmid Electroporations (E. coli)

The HPRM15772-PG02 plasmid was transformed into *E.coli* DH5-α via the following transformation protocol: 50 µl of thawed electrocompetent DH5-α was mixed with 1 µl of purified HPRM15772-PG02 plasmid (5 ng/µl) . The cell and plasmid mixture was then transferred to a sterile 1mm gap cuvette (Bio-Rad, Mississauga, ON, CAN) and electroporation performed using 25µF, 1.8kV and 200 Ω resistance using a Gene Pulser (BioRad). Immediately following the pulse, the cells were then transferred to 1 ml of fresh, warmed LB broth and incubated at 37°C with aeration for two hours. A 100 µl aliquot was then plated on an LB plate supplemented with 50 µg/ul kanamycin for antibiotic resistance marker selection at 37°C. The following day, a single colony was picked and restreaked onto LB agar, again supplemented with 50 µg/ul kanamycin and incubated overnight at 37°C. Freezer stocks were then prepared as described previously.

2.4.7 RNA Extraction

RNA isolation from HEK293 cells for subsequent use in reverse transcription (rt-PCR) and quantitative real time PCR (qRT-PCR) assays was carried out as suggested by the kit manufacturer (Qiagen – RNeasy Plus), and eluted in RNase free water. Following RNA quality/quantity assessment, 0.5 µl (10U) of an RNase inhibitor (SUPERase-In) was added to each sample.

2.4.8 Quality/Quantity Assessment of Total RNA

Purified total RNA was assessed using a NanoDrop and Absorbance at 260nm and 280nm were recorded for both quantification and assessment of the purity of the total RNA extracted.

2.4.9 Reverse Transcription (RT-PCR)

cDNA synthesis was carried out as suggested by the kit manufacturer, using Applied Biosystems High Capacity cDNA Reverse Transcription Kit. Each reverse transcription was run in 20 µl volume reactions using 2 µg of purified RNA. Cycle conditions were as follows:

Step 1 (x 1): Ten minutes at 25 ^{o}C
Step 2 (x 1): Two hours at 37 ^{o}C
Step 3 (x 1): Five minutes at 85 ^{o}C
Step 4 (x 1): Hold at 4 ^{o}C

2.5 Assays

2.5.1 Chemical treatment of THP-1

An aliquot of the THP-1 monocyte cell cultures were counted using a Neubauer hemocytometer following 1:1 Trypan Blue (Gibco, Burlington, ON, CAN) exclusion staining and then resuspended at a concentration of 1.0 x 10^6 cells/ml. In a 96-well cell culture plate (Greiner, Frickenhausen, GER), 100 µl of resuspended THP-1 cells were then seeded and incubated overnight at 37˚C and 5% CO_2. The following morning, the cells were then pelleted by centrifugation (1,100 rpm; five minutes) and seeding medium was removed via vacuum aspiration and immediately replaced by 100 µl of fresh culture media with 2-fold serial dilutions of each contact allergen and irritant; prepared as described previously. The THP-1 monocytes were then exposed to each chemical for twenty-four hours, at 37˚C and 5% CO_2 incubation.

2.5.2 Chemical treatment of Null & hTLR4 HEK-293

Following detachment in cold PBS, aliquots of both HEK293 cell cultures were counted using a Neubauer hemocytometer following 1:1 Trypan Blue (Gibco, Burlington, ON, CAN) exlusion staining and then resuspended at a concentration of 2.5 x 10^5 cells/ml. In a 96-well cell culture plate (Greiner, Frickenhausen, GER), 100 µl of resuspended parental (null2) or hTLR4 transfected HEK293 cells were then seeded and incubated overnight at 37˚C and 5% CO_2. The following morning, the cells were then pelleted by centrifugation (1,100 rpm; five minutes) and seeding medium was removed via vacuum aspiration and immediately replaced by 100 µl of fresh culture media with two-fold serial dilutions of each contact allergen and irritant; prepared as described previously. The HEK293 cells were then exposed to each chemical for six hours during 37˚C and 5% CO_2 incubation.

2.5.3 Assessing Cellular Viability

Contact allergen and contact irritant cytotoxicity was assessed using a CellTitre-Glo Luminescence Cell Viability Kit (Promega, Madison, WI, USA). Kit components were prepared as per manufacturer's instructions and viability was measured as briefly described: Following chemical treatment, cells were pelleted via centrifugation (1,100 rpm; five minutes) and 50 µl of cell supernatant was removed and saved for subsequent inflammatory cytokine assays. The remaining 50 µl and cell pellet were then lysed following the addition of 50 µl CellTitre-Glo reagent along with gentle agitation using a rotary plate shaker (700 rpm; two minutes). Following ten minute incubation at room temperature, the contents of each well were then transferred to a black 96-well flat-

bottom plate and the amount of ATP present was quantified by reading the luminescent signal over an integration time of 0.25 sec/well. Cell viability was then expressed as a percentage of available ATP relative to untreated (medium only) controls. LD_{50} measurements were then made from the cytotoxicity dose curves by interpolating the concentration of allergen which would correspond to 50% cellular viability.

2.5.4 Inflammatory Cytokine Assays

Inflammatory cytokines IL-1β, IL-6, IL-8 & TNF-α were assessed using highly sensitive electrochemiluminescence-based ELISA plates from Human Proinflammatory-4 II and Human IL-8 Tissue Culture Kits (Meso Scale Discovery, Rockville, MD, USA). Kit components were prepared as per the manufacturer's instructions and cytokines were detected as briefly described: Following chemical treatment, cells were pelleted via centrifugation (1,100 rpm; five minutes) and 50 µl of cell supernatant was removed and saved for inflammatory cytokine assays. For each sample, 25 µl of supernatant was then dispensed into the MSD plate, covered, and incubated at room temperature for two hours. To each well, 25 µl of detection antibody was added and the plate(s) were again covered and incubated at room temperature for two hours. Plates were then washed three times in 0.05% Tween-20 PBS solution followed by the addition of 200 µl of read buffer to each well prior to reading. The electrochemiluminescent signal was then quantified using a Sector Imager 2400 (Meso Scale Discovery, Rockville, MD, USA) and analyte concentrations were calculated relative to cytokine standards using Discovery Workbench software (Meso Scale Discovery, Rockville, MD, USA).

2.5.5 IL-8 Luciferase Reporter Assays

Following chemical treatment, cells were pelleted via centrifugation (1,100 rpm; five minutes) and 50 µl of cell supernatant was removed and saved for subsequent IL-8 luciferase or inflammatory cytokine assays. For each sample, 20 µl of supernatant was then dispensed into a 96-well, black, flat bottom plate, followed by the addition of 100 µL 1x Buffer-GLS (prepared twenty minutes prior to assay, as per manufacturer's instructions). The plate was then covered and incubated at room temperature for one minute. IL-8 activation was assessed via luciferase production and was quantified by reading the luminescent signal over an integration time of 1.0 sec/well.

2.5.6 Secreted Embryonic Alkaline Phosphatase (SEAP) Inhibition Assays

An aliquot of the THP-1 XBlue cell cultures were counted using a Neubauer hemocytometer following 1:1 Trypan Blue (Gibco, Burlington, ON, CAN) exclusion staining and then resuspended at a concentration of 1.0×10^6 in 10 ml of growth medium and grown overnight at 37˚C and 5% CO_2, in the presence of LPS (10 µg/ml). The following morning, cell free SEAP-containing supernatant was then collected by pelleting the cells via centrifugation (1,100 rpm; five minutes) and filter-sterilizing (0.22µm filter). Two-fold serial dilutions were then prepared in fresh assay medium from an aliquot of the original supernatant. From each dilution, a 20 µl aliquot was then mixed into 200 µl of Quanti-Blue detection medium (prepared as per manufacturer's instructions) in duplicate and incubated for twenty-four hours at 37˚C and 5% CO_2. The expression of Secreted Embryonic Alkaline Phosphatase (SEAP) activity was then quantified via colormetic change by reading the absorbance (655nm) and the dilution of

original SEAP-containing supernatant corresponding to an absorbance of 1.0 was recorded. For each chemical being assayed, 5 ml of a 2x stock was then prepared, ensuring the final concentration of DMSO as a vehicle was always ≤ 1.0%, and then filter-sterilized (0.22μm filter). In a 96-well flat bottom plate, a series of five 100 μl two-fold dilutions were made for each chemical in duplicate. For each dilution, 50 μl of contact allergen was then mixed with 50 μl of the SEAP-containing supernatant dilution corresponding to an absorbance (655nm) of 1.0 and incubated overnight at 37˚C and 5% CO_2. The following morning, 20 μl of each dilution of allergen and SEAP mixture was then added into 200 μl of Quanti-Blue detection medium and incubated for twenty-four hours at 37˚C and 5% CO_2. The expression of SEAP activity in the presence of contact allergen was then quantified by reading the absorbance (655nm) in comparison to untreated (medium only) controls.

2.5.7 Quantitative Real Time-PCR (qRT-PCR)

Custom, pre-designed TaqMan primer and probe sets were ordered for IL-8 (Hs00174103_m1) and Ribosomal 18S RNA (Hs99999901_s1) from Applied Biosystems. qRT-PCR reactions were performed using PerfeCta qPCR FastMix with UNG and ROX ,using conditions as suggested by the manufacturer. Data was then analyzed using the comparative ΔΔCt method. Cycle conditions were as follows, using an Applied Biosystems 7900HT qRT-PCR System:

Cycle 1 (x 1): Two minutes at 45 ^{o}C
Cycle 2 (x 1): Thirty seconds at 95 ^{o}C
Cycle 3 (x 40): Step 1 – Two seconds at 95 ^{o}C
Step 2 – Twenty seconds at 60 ^{o}C

CHAPTER III

CONTACT ALLERGENS TRIGGER CYTOKINE RELEASE FROM THP-1 CELLS, WHEREAS CONTACT IRRITANTS DO NOT

INTRODUCTION

Traditional testing for the potential of any given chemical to elicit allergic contact dermatitis has been done on guinea pigs, assessing the degree of inflammation on a patch of skin that has been shaved prior to application of the test. More recently, the Guinea Pig Maximization Test (GPMT) has been replaced by the murine Local Lymph Node Assay (LLNA), which instead looks at the total proliferation within the draining lymph node following application of the test chemical to the skin of the ear, compared to mice similarly treated with vehicle only (Kimber, 1992). However, both of these in *vivo* assays only reveal limited information regarding the specific role of the innate immune system, and they also require a heavy animal burden which has prompted an EU directive to limit animal testing for chemical sensitivity (EU, 2003; Directive 2003/15/EC). Thus, there has been a drive to develop both reliable *in vitro* and *in silico* assays to test the sensitizing potential of contact allergens.

Current *in silico* methods are classified as Qualitative Structure-Activity Relationship (QSAR) analysis and attempt to predict sensitizing potential based upon chemical structure and the sensitization capacity or potency of known structurally related compounds. One such example of these computer based simulations is the TIMES

(Tissue Metabolism Simulator), which also incorporates the dynamics of skin metabolism of chemicals and generates predictions based upon both parent and predicted metabolites (Dimitrov, 2005). Here, the software was able to correctly predict ~80% of chemicals that were strong sensitizers, however the same software was not able to accurately predict which chemicals would be weak sensitizers.

While *in silico* analysis has been shown to have promising potential, especially with further improvements, several groups have also begun to establish *in vitro* assays to predict the sensitizing nature of chemicals. One assay that has highlighted the surprising ability of the innate immune system to differentiate between contact allergen and irritant is the human Cell Line Activation Test (h-CLAT) (Sakaguchi, 2006; Ashikaga, 2006). Human monocytic cell lines THP-1 & U-937, which model the APCs that may be found in the skin, were treated with a variety of contact allergens and irritants for twenty-four hours and costimulatory molecule expression analyzed via flow cytometry. The authors found that both cell lines had significant increased expression of CD86 (B7.2) and CD54 (ICAM-1) when treated with a variety of contact allergens, but not irritants, and that THP-1 cells had a higher sensitivity for these markers than the U-937 cell line. However, due to the low sensitivity and relatively labor intensive nature of measuring CD86 and CD54 via flow cytometry, this assay does not translate well into a high-throughput system and, as such, had a small sample size of contact allergens that were tested.

Therefore, our first objective was to establish a suitable *in vitro* cell line model to investigate the effects of contact allergens and irritants on innate immune cells, using the human monocytic leukemia cell line THP-1. Preliminary work done in the Elliott lab indicated significant proinflammatory cytokine release following treatment of these cells

using a few contact allergens, which indicated this method may have suitable sensitivity for future high-throughput investigations. Further, with ELISA assays being rapid and relatively easy to perform, the THP-1 cytokine model should alleviate the sensitivity issues noted when evaluating flow-cytometry based assays (i.e. as in the hCLAT assay above). To investigate the use of THP-1 as a model innate immune antigen presenting cell, we selected a number of contact allergens (including both classic experimental and modern clinically relevant), as well as several contact irritants, and looked at both the cytotoxicity and cytokine release following exposure to the various test chemicals.

RESULTS

Effect of contact allergens on THP-1 cells

Table 3.1 lists the panel of contacts allergens selected for these investigations, the majority of which are among the 25 most common contact allergens in the United States (Zug, 2009). THP-1 monocytes were then treated with serial dilutions of the various contact allergens, with duplicate wells for each allergen, using 96-well plates and 1 x 10^5 cells per 100 µl culture medium. After twenty-four hours, plates were centrifuged briefly and 50 µl of supernatant withdrawn, being careful not to disturb the cells below. The remaining 50 µl of culture media plus cells were then used directly for viability assessment, based on the total ATP present. The harvested supernatant was used in an electrochemiluminescence-based ELISA assay which simultaneously measured four different cytokines (IL-1β, IL-6, IL-8 and TNF-α). Figure 3.1 shows survival, TNF-α and IL-8 release curves for three representative allergens. By analyzing survival curves it was possible to estimate the concentration of allergen which corresponded to survival of 50%

of the cells after twenty-four hours (i.e. LD_{50} for Nickel was 121 μg/ml or 509 μM). Rather than show the curves for all of the allergens, Table 3.2 summarizes the LD_{50} values for all of the contact allergens tested. The cytokine release curves shown in the lower panels of Figure 3.1 indicate that a peak amount of TNF-α or IL-8 release occurred at a specific concentration of allergen, and a similar pattern was found for all allergens tested. Again, rather than showing cytokine release curves for all of the contact allergens tested, Table 3.2 summarizes these data. In this case, to provide the essential information about the various cytokine release curves, we have only listed the maximum amount of cytokine released, expressed as both the absolute amounts measured, and also relative to untreated wells with only cells and medium present (fold increases over untreated controls). In addition, we have listed the concentration of allergen that gave the peak of cytokine release, which was often, but not always, near the LD_{50}. Table 3.3 shows the equivalent data corresponding to the cytokine release curves for IL-6 and IL-1β.

Effect of contact irritants on THP-1 cells

Figure 3.2 shows survival, TNF-α and IL-8 release curves for three representative contact irritants. Although the irritants were also cytotoxic at higher concentrations as similar to the contact allergens, there was little to no cytokine release observed. These results are summarized in Table 3.4. Note that for comparison, Table 3.4 also shows results for Buthionine sulfoximine (BSO), a potent inducer of oxidative stress, which also did not result in appreciable cytokine release even at high concentrations corresponding to a loss of cell viability. Table 3.5 shows the equivalent data corresponding to the cytokine release curves for IL-6 and IL-1β, where in many cases the cytokine levels were

below detectable limits.

Contact allergens and contact irritants had similar cytotoxic effects on THP-1 cells

All contact allergens and contact irritants tested showed cytotoxicity at higher concentrations. The range of concentrations used in these experiments were deliberately chosen to give survival figures between about 100% and 0% viability, and this allowed for the estimation of LD_{50} values for each of the chemicals tested. To allow for meaningful comparisons, these concentrations were also expressed in molarities wherever possible. For the contact allergens tested, LD_{50} values ranged from 2.0 μM to 564 μM, with Thimerosal being the most toxic and Palladium being the least toxic. The order of toxicity (from most to least toxic) was: Thimerisol > Potassium dichromate > DNCB > DCP > MCI/MI > DMDM hydantoin > Diazolidinyl urea > MI > Citral > Cobalt > Cinnamic aldehyde > pPD > Nickel > Palladium. Except for SDS, the toxicity of all contact irritants and the oxidative stress inducer were several orders of magnitude lower, roughly in the mM range.

The cytokine release curves for contact allergens were complex, and had two possible forms

The two possibilities, plateau or peak, are exemplified by the curves for Nickel versus Diazolidinyl urea, respectively (Figure 3.1). A potential explanation for this is that since Nickel showed a more gradual decline in viability with increasing concentration, accumulation of cytokine occurs during the first eight to twelve hours of incubation, before the cells succumb to the toxic effects of Nickel. The time-dependent

cytotoxicity of these contact allergens will be addressed later in Chapter V. In contrast, Diazolidinyl urea is more acutely toxic, and thus at the highest concentration the cells do not survive long enough to produce any appreciable cytokine. The fact that the cytokine release curves are complex and have a peak (or in some cases a plateau) suggests that the immune activating effect and the cytotoxicity of each contact allergen are both contributing to the overall shape of the curve.

The concentration of contact allergen that gave peak cytokine release was in general near the LD_{50}

Based on the ideas in preceding paragraph, it was of interest to determine the relationship between the LD_{50} values and the concentration of contact allergen that gave peak cytokine release. In fact, for a number of contact allergens the two values were nearly the same (e.g. DNCB, DCP, DMDM hydantoin, MI and Nickel), or within a factor of two of each other (Cinammic aldehyde, Citral, Cobalt, Diazolidinyl urea, MCI/MI and Thimerosal). For the remainder of the allergens the two values were within a factor of three, except for Potassium dichromate where the concentration corresponding to maximal cytokine release was much higher than the LD_{50} (34.0 μM and 4.8 μM, respectively).

Contact allergens varied in their capacity to induce THP-1 cytokine release

To examine this statement, for each allergen we expressed peak cytokine release as fold increase over cytokine released by untreated control wells. For TNF-α, these ratios ranged from 332 to 13.8 (for Cobalt and MCI/MI, respectively), and for IL-8 these

ratios ranged from 824 to 15.4 (again for Cobalt and MCI/MI, respectively). The fold increases for all contact allergens tested are depicted graphically in Figure 3.3 and Figure 3.4, plotted from highest to lowest. Among all of the contact allergens, Cobalt, Nickel, and DCP – and always in that order – gave the highest cytokine release values, and Thimerosal giving the fourth highest value for all but IL-6, where it was the fifth highest. In addition, Palladium was found to be among the top five and six for all but IL-8 release. This suggests that these allergens are especially potent at activating THP-1 cells, and also may hint that different signaling mechanisms are occurring.

In contrast, other contact allergens appeared to give consistently low fold increases, including MCI/MI, Citral, and at least for some cytokines, DMDM hydantoin, pPD and Potassium dichromate. However, since the cytokine release ratios at the low end are all very similar, the specific order is not likely of much significance.

Contact irritants were cytotoxic to THP-1 cells, but did not induce cytokine release

We compared the fold increase of peak cytokine release induced by the least potent contact allergen with the corresponding results obtained for the three contact irritants tested (Figure 3.5 and Figure 3.6). Statistical analysis showed that the least potent contact allergen still induced significantly more cytokine release than any of the contact irritants. Based on these results, we concluded that contact irritants fundamentally differ from contact allergens by their intrinsic capacity to activate innate immune cells. This induction of cytokine release was not triggered by simple cell death, since the irritants were equally cytotoxic at high concentrations, yet still produced minimal to no cytokine release. Also, the cytokine release did not appear to be triggered by oxidative

stress alone, since treatment of the cells with high concentrations of BSO again did not result in the cytokine levels noted following exposure to contact allergens.

DISCUSSION

The results obtained here can be compared with those published by Sakaguchi and colleagues, using the h-CLAT assay (Sakaguchi, 2006; Ashikaga, 2006). These authors initially tested six contact allergens (Cobalt, DNCB, Mercaptobenzothiazole, Nickel, pPD, and Platinum) and three contact irritants (DMSO, SDS, and Tween-80) on THP-1 cells, examining the upregulation of CD54 (ICAM-1) and CD86 (B7.2) following exposure to various concentrations of the contact allergens or irritants. They also found that both contact allergens and irritants were cytotoxic, and LD_{50} concentrations of relevance for this chapter were as follows: Cobalt – 130 μg/ml [we found 44.3 μg/ml], DNCB – 5 μg/ml [we found 1.7 μg/ml], Nickel - 170 μg/ml [we found 121 μg/ml], pPD - 90 μg/ml [we found 32.7 μg/ml] and SDS - 90 μg/ml [we found 60.8 μg/ml]. Thus the LD_{50} values are all quite comparable, with our LD_{50} values being consistently slightly lower, likely related to the fact that Sakaguchi *et al.* instead used an MTT assay to measure viability.

Using the h-CLAT assay, Sakaguchi *et al*. also found that Cobalt and Nickel were the most potent contact allergens, as similar to our findings, and that none of the contact irritants caused cellular activation even at cytotoxic concentrations, which again parallels our results. However, a limitation of the h-CLAT assay was that even the most potent contact allergens could only result in a four-fold increase in signal over baseline, whereas using our cytokine secretion based assay, the least potent contact allergen still resulted in

a 15-fold increase (IL-8 secretion induced by MCI/MI). A further limitation of the original h-CLAT work was that a very limited set of contact allergens were tested, perhaps in part because of the labor intensive nature of the assay.

More recent papers describe the inter-laboratory performance of the h-CLAT assay, testing a wider array of contact allergens, but these do not provide any specific data about LD_{50} values, nor the concentration of contact allergens that induced peak THP-1 cell activation (Nukada, 2011). In these papers, a positive result was defined as a relative fluorescence intensity (RFI) value of > 150% over baseline for CD86 and > 200% over baseline for CD54, in at least two of the three independent experiments. Thus, the signal for the h-CLAT assay apparently continues to be relatively weak compared to this cytokine-based assay.

Toebak and colleagues have also published on a related subject. Here, these authors treated monocyte-derived dendritic cells grown from human blood with various concentrations of contact allergens or irritants, and measured chemokine release following forty-eight hour exposures (Toebak, 2006). The eight contact allergens tested included: Chrome, Cobalt, Copper, DNCB, Nickel, Palladium, Potassium dichromate, and pPD; the contact irritants included: 1-Propanol, Benzalkonium chloride, DMSO, and SDS; and the chemokines measured were: CXCL8 (IL-8), CCL5, CCL17, CCL18, CCL20, and CCL22 (Toebak, 2006). The authors found that all allergens except Potassium dichromate induced significant IL-8 secretion, whereas none of the contact irritants had this effect. In contrast, none of the other chemokines assayed showed consistent results across all of the contact allergens which were tested. While they did not measure any LD_{50} values to compare with, their reported peak IL-8 secretion were

obtained at the following concentrations of relevant allergens: Cobalt - 300 μM [we found 263 μM], DNCB - 12 μM [we found 6.2 μM], Nickel - 600 μM [we found 526 μM], Palladium - 300 μM [we found 1410 μM] and pPD - 200 μM [we found 925 μM]. In general these results are comparable with those described in this chapter, and this suggests that THP-1 cells respond in similar fashion to the dendritic cells which were derived from human blood. In addition, both this paper and our results suggest that measuring IL-8 provides a useful and sensitive indicator of innate immune cell activation following exposure to contact allergens.

Table 3.1. Prevalence and common uses of selected Contact Allergens

Contact Allergen	Remark	Common Uses
2,4-Dinitrochlorobenzene	Classic experimental contact allergen	Sensitization *in vivo*
Cinnamic Aldehyde*	Clinical contact allergen	Fragrances, Flavoring
Citral	Clinical contact allergen	Fragrances
Cobalt chloride*	Clinical contact allergen	Alloys, Jewelry, Pigmentation
Diazolidinyl urea*	Clinical contact allergen	Preservative (consumer products)
Diphenylcyclopropenone	Classic experimental contact allergen	Topical treatment of warts and alopecia
DMDM Hydantoin*	Clinical contact allergen	Preservative (consumer products)
Methylchloroisothiazolinone/ Methylisothiazolinone*	Clinical contact allergen	Preservative (consumer products)
Methylisothiazolinone‖	Clinical contact allergen	Preservative (consumer products)
Nickel chloride*‡	Clinical contact allergen	Alloys, Jewelry, Coins
Palladium chloride	Clinical contact allergen	Alloys, Jewelry
Potassium dichromate*	Clinical contact allergen	Masonry/Cements
p-Phenylenediamine*†	Clinical contact allergen	Hair-dye products
Thimerosalᴪ	Clinical contact allergen	Preservative (vaccines)

*Indicates contact allergens which are among the top 25 most common contact allergens in the united states (Zug, 2009); ‖2013 ACDS Contact Allergen of the Year (ACDS, 2014); †2006 ACDS Contact Allergen of the Year (ACDS, 2014); ‡2008 ACDS Contact Allergen of the Year (ACDS, 2014); ᴪ2002 ACDS Contact Allergen of the Year (ACDS, 2014)

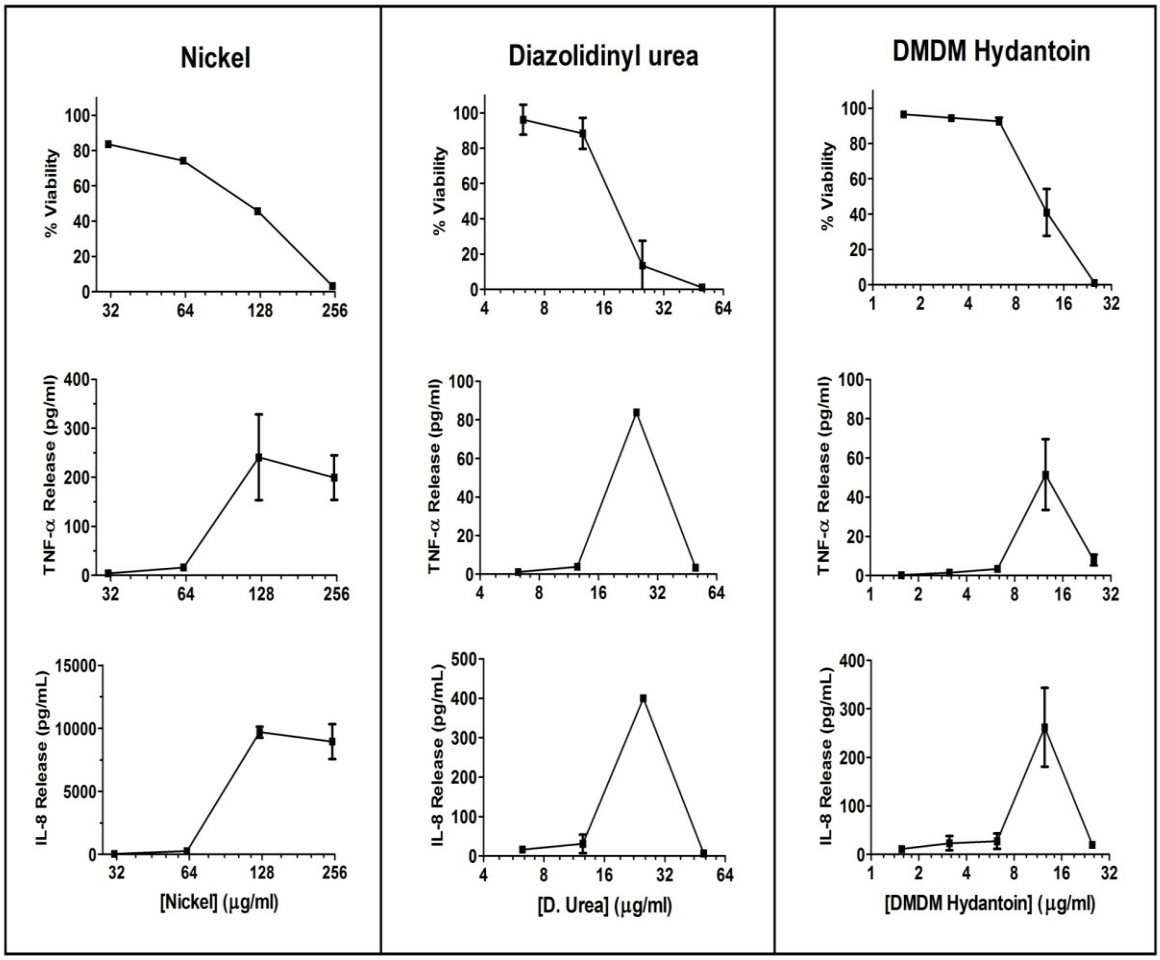

Figure 3.1. Example contact allergen viability, IL-8 release & TNF-α release characteristics. Following twenty-four hour exposure of THP-1 cells to contact allergens Nickel, Diazolidinyl urea and DMDM Hydantoin, cellular supernatants were collected and analyzed for cytokine secretion and cellular viability assessment was also performed. Data are presented as the mean ± s.d. of two independent experiments.

Table 3.2. THP-1 LD_{50} and peak TNF-α & IL-8 secretions for selected contact allergens

Contact Allergen (on THP-1 cells)	Maximum Cytokine Release		Conc. Of Allergen giving Max. Cytokine Release	Estimated LD_{50}
	TNFα (pg/10^6 cells/24hr)	IL-8 (pg/10^6 cells/24hr)		
2,4-Dinitrochlorobenzene	24.8 ± 5.4 (30.7)*	653 ± 312 (51.5)*	6.2 μM (1.3 μg/ml)	8.4 μM (1.7 μg/ml)
Cinnamic Aldehyde	21.1 ± 4.1 (26.0)*	461 ± 303 (36.4)*	99.3 μM (12.5 ppm)	197 μM (24.8 ppm)
Citral	16.4 ± 11.1 (20.3)*	346 ± 264 (27.3)*	147 μM (25.0 ppm)	95 μM (16.2 ppm)
Cobalt chloride	268 ± 128 (332)*	10,443 ± 475 (824)*	263 μM (62.5 μg/ml)	186 μM (44.3 μg/ml)
Diazolidinyl urea	83.9 ± 5.5 (104)*	400 ± 1.0 (31.6)*	89.9 μM (25.0 μg/ml)	59.3 μM (16.5 μg/ml)
Diphenylcyclopropenone	189 ± 37.6 (234)*	7,505 ± 1432 (592)*	12.1 μM (2.5 μg/ml)	10.2 μM (2.1 μg/ml)
DMDM Hydantoin	51.5 ± 18.0 (63.7)*	262 ± 81.4 (20.6)*	66.4 μM (12.5 ppm)	45.7 μM (8.6 ppm)
MCI/MI	11.2 ± 4.4 (13.8)*	196 ± 95.6 (15.4)*	18.0 μM (2.5 ppm)	11.5 μM (1.6 ppm)
Methylisothiazolinone	44.6 ± 15.1 (55.2)*	860 ± 595 (67.8)*	108.6 μM (12.5 μg/ml)	84.2 μM (9.7 μg/ml)
Nickel chloride	241 ± 87.4 (298)*	9,689 ± 446 (764)*	526 μM (125 μg/ml)	509 μM (121 μg/ml)
Palladium chloride	71.5 ± 1.0 (88.5)*	298 ± 34.6 (23.5)*	1,410 μM (250 μg/ml)	564 μM (100 μg/ml)
Potassium dichromate	15.1 ± 5.7 (18.7)*	427 ± 71.7 (33.7)*	34.0 μM (10.0 μg/ml)	4.8 μM (1.4 μg/ml)
p-Phenylenediamine	14.0 ± 2.5 (17.3)*	520 ± 58.1 (41.0)*	925 μM (100 μg/ml)	302 μM (32.7 μg/ml)
Thimerosal	110 ± 20.4 (136)*	2,358 ± 246 (186)*	3.1 μM (1.3 μg/ml)	2.0 μM (0.8 μg/ml)
Medium only	0.8 ± 0.8 (1.0)	12.7 ± 6.4 (1.0)	-----	------

Data are presented as the mean ± s.d. and (fold increases) from two independent experiments. *Fold Increase vs. untreated (medium only) controls.

Table 3.3. THP-1 peak IL-6 & IL-1β secretions for selected contact allergens

Contact Allergen (on THP-1 cells)	Maximum Cytokine Release		Conc. Of Allergen giving Max. Cytokine Release
	IL-6 (pg/10^6 cells/24hr)	IL-1β (pg/10^6 cells/24hr)	
2,4-Dinitrochlorobenzene	3.1 ± 1.0 (17.2)*	6.5 ± 2.6 (27.3)*	6.2 μM (1.3 μg/ml)
Cinnamic Aldehyde	1.9 ± 0.9 (10.6)*	3.0 ± 1.7 (12.8)*	99.3 μM (12.5 ppm)
Citral	1.5 ± 0.7 (8.2)*	2.1 ± 1.7 (9.0)*	147 μM (25.0 ppm)
Cobalt chloride	45.2 ± 8.5 (252)*	63.5 ± 11.1 (268)*	263 μM (62.5 μg/ml)
Diazolidinyl urea	2.8 ± 1.0 (15.4)*	5.0 ± 1.4 (21.2)*	89.9 μM (25.0 μg/ml)
Diphenylcyclopropenone	24.5 ± 2.7 (137)*	30.7 ± 5.1 (129)*	12.1 μM (2.5 μg/ml)
DMDM Hydantoin	1.9 ± 0.7 (10.5)*	2.7 ± 1.5 (11.5)*	66.4 μM (12.5 ppm)
MCI/MI	1.6 ± 0.9 (9.1)*	2.3 ± 1.5 (9.9)*	18.0 μM (2.5 ppm)
Methylisothiazolinone	4.5 ± 0.7 (25.0)*	8.8 ± 5.6 (37.1)*	108.6 μM (12.5 μg/ml)
Nickel chloride	31.1 ± 8.3 (174)*	43.2 ± 15.5 (182)*	526 μM (125 μg/ml)
Palladium chloride	13.3 ± 0.1 (74.1)*	15.9 ± 0.1 (67.2)*	2,820 μM (500 μg/ml)
Potassium dichromate	1.8 ± 0.2 (9.8)*	3.6 ± 0.3 (15.2)*	34.0 μM (10.0 μg/ml)
p-Phenylenediamine	0.0** (----)*	1.1 ± 0.2 (4.8)*	925 μM (100 μg/ml)
Thimerosal	10.2 ± 3.5 (56.8)*	17.0 ± 5.2 (71.7)*	3.1 μM (1.3 μg/ml)
Medium only	0.2 ± 0.1 (1.0)	0.2 ± 0.2 (1.0)	-----

Data are presented as the mean ± s.d. and (fold increases) from two independent experiments. *Fold Increase vs. untreated (medium only) controls. **Indicates assay replicates in which cytokine secretion was below detectable limits.

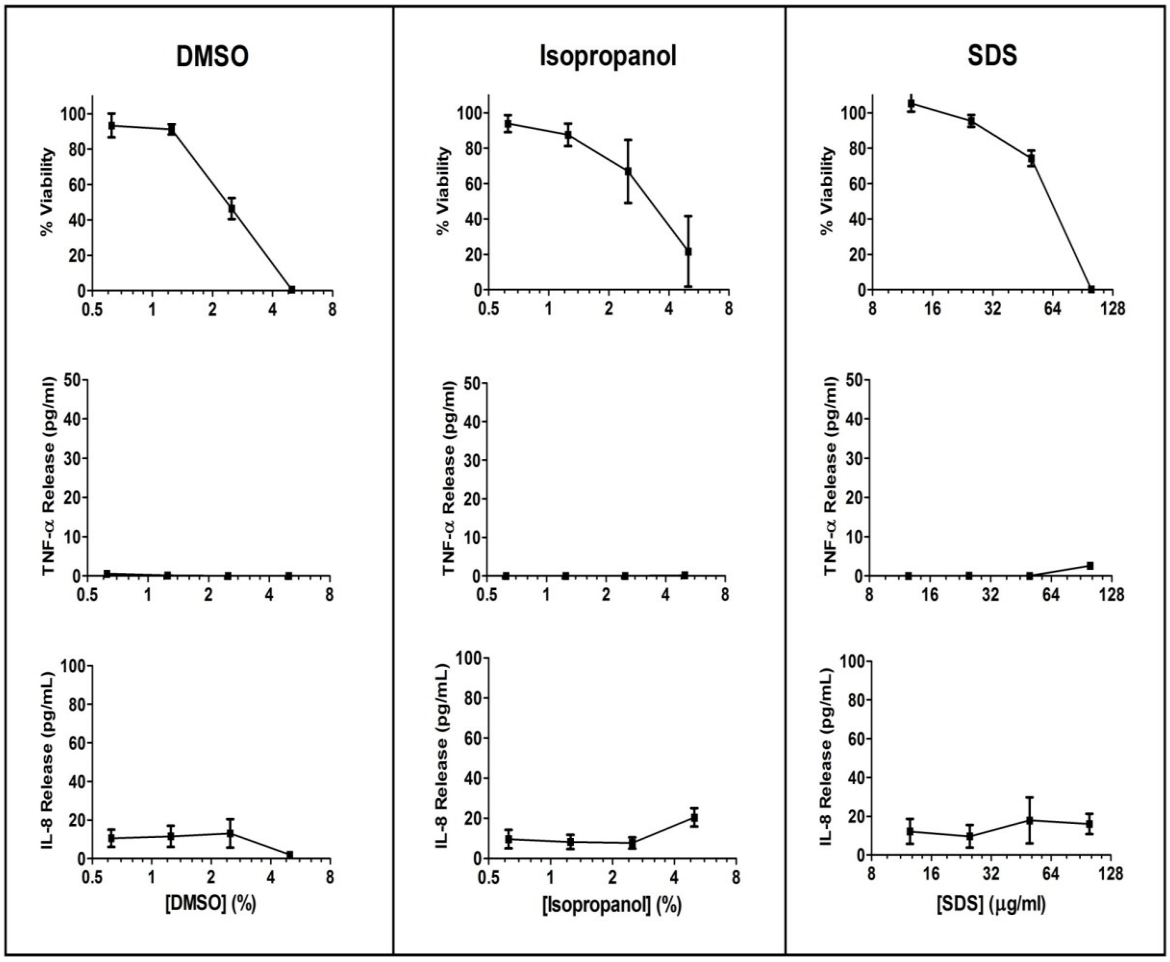

Figure 3.2. Example contact irritant viability, IL-8 release & TNF-α release characteristics. Following twenty-four hour exposure of THP-1 cells to contact irritants DMSO, Isopropanol, and SDS, cellular supernatants were collected and analyzed for cytokine secretion and cellular viability assessment was also performed. Data are presented as the mean ± s.d. of two independent experiments.

Table 3.4. THP-1 LD_{50} and peak TNF-α & IL-8 secretions for selected contact irritants

Contact Irritant (on THP-1 cells)	Maximum Cytokine Release		Conc. Of Irritant giving Max. Cytokine Release	Estimated LD_{50}
	TNF-α (pg/10^6 cells/24hr)	IL-8 (pg/10^6 cells/24hr)		
DMSO	0.1 ± 0.2 (0.1)*	13.0 ± 7.4 (1.0)*	2.5 %	3.0 %
Isopropanol	0.2 ± 0.4 (0.2)*	20.5 ± 4.7 (1.6)*	5.0 %	4.3 %
SDS	2.6 ± 0.5 (3.2)*	17.9 ± 11.9 (1.4)*	173 μM (50.0 μg/ml)	211 μM (60.8 μg/ml)
BSO	1.6 ± 1.0 (2.0)*	35.4 ± 11.9 (2.8)*	56.2 mM (12.5 mg/ml)	64.3 mM (14.3 mg/ml)
Medium only	0.8 ± 0.8 (1.0)	12.7 ± 6.4 (1.0)	-----	------

Data are presented as the mean ± s.d. and (fold increases) from two independent experiments. *Fold Increase vs. untreated (medium only) controls.

Table 3.5. THP-1 peak IL-6 & IL-1β secretions for selected contact irritants

Contact Irritant (on THP-1 cells)	Maximum Cytokine Release		Conc. Of Irritant giving Max. Cytokine Release
	IL-6 (pg/10^6 cells/24hr)	IL-1β (pg/10^6 cells/24hr)	
DMSO	0.0** (----)*	0.0** (----)*	-----
Isopropanol	0.0** (----)*	0.0** (----)*	-----
SDS	0.7 ± 0.2 (4.0)*	0.7 ± 0.1 (2.7)*	347 μM (100 μg/ml)
Medium only	0.2 ± 0.1 (1.0)	0.2 ± 0.2 (1.0)	-----

Data are presented as the mean ± s.d. and (fold increases) from two independent experiments. *Fold Increase vs. untreated controls (medium only). **Indicates assay replicates in which cytokine secretion was below detectable limits.

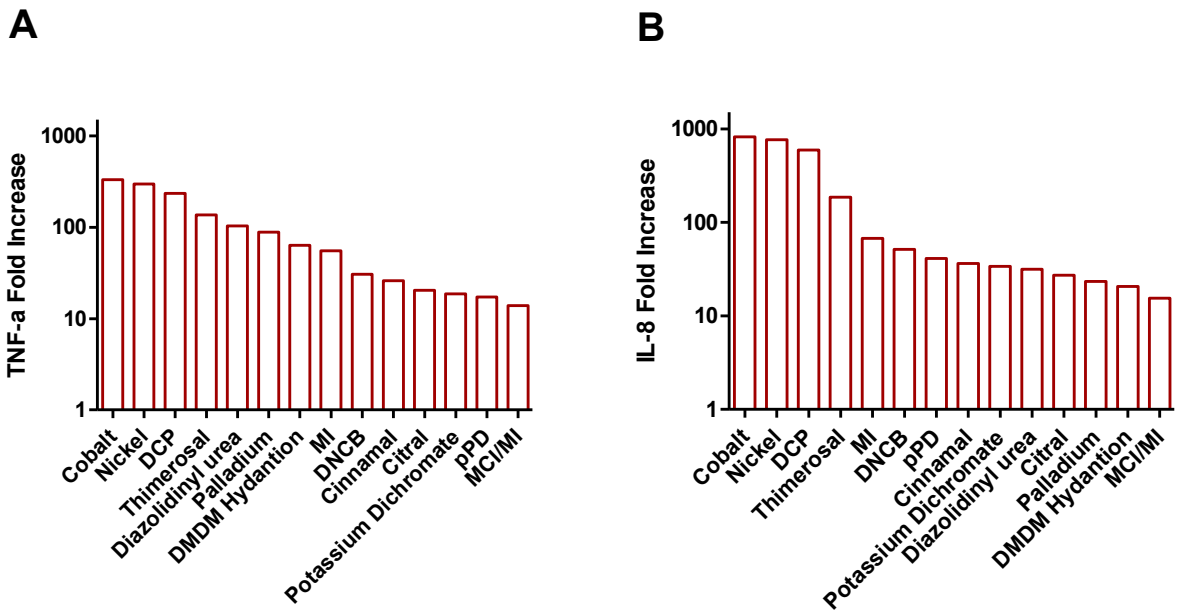

Figure 3.3. Comparison of TNF-α and IL-8 cytokine secretion profiles from THP-1 following exposure to selected contact allergens. THP-1 monocyte cell culture supernatants were harvested after exposure to a panel of contact allergens for twenty-four hours and TNF-α (A) or IL-8 (B) fold activations were calculated relative to untreated (medium only) controls. Data represents peak TNF-α or IL-8 fold secretions only and are presented as the mean of at least two independent experiments. For both A & B, all contact allergens yielded a P value < 0.001: unpaired t-test relative to medium only controls using measure cytokine values.

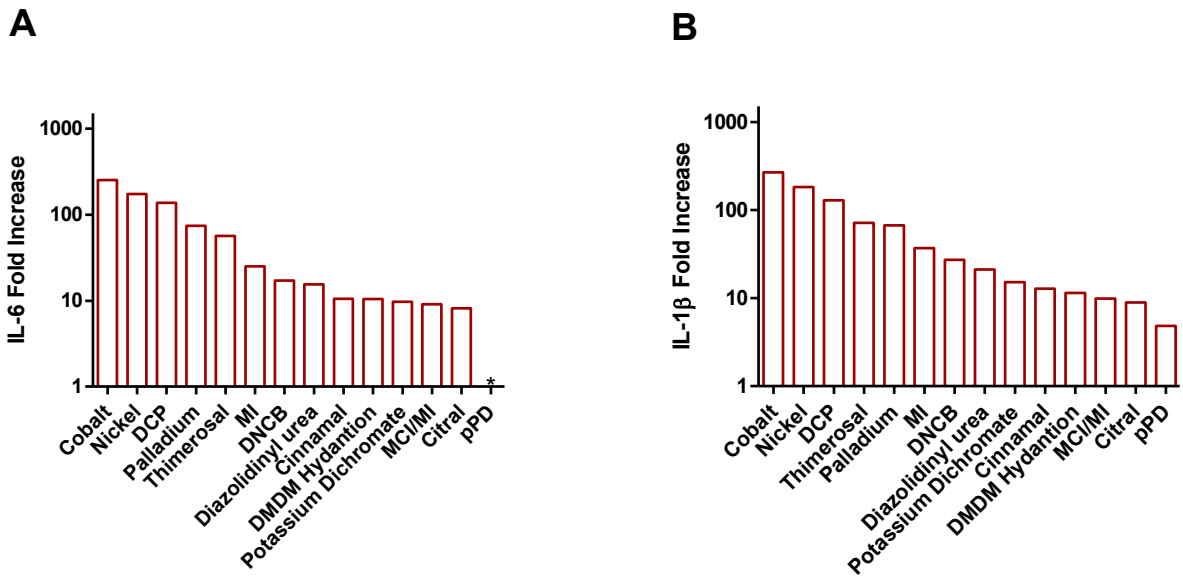

Figure 3.4. Comparison of IL-6 and IL-1β cytokine secretion profiles from THP-1 following exposure to selected contact allergens. THP-1 monocyte cell culture supernatants were harvested after exposure to a panel of contact allergens for twenty-four hours and IL-6 (A) or IL-1β (B) fold activations were calculated relative to untreated (medium only) controls. Data represents peak IL-6 or IL-1β fold secretions only and are presented as the mean of at least two independent experiments. For A & B, all contact allergens yielded a P value < 0.001, unless indicated (*): unpaired t-test relative to medium only control using measure cytokine values. *indicates cytokine values which were below detectable threshold.

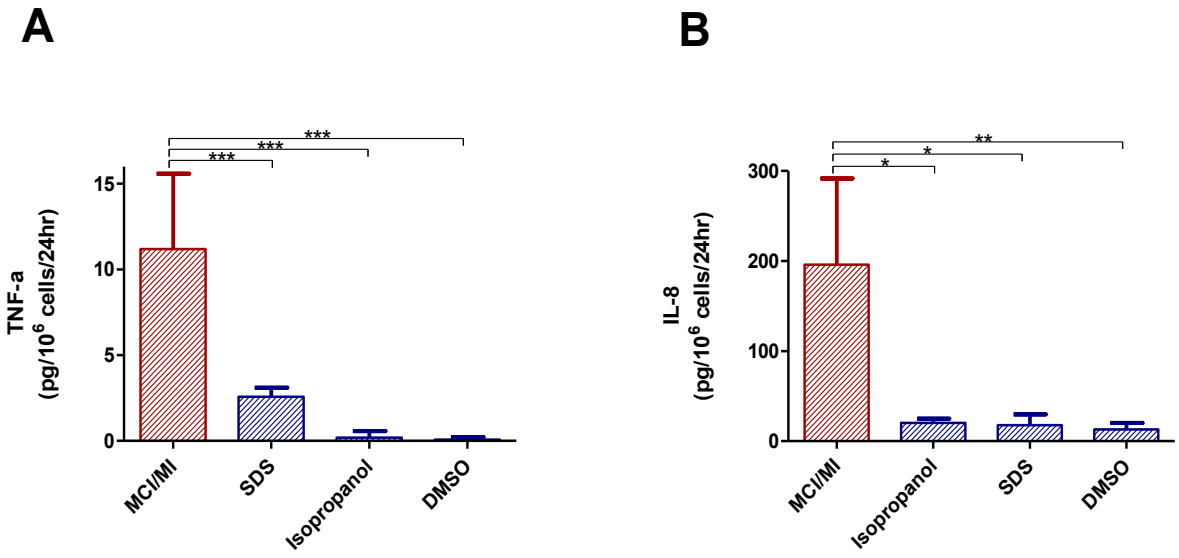

Figure 3.5. Comparison of TNF-α and IL-8 cytokine secretion profiles from THP-1 following exposure to selected contact irritants. THP-1 monocyte cell culture supernatants were harvested after exposure to a variety of contact irritants for twenty-four hours and TNF-α (A) or IL-8 (B) release was measured. The least potent contact allergen, MCI/MI, added for comparison. Data represents peak TNF-α or IL-8 release only and are presented as the mean ± s.d. of two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001: unpaired t-test.

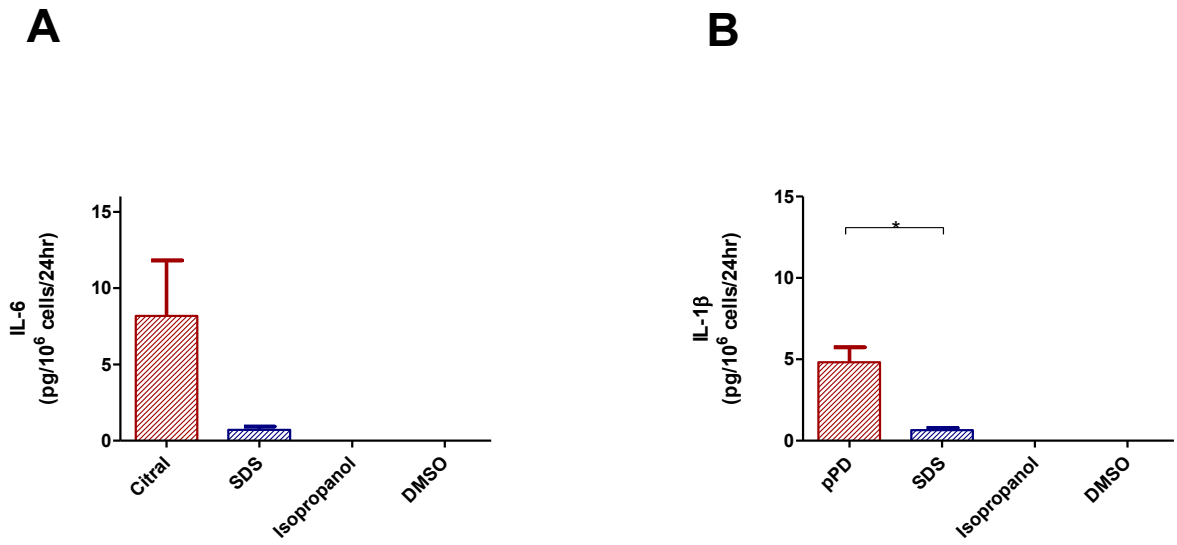

Figure 3.6. Comparison of IL-6 and IL-1β cytokine secretion profiles from THP-1 following exposure to selected contact irritants. THP-1 monocyte cell culture supernatants were harvested after exposure to a variety of contact irritants for twenty-four hours and IL-6 (A) or IL-1β (B) release was measured. The least potent contact allergen for each cytokine, Citral (IL-6) and pPD (IL-1β), added for comparison. Data represents peak IL-6 or IL-1β release only and are presented as the mean ± s.d. of two independent experiments. *P < 0.05: unpaired t-test.

CHAPTER IV

COBALT AND PALLADIUM ALSO SIGNAL THROUGH hTLR4

INTRODUCTION

Given the results of the previous chapter, it appears that contact allergens have the intrinsic capacity to activate innate immune cells. It is then reasonable to assume that this activation would be accomplished by signaling through one or more immune receptors. At the time this project was conceptualized, Nickel had just been demonstrated to signal through human, but not mouse, Toll-like receptor 4, resulting in the activation of NF-κβ and subsequent secretion of TNF-α and IL-8 (Schmidt, 2010). Furthermore, signaling through hTLR4 was shown to be independent of the origin of the MD2 co-receptor, as both human and mouse derived MD2 could pair with hTLR4 and respond to Nickel exposure (Schmidt, 2010). The authors proposed a model where dimerization of the human TLR4 was induced by Nickel coordinating to certain non-conserved histidine resides, present in human, but absent from mouse TLR4.

Given the results that Schmidt *et al.* obtained with Nickel, we hypothesized that other contact allergens might also signal through hTLR4. Considering the proximity of Cobalt and Palladium to Nickel on the periodic table (Cobalt immediately precedes Nickel and Palladium is immediately below) it would be then reasonable to hypothesize that these would behave similarly to Nickel. However, it was also of interest to

determine if any non-metal contact allergens additionally signaled through human Toll-like receptor 4.

To investigate this question, we utilized two different Human Embryonic Kidney (HEK) cell lines: wild-type HEK293 cells (which do not express hTLR4 and thus serve as a control for endogenous receptors), and HEK293-hTLR4 (transfected to express hTLR4 along with co-receptors MD2 and CD14). In these experiments, immune activation in either cell line was assessed by measuring acute-phase proinflammatory cytokine release.

RESULTS

Validation of the HEK293 cell lines

The hTLR4 transfected and parental HEK293 cell lines were initially tested using LPS as a positive control (Figure 4.1). The hTLR4 transfectants, but not parental HEK293 cells were able to respond to LPS by secreting IL-8, confirming both the absence and thus presence of functional hTLR4 complexes in the parental and transfected HEK293 cell lines, respectively. To reproduce exactly the data published by Schmidt *et al.,* the two cell lines were incubated with 356 μg/ml (1.5 mM) of Nickel, and IL-8 mRNA transcripts were measured after three hours using quantitative real-time PCR (qRT-PCR) (Figure 4.2). By exposing the cells to nickel for only three hours (as per Schmidt *et al.*), we found that cell death was negligible; a point which was not discussed in the Schmidt *et al.* paper. A significant increase in IL-8 mRNA transcripts in the hTLR4 transfected line was observed (79-fold increase over untreated control, both of which were normalized to 18S ribosomal RNA), whereas no increase in IL-8 mRNA was

noted from the parental HEK293 cell line. This indicates that Nickel indeed signals through hTLR4, and not through an endogenous receptor found in the original HEK293 cells.

Establishing assay conditions

While measuring cell activation at a transcript level was successful, we ultimately found that measuring protein following six hour incubations was less laborious and at least as sensitive. For example, IL-8 protein increased 810-fold after treatment with nickel for six hours (Table 4.1), compared to a 79-fold increase in IL-8 mRNA transcript after three hours. The six hour incubation time also decreased the cytotoxic effects of the contact allergens, compared to those observed with THP-1 cells after twenty-four hour exposure (see Chapter III), while still yielding significant cytokine release, as shown in Table 4.1.

Screening a panel of selected contact allergens

The same set of contact allergens used in Chapter III were then incubated with both the hTLR4 transfected and parental HEK293 cell lines, with viability and IL-8 release measurements made following six hours exposures (Table 4.1 and Table 4.2, respectively). As in Chapter III, the range of different concentrations for each contact allergen was selected to give viabilities ranging approximately between 100% and 0%. Viability curves and cytokine release curves were plotted, and the peak cytokine values, fold increases over untreated controls, concentrations of allergen giving peak cytokine release, as well as the LD_{50} values are summarized in Table 4.1 (for hTLR4 transfected

cells) and Table 4.2 (for parental HEK293 cells).

In comparison to the hTLR4 transfectants, the parental null2 HEK293 cell line had consistently lower basal levels of IL-8 secretion (see medium only values in Table 4.2 versus Table 4.1), presumably due to low levels of activation by trace amounts of endotoxin found in the FBS used during cell culture. Nonetheless, there was no significant increase in IL-8 secretion when the parental HEK293 cells were treated with any of the contact allergens (Table 4.2). In contrast, when the same contact allergens were screened on the hTLR4 transfected cell line, two additional contact allergens (Cobalt and Palladium) resulted in an increased secretion of IL-8 compared to untreated controls (medium only), along with the positive control Nickel. This data is also graphically represented in Figure 4.3.

To confirm our results with Cobalt and Palladium, which were the two additional contact allergens identified to signal through hTLR4 from our screens, a second cytokine (TNF-α) was also measured following exposure of these contact allergens on the hTLR4 transfected HEK293 cell line (Table 4.3 and Figure 4.4). Indeed, TNF-α was released in response to all three metals, albeit with fold increase values somewhat lower than those observed with IL-8 (i.e. Nickel, Cobalt and Palladium induced 810-, 118- and 13.6- fold increase in IL-8, respectively; whereas corresponding values were 26.1-, 9.7-, and 9.5-fold increases for TNF-α).

DISCUSSION

Despite chemical similarities and proximity on the periodic table, Nickel, Cobalt, and Palladium appear to have different capacities to trigger hTLR4-mediate activation. It

is possible then that these three different metals may vary in their capacity to induce dimerization of the hTLR4 receptors. For example, Palladium's larger size, compared to Nickel and Cobalt, may result in greater steric hindrance between the cross-linking histidine residues, and thus less efficient dimerization. However, another possible explanation for the lower potency of Palladium may relate to the fact that the Palladium salt used in our experiments (Palladium chloride) had to first be solubilized in concentrated hydrochloric acid. Therefore, even after dilution into the culture media and subsequent treatment of the cells, the pH of the medium remained mildly acidic resulting in a much lower viability of cells when treated with equimolar concentrations of Nickel or Cobalt.

After these experiments were completed, it became apparent that a different Palladium salt, Sodium tetrachloropalladate, has a much higher solubility at physiological pH, and has been previously reported to be better than Palladium chloride for use in patch testing (Muris, 2008). Thus, it is likely that if Sodium tetrachloropalladate had been used in our experiments, Palladium would have had a higher potency, more comparable to that of Nickel and Cobalt.

A recent clinical paper (Faurschou, 2011) reported a strong correlation between positive patch test responses to Nickel and Palladium. These authors reported that out of 10,778 patients patch tested with Palladium, 7.8% of these patients were positive for Palladium sensitivity, and of these only 0.2% failed to also give a positive response to Nickel. Further, other investigators have also reported similar findings (Bordel-Gomez, 2008). The most plausible explanation for these findings is that in virtually all patients, the memory T lymphocytes that confer sensitivity to Nickel by recognizing one or more

self-peptides modified by Nickel, also confer sensitivity to Palladium by recognizing precisely the same self-peptide(s) modified in an identical fashion, but by Palladium rather than Nickel. This clinical observation, and the associated ideas, supports to the hypothesis that Nickel and Palladium are nearly identical in the way they interact with proteins, with consequence for both the adaptive immune system (e.g. memory T cells), as well as the innate immune system (e.g. hTLR4 dimerization).

Results obtained from the screening experiments described in this chapter indicate that in addition to Nickel, the closely related metal allergens Cobalt and Palladium also signal through hTLR4. Soon after we obtained our results for Cobalt, a paper appeared in the literature describing the identical result (Raghavan, 2012). This work was done by the same group who reported that Nickel signals through hTLR4 (Schmidt, 2010). These authors also investigated the effects of Nickel and Cobalt on hTLR4 transfected HEK293 cell lines, measuring IL-8 protein secretion as a marker of activation. They also investigated the effects of Cobalt on primary human endothelial cells, as well as on dendritic cells established from human peripheral blood monocytes. They found that when primary human endothelial cells were incubated with Cobalt, transcripts for a number of different chemokines and cytokines were significantly upregulated. Interestingly, they showed that primary human keratinocytes did not respond to Nickel or Cobalt, presumably due to these cells lacking functional hTLR4. The Raghavan *et al.* report focused solely on Nickel and Cobalt, and contained no mention of Palladium.

However, a paper describing the fact that Palladium also signals through hTLR4 was published several months later (Rachmawati, 2013), after we had submitted our results in abstract form to the annual meeting of the Canadian Society of Investigative

Dermatology. Rachmawati and colleagues investigated the effects of seven different metals (Chromium, Cobalt, Copper, Iron, Nickel, Palladium, and Zinc) also on hTLR4 transfected and parental HEK293 cells, as well as induced dendritic cells established from human peripheral blood monocytes. They also found that Nickel, Cobalt, and Palladium all induced significant IL-8 release from the hTLR4 transfected, but not parental, HEK293 cells, and they obtained similar results with the blood-derived induced dendritic cells. Of note, in their assays these investigators used the Sodium tetrachloropalladate salt, which was discussed previously, rather than the Palladium chloride salt which we used. Interestingly, these authors found that copper induced IL-8 secretion from both the parental and hTLR4 transfected HEK293 cells, suggesting that this metal may signal through a receptor which is endogenous to HEK293.

Lastly, it is important to note that with the exception of Nickel, Cobalt, and Palladium, the remaining eleven contact allergens which were screened do not signal through hTLR4. However, the results of Chapter III clearly demonstrate that these eleven contact allergens do mediate activation of THP-1 cells, and likely other human innate immune cells. Therefore, if Toll-like receptor 4 is not mediating signaling for these other contact allergens, it raises the question as to which innate immune receptor(s) are responsible.

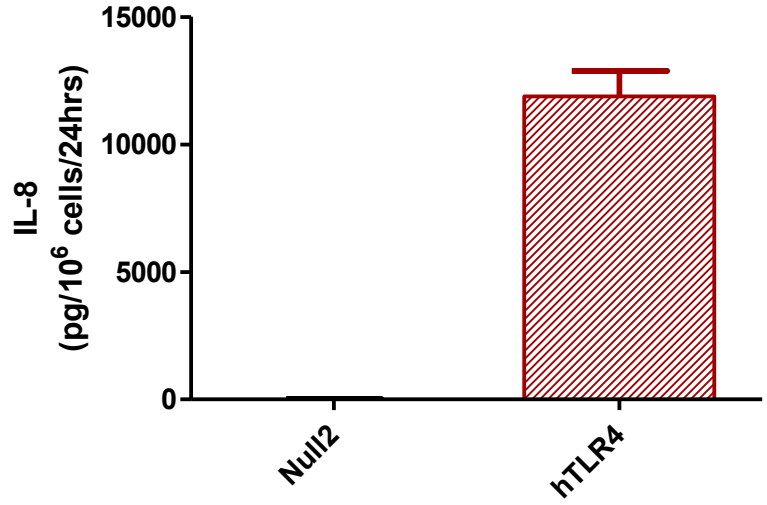

Figure 4.1. LPS triggers release of cytokine from hTLR4 transfected, but not parental, HEK293 cells. IL-8 secretions were analyzed from cellular supernatants collected from Null2 and hTLR4 transfected HEK cells following exposure to 5 ng/mL LPS for six hours. Data are presented as the mean ± s.d. of two independent experiments.

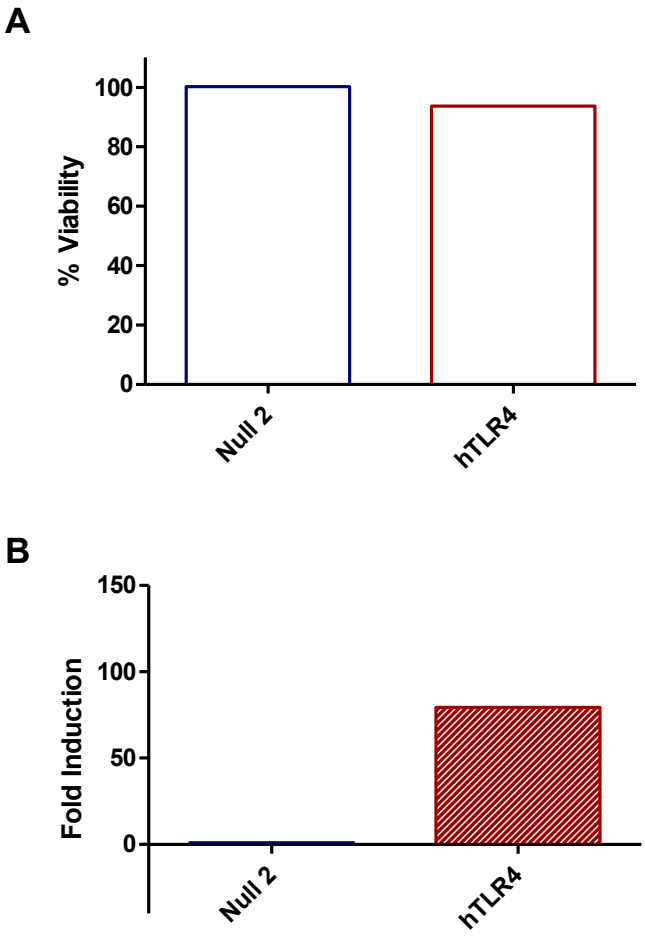

Figure 4.2. Nickel triggers upregulation of IL-8 mRNA transcript from hTLR4 transfected, but not parental, HEK293 cells. IL-8 mRNA transcripts were quantitated from cellular supernatants collected from parental and hTLR4 transfected HEK293 cells following exposure to 356 ug/mL (1.5 mM) Nickel for three hours. Data are presented as the mean from a single experiment performed in triplicate.

Table 4.1. HEK-hTLR4 LD_{50} and peak IL-8 secretion profiles following exposure to selected contact allergens

Contact Allergen (on HEK-hTLR4)	**Maximum IL-8 Release (pg/10^6 cells/6hr)**	**IL-8 Fold Increase**	**Conc. Of Allergen giving Max. IL-8 Release**	**Estimated LD_{50}**
2,4-Dinitrochlorobenzene	36.0 ± 11.8	0.9	6.2 μM (1.3 μg/ml)	17.3 μM (3.5 μg/ml)
Cinnamic Aldehyde	20.3 ± 8.4	0.5	199 μM (25.0 ppm)	767 μM (97.0 ppm)
Citral	35.4 ± 18.6	0.9	147 μM (25.0 ppm)	604 μM (103 ppm)
Cobalt chloride	4,846 ± 2774	118	1051 μM (250 μg/ml)	> 16,811 μM (> 4,000 μg/ml)
Diazolidinyl urea	32.0 ± 8.0	0.8	89.9 μM (25.0 μg/ml)	582 μM (162 μg/ml)
Diphenylcyclopropenone	19.1 ± 13.1	0.5	121 μM (25.0 μg/ml)	165 μM (34.0 μg/ml)
DMDM Hydantoin	18.6 ± 4.9	0.5	133 μM (25.0 ppm)	513 μM (96.6 ppm)
Methylchloroisothiazolinone / Methylisothiazolinone	47.5 ± 16.0	1.2	18.0 μM (2.5 ppm)	25.9 μM (3.6 ppm)
Methylisothiazolinone	48.6 ± 16.3	1.2	27.1 μM (3.1 μg/ml)	130 μM (15.0 μg/ml)
Nickel chloride	33,310 ± 9799	810	1052 μM (250 μg/ml)	> 8,414 μM (> 2,000 μg/ml)
Palladium chloride	560 ± 40.5	13.6	705 μM (125 μg/ml)	716 μM (127 μg/ml)
Potassium dichromate	58.4 ± 16.8	1.4	17.0 μM (5.0 μg/ml)	> 34.0 μM (> 10.0 μg/ml)
p-Phenylenediamine	59.6 ± 42.7	1.5	2312 μM (250 μg/ml)	5,225 μM (565 μg/ml)
Thimerosal	23.6 ± 11.4	0.5	15.4 μM (6.3 μg/ml)	33.6 μM (13.6 μg/ml)
Medium only	41.1 ± 13.5	1.0*	-----	------

Data are presented as the mean ± s.d. and fold increases from two independent experiments. *Fold Increase vs. untreated (medium only) controls.

.

Table 4.2. Parental HEK293 LD_{50} and peak IL-8 secretion profiles following exposure to selected contact allergens

Contact Allergen (on parental HEK293)	**Maximum IL-8 Release (pg/10^6 cells/6hr)**	**IL-8 Fold Increase**	**Conc. Of Allergen giving Max. IL-8 Release**	**Estimated LD_{50} for**
2,4-Dinitrochlorobenzene	1.5 ± 1.0	2.0	6.2 μM (1.3 μg/ml)	18.1 μM (3.7 μg/ml)
Cinnamic Aldehyde	0.3 ± 0.3	0.4	199 μM (25.0 ppm)	1,183 μM (149 ppm)
Citral	1.1 ± 0.8	1.5	147 μM (25.0 ppm)	768 μM (131 ppm)
Cobalt chloride	1.9 ± 0.6	1.3	1,051 μM (250 μg/ml)	> 16,811 μM (> 4,000 μg/ml)
Diazolidinyl urea	0.0*	-----	89.9 μM (25.0 μg/ml)	521 μM (145 μg/ml)
Diphenylcyclopropenone	0.7 ± 0.3	1.0	121 μM (25.0 μg/ml)	179 μM (36.9 μg/ml)
DMDM Hydantoin	0.7 ± 0.8	0.9	133 μM (25.0 ppm)	472 μM (88.9 ppm)
Methylchloroisothiazolinone / Methylisothiazolinone	0.7 ± 1.3	0.9	18.1 μM (2.5 ppm)	18.6 μM (2.6 ppm)
Nickel chloride	0.9 ± 0.6	1.3	1,052 μM (250 μg/ml)	> 8,414 μM (> 2,000 μg/ml)
Palladium chloride	1.1 ± 1.7	1.5	705 μM (125 μg/ml)	744 μM (132 μg/ml)
Potassium dichromate	1.2 ± 1.0	1.6	17.0 μM (5.0 μg/ml)	> 34.0 μM (> 10.0 μg/ml)
p-Phenylenediamine	0.3 ± 0.4	0.5	2312 μM (250 μg/ml)	6,001 μM (649 μg/ml)
Thimerosal	0.0**	-----	15.4 μM (6.3 μg/ml)	22.2 μM (9.0 μg/ml)
Medium only	0.7 ± 1.0	1.0*	-----	------

Data are presented as the mean ± s.d. and fold increases from two independent experiments. *Fold Increase vs. untreated (medium only) controls. **Indicates assay replicates in which cytokine secretion was below detectable limits.

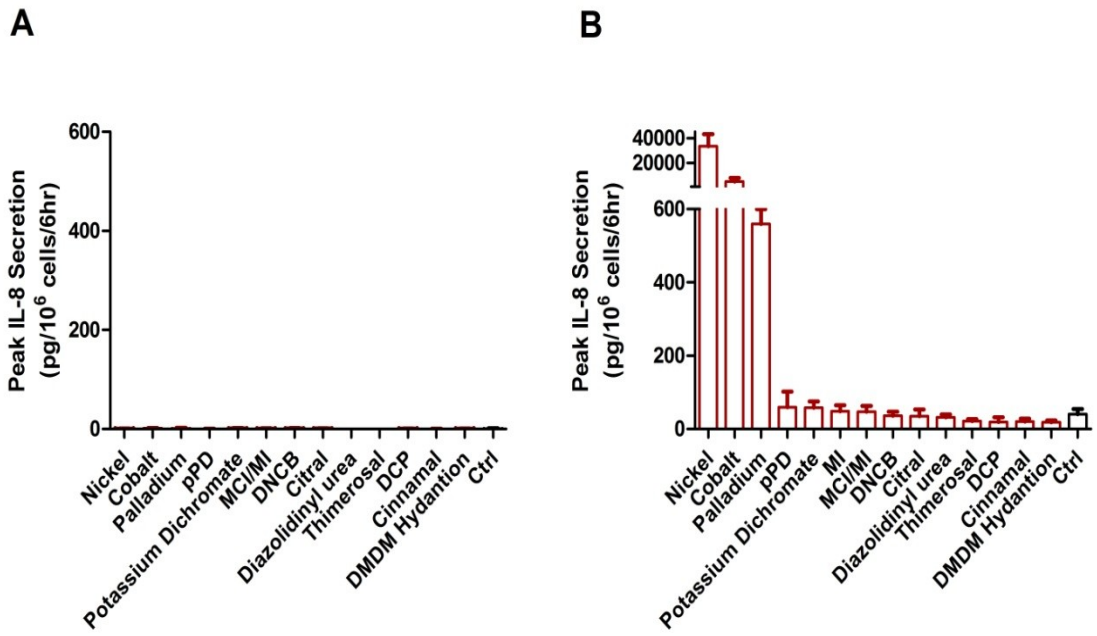

Figure 4.3. Cobalt and Palladium also result in increased cytokine expression from hTLR4 transfected HEK293 cells. IL-8 secretions were analyzed from cellular supernatants collected from either parental (A) or hTLR4 transected (B) HEK293 cells following exposure to known hTLG4 agonist Nickel and other experimental or clinically common contact allergens for six hours. Data represents peak IL-8 release only compared to untreated controls (Ctrl) and are presented as the mean ± s.d. of two independent experiments.

Table 4.3. HEK-hTLR4 TNF-α secretion when treated with metal contact allergens Nickel, Cobalt and Palladium

Contact Allergen (on HEK-hTLR4)	**Maximum TNF-α Release (pg/10^6 cells/6hr)**	**Fold Increase TNF-α**	**Conc. Of Allergen giving Max. TNF-α Release**
Cobalt chloride	414 ± 88.7	9.7	1,051 μM (250 μg/ml)
Nickel chloride	1,113 ± 363	26.1	2,104 μM (500 μg/ml)
Palladium chloride	406 ± 13.9	9.5	2,820 μM (500 μg/ml)
Medium only	42.7 ± 7.5	1.0*	-----

Data are presented as the mean ± s.d. and fold increases from two independent experiments. *Fold Increase vs. untreated (medium only) controls.

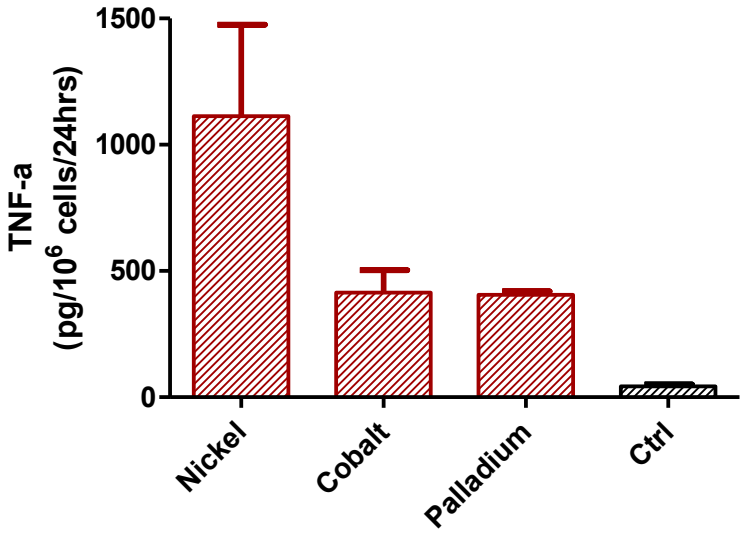

Figure 4.4. HEK-hTLR4 TNF-α cytokine secretion when treated with metal contact allergens Nickel, Cobalt and Palladium. Data represents peak TNF-α secretions only compared to untreated controls (Ctrl) and are presented as the mean ± s.d. of two independent experiments.

CHAPTER V

CHARACTERIZATION OF TWO THP-1 DERIVED CELL LINES CONTAINING NF-κβ OR IL-8 REPORTERS

INTRODUCTION

Based on the results of the previous chapters, it is apparent that all of the fourteen contact allergens we examined have the capacity to activate innate immune cells, as modeled by THP-1. However, only three of these allergens (Nickel, Cobalt, and Palladium) appear to signal through human Toll-like receptor 4. Thus, one of the outstanding questions in the field is: Which receptor(s) mediate activation of THP-1 cells for the remaining eleven contact allergens? To approach this question, it would be valuable to have a high-throughput assay which is both sensitive and economical. A potential solution would be a THP-1 cell line stably transfected with an appropriate promoter:reporter construct.

Background relevant to this project includes work by Viemann and colleagues, which demonstrated that Nickel induces the expression of a variety of cytokine and adhesion molecules via an NF-κβ dependant pathway (Viemann, 2007). Here, the authors found that Nickel mediated signaling could be abrogated through the expression of a dominant negative IKK complex, as detected by qRT-PCR for various targets of NF-κβ transcription, as well as through an NF-κβ:luciferase reporter. In addition, it was shown that Nickel also activates HIF-1α (Hypoxia-Inducible Factor 1-α), independent of NF-κβ

signaling, and HIF-1α was responsible for inducing expression of IL-6 from primary endothelial cells (Viemann, 2007).

Subsequent work by Schmidt and colleagues, also described in previous chapters, indicated that Nickel induces NF-κβ signaling through human Toll-like Receptor 4 (hTLR4) (Schmidt, 2010). This receptor had been previously characterized for its ability to detect lipopolysaccharide (LPS), a component of gram negative bacterial membranes. This is of particular interest because NF-κβ activation is not exclusive to just TLR4 signaling. In fact, the signaling adaptor molecule MyD88, which is upstream of NF-κβ activation, is utilized by nearly all TLRs, with the exception of TLR3 (Kawai, 2006). This raises the idea that NF-κβ may be involved with innate immune cell signaling for other contact allergens.

We then postulated that like Nickel, other contact allergens may similarly signal through the transcription factor NF-κβ, and that this signaling could be detected using an NF-κβ reporter assay. To pursue this idea, a commercially available THP-1 reporter cell line was purchased from Invivogen. This cell line, THP-1 XBlue, is stably transfected with an NF-κβ reporter element upstream of a secreted embryonic alkaline phosphatase (SEAP) reporter. The amount of SEAP that accumulates in the culture supernatant can then be easily quantified by colorimetric analysis using Quanti-Blue medium, as described in the Materials & Methods. In the first half of this chapter, we characterize the behaviour of this THP-1 XBlue cell line using our panel of contact allergens and irritants, over a range of concentrations, in order to assess if this cell line would be suitable for use in future screening studies.

The second part of this chapter describes the construction and characterization of

a THP-1 derived IL-8 reporter cell line. Because IL-8 gave a strong signal both at the transcript and protein levels (based on the results obtained in Chapters III and IV, as well as those described in the literature), this was a logical cytokine promoter to utilize. The reporter gene in this cell line was a secreted *Gaussia* luciferase, the plasmid construct for which was commercially available, complete with the human IL-8 promoter already inserted upstream.

RESULTS AND DISCUSSION

Effects of contact allergens and contact irritants on THP-1 XBlue cells

To establish a time course, six identical cell culture plates containing serial dilutions of the various contact allergens, or contact irritants, were simultaneously seeded with THP-1 XBlue cells. One plate (and all corresponding supernatants) was then harvested at each time point (i.e. 1, 3, 6, 9, 12 and 24 hours) and cell viabilities and SEAP signals were measured. Viability and the SEAP reporter activity curves were plotted, with the data for three representative contact allergens (DCP, Thimerosal, and Nickel) shown in Figure 5.1, and data for all allergens summarized in Table 5.1. For those contact allergens that did elicit SEAP production, this signal was detectable as early as six hours, and was maximal after twenty-four hours. However, for some contact allergens, twelve hours of incubation also gave a near maximal signal (e.g. DCP). Surprisingly, there was no SEAP signal detected from the Nickel treated cells, and as shown in Table 5.1, a similar result was also obtained for six other contact allergens (Cinnamic aldehyde, Citral, Cobalt, Diazolidinyl urea, MCI/MI and Potassium dichromate). All of the allergens tested were cytotoxic at higher concentrations and longer incubation times,

although for incubation times less than six hours these effects were minimal. The LD_{50} values obtained at twenty-four hours are shown in Table 5.1, and these values were similar to those found in Chapter III. As with the cytokine secretion curves shown in Chapter III (Figure 3.1), the SEAP secretion curves also showed a peak production at certain concentration of contact allergen, and decreased at higher concentrations due to the cytotoxicity.

Two different contact irritants (DMSO and SDS) and one oxidative stressor (BSO) were also tested on the THP-1 XBlue cells using the same experimental format, and these results are shown in Figure 5.2, and summarized for all irritants in Table 5.2. Regardless of exposure time, the contact irritants again did not induce appreciable reporter expression, with all SEAP activities less than two-fold over untreated controls. However, since seven of the fourteen contact allergens tested gave a similar result (shown graphically in Figure 5.3), the results for the contact irritants are more challenging to interpret than those described in Chapter III.

Assessment of the results obtained using the THP-1 XBlue assay

In comparison to the results obtained in Chapter III, measuring the robust secretion of proinflammatory cytokines, the THP-1 XBlue reporter cell line failed to perform as expected, with half of the allergens yielding no SEAP reporter expression above untreated controls. This was particularly surprising with regards to Nickel, since previous reports had found that Nickel treatment induced NF-κβ activation (Viemann, 2007; Schmidt, 2010). One possible explanation for these negative results would be that the contact allergens, still present in the harvested supernatants, directly inhibit the

phosphatase activity of the SEAP enzyme, and thus inhibiting the colorimetric change expected. To exclude this possibility, we then incubated the panel of contact allergens, with the exception of DCP as it gave a maximal signal, with a known amount SEAP for twenty-four hours and subsequently assayed for SEAP activity (Table 5.3). Notably, for some of the contact allergens, such as DMDM Hydantoin and Cobalt, moderate to strong inhibition of the SEAP signal was observed, and indeed this may account for the lack of reporter activity from these allergens in the THP-1 XBlue assay. Surprisingly, Nickel showed only minimal inhibition of the SEAP enzyme, and thus phosphatase inhibition cannot explain the complete lack of signal observed in the THP-1 XBlue assay from this otherwise potent contact allergen. A possible explanation, although remote and could not be excluded by our experiments, might be that Nickel interferes with the translation and/or folding of the SEAP reporter enzyme. Thus, inhibition of SEAP enzyme which was produced previously, in the absence of Nickel, would not be affected with subsequent addition of Nickel. In any case, the THP-1 XBlue assay appears to be of limited utility. From our panel, the contact allergens for which it could possibly be used include only DCP, Thimerosal, and DNCB, which resulted in signals greater than 5-fold over untreated controls.

Creation and characterization of a THP-1 IL-8 luciferase reporter cell line

Given the limitations of the NF-κβ based THP-1 XBlue reporter cell line; it was decided to create a new reporter cell line using a plasmid construct purchased from GeneCopoeia. This plasmid contained 1346 bp of DNA upstream of the human IL-8 gene, which was inserted next to encoded secreted *Gaussia* luciferase (Figure 5.4). The

linearized plasmid was electroporated into THP-1 cells and nine different puromycin resistant clones were obtained. The individual clones were expanded and luciferase production was assayed from supernatants following six hour exposure to LPS and to Nickel. The clone which gave the highest signal (25-fold for LPS and 12-fold for Nickel) in these preliminary assays was chosen for cryopreservation and further characterization.

As with previous experiments, the THP-1 IL-8 luciferase reporter cell line was again screened with our panel of contact allergens, over a range of concentrations, for twenty-four hours and luciferase production was measured. Consistent with previous results, peak luciferase secretion was noted at specific concentrations for each contact allergen, and the maximum fold increase and corresponding concentration of allergen where this occurred are summarized in Table 5.4, and shown graphically in Figure 5.5A. Here, only four allergens gave luciferase signals greater than 5-fold over untreated controls (Thimerosal, Nickel, DCP, and MI) and with DNCB giving greater than a 4-fold increase. The highest signal (17.8-fold increase) was obtained for Thimerosal, compared to the 5.5-fold increase obtained with the SEAP based NF-κβ reporter. Interestingly, Nickel did give a strong signal in this THP-1 IL-8 reporter cell line (15.3-fold increase), in contrast to the complete absence of signal in the THP-1 XBlue assay. Three different contact irritants (DMSO, Isopropanol, and SDS) and one oxidative stressor (BSO) were also tested on the THP-1 IL-8 luciferase reporter cell line, and these results are shown in Table 5.5 and Figure 5.5B. Again, these agents did not induce any reporter signal.

Assessment of results obtained using the THP-1 IL-8 luciferase reporter assay

Ultimately, the IL-8 reporter cell line was again unsatisfactory, as we could not

detect any signal from most of the contact allergens tested, including MCI/MI, Citral, DMDM Hydantoin, Potassium Dichromate, Palladium, Cinnamic aldehyde and Diazolidinyl urea. Other allergens displayed only marginal signals (pPD, Cobalt) and these also had a relatively high variance, due in part to the rapid loss of the luciferase signal that is characteristic of the *Gaussia* luciferase reporter system, which needs to be read within five minutes following the addition of luciferase substrate. Low or absent signaling from certain chemicals such as Cobalt, DMDM Hydantoin or pPD may again be due to direct inhibition of the luciferase enzyme (as was observed with SEAP), but this question was not explored further. Contact allergens for which the THP-1 IL-8 luciferase reporter cell line could possibly be used include only Thimerosal, Nickel, DCP, and MI. Here, if this reporter system was to be used in an siRNA screen, Nickel could at least serve as a positive control for siRNA's against hTLR4. Although use of this reporter assay for this purpose is unlikely.

While the IL-8 luciferase assay was more sensitive than the SEAP-based NF-κβ reporter, and was also able to detect Nickel mediated signaling, it did not parallel the impressive fold increases obtained when measuring IL-8 protein directly. While luciferase reporter inhibition may be occurring for some allergens, this probably does not fully account for the overall lack of sensitivity observed. Therefore, we investigated the literature regarding regulatory elements within the IL-8 promoter for an explanation for the poor performance of the IL-8 luciferase reporter assay. One report which looked at various truncations of the IL-8 promoter, showed that there are 4 regulatory binding sequences, all of which are within 400bp of the transcriptional start site (TSS) (Kikuchi, 2002). Further, the AP-1 and NF-κβ regulatory elements, which were found to be

essential in mediating transcription following LPS treatment, are within 130bp of the TSS, and truncations upstream of this point had little effect on the LPS response (Kikuchi, 2002). In fact, shortening the promoter from 1481bp to 335bp slightly increased promoter efficiency, suggesting the presence of either negative regulatory elements or simply that excess sequence may decrease promoter efficiency. Therefore, it seems likely that the 1349bp promoter sequence that was used in our IL-8 reporter cell line was not missing essential elements of the native IL-8 promoter, and thus not responsible for the lack of luciferase signaling seen for many of the contact allergens. However, it is worth noting that in the report describing various truncations of the IL-8 promoter, all lengths of the promoter:reporter clones were also much less sensitive when compared to measuring the IL-8 protein via ELISA (Kikuchi, 2002).

Overall, based on the results obtained in this Chapter, it would appear that the construction and use of reporter cell lines to characterize innate immune signaling pathways used by contact allergens is not a trivial matter. However, future experimentation, which measures IL-8 protein secretion directly from THP-1, for the purposes of siRNA screens or related approaches to investigate novel signaling pathways should prove to be successful.

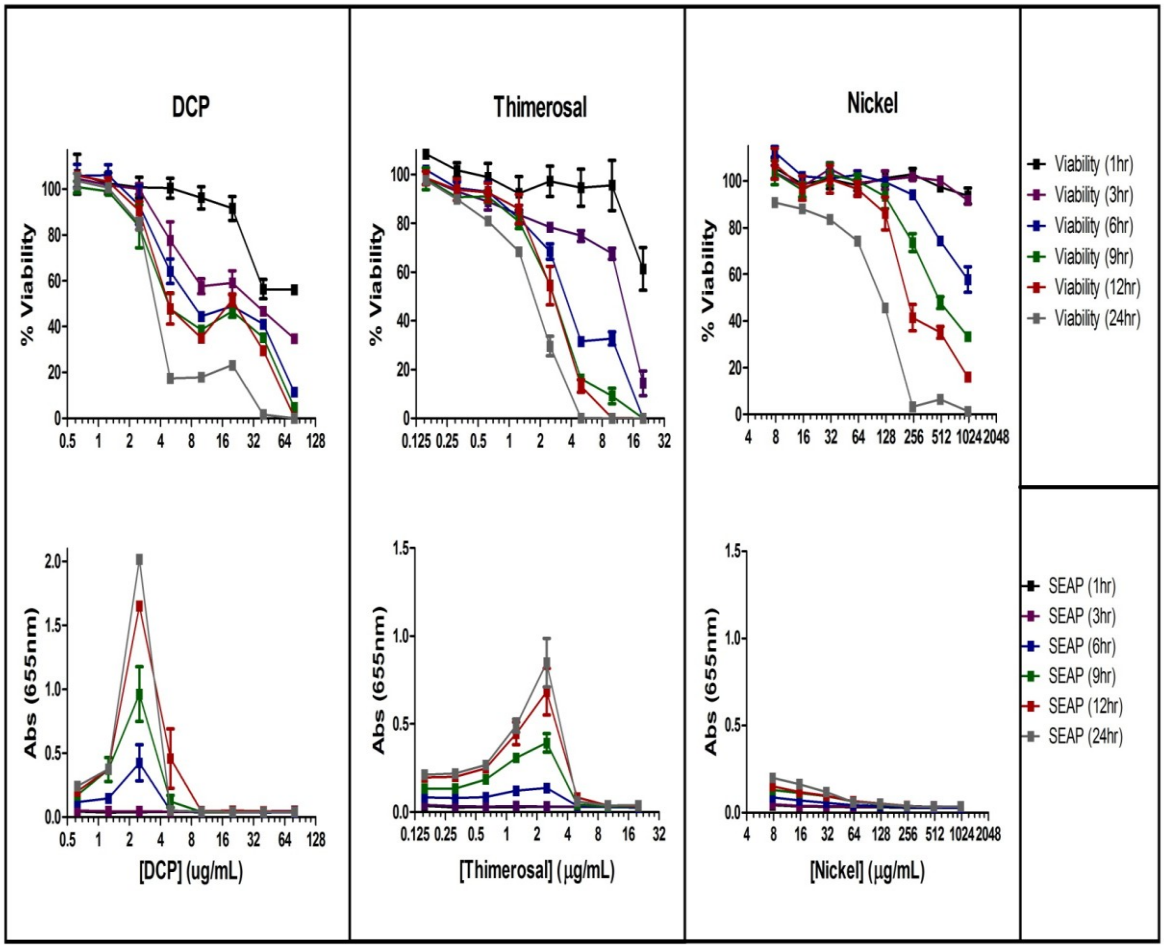

Figure 5.1. Viability and NF-κβ SEAP reporter characterization for three representative contact allergens. Following twenty-four hours exposure of THP-1 XBlue to contact allergens Diphenylcyclopropenone, Thimerosal and Nickel, supernatants were analyzed for secreted embryonic alkaline phosphatase reporter activity and viability assessment was also performed. Data are presented as the mean ± s.d. of two independent experiments.

Table 5.1. THP-1 XBlue LD_{50} and NF-κβ SEAP reporter expression for selected contact allergens

Contact Allergen (on THP-1 XBlue)	SEAP Reporter Fold Increase	Conc. Of Allergen giving Max. SEAP Reporter	Estimated LD_{50}
2,4-Dinitrochlorobenzene	5.1	6.2 μM (1.3 μg/ml)	13.3 μM (2.7 μg/ml)
Bronopol	2.2	62.5 μM (12.5 μg/ml)	92.5 μM (18.5 μg/ml)
Cinnamic Aldehyde	1.1	199 μM (25.0 ppm)	298 μM (37.5 ppm)
Citral	0.7	147 μM (25.0 ppm)	141 μM (24.0 ppm)
Cobalt chloride	1.0	1051 μM (250 μg/ml)	736 μM (175 μg/ml)
Diazolidinyl urea	1.9	89.9 μM (25.0 μg/ml)	64.7 μM (18.0 μg/ml)
Dimethyl Fumarate	4.0	43.4 μM (6.3 μg/ml)	164 μM (23.6 μg/ml)
Diphenylcyclopropenone	13.2	12.1 μM (2.5 μg/ml)	17.0 μM (3.5 μg/ml)
DMDM Hydantoin	2.5	133 μM (25.0 ppm)	59.0 μM (11.1 ppm)
Methylchloroisothiazolinone / Methylisothiazolinone	1.2	18.0 μM (2.5 ppm)	31.7 μM (4.4 ppm)
Nickel chloride	1.3	1052 μM (250 μg/ml)	475 μM (113 μg/ml)
Potassium dichromate	1.2	17.0 μM (5.0 μg/ml)	4.8 μM (1.4 μg/ml)
p-Phenylenediamine	4.2	2312 μM (250 μg/ml)	296 μM (32.0 μg/ml)
Thimerosal	5.5	15.4 μM (6.3 μg/ml)	4.2 μM (1.7 μg/ml)
Medium only	1.0*	-----	------

Data are presented as SEAP reporter fold increases from two independent experiments. *Fold Increase vs. untreated controls (medium only).

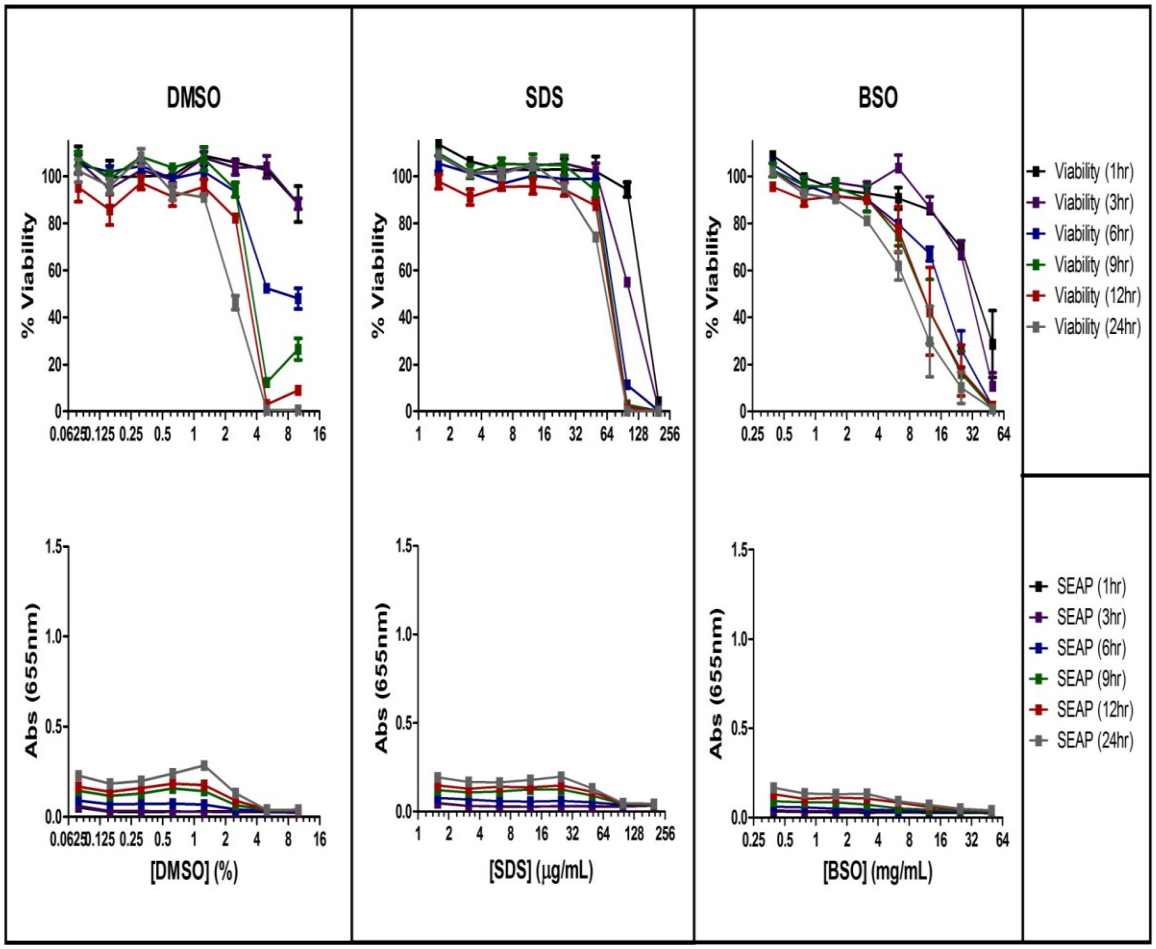

Figure 5.2. Viability and NF-κβ SEAP reporter characterization for two representative contact irritants and one oxidative stressor. Following twenty-four hours exposure of THP-1 XBlue to contact irritants DMSO, SDS and oxidative stressor Buthionine sulfoximine, supernatants were analyzed for secreted embryonic alkaline phosphatase reporter activity and viability assessment was also performed. Data are presented as the mean ± s.d. of two independent experiments.

Table 5.2. THP-1 XBlue LD_{50} and NF-κβ SEAP reporter expression for selected contact irritants and one oxidative stressor

Contact Irritant (on THP-1 XBlue)	SEAP Reporter Fold Increase	Conc. Of Allergen giving Max. SEAP Reporter	Estimated LD_{50}
Buthionine sulfoximine	1.1	1.8 mM (0.4 mg/ml)	36.9 mM (8.2 mg/ml)
DMSO	1.9	1.3 %	2.4 %
Isopropanol	1.1	0.2 %	3.3 %
SDS	1.3	86.7 μM (25.0 μg/ml)	215 μM (62.0 μg/ml)
Tween 80	0.5	59.6 μM (78.1 μg/ml)	1.3 mM (1.7 mg/ml)
Medium only	1.0*	-----	------

Data are presented as SEAP reporter fold increases from two independent experiments. *Fold Increase vs. untreated controls (medium only).

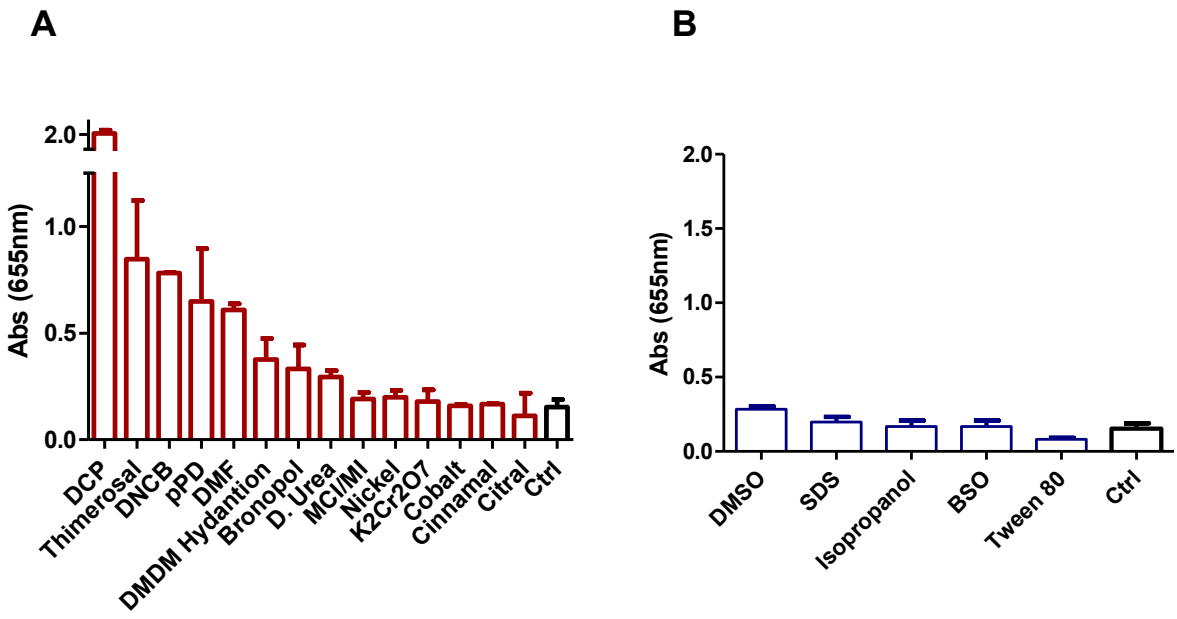

Figure. 5.3. THP-1 XBlue NF-κβ SEAP reporter characterization following exposure to selected contact allergens and irritants. THP-1 XBlue cell culture supernatants were harvested after exposure to a panel of contact allergens (A) or contact irritants (B) for twenty-four hours and the amount of secreted embryonic alkaline phosphatase reporter was quantified and compared relative to untreated controls (Ctrl). Data represents peak SEAP reporter secretions only and are presented as the mean ± s.d. of two independent experiments.

Table 5.3. Inhibition of the secreted embryonic alkaline phosphatase (SEAP) reporter

Chemical (on THP-1 XBlue)	SEAP Inhibition	Chemical (on THP-1 XBlue)	SEAP Inhibition
2,4-Dinitrochlorobenzene	-	DMDM Hydantoin	++
Bronopol	-	Methylchloroisothiazolinone / Methylisothiazolinone	-
Cinnamic Aldehyde	+	Nickel chloride	+
Citral	-	Potassium dichromate	-
Cobalt chloride	+++	p-Phenylenediamine	+
Diazolidinyl urea	-	Thimerosal	-
Dimethyl Fumarate	-	Medium only	-

The activity of a previously quantified amount of SEAP reporter enzyme was assessed in the presence of selected contact allergens and compared relative to medium only treated controls. Data represents NF-κβ SEAP reporter inhibitions using maximal contact allergen concentrations and represents two independent experiments. (100-90% Activity); + (90-70% Activity); ++ (70-50% Activity); +++ (< 50% Activity).

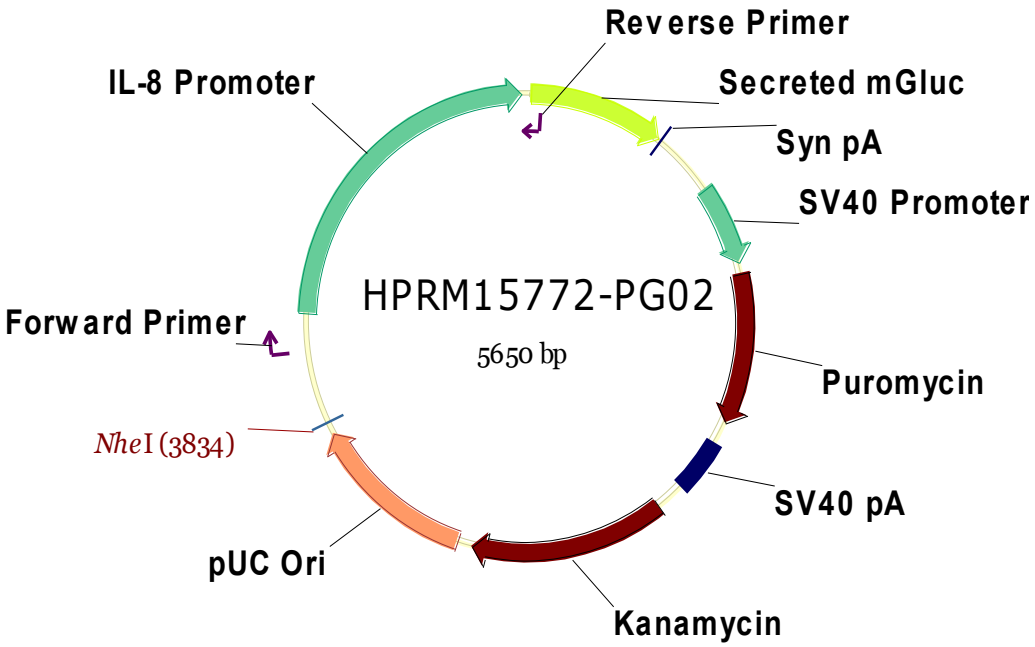

Figure 5.4. Annotation of the IL-8 luciferase reporter plasmid HPRM15772-PG02. The NheI (3834) cut-site, which was used to linearize the plasmid prior to transfection in generating a stable transfectant, is also shown.

Table 5.4. THP-1 IL-8 luciferase reporter induction for selected contact allergens

Contact Allergen (on THP-1 IL-8)	IL-8 Luciferase Fold Increase	Conc. Of Allergen giving Max. Luciferase Reporter
2,4-Dinitrochlorobenzene	4.2	6.2 μM (1.3 μg/ml)
Cinnamic Aldehyde	1.1	24.8 μM (3.1 ppm)
Citral	0.9	36.7 μM (6.3 ppm)
Cobalt chloride	2.4	263 μM (62.5 μg/ml)
Diazolidinyl urea	1.0	11.2 μM (3.1 μg/ml)
Diphenylcyclopropenone	6.8	12.1 μM (2.5 μg/ml)
DMDM Hydantoin	0.9	8.3 μM (1.6 ppm)
Methylchloroisothiazolinone / Methylisothiazolinone	0.8	4.5 μM (0.6 ppm)
Methylisothiazolinone	6.7	54.3 μM (6.3 ppm)
Nickel chloride	15.3	1052 μM (250 μg/ml)
Palladium chloride	1.0	176 μM (31.3 μg/ml)
Potassium dichromate	1.0	17.0 μM (5.0 μg/ml)
p-Phenylenediamine	1.7	462 μM (50.0 μg/ml)
Thimerosal	17.8	3.1 μM (1.3 μg/ml)
Medium only	1.0*	-----

Data are presented as reporter fold increases from two independent experiments. *Fold Increase vs. untreated controls (medium only).

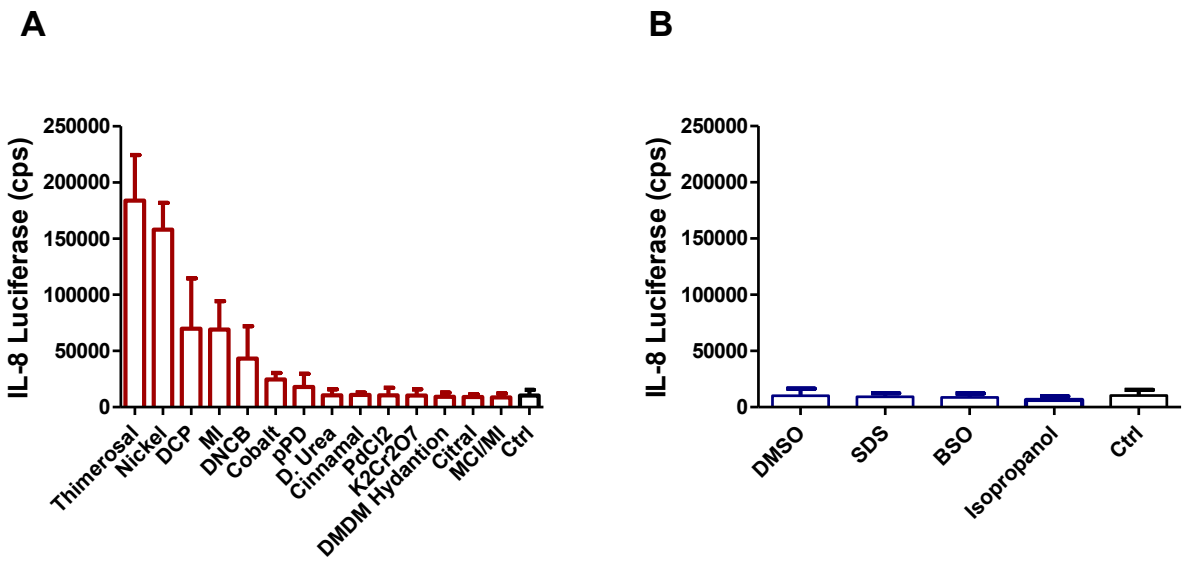

Figure 5.5. THP-1 IL-8 luciferase reporter characterization following exposure to selected contact allergens and irritants. THP-1 IL-8 monocyte cell culture supernatants were harvested after exposure to a panel of contact allergens (A) or contact irritants (B) for twenty-four hours and the amount of luciferase reporter was quantified and compared relative to untreated controls (Ctrl). Data represents peak luciferase reporter signals only and are presented as the mean ± s.d. of two independent experiments.

Table 5.5. THP-1 IL-8 luciferase reporter induction for selected contact irritants and one oxidative stressor

Contact Allergen (on THP-1 IL-8)	**IL-8 Luciferase Fold Increase**	**Conc. Of Irritant giving Max. Luciferase Reporter**
Buthionine sulfoximine	0.8	14.1 mM (3.1 mg/ml)
DMSO	1.0	0.6 %
Isopropanol	0.6	0.6 %
SDS	0.9	173 μM (50.0 μg/ml)
Medium only	1.0*	-----

Data are presented as reporter fold increases from two independent experiments. *Fold Increase vs. untreated controls (medium only).

CHAPTER VI

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The properties which render a chemical benign, irritating or reactive to the extent of producing an allergic response are complex. Characterizing this phenomena becomes increasingly difficult when also considering that not every person is allergic to a given contact allergen. While it is true that certain genetic factors exist which may predispose an individual to developing allergic contact dermatitis toward a specific chemical, evidence has yet to be found correlating HLA haplotypes with ACD. Therefore, individual susceptibility may often be attributed to whether or not sufficient sensitization conditions are met. These conditions may include never encountering a specific contact allergen, or coming in contact with an insufficient concentration of allergen to penetrate the skin and initiate sensitization. It is also possible to theorize that sensitization in some individuals could have occurred due to confounding conditions at the time of contact, such as coincidental infection or a damaged skin barrier. In either case, sensitization may have then occurred at concentrations of allergen which would have otherwise not resulted in the development of an allergic response.

Regardless of the potential genetic and idiopathic conditions which may aid in the development of contact allergies, the results of these investigations overall highlight the intimate roll between contact allergens and the innate immune system, which also support the recent trend of increasing interest to examine the role of innate immunity in Allergic

Contact Dermatitis. More specifically, we have found that every contact allergen tested resulted in robust secretion of inflammatory cytokines, including TNF-α, IL-6, IL-1β and most potently, IL-8. In contrast, treatment of the same model cell lines with all contact irritants resulted in only minimal or no inflammatory cytokine release. These results further speak to the idea that contact irritants can produce skin irritation, which clinically, can appear quite similar to the inflammation noted from allergic reactions. However, these irritation symptoms are usually distinguished as being short lived, without memory, and requiring a significantly higher dose of chemical to elicit inflammation compared to that of an allergic contact dermatitis reaction. One basic future direction would then be to characterize additional contact allergens and contact irritants using the THP-1 cytokine assay. A larger sample size of both groups would add further confidence to our claims and support the THP-1 cytokine release assay as a suitable model of innate immune cell activation by contact allergens. Such results would also support the idea that this assay could be used to characterize the sensitization potential of new and uncharacterized chemicals.

Another interesting finding from the THP-1 cytokine-based assay was the increased potency of Methylisothiazolinone (MI) compared to the mixture of Methylchloroisothiazolinone and Methylisothiazolinone (MCI/MI). Previously, consumer products contained these contact allergens in a 3:1 ratio of MCI:MI, a preservative blend commercially known as Kathon CG. However, just in the last few years, Methylisothiazolinone has been approved for use without Methylchloroisothiazolinone, as it was thought it may reduce the incidence of allergic contact reactions. Unfortunately, the exact opposite is now being observed, as rates of both MCI/MI and MI contact

allergies are increasing, even being described as a 'new epidemic' and MI was the American Contact Dermatitis Society 2013 contact allergen of the year (Scherrer, 2014; Urwin 2013; Castanedo-Tardana, 2013). Therefore, it is interesting that the clinical patch-test data from the general public parallels our results, as MI alone elicited nearly 4-fold higher TNF-α and IL-8 productions, compared to the MCI/MI mixture. Thus, this data may in part provide an explanation for these recent clinical observations.

While the specific receptor pathways which signal the presence of the vast majority of contact allergens remain unknown, we did attempt to further characterize the role of human Toll-like receptor 4 in the signaling of other, non-Nickel, contact allergens. While we were unknowingly in competition to publish results with groups in Germany and the Netherlands, we independently found that Cobalt and Palladium also signal through hTLR4. As the signaling of these metal contact allergens can probably be explained through a model in which the dimerization of hTLR4 receptors occur through the coordination between non-conserved histidine residues found in human, but not mouse, TLR4 (as described by *Schmidt et al.)*; it raises the question regarding the effects of polymorphisms in hTLR4 and the ability to become sensitized to Nickel. Toll-like receptor 4 has been shown to be highly polymorphic and these polymorphisms have already been implicated in moderating the immune responses to bacterial infections, and may be involved in a variety of other conditions such as periodontal disease, cardiac disease, asthma and diabetic neuropathy (as reviewed by Noreen, 2012). Therefore, one could conceive a future project hypothesis where polymorphisms resulting in the loss or change of one or more of these histidine residues at amino acid positions 431, 456 and 458 of hTLR4 may confer protection against Nickel allergies. Conversely, perhaps a

substitution of histidine residues within this region would allow for more efficient coordination between Nickel (or Cobalt and Palladium) and the hTLR4 dimers, rendering affected individuals even more susceptible to Nickel allergy.

We then attempted to find a THP-1 reporter assay which would be both sensitive and economical for future projects to investigate alternate signaling pathways besides hTLR4. Here we characterized a commercially available THP-1 based NF-κβ reporter assay (Invivogen) and created our own stably transfected THP-1 cell line with an IL-8 promoter:luciferase reporter construct (Genecopoeia). Ultimately, both reporter cell lines proved orders of magnitude less sensitive than measuring cytokine protein directly. Moreover, both cell lines could only reliably show THP-1 activation for a fraction of our contact allergen panel and therefore are of limited use. However, while measuring inflammatory cytokine release in the supernatant is more expensive and data acquisition is less rapid than the reporter cell lines we tried to create/characterize, this approach has the advantage of superior sensitivity. This will allow for more confident interpretation of data considering the typical background noise which occurs with RNAi or receptor-blockade experiments. Particularly interesting was a recent publication which looked at genome-wide transcriptional regulation following treatment by twenty contact allergens and contact irritants (Johansson, 2011). Here, a biomarker index was created of the top two hundred genes whose expression was regulated by contact allergens, but not by irritants. Among these included both human TLR6 and TLR9, and thus both of these would make interesting targets for a future RNAi or a receptor-blockade project. Further, while the NF-κβ reporter THP-1 XBlue cell line was not sensitive enough to be useful, and could not detect activation following Nickel exposure (in contrast to some previous

reports), the concept of exploiting NF-κβ activation, which is common to nearly all TLRs, remains valid. In fact, Invivogen has recently developed a luciferase-based NF-κβ reporter, also within THP-1 (THP-1 Lucia), and it would be interesting to see if NF-κβ activation could be readily detected from this reporter while also avoiding the issue of SEAP inhibition entirely. Another approach, which would be cheaper and faster than purchasing and characterizing yet another cell line, would be to detect NF-κβ subunit activation from our existing THP-1 cytokine model in an ELISA-like, plate-based assay, which is also commercially available.

Lastly, our data revealed that all contact allergens tested were cytotoxic and that the peak cytokine release or reporter activity observed was often at near toxic concentrations (LD_{50} values), presumably indicating increased activation with increased dose, until an extreme loss of viability resulted in an absence of cytokine release. However, the mechanism of cell death for most these contact allergens, whether via an apoptotic mechanism or simple necrosis, is unknown. While we generally look at apoptosis and the phagocytosis of apoptotic bodies as an immunosuppressive or anti-inflammatory process, it has been shown that the phagocytosis of apoptotic cells in the presence of LPS (a TLR4 ligand) generates both TGF-β and IL-6 innate immune responses and together mediate T_H17 cell subset differentiation (Torchinsky, 2009). Therefore, elucidation of the mechanism of cell death caused by a variety of contact allergens, which also possess the ability to stimulate innate immune cells (even through TLR4 as with Nickel, Cobalt and Palladium), should shed light on the immune mechanisms occurring in the skin following exposure to contact allergens. To determine the mechanism of cell death, specifically if apoptosis is involved, there are several well

documented procedures with corresponding commercially available kits. For example, viability loss and apoptosis induction could be monitored using an annexin V/Alexa Fluor488 flow cytometry staining kit (Invitrogen), coupled with a plate based assay to detect activation of executioner caspases 3 and 7 (Promega) to confirm results. Further, as the contact allergen concentrations corresponding to LD_{50} values have already been determined for our panel of allergens, these assays could be readily conducted with THP-1 and would require minimal assay condition optimization. If cell death does occur by apoptosis, this might explain in part why neutrophils are a prominent cell type present in the acute lesions of allergic contact dermatitis, reflecting a T_H17 response.

REFERENCES

Acuto, O., & Michel, F. (2003). CD28-mediated co-stimulation: A quantitative support for TCR signalling. *Nature Reviews.Immunology, 3*(12), 939-951.

Albanesi, C., Cavani, A., & Girolomoni, G. (1999). IL-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: Synergistic or antagonist effects with IFN-gamma and TNF-alpha. *Journal of Immunology (Baltimore, Md.: 1950), 162*(1), 494-502.

Alvarez-Sanchez, R., Basketter, D., Pease, C., & Lepoittevin, J. P. (2003). Studies of chemical selectivity of hapten, reactivity, and skin sensitization potency. 3. synthesis and studies on the reactivity toward model nucleophiles of the 13C-labeled skin sensitizers, 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI). *Chemical Research in Toxicology, 16*(5), 627-636.

Alvarez-Sanchez, R., Basketter, D., Pease, C., & Lepoittevin, J. P. (2004). Covalent binding of the 13C-labeled skin sensitizers 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI) to a model peptide and glutathione. *Bioorganic & Medicinal Chemistry Letters, 14*(2), 365-368.

Alvarez-Sanchez, R., Divkovic, M., Basketter, D., Pease, C., Panico, M., Dell, A., Morris, H., & Lepoittevin, J. P. (2004). Effect of glutathione on the covalent binding of the 13C-labeled skin sensitizer 5-chloro-2-methylisothiazol-3-one to human serum albumin: Identification of adducts by nuclear magnetic resonance, matrix-assisted laser desorption/ionization mass spectrometry, and nanoelectrospray tandem mass spectrometry. *Chemical Research in Toxicology, 17*(9), 1280-1288.

American Contact Dermatitis Society. ACDS allergens of the Year. Retrieved 2014, from http://www.contactderm.org/i4a/pages/index.cfm?pageid=3467

Asherson, G. L., Dieli, F., Sireci, G., & Salerno, A. (1996). Role of IL-4 in delayed type hypersensitivity. *Clinical and Experimental Immunology, 103*(1), 1-4.

Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., & Toyoda, H. (2006). Development of an in vitro skin sensitization test using human cell lines: The human cell line activation test (h-CLAT). I. optimization of the h-CLAT protocol. *Toxicology in Vitro : An International Journal Published in Association with BIBRA, 20*(5), 767-773.

Berg, D. J., Leach, M. W., Kuhn, R., Rajewsky, K., Muller, W., Davidson, N. J., & Rennick, D. (1995). Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *The Journal of Experimental Medicine, 182*(1), 99-108.

Bordel-Gomez, M. T., Miranda-Romero, A., & Castrodeza-Sanz, J. (2008). Isolated and concurrent prevalence of sensitization to transition metals in a spanish population. *Journal of the European Academy of Dermatology and Venereology : JEADV, 22*(12), 1452-1457.

Bos, J. D., & Meinardi, M. M. (2000). The 500 dalton rule for the skin penetration of chemical compounds and drugs. *Experimental Dermatology, 9*(3), 165-169.

Bryce, P. J., Miller, M. L., Miyajima, I., Tsai, M., Galli, S. J., & Oettgen, H. C. (2004). Immune sensitization in the skin is enhanced by antigen-independent effects of IgE. *Immunity, 20*(4), 381-392.

Budinger, L., & Hertl, M. (2000). Immunologic mechanisms in hypersensitivity reactions to metal ions: An overview. *Allergy, 55*(2), 108-115.

Carroll, J. M., Crompton, T., Seery, J. P., & Watt, F. M. (1997). Transgenic mice expressing IFN-gamma in the epidermis have eczema, hair hypopigmentation, and hair loss. *The Journal of Investigative Dermatology, 108*(4), 412-422.

Castanedo-Tardana, M. P., & Zug, K. A. (2013). Methylisothiazolinone. *Dermatitis : Contact, Atopic, Occupational, Drug, 24*(1), 2-6.

Cavani, A., Mei, D., Guerra, E., Corinti, S., Giani, M., Pirrotta, L., Puddu, P., & Girolomoni, G. (1998). Patients with allergic contact dermatitis to nickel and nonallergic individuals display different nickel-specific T cell responses. evidence for the presence of effector CD8+ and regulatory CD4+ T cells. *The Journal of Investigative Dermatology, 111*(4), 621-628.

Chong, S. Z., Tan, K. W., Wong, F. H., Chua, Y. L., Tang, Y., Ng, L. G., Angeli, V., & Kemeny, D. M. (2014). CD8 T cells regulate allergic contact dermatitis by modulating CCR2-dependent TNF/iNOS-expressing Ly6C+ CD11b+ monocytic cells. *The Journal of Investigative Dermatology, 134*(3), 666-676.

Cumberbatch, M., Dearman, R. J., & Kimber, I. (1997). Langerhans cells require signals from both tumour necrosis factor-alpha and interleukin-1 beta for migration. *Immunology, 92*(3), 388-395.

Cumberbatch, M., Griffiths, C. E., Tucker, S. C., Dearman, R. J., & Kimber, I. (1999). Tumour necrosis factor-alpha induces langerhans cell migration in humans. *The British Journal of Dermatology, 141*(2), 192-200.

de Jongh, C. M., Verberk, M. M., Spiekstra, S. W., Gibbs, S., & Kezic, S. (2007). Cytokines at different stratum corneum levels in normal and sodium lauryl sulphate-irritated skin. *Skin Research and Technology : Official Journal of International Society for Bioengineering and the Skin (ISBS) [and] International*

Society for Digital Imaging of Skin (ISDIS) [and] International Society for Skin Imaging (ISSI), 13(4), 390-398.

Dimitrov, S. D., Low, L. K., Patlewicz, G. Y., Kern, P. S., Dimitrova, G. D., Comber, M. H., Phillips, R. D., Niemela, J., Bailey, P. T., & Mekenyan, O. G. (2005). Skin sensitization: Modeling based on skin metabolism simulation and formation of protein conjugates. *International Journal of Toxicology, 24*(4), 189-204.

Effendy, I., Loffler, H., & Maibach, H. I. (2000). Epidermal cytokines in murine cutaneous irritant responses. *Journal of Applied Toxicology : JAT, 20*(4), 335-341.

Elias, P. M. (2005). Stratum corneum defensive functions: An integrated view. *The Journal of Investigative Dermatology, 125*(2), 183-200.

Elias, P. M. (2007). The skin barrier as an innate immune element. *Seminars in Immunopathology, 29*(1), 3-14.

Enk, A. H., & Katz, S. I. (1992). Identification and induction of keratinocyte-derived IL-10. *Journal of Immunology (Baltimore, Md.: 1950), 149*(1), 92-95.

Epling, G. A., Wells, J. L., & Yoon, U. C. (1988). Photochemical transformations in salicylanilide photoallergy. *Photochemistry and Photobiology, 47*(2), 167-171.

Esser, P. R., Wolfle, U., Durr, C., von Loewenich, F. D., Schempp, C. M., Freudenberg, M. A., Jakob, T., & Martin, S. F. (2012). Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PloS One, 7*(7), e41340.

Fallon, P. G., Sasaki, T., Sandilands, A., Campbell, L. E., Saunders, S. P., Mangan, N. E., Callanan, J. J., Kawasaki, H., Shiohama, A., Kubo, A., Sundberg, J. P., Presland, R. B., Fleckman, P., Shimizu, N., Kudoh, J., Irvine, A. D., Amagai, M., & McLean, W. H. (2009). A homozygous frameshift mutation in the mouse flg gene facilitates enhanced percutaneous allergen priming. *Nature Genetics, 41*(5), 602-608.

Faurschou, A., Menne, T., Johansen, J. D., & Thyssen, J. P. (2011). Metal allergen of the 21st century--a review on exposure, epidemiology and clinical manifestations of palladium allergy. *Contact Dermatitis, 64*(4), 185-195.

Ferguson, T. A., Dube, P., & Griffith, T. S. (1994). Regulation of contact hypersensitivity by interleukin 10. *The Journal of Experimental Medicine, 179*(5), 1597-1604.

Franot, C., Benezra, C., & Lepoittevin, J. P. (1993). Synthesis and interaction studies of 13C labeled lactone derivatives with a model protein using 13C NMR. *Bioorganic & Medicinal Chemistry, 1*(5), 389-397.

Fuhlbrigge, R. C., Kieffer, J. D., Armerding, D., & Kupper, T. S. (1997). Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. *Nature, 389*(6654), 978-981.

Gober, M. D., Fishelevich, R., Zhao, Y., Unutmaz, D., & Gaspari, A. A. (2008). Human natural killer T cells infiltrate into the skin at elicitation sites of allergic contact dermatitis. *The Journal of Investigative Dermatology, 128*(6), 1460-1469.

Goebeler, M., Meinardus-Hager, G., Roth, J., Goerdt, S., & Sorg, C. (1993). Nickel chloride and cobalt chloride, two common contact sensitizers, directly induce expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM-1) by endothelial cells. *The Journal of Investigative Dermatology, 100*(6), 759-765.

Grimbaldeston, M. A., Nakae, S., Kalesnikoff, J., Tsai, M., & Galli, S. J. (2007). Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nature Immunology, 8*(10), 1095-1104.

Gunn, M. D., Tangemann, K., Tam, C., Cyster, J. G., Rosen, S. D., & Williams, L. T. (1998). A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America, 95*(1), 258-263.

He, D., Wu, L., Kim, H. K., Li, H., Elmets, C. A., & Xu, H. (2006). CD8+ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. *Journal of Immunology (Baltimore, Md.: 1950), 177*(10), 6852-6858.

Hoath, S. B., & Leahy, D. G. (2003). The organization of human epidermis: Functional epidermal units and phi proportionality. *The Journal of Investigative Dermatology, 121*(6), 1440-1446.

Illing, P. T., Vivian, J. P., Dudek, N. L., Kostenko, L., Chen, Z., Bharadwaj, M., Miles, J. J., Kjer-Nielsen, L., Gras, S., Williamson, N. A., Burrows, S. R., Purcell, A. W., Rossjohn, J., & McCluskey, J. (2012). Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature, 486*(7404), 554-558.

Jakob, T., Ring, J., & Udey, M. C. (2001). Multistep navigation of Langerhans/dendritic cells in and out of the skin. *The Journal of Allergy and Clinical Immunology, 108*(5), 688-696.

Jakob, T., & Udey, M. C. (1998). Regulation of E-cadherin-mediated adhesion in langerhans cell-like dendritic cells by inflammatory mediators that mobilize langerhans cells in vivo. *Journal of Immunology (Baltimore, Md.: 1950), 160*(8), 4067-4073.

Johansson, H., Lindstedt, M., Albrekt, A. S., & Borrebaeck, C. A. (2011). A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. *BMC Genomics, 12*, 399-2164-12-399.

Kalish, R. S. (1990). The use of human T-lymphocyte clones to study T-cell function in allergic contact dermatitis to urushiol. *The Journal of Investigative Dermatology, 94*(6 Suppl), 108S-111S.

Kang, K., Kubin, M., Cooper, K. D., Lessin, S. R., Trinchieri, G., & Rook, A. H. (1996). IL-12 synthesis by human langerhans cells. *Journal of Immunology (Baltimore, Md.: 1950), 156*(4), 1402-1407.

Kaplan, D. H., Igyarto, B. Z., & Gaspari, A. A. (2012). Early immune events in the induction of allergic contact dermatitis. *Nature Reviews.Immunology, 12*(2), 114-124.

Kawai, T., & Akira, S. (2006). TLR signaling. *Cell Death and Differentiation, 13*(5), 816-825.

Kikuchi, T., Hagiwara, K., Honda, Y., Gomi, K., Kobayashi, T., Takahashi, H., Tokue, Y., Watanabe, A., & Nukiwa, T. (2002). Clarithromycin suppresses lipopolysaccharide-induced interleukin-8 production by human monocytes through AP-1 and NF-kappa B transcription factors. *The Journal of Antimicrobial Chemotherapy, 49*(5), 745-755.

Kimber, I., & Cumberbatch, M. (1992). Stimulation of langerhans cell migration by tumor necrosis factor alpha (TNF-alpha). *The Journal of Investigative Dermatology, 99*(5), 48S-50S.

Kish, D. D., Li, X., & Fairchild, R. L. (2009). CD8 T cells producing IL-17 and IFN-gamma initiate the innate immune response required for responses to antigen skin challenge. *Journal of Immunology (Baltimore, Md.: 1950), 182*(10), 5949-5959.

Larsen, J. M., Bonefeld, C. M., Poulsen, S. S., Geisler, C., & Skov, L. (2009). IL-23 and T(H)17-mediated inflammation in human allergic contact dermatitis. *The Journal of Allergy and Clinical Immunology, 123*(2), 486-492.

Lepoittevin, J. P. (2006). Metabolism versus chemical transformation or pro- versus prehaptens? *Contact Dermatitis, 54*(2), 73-74.

Lepoittevin, J., & Benezra, C. (1992). 13C-enriched methyl alkanesulfonates: New lipophilic methylating agents for the identification of nucleophilic amino acids of proteins by NMR. *Tetrahedron Letters, 33*(27), 3875-3878.

Loser, K., Mehling, A., Loeser, S., Apelt, J., Kuhn, A., Grabbe, S., Schwarz, T., Penninger, J. M., & Beissert, S. (2006). Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells. *Nature Medicine, 12*(12), 1372-1379.

Ma, J., Wang, J. H., Guo, Y. J., Sy, M. S., & Bigby, M. (1994). In vivo treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal langerhans cells to regional lymph nodes. *Cellular Immunology, 158*(2), 389-399.

Macatonia, S. E., Knight, S. C., Edwards, A. J., Griffiths, S., & Fryer, P. (1987). Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. functional and morphological studies. *The Journal of Experimental Medicine, 166*(6), 1654-1667.

Martin, S. F., Dudda, J. C., Bachtanian, E., Lembo, A., Liller, S., Durr, C., Heimesaat, M. M., Bereswill, S., Fejer, G., Vassileva, R., Jakob, T., Freudenberg, N., Termeer, C. C., Johner, C., Galanos, C., & Freudenberg, M. A. (2008). Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. *The Journal of Experimental Medicine, 205*(9), 2151-2162.

McFadden, J. P., & Basketter, D. A. (2000). Contact allergy, irritancy and 'danger'. *Contact Dermatitis, 42*(3), 123-127.

Moed, H., Boorsma, D. M., Stoof, T. J., von Blomberg, B. M., Bruynzeel, D. P., Scheper, R. J., . . . Rustemeyer, T. (2004). Nickel-responding T cells are CD4+ CLA+ CD45RO+ and express chemokine receptors CXCR3, CCR4 and CCR10. *The British Journal of Dermatology, 151*(1), 32-41.

Muris, J., Kleverlaan, C. J., Feilzer, A. J., & Rustemeyer, T. (2008). Sodium tetrachloropalladate (Na2[PdCl4]) as an improved test salt for palladium allergy patch testing. *Contact Dermatitis, 58*(1), 42-46.

Nakamura, T., Lee, R. K., Nam, S. Y., Podack, E. R., Bottomly, K., & Flavell, R. A. (1997). Roles of IL-4 and IFN-gamma in stabilizing the T helper cell type 1 and 2 phenotype. *Journal of Immunology (Baltimore, Md.: 1950), 158*(6), 2648-2653.

Nasir, A., Ferbel, B., Salminen, W., Barth, R. K., & Gaspari, A. A. (1994). Exaggerated and persistent cutaneous delayed-type hypersensitivity in transgenic mice whose epidermal keratinocytes constitutively express B7-1 antigen. *The Journal of Clinical Investigation, 94*(2), 892-898.

Nataf, S., Davoust, N., Ames, R. S., & Barnum, S. R. (1999). Human T cells express the C5a receptor and are chemoattracted to C5a. *Journal of Immunology (Baltimore, Md.: 1950), 162*(7), 4018-4023.

Nieuwenhuis, P., & Ford, W. L. (1976). Comparative migration of B- and T-lymphocytes in the rat spleen and lymph nodes. *Cellular Immunology, 23*(2), 254-267.

Nishibu, A., Ward, B. R., Boes, M., & Takashima, A. (2007). Roles for IL-1 and TNFalpha in dynamic behavioral responses of langerhans cells to topical hapten application. *Journal of Dermatological Science, 45*(1), 23-30.

Norcross, M. A., Luo, S., Lu, L., Boyne, M. T., Gomarteli, M., Rennels, A. D., Woodcock, J., Margulies, D. H., McMurtrey, C., Vernon, S., Hildebrand, W. H., & Buchli, R. (2012). Abacavir induces loading of novel self-peptides into HLA-B*57: 01: An autoimmune model for HLA-associated drug hypersensitivity. *AIDS (London, England), 26*(11), F21-9.

Noreen, M., Shah, M. A., Mall, S. M., Choudhary, S., Hussain, T., Ahmed, I., Jalil, S. F., & Raza, M. I. (2012). TLR4 polymorphisms and disease susceptibility. *Inflammation Research : Official Journal of the European Histamine Research Society ...[Et Al.], 61*(3), 177-188.

Nukada, Y., Ashikaga, T., Sakaguchi, H., Sono, S., Mugita, N., Hirota, M., Miyazawa, M., Ito, Y., Sasa, H., & Nishiyama, N. (2011). Predictive performance for human skin sensitizing potential of the human cell line activation test (h-CLAT). *Contact Dermatitis, 65*(6), 343-353.

O'Leary, J. G., Goodarzi, M., Drayton, D. L., & von Andrian, U. H. (2006). T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nature Immunology, 7*(5), 507-516.

Orteu, C. H., Poulter, L. W., Rustin, M. H., Sabin, C. A., Salmon, M., & Akbar, A. N. (1998). The role of apoptosis in the resolution of T cell-mediated cutaneous inflammation. *Journal of Immunology (Baltimore, Md.: 1950), 161*(4), 1619-1629.

Ostrov, D. A., Grant, B. J., Pompeu, Y. A., Sidney, J., Harndahl, M., Southwood, S., Oseroff, C., Lu, S., Jakoncic, J., de Oliveira, C. A., Yang, L., Mei, H., Shi, L., Shabanowitz, J., English, A. M., Wriston, A., Lucas, A., Phillips, E., Mallal, S., Grey, H. M., Sette, A., Hunt, D. F., Buus, S., & Peters, B. (2012). Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. *Proceedings of the National Academy of Sciences of the United States of America, 109*(25), 9959-9964.

Oyoshi, M. K., Murphy, G. F., & Geha, R. S. (2009). Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *The Journal of Allergy and Clinical Immunology, 124*(3), 485-93, 493.e1.

Price, A. A., Cumberbatch, M., Kimber, I., & Ager, A. (1997). Alpha 6 integrins are required for langerhans cell migration from the epidermis. *The Journal of Experimental Medicine, 186*(10), 1725-1735.

Probst, P., Kuntzlin, D., & Fleischer, B. (1995). TH2-type infiltrating T cells in nickel-induced contact dermatitis. *Cellular Immunology, 165*(1), 134-140.

Quezada, S. A., Jarvinen, L. Z., Lind, E. F., & Noelle, R. J. (2004). CD40/CD154 interactions at the interface of tolerance and immunity. *Annual Review of Immunology, 22*, 307-328.

Rachmawati, D., Bontkes, H. J., Verstege, M. I., Muris, J., von Blomberg, B. M., Scheper, R. J., & van Hoogstraten, I. M. (2013). Transition metal sensing by toll-like receptor-4: Next to nickel, cobalt and palladium are potent human dendritic cell stimulators. *Contact Dermatitis, 68*(6), 331-338.

Raghavan, B., Martin, S. F., Esser, P. R., Goebeler, M., & Schmidt, M. (2012). Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO Reports, 13*(12), 1109-1115.

Randolph, G. J. (2001). Dendritic cell migration to lymph nodes: Cytokines, chemokines, and lipid mediators. *Seminars in Immunology, 13*(5), 267-274.

Romagnoli, P., Labhardt, A. M., & Sinigaglia, F. (1991). Selective interaction of ni with an MHC-bound peptide. *The EMBO Journal, 10*(6), 1303-1306.

Saeki, H., Moore, A. M., Brown, M. J., & Hwang, S. T. (1999). Cutting edge: Secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *Journal of Immunology (Baltimore, Md.: 1950), 162*(5), 2472-2475.

Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama, K., Hirota, M., Itagaki, H., Toyoda, H., & Suzuki, H. (2006). Development of an in vitro skin sensitization test using human cell lines; human cell line activation test (h-CLAT). II. an inter-laboratory study of the h-CLAT. *Toxicology in Vitro : An International Journal Published in Association with BIBRA, 20*(5), 774-784.

Schaefer, H., & Lademann, J. (2001). The role of follicular penetration. A differential view. *Skin Pharmacology and Applied Skin Physiology, 14 Suppl 1*, 23-27.

Scharschmidt, T. C., Man, M. Q., Hatano, Y., Crumrine, D., Gunathilake, R., Sundberg, J. P., Silva, K. A., Mauro, T. M., Hupe, M., Cho, S., Wu, Y., Celli, A., Schmuth, M., Feingold, K. R., & Elias, P. M. (2009). Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants

and haptens. *The Journal of Allergy and Clinical Immunology, 124*(3), 496-506, 506.e1-6.

Scheibner, K. A., Lutz, M. A., Boodoo, S., Fenton, M. J., Powell, J. D., & Horton, M. R. (2006). Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *Journal of Immunology (Baltimore, Md.: 1950), 177*(2), 1272-1281.

Scheper, R. J., van Dinther-Janssen, A. C., & Polak, L. (1985). Specific accumulation of hapten-reactive T cells in contact sensitivity reaction sites. *Journal of Immunology (Baltimore, Md.: 1950), 134*(3), 1333-1336.

Scherrer, M. A., & Rocha, V. B. (2014). Increasing trend of sensitization to Methylchloroisothiazolinone/methylisothiazolinone (MCI/MI). *Anais Brasileiros De Dermatologia, 89*(3), 527.

Schmidt, M., Raghavan, B., Muller, V., Vogl, T., Fejer, G., Tchaptchet, S., Keck, S., Kalis, C., Nielsen, P. J., Galanos, C., Roth, J., Skerra, A., Martin, S. F., Freudenberg, M. A., & Goebeler, M. (2010). Crucial role for human toll-like receptor 4 in the development of contact allergy to nickel. *Nature Immunology, 11*(9), 814-819.

Schnuch, A., Westphal, G. A., Muller, M. M., Schulz, T. G., Geier, J., Brasch, J., Merk, H. F., Kawakubo, Y., Richter, G., Koch, P., Fuchs, T., Gutgesell, T., Reich, K., Gebhardt, M., Becker, D., Grabbe, J., Szliska, C., Aberer, W., & Hallier, E. (1998). Genotype and phenotype of N-acetyltransferase 2 (NAT2) polymorphism in patients with contact allergy. *Contact Dermatitis, 38*(4), 209-211.

Schwarz, A., Grabbe, S., Riemann, H., Aragane, Y., Simon, M., Manon, S., Andrade, S., Luger, T. A., Zlotnik, A., & Schwarz, T. (1994). In vivo effects of interleukin-10 on contact hypersensitivity and delayed-type hypersensitivity reactions. *The Journal of Investigative Dermatology, 103*(2), 211-216.

Schwarzenberger, K., & Udey, M. C. (1996). Contact allergens and epidermal proinflammatory cytokines modulate langerhans cell E-cadherin expression in situ. *The Journal of Investigative Dermatology, 106*(3), 553-558.

Shimizu, Y., Newman, W., Gopal, T. V., Horgan, K. J., Graber, N., Beall, L. D., van Seventer, G. A., & Shaw, S. (1991). Four molecular pathways of T cell adhesion to endothelial cells: Roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *The Journal of Cell Biology, 113*(5), 1203-1212.

Spiekstra, S. W., Toebak, M. J., Sampat-Sardjoepersad, S., van Beek, P. J., Boorsma, D. M., Stoof, T. J., von Blomberg, B. M., Scheper, R. J., Bruynzeel, D. P., Rustemeyer, T., & Gibbs, S. (2005). Induction of cytokine (interleukin-1alpha and tumor necrosis factor-alpha) and chemokine (CCL20, CCL27, and CXCL8) alarm

signals after allergen and irritant exposure. *Experimental Dermatology, 14*(2), 109-116.

Suto, H., Nakae, S., Kakurai, M., Sedgwick, J. D., Tsai, M., & Galli, S. J. (2006). Mast cell-associated TNF promotes dendritic cell migration. *Journal of Immunology (Baltimore, Md.: 1950), 176*(7), 4102-4112.

Tang, A., Amagai, M., Granger, L. G., Stanley, J. R., & Udey, M. C. (1993). Adhesion of epidermal langerhans cells to keratinocytes mediated by E-cadherin. *Nature, 361*(6407), 82-85.

Termeer, C., Benedix, F., Sleeman, J., Fieber, C., Voith, U., Ahrens, T., Miyake, K., Freudenberg, M., Galanos, C., & Simon, J. C. (2002). Oligosaccharides of hyaluronan activate dendritic cells via toll-like receptor 4. *The Journal of Experimental Medicine, 195*(1), 99-111.

Toebak, M. J., Pohlmann, P. R., Sampat-Sardjoepersad, S. C., von Blomberg, B. M., Bruynzeel, D. P., Scheper, R. J., Rustemeyer, T., & Gibbs, S. (2006). CXCL8 secretion by dendritic cells predicts contact allergens from irritants. *Toxicology in Vitro : An International Journal Published in Association with BIBRA, 20*(1), 117-124.

Torchinsky, M. B., Garaude, J., Martin, A. P., & Blander, J. M. (2009). Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature, 458*(7234), 78-82.

Urwin, R., & Wilkinson, M. (2013). Methylchloroisothiazolinone and methylisothiazolinone contact allergy: A new 'epidemic'. *Contact Dermatitis, 68*(4), 253-255.

Viemann, D., Schmidt, M., Tenbrock, K., Schmid, S., Muller, V., Klimmek, K., Ludwig, S., Roth, J., & Goebeler, M. (2007). The contact allergen nickel triggers a unique inflammatory and proangiogenic gene expression pattern via activation of NF-kappaB and hypoxia-inducible factor-1alpha. *Journal of Immunology (Baltimore, Md.: 1950), 178*(5), 3198-3207.

Vocanson, M., Hennino, A., Cluzel-Tailhardat, M., Saint-Mezard, P., Benetiere, J., Chavagnac, C., Berard, F., Kaiserlian, D., & Nicolas, J. F. (2006). CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. *The Journal of Investigative Dermatology, 126*(4), 815-820.

Waldorf, H. A., Walsh, L. J., Schechter, N. M., & Murphy, G. F. (1991). Early cellular events in evolving cutaneous delayed hypersensitivity in humans. *The American Journal of Pathology, 138*(2), 477-486.

Watanabe, H., Gaide, O., Petrilli, V., Martinon, F., Contassot, E., Roques, S., Kummer, J. A., Tschopp, J., & French, L. E. (2007). Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *The Journal of Investigative Dermatology, 127*(8), 1956-1963.

Weinlich, G., Heine, M., Stossel, H., Zanella, M., Stoitzner, P., Ortner, U., Smolle, J., Koch, F., Sepp, N. T., Schuler, G., & Romani, N. (1998). Entry into afferent lymphatics and maturation in situ of migrating murine cutaneous dendritic cells. *The Journal of Investigative Dermatology, 110*(4), 441-448.

Weiss, J. M., Sleeman, J., Renkl, A. C., Dittmar, H., Termeer, C. C., Taxis, S., Howells, N., Hofmann, M., Kohler, G., Schopf, E., Ponta, H., Herrlich, P., & Simon, J. C. (1997). An essential role for CD44 variant isoforms in epidermal langerhans cell and blood dendritic cell function. *The Journal of Cell Biology, 137*(5), 1137-1147.

Westphal, G. A., Reich, K., Schulz, T. G., Neumann, C., Hallier, E., & Schnuch, A. (2000). N-acetyltransferase 1 and 2 polymorphisms in para-substituted arylamine-induced contact allergy. *The British Journal of Dermatology, 142*(6), 1121-1127.

Williams, I. R., Ort, R. J., & Kupper, T. S. (1994). Keratinocyte expression of B7-1 in transgenic mice amplifies the primary immune response to cutaneous antigens. *Proceedings of the National Academy of Sciences of the United States of America, 91*(26), 12780-12784.

Wood, L. C., Elias, P. M., Calhoun, C., Tsai, J. C., Grunfeld, C., & Feingold, K. R. (1996). Barrier disruption stimulates interleukin-1 alpha expression and release from a pre-formed pool in murine epidermis. *The Journal of Investigative Dermatology, 106*(3), 397-403.

Woodland, D. L., & Kohlmeier, J. E. (2009). Migration, maintenance and recall of memory T cells in peripheral tissues. *Nature Reviews.Immunology, 9*(3), 153-161.

Zhang, X., Brunner, T., Carter, L., Dutton, R. W., Rogers, P., Bradley, L., Sato, T., Reed, J. C., Green, D., & Swain, S. L. (1997). Unequal death in T helper cell (th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *The Journal of Experimental Medicine, 185*(10), 1837-1849.

Zug, K. A., Warshaw, E. M., Fowler, J. F.,Jr, Maibach, H. I., Belsito, D. L., Pratt, M. D., Sasseville, D., Storrs, F. J., Taylor, J. S., Mathias, C. G., Deleo, V. A., Rietschel, R. L., & Marks, J. (2009). Patch-test results of the north american contact dermatitis group 2005-2006. *Dermatitis : Contact, Atopic, Occupational, Drug, 20*(3), 149-160.