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**Novel Immunoregulatory Elements in a Murine Model of Epicutaneous Chemical
Sensitization: A Role for Regulatory T Cells and microRNA 210**

Carrie Mae Long

Dissertation submitted to the School of Medicine
at West Virginia University
in partial fulfilment of the requirements
for the degree of

Doctor of Philosophy
In
Immunology and Microbial Pathogenesis

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ABSTRACT

Novel Immunoregulatory Elements in a Murine Model of Epicutaneous Chemical Sensitization

Carrie Mae Long

Toluene diisocyanate (TDI) is a low molecular weight chemical sensitizer and a major cause of occupational allergic disease and asthma. The roles of immunomodulatory elements such as microRNAs, which alter gene expression, and regulatory T cells (T_{regs}), which suppress T cell-mediated responses, in chemical sensitization are not fully elucidated. In order to further investigate the functional role of miR-210 and T_{regs} during TDI sensitization, BALB/c mice were dermally exposed to a single, sensitizing dose of TDI (0.5-4% v/v) and evaluated for immunologic endpoints. dLN T_{regs} were phenotyped during TDI sensitization and a heterogeneous, expanded population was observed. To examine T_{reg} functionality, a carboxyfluorescein succinimidyl ester-based T_{reg} suppression assay was conducted. T_{regs} isolated from the dLNs of TDI sensitized mice were more suppressive than their control counterparts. The T_{reg} population was then depleted prior to TDI sensitization by *in vivo* administration of a CD25-targeting antibody. The sensitization response was intensified following T_{reg} depletion, as evidenced by amplified total dLN cellularity, Th2 expansion, and IL-4 mRNA production. This data suggests that T_{regs} are important negative regulators of the TDI sensitization response.

We have previously shown that miR-210 expression in the draining lymph node (dLN) is augmented during TDI sensitization and several putative mRNA targets identified for this miRNA (*foxp3* and *runx3*) were directly related to T_{reg} differentiation and function. Therefore, we hypothesized that miR-210 targets transcription factors central to T_{reg} development and function, antagonizing the development and functional role of this subset during TDI sensitization. In this work, we show that miR-210 expression is increased in the mouse dLN and T_{reg} subsets during TDI sensitization. Putative miR-210-target/key player interactions were examined using an *ex vivo* stimulation system in which miR-210 mimics were transfected into primary, stimulated dLN cells; decreases in multiple T_{reg} -related genes were observed via qPCR analysis. Alterations in dLN and T_{reg} mRNA and protein expression of *foxp3*, *runx3*, *ctla4*, and *cd25* were observed at multiple time points following TDI exposure. These molecules are directly involved in T_{reg} expansion and function; therefore, miR-210 may potentially negatively target T_{reg} differentiation and function during TDI sensitization. In order to examine the effect of miR-210 modulation on T_{reg} functionality we performed a carboxyfluorescein succinimidyl ester-based T_{reg} suppression assay using dLN T_{regs} from naïve mice transfected with miR-210 mimic. While the suppressive ability of TDI/mimic T_{regs} was increased compared to the acetone/mimic counterparts, the TDI/mimic samples had less expression of miR-210 compared to the acetone/mimic controls suggesting other factors, such as additional miRNAs, might be involved in the regulation of the functional capabilities of these cells. These novel findings indicate that T_{regs} likely play an important role in modulating allergic responses during TDI sensitization and that miR-210 may have an inhibitory role in this process. Because the functional roles of miRNAs and T_{regs} have not been previously elucidated in a model of chemical sensitization, these data contribute to the understanding of the immunologic mechanisms of chemical induced allergic disease and may aid in the development of preventative strategies.

DEDICATION

I dedicate this work to my parents, Drs. Jim and Cathy Long. Their intelligence, success, humility, and character have been a source of inspiration throughout my life. Their unconditional love and unwavering support have enabled me to pursue my dreams.

I would also like to dedicate this work to my grandparents, Dr. and Mrs. Jim Long and Mr. and Mrs. Charles Romine. My life has been enriched because of your tremendous love.

I would like to thank my fiancé Evan Brusich for always making me laugh, reminding me to have perspective, and for helping me get through the ups and downs of graduate school.

Thank you to Katie Dunnick, Katie Roach, Breanne, Melissa, Donna, Colleen, my volleyball family, WVU track teammates, and everybody else that has helped make my time in Morgantown so special.

“For God hath not given us the spirit of fear; but of power, and of love, and of a sound mind.”

-2 Timothy 1:7.

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My mentor, Dr. Stacey Anderson has enabled me to perform this research by being a supportive and caring advisor. I could not have asked for a better mentor. I am honored to be her first doctoral student and I know she will be an excellent mentor to the many students that come after me. Dr. Nikki Marshall has also been instrumental in my journey. I would like to thank Nikki for being a flow cytometry and immunology guru and one of my best friends. Dr Barbara Jean Meade has exhibited prolific kindness and intelligence in our interactions; I will use the lessons she has taught me throughout my entire life. I would also like to thank Ewa Lukomska for being an excellent lab manager and friend. The other members of our research team have also been a huge part of this project and my life for the past five years; thank you Hillary, Jodi, and Antonella.

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Lastly, it seems appropriate to include a poignant statement made by A. Munn (1965) regarding isocyanates as these chemicals have been a captivating and productive subject of research.

“The more one knows about these fascinating compounds the more fascinated one becomes. So diverse are their uses, it is obvious that they are here to stay, and that their use will increase. So numerous have been the accounts of their effects, it is obvious not merely they are hazardous but that the nature and extent of their hazard has not always been fully appreciated.”

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LIST OF SYMBOLS, ABBREVIATIONS, AND NOMENCLATURE

³ H-dT	Tritiated Thymidine
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
ACGIH	American Conference of Governmental Industrial Hygienists
ADCC	Antibody-dependent cell-mediated cytotoxicity
AHR	Aryl hydrocarbon receptor
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AID	Activation-induced deaminase
AMPK	adenosine 3',5'-monophosphate -activated protein kinase
AO/PI	Acridine Orange/Propidium Iodide
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
CTLA4	Cytotoxic T lymphocyte-associated protein-4
dLN	Draining lymph node
DNCB	2,4-dinitrochlorobenzene
DNFB	Dinitrofluorobenzene
DNTB	2,4-dinitrothiocyanobenzene
DPM	Disintegrations per minute
DPRA	Direct Peptide Reactivity Assay
ECM	Extracellular matrix
ECVAM	European Center for the Validation of Alternative Methods
FMO	Fluorescence Minus One
Foxp3	Forkhead box p3

GARD	Genomic Allergen Rapid Detection Assay
GI	Gastrointestinal
GPMT	Guinea Pig Maximization Test
GRPS	GARD Respiratory Prediction Signature
HDI	Hexamethylene Diisocyanate
HIF-1 α	Hypoxia inducible factor 1 α
HLA	Human leukocyte antigen
HMW	High molecular weight
HRIPT	Human Repeated Insult Patch Test
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICOS	Inducible T-cell costimulator
IDLH	Immediately Dangerous to Life and Health
IDO	Indolamine 2,3-dioxygenase
IFN- γ	Interferon gamma
ILC	Innate lymphoid cell
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iTCR	Invariant TCR
kDa	Kilodalton
LCR	Locus control region
LLNA	Local Lymph Node Assay
LMW	Low molecular weight
lncRNA	Long non-coding RNA
LO	Lipofectamine only
MAC	Membrane attack complex

MBL	Mannose-binding lectin
MDI	Diphenyl-methane diisocyanate
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger RNA
miRNA	MicroRNA
MMP	Matrix Metalloproteinase
NA	Nonanoic acid
Ni ²⁺	Nickel
NIOSH	National Institute for Occupational Safety and Health
NK	Natural Killer Cells
NKT	Natural Killer T Cells
OECD	Organization for Economic Cooperation and Development
OEL	Occupational Exposure Limit
OPA	Ortho-phthalaldehyde
OSHA	Occupational Safety and Health Administration
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
ppb	Parts per billion
PCR	Polymerase chain reaction
PenH	Enhanced Pause
PFOA	Perfluorooctanoic acid
PPE	Personal Protective Equipment
RAST	Radioallergosorbent tests
RISC	RNA-induced Silencing Complex

ROS	Reactive oxygen species
RT	Reverse Transcription
SCID	Severe Combined Immunodeficiency
SI	Stimulation Index
TCR	T cell receptor
TDA	Toluenediamine
TDI	Toluene diisocyanate
T _{EM}	Effector memory T cell
Th	T-helper
TMA	Trimellitic anydride
TNCB	Trinitrochlorobenzene
TNF- α	Tumor necrosis factor alpha
TNP	Trinitrophenyl
T _{reg}	Regulatory T Cell
TSLP	Thymic stromal lymphopoetin
U.S.	United States
VEGFA	Vascular endothelial growth factor A

CHAPTER 1

Occupational Allergic Disease and Hypersensitivity

1.1. INTRODUCTION

Occupational allergic disease is a serious public health burden whose prevalence is growing worldwide. Workplace exposures can result in a number of allergic diseases and approximately 11 million American workers are potentially exposed to agents that can cause allergic disease (1). Asthma, allergic contact dermatitis, rhinitis, hypersensitivity pneumonitis, urticaria, eczema, and folliculitis can occur as a result of respiratory and dermal exposures in the workplace (1). Specifically, occupational exposures are responsible for approximately 9-25% of all adult onset asthma cases (2, 3) while occupational contact dermatitis represents 90% of all cases of work-related cutaneous disorders (4). These conditions may be detrimental to a worker's health and workplace performance, causing economic losses (5). Occupational asthma is sometimes more severe than general asthma as evidenced by persistence of symptoms despite treatment, reports of deaths following sensitization and repeated exposures, and the significantly increased use of medical resources by occupational asthmatics compared to those with non-occupationally related conditions (6).

A variety of agents can cause occupational allergic disease, including animal products (7), chemicals (1), drugs (8), foods (9), metals (4), molds (10), plant products (4), and venoms (11). Although both irritant and allergic conditions can be induced by occupational allergens, this work will focus on immune-mediated conditions such as allergic contact dermatitis and asthma caused by exposure to allergic sensitizers. Over 250 causative agents of occupational asthma have been reported (12), approximately 400 allergens are available for contact dermatitis patch testing in humans (4), and over 3000 contact allergens have been identified (13), demonstrating the breadth of potential allergens found in the workplace. Typically, these agents are classified as high or low molecular weight (HMW >1 kDa; LMW <1 kDa) and their size is thought to play a significant role

in their allergenicity, dermal penetration, and allergic mechanism of action. Many workplace factors may influence allergic disease, including barrier dysregulation caused by repetitive movements and irritants such as hand washing and contact with disinfectants, and occupational-induced stress which may contribute to the development and/or persistence of immune responses (14).

1.2. TYPES OF HYPERSENSITIVITY REACTIONS

Many occupationally-relevant allergic agents are known as sensitizers, indicating that repeated exposures will result in immune sensitization followed by the elicitation of allergic disease. This biphasic allergic process is characterized by two distinct phases: sensitization and elicitation. Sensitization is thought to occur because of numerous complex host and allergen-associated factors. The interplay of mucosal surfaces and allergens is an important concept directly related to the development of sensitization and many cell types are thought to be involved in this response, depending on the site of exposure and the individual's unique immune microenvironment.

Following epithelial access by an allergen, a variety of downstream effects can occur. In the context of hypersensitivity reactions, which indicate an augmented and potentially damaging immune response, there are four basic hypersensitivity reactions as classified by Patrick Gell and Robin Coombs (15). These distinct responses were characterized based on the primary effector molecules and immune cells thought to be involved in each reaction. A summary of the four hypersensitivity reactions can be found in Table 1.1. These reactions are grouped based on humoral (I-III) or cell-mediated (IV) primary effector mechanisms. Although it was proposed over 50 years ago and several revision attempts have been made to update the classification scheme, the Gell and Coombs hypersensitivity classification is still widely accepted as the most valid scheme for interpreting hypersensitivity reactions.

	Type I	Type II	Type III	Type IV
Name	IgE-Mediated	Antibody-Mediated Cytotoxic	Immune Complex-Mediated	T Cell-Mediated
Primary Effector Molecule/Cell	IgE and Mast Cells	IgG or IgM and Complement	Complement, Mast Cells, IgG or IgM, and Leukocytes	T cells (Th1 and CD8 ⁺) and Macrophages
Humoral or Cellular	H	H	H	C
Description	Mast cell-bound IgE is engaged with its cognate antigen, mast cells degranulate and release allergic mediators (e.g. histamine (immediate) and leukotrienes (delayed))	Antibody- antigen recognition, opsonization, complement activation, neutrophil chemotaxis	An excess of soluble antigen leads to the formation of small immune complexes which insert themselves into blood vessels, joints, and glomeruli. Precipitation results in complement activation and a pathogenic inflammatory response	CD4 ⁺ T cells recognize MHCII-Antigen complex on APCs and begin to produce Th1 cytokines. Cytotoxic CD8 ⁺ cells kill target cells and macrophages produce hydrolytic enzymes, causing tissue damage.
Clinical Symptoms	Anaphylactic shock, angiodema, conjunctivitis, urticaria, hives, wheal and flare	Hemolytic anemia, granulocytopenia, thrombocytopenia	Arthritis, chills, edema, fever, glomerulonephritis, rash	Erythema
Associated Conditions	Anaphylaxis (systemic or local), asthma, eczema, food allergy, hay fever	Blood transfusion reaction, Drug-induced hemolytic anemia, Rh antigen reaction (erythroblastosis fetalis)	Hypersensitivity Pneumonitis, Glomerulonephritis, Serum Sickness	Contact Dermatitis, Hashimoto's thyroiditis, Multiple sclerosis

Table 1.1: *Gell and Coomb's Hypersensitivity Reactions*

1.2.1. Type I Hypersensitivity Reaction

Type I hypersensitivity reactions are perhaps the most recognizable, due to an association with a number of prevalent allergic diseases and atopy. Type I reactions are mediated by IgE and mast cells and are sometimes called immediate-type hypersensitivity reactions. The progression of antigen recognition, T cell activation, and B cell antibody production is very similar to the classic mechanism that is observed in a general IgG-mediated humoral response; however, in a Type I hypersensitivity response, the primary cytotoxic antibody produced in response to antigen is IgE. A type I reaction can be described beginning with the recognition and uptake of allergen by APCs, presentation of antigen fragments to CD4⁺ T cells via MHC II, and the initiation of a Th2 allergic response. Th2 cytokines such as IL-4 and IL-13 are produced and secreted by CD4⁺ T cells, leading to antibody class switching and IgE production by B cells. Once activated by the Th2 microenvironment, basophils are thought to play an important role in the secretion of allergic cytokines and antibody class switching (16).

Antibody class switching is a dynamic process that involves several steps; for IgE in particular two signals are required (16). The first is provided by IL-4 or IL-13 through binding to their respective receptors on B cells, leading to activation of transcription at the IgE-specific S_ε switch region. The second signal is provided by CD40 (on B cells) – CD40 ligand (on T cells) binding, which activates DNA switch recombination. Additional factors such as the expression of activation-induced deaminase (AID) influence class switching to IgE. IgE class switching is thought to occur primarily in the germinal centers of secondary lymphoid organs such as the lymph nodes during allergic responses (16). It is important to note that atopic individuals who are particularly susceptible to develop Type I reactions have high circulating levels of baseline IgE, which may be present prior to activation against a particular antigen initiating a Type I response. IgE is considered

an allergic antibody due to its ability to bind to high affinity FcεR1 receptors on mast cells and basophils (16). Once IgE is produced and secreted by plasma cells, it binds to mast cells and basophils via the high affinity FcεR1 receptor. Allergen binds to cell-bound IgE, crosslinking the IgE and activating these cells.

Once activated, mast cells and basophils degranulate and release soluble allergic mediators such as histamine, heparin, cytokines, prostaglandins, leukotrienes, and proteases which act on smooth muscles, sensory nerves, mucous glands, arteries, and eosinophils (16, 17). The mechanisms of action of these mediators are listed in further detail in Table 1.2. Common clinical outcomes of Type I reactions include increased vascular permeability, smooth muscle cell contraction, and vasodilation, which can be experienced in one or more organs, depending on the site(s) and frequency of allergen exposure. From a broad perspective, it appears that Type I responses may have evolved in order to prevent pathogens from surviving in the respiratory and gastrointestinal (GI) tracts (18). Clinically, IgE-mediated responses are common in response to helminthic and parasitic infections but are not usually involved in responses to non-parasitic antigens. It is imperative to note that Type I reactions typically require a sensitization phase of initial antigen exposure in order to allow the immune system to develop clonal specificity and IgE responses upon secondary exposure, allowing for the effector phase of the Type I response.

Mediator	Primary or Secondary*	Receptor	Target Cells	Clinical Effects
Bradykinin	Secondary	Kinin B ₁ and B ₂ receptors	Endothelial cells	Increased vascular permeability (vasodilation), Neutrophil recruitment (via CXCL5)
Cytokines	Secondary	Varies	Varies	Inflammatory cell influx
Eosinophil Chemotactic Factor (IL-5)	Primary	Eosinophil Chemotactic Factor Receptor (IL-5R)	Eosinophils	Eosinophil recruitment
Histamine	Primary	H Receptors	Smooth muscle and endothelial	Smooth muscle contraction, Increased vascular permeability (vasodilation), Increased goblet cell mucus secretion
Leukotrienes	Secondary	BLT1, BLT2, CysLT1, CysLT2	Leukocytes, Endothelial cells	Bronchoconstriction, increased vascular permeability (vasodilation), increased mucus secretion, Smooth muscle contraction
Neutrophil Chemotactic Factor (IL-8)	Primary	CXCR1, CXCR2	Neutrophils (primary), endothelial cells, other granulocytes, macrophages	Neutrophil recruitment, Can also recruit other granulocytes
Platelet-activating Factor	Secondary	Platelet-activating factor receptor	Endothelial cells, leukocytes, and platelets	Increased vascular permeability (vasodilation), Leukocyte chemotaxis
Prostaglandins	Secondary	DP, EP, FP, IP, and TP receptor families	Smooth muscle cells	Increased smooth muscle contraction, Increased vascular permeability (vasodilation)
Proteases	Primary	Target peptide bonds	Blood vessels, Complement proteins	Increased vascular permeability (basement membrane degradation), Catalyze the complement cascade

Table 1.2: *Secreted Mediators of Type I Hypersensitivity*

*Primary and Secondary Mediator indicates whether the substance is made before degranulation (primary) or synthesized after cellular activation/released by the breakdown of membrane components during degranulation (secondary)

Adapted from (17)

1.2.2. Type II Hypersensitivity Reaction

Type II hypersensitivity reactions are humorally-mediated, and are characterized by the production of antibodies that cause cellular destruction. Antibodies bound to cell surface antigens can activate the complement cascade, resulting in cellular damage via formation of pores in the cell membrane or antibody-dependent cell-mediated cytotoxicity (ADCC: natural killer cell or macrophage-mediated cell death) (17). Typically, neutrophils are recruited to opsonized antibody-antigen complexes and are major mediators of inflammatory symptoms. Clinical features of Type II reactions may include allergic hemolytic anemia, agranulocytosis, fever, purpura, and thrombocytopenia. An example of a Type II response is a penicillin reaction which is characterized by penicillin-red blood cell complexing, leading to an antibody response and activation of the complement cascade. Type II responses may have developed as a defense strategy against small extracellular pathogens that are susceptible to neutrophil-mediated killing (18).

1.2.3. Type III Hypersensitivity Reaction

Type III hypersensitivity reactions are mediated by the formation of antibody-antigen complexes, which typically lead to antigen clearance by phagocytic and red blood cells. In the case of a hypersensitivity reaction, large quantities of these immune complexes can lead to activation of the complement system and an inflammatory response characterized by neutrophil infiltration. This response can be very damaging to the body's tissues and both the magnitude and breadth of the reaction depends on the number of immune complexes and their location (localized or systemic). The immune complexes alone can cause tissue damage due to their deposition in blood vessel walls and other tissues. Additionally, the complement component C3b opsonizes the immune complexes, leading to attempted neutrophil binding. If the immune complexes are deposited on basement

membranes then phagocytosis is impeded, leading to the release of large amounts of lytic enzymes that are very destructive to the surrounding tissue (17). The membrane attack complex (MAC) can also be formed, causing additional tissue destruction and cellular damage. A classic example of a Type III hypersensitivity reaction is serum sickness, which is caused by the introduction of foreign antiserum into an organism, resulting in the formation of antiserum-specific antibodies and immune complexes comprised of both self-antibodies and foreign antiserum. Serum sickness may lead to arthritis, fever, rashes, and vasculitis as a result of immune complex deposition and inflammatory mechanisms (17). This response typically develops in 9-11 days following the transfer of sera (19).

1.2.4. Type IV Hypersensitivity Reaction

The final hypersensitivity classification is characterized by T cell-mediated and delay observed between antigen exposure and outward symptoms. Many intracellular pathogens and contact antigens can induce Type IV responses, including *Mycobacterium tuberculosis*, *Candida albicans*, *Variola spp.* (Smallpox), and poison ivy (17). Type IV responses are diverse and involve a number of effector cells and molecules.

An initial sensitization phase is required following the first contact with antigen in order to activate T cells and allow for their clonal expansion. APC-T cell interaction is required for the development of Type IV responses via MHC presentation. Typically CD4⁺ Th1 cells are the primary T cell subset involved in Type IV responses but Th2 and CD8⁺ T cells have also been implicated in the development of Type IV responses (1, 17). Following effective sensitization, subsequent antigenic exposures result in the elicitation or effector phase of the Type IV response, characterized by the secretion of pro-inflammatory cytokines (GM-CSF, IFN γ , IL-3, IL-12, and TNF- β) by Th1 cells that activate and recruit macrophages and other immune cells. Due to the time it takes for these

cytokines to attract and activate macrophages at sites of exposure the effector phase typically occurs 24 hours following exposure and it generally peaks in magnitude at 48-72 hours after exposure (17). This phase of the response is characterized by the action of both clonally-specific Th1 cells and non-specific immune effectors such as macrophages. A well-managed Type IV response countering a pathogen can be beneficial to the host, resulting in pathogen clearance and minimal host cell damage; however, if the response is prolonged and not well controlled (as is the case in a hypersensitivity reaction) then severe tissue damage can occur. Contact dermatitis is an example of an allergen-induced Type IV hypersensitivity reaction. This condition can be caused by a variety of molecules, including metals, chemicals, and natural substances such as poison ivy. Many of these allergens are small in size and are able to permeate the skin and combine with skin proteins, forming antigen-protein complexes. These complexes are recognized by the immune system, allowing a Type IV response to develop.

Due to the known plasticity of T cell development and function, it can be assumed that numerous T-helper (Th) subsets can potentially be involved in Type IV responses. As previously mentioned, Th1, Th2, and cytotoxic CD8⁺ T cells have been associated with Type IV hypersensitivity reactions. Accordingly, four Type IV subclasses have been proposed due to the wide breadth of responses involved in this type of hypersensitivity. These subclasses include IVa-d. IVa reactions are thought to involve Th1 cells and monocytes and rely on the secretion of IL-1 and IL-2. IVb reactions are associated with Th2 cells and eosinophils along with the expression of IL-4, IL-5, and IL-13. Skin sensitization and asthmatic reactions to toluene diisocyanate (TDI) likely involve Type IVb components, based on the strong Th2 response paired with the disputed role of IgE. The major effector cells of type IVc reactions are cytotoxic CD8⁺ T cells which utilize FasL, granzyme, and perforin for their cell contact-mediated effector functions. Lastly, Type IVd

reactions involve T cells, neutrophils, and the production of IL-8, a neutrophil chemotactic factor. The necessity of multiple Type IV subtypes illustrates the diverse effector pathways involved in Type IV reactions. Even classical Type IV reactions encompass both IVa and IVc reactions, illustrating the complexity of these responses and the need for elucidation of the complete immunologic mechanisms underlying disease as diagnosis and treatment of these conditions will be greatly enhanced by increased understanding of these complex mechanisms.

1.2.5. Gell and Coomb's Classification: Modern Validity?

Although the Gell and Coomb's classification is a seminal work which has withstood decades of evolution in immunologic knowledge, its current validity has been questioned. The landscape of immunology has been dramatically altered since the 1960s when the classification was proposed; therefore, one would expect the need for mechanistic updates to the classification, which have been absent. In the context of drug reactions and chemical allergy, the Gell and Coombs classification does not encompass all reactions, including hypersensitivity pneumonitis and certain instances of chemical-induced asthma (19). The idea that more than one defined hypersensitivity mechanism may be involved in a given reaction has been proposed as well. Penicillin reactions have been shown to encompass a wide variety of defined hypersensitivity reactions, including anaphylaxis, angioneurotic edema, eosinophilia, fever, interstitial nephritis, respiratory symptoms, serum sickness, and skin rashes (20). This does not completely invalidate the hypersensitivity mechanisms presented by Gell and Coombs; rather, it emphasizes the flexibility of the classification system, which is not generally emphasized.

The Gell and Coombs classification is still valid for a number of reactions that are classically defined by the parameters initially set out by these two pioneers. While this is true for a number of

conditions, the inverse also holds true; many conditions do not fit into the current classification based on specific immune effectors as defined by Gell and Coombs. The broad clinical usage of this classification presents several concerns, including the dismissal of underlying immune mechanisms involved in certain conditions based on their simple classification. Admittedly, the understanding of immunologic mechanisms underlying many hypersensitivity reactions remains limited; however, current knowledge points to the need for a reevaluation of the current classification system based on the complexity of hypersensitivity reactions. Type I-III reactions are defined based on the involvement of antibody. While this effector mechanism remains valid, the T cell response preceding the humoral reaction (and acting in concert with this reaction) is also of significance. Our current knowledge of immunology has revealed the dynamic nature of the immune system; the discovery of a multitude of CD4⁺ T cell subsets involved in a wide spectrum of immune responses has necessitated the examination of this component of the immune system for inclusion in hypersensitivity classification. The model proposed by Gell and Coombs is still valid for many conditions but should not be utilized as a “one size fits all” classification system, as many conditions do not fall into the classification and much knowledge has been gained regarding the mechanisms of related immune responses.

1.3. CHEMICAL-INDUCED SENSITIZATION

As previously stated, allergens are commonly categorized as HMW or LMW; generally, protein allergens fall into the HMW range and chemical allergens are in the LMW range. These two classes of molecules differ in their reactivity and antigenicity. HMW protein allergens are functionally immunogenic due to their large size whereas LMW chemical allergens are not innately immunogenic due to their small size and are thought to react with self-proteins, forming

immunogenic hapten complexes that serve as functioning antigens (21). Typically, IgE-mediated allergic responses are induced by HMW protein antigens but this type of immunologic response is also seen following exposure to certain LMW chemical allergens. Human studies have revealed that HMW-induced occupational asthma cases are generally characterized by an early or dual asthmatic reaction, while LMW-induced occupational asthma cases generally exhibit delayed reactions (6). Additionally, a recent study suggested that occupational asthma induced by LMW agents may be more severe than that caused by HMW agents, based on specific inhalation challenge results and diagnostic severity classification in a cohort of patients with asthma of each etiology (6).

Metal ions constitute an additional type of allergen which includes chromium, cobalt, copper, nickel, palladium, platinum, and potassium dichromate; compared to protein allergens much less is known regarding the immune response underlying metal allergy (22, 23). Several metals including nickel sulfate, cobalt chloride, and potassium dichromate are significant causative agents of allergic contact dermatitis (22). Much like LMW chemical allergens, metal allergens such as nickel have displayed binding capabilities with MHC-bound proteins (usually via histidine residues) and in the case of nickel, this binding is hypothesized to occur on metal-binding motifs on self-peptides complexed with the MHC molecule (22). This concept of direct and/or reversible MHC or TCR-to-drug binding (non-covalent) has been termed “p-i” or pharmacological interaction with immune receptors and has been well studied in terms of drug-induced hypersensitivity reactions; ultimately, this process may result in the activation of drug-specific T cells (24). Other metal allergens may induce these changes via MHC peptide stripping (palladium) or more indirect modifications of the self-peptide-MHC complexes (beryllium) (22). Common haptentation mechanisms may be at play for certain metal allergens; for example, nickel-reactive human CD4⁺ T cell clones display cross-reactivity with copper and palladium (25). Although generally protein,

LMW chemical, and metal allergens likely utilize divergent mechanisms to cause allergic disease, co-exposures to multiple chemical classes and metals is a reality in the workplace and this may produce responses of altered severity with unique mechanisms.

Although many substances can cause allergic and hypersensitivity reactions mediated by sensitization, LMW chemical sensitizers represent a substantial portion of causative agents of allergic diseases. Typically, LMW chemical allergens cause dermal or respiratory allergic manifestations including contact dermatitis and asthma. While the most common cause of dermal sensitization leading to contact dermatitis is exposure to metal salts, exposure to various LMW chemicals such as rubber additives, plastics and resins, biocides, and cosmetics are also significant causes of allergic contact dermatitis (4), which accounts for 10-15% of all occupational diseases (3). Additionally, it is estimated that the prevalence of contact allergy due to at least one sensitizing chemical in North America and Western Europe is approximately 20% (26). Allergic respiratory diseases are associated with high levels of worker morbidity and are a global health burden; it is estimated that 9-25% of all cases of adult-onset asthma are associated with occupational exposures, which is equivalent to 250-300 cases per million of occupational-related adult-onset asthma each year (3). Flour was the most common cause of occupational asthma in the UK in 1998; however, isocyanates represented the second most common cause, illustrating the relevance of chemicals as causative agents of occupational allergic disease. The range of LMW chemicals that can cause occupational allergy is wide and is summarized in Table 1.3. This table is not exhaustive yet it illustrates the fact that these sensitizing chemicals are utilized in a wide variety of industries and for a number of applications.

	Specific Classification	Chemical Name	Product	Occupation	Allergic Disease Caused
Acrylic Resin Monomers					
	Acrylates		Paints, Varnishes, Glues, Inks	Painters, Printers, Cosmetics, Health Care , Automobile workers	Asthma (12), ACD (27)
	Acrylamides		Dyes, Polyacrylamide synthesis	Polymer synthesis, Paper and Textile, Waste Treatment	ACD (27)
	Cyanoacrylates		Glues	Dentistry	Asthma (28), ACD (27)
	Methacrylates				
		2-hydroxyethyl methacrylate (2-HEMA)	Glues, orthopedic and dental surgical devices/fillings	Medicine and Dentistry	ACD (27)
		Ethyleneglycol dimethacrylate (EGDMA)	Glues, Polymer synthesis intermediate	Dentistry, Polymer synthesis	ACD (27)
		2-hydroxypropyl methacrylate (2-HPMA)	Glues, Drug carrier	Dentistry, Pharmaceutical	ACD (27)
Amines					
	Aliphatic Amines and Polyamines			Chemical Workers	Asthma (12), ACD (29)
	Cycloaliphatic Polyamines			Chemical Workers	ACD (29)
	Aromatic Amines			Textiles, Pesticides	ACD (29)
Anhydrides					
	Dioctyl-phthalate		Plastics, Wood coatings, Medical devices	PVC Production	Asthma (12)
	Acid Anhydrides		Plastics, Epoxy resins	Chemical workers, Pharmaceuticals	Asthma (30). ACD (31)
Biocides					
	Aldehydes				
		Formaldehyde and Formaldehyde-based materials	Adhesive resin, Fiber board, Plywood, Glues, Medical preservative, Sterilizers	Healthcare, Textile, Automobile, Hairdressers, Health Care, Lab, Construction	Asthma (12, 32), ACD (33)
		Furfuryl Alcohol	Rocket engine fuel, Solvent, Intermediate in the manufacture of various products	Foundry Operations, Paints	Allergic Airway Hyperreactivity (Murine) (34)
	Chloramine T	N-chloro 4-methylbenzenesulfonamide sodium salt	Surface Disinfectant	Manufacturing, Chemical Factory, Healthcare	Asthma (35, 36)
	Dialdehydes				
		Glutaraldehyde	Disinfectant	Healthcare, Lab	Asthma (12, 32), ACD (37)

		Ortho-phthalaldehyde	Disinfectant	Healthcare, Lab	Asthma and ACD (38)
	Fungicides				
		1,2-benzisothiazolin-3-one	Paints	Chemical Workers, Painters	Asthma (12), ACD (39)
		Dyrene (2,4-dichloro-6-(D-chloroanilino)-s-triazine)	Pesticide	Lawn Care	ACD (40)
		Sulfites	Food additive, Preservative	Factory, Food Processing, Water Treatment, Pharmaceuticals	Asthma (12), ACD (41)
	Isothiazolinones		Antimicrobials	Various Applications (Cleaning Agent)	Asthma (36, 42), ACD (43)
	Quaternary Ammonium Compounds		Antimicrobials	Healthcare, Cleaning Agent	Asthma (36, 44)
Cosmetics					
	Cocamidopropyl betaine		Surfactant	Hairdressers	ACD (45)
	Dyes, Fragrances, and Perfumes			Sales, Hairdressers	Asthma (2, 12), ACD (46)
	Glyceryl thioglycolate		Hair Perm Solutions	Hairdressers	ACD (47)
	Methyldibromo glutaronitrile		Preservative		ACD (48)
	Parabens		Preservative		ACD (49, 50)
	p-Phenylenediamine		Dye	Hairdressers, Polymer synthesis	ACD (51)
	Persulfates		Denture cleaner, Curing agent, Bleach, Detergent	Hairdressers, Food Processing, Polymer synthesis	Asthma (12, 52), ACD (53)
Isocyanates					
	Diisocyanates				
		Hexamethylene Diisocyanate (HDI)	Polyurethane synthesis, Paints, Foams, Coatings, Insulation, and Varnishes	Automobile, Painters, Builders, Manufacturing	Asthma (12, 54), ACD (55)
		Methylene Bisphenol Isocyanate (MDI)	Polyurethane synthesis, Paints, Foams, Coatings, Insulation, and Varnishes	Automobile, Painters, Builders, Manufacturing	Asthma (12), ACD (56)
		Toluene Diisocyanate (TDI)	Polyurethane synthesis, Paints, Foams, Coatings, Insulation, and Varnishes	Automobile, Painters, Builders, Manufacturing	Asthma (12, 57), ACD (58)
Hardeners					
	Polyfunctional aziridine		Paints, Coatings, Varnishes, and Inks	Painters, Builders	Asthma (59), ACD (60)
	Triglycid isocyanurate		Paints, Laminates, and Varnishes	Painters	Asthma (61), ACD (62)
	Acrylates		Paints, Varnishes, Glues, Inks	Painters, Printers, Automobile Workers	Asthma (12), ACD (27)

	Acrylamides			Polymer synthesis, Paper and Textile, Waste Treatment	ACD (27)
	Cyanoacrylates		Glues	Dentistry	Asthma (28), ACD (27)
	Methacrylates				
		2-hydroxyethyl methacrylate (2-HEMA)	Glues	Dentistry	ACD (27)
		Ethyleneglycol dimethacrylate (EGDMA)	Glues	Dentistry	ACD (27)
		2-hydroxypropyl methacrylate (2-HPMA)	Glues	Dentistry	ACD (27)
	Aliphatic Amines				
		Aliphatic Polyamines		Chemical Workers	Asthma (12), ACD (29)
		Cycloaliphatic Polyamines		Chemical Workers	ACD (29)
	Aromatic Amines			Textiles, Pesticides	ACD (29)
	Anhydrides				
		Dioctyl-phthalate		PVC Production	Asthma (12)
		Acid Anhydrides		Chemical workers, Pharmaceuticals	Asthma (30). ACD (31)
	Epoxy and phenolic monomers		Paints, Flooring Products	Painters, Builders	Asthma (63), ACD (64)
Rubber Additives					
	Accelerators				
		Carbamates (ex: Iodopropynyl butylcarbamate)	Cosmetics, Insecticides, Polyurethane Synthesis, Preservatives	Farmer, Chemical Workers	ACD (65)
		Mercaptobenzothiaz ole	Lubricants, Metalworking Fluids, Plastic and Rubber Synthesis	Automobile, Metallurgy, Chemical Workers	ACD (66)
		Thioureas	Organic Synthesis, Rubber Synthesis	Chemical Workers	ACD (67)
		Thiurams	Rubber Synthesis	Chemical Workers	ACD (68, 69)
	Antioxidants				
		N-phenyl-N- isopropyl- paraphenylenediami ne	Rubber Synthesis, Food Additives	Chemical Workers, Food Workers	ACD (70)

Table 1.3: Summary of LMW Chemicals Causing Occupational Allergy

The initiation of sensitization begins with exposure and antigen recognition. In order to gain access to the immune cells responsible for the commencement of sensitization, allergens must

penetrate the epithelium. In the skin, LMW chemical allergens may be absorbed through the stratum corneum, hair follicles, and sebaceous glands (71), accessing internal cells without physical alteration of the epithelium due to their small size. This is a departure from the mechanism of HMW protein allergens which can physically disrupt the epithelium through the utilization of factors like proteases (72). Both HMW and LMW sensitizers may take advantage of a compromised epithelium. Absorbance of LMW chemical allergens may be increased following exposure to solvents which are commonly used in a variety of workplaces (1). A widely-accepted concept explaining the immunogenicity of LMW chemical allergens involves a haptentation step, combining with and altering a self-protein, causing an allergic response following recognition by antigen presenting cells (APCs) (21, 73, 74). According to this hypothesis, in order for a LMW chemical to cause sensitization, skin penetration should occur at a rate that is quick enough to ensure that an ample amount of chemical may combine with self-proteins to produce sufficiently immunogenic conjugates sometimes involving conformational changes of the LMW chemical and the self-protein. Epithelial activation and disruption is an initial step in the activation of innate immune cells by secreted factors such as cytokines and chemokines, eventually involving APCs followed by an adaptive component. Following the establishment of sensitization, subsequent exposures may lead to elicitation, which is manifested in the form of allergic diseases like asthma and contact dermatitis. LMW chemicals have been reported to cause both contact dermatitis and occupational asthma, in the form of early and late asthmatic responses (75).

It should be noted that two additional classes of non-reactive chemical allergens exist; prohaptens are converted to reactive contact allergens by enzymes including those of the cytochrome P450 family in keratinocytes and dendritic cells and may be transported out of these cells via the

multidrug-resistance related proteins and prehaptenes may acquire allergenicity via auto-oxidation (13).

1.3.1. Dermal and Respiratory Chemical Sensitizers

As evidenced by Table 1.3, LMW chemical allergens are diverse in structure, reactivity, and application; however, there are several common attributes that are associated with immunogenicity including haptenation potential (protein reactivity), ability to access the epithelium, and irritancy induction (76). The route of exposure, exposure intensity, and duration/frequency of exposure are additional factors that impact chemical allergenicity (3). Traditionally, LMW chemical sensitizers have been classified as either respiratory or dermal sensitizers, based on their ability to sensitize via the respiratory tract or skin and their ability to induce Th2 or Th1-dominated immune responses, respectively (77). Thousands of chemicals have been identified as causative agents of skin sensitization resulting in allergic contact dermatitis such as dinitrochlorobenzene, while substantially fewer respiratory chemical allergens (<100) have been identified as causative agents of respiratory sensitization preceding asthma (78, 79). Major chemical respiratory sensitizers include acid anhydrides, chloroplatinate salts, diisocyanates, and certain reactive dyes (80). In general, the majority of chemical sensitizers are capable of causing dermal sensitization but do not cause respiratory sensitization (76); however, many respiratory sensitizers can also cause skin sensitization, leading to both skin allergy and/or asthma following skin sensitization. These observations are intriguing, given the rigid skin/respiratory classification scheme that is commonly used to identify these agents based on their sensitization potential. It is important to note that many respiratory sensitizers that have not been observed to cause dermal allergy in humans (despite high potential for skin exposure) elicit positive reactions in animal-based models designed to screen for

skin sensitization (76). Phthalic anhydride is a poignant example of this concept, as it has rarely been observed to be a human skin sensitizer yet it elicits a positive response in both guinea pig and mouse-based skin sensitization tests (76). The cause of this discrepancy has not yet been elucidated.

The juxtaposition between dermal and respiratory sensitizers has been investigated in a variety of contexts. This concept has been inspected in the realm of protein binding selectivity. It has been shown that contact sensitizers preferentially associate with cellular proteins, while respiratory sensitizers prefer to bind to soluble proteins (81). These observations require further analysis from a biologic perspective; however, protein binding selectivity may influence downstream allergic responses. In a related vein, both contact and respiratory sensitizers have been investigated in the context of preferential chemical reaction and mechanism. It has been suggested that the presence of multiple functional groups on a chemical may be directly related to its ability to cause respiratory sensitization. Contrary to this hypothesis several respiratory chemical sensitizers do not contain multiple functional groups such as abietic acid, phthalic anhydride, and vinyl benzene (82). Further investigating the electrophilic chemical reactions associated with respiratory sensitization potential, one *in silico* study profiled the chemical reactions associated with 10 respiratory chemical sensitizers (82). This study revealed diverse mechanisms involved in chemical-protein interaction among respiratory sensitizers along with varying electrophilicity/cross-linking reactivity potential requirements needed to cause respiratory sensitization specific to each class of electrophilic reaction.

The divergence between dermal and respiratory sensitizers has also been investigated in the context of Langerhans cell activity following topical administration of a dermal (2,4-dinitrochlorobenzene (DNCB)) and respiratory (trimellitic anhydride (TMA)) sensitizer (83). TMA was associated with early increased IL-10 expression (presumably resulting in decreased IL-12 levels, leading to decreased Th1 differentiation in the dLN) while DNCB resulted in early IL-1 β

expression by Langerhans cells (leading to increased Th1 and Th17 differentiation in the dLN), illustrating early divergence in the innate immune response to both types of sensitizer. Langerhans cells exhibited delayed migration in response to TMA compared to DNCB; this was confirmed to be due to increased immunosuppressive IL-10 and decreased IL-1 β expression following TMA exposure. The authors hypothesized that these dichotomous epidermal responses resulted in the altered polarization of the immune response following exposure to each of the representative sensitizers. Approaching the same concept from a different perspective, the DNA methylation status of the lymph node following cutaneous application of DNCB or TMA was investigated in a recent work by Chapman, et al. (84). This data revealed distinct DNA methylation patterns induced by chemical exposure, specifically in regard to key Th1 and Th2 -associated genes, implicating epigenetic factors in the development of contact or respiratory sensitization. In any case, the factors differentiating dermal and respiratory sensitizers are a subject of much discussion.

It has been established that dermal sensitizers preferentially elicit a Th1-mediated cellular immune response, while respiratory sensitizers preferentially elicit a Th2-mediated cellular immune response (85-87), yet the specific chemical properties that define each type of sensitizer have not been identified. Dermal sensitizers are thought to provoke a Th1-based immune response, grossly conforming to the Type IV hypersensitivity reaction described by Gell and Coombs (88). CD4⁺ Th1 and Th17 cells along with CD8⁺ T cells are commonly implicated as key players involved in the dermal sensitization response. Alternatively, respiratory sensitizers are thought to elicit a Th2-based immune response and are generally associated with the Type I atopic hypersensitivity reaction (88). These observations are true for both humans and rodents. The controversial role of IgE in the pathogenesis of many respiratory chemical sensitizers along with the mixed Th1/Th2 response observed complicates this categorization of chemical respiratory sensitizers. As emphasized before,

some of these reactions do not fit perfectly within the Gell and Coombs classification due to the complicating presence of a mixed T cell response in the case of certain respiratory sensitizers. Clearly, the dermal/respiratory classification scheme is useful in many regards but may be confusing and invalid as a rigid, mutually exclusive categorization system for all chemical sensitizers.

1.3.2. Hazard Identification Strategies for Chemical Sensitizers

Due to the environmental, occupational, and clinical significance of chemical sensitizers, the use of rapid and sensitive methods for hazard identification are necessary. A human -based assay known as the human repeated insult patch test (HIRPT) has been used to identify skin allergens; however, due to ethical concerns and alternate methods the use of this assay has been eliminated in many countries (87). Two guinea pig-based assays have been utilized to predict chemical sensitizers including the guinea pig maximization test (GPMT) and the Buehler assay; these assays are recommended for use by the Organization for Economic Cooperation and Development (OECD) guideline #406 (89). The GPMT utilizes intradermal administration of a test chemical combined with or without Freund's complete adjuvant followed by topical administration of the test chemical. Two weeks post topical dosing the animals are challenged by patch test of the flank and the allergic reaction on the skin is assessed to measure sensitization potential (89). The Buehler assay also utilizes a guinea pig model of elicitation, animals are exposed to the test agent for 6 hours for three consecutive weeks and two weeks after the final patch exposure the animals are challenged by patch test of the flank for 6 hours (89). The GPMT and Buehler assays measure the elicitation phase of allergy, complicating these test models beyond the sensitization phase, which is a noted limitation of these assays. Further limitations of these assays include limited dose selection range (based on skin irritation threshold) and the use of single induction and challenge concentrations, providing no

dose response or threshold data (90). In spite of these limitations, guinea pig-based sensitization assessment assays also have significant utility, including a substantial database of test data, allowing for comparisons to new test agents and information regarding the challenge phase of allergy (90).

Currently, the gold standard for hazard identification of dermal sensitizers is the local lymph node assay (LLNA). This assay has been validated by among independent laboratories (91, 92), with the USA-based Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (93) and the European-based European Center for the Validation of Alternative Methods (ECVAM) (87). Additionally, in April 2002 the LLNA was incorporated into the Test Guideline 429 by the OECD (94). The LLNA is based on the concept that repeated dermal exposure to sensitizers causes lymphocyte proliferation in lymph nodes draining the site of chemical application and that this proliferation can be quantified by utilizing tritiated thymidine (^3H) which is incorporated into dLN DNA. Figure 1.1 illustrates the basic protocol of the LLNA. The assay utilizes female mice (preferably of the CBA/Ca or CBA/J strain) at a minimum of four per group. These mice are dermally exposed to 25 μL of vehicle, increasing concentrations of the test agent (a minimum of three concentrations), and a positive control per ear for three consecutive days. After three days of rest (on day six) the mice are intravenously injected with ^3H . Five hours following ^3H injection mice are euthanized and the dLNs are excised and processed into a single cell suspension. These suspensions are tested for ^3H incorporation based on disintegration per minute (DPM) readings as quantified using a liquid β -scintillation counter. Results are expressed as the stimulation index (SI) which is obtained by dividing the radioactive incorporation for each treatment group over each vehicle control group or alternatively, by dividing mean DPM/mouse within each treatment group by the mean DPM/mouse for the vehicle control group. A test substance is generally considered a sensitizer if it yields a stimulation index ≥ 3 and exhibits a dose-responsive increase in dLN

proliferation. This value indicates a three-fold or greater increase in dLN cell proliferation compared with vehicle-control mice. In general, predictive tests exhibit certain limitations and results must be interpreted accordingly. The LLNA is not effective in identifying nickel salts (presumably due to variation in TLR4 signaling between mice and humans), exhibits false positives (specifically regarding non-sensitizing irritants) (94, 95), and cannot distinguish dermal and respiratory sensitizers. Regardless, the LLNA exhibits many advantages over other sensitization assays, including quick turnover and cost effectiveness, less animal trauma, an end point directly associated with sensitization, dose response analysis yielding an index of potency (EC3 value), and close correlation between EC3 and human skin sensitization data (90).

Figure 1.4 Pictorial overview of the LLNA

Recently, much experimental focus has been allocated to the development of non-animal alternatives to the LLNA. The major challenge in the development of *in chemico*, *in silico*, and *in vitro* alternatives for hazard identification of skin sensitizers is the ability to recapitulate the complex

in vivo environment of an organism undergoing sensitization. In an effort to address this concept, several assays are being developed to model haptenation, keratinocyte activation, and dendritic and T cell proliferation and interaction (87). *In silico* methods focused on predicting chemical reactivity based on the known *in vivo* reactivity of chemicals bearing structural significance are known as quantitative structural activity relationship. Additional efforts have been made to determine the sensitization potential of a chemical based on its electrophilic reactivity (96). Perhaps the most well-developed *in chemico* assay showing potential for differentiation of respiratory chemical sensitizers is the Direct Peptide Reactivity Assay (DPRA). This assay characterizes the reactivity of chemicals with model peptides (synthetic cysteine and lysine solutions) as surrogate nucleophiles, mimicking the covalent binding of electrophilic chemical allergens to nucleophilic centers in self-proteins based on the hapten hypothesis of chemical immunogenicity. This assay was initially developed by Gerberick, et al in 2004 and has been refined since then (97). In 2013 this assay was recommended for use by the ECVAM in skin sensitization testing (98) and draft guidelines were approved in early 2015 (99). These guidelines recommended use of this assay with additional tests due to the complex nature of skin sensitization prediction. Several *in vitro* assays are in the process of development for the purpose of skin sensitization hazard identification. Many of these assays are based on immunologic events that take place during sensitization including keratinocyte and dendritic cell activation (87). Endpoints such as cytokine production and cellular phenotyping are frequently utilized with these assays. While these approaches are promising, the LLNA remains the preferred method of hazard identification for dermal chemical sensitizers.

1.3.3. Challenges Regarding the Investigation of Respiratory Chemical Sensitizers

Not surprisingly, several characteristics of dermal and respiratory chemical sensitizers make these substances difficult to regulate. The lack of information regarding the immunologic mechanisms of respiratory sensitizers paired with the difficulty of setting reasonable exposure limits for these chemicals pose significant challenges for risk assessors and hazard identifiers when working with sensitizing chemicals. As described above, several validated hazard identification assays exist for skin sensitizers including the guinea pig maximization test and LLNA, among other assays (87). Although these assays are relatively effective and sensitive for dermal sensitizers, they are not validated for respiratory sensitizers.

Positive results in the LLNA are usually ascertained for respiratory sensitizers but this assay is not specific for respiratory responses and is conducted utilizing a dermal exposure model, creating problems regarding the interpretation and application of this assay to respiratory sensitizers. Modified lymph node proliferation-based assays have been tested for protein allergens following intratracheal and oropharyngeal aspiration exposures (100); however, these assays are hampered by concerns regarding vehicle and have yet to be established for respiratory chemical sensitizers. Another complicating factor contributing to the failure to develop a respiratory chemical allergen screening assay is the limited understanding of the mechanisms involved in sensitization (101). Because of this, there have been numerous experimental efforts to develop an assay capable of distinguishing respiratory sensitizers but none have been validated or universally applied.

Antibody production has been proposed as an endpoint for the identification of respiratory chemical sensitizers. The mouse IgE test is characterized by increases in total serum IgE following topical application as a function of a chemical's ability to cause respiratory sensitization. This test was initially developed in the early 1990s (102) and was complicated by variability in IgE expression

among non-respiratory sensitizers and among different mouse strains (103). In a similar fashion, Maentz and Meade developed a comprehensive flow cytometry-based assay in 1999 meant to identify and differentiate chemicals based on their ability to elicit irritation, IgE- (respiratory), or T cell-(dermal) mediated hypersensitivity responses (103). This assay is based on ear swelling and the phenotype of dLN B220⁺ B cells, specifically, their expression of IgE following 10 days of dermal exposure to the chemical of interest. These studies revealed discriminatory expression of B220 and IgE, allowing for identification of respiratory or dermal sensitizers. B220 has also been utilized as a discriminatory marker to distinguish allergens and irritants in the LLNA as irritants may induce significant dLN proliferation, causing false positives in the LLNA (104).

Cytokine profiling, T cell fingerprinting, and dLN gene expression have been proposed as methods of differentiating dermal and respiratory chemical allergens due to the dichotomy in Th1 and Th2 responses observed for each class of chemical. A variety of murine *in vivo* experiments have demonstrated preferential lymph node protein expression of IFN- γ and IL-12 (Th1-dermal) vs IL-4, IL-5, IL-10, and IL-13 (Th2-respiratory) following topical exposure to various panels of chemical sensitizers (101). Plitnick, et al. described a ribonuclease protection assay that quantified dLN cytokine messenger RNA (mRNA) levels from dermally exposed mice in an effort to distinguish contact and respiratory sensitizers (105, 106). These studies demonstrated differential Th1 and Th2-skewed dLN cytokine mRNA profiles following dermal and respiratory sensitizer application, suggesting the potential utility of this assay in the identification of respiratory chemical sensitizers. Unfortunately, dose selection parameters were difficult to pair with LLNA data and necessitated further modification. Additional efforts have been made to examine cytokine fingerprinting in the dLN following both respiratory and skin exposure to a panel of respiratory and dermal chemical sensitizers. Jong, et al. found that dLN IL-4 expression following either inhalation

or epicutaneous exposure was predicative of most respiratory sensitizers (107). Additional studies measured gene expression responses in the dLN following the LLNA exposure protocol revealing differential gene expression for mast cell proteases in respiratory sensitizers compared to contact sensitizers (108). Accordingly, several authors have noted that an initial LLNA conducted prior to cytokine fingerprinting may aid in dose selection rationale and prescreening. This method was initially proposed over 15 years ago and has not been validated, presumably due to variability associated with cytokine detection methods and results.

While many *in vitro* assays have been proposed for skin sensitization as a replacement for *in vivo* assays such as the LLNA, few comparable assays have been developed specifically for respiratory chemical sensitizers. The DPRA has revealed altered amino acid preferences in some cases when comparing dermal and respiratory sensitizers; which seem to prefer to binding to cysteine and lysine residues, respectively (109). While this trend was repeated, it was not always followed by known respiratory sensitizers; diisocyanates displayed low levels of lysine depletion in comparison to cysteine depletion, indicating that these chemicals had a reactivity profile similar to dermal sensitizers (78). This phenomena may be explained by a process known as “isocyanate exchange” characterized by reversible covalent conjugation to cysteine thiol followed by hydrolysis and transfer to a lysine residue (110, 111). Thus, the reactivity between multiple nucleophiles must be considered when evaluating the DPRA as an assay for identification of respiratory sensitizers based on lysine preference. The data presented here show that the DPRA may be a useful supplemental assay in the identification of respiratory sensitizers based on reactivity profiling. It is important to note that this assay was validated for use in dermal sensitization studies, as mentioned in the previous section, but not specifically for respiratory sensitizers.

Another recently developed *in vitro* assay has shown considerable promise in the identification of respiratory sensitizers; this assay is known as the Genomic Allergen Rapid Detection (GARD) assay. This assay was developed by Johansson, et al. and utilizes a human myeloid cell line, MUTZ-3, and a genomic biomarker signature following chemical stimulation (112). This assay was originally developed as an *in vitro* alternative to the LLNA but has shown utility in the identification of respiratory sensitizers with the use of a separate GARD Respiratory Prediction Signature (GRPS) (113). This assay demonstrated sensitivity, specificity, and accuracy values of 67%, 89%, and 84%, respectively, respectable metrics that surpass other attempted methods of hazard identification classification of respiratory sensitizers (113).

Although there continues to be progress made in the development of chemical sensitization identification assays, both *in vitro* and *in vivo*, the limited knowledge of the immunologic mechanisms of sensitization induced by chemical sensitizers continues to complicate the development of these assays. Currently, the LLNA remains the gold standard for LMW sensitizer hazard identification and additional strategies need to be investigated in order to differentiate respiratory and dermal sensitizers beyond this assay. Novel molecules and mechanisms involved in allergic disease should be examined in order to elucidate specific entities that can be utilized for the development of hazard identification assays for respiratory sensitizers.

1.4. NOVEL MEDIATORS OF ALLERGIC DISEASE

Recently, the emergence of many novel cellular subsets and molecules involved in immunological responses has occurred, shedding light on unexplored realms of the immune system and their potential involvement in a variety of disease states, including allergic disease. These novel mediators include innate lymphoid cells (ILCs), microRNAs (miRNAs), and additional Th subsets.

Currently, the general understanding of the mechanisms of LMW chemical sensitization are limited to Gell and Coombs classification. Typically, dermal sensitizers are considered Type IV inducers and respiratory sensitizers are considered Type I inducers. While the respective immune mechanisms may be valid for certain chemical sensitizers, many do not induce immune responses that fit into these categories. For example, TDI is a potent respiratory sensitizer that evokes a mixed Th1/Th2 immune response, leading to sensitization via the skin or respiratory tract, and ultimately asthma. In murine models, IgE is a major player in the sensitization and elicitation process, but in human TDI asthmatics the role of IgE is contested, complicating the Type I classification of the immune response caused by this chemical. Due to the complexity of mechanisms involved in chemical allergy and the lack of knowledge regarding these mechanisms, the investigation of novel cellular subsets and molecules involved is imperative for the greater understanding of these conditions and the development of hazard identification strategies for chemical sensitizers.

T cells are recognized as important mediators of allergic responses and chemical sensitization. Many factors can influence the phenotype and function of a T cell, including the immune microenvironment, the level and duration of TCR signaling, and the presence of signaling molecules such as cytokines. The cellular phenotype and effector functions of a T cell help determine its unique subset. Our knowledge of T cells has increased exponentially in the past thirty years, since Mosmann and Coffmann proposed the seminal T cell classification scheme, including Th1 and Th2 cells (114). The existence of regulatory T cells (T_{regs}) had also been recognized at this time and this subset was known as the suppressor T cell subset. Currently, there are many generally recognized T cell subsets, including the $CD4^+$ Th1, Th2, Th9, Th17, Th22, T follicular helper (T_{FH}), and T_{regs} , natural killer T cells (NKT), and cytotoxic $CD8^+$ cells (See Table 1.4).

Subset	Master Transcription Factor or Marker	Effector Function	Inducer Cytokines/Stimulus	Cytokines Produced
Th1	Stat4-Tbet	Intracellular pathogen defense	IL-12	IFN γ , IL-10, TNF- β
Th2	Stat6-Gata3	Large extracellular pathogen defense, Allergic reactions	IL-4	IL-4, IL-5, IL-9, IL-10, IL-13, IL-25
Th9	Batf (Stat6-Gata3), IRF4 (TCR signaling), and PU.1 (encoded by Sfp1)	Parasitic helminth defense, Allergic reactions	IL-2, IL-4, TGF- β	IL-9, IL-13
Th17	Stat3-Ror γ t	Mucosal immunity, Autoimmunity	IL-6 and TGF- β	IL-8, IL-17A and F, IL-21, IL-22
Th22	AHR	Skin immunity and inflammation	IL-1 β , IL-6, and TNF- β	IL-13, IL-22, TNF- β
T _{FH}	Bcl6 (along with CXCR5, ICOS, and PD-1)	Mediate the development of antigen-specific B cell immunity originating in germinal centers	ICOS, IL-6, IL-21	IFN- γ , IL-2, IL-4, IL-10, IL-17, and IL-21
T _{reg}	Stat5-Foxp3	Maintenance of immune tolerance, regulation of adaptive immune responses	IL-2 and TGF- β	IL-10, TGF- β , IL-35
NKT	iTCR	Prevention of autoimmunity and infection, involved in allergic reactions	Glycolipids presented by CD1d	IFN- γ , IL-2, IL-4, TNF- α
$\gamma\delta$	$\gamma\delta$ -TCR	Pathogen response	Non-MHC I/II-bound antigens, IL-2	IFN- γ , IL-17, TNF
CD8 ⁺	CD8	General infection and immunity	IFN- γ , IL-2	Granzyme B, IFN γ , Perforin, XCL1

Table 1.4: *T Cell Subsets*

While Th1, Th2, and cytotoxic CD8⁺ T cells have a recognized role in allergic disease and chemical sensitization, the roles of the remaining T cell subsets are largely unexplored. Th17 cells have been implicated in the pathogenesis of autoimmune diseases and the clearance of extracellular pathogens due to their ability to recruit and activate neutrophils, activate macrophages, and cause

acute and chronic inflammation (115). Recently, these cells have also been associated with allergic diseases such as contact dermatitis and allergy. Increased IL-17 levels and neutrophils have been found in the lungs of patients with asthma and murine models suggest that Th17 cells can act in concert with Th2 cells to promote allergic responses and airway hyperresponsiveness (115). Additionally, IL-17 appears to be important in the recruitment of neutrophils to the mouse airway and overall pathogenesis following TDI exposure (116, 117). Th17 cells may also play a significant role in the pathogenesis of allergic contact dermatitis. IL-17 was first associated with skin allergy in 1999 in a report utilizing Ni²⁺-specific T cells isolated from patients with allergic contact dermatitis (118). Both human-derived Ni²⁺-specific T cells and keratinocytes were able to express IL-17 and *il17r*, respectively. This study suggested that IL-17 was involved in T cell-mediated allergic responses in the skin. Several years later, IL-17-deficient mice were utilized in several allergic disease models in an effort to define the role of Th17 cells in these conditions (119). Chemical-induced contact hypersensitivity and an ovalbumin (OVA) -specific airway hypersensitivity model were reduced in magnitude in the absence of IL-17 and this was attributed to impaired antigen-specific T cell activation, further implicating Th17 cells and IL-17 as major players in allergic disease. The requirement of TGF- β in Th17 cell differentiation is notable, especially considering it is also important for T_{reg} differentiation and signaling. True to their name, T_{regs} are a subset of T cells that are involved in the maintenance of immune tolerance and homeostasis. T_{regs} have been implicated as key players in allergic disease as well as potential therapeutic targets in a variety of disease states and will be discussed in more detail in Chapters 3 and 5.

Th9 cells are characterized based on their production of IL-9, a cytokine that is recognized for the promotion of allergic responses. Th9 induction has been shown to be dependent on TGF- β and IL-4 expression (120), two cytokines that are also positively involved in T_{reg} and Th2

development, respectively. In a murine model of ovalbumin-induced airway inflammation IL-9 neutralization resulted in reduced allergic symptoms and inflammation (121). Atopic human patients exhibit increased Th9 cell numbers compared to non-atopic patients, correlating with increased IgE levels (122). In the same study, a murine model of house dust mite allergic airway inflammation was established and revealed rapid Th9 expansion (quicker than Th2 cells) resulting in mast cell recruitment and activation. This concept has been demonstrated experimentally, with IL-9 enhancing the survival of mast cells, stimulating the production of mast cell proteases, and generally priming the mast cells response in an allergic environment (120). Th9 cells can be identified in the peripheral blood of allergic patients and in healthy and inflamed skin (123). The stability of Th9 cells *in vivo* is a contested matter; however, the recent development of an IL-9 fluorescent reporter mouse will aid in studies designed to address this issue. There is still much to be learned about Th9 cells, both in general and in the context of allergic disease. Further characterization of this subset and increased knowledge of its role in allergic disease is important to our understanding of the mechanisms involved in allergic disease and chemical sensitization.

Another newly characterized T cell subset thought to play a role in allergic disease is the Th22 subset. Th22 cells are identified by production of IL-22 and their differentiation and activation depends on engagement of the aryl hydrocarbon receptor (AHR), leading to an increase in IL-22 production and Th22 expansion (124). Along with allergic disease, Th22 cells have been implicated in arthritis, autoimmunity, cancer, and infection. IL-22 levels are increased in the skin of patients with atopic dermatitis, allergic contact dermatitis, and allergic asthma (124). In a murine model of ovalbumin-induced allergic asthma, the external addition of IL-22 results in decreased asthmatic symptoms, implicating this cytokine as a protective factor in asthma which may be mediated through the inhibition of CCL17 and IL-25 from lung epithelial cells (124). Much remains to be investigated

regarding the role of Th22 cells in allergic disease, including the overall role of this cell type, the specific effector mechanisms involved in modulating allergic disease, and the potential interaction with additional cell types with recognized involvement in allergic disease.

Humoral immunity plays an important part in many immune responses, including multiple types of allergic disease. While B cells and antibody production are the main effector molecules involved in this type of immune reaction, T cells are known to be a prerequisite for the development of humoral immunity in many situations. T_{FH} cells are a recently characterized T cell subset that are named based on their presence in germinal centers within B cell follicles of secondary lymphoid organs, where they exert their effector function of aiding the development of B cell antibody responses. T_{FH} cells are considered imperative for the generation of almost all isotype switched and affinity matured antibodies, demonstrating their significant role in a variety of immune responses including allergy, autoimmunity, vaccine-induced immunity, and protective responses against pathogens (125). Regarding allergy, T_{FH} cells have been loosely implicated in the pathogenesis of allergic protein-induced asthma, highlighted in a recent work illustrating the role of T_{FH} cells in the process of IL-4 and IL-13 production and eosinophil recruitment, contributing to the overall pathogenesis of house dust mite antigen-induced asthma (126). Further emphasizing the integral role for T_{FH} cells in the development of allergic asthma, T_{FH} depletion during the sensitization phase of the response prevented Th2-mediated responses in a house dust mite allergy model (assessed via Th2 allergic cytokine expression) (126). This study demonstrates the important role of T_{FH} cells in the development of Th2 responses during sensitization to protein allergens and in the overall pathogenesis of allergic asthma. The role of this subset should be further investigated in additional models of allergic disease, including skin disorders and chemical-induced allergy. This data may

also help to elucidate the role of B cells and antibody in chemical sensitization, which is currently contested for sensitizers such as TDI.

Lastly, NKT cells are a unique subset of T cells that exhibit features of both T cells and natural killer cells. NKT cells express the $\alpha\beta$ T cell receptor and can be single positive or double negative for CD4 and/or CD8 (127). These cells also express an invariant TCR (iTCR) and recognize glycolipid antigens via CD1d on APCs, bypassing the traditional MHC-mediated antigen presentation strategy utilized by other T cell subsets (127). iTCR activation results in rapid and large-scale production of cytokines like IFN- γ and IL-4 (127), allowing these cells to participate in innate immune responses and help initiate adaptive responses. NKT cells have been shown to play a part in a variety of disease states, including allergic disease, autoimmunity, infection responses, systemic lupus erythematosus, and transplant tolerance (127). In the context of allergy, NKT cells have been implicated as key players most likely due to their ability to rapidly secrete large amounts of effector cytokines. Specifically, studies have solidified their pathogenic role in animal models of airway hyperreactivity and human patients with asthma, even demonstrating that NKT cells alone are sufficient to induce airway hyperreactivity following a glycolipid challenge in a murine model (127). Chemical-induced delayed type hypersensitivity skin reactions are partially mediated by NKT cells, which stimulate B-1 cells to produce IgM, leading to T cell recruitment to participate in the hypersensitivity response (128). Further research on natural ligands of the iTCR, therapeutic targeting of NKT cells, and methods of NKT activation and effector mechanisms are necessary in order to fully understand the mechanisms responsible for NKT cell involvement in allergic diseases.

NKT cells represent a class of cells that primarily exert their effector mechanism during the innate phase of the immune response, leading to the development of an adaptive response. Innate lymphoid cells (ILCs) are another class of cells in this same functional categorization, encompassing

the innate response and helping to initiate the adaptive one. ILCs are non-T, non-B cells that do not possess rearranged antigen-specific receptors; they participate in innate reactions in response to a large range of innate signals (129). ILCs are classified based on their phenotype and effector functions, and the current classification scheme is reflective of the CD4⁺ Th paradigm in the context of Th1, Th2, Th17, and Th22 cells. The commonalities between ILCs and CD4⁺ Th cells are fascinating and illustrate the worlds of innate and adaptive immunity, which coexist both uniquely and synergistically, influencing each other yet performing separate functions via unique mechanisms. Current ILC subclasses include natural killer (NK) cells, ILC1s, ILC2s, and ILC3s. NK cells produce IFN- γ , express *tbet*, and exhibit cytolytic activity (129). Based on these functional attributes, NK cells have been implicated in innate viral defense, tumor surveillance, and both organ and hematopoietic stem cell transplantation (130). ILC1s are related to NK cells, which is evident based on their production of IFN- γ and expression of *tbet*, but are distinct as they do not express granzyme B, killer-cell immunoglobulin-like receptors, nor perforin, and they respond to IL-7 rather than IL-15 (129). ILC1s are involved in inflammatory responses, including pathogen defense. ILC2s have been called nuocytes or natural helper cells, required *ror α* and *gata3* transcription factors for their expression, they produce IL-5, IL-9, and IL-13 (129), explaining their involvement in helminth infection and allergy. ILC3s require *ror γ t* and *gata3* transcription factors for their development and are divided into several subtypes. Overall, ILC3s can produce IL-17 and IL-22 and may be involved in lymphoid organogenesis, host defense at mucosal sites, and inflammatory and allergic diseases (129).

ILC2s are the ILC subtype most commonly associated with allergic disease. Initially this subset was described fairly rudimentarily, as a non-T not B cell subset associated with a Th2 response; later, this subset was further investigated and was found to express CD25 (high affinity

IL-2 receptor α), CD90 (Thy1), CD117 (c-kit), CD127 (IL-7 receptor α), CD278 (Inducible T-cell costimulator (ICOS)), ST2 (IL-33 receptor), and IL-17 receptor β and to produce IL-5 and IL-13 in response to IL-25 and IL-33 (129). ILC2s increase during helminth infections, inflammatory lung disease, and allergic conditions, and proliferate in response to pathogenic insults. In a murine model of OVA-induced allergic asthma, ILC2-derived IL-33 was found to be important for maintenance of allergic cytokine secretion, inflammatory, and airway hyperreactivity, although not as important as Th2 responses (129). Ultimately, ILC2s have been shown to be present and involved in models of allergic lung disease as well as in human patients; however, it remains to be seen whether these cells are absolutely required for the development and maintenance of these conditions. ILC1 and ILC3s may also have specific roles in allergic disease; however, they have not been studied extensively in this context. Further study of all subtypes of ILCs in the realm of allergic disease and chemical sensitization will greatly contribute to our understanding of the mechanisms responsible for these conditions and potential therapeutic strategies for allergic diseases. The scarcity of research regarding the role of ILCs in chemical-induced allergy is striking and as more experimental tools are available to facilitate the study of these cells they should be characterized in relevant models of chemical-induced asthma and skin disease, as they likely play important roles in these conditions.

Epigenetic mechanisms are commonly defined as alterations in gene expression due to changes in DNA binding elements (which can be influenced by environmental factors) as opposed to changes in the basic DNA sequence (131). These mechanisms include DNA methylation, histone modification, and noncoding RNA regulation. Yang and Schwartz suggest that epigenetic changes may be at least partially responsible for the recent increasing prevalence of asthma. In a related murine study, Th2 cytokine genes (such as IL-4, -5, and -13) and the pathogenesis of allergic asthma were shown to be regulated by the Th2 locus control region (LCR), involving histone modification

and DNA methylation (132). Numerous studies have demonstrated the link between histone modification and DNA methylation and allergic asthma in animal models. There have also been demonstrations of the link between miRNAs and allergic asthma, which will be discussed below. In human studies, the statistical association between epigenetic elements including DNA methylation, histone modification, and noncoding RNAs and asthma has been demonstrated (131); however, there remains much ground to be covered in the investigation of these components of allergic disease and the study of epigenetics itself is complicated by many factors such as diet, environmental influences, *in utero* exposures, aging, and comorbidities.

Noncoding RNA elements such as microRNAs (miRNA), transfer RNAs, small interfering RNAs, ribosomal RNAs, and long noncoding RNAs (lncRNA) are molecules that have exhibited great regulatory potential in a variety of conditions and models. Many of these noncoding RNA structures have been shown to exhibit particular functions, contributing to homeostasis, regulatory processes, and a variety of disease states. Recently the perspective on eukaryotic noncoding RNAs has shifted from that of performing generic cellular functions (for example: ribosomal and transfer RNA involved in mRNA translation, small nuclear RNAs involved in RNA splicing, and small nucleolar RNAs involved in ribosomal RNA modification) to performing diverse regulatory and biological functions normally reserved for proteins (133). These RNA molecules are not translated into protein like messenger RNA (mRNA) typically would be. Noncoding RNAs can exert functional effects by regulation of gene expression during chromatin remodeling, transcription, RNA splicing, editing, translation, and turnover (133). Specifically, regulatory RNA molecules function by base pairing with complementary sequences on other RNAs or DNAs to form complexes that are recognized by RNA-induced silencing complexes (RISCs) or RNA-editing enzymes (133). The role of miRNAs in allergic disease has been demonstrated in a number of studies including the elucidation

of the role of miR-126 in a model of house dust mite allergy and miR-145 in eosinophilic inflammation, mucus production, and Th2 cytokine production. The functional role of noncoding RNA molecules in chemical sensitization and allergy has not been investigated. Anderson et al. investigated the global dLN miRNA expression profile in a murine model of dermal TDI sensitization (134); however, the specific roles for these differentially expressed miRNAs were not identified. Pertinent functional investigations are carried out in Chapter 4.

1.5. CONCLUSIONS

The burden of occupational allergic disease is widespread. Occupational allergic conditions are multifactorial and are the result of complicated immunologic events. As illustrated in Section 1.1, a plethora of allergic diseases can be caused by workplace exposures to both protein and chemical allergens. Although Gell and Coombs hypersensitivity classification is helpful in the description of the mechanisms of many allergic reactions, this system is not fully inclusive. Many allergic reactions, especially those precipitated by drug and chemical exposures cannot be comprehensively defined within the constraints of this classification system. This illustrates the need for increased research pertaining to the mechanisms behind sensitization and allergic disease as well as the development of updated hypersensitivity classifications.

Chemical sensitizers are utilized in virtually every industry worldwide. Typically these compounds are LMW molecules that are known as dermal or respiratory sensitizers, depending on the route of sensitization. Although this classification typically predicts the type of allergic response induced (Th1 vs Th2, respectively), this paradigm is not always valid. Many chemical sensitizers can induce sensitization via dermal and respiratory routes and/or induce a mixed Th1/Th2 response. Furthermore, there are no validated hazard identification assays for respiratory sensitizers. The

perennial question of “what makes a chemical an allergen” is persistently asked and may be answered with diligent and focused mechanistic research. This inquiry is given greater weight due to the current focus on developing *in vitro* assays for the identification of chemical sensitizers. The identification of novel mechanisms of allergic disease is paramount to the objective of answering the questions of what makes a chemical an allergen and what are the immunologic events that differentiate contact and respiratory allergens. Furthermore, the investigation of innate events preceding full sensitization such as danger signals, cytokine and chemokine signaling, and APC phenotype and function is also necessary to answer this question.

As exemplified above, the discovery of novel mechanisms of allergic disease, specifically relating to chemical sensitization, is necessary to further the development of preventative and therapeutic strategies to combat allergic disease. Having a more complete view of the immunologic events taking place during both sensitization and elicitation will be achieved by investigating novel cellular subsets and players potentially involved in these events. There are numerous “suspects” in this regard as mentioned above; however, many of these have not been investigated in chemical allergy. The role of T_{regs} and miRNAs are explored in this work yet these represent only a fraction of the immunological players involved. Optimistically, the discovery of novel players will broaden the general classification schemes and views of chemical allergy as a whole. This could represent a paradigm shift that may greatly improve allergy research and understanding by clinicians, toxicologists, basic researchers, students, etc.

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CHAPTER 2

Toluene Diisocyanate (TDI)

2.1. INTRODUCTION

Isocyanates are a group of chemicals that contain at least one isocyanate group (-N=C=O). These chemicals are utilized in a variety of industries due to their high reactivity. This characteristic is attributed primarily to their isocyanate side chains, which are capable of reacting with active hydrogen atoms, readily forming crosslinks (1). Common reactions of the isocyanate group encompass interactions with hydroxyls resulting in the formation of urethanes, with amines resulting in urea derivatives, and with water forming carbon dioxide and the corresponding amine (2). Isocyanates are primarily utilized in the manufacture of polyurethane and associated products and workers may be exposed in a variety of jobs, including: painting, polyurethane manufacture (factory/chemical plant) and foam application. Many isocyanates can cause allergic disease and some may also be carcinogenic (3). Commonly used diisocyanates include hexamethylene diisocyanate (HDI), methyl diphenyl diisocyanate (MDI), and toluene diisocyanate (TDI). For the purposes of the work reported in this dissertation, TDI will be the primary focus due to its establishment in animal models of allergic disease, occupational relevance, and correlation with human allergic illness.

TDI is a low molecular weight diisocyanate utilized in the automobile industry and in the manufacture of polyurethane foams, paints, elastomers, and coatings. The two most common isomers of TDI are 2,4 and 2,6 which vary in the location of their isocyanate side groups; typically a mix of these isomers are utilized in a workplace setting. At room temperature TDI is a clear liquid with a pungent odor. Pure TDI will decompose in water but is very soluble in acetone and benzene and is miscible with additional chemicals (4). TDI is widely used throughout the United States and around the globe; the U.S. Environmental Protection Agency reports that U.S. production and importation of 2-4 and 2-6 TDI isomers rose above 1 billion pounds in 2006 (4). TDI is a potent

allergen and irritant; dermal and inhalation exposure can lead to a variety of allergic diseases including asthma, hypersensitivity pneumonitis, rhinitis, and contact dermatitis (1, 5). These diseases can be disabling and extremely severe, potentially resulting in lifelong illness and/or death (6). TDI is generally classified as primarily a respiratory (IgE/Th2)-mediated sensitizer with a Th1-mediated component (7), yet the pathogenic mechanisms of sensitization are not fully understood (8).

2.2. OCCUPATIONAL EXPOSURE TO TDI

As a result of its widespread industrial use and potent sensitizing capability, TDI is a major causative agent of occupational asthma (1, 9). Although exact worker exposure numbers are currently unknown, NIOSH has estimated that 280,000 US workers are potentially exposed to isocyanates (10) and this estimate has likely increased due to the recent increased industrial use of isocyanates (5). Both the respiratory tract and skin are documented sites of diisocyanate exposure, which can occur in aerosol, liquid, or vapor forms (11). Recently, the potential for dermal diisocyanate exposure leading to the elicitation of diisocyanate-induced asthma has been noted and there has been an increasing amount of data being released regarding this concept (5). This will be further addressed in Section 2.4. Due to its high volatility, TDI exposure may occur in all stages of manufacture and use; workers may be exposed to TDI during production, handling, and processing of TDI or during the production of products made with TDI such as foams and coated fabrics (4). Worker exposure has been confirmed via TDI adducts in the plasma and urine; toluene diamine, a hydrolysis product of TDI, has been measured in the plasma of exposed workers after acid hydrolysis as an alternate marker of exposure (4). It is difficult to monitor and control occupational TDI exposures due to the mixed species of TDI commonly used in workplaces along with the lack of

well-defined exposure data (11). Despite gaps in the literature regarding TDI exposure it is clear that reduced exposure levels lead to lower cases of TDI-induced asthma.

Limiting the development and incidence of occupational allergic disease is key to maintaining the health, safety, and vitality of workers. One strategy utilized in the pursuit of this goal is the establishment of occupational exposure limits (OEL). The current TDI OEL set by the Occupational Safety and Health Administration (OSHA) is 0.02 ppm (approximately 140 $\mu\text{g}/\text{m}^3$) and 2.5 ppm is considered Immediately Dangerous to Life and Health (IDLH). Workplace air concentrations can range from < 1 to $> 1,000 \mu\text{g}/\text{m}^3$, based on various reports (4). Typically these limits are set by risk assessors based on experimental data and human health studies (covering effects and exposures) encompassing carcinogenesis, portal of entry irritation, and systemic effects (12). Frequently OELs are not adequate for protecting workers from chemical-induced allergy due to limited human data and the complexity of the sensitization response. Several of the key challenges associated with setting OELs for chemical allergens are: selection of sensitization or elicitation to serve as the basis of the OEL, temporal factors involved in the allergic response, individual susceptibility and reaction to sensitization, and examination of the relevant routes of exposure (12). Furthermore, once sensitization has occurred, exposure to very low levels of TDI (below the accepted OELs) may elicit symptoms, making regulation extremely difficult (11). Asthmatic responses have been demonstrated in sensitized workers following inhalation challenge of ≤ 1 -5 parts per billion (ppb) TDI, which is at or below the current American Conference of Governmental Industrial Hygienists (ACGIH) threshold limit value time weighted average of 5 ppb (12). Clearly, prevention of sensitization is the ideal outcome of regulation; however, if this isn't feasible early diagnosis and removal from any exposure would be the best course of action for a sensitized worker.

2.3. TDI SENSITIZATION

The TDI sensitization response is characterized by a tightly regulated and coordinated immune response involving a variety of cellular subsets and signaling molecules (13); however, much is still unknown regarding the immunologic events that take place following TDI exposure and during sensitization. Both dermal and respiratory sensitization are capable of priming the body for TDI asthma, highlighting the systemic nature of sensitization (14, 15). The realization that dermal sensitization may result in respiratory allergic disease has significant implications for risk management and preventative measures in the workplace, as skin protection may be just as, or more important than respiratory protection, which is discussed in further detail in Section 2.4.

In a typical T-cell mediated response involving dermal or respiratory exposure to a protein allergen, following access to the epithelium, allergen fragments are taken up by APCs. It is believed that LMW chemicals like TDI pass through the stratum corneum then form complexes with self-proteins, acquiring immunogenicity that is similar to that intrinsically exhibited by peptide fragments of protein allergens. Following APC-antigen uptake these specialized cells migrate to the lymphatics, particularly the dLNs, where the allergen fragments are presented to T cells via MHC-mediated antigen presentation. This is thought to be the case for protein and chemical allergens. APC activation and mobilization are important events that may play an important part in the development of sensitization. These events are modulated primarily via increased local cytokine and chemokine expression such as caspase-1, IL-1 β , IL-18, and TNF- α along with danger signaling following chemical exposure in the case of sensitization precluding contact dermatitis in both humans and mice (14, 16). In mouse skin, three major types of dendritic cells are thought to be important APCs involved in chemical sensitization: Langerhans cells (Langerin⁺CD103⁺), and two subsets of dermal dendritic cells (Langerin⁻CD103⁻ and Langerin⁺CD103⁻). This event causes T cells

to proliferate and differentiate into a number of different CD4⁺ T-helper (Th) and CD8⁺ effector subsets including Th1, Th2, Th17, T_{reg}, and cytotoxic T cells. In a chemical-induced contact dermatitis, preferential expansion of Th1, T_{reg}, and cytotoxic CD8⁺ cells is observed (16). These activated T cells can migrate through the lymphatics, blood, and local tissues; orchestrating an adaptive immune response against the allergen. This process characterizes the sensitization response and repeated exposures can cause the sensitized T cells to produce specific cytokines capable of skewing the immune response to a Th1/cytotoxic (contact dermatitis-dermal sensitizer) or Th2 (asthma-respiratory sensitizer) –mediated pathway. Th1 cells are characterized by production and secretion of interferon- γ (IFN- γ), IL-2, and tumor necrosis factor α (TNF- α), while Th2 cells are characterized by production and secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 (17). A mixed Th1/Th2 response is observed in both animal models and humans exposed to diisocyanates like TDI (18). Diisocyanate-specific T cells and Th1 and Th2 cytokines have been observed in the respiratory mucosa of diisocyanate-exposed workers and this concept has been ratified in animal models of TDI sensitization and asthma, furthering the importance of Th1 and Th2 cells and associated cytokines. Activated T-cells and an allergic microenvironment may also stimulate B cells to become plasma cells and secrete antibody.

Once the elicitation phase begins, a coordinated cellular response occurs and the allergic response ensues. This response involves re-exposure to the haptenated complex as an antigen, causing crosslinking of the antigen with antibody, binding to mast cells and basophils, leading to degranulation, the production of additional antigen-specific allergic antibody, and the proliferation of antigen-specific T cells and the involvement of memory T cells. The elicitation response is responsible for the symptoms of allergic disease such as asthma, which may involve airway hyperreactivity, inflammation, and mucus secretion. The clinical pathogenesis of TDI-induced

asthma is not fully understood; however, observational data has been collected describing the physiological features of individuals suffering this condition. Following an initial period of sensitization and latency which may last for months to years following initial TDI exposure, asthmatic symptoms typically begin to appear. Overall, the clinical presentation of TDI-induced asthma is variable, which often complicates diagnosis and treatment (11). Early symptoms include chest tightness, cough, shortness of breath, and wheezing and may appear anywhere from <1 hour to 2-4 hours following workplace TDI exposure (11). Clinical features of TDI-induced asthma are similar to those of atopic asthma and include bronchial hyperreactivity and inflammatory infiltrate, goblet cell hyperplasia, increased airway mucus production, and subepithelial fibrosis (11). An ideal animal model of TDI asthma would recapitulate airway narrowing and bronchial inflammation along with hyperreactivity following sensitization and airway-based challenge (19). TDI-induced asthma can be fatal; in one case, an autopsy of a patient with TDI-induced asthma revealed symptoms similar to those found in status asthmaticus (2). These findings included excessive mucus production, overinflated lungs, epithelial desquamation, bronchial wall thickening, and lymphocyte infiltration in the lamina propria. Allergic rhinitis has also been reported as a comorbidity and sometimes a preceding condition in patients with TDI asthma and rhinitis may cost more in terms of lost productivity and time at work compared to asthma (18). Due to the similarities between the upper and lower respiratory tracts in regards to fundamental allergic susceptibility many of the factors involved in TDI sensitization and asthma are thought to play a role in TDI rhinitis.

Additional factors have been implicated as risk factors for human TDI sensitization including cigarette smoking and polymorphisms in the glutathione *S*-transferase, N-acetyltransferase, and MHC class II genes (14), shedding light on potential mechanisms and pathways involved in this process. Interestingly, there has been no consistent link between IgE or atopy and TDI in humans.

The lack of association between IgE production and TDI sensitization and asthma is intriguing considering the well-established link between atopy and other chemical allergens, including acid anhydrides, platinum salts, and dyes (14).

2.4. DERMAL TDI SENSITIZATION

The epithelium plays a significant role in the early stages of sensitization, acting as a physical barrier to allergens and a site of immune surveillance (20). The skin, in particular, serves as a protective layer for our bodies from the outside environment. As the largest organ in the body, the skin is an extremely important player in relation to allergic disease. The skin is made up of the epidermis and the dermis. The epidermis represents the outermost portion of the skin and is comprised of cornified lipids and proteins such as keratin and filaggrin (21), forming a protective barrier for the rest of the body. The inner layer, or dermis, is comprised of lymphatic vessels, hair follicles, sebaceous glands, nerve fibers, connective tissue, and blood vessels (21), effectively cellularly connecting the skin to the rest of the body. The presence of multiple innate immune factors present in the skin including leukocytes, complement factors, antimicrobial peptides, and pattern recognition receptors (22) allow the skin to be a site of immune surveillance and tolerance yet these factors may also contribute to the development of allergic disease. Not only is the skin involved in the development of dermal sensitization, it has recently been implicated in the development of systemic sensitization leading to elicitation at various sites in the body, including the respiratory tract (5). This has been demonstrated in animal studies involving both protein and chemical allergens. Ovalbumin and isocyanates are perhaps the most well-studied models exhibiting skin sensitization leading to asthma (21, 23) but this has been demonstrated in a rat model of TMA sensitization as well (24). In humans, several studies have linked isocyanate skin exposure to asthma risk and prevalence (21, 25). These studies face severe limitations, including the limited methods of skin

exposure quantitation, timing of sample collection, the diverse nature of dermal exposures and chemical composition (including mixed exposures), influence of personal protective equipment (PPE), and risk assessment challenges (21). Regarding isocyanates specifically, the reliance upon free unreacted isocyanate groups for sampling methods further complicates the accurate documentation of isocyanate skin exposure (26).

Due to occupational activities, the epithelium may be compromised and workers may be exposed to a variety of potential allergens, leading to increased risk of an allergic reaction to these substances. Loss of barrier function has been identified as an important factor in the development of various allergic diseases including asthma, atopic dermatitis, and sensitization (21). This concept has been elegantly demonstrated with animal and human-based studies involving filaggrin, a protein involved in the formation of the epidermis (27-29). Genetic variability in barrier protein genes, solvent and soap exposure, excessive hand washing, existing skin conditions, humidity, temperature, physical trauma, and glove use are all factors that can influence skin barrier function, which is a known factor in the development of allergic disease (21). Barrier disruption and dysfunction followed by allergen exposure may allow the perturbing agent to activate the epithelium, leading to the production of cytokines such as IL-33 and thymic stromal lymphopoietin (TSLP), which are directly involved in the stimulation of Th2 responses (30, 31). Barrier dysfunction also allows dermal dendritic cells and Langerhans cells to come into contact with allergens and become activated, also potentially impacting the development of Th2 responses.

Dermal isocyanate exposure has been a fairly recent topic of concern in relation to the historical perception that respiratory exposure primarily leads to asthma. Several cases of skin sensitization leading to asthma have been surmised in humans and this concept has been demonstrated in animal models (21). In a workplace setting, the likelihood of dermal contact with

isocyanates may be high, further supporting the idea that dermal exposures may lead to respiratory allergic disease. In addition to their probability of occurrence in the workplace, isocyanate skin exposures also have the potential for high dose-delivery in comparison to inhalation exposures (5). From a regulatory perspective, there are few standards for skin isocyanate exposure in comparison to respiratory guidelines (5). Although skin exposure as a route of sensitization has been noted and is a generally accepted concept among experts, there are no dermal exposure limits for di- or poly-isocyanates including TDI.

Chemical potency in relation to sensitization potential may also be affected by the route of exposure. The studies described in this dissertation utilize a single exposure TDI sensitization model. This model was developed on the basis of extensive experimental sensitization confirmation as well as historical literature suggesting that single isocyanate exposure is sufficient to lead to sensitization in mouse models (5, 21). Several murine studies have demonstrated the ability of a single dermal exposure of TDI (1% and 0.3%) to induce respiratory allergic symptoms such as lung associated lymph node cytokine secretion, serum IgE, and airway inflammation and hyperreactivity after subsequent respiratory challenge (32, 33). Similar results have been ascertained utilizing HDI (34) and MDI (35), two related diisocyanates. As our studies and others' have demonstrated, doses ranging from 1-100 μ mol isocyanate in the form of 1-10% solution are sufficient to induce sensitization in a murine model and are representative of a realistic occupational exposure of a few droplets of diluted diisocyanate solution (5). These realistic exposure scenarios are compounded by human case reports suggesting development of diisocyanate asthma in occupational settings with very low or undetectable air isocyanate levels, implicating dermal sensitization in these workers (5). In addition to animal models and human case reports, several epidemiologic studies have linked skin diisocyanate exposure and asthma risk together, further strengthening the concept of dermal

sensitization causing a systemic immune response that can lead to asthma and other Th2-mediated allergic diseases. This data emphasizes the need for more focused research in order to elucidate mechanisms involved in this phenomenon. The lack of suitable workplace exposure limits for respiratory sensitizers that may sensitize dermally as well is a problem that needs to be addressed in order to limit unsafe exposures. Clearly, the study of skin exposure-respiratory elicitation relationships has revealed interesting data yet there is still much to be researched in order to properly protect workers utilizing chemical and protein sensitizers.

2.4.1. Animal models of Dermal TDI sensitization

Karol, et al first demonstrated that TDI skin sensitization is capable of priming the immune system for subsequent respiratory asthmatic responses in guinea pigs (15). Following this study, other investigators have demonstrated skin exposure to TDI leading to sensitization, followed by inhalation challenge, resulting in an asthmatic response (19, 32, 36, 37). Vanoirbeek, et al. proposed a model of dermal TDI sensitization (ear; three consecutive days of dosing with 0.3% or 3% TDI or one single dose of 1% TDI) followed by a dermal “boost” at day 7 (same concentration as initial dosing) and intranasal challenge at day 10 (0.1% TDI or vehicle) (19). These dosing regimens were followed by whole body plethysmography (40 min following intranasal challenge) and methacholine challenge (24 hours later). Terminal endpoints were bronchoalveolar lavage (BAL) and histology. All mice that were dermally sensitized with TDI exhibited elevated enhanced pause (PenH) following intranasal TDI and/or methacholine challenge. This alteration in ventilatory function was paired with increased neutrophil inflammation with the exception of mice dermally sensitized with 3% TDI (high dose) and given the day 7 dermal “boost”. This observation led the authors to conclude that immunological tolerance may be responsible for the lack of a respiratory

response following intranasal challenge in this group, introducing an important concentration-dependent response relationship between TDI and allergic reactions, as has been noted for protein allergens. The efficacy of dermal sensitization has also been demonstrated for other chemicals such as trimellitic anhydride and protein allergens such as ovalbumin that can cause respiratory allergic disease (5, 24, 38). Another notable study was performed by Matheson, et al. that involved repeat subcutaneous TDI sensitization followed by repeat inhalation challenge (39). This model revealed increased methacholine airway reactivity and nasal and tracheal inflammation in wild-type mice sensitized with TDI, exhibiting the effective sensitizing potential of the subcutaneous route.

Some important considerations regarding murine models of dermal TDI sensitization include potential differences in skin permeability between mice and humans and the relevance of exposure site and dose compared to real world exposures. While the ability of TDI to penetrate both mouse and human skin is not in question, potential variability in skin permeability between the two organisms are important to remember when translating the value of these studies. In a manuscript examining dermal penetration of perfluorooctanoic acid in mouse and human epidermal membranes Anderson, et al. observed slight variations in chemical penetration in mouse (39%) and human (24%) skin following perfluorooctanoic acid (PFOA) application (40). Additionally, the total absorbable amount was 50% in mouse skin and 69% in human skin. These findings illustrate potential discrepancies between mouse and human skin permeability and chemical uptake, which are important to consider when choosing doses for experimental studies and extrapolating murine results to human data. The route of exposure and site of exposure should also be kept in consideration when interpreting murine studies. Many dermal TDI sensitization models are carried out using ear pinnae exposures, which are experimentally sound and necessitated yet may not accurately represent real-world exposures which presumably take place at other sites on the body (hands, arms, etc.).

Additionally, subcutaneous injection models are a departure from most realistic exposure scenarios but may mimic exposure in the presence of skin abrasions and epidermal lesions. Regardless of the site and route of exposure, dermal TDI sensitization models are valuable tools utilized for the investigation of this immune response. Dose selection is also an important aspect of murine TDI sensitization models. For many studies, the aim of dose selection is to induce a significant state of sensitization, while others seek to mimic real-world exposures. We should be careful in interpreting exact doses from murine to human exposures due to the factors mentioned above including dermal penetration variability and route/site of exposure in mice and humans.

Skin sensitization has been proposed to be an extremely effective route of allergic sensitization, in some cases it has been suggested to be more effective than respiratory sensitization regarding the downstream elicitation of asthma. In a study evaluating four different models of MDI-induced respiratory sensitization in guinea pigs, epicutaneous and intradermal MDI exposure were reported to be more effective at sensitizing the respiratory tract than inhalation MDI exposure (41), indicating that skin sensitization is a relevant and powerful route of exposure in relation to respiratory chemical sensitization. More recent studies have bolstered this concept, including a TDI sensitization study that focused on a variety of sensitization exposure scenarios (epicutaneous, subcutaneous, and intradermal) followed by respiratory challenge in mice, revealing that epicutaneous sensitization resulted in the most clinically relevant pathophysiological response in the lungs while inhalation sensitization was, in comparison, not an effective route of sensitization (32). Observations with formaldehyde suggested that dermal sensitization was far more effective than respiratory sensitization, which did not occur following comparable formaldehyde exposures yielding similar irritation responses (42). Similar investigations were completed utilizing the potent respiratory sensitizer glutaraldehyde and they revealed clear skin sensitization and the absence of respiratory

sensitization, despite an irritant response in both tissues (42). The authors hypothesized that the hydrophilic and highly reactive glutaraldehyde dissolves in the mucus of the airways where it reacts with the dissolved lysine-rich proteins in this fluid, preventing immunologic activation by APCs which are required to induce sensitization (42). This observation may be unique to aldehydes; other LMW sensitizers may be less reactive and more lipophilic and in turn less affected by the mucus layer in terms of sensitization potential (42). In contrast, the skin does not contain a mucus layer, allowing LMW chemicals to more readily access APCs and initiate sensitization. Interestingly, it has been reported that thiol-rich proteins in the stratum corneum may act as a barrier against dinitrothiocyanobenzene (DNTB) which contains highly reactive –SCN groups (42). It appears that the unique environments of the skin and the respiratory tract are important factors in the development of LMW chemical-induced sensitization and these tissues exhibit unique characteristics that may impact the sensitization potential of chemicals depending on a number of factors.

2.5. IMMUNOLOGIC FACTORS INVOLVED IN TDI-INDUCED ALLERGIC DISEASE

TDI-induced sensitization and asthma are complex and multi-factorial conditions. TDI sensitization is characterized by repeated exposures leading to the establishment of the allergic threshold. Typically, these exposures are cumulative and may occur for weeks to years, allowing the sensitization response to develop. Once the elicitation threshold has been met, exposure to minute amounts of TDI can lead to severe asthmatic responses. Although pathologically similar to atopic asthma, several key discrepancies have been identified in clinical studies, including a mixed Th1/Th2 response, low association with atopy and specific IgE antibodies, and high numbers of antigen-specific CD8⁺ T cells (7). While the mechanisms of sensitization and elicitation have not

been fully elucidated for TDI-induced allergic disease, there are a number of immune mediators that have been suggested to play a role in these processes.

2.5.1. Antigenicity and APCs

TDI-induced asthma differs in the process of antigenicity compared to protein allergens, presumably by forming haptens with self-proteins such as albumins and keratins. The high cross-linking potential of diisocyanates like TDI contribute to their industrial utility but also to their immunotoxicity. The electrophilic nature of TDI allows this chemical's isocyanate side chains to react with hydroxyl, amino, and thiol groups on proteins, forming covalent bonds with self-proteins (11); in a process known as haptenation. This event likely alters the mechanism of sensitization compared to that of protein allergens. TDI is thought to bind to actin, albumin, glutathione, hemoglobin, keratin, laminin and tubulin *in vivo* based on numerous studies (11, 43, 44). This binding may result in cellular damage and immune cell recognition of TDI-protein conjugates, fully initiating the TDI sensitization immune response. The isocyanate groups in TDI can react with amine groups to form ureas, hydroxyl groups to form urethanes, and/or thiol groups to form thioureas (43). The antigenic portion of TDI-hapten complexes are poorly understood along with the overall process *in vivo*. This is a challenging subject as TDI and reactive diisocyanates can self-polymerize and haptenate proteins at single or multiple locations, creating a complex chemical environment (43). A greater understanding of this process will likely aid in the understanding of the immunologic basis for TDI sensitization and that of LMW chemicals in general.

It has been demonstrated that TDI-protein complexes interact with Langerhans cells in the skin (specifically, in the hair follicles) and dendritic cells in the dLN in a murine model of epicutaneous TDI sensitization (45). This interaction illustrates the initial step in the initiation of an

adaptive immune response, antigen presenting cell-antigen interaction. In an elegant study conducted to analyze binding of the hapten-major histocompatibility complex class II (MHC II) complexes to antigen-specific T cells, Kohler, et al. demonstrated that hapten-peptide complexes of trinitrophenyl (TNP) bound to a variety of proteins (both mouse self-proteins such as albumins as well as non-mouse proteins) accounted for the majority of epitopes recognized by TNP-specific CD4⁺ T cells (46). This landmark study demonstrated the role of the chemical-peptide hapten complex in the induction of sensitization. Following APC uptake of the hapten complex, these cells may migrate to the lymphatics and interact with T cells, initiating an adaptive response. Uncertainty regarding the exact structure and identity of TDI hapten complexes that are relevant *in vivo* have posed challenges for diagnosis and understanding of the mechanisms driving TDI-induced sensitization and asthma.

2.5.2. The Role of IgE in Respiratory Chemical Sensitization and Asthma

Unlike atopic asthma induced by protein allergens, IgE has not been definitively implicated as an integral immune mediator in all cases of chemical-induced asthma, even though clinical symptoms are similar for both types of asthma (47). For example, the consistent expression of IgE in murine models of TDI allergy (48) is not reflected in human studies, as many individuals with diagnosed TDI-induced asthma do not have increased levels of TDI-albumin conjugate-specific IgE in their blood (49). As few as 5-30% of symptomatic workers have detectable levels of diisocyanate-specific IgE (11). Levels of diisocyanate-specific antibodies typically do not correlate with development of reactive airway disease (50) and atopy has not been identified as a risk factor for diisocyanate-induced asthma (11). Additionally, during the elicitation phase of diisocyanate-induced asthma, isolated “early” reactions occurring < 1 hour following diisocyanate exposure are

less prevalent than that observed in protein induced asthma (11) (although they have been reported) suggesting that diisocyanate induced asthma is not solely IgE-mediated, as this response alone would be immediate. As IgE is a hallmark of the humoral response induced by Th2 responses (51), this data suggests that either TDI-specific IgE is not capable of being properly detected or an IgE-independent allergic mechanism is involved in human TDI sensitization. The hypothesis regarding limited TDI detection is an interesting concept, given the uncertainty regarding the specific identification of the TDI-hapten complex responsible for antigenicity. Several components of antibody composition, including appropriate selection of hapten-protein conjugates and the molar substitution ratio, along with the time of blood collection in relation to chemical exposure are important in the detection of IgE in chemical allergy (14). The temporal component of IgE detection also seems to play an important role in this process, in serum radioallergosorbent tests (RAST) from patients with documented diisocyanate-induced asthma specific IgE measures were more likely to be detected if the samples had been collected within 30 days of the last exposure to diisocyanate (52). The requirement of a Th2 response for respiratory chemical sensitization and allergy, which has been well demonstrated in animal models and humans, suggests that IgE-independent mechanisms could be at play. Further complicating this concept, a strong association between IgE and chemical-induced asthma has been made for several chemicals, including methyltetrahydrophthalic anhydride (53). The relationship between IgE and TDI sensitization is complicated and is not fully understood. There remains doubt as to the efficiency of hapten-specific IgE detection as well as the true role of IgE in TDI sensitization and allergic disease. Further insight into this concept will be valuable for toxicological evaluation, prediction, and risk assessment.

2.5.3. Immune Factors Involved in TDI Sensitization and Elicitation

A hallmark of isocyanate-induced allergic disease in both humans and mice is an active Th2 adaptive immune response. This response also involves a Th1 component, but this is generally thought to be less dominant. Increased levels of a variety of cytokines (IL-1 β , IL-4, IL-5, IL-6, IL-15, and TNF- α) and immune cell subsets (CD4⁺ and CD8⁺ T cells) have been documented in the BAL and sputum of patients with diisocyanate-induced asthma (2). These data illustrate the mixed Th1/Th2 response that takes place in this response. In a murine model of inhalation sensitization (6 weeks, 5 days/week, 4 hours/day) and challenge (following a 14 day rest period, 1 hour), adaptive immune components of TDI asthma were examined (7). Following TDI sensitization and challenge CD4- and CD8-deficient mice displayed significant reductions in PenH, an indirect measure of airway hyperreactivity, following a nonspecific methacholine challenge. CD4 knockout mice exhibited reduced serum total IgE and undetectable total IgG levels following TDI sensitization and challenge compared to increased levels of both antibodies in TDI sensitized and challenged control animals. CD8 knockout mice displayed similar reductions in IgE but did not show any significant decrease in total IgG levels nor any changes in specific IgG isotypes. CD4 and CD8 knockout mice sensitized and challenged with TDI also experienced reduced levels of lung histopathological changes that were seen in TDI sensitized and challenged control mice; these changes include goblet metaplasia, epithelial damage, and cellular infiltration. Th1 and Th2 cytokine mRNA expression was not elevated in CD4 and CD8 knockout mice following TDI sensitization and challenge, while control mice undergoing TDI sensitization and challenge exhibited increased levels of both Th1 and Th2 cytokine mRNA. The authors also utilized mice deficient in IFN γ , IL-4, IL-13, and IL-4+IL-13 in their TDI sensitization and challenge model in an effort to establish the importance of these signaling molecules in the pathogenesis of TDI-induced asthma. Following TDI sensitization and challenge all cytokine knockout mice exhibited reduced airway hyperreactivity following a

nonspecific methacholine challenge compared to control mice. Total IgE antibodies were significantly decreased in the serum of IFN γ , IL-4, and IL-4+IL-13 knockout mice following TDI sensitization and challenge, while all knockout groups exhibited lower IgG levels. The histopathologic changes seen in cytokine knockout groups were less pronounced than that of the control TDI sensitized and challenged control mice. A decrease in BAL eosinophilia was noted in CD4, CD8, IL-4, IL-13, and IL-4+IL-13 knockout mice following TDI sensitization and challenge, indicating that both CD4 and CD8-mediated mechanisms may play a role in TDI-induced asthma pathogenesis. In summary, this seminal study emphasizes the importance of the T cell response in the pathogenesis of TDI-induced sensitization and asthma. Specifically, this work demonstrated the requirement of the CD4⁺ Th2-mediated response and the less critical but still significant role of CD4⁺ Th1 cells and CD8⁺ T cells in this response. The synergistic role of Th1 and Th2 cells in asthma pathogenesis has been proposed in models of protein allergy and appears to be a component of this study.

Additionally, the role of B cells in TDI-induced asthma is a relevant topic, considering the controversial role of IgE in this condition. In a recent work by De Vooght, et al., a dermal, murine TDI sensitization model was utilized to exhibit the importance of B cells in the allergic response following TDI challenge (54). Adoptive transfer of B cells from TDI-sensitized mice into naïve, B-cell knockout, and severe combined immunodeficiency (SCID) mice resulted in asthmatic symptoms following oropharyngeal TDI challenge; these observations suggest a T cell-independent capability of B cells to induce allergic outcomes. Between this data and the questionable role of IgE in diisocyanate-induced asthma, B cells should be further investigated in chemical-induced allergic disease, specifically in human patients or human-like animal models in order to recapitulate the specific mechanisms of B cell activity in these conditions.

TNF- α is a Th1-associated cytokine that is pleiotropic and is a central regulator of inflammatory responses. This cytokine has been implicated as a significant player in TDI-induced asthma due to its inflammatory nature and its potential effects on dendritic cells. In a murine model of subcutaneous TDI sensitization and inhalation challenge TNF- α neutralization (via TNF-targeting antibody or TNFR1/2 knockout) reduced airway dendritic cell migration, inflammation, pathology, and hyperreactivity (following a methacholine challenge) (55). Interestingly, serum TDI-specific IgG and total IgE levels were not affected by TNF- α neutralization, suggesting a role for this cytokine in the early inflammatory phase of the allergic response. This data along with additional studies demonstrate essential role for Th1 cells in the progression of TDI-induced asthma. Although there has been limited investigation regarding the potential role of other T cell subsets in TDI sensitization and allergic disease, upregulation of T_{reg} and Th17-associated cytokines (IL-10 and IL-17, respectively) were observed in the auricular lymph nodes of dermally sensitized mice receiving a single oropharyngeal aspiration challenge of TDI (56). This data suggests a potential role for additional T cell subsets in TDI allergic disease and prompts investigation of these subsets in both animal models and humans.

Neutrophils, mast cells, and eosinophils are additional immune cell populations thought to play a role in TDI-induced sensitization and asthma pathogenesis. Clinical human studies support this concept, finding increased neutrophil activation and IL-8 levels in sputum samples (8), elevated eosinophil levels in the bronchial mucosa (47), and elevated mast cell numbers in the airway mucosa of TDI asthmatics (57). Neutrophil involvement marks a key difference in TDI-induced and protein-induced asthma; several studies have demonstrated the presence of airway neutrophilia during TDI asthma in both mice and humans (11), suggesting that the pathogenic mechanisms differ between these two conditions. In a murine model of TDI asthma de Vooght, et al. depleted granulocytes and

neutrophils prior to airway (oropharyngeal aspiration) TDI challenge using a variety of strategies including chemical and antibody-mediated depletion (58). Neutrophil depletion ameliorated airway hyperreactivity, suggesting that this cellular compartment is involved in the pathogenesis of TDI-induced asthma.

A loss of functional airway epithelium is also observed in isocyanate asthma. An *in vitro* study demonstrated disintegration of tight junctions between human bronchial epithelium cells, cytotoxicity, and increased mucus secretion following exposure to TDI. This study also found damage to cilia and impairment of their function at increasing concentrations of TDI, explained by an interaction between the isocyanate groups of TDI and structural filaments like tubulin (59). Another factor that may contribute to airway epithelium damage is isocyanate-glutathione binding (2). This action may lead to the loss of glutathione-mediated protection against oxidative stress. Interestingly, several studies have demonstrated a connection between polymorphisms in glutathione S-transferase supergene family genes (GSTM1, GSTP1) and the risk for developing diisocyanate-induced asthma (2). These enzymes are responsible for catalyzing the conjugation of glutathione to electrophilic substances like TDI (60), indicating that glutathione may play a protective role in the pathogenesis of TDI-induced asthma. The extracellular matrix (ECM) of the lungs can be disturbed during asthma, characterized by bronchial remodeling. Matrix metalloproteinases (MMPs) are proteases whose primary function is to break down the ECM (61); MMPs and their inhibitors are important players in the homeostasis of the ECM under normal and perturbed conditions. MMP-9 is known to cause bronchial remodeling in allergic asthma and it also induces the movement of granulocytes across basement membranes (62). Due to the known roles of MMPs in allergic asthma, the role of these enzymes in a murine model of TDI-induced asthma was investigated by Lee, et al. (62). Mice were sensitized to TDI by intranasal administrations for five consecutive days; following

three weeks of rest, mice were further sensitized for 5 more days. A week after the second sensitization exposure regimen mice were challenged via inhalation. After establishing their model of TDI asthma, the authors observed increased levels of MMPs (including MMP-9) in BAL of TDI-exposed mice. After treating mice with a MMP-9 inhibitor three times at one day intervals beginning 30 minutes before TDI challenge the lung inflammatory cell infiltrate was decreased (total cells, neutrophils, and eosinophils) and mice displayed reduced airway hyperreactivity following a nonspecific methacholine challenge compared to mice exposed to TDI and not receiving MMP-9 inhibitor. This data demonstrates a role for MMPs, specifically MMP-9, in the pathogenesis of TDI-induced asthma. Based on known mechanistic data, the authors postulate that MMP-9 activity alters the ECM, allowing the migration of inflammatory cells into the airways (62).

Oxidative stress is known to play a pathogenic role in a variety of conditions, including respiratory illnesses such as contact dermatitis, asthma, and chronic obstructive pulmonary disease (63, 64). The anti-oxidant defenses in the body exist to protect various tissues such as the lungs and the skin from oxidant insults such as cigarette smoke, UV radiation, combustion products, and homeostatic cellular processes. These defenses include glutathione, vitamins A and E, catalase, superoxide dismutase, transferrin, haem oxygenase-1, and redox proteins (63, 65). Elevated transferrin and decreased ferritin levels were observed in the serum of TDI asthmatics compared to asymptomatic exposed control subjects and non-exposed control subjects (63, 66). Ferritin's role in detoxifying inflammatory-induced oxidative stress and transferrin's antioxidant capabilities suggest altered oxidative defense capabilities during TDI asthma. Following further investigation, expression of ferritin light chain and the antioxidant protein heme oxidase-1 was inhibited in human airway epithelial cells in culture following TDI exposure (63). This was demonstrated to take place via MAPK signaling inhibition which resulted in defective downstream Nrf2 nuclear translocation

(63). These TDI-induced signaling alterations may result in a significantly impaired anti-oxidant defense repertoire due to the inability of Nrf2 to translocate to the nucleus, bind to anti-oxidant response elements, and induce transcription of numerous genes important to cellular defense against oxidative stress (67). Importantly, cellular treatment with a PPAR- γ agonist, activating another important antioxidant signaling molecule, was able to rescue the expression of heme oxygenase-1 and ferritin light chain which was formerly inhibited by TDI (63). Additional studies investigating the therapeutic potential of epigallocatechin-3-gallate, a compound found in tea that has exhibited anti-allergic properties, revealed that this compound reduced murine TDI-induced asthma severity via regulation of inflammatory cell migration (68). This may be mediated via suppression of MMP-9 and reactive oxygen species (ROS) (68), two key mediators of ECM injury and oxidative stress which are known to play a role in TDI asthma (62). These studies reveal the therapeutic potential of targeting antioxidant mechanisms in the treatment of TDI-induced asthma.

In contrast to TDI elicitation, little is known regarding the role of oxidative stress during TDI sensitization. Oxidative stress is known to cause apoptosis, specifically in T_{reg} s, which have been shown to be critically involved in a murine model of epicutaneous TDI sensitization (69). Additionally, oxidative stress induced by food contaminants and environmental chemicals contribute to the adjuvant effect of these molecules during the sensitization phase of OVA-induced allergic airway inflammation (70). Both *in vitro* and *in vivo* studies reveal that exposure to these contaminants promote Th2 differentiation and induce Th1 and T_{reg} apoptosis via induction of oxidative stress (specifically by glutathione depletion), ultimately promoting Th2-mediated allergic inflammation (70). Interestingly, *in vivo* studies involved oral exposure to these contaminants, leading to oxidative stress in secondary lymphoid organs (70). The effects of these compounds on sensitization were not observed when exposure occurred during the elicitation phase of allergic

disease, suggesting a potential role for these antioxidant defense supplementation during sensitization not elicitation.

AMP-activated protein kinase (AMPK) is a widely expressed molecule involved in a variety of cellular processes including ATP depletion, apoptosis, proliferation, and protein synthesis (71). AMPK can influence inflammatory signaling by suppressing proinflammatory responses, promoting anti-inflammatory macrophage polarization, and inhibiting production of ROS in neutrophils (71). AMPK can be activated by 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), which enters cells, is converted to the equivalent monophosphorylated nucleotide, ZMP, which mimics AMP and activates AMPK without changing levels of ATP, ADP, or AMP (71). AMPK activation via AICAR administration has been shown to reduce inflammation and tissue injury in a number of experimental models including experimental autoimmune encephalomyelitis and LPS- and viral-induced lung inflammation. Another signaling pathway thought to be involved in inflammation, hyperresponsiveness, and remodeling, specifically in the airway, is mediated by vascular endothelial growth factor A (VEGFA) (71). VEGFA is produced by human airway smooth muscle cells in response to a variety of Th2-related cytokines and has been implicated as a major component of TDI-induced asthma in a murine model (71). Using a murine model of TDI-induced asthma to investigate the role of AMPK and VEGFA on airway inflammation and other asthma-related symptoms, the authors found that activation of AMPK via AICAR resulted in decreased TDI-induced airway inflammation and hyperresponsiveness and increased levels of VEGFA (71). These data indicate that AMPK activation has a negative effect on airway inflammation during TDI-induced asthma and this is mediated by the VEGFA pathway, primarily by reducing vascular permeability and ROS production. This study reveals a potential therapeutic avenue for the treatment of TDI-induced asthma and outlines significant regulatory processes involved in this condition.

Several genetic factors have been investigated as sources of susceptibility to isocyanate-induced allergic disease. Because of the known role of APCs in the uptake and presentation of TDI-protein complexes (45) the influence of human leukocyte antigen (HLA) genes, which encode the major histocompatibility complex (MHC) in humans, have been investigated in the context of susceptibility to diisocyanate asthma. One study carried out by Mapp, et al. involving TDI-exposed workers demonstrated that the HLA class II genotypes *DQA1*0104* and *DQB1*0503* were overrepresented in asthmatic workers compared to asymptomatic workers (72). An earlier study by the same group demonstrated a positive association of *DQB1*0503* with TDI asthmatics compared to individuals in control groups who had been exposed to TDI but were asymptomatic or had never been exposed to TDI and were asymptomatic (73). This study utilized a different cohort of workers and contributed to the hypothesis that HLA-*DQB1*0503* is involved in conferring susceptibility to TDI-induced asthma. Additionally, the authors discovered that residue 57 of the *DQB1* allele may play a particularly important role in this process. These studies, among others, have demonstrated the involvement of HLA variability in TDI-induced asthma susceptibility. TCR expression of $V\beta$ gene repertoires was investigated in a cohort of diisocyanate asthmatics in an effort to investigate the hypothesis that diisocyanate asthma-susceptible workers process antigen differently than nonsymptomatic workers due to differences in TCR $V\beta$ gene repertoires (74, 75). Following coincubation with diisocyanate-conjugated proteins, mRNA was isolated from peripheral blood-derived lymphocytes from diisocyanate asthmatics, asymptomatic diisocyanate-exposed workers, subjects without occupational asthma, and unexposed subjects free of symptoms. Polymerase chain reaction (PCR) was performed before and after diisocyanate stimulation in order to profile each TCR on the basis of $V\beta$ gene segment expression. Before *in vitro* diisocyanate stimulation lymphocytes from diisocyanate asthmatics exhibited significantly decreased $V\beta 1$ and $V\beta 5$ expression compared to

control groups; however, following stimulation these cells exhibited selective increases in $V\beta 1$ and $V\beta 5$ expression compared to controls. These studies suggest that antigen-specific T cells may express varying TCR $V\beta$ gene expression in response to diisocyanate exposure.

Additional genetic risk factors that have been identified for diisocyanate-induced asthma are mannose-binding lectin (MBL) polymorphisms (76). MBL is a component of the complement pathway, serving as the initiation step of the innate lectin complement cascade that follows the opsonization of a pathogen-derived carbohydrate structure. Increased levels of MBL have been found in the BAL fluid of children with airway infections and this compound appears to be protective in inflammatory environments, reducing inflammation induced by oxidative stress (76). MBL2 polymorphisms have been linked to altered MBL serum levels and were shown to be associated with increased risk for severe airway hyperresponsiveness in diisocyanate asthmatics. Additionally, there was an increased frequency of haplotype 2 expression among diisocyanate asthmatics compared to asymptomatic exposed controls (76). This data sheds an important light on the influence of genetic variability on TDI-induced asthma as well as potential effector mechanisms involved in this condition.

2.6. CONCLUSIONS

TDI is a potent allergic sensitizer widely used in the manufacture of a variety of polyurethane products. Workplace exposures result in a high number of occupational allergic disease cases each year. The impact of isocyanate-induced allergic disease is geographically broad; exposures occur worldwide in a variety of industries and occupations. The high reactivity of isocyanate groups on TDI molecules allows this chemical to bind to self-proteins following dermal or respiratory exposure, leading to immunogenicity and allergic sensitization. The concept of dermal sensitization

leading to respiratory elicitation is an important one. This has been proven in animal models and human epidemiological data supports this concept. The lack of exposure limits relevant to skin exposure and the limited understanding of the immunologic mechanisms involved in this process necessitate further investigation into this concept in both animal models and observational human studies.

In rodents and humans TDI is considered to be a “mixed” Th1/Th2 sensitizer, exhibiting increased Th1 and Th2 T cell polarization, cytokine secretion, and effector mechanisms leading to conditions like asthma. This mixed response is fairly unique among allergic sensitizers and is not currently encompassed by any formal hypersensitivity classification, which is a confounding variable in the study and treatment of TDI-induced allergic disease. Additionally, the problem of not knowing the true TDI-hapten “antigen” structure contributes to the limited understanding of the mechanisms of sensitization of TDI and chemical allergens in general. Although monoclonal antibodies against TDI-self-protein antigens have been produced and albumin is thought to be a primary soluble protein isocyanate target (77), the comprehensive *in vivo* TDI-hapten antigen landscape has not been fully elucidated and remains hampered by several factors including variation in murine and human TDI sensitization responses and limited isocyanate-specific reagent availability. Another contentious concept in the TDI community is the IgE conundrum; this refers to the lack of consistent TDI-specific IgE in TDI asthmatics regardless of the proven importance of this isotype in murine models of TDI sensitization and asthma. Whether this antibody is not able to be detected due to reagent limitations, testing is done at times when IgE levels are not detectably above baseline, or there is truly no TDI-specific IgE increase in this condition, the IgE conundrum remains unsolved. Consistent increases in IgG suggest that this isotype may be a main effector antibody involved in TDI sensitization and asthma in humans, or at least a potential marker of TDI

exposure. This may be reflective of the influence of the Th1 component of the immune response. The role of IgE in TDI sensitization and allergic disease needs to be investigated further in order to fully elucidate the immunologic mechanisms of this condition.

The lack of knowledge regarding the complete immunologic mechanisms of TDI sensitization and elicitation paired with the lack of validated hazard identification assays for respiratory sensitizers like TDI illustrate the need for the investigation of novel immune mediators of TDI-induced allergic disease. As discussed in Chapter 1, there are a plethora of potential novel mediators of allergic disease that may be involved in TDI-induced conditions. While some of these players such as Th1 and Th2 cells have been thoroughly investigated and others like miRNAs (78) and T_{regs} (69) have been explored (preliminarily, much work remains to be done), many others such as T_{FH} and ILCs have not been investigated in TDI sensitization. Work involving these novel molecules should be supported and pursued as it may lead to the further understanding of factors involved in TDI-induced allergic disease. This, in turn, may aid in the development of hazard identification strategies for respiratory sensitizers, preventative, and therapeutic strategies for sensitizers like TDI. Despite the many unresolved issues relevant to the study of isocyanate-induced allergic disease much progress has been made in the investigation of this condition both due to the use of animal models and observations in human patients.

As previously stated, the pathologic features of TDI-induced asthma are typically similar to those found in atopic asthma. TDI asthmatics typically express high numbers of inflammatory cells in the airway mucosa, increased eosinophils, mast cells, and leukocytes in the epithelium, and mast cell degranulation (79). To reiterate, several key differences between TDI-induced and atopic asthma should be noted, including: low association with atopy and IgE, a mixed Th1/Th2 response, and high numbers of antigen-specific CD8⁺ T cells (7). These discrepancies, along with a lack of

comprehensive knowledge of the pathogenic mechanisms of TDI-induced asthma warrant further study of the immunological mechanisms of TDI-induced asthma.

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CHAPTER 3

General Expression Kinetics, Phenotype, and Functional Characterization of T_{regs} During TDI Sensitization

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A Role for Regulatory T Cells in a Murine Model of Epicutaneous Toluene Diisocyanate Sensitization

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ABSTRACT

Toluene diisocyanate (TDI) is a leading cause of chemical-induced occupational asthma which impacts workers in a variety of industries worldwide. Recently, the robust regulatory potential of regulatory T cells (T_{regs}) has become apparent, including their functional role in the regulation of allergic disease; however, their function in TDI-induced sensitization has not been explored. To elucidate the kinetics, phenotype, and function of T_{regs} during TDI sensitization, BALB/c mice were dermally exposed (on each ear) to a single application of TDI (0.5-4% v/v) or acetone vehicle and endpoints were evaluated via RT-PCR and flow cytometry. The draining lymph node (dLN) T_{reg} population expanded significantly 4, 7, and 9 days after single 4% TDI exposure. This population was identified using a variety of surface and intracellular markers and was found to be phenotypically heterogeneous based on increased expression of markers including CD103, CCR6, CTLA4, ICOS, and Neuropilin-1 during TDI sensitization. T_{regs} isolated from TDI-sensitized mice were significantly more suppressive compared to their control counterparts, further supporting a functional role for T_{regs} during TDI sensitization. Lastly, T_{regs} were depleted prior to TDI sensitization and an intensified sensitization response was observed. Collectively, these data indicate that T_{regs} exhibit a functional role during TDI sensitization. Because the role of T_{regs} in TDI sensitization has not been previously elucidated, these data contribute to the understanding of the immunologic mechanisms of chemical induced allergic disease.

Key Words: Chemical sensitization, isocyanate, TDI, chemical allergy, regulatory T cells, hypersensitivity

3.1. INTRODUCTION

Toluene diisocyanate (TDI) is a low molecular weight, highly reactive chemical utilized in the automobile industry and in the manufacture of polyurethane, foams, paints, and coatings. The U.S. Environmental Protection Agency reports that domestic production and importation of 2,4- and 2,6- TDI isomers rose above 1 billion pounds in 2006 (1). TDI is a potent sensitizer and irritant; repeat dermal and/or inhalation exposure can lead to a variety of allergic diseases including asthma, hypersensitivity pneumonitis, rhinitis, and contact dermatitis (2-4). These diseases can be disabling and extremely severe, potentially resulting in lifelong illness and/or death (5). TDI is generally classified as primarily a respiratory, Th2-mediated sensitizer with a Th1 component (6). Although the murine local lymph node assay (LLNA) is validated for the identification of chemicals that preferentially elicit dermal allergic disease, it is not validated for chemicals that preferentially elicit respiratory disease (7). In light of this challenge, further mechanistic insight into the role of immunologically relevant cellular subsets (in addition to Th1 and Th2), involved in respiratory sensitization may ultimately contribute to the development of improved predictive models based on cellular phenotype, secreted cytokine expression, or other related parameters. Recent immunological developments such as the discovery of novel T cell subsets including Th17, T follicular helper, Th22, Th9, and T_{regs}, cells support the potential for the utilization of novel mediators of allergic disease (8).

Classical T_{regs} (CD4⁺CD25⁺) were initially identified based on their suppressive capabilities which contributed to the maintenance of immune tolerance in mice (9). Following this discovery, a transcription factor known as forkhead box p3 (Foxp3) was identified as the master transcription factor of T_{regs}, allowing for their identification and functional manipulation (10). Both naturally occurring T_{regs} (nT_{regs}) that develop in the thymus and inducible T_{regs} (iT_{regs}) that develop in the periphery are involved in the maintenance of immune tolerance. Functionally, T_{regs} have

demonstrated a critical role in the development of immune tolerance and can serve as effectors helping to prevent overzealous adaptive responses to foreign antigens and allergens (11, 12). This suppressive function is mediated by a variety of mechanisms including, but not limited to, the control of conventional T cell proliferation through the inhibition of co-stimulation via cytotoxic T-lymphocyte associated protein 4 (CTLA4) expression and/or IL-2 consumption, immunosuppressive cytokine secretion (IL-10 and TGF- β), metabolic interference, and disruption of dendritic cell function (12-14).

Although a functional role for T_{regs} has been suggested in models of chemical-induced contact hypersensitivity (15), this cellular subset has not been investigated in TDI sensitization. Vanoirbeek and colleagues observed suspected immune tolerance induced by high dose (3% TDI in acetone-olive oil) dermal sensitization followed by intranasal challenge resulting in the absence of airway hyperreactivity, contrasted with low dose (0.3% TDI in acetone-olive oil) dermal sensitization resulting in airway hyperreactivity following intranasal challenge (16). Although not explicitly stated in the manuscript, this seminal study suggested a role for T_{regs} in TDI sensitization. The collection of data regarding T_{regs} and chemical allergy is growing but remains limited. In order to elucidate the immunologic mechanisms involved in TDI sensitization, the biological functions of pertinent immune cell subsets need to be delineated. It is important to note that although a variety of regulatory T cells may be involved in chemical sensitization, this study focuses on classical T_{regs} (CD3⁺CD4⁺CD25⁺Foxp3⁺) due to their well-documented regulatory potential in a variety of disease states, including allergy and asthma (17), and to the lack of data regarding these cells in models of low molecular weight chemical allergy. Here, we utilize a murine model of dermal TDI sensitization in order to elucidate the expression kinetics, phenotype, and functional role of T_{regs} during chemical sensitization.

3.2. MATERIALS AND METHODS

3.2.1. *Mice*

Female BALB/c mice (6-8 weeks of age) purchased from Taconic (Germantown, NY) were acclimated for 5 days and then randomly assigned to treatment group. Homogenous weight distribution was insured across treatment groups. BALB/c mice were selected on the basis of their Th2 bias, robust IgE production, and the historical use of these mice in the laboratory to investigate chemical sensitization (18). Mice were housed in ventilated plastic shoebox cages with hardwood chip bedding at a maximum of five animals per cage. A NIH-31 modified 6% irradiated rodent diet (Harlan Teklad) and tap water were administered *ad libitum*. Housing facilities were maintained at 68-72°F and 36-57% relative humidity, and a 12 hour light-dark cycle was maintained. All animal experiments were performed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited National Institute for Occupational Safety and Health (NIOSH) animal facility in accordance with an Institutional Animal Care and Use Committee-approved protocol.

3.2.2. *TDI Sensitization Model*

Toluene 2,4-diisocyanate (TDI, CAS# 584-84-9) was purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI). Animals (n=4-5) were exposed to a single dose of 0.5-4% TDI (v/v) on the dorsal surface of each ear (25 µL per ear). The highest concentration (4% v/v; the maximum sensitizing concentration with minimal toxicity) and dosing regimen was previously shown to induce sensitization (19) and sensitization was confirmed in the lower dose based on total serum IgE levels and dLN allergic cytokine mRNA expression (0.5% v/v; data not shown). Acetone

was selected as the vehicle control to minimize chemical reactivity as diisocyanates react with OH groups which are present in other potential vehicles such as olive oil. Ear thickness was measured 4 days following TDI exposure using a modified engineer's micrometer (Mitutoyo Corporation, Japan) and measurements were collected in millimeters (mm). Average ear swelling was calculated as previously described (20). Mice were euthanized via CO₂ asphyxiation at time points ranging from 1 – 11 days post chemical exposure.

3.2.3. Euthanasia, Tissue Collection, and Processing

Animals were weighed, euthanized via CO₂ asphyxiation, and examined for gross pathology at the designated time point. Left and right superficial parotid draining lymph nodes (dLNs; drain site of chemical application (21)) were collected in sterile phosphate-buffered saline (pH 7.4) and manually dissociated using the frosted ends of two microscope slides. dLN cellularity was determined using a Cellometer (Nexcelom Bioscience, Lawrence, Massachusetts) with size exclusion parameters (3.5 to 36 μ m) and a combined Acridine Orange/Propidium Iodide (AO/PI) solution to identify viable cells. Blood was collected via cardiac puncture, placed into serum collection tubes, centrifuged, and serum was removed and stored at -80°C for subsequent total IgE analysis via ELISA. Ears were collected in 1 mL RNALater (Ambion, Pittsburgh, PA), stored at -80°C for subsequent gene expression analysis or processed for flow cytometric analysis. For flow cytometric analysis of the skin (Figure 5.4 and Appendix 5.1), ear cell suspensions were prepared by splitting ears into ventral and dorsal halves, mincing into 1cmx1cm pieces followed by an enzymatic digestion for 90 min at 37°C with 0.25mg/ml Liberase-TL Research grade (Roche) in RPMI with 100ug/ml DNase I (Sigma). Following digestion cells were passed through a 70 μ m cell

strainer to make a single cell solution, washed with RPMI + 10% FBS, then live cells were counted on a Cellometer (Nexcelom) using AO/PI.

3.2.4. RNA Isolation and Reverse Transcription

Ears were processed for RNA isolation using a Tissue Lyser II in Qiagen lysis buffer. Total RNA was isolated from the ears and dLN using the Qiagen RNeasy and miRNeasy kits respectively. A QiaCube (Qiagen, Hilden, Germany) automated RNA isolation machine was utilized in conjunction with the specified RNA isolation kits. A DNase treatment was performed for removal of residual DNA. The concentration and purity of the RNA was determined using a ND-1000 spectrophotometer (Thermo Scientific Nanodrop, Wilmington, DE). First strand cDNA synthesis was performed using a High-Capacity cDNA Synthesis Kit (Applied Biosystems, Carlsbad, California) according to manufacturer recommendations. Ultimately, the cDNA was analyzed for mRNA expression as described in the real-time PCR methods section below.

3.2.5. Real Time PCR (RT-PCR)

For analysis of mRNA expression, TaqMan Universal Fast master mix (Applied Biosystems, Carlsbad, California), cDNA, and mouse-specific mRNA primers (TaqMan Custom PCR Arrays, Carlsbad, California) were combined and PCR was performed according to manufacturer protocol. Primers used include: *il-1 β* , *il-6*, and *tnf- α* . Master mix, primers, and cDNA were added to a MicroAmp Fast Optical 96-well reaction plate and analyzed on an Applied Biosystems 7500 Fast Real Time PCR system using cycling conditions as specified by the manufacturer. β -actin was used as the endogenous reference control gene as expression was determined to be stable following chemical exposure (data not shown). RT-PCR data were collected and represented as relative fold

change over vehicle control, calculated by the following formula: $2^{-\Delta\Delta Ct} = \Delta Ct_{\text{Sample}} - \Delta Ct_{\text{Control}}$. $\Delta Ct = Ct_{\text{Target}} - Ct_{\beta\text{-ACTIN}}$, where Ct = cycle threshold.

3.2.6. Flow Cytometric Analysis

Single cell suspensions were prepared from tissues and a minimum of 150,000 dLN and 1,000,000 ear skin cells were aliquoted into 96-well U-bottom plates and washed in FACS staining buffer (PBS + 1% bovine serum albumin + 0.1% sodium azide). Cells were resuspended in staining buffer containing anti-mouse CD16/32 antibody (clone 2.4G2; BD Biosciences, San Jose, CA) for blocking of F_c receptors to minimize nonspecific binding. Cells were resuspended in staining buffer containing a cocktail of fluorochrome-conjugated antibodies specific for cell surface antigens including: CCR6 (clone: 29-2L17, fluorophore: BV605, BioLegend), CD3 (500A2, V500, BD), CD4 (RM4-5, AF700, BD), CD8a (53-6.7, AF488, BioLegend), CD25 (PC61, APC Cy7, BioLegend), CD45 (30-F11, PE, BD), CD103 (2E7, PerCP Cy5.5, BioLegend), ICOS (C938.4A, PE Cy7, eBioscience), Neu-1 (3E12, PE, BioLegend), and Ter-119 (TER-119, FITC, eBioscience). Following surface staining, cells were washed in staining buffer and fixed using the Foxp3 fixation buffer set (eBioscience, San Diego, CA). After overnight incubation in staining buffer, cells were permeabilized using the Foxp3 fixation buffer set (eBioscience, San Diego, CA) and re-suspended in permeabilization buffer containing a cocktail of fluorochrome-conjugated antibodies specific for intracellular antigens including: CTLA4 (UC10-4B9, BV421, BioLegend), Foxp3 (FLK-16s, eF450 and APC, eBioscience), Gata3 (TWAJ, eF660, eBioscience), Ror γ t (Q31-378, PerCP Cy5.5, eBioscience), and Tbet (4B10, PE Cy7, eBioscience). Following staining, cells were re-suspended in staining buffer and analyzed on an LSR II flow cytometer using FACS Diva software (BD Biosciences). Data analysis was performed with FlowJo 10.0 software (TreeStar Inc., Ashland, OR).

A minimum of 10,000 events were captured for each sample. Leukocytes were first identified by their expression of CD45. The T_{reg} subset was further identified as CD3⁺CD4⁺CD8⁻CD25⁺Foxp3⁺. Numerical population values were calculated by applying subset frequencies to the initial cell count obtained following lymph node homogenization. Compensation controls were performed using single stained cellular suspensions and OneComp beads (eBioscience, San Diego, CA) and fluorescence minus one (FMO) staining controls were included to help set gating boundaries.

3.2.7. T_{reg} Suppression Assay

The suppressive ability of T_{regs} was analyzed using an *ex vivo* T_{reg} suppression assay as described by Kruisbeek, et al (22) and Marshall, et al (23) with some modifications. This assay evaluates the ability of naïve, conventional dLN-derived T cells (T_{cons}) to proliferate in the presence of varying numbers of T_{regs} isolated from acetone- or TDI-exposed mice. Mice were exposed to acetone (n=7-11) or TDI (4%) (n=4-5) as previously described and following sacrifice at 4 and 7 days post TDI (peak of the expansion) exposure the dLN and spleens were removed. T_{regs} (CD4⁺CD25⁺) and T_{cons} (CD4⁺CD25⁻) were isolated from the lymph nodes and CD4⁻ accessory cells were isolated from naïve spleens using CD4 negative and CD25 positive selection-based magnetic separation kits (Stemcell, Vancouver, BC). Average T_{reg} purity is as follows for 4 days: Acetone-70.65% of CD3⁺CD4⁺ cells and 4% TDI-63.05% of CD3⁺CD4⁺ cells and 7 days: Acetone-91.35% of CD3⁺CD4⁺ cells and 4% TDI-82.8% of CD3⁺CD4⁺ cells post 4% TDI exposure. Following isolation from naïve mouse dLNs, T_{cons} were labeled with 2 µM carboxyfluorescein succinimidyl ester (CFSE). T_{cons} and T_{regs} were cultured in a 96-well U-bottom plate with anti-CD3 (0.2 µg/mL; BD Biosciences) and accessory cells at a variety of T_{con}:T_{reg} ratios (1:1, 2:1, 4:1, and 8:1). Naïve CD4⁻ splenocytes were treated with Mitomycin C (Sigma Aldrich, Saint Louis, MO) and utilized as

accessory cells. Additional controls included stimulated T_{cons} only to assess baseline proliferation, T_{regs} only, accessory cells only, and T_{cons} only with no stimulation nor accessory cells. Cells from each treatment group were pooled and added to triplicate wells of the culture plate. 72 hours following plating, cells were stained with anti-CD4 and Live/Dead Violet (Life Technologies, Carlsbad, CA). T_{cons} were defined as $CD4^+ CFSE^+$ cells and suppression was measured based on changes in the frequency of dividing $CFSE^+$ cells based on the dilution of CFSE. T_{regs} were analyzed for purity based on their expression of CD3, CD4, and Foxp3 as determined by flow cytometric analysis as previously described.

3.2.8. *In vivo* Anti-CD25 Antibody Treatment

In order to deplete T_{regs} before and during TDI sensitization, *InVivo*MAb anti m CD25 (BioXCell, PC-61.5.3) was administered *in vivo*. The use of this antibody as a T_{reg} depletion strategy is validated *in vivo* (24, 25). *InVivo*MAb Rat IgG₁ (Bioxcell, HRPN) was utilized as a negative, isotype control. Treatment groups for this study (n=5 mice) are as follows: isotype/acetone, isotype/0.5% TDI, isotype/2% TDI, anti-CD25/acetone, anti-CD25/0.5% TDI, and anti-CD25/2% TDI. 200 μ g of the respective antibody in USP grade saline was administered intraperitoneally at days -11 and -8 during the depletion study (see Figure 6A for study timeline). Animal weights were recorded throughout the duration of the experiment to monitor potential toxicity. In order to confirm the effectiveness of the antibody, blood was collected from the lateral tail vein at days -2 and 7, and T_{regs} were measured by flow cytometry as previously described. Baseline blood T_{reg} levels were assayed at day -12 to ensure equal pre-treatment frequencies across all groups. Mice were exposed to a single dermal application of 0.5% or 2% TDI on day 0. The high dose of 2% TDI was selected in an effort to allow for a measurable increase in the sensitization response, as 4% TDI elicits a

maximum sensitization response. Mice were euthanized 7 days following dermal chemical exposure. Specific measures of sensitization were evaluated, including examination of the Th2 population by flow cytometry (CD3⁺CD4⁺Gata3⁺), IL-4 dLN mRNA levels by RT-PCR, and total IgE levels in the serum. Total serum IgE was quantified following serum separation from whole blood (centrifugation) and analysis using a Mouse IgE Ready-SET-Go! Kit (Affymetrix eBioscience, San Diego, CA) according to the manufacturer's protocol. Absorbance was determined using a Spectramax Vmax plate reader (Molecular Devices, Sunnyvale, California) at 450 and 650 nm. Data analysis was performed using the IBM Softmax Pro 3.1 program (Molecular Devices, Sunnyvale, California) and the IgE concentrations for each sample were interpolated from a standard curve derived from multipoint analysis.

3.2.9. Statistical Analysis

Statistical analyses were generated using SAS/STAT software, version 9.3 (SAS Institute, Cary, NC) and GraphPad Prism version 5.0 (San Diego, CA). For irritancy and inflammatory gene expression analysis (Figure 1), a one way analysis of variance (ANOVA) was conducted. If the ANOVA showed significance at $p < 0.05$ or less, the Dunnett's Multiple Comparison Test was used to compare values from groups of mice treated with varying concentrations of TDI to the acetone control group. Figures 2-6 and Table 2 were analyzed by analysis of variance using PROC MIXED. In some cases, data were transformed using the natural log to meet the assumptions of the analysis. Significant interactions were explored utilizing the "slice" option in PROC MIXED and pairwise differences were assessed using a Fishers Least Significant Difference Test. Supplemental data was analyzed by a Student *t*-test comparing groups as indicated in the figure legends. All differences

were considered significant at $p < 0.05$; representative significance symbols varied by figure, as indicated in the figure legend.

3.3. RESULTS

3.3.1. Examination of Sensitization and Skin Irritancy Potential of TDI

To confirm that a single dose exposure to TDI (0.5 and 4%) would sensitize animals, total serum IgE was evaluated following TDI exposure. Although not initially statistically significant, IgE levels appeared to increase in a dose-dependent manner, reaching significance following 4% exposure (Figure 3.1 A). Because TDI is a known irritant (26), the selected doses of TDI (0.5-4% v/v) were assayed for dermal irritancy potential via ear swelling measurements and ear inflammatory cytokine mRNA production quantified via RT-PCR. Average ear swelling was significantly increased four days following 2 and 4% TDI exposure (Figure 3.1 B); however, neither 0.5 nor 1% TDI exposure induced significant increases in ear swelling (Figure 3.1 B). Inflammatory cytokine (IL-1 β , IL-6, and TNF- α) mRNA levels were significantly increased in the ear four days following 4% TDI exposure (Figure 3.1 C-E). These data suggest that 2 and 4% TDI exposure induce a significant irritation response in the ear compared to 0.5 and 1% TDI exposure. Based on this data, 0.5 and 4% TDI were selected as the exposure concentrations in subsequent studies to represent sensitizing concentrations that encompassed both a low dose exhibiting a lack of irritation (0.5%) along with a high dose exhibiting significant irritation (4%).

Figure 3.1: Confirmation of Sensitization and Evaluation of Skin Irritancy Following Dermal TDI Exposure

ELISA analysis of total serum IgE levels 11 days following single TDI exposure at the indicated concentration (A). Percent change in ear thickness as determined 4 days following TDI exposure (B). Ear mRNA expression of the inflammatory cytokines *IL-1 β* (C), *il-6* (D), and *tnf- α* (E) as determined 4 days following TDI exposure via RT-PCR. Bars represent mean ($\pm$ SE) of 4-5 mice per group. Statistical significance is indicated by (*) at a p value <0.05 and (**) at a p value <0.01 .

3.3.2. dLN T_{reg} Expression Kinetics Reveal an Expansion of this Population During TDI Sensitization

In order to profile the expression kinetics of the T_{reg} subset during TDI sensitization we examined dLN cell populations at 1, 2, 4, 7, and 9 days post TDI exposure (See Table 3.1 for markers utilized). T_{regs} were identified as $CD3^+CD4^+CD25^+Foxp3^+$ cells by flow cytometry (Figure 3.2 A). The frequency of T_{regs} was unchanged compared to control cells from acetone-treated mice during 0.5% TDI sensitization; however, T_{reg} frequency increased significantly at 4, 7, and 9 days post 4% TDI exposure (Figure 3.2 B). T_{regs} also significantly increased in number at all time points analyzed during 0.5 and 4% TDI sensitization (Figure 3.2 C). The peak numbers of T_{regs} in the dLN appeared to occur at day 4 post TDI exposure for both concentrations (mean $\pm$ SEM; 1.17×10^6 cells $\pm$ 0.08 (0.5%) and 1.7×10^6 cells $\pm$ 0.08 (4%)) compared to the acetone control (0.18×10^6 cells $\pm$ 0.02). The T_{reg} population remained elevated compared to the acetone control, but began to retract at days 7 (0.5% TDI- $0.88 \times 10^6 \pm 0.09$, and 4% TDI- $1.39 \times 10^6 \pm 0.1$) and 9 (0.5% TDI- $0.37 \times 10^6 \pm 0.06$, and 4% TDI- $0.79 \times 10^6 \pm 0.05$) following TDI exposure relative to their peak at day 4.

In order to better elucidate the origin of the T_{regs} involved in the TDI sensitization response, natural T_{regs} (nT_{regs}) were identified based on their expression of neuropilin-1 (27). Interestingly, the frequency of the nT_{reg} subset as a percentage of total T_{regs} significantly decreased at 7 and 9 days during 0.5% and 4% TDI sensitization (Figure 3.2a C). Although the frequency of this subset decreased in relation to the acetone control group, the numbers of nT_{regs} in the dLN increased during 0.5 and 4% TDI sensitization (Figure 3.2a D). This increase was significant for 4-9 days during 0.5% TDI sensitization and at all time points measured during 4% TDI sensitization. It is important to note that both the nT_{reg} and the neuropilin-1^{neg} T_{reg} population (presumably induced T_{regs} (iT_{regs})) were represented as co-expressing populations examined using additional markers.

Marker	Abbreviation	Surface or Intracellular Detection	Significance	T Cell Populations Expressing
Cluster of Differentiation 3	CD3	S	Pan T cell marker	All T cells
Cluster of Differentiation 4	CD4	S	CD4 ⁺ T cell marker	CD4 ⁺ T cells
Cluster of Differentiation 25 / IL2 α	CD25	S	High affinity IL-2 receptor α , T and B cell growth factor (via IL-2 binding) (28, 29)	T _{regs} , some activated T cells (non-T _{regs})
Forkhead Box Protein 3	Foxp3	IC	Master T _{reg} transcription factor (10)	T _{regs}
Chemokine (CC-Motif) Receptor 6	CCR6	S	Lymphocyte chemoattractant CCL20 is its ligand (30)	Migratory Effector T _{regs} (31, 32) and CD8 ⁺ T cells (33)
Cluster of Differentiation 103	CD103	S	Integrin involved in epithelial T cell migration and retention (34)	Migratory Effector T _{regs} (35) and tissue resident CD8 ⁺ T Cells (36)
Cytotoxic T-Lymphocyte Associated Protein 4	CTLA4/CD152	IC	Member of the CD28 family that is a potent inhibitor of T cell costimulation (37)	T _{regs} and activated T cells (non-T _{regs})
Inducible T-Cell Costimulator	ICOS/CD278	S	Member of the CD28 family that has costimulatory functionality during T cell activation (38)	Migratory Effector T _{regs} (39-41) and activated T cells (41)
Neuropilin-1	Neuropilin-1	S	Receptor for vascular endothelial growth factors and semaphorins (42)	nT _{regs} (11, 43), T _{FH} cells (44), and CD8 ⁺ T cells (45)

Table 3.1 *T_{reg} Flow Cytometry Phenotyping Marker Guide*

dLN T_{reg} subsets were further phenotyped by flow cytometry (Table 1). The phenotyping analysis revealed a number of T_{reg} subpopulations that exhibited the potential to be functionally diverse in relation to their mechanism(s) of suppression (Table 1 and A3.1). An expansion in the frequency and number of the CTLA-4⁺ T_{reg} population was observed at all measured time points following both 0.5 and 4% TDI exposure (Figure 3.2a A and B). This population peaked in

expression of both frequency (Figure 3.2a A) and number (Figure 3.2a B) at day 4 post TDI exposure. The emergence of the CTLA4⁺ T_{reg} population during TDI sensitization suggests the engagement of CTLA-4, a negative costimulatory molecule, as a potential suppressive mechanism during this response (46). As expected, presumably due to compensatory mechanisms during T cell activation, there was also an increase in CTLA4 expression in CD4⁺ non-T_{regs}; however, at the peak of expression (4 days) the mean frequency of expression among all CD4⁺ cells was 0.54% ± 0.024 for acetone, 4.6% ± 0.24 for 0.5% TDI, and 7.85% ± 0.66 for 4% TDI groups. Compared to the T_{regs} expressing CTLA4 at the same time point, these numbers reveal a sizably smaller frequency of non-T_{regs} expressing this marker, emphasizing the specificity of this marker to the T_{reg} population during this response.

In addition to the CTLA4⁺ T_{regs} and nT_{regs}, cells expressing the homing molecules CD103, CCR6, and ICOS were analyzed during TDI sensitization. T_{reg} expression of CD103, CCR6, and ICOS increased in both frequency and number throughout 0.5 and 4% TDI sensitization (Table 3.2). The CCR6⁺ population's expression frequency was highest at 7 days post TDI exposure, while the CD103⁺ and ICOS⁺ populations' frequency peaked at 4 days post exposure. It is important to note that while there are significant changes in these subsets, they represent a small portion of the total CD4⁺ population, as their expression is presented on T_{regs}, which themselves constitute a minority of the total CD4⁺ population. Although there was robust T_{reg} expression of CD103, CCR6, and ICOS as single markers, there was a significant population of dLN T_{regs} that co-expressed these molecules (Figure 3.2b A and B) with kinetics were similar to those exhibited by each single marker. This population likely represents a migratory subset of effector T_{regs} that have been activated during TDI sensitization. This co-expressing population expanded in the dLN in both number and percent during TDI sensitization and represented 6.34% ± 1.2 of T_{regs} following acetone exposure, 12.82% ± 1.2 of

T_{regs} during 0.5% TDI sensitization and 20.7% ± 1.8 of T_{regs} during 4% TDI sensitization at 7 days post exposure (peak expression). Ear-derived T_{regs} were also examined by flow cytometric analysis and the corresponding data (Figure 5.4) will be discussed in Chapter 5.

Figure 3.2 *T_{reg} Subset Expands During Dermal TDI Sensitization*

Flow cytometric analysis of T_{regs} following dermal TDI sensitization (A) T_{regs} were first gated on their expression of CD3 and CD4, then were further identified by CD25 and Foxp3 expression at indicated time points. dLN T_{reg} frequency (B) and number (C) were determined based on flow cytometry analysis and extrapolation of this data with total dLN cellularity. Graph symbols represent

mean ($\pm$ SE) of 5 mice per group. *P* values are represented by ^ (0.5% TDI) and asterisks (4% TDI) ($P < 0.05$). Significance is indicated as follows: $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****) for 4% TDI or $P \leq .05$ (^), $P \leq .01$ (^ ^), $P \leq .001$ (^ ^ ^), and $P \leq .0001$ (^ ^ ^ ^) for 0.5% TDI. Dermal treatment groups are indicated by the following symbols: circle- acetone, square- 0.5% TDI, and triangle- 4% TDI.

Figure 3.2a Expansion of CTLA4⁺ and nT_{reg} Populations During TDI Sensitization

Flow cytometric analysis of dLN CTLA4⁺ T_{reg} frequency (A) and number (B) and nT_{reg} (Neuropilin-1⁺) frequency (C) and number (D) following TDI sensitization. Bars represent mean ($\pm$ SE) of 5 mice per group. *P* values are represented by ^ (0.5% TDI) and asterisks (4% TDI) (*P*<0.05). Significance is indicated as follows: *P* $\leq$.05 (*), *P* $\leq$.01 (**), *P* $\leq$.001 (***), and *P* $\leq$.0001 (****) for 4% TDI or *P* $\leq$.05 (^), *P* $\leq$.01 (^^), *P* $\leq$.001 (^^^), and *P* $\leq$.0001 (^^^^) for 0.5% TDI. Dermal treatment groups are indicated by the following symbols: circle- acetone, square- 0.5% TDI, and triangle- 4% TDI.

CCR6⁺ % (of T_{regs})	<u>1 day</u>	<u>2 day</u>	<u>4 day</u>	<u>7 day</u>	<u>9 day</u>
Acetone	11.14 ± 0.7	12.72 ± 1.03	10.86 ± 0.55	18.22 ± 1.36	9.23 ± 0.8
0.5% TDI	10.22 ± 0.68	17.7 ± 1.64**	20.58 ± 0.84****	26.54 ± 0.68****	14.82 ± 0.99**
4% TDI	10.02 ± 0.92	10.2 ± 1.1	30.7 ± 1.75****	40.42 ± 1.87****	25.02 ± 1.98****
CCR6⁺ # (x 10⁵ cells)					
Acetone	0.2 ± 0.05	0.14 ± 0.04	0.19 ± 0.02	0.24 ± 0.04	0.16 ± 0.03
0.5% TDI	0.34 ± 0.06*	0.61 ± 0.12****	2.41 ± 0.19****	2.35 ± 0.28****	0.57 ± 0.13****
4% TDI	0.533 ± 0.05****	0.3 ± 0.07**	5.25 ± 0.48****	5.59 ± 0.41****	1.99 ± 0.2****
CD103⁺ % (of T_{regs})	<u>1 day</u>	<u>2 day</u>	<u>4 day</u>	<u>7 day</u>	<u>9 day</u>
Acetone	22.86 ± 0.75	18.88 ± 4.24	20.82 ± 0.94	28.44 ± 2	21.18 ± 0.92
0.5% TDI	18.38 ± 0.84	24.44 ± 2.99*	35.88 ± 0.84****	38.8 ± 1.1***	32.72 ± 2.23****
4% TDI	19.62 ± 1.25	16.5 ± 1.64	43.18 ± 1.78****	52.12 ± 1.36****	44.64 ± 1.45****
CD103⁺ # (x 10⁵ cells)					
Acetone	0.41 ± 0.09	0.16 ± 0.03	0.37 ± 0.04	0.38 ± 0.07	0.36 ± 0.06
0.5% TDI	0.59 ± 0.1	0.83 ± 0.15****	4.21 ± 0.33****	3.47 ± 0.45****	1.22 ± 0.23****
4% TDI	1.04 ± 0.06****	0.48 ± 0.11****	7.4 ± 0.63****	7.23 ± 0.56****	3.53 ± 0.23****

ICOS ⁺ % (of T _{regs})	<u>1 day</u>	<u>2 day</u>	<u>4 day</u>	<u>7 day</u>	<u>9 day</u>
Acetone	13.38 ± 0.36	15.92 ± 1.21	13.56 ± 0.41	18.7 ± 1.22	15.98 ± 1.24
0.5% TDI	14.06 ± 1.03	36.42 ± 2.16****	47.8 ± 1.15****	38.38 ± 1.72****	33.36 ± 2****
4% TDI	16.68 ± 1.45	33.18 ± 1.27****	62.64 ± 1.03****	55.02 ± 2.12****	45.54 ± 1.78****
ICOS ⁺ # (x 10 ⁵ cells)					
Acetone	0.23 ± 0.05	0.17 ± 0.04	0.24 ± 0.02	0.25 ± 0.04	0.28 ± 0.5
0.5% TDI	0.46 ± 0.09**	1.31 ± 0.27****	5.61 ± 0.45****	3.42 ± 0.45****	1.27 ± 0.26****
4% TDI	0.88 ± 0.09****	0.94 ± 0.18****	10.68 ± 0.62****	7.62 ± 0.62****	3.62 ± 0.29****

Table 3.2 *dLN Migratory Effector T_{reg} Population Expands During TDI Sensitization*

Data represents mean frequency and number of each T_{reg} population 1-9 days following chemical exposure. Significance is indicated as follows: P ≤ .05 (*), P ≤ .01 (**), P ≤ .001 (***), and P ≤ .0001 (****) for each group compared to the acetone control value from the matching time point.

Figure 3.2b *dLN Migratory Effector T_{reg} Population Expands During TDI Sensitization (Co-Expression)*

dLN CCR6⁺ CD103⁺ ICOS⁺ T_{reg} frequency (A) and number (B) were determined based on flow cytometry analysis and extrapolation of this data with total dLN cellularity. Bars represent mean ($\pm$ SE) of 5 mice per group. *P* values are represented by ^ (0.5% TDI) and asterisks (4% TDI) (*P* < 0.05). Significance is indicated as follows: *P* $\leq$.05 (*), *P* $\leq$.01 (**), *P* $\leq$.001 (***), and *P* $\leq$.0001 (****) for 4% TDI or *P* $\leq$.05 (^), *P* $\leq$.01 (^ ^), *P* $\leq$.001 (^ ^ ^), and *P* $\leq$.0001 (^ ^ ^ ^) for 0.5% TDI. Dermal treatment groups are indicated by the following symbols: circle- acetone, square- 0.5% TDI, and triangle- 4% TDI.

3.3.3. T_{regs} Exhibit Potent Suppressive Ability During TDI Sensitization

Because the T_{reg} population expanded during TDI sensitization, the functional role of T_{regs} was further examined by performing a CFSE-based T_{reg} suppression assay with T_{regs} isolated from acetone or TDI-treated mice (Figure 3.3 A). T_{regs} from acetone-treated mice were significantly suppressive at all T_{con}:T_{reg} ratios tested when isolated at 4 (Figure 3.3 B) and 7 days (Figure 3.3 C) post exposure. Acetone T_{reg}-induced suppression was found to be equivalent to naïve-derived T_{reg}-induced suppression (data not shown). T_{regs} from TDI-exposed mice exhibited increased suppressive ability compared to the acetone controls when isolated at both 4 (Figure 3.3 B) and 7 days (Figure 3.3 C) at all T_{con}:T_{reg} ratios tested. The heightened suppressive ability of T_{regs} was surprising, given the progression of sensitization at the selected concentrations of TDI. This data suggested a functional, suppressive role for T_{regs} in TDI sensitization.

Figure 3.3 *T_{reg} Suppression Assay Reveals Increased Suppressive Ability of T_{regs} During TDI Sensitization*

A CFSE-based T_{reg} suppression assay was performed and percent dividing CFSE⁺ cells were quantified as illustrated in (A). The zero peak represents the cell population that retained all original CFSE stain. The percent dividing CFSE⁺ cells (T_{cons}) are represented in combination with T_{regs} from

mice 4 (B) and 7 (C) days following TDI exposure at a variety of $T_{con}:T_{reg}$ ratios. For (B) and (C) 3 plate replicates were utilized from groups of 4-11 mice, as described in the materials and method's section. Significance is indicated as follows: $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****). *P* values are represented by asterisks (comparison of each treatment group to T_{con} only from the same chemical treatment group) or horizontal bars with asterisks above (comparison of identical ratios between different chemical treatment groups).

3.3.4. Depletion of T_{regs} Before and During TDI Sensitization Augments the T cell-mediated Allergic Response

In order to analyze the functional potential of T_{regs} *in vivo* during TDI sensitization, this subset was depleted by injecting mice with anti-CD25 antibody days -11 and -8 prior to TDI exposure (Figure 3.4 A). The high dose of 2% TDI was selected in an effort to allow for a measurable increase in the sensitization response, as 4% TDI elicits a maximum sensitization response. The basal levels of T_{regs} in the blood of mice were determined to be comparable among all groups prior to dosing (A3.2 A) and depletion was confirmed in the blood (A3.2 A) at days -2 and 7 and in the dLN (A3.2 B) at day 7. Mice dosed with 2% TDI that received anti-CD25 lost significantly more body weight (grams; mean decrease $5.33\% \pm 3.9$) than mice exposed to 2% TDI and isotype control antibody (mean increase $1.01\% \pm 4.3$), suggesting enhanced toxicity following T_{reg} depletion and high-dose TDI administration. Because local irritation is thought to influence allergic sensitization (47) and TDI is a known irritant (48), we analyzed the dermal irritation response at the site of TDI exposure by ear swelling measurements following T_{reg} depletion. As expected, there was a dose-responsive increase (Linear trend test $p < 0.01$) in ear swelling following exposure to TDI for both

the isotype control and anti-CD25 groups (Figure 3.4 B). Although not statistically significant, ears from animals treated with anti-CD25 and either concentration of TDI exhibited increased swelling ($12.4\% \pm 3.7$ for 0.5%; $54.4\% \pm 10.7$ for 2% TDI) compared to their isotype-treated counterparts ($1.4\% \pm 6.6$ for 0.5%; $35.6\% \pm 8.9$ for 2% TDI). Compared to values at day -11, total body weight decreased significantly in both groups treated with 2% TDI; however, the anti-CD25-treated group exhibited weight loss that was significant beyond that of the isotype control (A3.3 A). At day 7, dLN cellularity increased dose-responsively (Linear trend test $p < 0.05$) for both the isotype control and anti-CD25-treated groups during TDI sensitization (Figure 3.4 C). For both the 0.5% and 2% TDI-treated groups statistically significant increases in the dLN cellularity with anti-CD25 treatment compared to isotype were observed. Another effector $CD4^+$ T cell subset, Th2, which play an important role in allergic responses, was identified by GATA-3 expression and was found to dose-responsively expand in number during 0.5% and 2% TDI sensitization for both the isotype and anti-CD25-treated groups (Figure 3.4 D; Linear trend test $p < 0.01$). When comparisons were made between the isotype control and anti-CD25 treated groups this population appeared to further expand during TDI sensitization, although these perceived trends were not statistically significant. Similar observations were made for the Th2 population's frequency (A3.3 B). Interestingly, the Gata3 mean fluorescence intensity (MFI) was significantly augmented following anti-CD25 treatment in the 0.5 and 2%-treated groups, indicating increased brightness on a cell-per-cell basis (A3.3 C). IL-4 mRNA expression levels increased dose-responsively in the dLN following 0.5% and 2% TDI exposure (Linear trend test $p < 0.05$; isotype and anti-CD25 groups) and were significantly augmented in groups treated with anti-CD25 compared to isotype-treated controls (Figure 3.4 E). Similarly, dose-responsive increases in total IgE levels in the blood appeared to occur at 7 days following exposure to TDI in mice treated with both isotype control and anti-CD25, although significance was

only observed for the 2% TDI anti-CD25-treated group (Linear trend test $p < 0.01$; Figure 3.4 F). Statistically significant increases in serum IgE levels were observed after anti-CD25 treatment following exposure to 0.5 and 2% TDI.

Other T cell-related dLN phenotyping was performed at day 7 including CD3, CD4, and CD8 expression, along with other effector $CD4^+$ Th1 and Th17 populations based on their expression of Tbet and Ror γ t, respectively (A3.4). $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, Th1 and Th17 cells dose-responsively expanded in number during 0.5% and 2% TDI sensitization and anti-CD25 depleted animals exhibited higher numbers of cells during TDI sensitization (A3.4 B, D, F, H, J). dLN $CD3^+$ cellular frequency decreased in isotype and anti-CD25-treated groups during 0.5% and 2% TDI sensitization compared to their respective acetone control (A3.4 A). Interestingly, $CD3^+$ cellular frequency was significantly increased in groups treated with anti-CD25 compared to isotype controls for acetone and 0.5% TDI, indicating that anti-CD25 treatment did not affect general T cell frequencies in the dLN. $CD4^+$ and $CD8^+$ T cell frequency (as a percentage of $CD3^+$ cells) remained stable during TDI sensitization; however, the $CD4^+$ frequency decreased following anti-CD25 treatment while the $CD8^+$ frequency increased A3.4 C and E). The Th1 dLN population significantly expanded in frequency during TDI sensitization (A3.4 C). This subset further expanded in mice treated with anti-CD25 in frequency (2%) compared to the isotype control groups. The Th17 subset was also profiled and was not significantly altered in frequency during TDI sensitization in the isotype control treated groups; however, the frequency of Th17 cells was significantly increased above both the corresponding acetone and isotype-treated control groups during 2% TDI sensitization following anti-CD25 treatment (A3.4 D).

Figure 3.4 *The Severity of the Sensitization Response is Intensified in the Absence of T_{regs} During TDI Sensitization*

T_{regs} were depleted in mice prior to and during dermal TDI sensitization (A). Evaluation of dermal irritancy (B), dLN cellularity (C), dLN Th2 population (D), dLN IL-4 gene expression (E), and

serum total IgE (F) were evaluated following a single exposure of 0.5% or 2% TDI with isotype control or anti-CD25. Bars represent mean ($\pm$ SE) of 5 mice per group. Significance is indicated as follows: $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****). *P* values are represented by asterisks (comparison of acetone to TDI-exposed group from the same antibody treatment regimen) or horizontal bars with asterisks above (comparison of antibody and isotype-treated groups receiving identical chemical treatment).

3.4. DISCUSSION

Occupational exposure to sensitizing chemicals that are capable of inducing allergic disease is increasing globally and is an important public health concern. Although there continues to be progress made in the area of hazard identification, the limited knowledge of the immunologic mechanisms of sensitization induced by respiratory sensitizers such as TDI, continues to complicate the development of these assays. Novel molecules and mechanisms involved in allergic disease need to be investigated in order to elucidate specific entities that can be utilized for the development of hazard identification assays for respiratory sensitizers. Due to the recognized role of T_{regs} in related allergic disease states, we chose to investigate the expression kinetics, phenotype, and functional capability of T_{regs} in a murine model of dermal TDI sensitization. To our knowledge, this is the first study that investigates the expression and functionality of T_{regs} in Th2-mediated chemical sensitization.

T_{reg} involvement has been suggested in the prevention of the development of allergic disease in both mouse and human models of allergy, specifically impacting Th2-related responses (49). Murine $CD4^+CD25^+$ T cells can prevent the transition of naïve $CD4^+$ T cells to Th2 cells *in vitro* (50). In a model of house dust mite antigen-induced airway inflammation decreased airway

pathology and *ex vivo*-derived splenic IL-4 and IL-13 levels were observed following T_{reg} adoptive transfer (51). Additionally, T_{regs} have been implicated in *in vivo*-induced tolerance following inhaled ovalbumin exposure (52), highlighting the importance of these cells in the prevention of allergic disease. Supporting human studies have demonstrated the expansion of T_{regs} in the nasal mucosa following allergen immunotherapy and noted their association with the efficacy of treatment, suggesting a role for T_{regs} in the development of antigen-specific tolerance in allergic humans (53). In addition to their direct actions on Th2 cells, T_{regs} have also demonstrated the ability to suppress allergic disease by influencing granulocytes, antibody-producing B cells, and resident tissue cells (54). In addition, T_{regs} have demonstrated involvement in maintaining tolerance and controlling the allergic response during the sensitization phase of both ovalbumin-induced allergic airway inflammation (55) and hapten-induced CD8⁺ T cell-dependent contact hypersensitivity response (Christensen *et al.*, 2015).

In an effort to explore the role of T_{regs} in TDI sensitization, this population was profiled following epicutaneous TDI exposure. Several basic T_{reg} markers (CD3/CD4/CD25/Foxp3) were used to define the classical T_{reg} population but it is important to note that some T_{reg} subsets do not express CD25 and/or Foxp3 such as Tr1 (56) and Th3 (57) cells which typically exhibit TGF- β and IL-10-mediated suppression. Due to the use of CD25 as a functional tool in these studies, we have chosen to focus on classical T_{regs}. The basic T_{reg} population encompasses numerous subpopulations including, but not limited to, nT_{regs}, iT_{regs}, migratory and homing T_{regs}, and IL-10 secreting T_{regs}. Beyond the nT_{regs} and iT_{regs}, these subsets are not mutually exclusive, so while phenotyping markers may be presented alone, many of these populations are co-expressers of a variety of markers. In general, the T_{reg} populations (basic and specialized; Table 1) were identified to expand in both number and frequency, peaking around 4-7 days and beginning to retract around 7-9 days following

TDI exposure. This retraction may be due to the early importance of this population in the dLN or may also be mediated by other compensatory or regulatory elements. Interestingly, the general T_{reg} population's frequency did not significantly increase following 0.5% TDI exposure (Figure 3.2 B), potentially implicating a role for the irritant response in both the induction of T_{regs} and as a precursor to the initiation of sensitization.

Further support for a role for T_{regs} in TDI sensitization was identified following *in vivo* administration of anti-CD25 treatment. In this T_{reg} depletion study, augmentations in traditional Th2 allergic markers (dermal irritation, dLN cellularity, Th2 population, IL-4 mRNA expression, and serum total IgE production) were observed. Dose-responsive increases in dLN cellularity, IL4, Th2 cells, and IgE were also observed and were further augmented following anti-CD25 treatment indicating increased sensitization in the absence of T_{regs} . dLN cellularity was measured as a gross marker of sensitization intensity, as this parameter is traditionally utilized in the standard sensitization assay known as the local lymph node assay (Anderson *et al.*, 2011). The dLN Th2 cellular subset was examined as the main effector T cell subset traditionally thought to be involved in allergic responses, with the allergic cytokine IL-4 being primarily produced by this subset, influencing the development and homeostasis of the allergic microenvironment (58). IgE expression indicates the development of an allergic humoral response. While T_{reg} depletion was identified to promote a Th2 response, the findings from the T_{reg} suppression assay suggested that T_{regs} isolated from the TDI-sensitized animals were more suppressive compared to their acetone counterparts. These data suggest a significant role for T_{regs} in the regulation of chemical-induced allergy, implying that their presence may delay or reduce the intensity of sensitization.

Typically, the development of allergic disease is thought to involve an imbalance between Th1 and Th2 responses to allergens (59), resulting in an “overzealous” Th2 response. While the Th2 response

is an important mediator of TDI sensitization, it is important to note that TDI sensitization and resultant allergic disease also contain Th1 and Th17 components (A 3.4 G-J) (8). T_{regs} are capable of suppressing both Th1 (60, 61) and Th17 (62) –mediated responses, indicating that they may be influencing these components of the TDI sensitization response. Selective suppression of the Th1 response during TDI sensitization could further enhance the development of Th2-mediated sensitization based on the Th1/Th2 imbalance hypothesis.

While T_{regs} isolated from TDI sensitized mice were identified to be more suppressive than acetone-derived T_{regs} , this was reported based as a combined function for all populations of T_{regs} . This is important to note because TDI-induced T_{regs} were identified to be phenotypically heterogeneous. While the kinetics of the specialized T_{reg} populations generally mimic that of the basic T_{reg} population several discrepancies were identified that may provide insight into the mechanisms driving this response. The CTLA4^+ T_{reg} population was identified to increase in number and frequency earlier than the general T_{reg} population with significant increases identified one day post exposure (Fig 3.2a A and B). The early expansion of this subset was intriguing, given the lack of any data implying a role for CTLA4-mediated suppression in chemical-induced allergy, although this population has been noted to play an immunosuppressive role in the sensitization phase of the ovalbumin-induced allergic response (63). This population participates in contact-mediated suppression by binding to the B7 costimulatory complex expressed on APCs, thus inhibiting T cell activation in a highly suppressive manner (64). Based on the early expansion of this subset, it is possible that these cells are interacting with APCs in the early phase of the sensitization response. nT_{regs} are thymus-derived cells whose counterparts are inducible T_{regs} (iT_{regs}) generated in the periphery; nT_{regs} are identified by neuropilin-1 expression (11, 43). Interestingly, the nT_{reg} frequency significantly decreased between 7-9 days following TDI exposure compared to the acetone-treated

population (Fig 3.2a C). This decrease may be attributed to the efflux of cells from the dLN into peripheral tissues and/or the influx and expansion of iT_{regs} in the dLN. Because certain populations of T_{regs} are known to possess migratory capabilities, the T_{reg} phenotyping panel included several markers associated with T_{reg} migration and effector capabilities including CCR6, CD103, and ICOS. T_{regs} expressing any or all of these markers were defined as migratory effector T_{regs} due to the noted homing capabilities and the effector functions (31, 32, 35, 39, 65). The expansion of T_{regs} expressing CCR6, CD103, and/or ICOS (Table 3.2) may represent subsets that have acquired effector functions, such as IL-10 production, and possess migratory capabilities. Taken together, these findings suggest that T_{regs} likely utilize a variety of suppressive mechanisms as indicated by their phenotypic diversity. This could be a potential explanation for why TDI sensitized T_{regs} were identified as more suppressive than their acetone control counterparts.

These studies reveal an important role for T_{regs} in a murine model of TDI sensitization. Elucidation of the role of T_{regs} in this response will result in a better understanding of the immunologic mechanisms involved in chemical sensitization. Due to the complexity of mechanisms involved in chemical allergy, the investigation of novel cellular subsets and mediators of allergic disease is imperative for the greater understanding of these conditions and the development of hazard identification strategies for respiratory chemical sensitizers. In conclusion, we have demonstrated that T_{regs} are a phenotypically heterogeneous population that expand, suppress, and play a part in controlling the allergic response during TDI sensitization.

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CHAPTER 4

Investigation of the Expression and Potential Targets of microRNA 210 During TDI Sensitization

In preparation – Invited Submission. Genes.

Potential Inhibitory Influence of miRNA 210 on Regulatory T Cells During Epicutaneous Chemical Sensitization

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Running Title (40 characters): miR-210 May Target T_{regs} in TDI Sensitization

ABSTRACT

Toluene diisocyanate (TDI) is a potent low molecular weight chemical sensitizer and a leading cause of chemical-induced occupational asthma. The regulatory potential of microRNAs (miRNAs) has been recognized in a variety of disease states, including allergic disease; however, the roles of miRNAs in chemical sensitization is unknown. In a previous work, increased expression of multiple miRNAs during TDI sensitization was observed and several putative mRNA targets identified for these miRNAs were directly related to T_{reg} differentiation and function including *foxp3* and *runx3*. In this work, we show that miR-210 expression is increased in the mouse dLN and T_{reg} subsets during TDI sensitization. Alterations in dLN mRNA and protein expression of T_{reg} related genes/putative miR-210 targets (*foxp3*, *runx3*, *ctla4*, and *cd25*) were observed at multiple time points following TDI exposure and in *ex vivo* systems. A T_{reg} suppression assay, including a miR-210 mimic, was utilized to investigate the suppressive ability of T_{regs}. TDI derived cells treated with miR-210 mimic had less expression of miR-210 compared to the acetone control suggesting other factors, such as additional miRNAs, might be involved in the regulation of the functional capabilities of these cells. These novel findings indicate that miR-210 may have an inhibitory role in T_{reg} function during TDI sensitization. Because the functional roles of miRNAs have not been previously elucidated in a model of chemical sensitization, these data contribute to the understanding of the immunologic mechanisms of chemical induced allergic disease and may aid in the development of preventative strategies.

4.1. INTRODUCTION

Occupational allergic disease is a significant health burden. A variety of diseases can be caused by workplace chemical exposures, including asthma, conjunctivitis, dermatitis, rhinitis, and urticaria (1). Diisocyanates are a group of highly reactive chemicals characterized by the presence of double isocyanate functional groups; many of these chemicals are potent sensitizers and major causative agents of occupational allergic disease (2-4). Toluene diisocyanate (TDI) is frequently utilized in the automobile industry and in the manufacture of polyurethane foams, paints, and coatings (5-8). TDI is widely used throughout the United States (U.S.) and around the globe; the U.S. Environmental Protection Agency reports that U.S. production and importation of 2-4 and 2-6 TDI isomers rose above 1 billion pounds in 2006 (6). The principal routes of human exposure to TDI are inhalation and dermal contact (6). While TDI has generally been classified as a T-helper type 2 (Th2) sensitizer, the immune response following exposure is more accurately characterized as a mixed Th1/Th2 response in both rodents and humans (1, 2, 9). Both respiratory and dermal sensitization have been documented (7, 10) and can lead to allergic disease once the elicitation threshold has been reached. Skin sensitization is thought to be sufficient for subsequent allergic disease of the respiratory tract (7), illustrating the systemic nature of TDI sensitization. Although TDI-induced asthma is an extremely relevant occupational health concern, the pathogenic mechanisms of diisocyanate-induced asthma are not fully understood (5) and the identification of novel mediators of allergic disease may be necessary to obtain a full and complete understanding of the disease. Because of the clinical significance of and lack of validated identification strategies for chemical respiratory sensitizers like TDI (4, 11), it is necessary to investigate and identify functional pathways and mechanisms that are involved in TDI sensitization. Specific understanding of disease mechanism may have direct implications in risk assessment, hazard communication and guidance

used in the selection of safe products, the identification of safer alternatives, and preclinical biomarker discovery.

An emerging class of epigenetic regulatory elements that have been the subject of recent scientific focus are microRNAs (miRNAs). These molecules are single stranded, noncoding RNA molecules that are approximately 19-23 nucleotides long (12). They are processed by Drosha and Dicer nucleases preceding their mature form (13). MiRNAs exhibit functional significance through posttranscriptional gene regulation due to their ability to bind to the target messenger RNA (mRNA) and destabilize and decrease protein translation when in complex with argonaute proteins and the RNA-induced silencing complex (RISC). The seed sequence of a mature miRNA can interact with a portion of the target mRNA, typically within the 3' untranslated region, nucleotide base pairing can occur, and this may lead to the translational repression or degradation of the target mRNA, influencing gene expression. Recently, it has been shown that miRNAs play a major role in a variety of immune responses (14-17). These emerging regulatory factors have not been functionally investigated in the context of chemical sensitization, with the exception of recent work by our group that profiled the general expression kinetics of miRNAs involved in TDI sensitization (1) and research profiling miRNA expression in both human and murine diphenylcyclopropanone-induced dermatitis (18).

The identification of increased expression of several miRNAs in the draining lymph nodes (dLN) of mice during epicutaneous TDI sensitization prompted further investigation into the potential functional roles of these molecules in the allergic response. Target analysis for these upregulated miRNAs revealed several putative and confirmed regulatory T cell (T_{reg})-related targets for miR-21, -22, -27b, -31, -155, -210, and -301a in murine and human genomes (1). Several molecules including the transcription factors *foxp3* and *runx3* and the signaling molecules CD25

and CTLA4 are integral to T_{reg} differentiation and function. The expression of these molecules allows this subset to differentiate in response to allergens and exert immune regulatory functions, dampening inappropriate inflammatory and adaptive immune responses. Additional miRNAs potentially involved in TDI sensitization have been implicated as regulators of T_{regs} in a variety of contexts including miR-31 and -155 (19, 20). A role for T_{regs} has been suggested in models of chemical-induced contact hypersensitivity (21, 22) and in a recent manuscript, Long, et al. demonstrated the increased expression and functional capability of T_{regs} during TDI sensitization (22). While the collection of data regarding T_{regs} and chemical allergy is growing, it is still limited. Similarly, data regarding the role of miRNAs in chemical allergy is very limited. While our data suggests an important role for T_{regs} in dermal TDI sensitization, the interaction between these cells and selected miRNAs has not been investigated. While miR-210 is well characterized in the hypoxia response, its specific role in allergic disease has not yet been defined. In the present study we utilized a murine model of epicutaneous TDI sensitization in order to elucidate the expression kinetics and role of miR-210 and its putative mRNA targets in a murine model of epicutaneous TDI sensitization, specifically in relation to the T_{reg} subset.

4.2. MATERIALS AND METHODS

4.2.1. Mice

Female BALB/c mice (6-8 weeks of age) obtained from Taconic (Germantown, NY), acclimated for 5 days, and then randomly assigned to treatment group; homogenous weight distribution was insured across treatment groups. BALB/c mice were selected on the basis of their Th2 bias, robust IgE production, and the historical use of these mice in the laboratory to investigate

TDI sensitization (1, 23). Mice were housed in ventilated plastic shoebox cages with hardwood chip bedding at a maximum of five animals per cage. A NIH-31 modified 6% irradiated rodent diet (Harlan Teklad) and filtered tap water were administered *ad libitum*. Housing facilities were maintained at 68-72°F and 36-57% relative humidity, and a 12 hour light-dark cycle was maintained. All animal experiments were performed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited National Institute for Occupational Safety and Health (NIOSH) animal facility in accordance with an Institutional Animal Care and Use Committee-approved protocol.

4.2.2. TDI Sensitization Model

Toluene 2,4-diisocyanate (TDI, CAS# 584-84-9) was obtained from Sigma-Aldrich (Milwaukee, WI). Animals were exposed to a single dose of 0, 0.5, and 4% TDI (v/v) on the dorsal surface of each ear (25 µL per ear). The chosen TDI concentrations (0.5 and 4% v/v) and dosing regimen was previously shown to induce sensitization (1, 22) and 4% TDI was previously reported as the minimum single dose concentration of TDI that could induce maximum sensitization in the absence of systemic toxicity (1). Acetone was selected as the vehicle control and has been historically utilized in our laboratory to evaluate chemical sensitization (22-24).

4.2.3. Euthanasia, Tissue Collection, and Processing

Animals were weighed, euthanized via CO₂ asphyxiation at time points ranging from 1 to 11 days post chemical exposure, and examined for gross pathology. Left and right auricular draining lymph nodes (dLNs; drain the site of chemical application) were collected in 4 mL sterile phosphate-buffered saline (pH 7.4) and manually dissociated using the frosted ends of two microscope slides.

Cells were counted using a Cellometer (Nexcelom Bioscience, Lawrence, Massachusetts) and size exclusion parameters (3.5 to 36 μm) with a combined acridine orange/propidium iodide solution to identify viable cells. For isolation of specific cellular subsets Stemcell magnetic isolation kits (Vancouver, BC) were utilized, including the T_{reg} isolation kit (CD4 negative and CD25 positive selection).

4.2.4. *Ex vivo miRNA Transfection Assays*

Following dLN processing and counting miR-31, -155, and -210 mimics (25 pmol in Lipofectamine transfection reagent, ThermoFisher, Waltham, MA) were reverse transfected in flat-bottom 24-well plates. 1.5×10^6 naïve or acetone-treated dLN cells/mL (in RPMI-1640 media) were added to plates followed by general T cell stimulation (2 $\mu\text{g/mL}$ α -CD3, 1 $\mu\text{g/mL}$ α -CD28). Plates were incubated for 72 hours at 37°C and 5% CO₂. Following a 72 hour incubation, cells were washed with RPMI-1640 and RNA was isolated as described in the following section. RNA was assayed for intracellular miRNA expression to conform the intracellular uptake of miRNA mimics as well as T_{reg}-related gene expression via RT-PCR.

4.2.5. *RNA Isolation, Reverse Transcription, and RT-PCR*

Total RNA was isolated from the ears and dLN using the Qiagen RNeasy and miRNeasy kits respectively according to the manufacturer's directions. A QiaCube (Qiagen, Hilden, Germany) automated RNA isolation machine was utilized in conjunction with the specified RNA isolation kits. A DNase treatment was included in the RNeasy protocol for the removal of residual DNA. The concentration and purity of the RNA was determined using a ND-1000 spectrophotometer (Thermo Scientific Nanodrop, Wilmington, DE). For gene and primary miRNA expression analysis, first

strand cDNA synthesis was performed using a High-Capacity cDNA Synthesis Kit (Applied Biosystems, Carlsbad, California) according to manufacturer recommendations. For mature miRNA reverse transcription TaqMan MicroRNA Assays (looped-primer RT-PCR; Applied Biosystems, Carlsbad, California) were utilized according to manufacturer recommendations (both multiplex and singleplex protocols were utilized).

For analysis of mRNA and primary miRNA expression, TaqMan Universal Fast master mix (Life Technologies, Calsbad, California), cDNA, and mouse-specific mRNA primers (TaqMan Custom PCR Arrays, Carlsbad, California) were combined and PCR was performed according to manufacturer protocol (TaqMan Gene Expression Analysis). For analysis of miRNA expression, TaqMan Universal 2x master mix, No AmpErase UNG (Life Technologies, Calsbad, California), cDNA, and mouse-specific miRNA primers (TaqMan Custom PCR Arrays, Carlsbad, California) were combined and PCR was performed according to manufacturer protocol (TaqMan miRNA Assays both Single- and Multi-Plex). Primers used include: *β-actin*, *cd25 (il2ra)*, *ctla4*, *foxp3*, *mature miR-21*, *-22*, *-27b*, *-31*, *-126*, *-155*, *-210*, and *-301a*, *primary miR-210*, *runx3*, and *sno234*. MicroAmp Fast Optical 96-well reaction plates were analyzed on an Applied Biosystems 7500 Fast Real Time PCR system using cycling conditions as specified by the manufacturer. *β-actin* (mRNA and primary miRNA) and *snoRNA234* (miRNA) were used as the endogenous reference control gene as expression was determined to be stable following chemical exposure (data not shown). RT-PCR data were collected and represented as relative fold change over vehicle control, calculated by the following formula: $2^{-\Delta\Delta Ct} = \Delta Ct_{\text{Sample}} - \Delta Ct_{\text{Control}}$. $\Delta Ct = Ct_{\text{Target}} - Ct_{\beta\text{-ACTIN}}$, where Ct = cycle threshold as defined by manufacturer.

4.2.6. Flow Cytometric Analysis and T_{reg} Phenotyping

Single cell suspensions were prepared from tissues and a minimum of 150,000 dLN cells were aliquoted into 96-well U-bottom plates and washed in FACS staining buffer (PBS + 1% bovine serum albumin + 0.1% sodium azide). Cells were resuspended in staining buffer containing anti-mouse CD16/32 antibody (clone 2.4G2; BD Biosciences, San Jose, CA) for blocking of F_c receptors to minimize nonspecific binding. Cells were resuspended in staining buffer containing a cocktail of fluorochrome-conjugated antibodies specific for cell surface antigens including: CD3 (500A2, V500, BD Biosciences, Franklin Lanes, NJ), CD4 (RM4-5, AF700, BD), CD8a (53-6.7, AF488, BioLegend, San Diego, CA), CD25 (PC61, APC Cy7, BioLegend), CD45 (30-F11, PE, BD). Following surface staining, cells were washed in staining buffer and fixed using the Foxp3 fixation buffer set (eBioscience, San Diego, CA). After overnight incubation in staining buffer, cells were permeabilized using the Foxp3 fixation buffer set (eBioscience, San Diego, CA) and re-suspended in permeabilization buffer containing a cocktail of fluorochrome-conjugated antibodies specific for intracellular antigens including: RUNX3 (R3-5G4, PE, BD), Foxp3 (FLK-16s, eF450 and APC, eBioscience, San Diego, CA). Following staining, cells were re-suspended in staining buffer and analyzed on an LSR II flow cytometer using FACS Diva software (BD Biosciences). Data analysis was performed with FlowJo 10.0 software (TreeStar Inc., Ashland, OR). A minimum of 10,000 events were captured for each sample. Leukocytes were first identified by their expression of CD45. The T_{reg} subset was further identified as $CD3^+CD4^+CD8^-CD25^+Foxp3^+$. Numerical population values were calculated by applying subset frequencies to the initial cell count obtained following lymph node homogenization. Compensation controls were performed using single stained cellular suspensions and OneComp beads (eBioscience, San Diego, CA) and fluorescence minus one (FMO) staining controls were included to help set gating boundaries.

4.2.7. *T_{reg}* Suppression Assay

The suppressive ability of T_{regs} was analyzed using an *ex vivo* T_{reg} suppression assay as described by Long, et al. (22). This assay evaluates the ability of naïve, conventional dLN-derived T cells (T_{cons}) to proliferate in the presence of varying numbers of T_{regs} isolated from acetone- or TDI-exposed mice. Mice were exposed to acetone (n=7-11) or TDI (4%) (n=4-5) as previously described and following sacrifice at 7 days post TDI (peak of the expansion) exposure the dLN and spleens were removed. T_{regs} ($CD4^+CD25^+$) and T_{cons} ($CD4^+CD25^-$) were isolated from the lymph nodes and $CD4^-$ accessory cells were isolated from naïve spleens using $CD4^-$ negative and $CD25^+$ positive selection-based magnetic separation kits (Stemcell, Vancouver, BC). Average T_{reg} purity is as follows for 7 days: Acetone-96.5% $\pm$ 0.8 of $CD3^+CD4^+$ cells and 4% TDI-97.35% $\pm$ 0.25 of $CD3^+CD4^+$ cells. Following isolation from naïve mouse dLNs, T_{cons} were labeled with 2 μM carboxyfluorescein succinimidyl ester (CFSE). miR-210 mirVana mimic (2 pmol; ThermoFisher) in Lipofectamine RNAiMAX (ThermoFisher) or Lipofectamine only control (LO) were added to a 96-well U-bottom plate in order to reverse transfect cells. T_{cons} and T_{regs} were cultured in a 96-well U-bottom plate with anti-CD3 (0.2 $\mu\text{g/mL}$; BD Biosciences) and accessory cells (naïve $CD4^-$ splenocytes treated with Mitomycin C) at a variety of $T_{\text{con}}:T_{\text{reg}}$ ratios (1:1, 2:1, 4:1, and 8:1). Additional controls included stimulated T_{cons} only to assess baseline proliferation, T_{regs} only, accessory cells only, and T_{cons} only with no stimulation nor accessory cells. Cells from each treatment group were pooled and added to triplicate wells of the culture plate. 72 hours following plating, cells were stained with anti-CD4 and Live/Dead Violet (Life Technologies, Carlsbad, CA). T_{cons} were defined as $CD4^+CFSE^+$ cells and suppression was measured based on changes in the frequency of dividing $CFSE^+$ cells based on the dilution of CFSE. T_{regs} were analyzed for purity

based on their expression of CD3, CD4, and Foxp3 as determined by flow cytometric analysis as previously described.

4.2.8. Statistical Analysis

Statistical analyses were generated using GraphPad Prism version 5.0 (San Diego, CA). Data was analyzed by a Student *t*-test comparing groups as indicated in the figure legends. Figure 4.4 C was analyzed by analysis of variance using PROC MIXED. In some cases, data were transformed using the natural log to meet the assumptions of the analysis. Significant interactions were explored utilizing the “slice” option in PROC MIXED and pairwise differences were assessed using a Fishers Least Significant Difference Test. All differences were considered significant at $p < 0.05$; representative significance symbols varied by figure, as indicated in the figure legend.

4.3. RESULTS

4.3.1. Examination of miR-210 Expression during TDI Sensitization

The kinetics of mature miR-210 expression were investigated via RT-PCR in the dLN following 0.5% and 4% TDI exposure. As previously reported (1), dLN miR-210 increased at various time points during 0.5 and 4% TDI sensitization, including 4 (4%), 7, and 9 (0.5 and 4%) days post single exposure, with expression appearing to peak at 4 days post 4% TDI exposure (Figure 4.1A). Primary miRNA (pri-miRNA) transcripts represent the early precursor stage of mature miRNA structure prior to modification and cleavage by Drosha and Dicer. Pri-miR-210 levels were analyzed in the dLN 4 days post TDI exposure, which represents the relative peak of dLN miR-210 expression. Although expression of pri-miR-210 was detected in the dLN, no significant alterations

were observed. Alternatively, pri-miR-210 expression significantly decreased in the CD4⁺ subset during 4% TDI sensitization (Figure 4.1 B)

Figure 4.1 Mature miR-210 expression increases during TDI sensitization

RT-PCR analysis of mature miR-210 expression in the dLN at various time points post TDI exposure (A). Primary miR-210 expression in the whole dLN and dLN CD4⁺ subset 4 days post TDI exposure (B). Cellular purity was assessed via flow cytometric staining for CD4⁺ Cells (% CD4⁺ of all cells; Acetone: 89.6% ± 2.2, 0.5% TDI: 91.5% ± 0.3, and 4% TDI: 90.4% ± 0.3). Bars represent mean relative fold change (± SE) of 3-5 mice per group. *P* values are represented by ^ (0.5% TDI) and * (4% TDI) (*P*<0.05). Dermal treatment groups are indicated by the following symbols: circle- acetone, square- 0.5% TDI, and triangle- 4% TDI.

4.3.2. Ex vivo miR-210 Mimic Transfection Reveals Potential Inhibitory Effect of miR-210 on T_{reg} -related Genes

Due to the putative link between miR-210 and T_{reg} -related targets in both human and mouse-based studies and target algorithms (1), an *ex vivo* transfection and stimulation assay was set up to directly examine miR-210 and T_{reg} -related gene expression. Analysis of T_{reg} -related gene expression induced by the addition of excess levels of miR-210 was examined. Although not statistically significant, Figure 4.2 A reveals increased miR-210 expression in cells treated with miR-210 mimic (101 fold) as compared to lipofectamine only (LO) control (1.2 fold). Analysis of T_{reg} -related genes revealed decreased trends of expression of T_{reg} -related genes following miR-210 mimic transfection compared to the LO control, including *foxp3* (~ -2.0 fold compared to 0.6 fold) and *cd25* ((~ -3.2 fold compared to 0.2 fold) (Figure 4.2 B)). No apparent changes in *runx3* nor *ctla4* were observed (data not shown).

Figure 4.2 *Ex vivo* Data Reveals Potential Inhibitory Effect of miR-210 on T_{reg} -related Genes

Intracellular miR-210 (A) was quantified via RT-PCR in *ex vivo* stimulated samples 72 hours following transfection with lipofectamine control (LO) or miR-210 mimic. In (B) *foxp3* and

cd25 (il2ra) were investigated as potential miRNA targets via RT-PCR. Bars represent mean relative fold change ($\pm$ SE) of 3 replicates per group. Data represents two independent experiments.

4.3.3. *In vivo* dLN Target Expression Reveals Decreased Expression of Several Key T_{reg}-related Genes

The expression of potential miR-210 targets and key T_{reg} genes potentially affected by miR-210 was investigated in the whole dLN at various time points following 4% TDI exposure. Similar to the findings of the *ex vivo* study and previously reported findings, dLN target/key player mRNA expression was significantly decreased at 4 (*runx3*), 7 (*foxp3*), and 9 (*foxp3*) days post 4% TDI exposure (Figure 4.3 A and B). *Foxp3* expression was decreased by approximately threefold in comparison to the acetone control at 7 and 9 days post TDI exposure (Figure 4.3 A). Early statistically significant increases in *foxp3* (1day) and *run3* (2 day) were also observed but only for a single time point and not determined to be of biological significance. dLN *ctla4* and *cd25* expression significantly increased with peak expression at 4 and 2 days post 4% TDI exposure, respectively. However, these increases in expression were not maintained at later time points (Figure 4.3 C and D).

Figure 4.3 In vivo Target mRNA Expression

RT-PCR analysis of *foxp3* (A), *runx3* (B), *ctla4* (C), and *cd25* (D) expression in the whole dLN at various time points post 0 and 4% TDI exposure. Bars represent mean relative fold change of 5 mice per group. Statistical significance is represented by * ($P < 0.05$).

4.3.4. T_{reg}-Specific miRNA and Gene Expression

Due to the suspected influence of miR-210 on T_{regs} the levels of this miRNA were examined in T_{regs} (CD4⁺CD25⁺) from the dLN of mice treated with 0, 0.5, and 4% TDI at 2 and 7 days post exposure. These time points were selected to reflect early and peak miR-210 and T_{reg} responses in the dLN (Figure 4.1 A and (1)). While no statistically significant changes were observed following 2 days of exposure, miR-210 levels were significantly increased in T_{regs} during 0.5 and 4% TDI sensitization in a dose responsive fashion at 7 days post exposure (Figure 4.4 A). Expression of T_{reg}-related genes was also examined in isolated T_{regs} following TDI exposure. Significant decreases in *foxp3*, *ctla4*, *runx3*, and *cd25* were observed at both concentrations (except for *ctla4* at 4%) at 2-days post exposure. Similarly, significant decreases in *foxp3*, *ctla4*, and *cd25* mRNA were observed in T_{regs} from 4% TDI-exposed mice at 7 days post 4% TDI exposure (Figure 4.4 B), further indicating that these genes may be influenced by miRNA (Figure 4.1 A). T_{reg}-specific expression of Runx3 was assayed in dLN T_{regs} during TDI sensitization. In contrast to the mRNA levels, increases in frequency and numbers of Runx3⁺ T_{regs} at all time points were observed following 0.5 and 4% TDI exposure (Figure 4.4 C).

A

T_{reg} miR-210 Expression

B

C

Figure 4.4 *Mature miRNA expression increases in T_{regs} during TDI sensitization*

RT-PCR analysis of -210 (A and B) and T_{reg} -related gene (C and D) expression in the dLN T_{reg} subset at 2 and 7 days post TDI exposure. T_{regs} were isolated as $CD4^+CD25^+$ cells and cellular purity was assessed via flow cytometric staining for $CD3^+CD4^+Foxp3^+$ events (2 days: % T_{regs} of all $CD4^+$ events, mean, n=4-5 per group: Acetone: $83.8\% \pm 1.6$, 0.5% TDI: $67.1\% \pm 4.2$, and 4% TDI: $72.3\% \pm 1.9$; 7 days: % T_{regs} of all $CD4^+$ events, one representative sample: Acetone: 91%, 0.5% TDI: 88.4%, and 4% TDI: 86.7%). Flow cytometric analysis of dLN Runx3⁺ T_{reg} frequency and number (C) following TDI sensitization. Bars represent mean relative fold change ($\pm$ SE) of 4-5 mice per group. For A and B, *P* values are represented by ^ (0.5% TDI) and asterisks (4% TDI) (*P*<0.05). For C, Significance is indicated as follows: *P* ≤ .05 (*), *P* ≤ .01 (**), *P* ≤ .001 (***), and *P* ≤ .0001 (****) for 4% TDI or *P* ≤ .05 (^), *P* ≤ .01 (^^), *P* ≤ .001 (^^^), and *P* ≤ .0001 (^^^^) for 0.5% TDI. Dermal treatment groups are indicated by the following symbols: circle- acetone, square- 0.5% TDI, and triangle- 4% TDI.

4.3.5. The *Ex Vivo* Suppressive Capability of T_{regs} is Influenced by miR-210 Levels

In order to examine the suspected inhibitory role of miR-210 on T_{regs} during TDI sensitization, the functional capabilities of T_{regs} were tested in an *ex vivo* suppression assay. miR-210 mimic was transfected in selected wells utilizing a lipid-based transfection strategy and intracellular miR-210 mimic uptake was confirmed in these wells at the time of cellular harvest (Figure 4.5 A). Interestingly, following addition of the miR-210 mimic, miRNA-210 levels were almost 10-fold higher in wells containing acetone T_{regs} compared to those with TDI T_{regs} (Figure 4.5 A). No differences were noted between miR-210 levels among the acetone and TDI groups treated with LO (Figure 4.5 A). Significant baseline T_{reg} suppressive ability was observed in wells with

Lipofectamine only and acetone-derived T_{regs} as well as 4% TDI-derived T_{regs} (data not shown). This was not altered significantly upon the addition of miR-210 mimic with the exception of the 1:1 ratio in wells with 4% TDI-derived T_{regs} (data not shown). As previously evidenced (22), TDI-derived T_{regs} exhibited greater suppressive capability than acetone-derived T_{regs} in mimic-treated wells for the 1:1, 2:1, 4:1, and 8:1 ratios (Figure 4.5 B). This increased function was observed in the presence of lower miR-210 levels suggesting that TDI might induce additional factors that might influence that functional capabilities of the T_{regs}.

A

B

Figure 4.5 *Increased T_{reg} Suppression is Associated with Decreased miR-210 levels*

A CFSE-based T_{reg} suppression assay was performed with T_{regs} from mice treated with acetone or TDI (7 days post exposure) and the addition of miR-210 mimic. RT-PCR analysis of intracellular miR-210 expression (A) in pooled wells from each indicated group was performed (one replicate from each ratio was included for the 1:0, 1:1, 2:1, and 4:1 $T_{con}:T_{reg}$ ratios.) A CFSE-based T_{reg} suppression assay with the addition of miR-210 mimic was performed and percent dividing CFSE⁺ cells were quantified (B). For (B) 3 plate replicates were utilized from groups of 5-10 mice, as described in section 4.2. *P* values are represented by asterisks ($P<0.05$; comparison of each treatment group to T_{con} only from the same chemical treatment group) or horizontal bars with asterisks above (comparison of identical ratios between different LO/mimic treatment groups). Bars represent mean relative fold change ($\pm$ SE) of 3 replicates per group. *P* values are represented by horizontal lines and asterisks ($P<0.05$).

4.3.6. Further Investigation of miR-31 and -155 Suggest Additional Regulation of the TDI Sensitization Response, Potentially Impacting miR-210 and T_{regs}

Evaluation of additional miRNAs was performed in an attempt to identify other potential factors that might influence miR-210 and T_{reg} function. MiR-31 and -155 were selected since they have been identified to significantly increase in dLN expression earlier in TDI sensitization compared to miRNA-210 (Figure 4.6 A and B). *Ex vivo* target analysis was performed utilizing additional miRNA mimics including miR-31 and -155 in order to further pursue the potential regulation of the T_{reg} subset by these miRNAs in addition to miR-210. Significantly increased intracellular levels of -31 (Figure 4.6 C) and -155 (Figure 4.6 D) were confirmed 72 hours following transfection and stimulation. Although not statistically significant, apparent decreases in *foxp3*

expression were observed following miR-31 (Figure 4.6 E) and -155 (Figure 4.6 F) mimic transfection. No changes in expression were observed for *runx3*, *ctla4*, or *cd25* following mimic addition (data not shown).

E **F**

Figure 4.6 *In vivo* dLN miR-31 and -155 Levels Increase During TDI Sensitization

Whole dLN miR-31 (A) and -155 (B) levels were quantified via RT-PCR at various time points post TDI sensitization. Intracellular miR-31 (C), -155 (D) was quantified via RT-PCR in *ex vivo* stimulated samples (with miR-31 or -155 mimic addition, respectively) after 72 hours. In D-F *foxp3* expression was investigated in cultures following miR-31 (D) and -155 (E) mimic treatment. Bars represent mean relative fold change ($\pm$ SE) of 3 replicates per group. *P* values are represented by ^ (0.5% TDI) and * (4% TDI) ($P < 0.05$).

T_{reg} -specific expression of miR-31 and miR-155 were also analyzed, revealing increased miR-31 levels 2 and 7 days post 0.5% TDI exposure (Figure 4.7 A) and increased miR-155 levels 7 days post 4% TDI exposure (Figure 4.7 B). Since miR-31 was identified to increase in expression earlier than miR-210 in the dLN and T_{regs} , miR-210 expression was evaluated in dLN cells treated with 31 and 155 mimics. However no changes in expression were observed. Similarly, expression of miR-31 and -155 was analyzed in cells treated with -210 mimic but no significant changes in expression were observed (data not shown).

Figure 4.7 Mature miR-31 and -155 expression increases in *T_{regs}* during TDI sensitization

RT-PCR analysis of miR-31 (A) and -155 (B) expression in the dLN *T_{reg}* subset at 2 and 7 days post TDI exposure. *T_{regs}* were isolated as CD4⁺CD25⁺ cells and cellular purity was assessed via flow cytometric staining for CD3⁺CD4⁺Foxp3⁺ events (2 days: % *T_{regs}* of all CD4⁺ events, mean, n=4-5 per group: Acetone: 83.8% ± 1.6, 0.5% TDI: 67.1% ± 4.2, and 4% TDI: 72.3% ± 1.9; 7 days: % *T_{regs}* of all CD4⁺ events, one representative sample: Acetone: 91%, 0.5% TDI: 88.4%, and 4% TDI:

86.7%). Bars represent mean relative fold change ($\pm$ SE) of 4-5 mice per group. *P* values are represented by * ($P < 0.05$).

4.4. DISCUSSION

The occupational use of sensitizing chemicals such as TDI remains a significant public health concern. There are no validated hazard identification strategies for respiratory sensitizers like TDI and the complete immunologic mechanisms of sensitization have not been elucidated for these agents, hindering development of appropriate preventative assays. This justifies research pertaining to the identification of novel cellular subsets and epigenetic regulatory mechanisms such as miRNAs that may be involved in the respiratory chemical sensitization process. Following the identification of several upregulated dLN miRNAs during TDI sensitization, these molecules were investigated during this response, specifically in relation to T_{reg} development and functionality. To our knowledge, this is the first work that functionally investigates miRNAs in a model of Th2-mediated chemical sensitization.

miRNAs are powerful regulatory molecules which have been implicated in a number of immunologic states and conditions, including allergic disease (25-27). Specifically, miR-155 has demonstrated a critical role in the development of antibody responses and germinal center function (28), miR-326 has been shown to regulate Th17 differentiation, exhibiting critical involvement in multiple sclerosis pathogenesis (29), and *in vivo* miR-126 inhibition reduces a house dust mite-induced asthmatic phenotype, demonstrating the importance of this miRNA in the regulation of Th2 responses and allergic asthma (25). Vennegaard, et al. described upregulation of several miRNAs, including miR-21, in skin biopsies from patients with allergic responses to diphenylcyclopropanone and in a murine model of dinitrofluorobenzene (DNFB) allergic contact dermatitis (18).

Additionally, previous work from our groups identified upregulation of several miRNAs, including miR-31, -155, and -210, in a murine model of epicutaneous TDI sensitization (1). These ubiquitous signaling molecules are well-established mediators of many of signaling pathways in a number of cell types; however, their role in chemical sensitization is not well understood. For the work described in this manuscript, miR-210 was selected for additional investigation since it has been predicted and demonstrated to target T_{reg}-related genes. In addition, its role in chemical sensitization and T_{reg} regulation has not yet been described.

The expression of miR-210 was quantified in a variety of tissues and cellular subsets in a murine model of TDI sensitization. Consistent with previous findings (1), increased expression of miR-210 in the dLN was also demonstrated in the present study during TDI sensitization (Figure 4.1 A). In addition, increased expression of miR-210 was also identified in T_{regs} during TDI sensitization (Figure 4.4 A). Since miRNA can be transported to cells via mechanisms such as exosomal transport, experiments to determine if cells are actively producing miR-210 following TDI exposure were conducted. The expression of pri-miRNA indicates gene level expression, presumably within the cell type tested. Pri-miR-210 was detected in the dLN and CD4⁺ subsets 4 days post TDI exposure (Figure 4.1 B), indicating that miR-210 is being expressed in this tissue by CD4⁺ T cells. Interestingly, although they were detectable, pri-miR-210 levels significantly decreased in CD4⁺ T cells 4 days post 4% TDI exposure compared to equivalent cells in acetone-exposed mice, potentially indicating that the majority of mature miR-210 in the dLN is being produced by another cell type, is being transported from another tissue, or is being transcriptionally downregulated at this point, the peak of mature miR-210 levels in the dLN. While pri-miR-210 levels in T_{regs} were not investigated in the current study, previous studies have demonstrated miR-210 expression following the polarization of naïve T cells into T_{regs} (30).

Since T_{regs} have been implicated as regulators of TDI sensitization (22) and miR-210 expression has been shown to increase in T_{regs} following TDI sensitization (Figure 4.4 A), an *ex vivo* target analysis system was designed in order to directly examine the effects of miR-210 on T_{reg} -related genes. Selected genes included *foxp3*, the master transcription factor of the T_{reg} subset and *runx3*, a transcription factor that signals upstream of *foxp3* by binding to this gene's promoter (31) and identified as a potential direct target of miR-210. Additionally, *cd25* and *ctla4* were investigated as important T_{reg} -related genes as they are involved in IL-2 signaling and proliferation along with direct suppressive functions, respectively. This setup revealed a potential role for miR-210 in the downregulation of T_{reg} -associated genes (*foxp3* and *cd25*; Figure 4.2 B) following the addition of miR-210 mimic. In addition *foxp3* and *runx3* dLN expression decreased in the dLN at various time points following TDI exposure (Figure 4.3 A and B) which is consistent with our previously reported findings (22). The earlier decrease in *runx3* expression observed at 4 days post 4% TDI exposure may be reflective of the upstream signaling activity of this transcription factor in relation to *foxp3* (Figure 4.3 B). While the findings for the *ex vivo* assay did not reach statistical significance, further support for T_{reg} -associated genes as miRNA targets was provided by *in vivo* data. Interestingly whole dLN expression of *ctla4* and *cd25* increased at 1, 2, and 4 days post 4% TDI exposure (Figure 4.3 C and D) which is likely a reflection of the activation of both T_{regs} and conventional T cells as elevated protein expression of these molecules is observed in T_{regs} at these time points (22) and would likely be increased in conventional T cells involved in TDI sensitization as well. In T_{regs} , decreases in *foxp3*, *cd25* and *ctla4* were observed at 2 and 7 days with *runx3* only being decreased at the earlier time point post TDI exposure (Figure 4.4 B). These early changes in T_{reg} factors provide further support that additional factors might be involved in T_{reg} regulation since peak increases in miR-210 occur later than 2 days; accordingly, miR-31 was shown to be increased at this time point. In contrast

to the transcript, the expression kinetics of Runx3 increased following TDI exposure (Figure 4.4 C). This expression pattern was similar to other T_{reg} proteins such as CD25 and Foxp3 (which are represented by the general T_{reg} population) and T_{reg} -specific CTLA4 expression which have previously been investigated during TDI sensitization (22). The kinetics of T_{regs} bearing these molecules tended to peak at 4 days post TDI exposure with a relative decrease in both cellular frequency and number at 7 days post TDI exposure (22). In relation to miR-210 expression kinetics, this data may suggest that miR-210 has a regulatory role on the T_{reg} subset, as its expression wanes in concert with the general T_{reg} population as well as CTLA4⁺ and Runx3⁺ T_{regs} . Additionally, miR-210 is a putative *runx3* target, suggesting a potential direct effect on this gene (1). Collectively, this data is suggestive of T_{reg} regulation with visible effects on the expression of proteins beginning at day 7 post TDI exposure. miR-210 expression remains elevated in the dLN throughout 9 days post 0.5 and 4% TDI exposure (Figure 4.1 A) and in T_{regs} at 7 days post 0.5 and 4% TDI exposure (Figure 4.4 A).

Due to the potential link between miR-210 and T_{reg} -related gene expression, the functional capabilities of T_{regs} (acetone and TDI -derived) were examined in the presence and absence of miR-210 mimic. Interestingly, it appeared that miR-210 levels were lower in wells containing T_{regs} from TDI-treated mice and miR-210 mimic compared to wells with acetone-derived T_{regs} and miR-210 mimic (Figure 4.5 A). The increased suppressive capability of TDI T_{regs} with miR-210 mimic (Figure 4.5 B) may be a reflection of reduced miR-210 levels, as we hypothesize that miR-210 is inhibiting T_{reg} differentiation and/or function. This finding suggested that other regulatory factors including other miRNAs might be involved in the regulation of T_{reg} function. Complex interactions and interplay have often been reported for other miRNAs (32), therefore this concept was evaluated in the current study. Typically direct miRNA-miRNA interactions are mediated by reverse

complementary binding, resulting in the formation of duplexes (32). Additionally, indirect miRNA-miRNA interaction may occur via target gene interaction; e.g. if a miRNA targets a gene that induces a different miRNA, this miRNA is being regulated by its own species.

miRNA-31 and -155 were further investigated for the potential to regulate the expression of miR-210 as they were identified to increase at early time points in TDI sensitization in the dLN (Figure 4.6 A (miR-31) and B (miR-155)) and Tregs (Figure 4.7 A (miR-31)). Similarly to miR-210, miR-31 and -155 were shown to potentially downregulate *foxp3* expression in this assay (Figure 4.6 E and F). Although limitations in the assay sensitivities did not reflect significant changes, this may be reflective of a direct effect on *foxp3* or an indirect effect on this gene via other signaling pathways such as miR-210. These alterations are in accordance with recent findings pertaining to miRNA-mRNA interactions. miR-31 may indirectly target *foxp3*, leading to suppressed iT_{reg} development (20), accounting for the potential decreases in this gene evidenced following miR-31 mimic transfection. Also miR-31 increases earlier when more persistent decreases in *foxp3* were observed. Additionally, miR-155 expression appears to be controlled by *foxp3* in T_{regs} via binding to the intron within the DNA sequence encoding Bic, the precursor transcript of miR-155; accordingly, T_{reg} miR-155 levels have been shown to be highly responsive to *foxp3* levels (33). This regulation may be interrupted by abnormally high levels of miR-155 in the mimic transfection system, resulting in decreased *foxp3* expression in these conditions via signaling feedback. This data suggests that miR-31 and -155 may be influencing the expression of miR-210 and/or T_{regs}, possibly acting as early signaling mediators in the TDI sensitization response.

The lack of significance associated with the *ex vivo* experiments conducted in this work could be a reflection of experimental variability associated with similar assays and temporal discrepancies associated with signaling events. The *ex vivo* system displays several limitations, explaining the

utilization of the *in vivo* TDI sensitization model in the target investigation as well. The T_{reg}-related gene alterations that were observed as a consequence of miRNA mimic transfections in this system may indicate direct and/or indirect targeting by the miRNA. We propose that for the majority of miRNA-mRNA interactions investigated in our model, regulation is indirect, as few, if any, putative binding sites were identified for many of the potential targets and the corresponding miRNA. For example, miR-210 is predicted to target the 3' UTR of *runx3* (1) and although we did not observe significant alterations in *runx3* expression following miR-210 mimic transfection in our *ex vivo* system, the *in vivo* expression kinetics of *runx3* suggest potential regulation. The ubiquitous nature of miRNAs and their involvement in various signaling processes accounts for their functional significance but can also cloud investigations into their mechanistic functions.

It is important to note that the increases in miRNA expression were not dependent on the irritant response, as dLN miR-210 levels significantly increased (Figure 4.1 A) following the non-irritating (22) 0.5% TDI exposure. As 4% TDI exposure causes significant dermal irritation (22), ear miR-210 expression was analyzed at both non-irritant (0.5%) and irritant (1, 2, and 4%) TDI concentrations, revealing significant increases in dLN and ear expression at both non-irritant and irritant doses (A 4.1 A). In addition other miRNAs including miR-22, -31, and -301a were also shown to increase significantly in expression regardless of the irritant status of the TDI dose (data not shown). This data prompts insight into the concept of the “two-signal” sensitization hypothesis which states that antigen delivery alone is insufficient for effective immunological priming but rather a second, innate signal is necessary to ensure the development of sensitization (34, 35). As noted in previous studies, the irritant response appears to be a prerequisite for strong sensitization responses in the case of dermal TDI sensitization (22). Regardless, the expression of multiple miRNAs in the dLN appears to be due to the sensitization response alone and not significantly influenced by the

irritant component of this response, which may be revealing as to their supposed functional roles in the sensitization response and may suggest potential utility as biomarkers of sensitization.

These studies reveal a potential role for miR-210 in a murine model of dermal TDI sensitization. Additionally, miR-31 and miR-155 were investigated for their regulatory potential in this response. The investigation of novel mediators of chemical-induced allergic disease is important for the overall understanding of the mechanisms involved in these responses. Therefore, this data may result in enhanced understanding of the mechanisms involved in chemical sensitization and could potentially aid in the development of hazard identification strategies for respiratory chemical sensitizers. In conclusion, we have demonstrated that miR-210 may negatively influence the differentiation and/or function of T_{regs} via direct targeting of *runx3* and/or indirect actions on other T_{reg}-related genes. These findings suggest that these miRNAs may work in concert to affect the differentiation and function of T_{regs} as well as the expression and function of miR-210.

4.5. REFERENCES

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CHAPTER 5

General Discussion

5.1 Introduction

Recent increases in the global burden of allergic disease have prompted study into the underlying immunologic mechanisms of these conditions. Encompassing a wide variety of conditions, the umbrella of allergic disease may include asthma, contact dermatitis, eczema, folliculitis, hypersensitivity pneumonitis, rhinitis, and urticaria. Although many factors may contribute to the development of allergic disease, including atopy, environmental exposures, epigenetics, genetics, and stress, occupational exposures are a major cause of allergic disease. It is evident that exposure to a variety of substances via multiple routes of exposure can lead to allergic disease, illustrating the significant breadth of occupational allergic disease.

While protein and metal allergens are important causative agents of allergic disease, this work focuses on TDI, an organic LMW chemical agent of allergic disease. In general, LMW chemical allergens are diverse and encompass a wide variety of chemical structures and are utilized in a number of industries worldwide (Table 1.1). While it may be easy to imagine significant chemical exposure as a factory worker in a chemical plant, these exposures may occur in virtually any industry. Many disinfectants utilized in the healthcare and cosmetology industries are known to cause asthma and contact dermatitis (e.g. eugenol, glutaraldehyde, ortho-phthalaldehyde (OPA), quaternary ammonium compounds) or may augment existing allergies (e.g. triclosan). Many of these chemicals are found in consumer products such as antimicrobial coatings and cleaning products, further illustrating the ubiquity of these products and potential for exposure.

As eloquently summarized by TV Rajan, the term “allergy” was initially utilized to identify both immune responses that were protective and deleterious to the host; this concept was also initially applied to the term “hypersensitivity” which was used to indicate the [typically] protective response that took place upon re-exposure to an infectious agent (1). The more recent view of

“hypersensitivity” as a host-deleterious response with an allergic component is illustrated in the Gell and Coomb’s hypersensitivity classification (Table 1.1). In this context, the Gell and Coomb’s classification is largely accurate, yet non-inclusive for a wide variety of reactions, particularly those caused by chemicals, metals, and drugs. In line with the origins of the term “hypersensitivity”, the four types of reactions classified by Gell and Coombs may be attributed to overarching mechanisms originating from those utilized by the immune system during pathogen defense (1). This view, proposed by TV Rajan, is based on the idea that immune reactions likely arose for host-protective reasons rather than solely to cause harm to the host, as seems to be the case when allergy is viewed from a separate origin from pathogen defense strategies (1). This understanding may aid in the interpretation of each reaction and appropriate revisions that could possibly be made to the classification, in light of the dramatically altered landscape of immunological knowledge. Essentially, the inappropriate activation and/or execution of these “protective” mechanisms may result in host-deleterious conditions. Type I hypersensitivity is characterized by IgE and mast cell activation; this reaction may have initially developed as a parasite-defense mechanism, indicated by similar IgE-mediated mechanisms and expulsive strategies manifested in symptoms such as vasodilation and mucin production (1). Type II hypersensitivity is characterized by complement activation (due to antibody-antigen recognition) and neutrophil invasion and these mechanisms are involved in extracellular pathogen clearance (1); antibody opsonization is a classic mechanism of pathogen clearance as is complement activation and neutrophil recruitment. The main effector mechanisms of type III hypersensitivity reactions are the formation of antibody-antigen complexes, deposition of these complexes in tissue, and complement and neutrophil recruitment (1); these mechanisms are proposed to be based on viral clearance strategies. Type IV hypersensitivity is characterized by delayed CD4⁺ Th1 and CD8⁺ cytotoxic T-cell action, leading to host cellular

destruction and conditions such as contact dermatitis and diabetes mellitus (1). This reaction is thought to be an inappropriate manifestation of both viral and intracellular bacterial host clearance strategies, which commonly involve the same effector cellular subsets. A strong epidemiological link has been noted between respiratory virus infection and asthma risk, suggesting that these conditions may share common risk factors and mechanisms (2). The inverse is also true; atopy has also been associated with increased severity of respiratory viral infections and wheezing (2). These associations emphasize both the common underlying mechanism of each condition as well as the potential common evolutionary origins of each disease state.

While viewing hypersensitivity reactions in light of their proposed evolutionary origins is helpful for the understanding of the mechanisms involved, there remains uncertainty regarding the validity of Gell and Coomb's classification in the context of new immunological insights into both allergic disease and pathogen defense. For many conditions, the current parameters of Gell and Coombs do not provide an accurate classification. These conditions include hypersensitivity pneumonitis and certain instances of chemical-induced asthma (3). The pathogenesis of hypersensitivity pneumonitis is similar to "traditional" atopic asthma (which is typically considered a Type I hypersensitivity reaction) in many ways, yet several key differences exist. Neutrophilia, alveolitis, Th1 involvement, and eventual fibrosis are all important components of hypersensitivity pneumonitis reactions that may not be present in conventional asthmatic responses (4). The acute phase of hypersensitivity pneumonitis can be summarized as an inflammatory process characterized by non-atopic neutrophilic inflammation caused by small organic particles, generally, nonspecific airway hyperreactivity and mucus production are not observed during this reaction as is typically the case with atopic asthma (4). The chronic symptoms associated with hypersensitivity pneumonitis are interstitial pneumonitis, lymphatic bronchiolitis (primarily CD8⁺ T cells), and

granulomas along with fibrosis of the alveoli and bronchioles (4). These pathological features differ from those associated with atopic asthma, clearly delineating these two conditions. Hypersensitivity pneumonitis has been considered both a Type III and IV hypersensitivity reaction due to the formation of IgG-antigen complexes and the involvement of CD8⁺ and Th1 cells, respectively (4, 5). It is important to note that, while hypersensitivity pneumonitis exhibits features of multiple classifications of hypersensitivity, leading to potential misunderstanding of the underlying mechanisms of disease. This condition may warrant its own subtype of hypersensitivity, due to its shared, yet unique characteristics. In either case, it is important to note that this condition transcends the Gell and Coomb's classification by encompassing multiple reactions at once.

Many cases of chemical-induced asthma cannot be accurately classified utilizing the Gell and Coomb's system. Specifically, diisocyanate-induced asthma exhibits immunologic and clinical features that are not accurately summarized by any of the four types of hypersensitivity reactions individually. While diisocyanate-induced asthma shares several commonalities with type I hypersensitivity reactions including eosinophilia, Th2-involvement, and clinical features of asthma, many other distinguishing characteristics do not fit within this classification. Neutrophil involvement, a Th1 component, IgG involvement, and the contested role of IgE are all components of diisocyanate-induced asthma that contribute to hypersensitivity classification uncertainty. Diisocyanate-induced asthma also shares similar features with hypersensitivity pneumonitis such as neutrophilia, Th1, CD8⁺ T cell, and IgG involvement, yet clinical symptoms are significantly divergent. LMW chemical-induced asthma seems to be related to both atopic asthma and hypersensitivity pneumonitis while remaining a unique entity. It may be classified as a mixed type I and IV response; however, this does not account for the condition as a whole. Additionally, as sensitization events precipitate elicitation and allergic symptoms, it is important to keep the

mechanisms of sensitization in perspective when setting classification guidelines for allergic reactions. With the investigation of additional mediators of allergic disease such as novel T cell subsets, this predicament will likely be amplified. This work identified the potential involvement of T_{regs} and Th17 cells in TDI sensitization, and although the functional roles of these cells need to be investigated further, particularly in the elicitation phase, these subsets are not addressed in the current classification.

When evaluating Gell and Coomb's classification in the context of modern immunologic knowledge, it is important to consider all significant aspects of each hypersensitivity reaction without getting "bogged down". All hypersensitivity reactions are unique in some way; therefore, emphasis of less functionally-relevant mechanisms may result in an overly complicated classification system, defeating the entire purpose of the system. Gell and Coomb's classification has withstood the test of time remarkably well, considering the massive influx of data and knowledge gained regarding hypersensitivity reactions. With this in mind, I propose a reevaluation of the current classification system, potentially resulting in further sub-classification of current types of hypersensitivity along with novel types of hypersensitivity reactions. The current sub classification of Type IV reactions is a poignant example of the potential diversity of reactions that fall in the same classification. Divided based on the cytokine milieu and effector cells present, four Type IV subclasses have been described (6) and these descriptive groupings aid in the understanding of each unique reaction. Following a basic survey of each of the four types of hypersensitivity reactions, the possible subcategorization of each type could be useful. In light of the known role of relatively new mediators such as Th17 cells, this reevaluation would serve as an update to the current system, potentially aiding student and clinicians in the understanding of these conditions. This also brings to light the necessity of further investigation into the mechanisms of allergic disease. Additional understanding

of the underlying mechanisms of hypersensitivity reactions may result in the re-classification of certain poorly understood conditions or the formation of novel types of hypersensitivity. Overall, the Gell and Coomb's classification system remains valid for a number of conditions but others remain un-classifiable with the current system. This should be taken into consideration by researchers and clinicians alike, as further investigation into the immune effectors of hypersensitivity reactions is needed and the current system should be interpreted with care.

LMW chemical allergens are an occupationally relevant class of compounds that are responsible for a variety of allergic diseases, perhaps the most notable being asthma and contact dermatitis. LMW chemical allergens are generally characterized as dermal (Th1) or respiratory (Th2) sensitizers, depending on the type of disease they elicit. While this classification is technically valid for some sensitizers, it is confusing in many cases. Certain LMW chemical sensitizers like TDI may elicit asthma following respiratory and/or dermal sensitization, yet this chemical is known as a respiratory sensitizer. This is a misnomer because although TDI exposure primarily causes respiratory disease, sensitization may occur by the dermal route. TDI may be more accurately classified as a systemic sensitizer and a respiratory allergen. Furthermore, respiratory sensitizers are generally associated with a Th2-dominant response with contact sensitizers being linked to Th1-dominant mechanisms. TDI induces a mixed Th1/Th2 response in both animal models and humans, further emphasizing the ambiguous nature of the current nomenclature. Nevertheless, there are no validated hazard identification strategies for LMW chemicals that are capable of causing respiratory allergic disease. This suggests that further research is necessary to elucidate the mechanisms of LMW chemical-induced sensitization and elicitation in both animal models and humans.

With recent expansions of immunology knowledge, specifically relating to novel mediators of allergic disease, the study of chemical-induced allergy has many new, exciting potential areas of

study. Some novel mediators include ILCs, miRNAs, and additional Th subsets. Currently, the general understanding of the mechanisms of chemical sensitization are sometimes defined by the Gell and Coombs classification; typically, dermal sensitizers are considered Type IV inducers and respiratory sensitizers are considered Type I inducers. This may be appropriate for certain sensitizers; however, many LMW chemicals induce responses that do not fit within these categories. TDI is an example of this conundrum, as previously described. This work identifies several novel immunoregulatory elements as being potentially involved in TDI sensitization. The suppressive role of T_{regs} is demonstrated both *ex vivo* and *in vivo*, the increased presence of Th17 cells is documented, and a potential immunomodulatory role for miR-210 is identified in this work. This data reveals the involvement of several T cell subsets along with epigenetic mechanisms that have been understudied in the context of chemical sensitization and allergy. Additionally, our lab has investigated the phenotype of ILCs during TDI sensitization, revealing increased activation of dermal ILC2s at 1-9 days post single TDI exposure (data not shown). While the role of these cells remains to be elucidated during the elicitation phase of allergy, the identification of these mediators during the sensitization response provides valuable mechanistic insight into the key events that take place prior to the establishment of the allergic threshold. Many additional innate and adaptive immune modulators need to be studied in models of chemical allergy including T cell subsets (e.g. Th9, Th22, and T_{FH} cells), ILCs, and epigenetic factors. Pertinent investigations will result in the expansion of current knowledge regarding immune mechanisms responsible for chemical allergy and may ultimately aid in the development of preventative and therapeutic strategies to combat disease caused by these agents.

With the increasing immunologic perspective into the mechanisms of chemical sensitization, T cell analysis paired with the LLNA seems to be a promising direction of future study for the

development of hazard ID strategies to differentiate respiratory and contact sensitizers. Because the Gell and Coomb's classification does not take into account the mixed responses that are observed during both types of sensitization, it is important to define common effector mechanisms and biomarkers of sensitization in order to utilize methods like T cell phenotyping and cytokine profiling along with the LLNA. Although much remains to be investigated regarding these responses, the growing body of experimental data is adding to our knowledge of the mechanisms underlying chemical sensitization and this can only enhance efforts to develop predictive assays for chemical sensitizers.

5.2 TDI Sensitization

TDI sensitization may occur via the skin or respiratory tract and is due to the coordinated action of innate and adaptive immunological cells and mediators (7), some of which have, presumably, not yet been identified nor investigated. TDI sensitization may be viewed as a systemic event, illustrated by the ability of dermal sensitization to lead to asthma following a single respiratory exposure post sensitization. According to the most recent compilation of experimental data, the TDI sensitization response is initiated by penetration of TDI through the stratum corneum and other dermal structures (e.g. hair follicles and sebaceous glands) followed by the haptenation of self-proteins with TDI molecules. This event emphasizes the reactivity of the isocyanate groups on TDI molecules and serves as the mechanism responsible for the formation of a TDI-protein complex that can be recognized by APCs, initiating the adaptive immune response. The unique formation of hapten complexes may be a critical determinant of the downstream immunological mechanisms involved in LMW chemical allergy; however, further investigation is needed regarding this subject. APC activation and mobilization are important events modulated primarily by local cytokine and

chemokine expression induced by danger signals and skin irritation. In mice, three primary dendritic cell types are purported to be involved in chemical sensitization, including Langerhans cells (Langerin⁺CD103⁺), and two additional subsets of dermal dendritic cells (Langerin⁻CD103⁻ and Langerin⁺CD103⁻). Following APC-antigen uptake and lymphatic migration, APCs are thought to present fragments of the TDI-protein conjugates to T cells via MHC-mediated antigen presentation, although this mechanism has not been fully confirmed in models of TDI sensitization. This action results in the proliferation of T cells into a number of effector subsets including CD4⁺ Th1, Th2, Th17, T_{reg}, and cytotoxic T cells. Data from our model of dermal TDI sensitization shows increased Th1, Th2, Th17, and T_{reg} cell numbers in the dLN following exposure (Figures 3.2, 3.4 and A3.4). A mixed Th1/Th2 response (based on T cells and associated cytokines) is observed in both animal models and humans exposed to diisocyanates like TDI (8). Additionally, the presence and importance of CD8⁺ T cells has been established in murine models of TDI sensitization (9). These activated T cells can migrate through the lymphatics, blood, and local tissues; orchestrating an adaptive immune response specific to the TDI-protein antigen. Activated T cells and the allergic microenvironment may serve as mediators of B cell activation. Although the role of IgE is contested in a portion of TDI asthma, IgG is thought to play a role in the development and pathogenesis of this condition (10). Both Th1 and Th2 cells are important mediators of antibody class switching due to cytokine production. Another T cell subset intimately involved in germinal center B cell maturation is the T_{FH} cell which remains uninvestigated in chemical allergy but may be heavily involved in the pathogenesis of this process. Collectively, these processes allow the immune system to develop an “arsenal” of TDI-specific cells; subsequent exposures may further augment this process and will eventually lead to the elicitation phase, which is typically represented by asthma. The immunologic progression of TDI sensitization is illustrated in Figure 5.1. The involvement of several components

of the response have not been fully proven such as T_{FH} cells and ILCs; however, existing data from models of other types of allergy indicate that these cell types may play an important role in the TDI sensitization response.

Figure 5.1 Overview of the Immune Mechanisms of TDI Sensitization

Upon initiation of the elicitation phase, allergic symptoms begin to appear. This response is thought to involve the TDI-protein complex and immune cell recognition (APCs, B cells, and T cells), potentially causing crosslinking of the TDI-protein antigen with antibody, leading to mast cell degranulation, the production of additional antigen-specific allergic antibody, the proliferation of antigen-specific T cells, and the involvement of memory T cells. These processes likely contribute to the manifestation of diverse symptoms observed in human TDI asthmatics such as airway inflammation, bronchial thickening, epithelial alterations, hyperreactivity, lymphocyte, neutrophil, and eosinophil infiltration and excessive mucus secretion (11-13). Following sensitization and a latency period which may last for months to years following initial TDI exposure, asthmatic

symptoms usually begin to appear. Chest tightness, cough, shortness of breath, and wheezing are frequently described anywhere from <1 to 4 hours following TDI exposure (13).

Unfortunately, TDI sensitization is a difficult response to fully characterize due to the difficulty of performing appropriate human studies and variation observed between human and mouse models. Accordingly, the clinical pathogenesis of TDI-induced asthma is not fully understood, beyond observational data collected from patients with this condition. The contested role of IgE in TDI sensitization and asthma is a poignant example of the lack of understanding of basic mechanisms involved in these conditions. The consistent appearance of IgE in murine models of TDI allergy is contrasted by the inconsistent appearance of this antibody (both total and specific) in human patients. The fact that IgE is associated with other chemical allergens and the pathogenesis of allergic asthma caused by these agents further complicates this question. When present, TDI-specific IgE serves as a predictive tool but this antibody is not detected in many TDI asthmatics who also frequently exhibit normal total IgE levels (14). It is possible that IgE-independent mechanisms of sensitization and asthma such as those mediated by Th1, Th17, and IgG are integral in the pathogenesis of TDI allergy. Regarding the IgE conundrum along with general mechanisms of disease, it is evident that additional understanding of the immunologic mechanisms of sensitization and elicitation will better enable the development of appropriate hazard identification strategies and therapeutic targets relevant to TDI sensitization and asthma.

5.3 The role of T_{regs} in TDI sensitization

Among the numerous regulatory strategies employed by the immune system, T_{regs} are one of the most functionally significant. These cells act as both general and specific immunoregulators; they are instrumental in the maintenance of tolerance and in the dampening of immune responses.

The rocky history of T_{regs} began in the 1970s when Gershon and Kondo identified suppressor T cells which were capable of dampening immune responses (15). In the 1980s, the study of suppressor T cells was shaken by scrutiny based on observations that the MHC gene did not encode the I-J region which was purported to be associated with the function of these cells (15). This observation, paired with difficulties in the identification of reliable markers for suppressor T cells and uncertainty regarding specific mechanisms of suppression resulted in the decline of suppressor T cell study (15). The study of nT_{regs} that arise naturally in the thymus, rather than experimentally-induced cells (15) aided in the “rebirth” of the suppressor T cell, now known as the T_{reg} . The identification of Foxp3 as the “master transcription factor” responsible for the differentiation and function of T_{regs} was prompted by the investigation of the genetic mechanisms responsible for the symptoms seen in patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and in scurfy mice which are mediated by loss-of-function mutations in *foxp3* (16, 17).

T_{regs} can be derived from the thymus (nT_{reg}) or induced in the periphery (iT_{regs}) (18), acquiring suppressive capabilities that are mediated through a diverse repertoire of mechanisms. T_{regs} aid in the mediation of self-reactive or overzealous immunity utilizing a variety of mechanisms. T_{regs} have a known role in a plethora of diseases and conditions mediated by the immune system including allergy, asthma, autoimmune disorders, cancer, and infections (19). Regarding allergic disease, impaired nT_{reg} inhibition of allergen-specific Th2 responses during hay fever responses, decreased levels of T_{regs} in patients with allergic disease compared to healthy individuals, and a protective role for T_{regs} in grass, milk, and nickel allergy have been demonstrated (19), emphasizing the functional importance of these cells in the control of allergic responses. Additionally, depletion of T_{regs} in a model of OVA atopic dermatitis lead to augmented lung responses (characterized by increased pathology, inflammation, IL-4, eosinophil, and $CD4^+$ T cell levels) following intranasal

OVA challenge in a murine model (20). The corresponding study found that T_{regs} are important in the regulation of both skin inflammation and allergen-specific immune responses, both of which are involved in TDI sensitization as well. Several studies have explored the potential action of IL-10 secretion by T_{regs} in a nickel allergy model and the presence of T_{regs} in murine models of urushiol 3-pentadecylcatechol and TNCB sensitization (7). Generally, T_{regs} are able to regulate immune response and their numbers and/or function appear to be impaired in various disease states.

Chapter 3 described several experiments demonstrating the role of T_{regs} in TDI sensitization. This subset expands in the dLN (Figure 3.2) and ears (Figure 5.4), was phenotypically diverse, and likely contained a number of functionally significant subsets. The expansion of the general T_{reg} population in the dLN (Figure 3.2) was significant in number following both 0.5 and 4% TDI exposure; however, increases in frequency were only observed for the 4%-exposed mice (Figure 3.2; significant at days 4-9). This may be indicative of T_{reg} migration and proliferation in response to the strongest sensitizing concentration and may also be influenced by the irritant nature of this dose, as cytokine and chemokine signaling would be augmented in response to both of these factors. This hypothesis is based on studies demonstrating IL-4 and IL-13-dependent T_{reg} differentiation (21), which is likely occurring in response to the Th2 component of TDI sensitization.

Thymic nT_{regs} are typically $CD25^{\text{hi}}CD62L^{+}CCR7^{+}$ and migrate to secondary lymphoid tissues such as lymph nodes following the completion of their development (22). Upon egress, a subset of these T_{reg} emigrants acquire high CD44 expression; this confers non-lymphoid tissue access to these cells (22). These $CD44^{\text{hi}} T_{\text{regs}}$ may be attributed to recognition of self-antigens in secondary lymphoid tissues (22). Their phenotypic divergence represents the innate diversity in the T_{reg} repertoire. It may also shed light upon their functional potential. High CD44 expression allows T_{regs}

to proliferate more quickly than their counterparts and their presence in non-lymphoid environments may allow them to incorporate diverse suppressive strategies.

Peripherally-induced iT_{regs} typically exhibit a more diverse phenotypic profile due to their origin. iT_{regs} may be generated during an active immune response that is skewed towards a certain Th phenotype or that involves diverse antigen presenting cells, influencing T_{reg} development. iT_{regs} are thought to be less stable than nT_{regs} , further emphasizing their apparent plasticity and response-specific nature. Both nT_{regs} and iT_{regs} were found in the dLN of mice during TDI sensitization, with the frequency of nT_{regs} actually decreasing at 7 and 9 days post 0.5 and 4% TDI exposure (Figure 3.2a). This may be a reflection of the reduced trafficking of these cells to the lymphatics at later time points post exposure or may simply be that these cells are “overshadowed” by the expanding iT_{reg} population, as the number of nT_{regs} is still significantly increased compared to the acetone control at these time points. The presence and expansion of both nT_{regs} and iT_{regs} is reflective of the systemic nature of TDI sensitization as well as the migratory and proliferative potential of T_{regs} in response to an antigenic challenge.

Recent data suggests the development of specialized T_{reg} phenotypes which allow these cells to respond to individual Th subsets (18, 22). In order to migrate to areas of Th action, T_{regs} have been shown to express transcription factors such as *tbet* (Th1), interferon regulatory factor-4 (Th17, Th2, and T_{FH}), *stat3* (Th17), and *gata3* (Th2) while retaining immunosuppressive functions such as the secretion of IL-10 (18). Steady-state T_{regs} have also been shown to dynamically express *tbet* and *gata3* in order to maintain homeostasis (23). This phenotypic diversity and plasticity is an important concept relevant to all types of T cells. A phenotypically varied T_{reg} population was observed in both tissues during TDI sensitization and this would be expected in terms of Th-specific transcription factor expression due to the assortment of Th subtypes involved in TDI sensitization. The additional

phenotyping of T_{regs} involved in TDI sensitization (in both the dLN and ears) may reveal diverse transcription factor expression; therefore, influencing T_{reg} -Th dynamics and the suppressive capacity of these cells in relation to the diverse Th response involved in TDI sensitization.

5.3.1. T_{regs} Suppressive Strategies

Investigations into the suppressive mechanisms of T_{reg} function during TDI sensitization are warranted based on the data in Chapter 3. These mechanisms generally raise the T cell activation threshold and/or inhibit dendritic cell function, preventing the priming of self-reactive T cells and aberrant immune responses. Cells that may be directly impacted by the suppressive actions of T_{regs} include B cells, $CD4^{+}$ and $CD8^{+}$ T cells, NK cells, and NKT cells (24). Not only can T_{regs} suppress the activation and expansion of naïve T cells but they can inhibit previously activated T cells (including memory and effector T cells) (25). T_{regs} may utilize a variety of mechanisms including secretion of immunosuppressive cytokines (IL-10, IL-35, and TGF- β), CTLA4 expression (via CD80/86 downregulation on dendritic cells), indolamine 2,3-dioxygenase (IDO) expression, IL-2 consumption, and perforin-dependent cytotoxicity of dendritic cells (Summarized in Figure 5.2). IL-10 secretion and CTLA-4 expression have been proven as imperative for T_{reg} function in T_{reg} -specific selective depletion models exposing spontaneous colitis and augmented immune responses (IL-10 depletion) and systemic multi-organ autoimmunity paired with dysregulated T cell activation profiles (CTLA-4 depletion) (22). Cell-to-cell suppression has also been documented between T_{regs} and dendritic cells; in some cases this may be dependent on LFA-1 dependent formation of T_{reg} -immature dendritic cell aggregates leading to LFA-1 and CTLA4-mediated downregulation of CD80/86 on dendritic cells (26). The type(s) of suppressive mechanism employed by T_{regs} may depend on many factors, including the site, type, and intensity of a reaction. The relationship

between T_{reg} function and localization remains complex and merits further investigation in a variety of environments, including during TDI sensitization.

Figure 5.2 *Mechanisms of T_{reg} Suppression*

T_{reg} phenotyping data implied that T_{regs} may employ a number of functional strategies to suppress the TDI sensitization response, including CTLA4 expression. The increased expression of CTLA4 was evident following 0.5 and 4% TDI exposure at all time points analyzed (Figure 3.2a A and B). This data suggests that CTLA4 expression may be utilized by T_{regs} in order to dampen TDI sensitization responses in a cell-to-cell manner, which should be investigated further.

The expression of CD103, ICOS, and chemokine receptors are also linked to effector functions of T_{regs} , as described in Chapter 3. The expression of both ICOS and CD103 increased among T_{regs} in the skin (Figure 5.4 F and G) and dLN (Table 3.2) during TDI sensitization. More specifically, T_{regs} co-expressing both ICOS and CD103 were identified in the skin and dLN during TDI sensitization (Figure 5.3 A and B). This subset was previously identified as a lymph node-

specific differentiation stage of T_{regs} (27). ICOS expression is likely connected to T_{reg} – migratory dendritic cell interactions (27) and is associated with activated T_{regs} and suppressive activity. As expected based on their lymph node specificity, the increase in frequency of $\text{ICOS}^+\text{CD103}^+T_{\text{regs}}$ was more pronounced in the dLN than in the ears (Figure 5.3 A and B). Interestingly, $\text{ICOS}^+\text{CD103}^+T_{\text{regs}}$ also slightly increased in the ear during TDI sensitization, possibly indicative of a migrating population of cells capable of suppression.

A

B

Figure 5.3 $\text{ICOS}^+\text{CD103}^+T_{\text{reg}}$ Population in the Ear and dLN during TDI sensitization

$\text{ICOS}^+\text{CD103}^+T_{\text{regs}}$ were identified in the ear (A) and dLN (B) of mice exposed to 0, 0.5, or 4% TDI at various points post exposure via flow cytometry. Bars represent mean relative fold change ($\pm$ SE)

of 5 mice per group. *P* values are represented by ^ (0.5% TDI) and * (4% TDI) (*P*<0.05). Dermal treatment groups are indicated by the following symbols: circle- acetone, square- 0.5% TDI, and triangle- 4% TDI.

5.3.2. Skin T_{regs} and Dendritic Cells

The epithelium is a barrier to allergens like TDI; specifically, the skin and its related structures, specifically, play an important role in the development of TDI sensitization. The skin has been implicated in the development of both dermal and respiratory sensitization (via dermal exposure) in a variety of models of allergic disease, encompassing both protein allergens such as OVA (28) and chemical allergens such as TDI (29). Occupational practices may compromise epithelial surfaces, further contributing to the development of allergic disease. The innate immune system houses many cells and surveillance systems in the skin and related tissues, presumably as a “first line defense” against pathogens. These cells include keratinocytes, T cells, dendritic cells, macrophages, monocytes, granulocytes, and innate lymphoid cells (30). Innate factors may also contribute to the development of allergic disease by precipitating, augmenting, and/or initiating the adaptive immune response that is characteristic of disorders like TDI allergy.

Skin T_{regs} were phenotyped (Gating strategy is shown in Appendix 5.1) during TDI sensitization in an effort to profile these cells and gauge their involvement in this response. T_{regs} increase in the skin in both frequency and number following 0.5 and 4% TDI exposure (Figure 5.4 D). These cells may be skin resident T_{regs} or they may be trafficked in from other tissues such as the dLN. T_{regs} are capable of expressing an abundance of chemoattractant and surface adhesion receptors, allowing them to be present in nearly all tissues in the body (22). Clearly, the presence of inflammation enhances T_{reg} recruitment and the specific signaling pathways involved are dictated by

the inflammatory environment present in the impacted tissue. During experimental autoimmune encephalomyelitis (EAE), Th17-mediated inflammation promotes epithelial expression of the chemokine CCL20, which is recognized by CCR6, leading to increased CCR6 expression on T_{regs} and recruitment to epithelial tissue (22). Alpha E integrin, or CD103, is expressed by a high frequency of skin T_{regs} (Figure 5.4 F) due to its importance in the migration and retention of these cells in the skin (22). Conversely, CCR7 is involved in T_{reg} migration to dLN and overall T_{reg} function as loss of this receptor results in loss of the ability of T_{regs} to migrate to the dLN and function in a murine model of colitis (22, 31). T_{reg} migration is highly coordinated by a number of signaling molecules and remains a highly temporal and site-specific process. Compartmentalization and proper localization have been shown to be imperative for T_{reg} function, with each unique subset working in concert in a “division of labor” to maintain tolerance and regulate immune responses (32). In light of this, therapeutic targeting of T_{reg} migration is not fully feasible and such efforts will require additional understanding into this process and the mechanisms involved.

During TDI sensitization ear T_{regs} expand in frequency at 2 and 4 (4%) and 4 and 7 (0.5%) days post exposure (Figure 5.4 D). They also expand in number during days 2-9 following exposure to both concentrations of TDI (Figure 5.4 D). This expansion exhibited similar kinetics to the dLN T_{regs} during this response, with an apparent peak in T_{reg} expression 4 days post TDI exposure. This congruency between tissues was intriguing and may reveal the systemic nature of sensitization and T_{reg} trafficking. As previously mentioned, CCR7 expression is involved with T_{reg} lymph node homing and the expression of this receptor was analyzed on T_{regs} during TDI sensitization (Figure 5.4 E). T_{reg} CCR7 expression was decreased in frequency during 4% TDI sensitization (2-7 days post exposure) but due to the general expansion of T_{regs} CCR7⁺ cells increased in number during 0.5 and 4% TDI sensitization. dLN-derived T_{regs} may be losing expression of their lymph node

migratory markers in order to move to the ear, the site of chemical application and of a significant inflammatory response in the 4% TDI-exposed mice. This hypothesis is supported by decreased expression of CD103, an epithelial T_{reg} marker, in skin T_{regs} during 0.5% (4 days post exposure) and 4% (1-9 days post exposure) TDI sensitization (Figure 5.4 F). $ICOS^{+}T_{regs}$ significantly increased in frequency and number during 0.5% and 4% TDI sensitization (Figure 5.4 G; 0.5%: 1-9 days post exposure and 4%: 2-9 days post exposure). This population accounted for more than 70% of skin T_{regs} in acetone-treated control mice and nearly 100% of skin T_{regs} in TDI-exposed mice, revealing the dermal T_{reg} population is probably capable of being functionally active and is at a high activation status as ICOS is a costimulatory molecule important to T_{reg} function (33). These data reveal an expansion of dermal T_{regs} at the site of TDI application during sensitization that is potentially functionally active and phenotypically diverse.

In line with the general migratory capabilities of lymphocytes (34), the T_{regs} identified in both the ear and dLN during TDI sensitization are potentially migratory, allowing for the regulation of the systemic sensitization response that develops in response to TDI. Comparison of the dLN and skin T_{reg} subsets lends important insight into the potential migration and action of T_{regs} during TDI sensitization. While the general kinetics of T_{reg} frequency appeared to be similar between dLN (Figure 3.2 B) and skin (Figure 5.4 D) T_{regs} , the overall percentages are very different for each tissue. Approximately 5-10% of $CD4^{+}$ T cells in the dLN are T_{regs} and approximately 10-30% of $CD4^{+}$ T cells in the ear are T_{regs} . This may be a reflection of the greater percentage of naïve and/or effector T cells in the lymph node as a secondary lymphoid organ. T_{reg} numbers varied greatly between the dLN (Figure 3.2 C) and skin (Figure 5.4 D), revealing up to approximately 1.6 million T_{regs} in the dLN and 50,000 T_{regs} in the skin of TDI-treated mice. This is likely a reflection of the status of the lymph node as a secondary lymphoid organ and a major site of lymphocyte retention. The decreased

expression of CCR7 following 4% TDI exposure in the ear T_{regs} (Figure 5.4 E) indicated that dLN T_{regs} were migrating to the ears. This was supported by increased dLN CCR6⁺ T_{reg} frequency during 0.5% (2-9 days post exposure) and 4% (4-9 days post exposure) TDI sensitization (Table 3.2). CCR6 is a lymphocyte chemotactic factor that causes T cell migration to inflammatory tissues (35) and its upregulation on dLN T_{regs} during TDI sensitization suggests that T_{regs} are moving from the dLN to the ear during TDI sensitization. CD103 was expressed on approximately 80% of acetone-treated T_{regs} in the ear (Figure 5.4 F) compared with approximately 20% of their counterparts in the skin (Table 3.2). This is in accordance with CD103's skin homing role in T_{regs} described above. CD103⁺ T_{regs} increased in frequency in dLN T_{regs} during 0.5% (2-9 days post exposure) and 4% (4-9 days post exposure) TDI sensitization (Table 3.2), while the frequency of this subset decreases in the ear following 0.5% (4 days post exposure) and 4% (1-9 days post exposure) TDI exposure (Figure 5.4 F). This increase in CD103⁺ T_{reg} frequency in the dLN may indicate T_{reg} migration to the skin as observed with CCR6 expression. Decreases in CD103⁺ T_{reg} frequency in the skin may be attributable to lymph node migration from the ear, particularly at later time points such as 4 days post exposure, where this population seems to be the most well represented during 4% TDI sensitization. Lastly, the ICOS⁺ T_{reg} subsets increased in both the dLN (Table 3.2; 2-9 days post exposure) and ear (Figure 5.4 G; 0.5%: 1-9 days post exposure and 4%: 2-9 days post exposure), suggesting an activated state in these T_{regs} . Overall, it appeared that more ear T_{regs} expressed ICOS (in both the basal and TDI-treated states) than dLN T_{regs} in terms of frequency. All ear T_{reg} subsets increased in number during TDI sensitization (Figure 5.4 E-G) with similar kinetics, indicating that regardless of percentage alterations, increased T_{regs} expressing each marker analyzed expanded in the skin due to the overall increase in T_{reg} numbers.

A

B

C

D

E

F

G

Figure 5.4 Ear T_{reg} Phenotyping

Flow cytometric analysis of dermal T_{regs} following dermal TDI sensitization. T_{regs} were first gated on their expression of CD45 (A), CD3 (B), and CD4 (C) then were further identified by CD25 and Foxp3 expression (T_{regs}) at indicated time points. T_{reg} frequency and number (D) were determined based on flow cytometry analysis and extrapolation of this data with total ear cellularity. T_{regs} were also analyzed for CCR7 (E), CD103 (F), and ICOS (G) expression. Graph symbols represent mean ($\pm$ SE) of 5 mice per group. *P* values are represented by ^ (0.5% TDI) and * (4% TDI) (*P*<0.05). Dermal treatment groups are indicated by the following symbols: circle- acetone, square- 0.5% TDI, and blue triangle- 4% TDI.

The T_{reg} phenotyping carried out in a murine model of TDI sensitization did not specifically characterize effector memory T_{regs}. This is primarily due to the fact that this model utilizes a single exposure and may not induce high numbers of effector memory T_{regs} by the assayed time points; however, this subset is likely important in the elicitation phase of allergic disease and upon repeated allergen exposures. While several of the migratory-related receptors may be expressed on these cells, complete phenotyping of effector memory T_{regs} would require the identification of CD127 (IL-7R), CD44, CD27, and CCR6 expression in mice (36). Skin-residing memory T_{regs} appear to express

lower levels of CD25 and higher levels of CD127 compared to T_{regs} in the dLN and IL-2^{-/-} and IL-7R α -blocking antibody studies indicate that murine skin memory T_{regs} require IL-7 but not IL-2 for their survival in this tissue (36). The IL-7 requirement was not evidenced with respect to human memory T_{regs} in the skin (37). Murine and human T_{regs} in the skin localize to hair follicles, in close proximity to the follicular epithelium while conventional T cells did not appear to preferentially localize to the hair follicle (37). Interestingly, in our epicutaneous murine TDI sensitization model, TDI-hapten complexes localize to several sites in the skin, including the hair follicles; these TDI-hapten complexes were shown to co-localize with CD11b⁺, Langerin⁺, and CD103⁺ cells in the hair follicles, presumably demonstrating APC-hapten interaction (38). The presence of T_{regs} at sites like the hair follicles may be a protective mechanism due to the accessibility of these structures by allergens. In order to compare the antigen-specificity of human skin memory T_{regs} and memory T cells deep sequencing of the TCR β chain was performed in each of these subsets and this analysis revealed that these two cell types likely recognize dissimilar antigens (37). Another unique attribute of human skin memory T_{regs} is that they are largely nonmotile, compared to memory conventional T cells which tend to express CCR7, a lymphatic homing marker (37). In a murine model of DNFB contact allergy, memory effector T_{regs} were found to be capable of circulating from the blood and skin, returning to the skin upon antigen exposure (39). This data suggests that memory effector T_{regs} are likely present in both the skin and in the circulation in our model of TDI sensitization, migrating throughout the body but able to respond to TDI exposure both locally and distally.

dLN and ear T_{reg} comparisons reveal potential insights into the function of these cells. Presumably, each tissue holds a diverse array of T_{regs} whose origins and functions are as unique as their home. Dendritic cell interactions play an important role in the differentiation and stimulation of T_{regs}, providing direct interactions and influencing the local cytokine and chemokine milieu. The

dendritic cell populations of the lymph nodes and skin are innately diverse yet contain many shared populations due to the normal migration of immune cells between barrier surfaces and dLNs. Typically, the murine lymph node dendritic cell population is made up of several main subsets including $CD4^+CD8^-DCE-205^-$, $CD4^-CD8^-DCE-205^-$, and $CD4^-CD8^+DCE-205^+$ dendritic cells along with skin-derived dendritic cells which may originate as dermal dendritic cells or Langerhans cells (40). Langerhans cells are the only dendritic cell subset present in the epidermis at a basal level and the dermis contains several dermal dendritic cell subtypes (41). Homeostatic migration of skin-derived dendritic cells is considered normal even in the absence of an active immune response (40). Additionally, skin T_{regs} may actively migrate to the lymph nodes and back, participating the maintenance of tolerance and in the regulation of immune responses such as TDI sensitization. The ability of immune cells to access the lymphatic network from barrier sites such as the skin is imperative in normal functioning of the immune system and is also a major contributor to the development of systemic sensitization. Dendritic cells are often regarded as the “bridge” between innate and adaptive immunity due to their antigen presentation abilities. Although they are involved in the activation of reactive Th cells, dendritic cells play a crucial role in T_{reg} induction. IL-10, IL-27, and TGF- β production and IOCSL signaling by dendritic cells promotes Tr1 differentiation and thymic dendritic cells produce thymic stromal lymphopoietin (TSLP) to promote nT_{reg} differentiation, generally from self-reactive T cells (42). iT_{reg} differentiation can be influenced dendritic cell-derived IDO and tryptophan depletion metabolites 3-hydroxyanthranilate and kynurenine, dendritic cell TGF- β , retinoic acid, and RANKL signaling (42).

The role of dendritic cells in relation to T_{regs} has been investigated in a murine model of DNTB contact hypersensitivity (41). This work utilized a weak hapten model in an effort to understand the mechanisms driving tolerance to weak haptens (e.g. DNTB) vs. strong haptens (e.g.

DNFB). DNTB cannot activate the inflammasome nor can it induce contact hypersensitivity following a single exposure in mice yet it generates similar self-peptide complexes upon skin contact as those associated with DNFB which behaves as a strong allergen (41). As a weak hapten, DNTB appears to promote tolerance to DNFB sensitization and this effect is thought to be mediated by T_{reg} induction (41). Additionally, Langerhans cells are able to cause anergy and deletion of DNTB-reactive $CD8^+$ T cells and promote the expansion of $ICOS^+ T_{regs}$ in order to suppress allergic contact dermatitis (41). In this model of DNFB allergy, Langerhans cells and dermal dendritic cells were able to present the hapten-protein complex within 24 hours of dermal exposure, emphasizing the quick response time of these cells and their important role in the allergic response (41). The importance of Langerhans cells in the maintenance of tolerance in this “weak hapten” model is relevant to “strong haptens” like TDI; although different dendritic cell populations may be mediating this effect (possibly monocyte-derived skin dendritic cells and/or $CD207^+$ dermal dendritic cells) (41), it is likely a part of the immune response involved in TDI allergy. Based on the importance of both MHC I and II in the induction of dendritic cell-initiated tolerance in the DNTB model (41), in the case of TDI sensitization, it is likely that this mechanism of dendritic cell tolerance establishment is skewed, resulting in the massive expansion of antigen-reactive T cells. The difference between tolerance established following “weak hapten” exposure and the allergic disease established following “strong hapten” exposure may be attributed to variant outcomes following dendritic cell antigen presentation in the lymph node.

In our TDI sensitization model, tolerance may not be established due to skin irritation causing danger signal induction and antigen presentation by dendritic cells resulting in activation of antigen-reactive T cells. Although T_{regs} do expand and some antigen-reactive T cells may be deleted, these events do not appear to be strong enough to overcome the sensitization response that has begun. The

local cytokine environment is a very important factor involved in the maintenance of tolerance or the induction of an allergic response. IL-35 has been shown to impact tolerance by promoting anti-inflammatory pathways; this cytokine acts as a regulatory signaling molecule in a murine model of OVA-induced allergic airway inflammation (43). IL-35 exerts these effects by a number of mechanisms, including the suppression of inflammatory dendritic cells at the site of inflammation as well as the dLNs (43). Another anti-inflammatory cytokine, IL-10, is known to downregulate pro-inflammatory cytokine (IL-1 β and IL-6) production if produced early in the innate immune response (44). IL-10 may be produced by T_{regs}, macrophages, dendritic cells, neutrophils, and keratinocytes (20) and may play a role in regulating the TDI sensitization response as early as the initiation of dendritic cell function. A study by Cumberbatch, et al. revealed dichotomous immune responses in the skin of mice exposed to contact (DNCB) and respiratory (TMA) allergens. Langerhans cell migration to the dLN was delayed in response to TMA compared to DNCB which was attributed to differential epidermal cytokine profiles, in particular altered IL-10 expression leading to reduced IL-1 β levels (45). This data reveals the importance of the cytokine environment in the development of allergic disease, as well as the potential importance of IL-10 in the development of Th2 sensitization. The increased inflammatory response observed in our murine TDI sensitization model (Figure 3.1) may influence the phenotype and functional capabilities of skin dendritic cells, leading to the development of an adaptive response which we know as sensitization. Clearly the local cytokine and chemokine environment can guide the dendritic cell response, which is also a crucial component of the induction of allergic disease. Further investigation into dendritic cell and T_{reg} activity during TDI sensitization may reveal valuable information regarding the mechanisms involved in the orchestration of sensitization and the balance between allergic disease and tolerance, as dendritic cells appear to be key players.

5.3.3. Therapeutic Potential of T_{regs}

The therapeutic potential of T_{regs} is an exciting prospect, yet in many cases this concept remains in its infancy in functional terms. General challenges regarding the development of T_{reg}-based therapeutics include T_{reg} isolation and identification issues, plasticity, high cost, and unintended, “off-target” effects on pathogen clearance or tumor immunity (46). Regardless, several Phase I clinical trials have been completed utilizing T_{regs} therapeutically in the context of graft versus host disease and type 1 diabetes (46). These studies revealed generally safe responses to supplemental T_{reg} therapy with immunosuppression being a major side effect (46). Several Phase I and II clinical trials are currently recruiting and will investigate the use of T_{reg} therapy in kidney and liver transplant, Crohn’s disease, and multiple sclerosis (46). Although a Phase I trial for the use of autologous polyclonal T_{regs} in steroid-resistant asthma is purported to be initiated soon, this has not begun (46). The subcutaneous and intradermal administration of insulin-like growth factor-1 helped to alleviate inflammation (ear swelling) in a murine model of DNFB contact hypersensitivity (47), demonstrating that T_{reg} expansion is helpful in the control of allergic responses in the skin. It seems that much remains to be learned about these cells before they may be used in the effective treatment of allergic disease. In terms of TDI allergy, the role of T_{regs} in TDI elicitation is currently unknown and should be elucidated to fully characterize the importance of these cells in a complete model of TDI allergy, encompassing both sensitization and elicitation. This would allow for a clear understanding of the temporal significance of T_{regs} during all phases of this response, which may have strong implications in regards to clinical uses of these cells.

In models of OVA-induced allergy, T_{regs} were shown to have a protective role in the sensitization but not the elicitation phase of this response (48, 49). The suppressive role of T_{regs} has also been investigated and confirmed in a model of DNFB-induced sensitization and contact

hypersensitivity (50). If T_{regs} play a role in TDI sensitization but not in elicitation, these cells may not have an immediately apparent therapeutic or preventative role. Because sensitization is typically not accompanied by any clinical symptoms or “outward” signs, the interventional significance of T_{regs} at this time in the development of the response may be difficult to discern. In line with the thoughts of Baru, et al. (49) the apparent lack of T_{reg} importance during OVA-induced elicitation does not automatically discredit the idea that T_{regs} may be able to help lessen allergic inflammation and pathology if administered exogenously or induced at this phase of the response. Exogenous T_{reg} administration has been shown to ameliorate tissue damage, remodeling, and pathology in murine models of LPS-induced acute lung injury (51) and OVA-induced allergic airway inflammation (52, 53). Also, adoptive transfer of preactivated OVA-specific T_{regs} into OVA-sensitized mice resulted in a blunted Th2 response in the lung in response to OVA challenge (54). Importantly, T_{reg} supplementation following the establishment of allergic symptoms did not reverse airway hyperreactivity, regardless of the decreased airway inflammation and eosinophilia observed following treatment (52). This data indicate that T_{regs} exhibit therapeutic potential in the regulation of allergic disease, even if administered once symptoms appear. Clearly, further investigation into the mechanisms and temporal influence of T_{regs} during TDI sensitization and elicitation is necessary in order to fully realize the potential utility of these cells. Ideally, therapeutic strategies involving T_{regs} such as vitamin D treatment leading to dexamethasone-induced IL-10 responsiveness (T_{reg} mediated) in glucocorticoid-resistant asthmatics (55) may be effective for TDI asthmatics.

5.4 The role of miRNAs in TDI sensitization

Just as T_{regs} are important in the regulatory milieu of the immune system, miRNAs have exhibited powerful regulatory functions in a number of disease states and conditions. It has been

estimated that approximately 30-92% of human genes are regulated by miRNAs (56). The ability of multiple miRNAs to regulate multiple genes and simultaneously impact target protein translation demonstrates the ubiquitous regulatory power of these tiny structures. These small RNA molecules originate from mRNA that is typically transcribed by RNA polymerase II or III, producing primary miRNA transcripts (56). miRNAs can originate from the activation of independent promoters as polycistronic transcripts from the introns of protein-coding genes (57). Primary RNA transcripts are then processed into pre-miRNA stem loops which contain approximately 60 nucleotides; this is mediated by the RNase III enzyme Drosha and DGCR8 (56). The pre-miRNA stem loops are transported out of the nucleus into the cytoplasm by Exportin-5 and are processed into mature miRNA molecules by the cytoplasmic RNase III ribonuclease Dicer (56). This mature miRNA molecule can then form a complex with RNA-induced silencing complex (RISC) machinery and impact the translation of target gene by sequence-specific targeting of mRNA, typically within the 3' UTR where their binding is the most efficient (57, 58). RISC-miRNAs bind through complementarity that may be partial (at least 6-8 nucleotides) or perfect, leading to repression of the target gene's translation or mRNA degradation via initiation of deadenylation (57). Mature miRNAs can be released from their cells of origin via exosomes, which are cell-membrane derived vesicles, by cellular degradation, or by complexing with proteins such as high-density lipoprotein and Argonaute (57). These extracellular miRNAs may be taken up by recipient cells bearing scavenger receptors (HDL-bound miRNAs) or passively (57).

miRNAs have integral functions in the development of the immune system, as conditional Dicer depletion in hematopoietic stem cells leads to dysfunction in the hematopoietic compartment and this setup in T or B cells causes lymphopenia and reduced functional capacity of these cells (58). The role of these regulatory elements is beginning to be explored in the context of allergic disease.

Several miRNAs have been shown to be differentially expressed in the airways of asthmatic patients and in the lymphocytes of mice in models of allergic asthma (57). miR-155 appears to be a major player in the development of Th2-mediated allergic disease as miR-155 deficient mice exhibit reduced eosinophilic inflammation and reduced Th2 cell numbers in a murine model of OVA-induced asthma (57). The upregulation of this miRNA during TDI sensitization (Figure 4.7 B) suggests that this miRNA may be involved in the development of TDI sensitization, which contains strong Th2 components. The inhibition of several miRNAs (including miR-106a, -126, -145, and -221) in murine models of allergic asthma has resulted in the reduction of pathogenic symptoms (57, 59), suggesting important roles for additional miRNAs in the development of allergic asthma. Alternatively, miR-24 and -27 suppress Th2 differentiation via IL-4 downregulation in T cells, acting as negative regulators of OVA-induced allergic disease (60). miR-21 is thought to target IL-12p35, leading to augmented Th2 responses in both murine and human models (57). miR-155 has also been found to be overexpressed in skin samples from patients with atopic dermatitis and from skin challenged with diphenylcyclopropenone (57). Additional miRNAs have been shown to be differentially expressed in models of allergic skin disease, including atopic dermatitis and allergic contact dermatitis (57). There is likely a complex network of miRNAs involved in the regulation of allergic disease, both augmenting and decreasing the response.

5.4.1. Overview of Selected miRNAs and their Relationship with T_{regs}

Although miR-210 was the focus of the miRNA-based studies included in this work, miR-31 and -155 were also investigated for their potential to induce miR-210 expression and to influence T_{reg} differentiation and function. Interestingly, miR-21, -155, and -301a made up a signature expression profile in stimulated splenic CD4⁺ T cells (both nonspecific and MOG₃₅₋₅₅ peptide-specific) derived from a murine EAE model (61). These three miRNAs were also increased in the

dLN of mice dermally sensitized to TDI (62). Due to obvious differences in treatment and exposures in each of the models, the temporal kinetics of each miRNA were variable; however, for each response, these miRNAs were significantly elevated at time points encompassing immunologically significant events. Collectively, this data emphasizes the importance of multiple miRNAs in immunologic processes and the conserved nature of these miRNAs in similar cell types and responses.

Thymic T_{reg} development is affected by miRNAs as conditional Dicer1 allele deletion at the double negative/double positive transition results in a significant decrease in $Foxp3^{+} nT_{reg}$ frequency in the LN, spleen, and thymus (63). The authors also investigated iT_{regs} in the context of Dicer depletion and upon stimulation and dicer-deficient, TGF- β stimulated $CD4^{+}CD25^{-}$ T cells produced less T_{regs} (13.5%) than Dicer-competent T cells (48.5%). In a recent work by Liston, et al. T_{reg} -specific miRNA depletion via Dicer caused a lethal autoimmune condition analogous to that observed in *foxp3*-deficient mice (64). These data show the importance of miRNAs in the development of T_{regs} . Murine nT_{regs} and T_{cons} exhibit distinct miRNA expression profiles; nT_{regs} exhibited increased expression of miR-21, -22, -27b, and -155 (among others) compared to T_{cons} (63). The human cord blood-derived nT_{reg} miRNA signature is similar with increased miR-21 and decreased miR-31 (65). The same study revealed that in human cord blood-derived non- T_{regs} miR-21 induces *foxp3* expression while in nT_{regs} miR-31 targets *foxp3*, antagonizing T_{reg} development. The impact of miRNAs on T_{reg} development is clearly significant, and may occur through positive or negative regulation. In light of their potential roles in T_{reg} differentiation and/or function, miR-210, -31, and -155 will be discussed in the context of current knowledge from the literature as well as experimental data presented in this work.

miRNA 210

miR-210 is upregulated in breast cancer, pancreatic tumors, and head and neck cancer (66). Functionally, this miRNA is known to be cytoprotective, increase angiogenesis, and inhibit apoptosis in hypoxic conditions, precluding its moniker, the “master hypoxamir” (66). miR-210 has also been associated with breast cancer tumor proliferation (67), identified as a potential human serum biomarker for pancreatic cancer (68), and has been suggested as a therapy for the treatment of ischemic heart disease (69). The relationship between miR-210 and T_{regs} is not well studied; however, most of the investigations into this concept have been carried out in humans. In a study involving patients with psoriasis vulgaris, miR-210 levels were increased in peripheral blood mononuclear cells (PBMCs) from patients with psoriasis (66). Foxp3 was identified as a miR-210 target and this interaction was correlated with downregulated IL-10 and TGF- β secretion by T_{regs}. This study suggested that miR-210 negatively regulates foxp3, leading to immune dysfunction. In T_{regs} isolated from healthy adult donor blood miR-210 was found to potentially target the 3' UTR of *foxp3* and *ctla4* (70). Additionally, foxp3 has been predicted to target *runx3* (62), a transcription factor that acts upstream of foxp3 and is imperative for iT_{reg} activation (71, 72). Our investigations showed increased dLN and T_{reg}-specific miR-210 expression and altered runx3 expression during TDI sensitization. Collectively, this data suggests that miR-210 may be targeting T_{regs} during TDI sensitization.

miR-210 may be induced by HIF-1 α and may also target this gene (73). Due to the hypoxic conditions in lymphoid tissues such as the lymph nodes and inflammatory sites such as TDI-exposed skin, it is logical to assume HIF-1 α is inducing the expression of miR-210 during TDI sensitization. HIF-1 α expression increases in allergic murine and human airways (74). HIF-1 α protein levels in ear lysates appear to increase during TDI sensitization (Figure 5.5 A), suggesting that this signaling

factor is increasing miR-210 expression in the ear. dLN levels of HIF-1 α should be investigated as primary miR-210 expression was noted in this tissue, indicating that miR-210 was being produced locally. Interestingly, the increased expression of miR-210 at non-irritant TDI concentrations in both the dLN (Figure 4.1) and ear (A4.1) suggests an alternative induction pathway for this miRNA.

In hypoxic conditions, HIF-1 α can induce *foxp3* expression by binding to the hypoxia response elements found within the *foxp3* promotor (75). Dissimilarly, an earlier study reported the negative effect of HIF-1 α on T_{reg} development via targeting *foxp3* for proteasomal degradation (76). This was elegantly refuted by studies utilizing *foxp3*-GFP transcriptional reporter mice which showed that the frequency of *foxp3* mRNA-expressing cells (GFP⁺) was tightly correlated with the frequency of *foxp3* protein-expressing cells in normoxic and hypoxic conditions (75). HIF-1 α has also been reported to affect Th17 cells by inducing transcription of *roryt*, promoting the differentiation of this subtype (73), emphasizing the global and diverse effects of this signaling molecule on T cells. If HIF-1 α is inducing the expression of miR-210 during TDI sensitization, it is possible that this is a regulatory mechanism to prevent the over proliferation of T_{regs} (and maybe Th17 cells as well) in hypoxic conditions, assuming miR-210's purported action on T_{regs} is valid.

miR-210 has been investigated in the context of *hif1 α* expression and T cell differentiation both at steady state conditions and in a model of experimental colitis (73). These studies revealed increased miR-210 levels in activated T cells and that HIF-1 α is both a target and important inducer of miR-210 (73). Additionally, miR-210 suppressed Th17 differentiation in specific oxygenation conditions and was able to reduce the severity of colitis, presumably due to targeting *hif1 α* (73). Although this data does not directly address T_{regs} in relation to miR-210, the authors show increased miR-210 levels in this subset following *in vitro* polarization of naïve T cells (73). Clearly regulatory elements like HIF-1 α and miR-210 are tightly regulated both temporally and in terms of the specific

immune response. While miR-210 may be suppressing Th17 differentiation during TDI sensitization this remains to be investigated. Overall, this data exposes the dynamic nature of miRNAs and their targets which may be variable among unique immune responses.

A

Figure 5.5 *HIF-1α* Protein Levels in Ear Lysates During TDI Sensitization

HIF-1α levels were quantified in lysates from ear cells collected at various time points during TDI sensitization utilizing a DNA-binding ELISA (A). Bars represent mean ($\pm$ SE) of 4 mice per group. *P* values are represented by * ($P < 0.05$).

dLN and CD4 expression of primary miR-210 during TDI sensitization (Figure 4.1 B) indicates that this tissue and cell type are capable of producing miR-210 at the transcriptional level. Additionally, mature miR-210 levels are detected (and increase) in T_{regs} following *ex vivo* T_{reg} polarization (73). This data suggests that miR-210 is expressed by CD4⁺ T cells in the dLN during TDI sensitization. Interestingly, although they were detectable, primary miR-210 levels significantly decreased in the CD4⁺ subset compared to the equivalent cells in acetone-exposed mice 4 days post

4% TDI exposure (Figure 4.1 B). This data may suggest that CD4⁺ T cells are not the primary producers of miR-210 in the dLN, as mature miR-210 levels significantly increase at this time point in the dLN (Figure 4.1 A). Because 4 days post TDI exposure appears to be the peak of mature miR-210 expression, decreasing primary miR-210 levels at this time may indicate the beginning of transcriptional downregulation of miR-210, which could be reflected by relative decreases in mature miR-210 expression at later time points (Figure 4.1 A). Another possibility is that miR-210 could be shuttled into the dLN via exosomes or other trafficking machinery which would result in increased mature miR-210 but not necessarily increased primary miR-210 levels. Regardless of the method or site of induction, it is clear that mature miR-210 is likely responsible for a regulatory functions during TDI sensitization as illustrated in Figure 5.6.

* Putative or confirmed **miR-210** target or key player influenced by miR-210

Figure 5.6 Potential sites of miR-210 action in T_{reg} Biology

*Indicates putative or confirmed targets of miR-210. This may be occurring through direct or indirect means.

miRNA 31

Due to the reported miR-31 binding site on foxp3 mRNA, this miRNA has been investigated for its potential role in the development and/or function of T_{regs}. In a murine model of EAE, increased miR-31 expression was observed in pathogenic CD4⁺ T cells along with stimulated CD4⁺ T cells from naïve mice, implicating TCR signaling as an inducer of this miRNA (77). In an effort to examine miR-31 expression in EAE-relevant T cell lineages, naïve T cells were polarized to Th1,

Th17, and T_{reg} subsets *in vitro* and each population's miR-31 expression was measured. miR-31 levels increased in Th1 and Th17 cells but decreased in T_{regs} when compared to naïve T cells; the authors also discovered that foxp3 was significantly recruited to the putative miR-31 promoter (via chromatin immunoprecipitation assay), suggesting that foxp3 may bind to the miR-31 promoter and downregulate its expression when T_{regs} are present and/or expanding. Gprc5a, or retinoic acid-inducible protein 3, was identified as a target of miR-31, its knockdown leading to iT_{reg} downregulation and increased EAE severity. These data suggest that miR-31 is a negative regulator of iT_{reg} generation via downregulation of Gprc5a expression.

miRNA 155

miR-155 is a very well-investigated molecule that was one of the initial miRNAs functionally identified in T cells and associated with TCR stimulation and inflammation (61, 78). miR-155 can be induced by a number of innate immune signaling elements, including LPS, Poly (I:C), interferons, TNF- α , CpG, and PMA (78). Autoimmune symptoms have been observed in miR-155-deficient mice and this has been attributed to a Th2 imbalance (61). miR-155 deficient mice exhibit dysfunctional Th1 and Th17 differentiation and are highly resistant to EAE (57, 79). The pro-inflammatory and pro-allergic role of miR-155 has been linked to its action in macrophages, where it targets IL-13RA, SOCS1, and SHIP1; upon reduction of miR-155 levels, macrophages assume a M2 phenotype, suggesting that miR-155 promotes the inflammatory M1 phenotype (57). miR-155 has also been linked to dendritic cell Th2 priming, as miR-155-deficient dendritic cells exhibit limited Th2 priming function *in vitro* and failed to induce airway inflammation following intratracheal transfer in a murine model of ovalbumin-induced allergic airway disease (80). These effects were shown to be mediated by miR-155 targeting of ATP-induced dendritic cell migration and activation (80). miR-155 has also been implicated as a player in IgG class switching, germinal

center B cell expansion, EAE pathogenesis, and dendritic cell function (56, 61), emphasizing the global importance and complexity of this miRNA. MiR-155 expression increases in response to various inflammatory stimuli, potentially explaining its early increases in response to irritant concentrations of TDI (Figure 4.6 B). These increases are typically attributed to TLR activation or inflammatory signaling mediated by cytokines such as tumor necrosis factors, interferons, and NF- κ B (58).

The intron encoding miR-155 (precursor mRNA, Bic) can be bound by Foxp3 (81, 82), showing that miRNA expression can also be regulated by T_{reg}-associated genes. miR-155 expression was found to increase in T_{regs} and this was dependent on sustained foxp3 expression (81). The authors also found that miR-155 had a positive effect on the numbers of T_{regs} and the proliferative potential of these cells; these effects were attributed to attenuated IL-2 signaling primarily due to elevated SOCS1 protein expression in miR-155-deficient T_{regs} as miR-155 was verified as a target of *socs1* (83). miR-155 was shown to target *socs1*, a negative regulator of IL-2R/STAT5 signaling, enhancing foxp3 expression (84). This study demonstrated the critical role of miR-155 in T_{reg} homeostasis in competitive IL-2 scenarios (but not in lymphopenic situations where IL-2 is dispensable) along with the crucial activity of foxp3 in the maintenance of miR-155 expression. Additionally, miR-155 deficient mice exhibit reduced thymic and peripheral T_{reg} numbers (84). Generally, it seems that miR-155 affects the proliferation and differentiation of T_{regs} but not the suppressive capability nor specific markers expressed by these cells, although miR-155 was shown to be a direct target of *ctla4*, which would affect T_{reg} function (84). miR-155 can also suppress c-Maf, leading to impaired IL-4 and Th2 responses and enhanced Foxp3 and T_{reg} responses (84). Overall, miR-155 positively regulates *tbet*, *foxp3*, and *ror γ t* while it negatively regulates *gata3*, further influencing T cell lineage specificity (83).

miR-31, -155, and -210 are all valid experimental focuses in future studies evaluating TDI sensitization. Their unique expression and functions hold much potential in terms of elucidating the underlying mechanisms of allergic disease as well as the identification of biomarkers for these conditions. Before confirming the functional roles of these miRNAs, the functional availability of these structures should be confirmed, either by Luciferase binding assays to evaluate the ability of these miRNAs to bind targets or by Argonaute immunoprecipitation assays which identify mature miRNAs, the RISC, and their target mRNA in active complex. Because the specific targets of these miRNAs have not been fully elucidated, these target-based mechanisms may not be feasible; however, it is important to consider miRNA degradation by nucleases (85) and natural circular RNA (86) in this context. Generally mature miRNAs are stable and degradation should not interfere with functional roles.

5.4.2. Therapeutic Potential of miRNAs

The quantifiable expression of miRNAs in a variety of tissues and fluids in the body suggest their potential utility as biomarkers of disease. Perhaps the most promising site of miRNA expression regarding the use of these molecules as biomarkers is the blood. miRNAs can be expressed in additional bodily fluids such as lavage fluids, saliva, and urine; however, blood miRNAs have been proposed as suitable biomarkers for cancer, inflammatory disease, and tissue injury (57). The blood miRNA profile of patients with chronic obstructive pulmonary disease, allergic rhinitis, and the BAL fluid miRNA profiles of patients with mild asymptomatic asthma have been shown to be unique and may have potential use as biomarkers of allergic disease (57, 87). When mice were dermally exposed to archetypal Th1 (DNCB and α -HCA) or Th2 (TDI and OPA) sensitizers dLN miR-210 expression was increased in response to the Th2 sensitizers but was

decreased in response to the Th1 sensitizers (data not shown). This suggests the potential utilization of miRNA expression as a tool to determine if a chemical is a dermal or respiratory sensitizer. Furthermore, miR-210 expression was investigated in a variety of tissues during TDI sensitization and expression was significantly increased in the ear at various time points post 4% TDI exposure (A 4.1 A). miR-210 expression in the ear was significantly increased 4 days post 0.5, 2, and 4% TDI exposure; however, expression following the strong irritant dose of 4% was much higher in relation to the less irritating doses, indicating that miR-210 expression in the ear is more dependent on dLN expression of miR-210 at the same doses (data not shown). miR-210 expression was also examined at day 4 post 4% TDI exposure in the blood (A 4.1 B). Blood miR-210 levels were not altered at this time point in response to TDI sensitization; this warrants further investigation at multiple time points post exposure in the study of miR-210 as a biomarker.

MicroRNA-based therapies are in their infancy, with several currently undergoing Phase I and II clinical trials. Regardless, these therapies exhibit great potential due to the ubiquitous functional power of miRNAs. miRNA-based therapies can involve the inhibition or overexpression of individual or multiple miRNAs. Miravirsen, the first miRNA-based therapeutic to undergo clinical trials, is a miR-122 inhibitor which inhibits Hepatitis C replication, acting as a potent anti-viral agent in nonhuman primate studies (57). Although no miRNA-based therapeutics are currently in clinical trials to combat allergic disease, intranasal inhalation of various miRNA inhibitors has been shown to reduce allergic airway inflammation in murine models (57), providing experimental evidence that miRNA-based therapies for allergic disease is a viable concept.

5.5 Other Conclusions

5.5.1. Irritancy and the Development of Allergic Disease

Ni^{2+} allergy is an example of the importance of innate signaling pathways in the development of allergy. Ni^{2+} is typically a false negative in the LLNA although it is an extremely common cause of allergic contact dermatitis in humans (88). The explanation for this phenomenon are species-specific variations in TLR4 histidine residues (positions 456 and 458 on human TLR4 but not on mouse TLR4) that allow Ni^{2+} to activate this receptor, if present (88). This response illustrates the necessity of the innate “danger signaling” in the initiation and sustainability of an adaptive allergic response. Local trauma, endotoxin, and additional adjuvants may serve as danger signals to allow for the initiation of adaptive responses that may have been suppressed in the absence of these signals; essentially, these events control the fine balance between tolerance and immunity. It is assumed that many chemical allergens are capable of inducing irritation or inflammation, supporting the initiation of an adaptive response and sensitization (88-90). As noted in section 5.1 regarding hypersensitivity classifications, innate mechanisms precluding allergy are remarkably similar to pathogen clearance responses. These signals may originate from epidermal cells which may produce inflammatory cytokines such as IL-1 β and IL-6 when activated (44). This is summarized in the “two signal” hypothesis which states that antigen delivery alone is insufficient for effective immunological priming but rather a second, innate signal is necessary to ensure the development of sensitization (88, 90). This concept is illustrated in the studies outlined in Chapter 3. The contrasting biological effects of 0.5 and 4% TDI are evident, with increased dermal inflammation, dLN cellularity, and augmented sensitization noted for the 4% dose (Figures 3.1-3.4). A key difference between the low and high dose utilized in these studies is the presence of strong (4%) and minimal (0.5%) dermal

irritation. As noted in Chapter 3, the irritant response appears to be a prerequisite for strong sensitization responses in the case of dermal TDI sensitization. This observation has been noted in models of contact dermatitis as well, Bonneville, et al. noted the conditioning role that acute skin irritation appeared to play in a murine model of DNFB contact dermatitis (44). Protein allergens typically require the exogenous administration of adjuvants or additional innate signaling to cause irritation (91), illustrating the unique ability of many LMW chemicals to serve as their own “second step” in the journey to allergenicity.

The mechanisms of innate responses involved in dermal sensitization have been investigated in a variety of models of contact hypersensitivity. The dual absence of TLR2 and 4 in a murine model of contact hypersensitivity prevented TNCB, oxazolone, and fluorescein isothiocyanate sensitization from occurring, revealing the importance of these two pattern recognition receptors in the development of contact hypersensitivity (92). This study also revealed that mice lacking both IL-12R β 2 and TLR2 or 4 were also resistant to contact hypersensitivity; this was not demonstrated in mice lacking only one of the following components (92). These results indicate that allergic sensitization may depend on several innate factors, including: an IL-12-dependent mechanism mediated in concert with TLRs and an IL-12-independent mechanism that involves TLR2 and 4 (92). IL-12 likely plays a role in dendritic cell and Th1 activation and function, further contributing to the pathogenesis of allergic disease. Interestingly, TNCB sensitization and contact hypersensitivity was sustained in germ free mice (92), suggesting a role for skin-derived TLR ligands in the innate response to chemical allergens.

While significantly augmented miR-210 expression was observed in the dLN (0.5 and 4% TDI) and ear (4% TDI) at various time points post chemical exposure, 4% TDI is a dermally-irritating concentration (93), indicating that miR-210 expression may be somewhat dependent on

this response. To investigate this, a range of TDI concentrations were selected to encompass both non-irritant (0.5%) and irritant (1, 2, and 4%) doses based on ear swelling and inflammatory cytokine mRNA levels in the ear, as reported by Long, et al. (93). dLN and ear miR-210 expression was quantified 4 days post 0.5 and 4% TDI exposure. dLN miR-210 levels were significantly upregulated at all TDI concentrations, regardless of irritant status (Figure 4.2A), indicating that dLN miR-210 expression is not dependent on dermal irritation. Ear miR-210 levels appeared to be more affected by the irritant status of the TDI concentration, revealing significantly increased miR-210 levels following 0.5, 2, and 4% TDI exposure yet the magnitude of miR-210 fold change is much higher in 4%-treated ears (Appendix 4.1 B). While irritation is considered by many to be an important component of sensitization, this is not unexpected; however, the increased expression of miR-210 in the 0.5%-treated ears further suggests a role for miR-210 in the sensitization response and not solely the irritant response. The expression of additional miRNAs that were previously shown to be upregulated during TDI sensitization (62) was investigated at non-irritant and irritant TDI levels and dLN miR-22 and -31 significantly increased at various time points post 0.5, 1, 2, and 4% TDI exposure (data not shown). An additional study was completed in order to further confirm the sensitization-related expression of miR-210. Dermal application of a known irritant, nonanoic acid (NA), which lacks sensitization capabilities, was carried out followed by confirmation of dermal irritation and no significant changes in dLN miR-210, -22, nor -31 expression following NA exposure and the development of an irritant response in the absence of sensitization (data not shown). Altogether, this data suggests that several miRNAs are significantly increased due to the TDI sensitization response as a whole and not singularly due to the irritant component of this response.

5.5.2. Of Mouse and Man: Important Considerations

Clearly, murine and human immunology share many conserved features, emphasizing the utility and popularity of experimental murine models. Mouse research has been a staple in many landmark immunologic discoveries of the past century. Mice, in particular, are very useful for the study of human diseases because of the highly conserved gene repertoire between the two species. While these models are extremely useful and can mimic the human disease state in many instances, they are not a perfect reflection of the human condition. Regarding immunology in particular, many key differences in the two organisms should be noted. Murine blood contains a high percentage of lymphocytes (75-90% of all cells) while human blood is mostly composed of neutrophils (50-70% of all cells) (94). This may be significant in systemic responses such as TDI sensitization, as a determinant of cellular responsiveness. Several innate immune structures are altered in mice and humans, including TLR expression, defensin production, and inducible nitric oxide synthase production by macrophages (94). In terms of adaptive immunity, humans express several FcRs that mice do not, there is variation in antibody isotype structure between the two species, several chemokines and receptors have been identified in one species but not the other (e.g. CXCR1, CCL6, and MCP-4), and T-helper skewing may not be as “clear cut” in humans as it is in mice (94). T cell activation is characterized by several key steps, one being inward calcium flux which is thought to be mediated by the outward flow of potassium via the Kv1.3 K⁺ channel; murine T cells do not express this channel, calling into question the mechanism of calcium flux sustainability by murine T cells (94). In addition to the basic mechanistic differences in murine and human models, many differences exist in the manifestation of disease in models of illness such as delayed type hypersensitivity, where the cellular components of the response are divergent (humans- neutrophils initially followed by T cells and macrophages and mice- more neutrophil rich throughout the

response) and where mice typically require higher antigen concentrations for the elicitation of the allergic response than humans (94). In terms of TDI-induced asthma, the choice of mouse strain determines the asthma phenotype (e.g. severity and cellular involvement) (95), demonstrating intraspecies variability. As previously noted, several important species-to-species disparities remain in the study of TDI-allergic disease, including the role of IgE (Ref Chapter 2). Additionally, T_{regs} vary considerably in rodents and humans; CD103 is robustly expressed on naïve and activated murine T_{regs} (Ref Chapter 3) yet this molecule is essentially nonexistent on human T_{regs} (96). This is an important consideration in regards to extrapolation of data from studies utilizing CD103 as a T_{reg} marker in mice.

It is very important to consider species-specific differences when utilizing animal models in both basic and preclinical research. One of the most haunting examples of failure to take into account these differences is the 2006 TGN1412 (CD28 superagonist) clinical trial which resulted in life threatening cytokine storms in six healthy young male volunteers. This agent, which was intended to stimulate the production of T_{regs} (97) and Th cells (98) underwent preclinical evaluation in rodents and nonhuman primates, showing no significant adverse effects. The unprecedented, nearly fatal cytokine storm (primarily IFN- γ , IL-2, and TNF) in Phase I human volunteers has been determined to be due to CD4⁺ effector memory T cells (T_{EM}) which are produced by exposure to infectious agents, which is not experienced by laboratory animals housed in pathogen-free conditions, resulting in a disadvantageous $T_{\text{reg}}:T_{\text{EM}}$ balance not seen in rodents utilized in preclinical studies (97). Nonhuman primates, which are virtually genetically analogous to humans, were also utilized in preclinical studies and cynomolgus macaques were administered TGN1412 at doses up to 500 times higher than the dose given to the human volunteers in the absence of negative effects (97). One important caveat that was overlooked is that macaque CD4⁺ T cells lose CD28 expression during

T_{EM} differentiation, while human T_{EM} cells retain CD28 expression (97), possibly accounting for the massive T_{EM} activation and cytokine release following TGN1412 administration. Additionally, the human CD28 extracellular domain varies by four amino acids from the macaque molecule, one of the substitutions occurring in the C'D binding loop which is the site of TGN1412 binding (98). This tragic event emphasizes the sometimes subtle yet critical species-specific differences between humans and model organisms that should be fully investigated and always considered both in basic and preclinical research. Additionally, retrospective studies with humanized mice revealed concerning effects of TGN1412 administration on the immune system of these mice, including rapid peripheral lymphopenia, which was also evidenced in the human volunteers during the clinical trial (98). This emphasizes the utility of humanized animal models, particularly in immunologic studies.

The divergent immune systems of humans and mice can be explained by natural selection; each unique mechanism was likely adopted as a selective advantage for each species based on their environment, lifespan, size, and specific pathogens encountered. These differences are inevitable and do not invalidate the use or validity of murine models in immunology research. Rather, the careful consideration of species-specific differences and the resultant thoughtful extrapolation of this data to human disease is required for these studies to reach their maximum impact on human health.

5.5.3 Summary

Overall, much progress has been made in the understanding of TDI sensitization and allergic disease; however, there is still a pressing need for hazard identification strategies for respiratory sensitizers like TDI. The involvement of novel mediators of sensitization like T_{regs} and miRNAs has implications on this burden. Not only is the data presented in this work relevant for TDI

sensitization, but presumably it is relevant to many chemical sensitizers. We have identified a functional role for T_{regs} in a murine model of epicutaneous TDI sensitization. This role appears to be significant in the dampening of allergic responses; however, the overall sensitization response is not abrogated due to the expansion of these cells. Although T_{regs} may be suppressive in the TDI sensitization response, the proliferation of allergic cells and their secretion of cytokines may be overwhelming to the T_{regs} that are present during the response. This concept has been applied to autoimmune responses in murine models of atherosclerosis, rheumatoid arthritis, and primary biliary cirrhosis where peak inflammatory conditions and peak T_{reg} levels occur at the same time, suggesting that the suppressive capabilities of these cells are overtaken by the strong autoimmune response that originally resulted in their induction (99). This is not to say that the T_{regs} aren't having a regulatory effect at all in conditions such as TDI sensitization and autoimmune disorders; however, their presence is not enough to diminish symptoms completely and stop the response "in its tracks".

The hypothesized action of miR-210 and other miRNAs such as miR-31 as T_{reg} antagonists suggest more intricate regulatory mechanisms at play than are currently understood in chemical sensitization. While T_{regs} expand and are functionally active during TDI sensitization, these cells are likely being regulated by a number of factors including miRNAs, as reported in this work. miRNAs display great promise as biomarkers of disease in a number of conditions and these molecules could potentially be investigated as sensitization biomarkers in chemical sensitization or utilized to distinguish contact and respiratory sensitizers. Additional miRNAs were upregulated in the dLN during TDI sensitization, including miR-21, -22, -126, and -301a (62). While these miRNAs were not investigated in this work, they may also play important regulatory roles in the TDI sensitization response. Further research characterizing the miRNome during TDI sensitization and elicitation would lend insight into the potential roles of the miRNAs thought to be involved.

Altogether, this work represents insight into the regulatory mechanisms involved in the complex TDI sensitization immune response. Both T_{regs} and miRNAs are significant in their regulatory functions and their newfound roles in TDI sensitization are illustrated in figure 5.1. These insights may aid in the further exploration of immunoregulatory mechanisms and novel effectors of chemical sensitization. Ultimately, this work may promote the development of novel hazard identification strategies that are designed in regard to the complex regulatory mechanisms that are a major component the chemical sensitization immune response.

5.6. REFERENCES

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APPENDIX

A 3.1 T_{reg} Phenotyping Gating Strategy

T_{reg} gating strategy for dLN flow cytometric analysis of CTLA4 (A), Neuropilin-1 (B), and CCR6, CD103, and ICOS (C) expression. Examples are shown for populations positive for each marker and a FMO control is shown for Neuropilin-1 staining in part B

A 3.2 T_{reg} Depletion Study Model Confirmation

(A) Blood T_{reg} gating strategy for flow cytometric analysis. Examples are shown for baseline (left panel) and depleted (right panel) samples. (B) Blood T_{reg} flow cytometric analysis is shown for

baseline (left panel), day -2 (center panel), and day 7 (right panel) samples and is presented as percent of CD45⁺ CD4⁺ or CD4⁺ cells. (C) dLN T_{reg} flow cytometric analysis is shown for day 7 as percentage of CD45⁺CD4⁺ cells (left panel) and number of cells (right panel) based on extrapolation of this data with total dLN cellularity. Bars represent mean ($\pm$ SE) of 5 mice per group. Statistical significance was determined by Student *t*-test and is indicated as follows: *p* value <0.05 and (**) *p* value <0.01. *P* values are represented by asterisks (comparison of acetone to TDI-exposed group from the same antibody treatment regimen) or horizontal bars with asterisks above (comparison of identical chemical treatment between antibody and isotype-treated groups).

A

B

C

A 3.3 *T_{reg}* Depletion Study Supplemental Data

Percent change in total body weight (tracked per individual mouse, presented as SEM) as determined from day -11 to 7 (A). dLN T cell phenotyping was performed 7 days following dermal TDI exposure as in Figure 3.4. Th2 (CD3⁺CD4⁺Gata3⁺) frequency (B) and Th2 (CD3⁺CD4⁺Gata3⁺Foxp3⁻) MFI (C) were determined based on flow cytometric analysis (frequency and MFI) and extrapolation of this data with total dLN cellularity. Bars represent mean ($\pm$ SE) of 5 mice per group. Statistical significance was determined by Student *t*-test and is indicated as follows: *p* value <0.05 and (**) *p* value <0.01. *P* values are represented by asterisks (comparison of acetone

to TDI-exposed group from the same antibody treatment regimen) or horizontal bars ($p < 0.05$; comparison of identical chemical treatment between antibody and isotype-treated groups).

A 3.4 *T_{reg}* Depletion Study General T Cell Endpoints

dLN T cell phenotyping was performed 7 days following dermal TDI exposure. CD3⁺ frequency (A) and number (B), CD3⁺CD4⁺ frequency (C) and number (D), CD3⁺CD8⁺ frequency (E) and number (F), Th1 (CD3⁺CD4⁺Tbet⁺) frequency (G) and number (H), and Th17 (CD3⁺CD4⁺Rorγt⁺) frequency (I) and number (J) were determined based on flow cytometric analysis and extrapolation of this data with total dLN cellularity. Bars represent mean (± SE) of 5 mice per group. Statistical significance was determined by Student *t*-test and is indicated as follows: *p* value <0.05 and (**) *p* value <0.01. *P* values are represented by asterisks (comparison of acetone to TDI-exposed group from the same antibody treatment regimen) or horizontal bars (*p* <0.05; comparison of identical chemical treatment between antibody and isotype-treated groups).

A

B

A 4.1 miR-210 Expression in a Variety of Tissues During 4% TDI Sensitization

RT-PCR analysis of miR-210 expression in the ears (A) and blood (B) 4 days post TDI exposure.

Bars represent mean relative fold change ($\pm$ SE) of 4-5 mice per group. *P* values are represented by asterisks ($P < 0.05$).

A

FMO Control

CD45

B

CD3

C

CD4

D

CD25

E

Foxp3

F

CCR7

G

CD103

H

Figure A 5.1 *Ear T_{reg} Phenotyping Gating Strategy*

T_{reg} gating strategy for dLN flow cytometric analysis of CD45 (A), CD3 (B), CD4 (C), CD25 (D), Foxp3 (E), CCR7 (F), CD103 (G), and ICOS (H) expression. Examples are shown for populations positive for each marker and a fluorescence minus one (FMO) control is shown for each marker.

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<u>Peer Tutor for GWU Learning Assistance Program</u>	2008-2010
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Subjects tutored: Organic Chemistry and Calculus I

SERVICE

Postdoctoral Representative (Pending Graduation) Dermal Toxicology Specialty Section (DTSS): Society of Toxicology (SOT)	2016-present
WVU Subcommittee for Graduate Faculty Mentor Award	2016-present
SOT Annual Meeting High School Program Volunteer Scavenger Hunt Guide	2016
Graduate Student Representative DTSS: SOT	2015-2016
Graduate Student Leadership Committee Member Programming Subcommittee Member	2015-2016
Volunteer, WVU Medicine Neonatal Intensive Care Unit and Pediatrics Total service hours: 157	2014-2016
SOT Continuing Education Volunteer	2014-present
WVU Health Sciences Center Graduate Student Association	2013-present
Summer Camp Counselor Camp Boggy Creek	2010
GWU Student Government Association Executive Treasurer Sophomore Class Senator	2008-2011

HONORS

Best Poster, Graduate Student Year 5 WVU Van Liere Convocation	2016
Charles River Graduate Student Travel Award DTSS: SOT	2016
Featured Graduate Student: Portraits of Excellence WVU Graduate Life and Education	2016
CDC-Charles Shepard Award Nominee Laboratory Science Nayak, et al. 2014 and Marshall, et al. 2015	2015 and 2016
Best Overall Methodology Allegheny-Erie SOT Regional Chapter Annual Meeting	2015
Selected Oral Presentation Allegheny-Erie SOT Regional Chapter Annual Meeting	2015
Three Minute Thesis Finalist WVU Office of Graduate Life and Education	2015
Battelle Research Award DTSS: SOT	2015
Best Presentation by a Graduate Student, First Place SOT Immunotoxicology Specialty Section	2015
Annual Meeting Graduate Student Travel Award Allegheny-Erie SOT Regional Chapter	2015
SOT Graduate Student Travel Award	2014
WVU Graduate Student Travel Award	2014
Selected Oral Presentation WVU Van Liere Convocation, Basic Science	2014 and 2016
AAAS/ <i>Science</i> Program for Excellence Nominee	2013
WVU Varsity Track and Field Letter winner WVU Academic-Athletic Honor Roll Big East Conference All-Academic Team	2011-2012

Graduated from GWU with a 4.0 GPA	
Senior Scholastic Award	2011
1-3 Year Academic Award Recipient	2008-2010
Big South Conference-NCAA Woman of the Year Nominee	2011
George A. Christenberry Award for Academic/Athletic Excellence	
Awarded by the Big South Conference	2011
Big South Conference Scholar Athlete of the Year	2010-2011
Indoor and Outdoor Track and Field	
Presidential Scholar and Dean's List	2007-2011
Varsity Track and Field Letterwinner	2007-2011
Team Captain ('10-'11)	
Atlantic Sun and Big South Conference	2008-2011
Women's Indoor and Outdoor Track and Field All-Academic and All-Conference Team	
NCAA Division 1 Academic All-American	2008
United States Track and Field and Cross Country Association	

SELECTED PEER-REVIEWED PUBLICATIONS

- Long CM**, Marshall, NB, Lukomska, E, Nayak A, Meade B, Anderson S. Potential Inhibitory Influence of miRNA 210 on Regulatory T Cells During Epicutaneous Chemical Sensitization. [In Preparation]
- Long, CM**, Marshall, NB, Lukomska E, Kashon M, Meade B, Shane H, Anderson S. A Role for Regulatory T Cells in a Murine Model of Epicutaneous TDI Sensitization. *Tox Sci*. doi: 10.1093/toxsci/kfw074
- Anderson S, Shane H, **Long C**, Lukomska E, Meade B, Marshall N. Evaluation of the Irritancy and Hypersensitivity Potential following Topical Application of Didecyldimethylammonium chloride. *J Immunot*. [Accepted]
- Marocco A, Meade BJ, **Long CM**, Lukomska E, Marshall, NB, Anderson SE [2015]. Immunotoxicity and Allergic Potential Induced by Topical Application of N-Butylbenzenesulfonamide in a Murine Model. *J Toxicol. Environ. Health Part A: Current Issues*. PMID: 26291892
- Marshall NB, Lukomska E, **Long CM**, Kashon ML, Sharpnack DD, Nayak AP, Anderson KL, Meade BJ, Anderson SE [2015]. The antimicrobial chemical triclosan induces thymic stromal lymphopoietin in skin promoting Th2 allergic responses. *Tox Sci*. PMID: 26048654

- Anderson SE, Meade BJ, **Long CM**, Lukomska E, Marshall NB [2015]. Investigations of Immunotoxicity and Allergic Potential Induced by Topical Application of Triclosan in Mice. *J Immunot.* 25812624
- Nayak, AP, Hettick, J, Siegel, PD, Anderson, SE, **Long, CM**, Green, B, Beezhold, D. [2014]. Toluene diisocyanate (TDI) disposition and co-localization of immune cells in hair follicles. *Tox Sci*, PMID: 24798378
- Anderson SE, Beezhold K, Lukomska E, Richardson J, **Long CM**, Anderson KL, Franko J, Meade BJ, Beezhold D [2013]. Expression Kinetics of miRNAs involved in Chemical Sensitization. *J Immunot*, PMID: 24063594
- Brock KL, Anderson SE, Lukomska E, **Long CM**, Anderson KL, Marshall NB, Meade BJ [2013]. Immune Stimulation Following Dermal Exposure to Unsintered Indium Tin Oxide. *J Immunot*, PMID: 24164313

PRESENTATIONS

- Shane HL, Marshall NB, Lukomska E, **Long CM**, Anderson SE. Evaluation of dermal immune responses following didecyldimethylammonium chloride-induced hypersensitivity. Allegheny-Erie Society of Toxicology Annual Meeting, Morgantown, WV. 2016
- Marshall N, Lukomska E, **Long C**, Anderson S. Dermal exposure to the antimicrobial triclosan affects the gut microbiome and augments Th2 allergic responses mediated through toll-like receptor 4. *Journal of Immunology*. American Association of Immunologists Annual Meeting, Seattle, WA. Poster. 2016
- Anderson S, Shane H, **Long C**, Lukomska E, Meade B, Marshall N. Irritancy and Allergic Responses Induced by Topical Application of Didecyldimethylammonium Chloride. *Toxicologist*. Society of Toxicology Annual Meeting, New Orleans, LA. Poster. 2016
- Long, CM**, Marshall NB, Lukomska E, Kashon M, Shane H, Siegel PD, Meade B, Anderson S. The Immunomodulatory Potential of Regulatory T Cells in a Murine Model of Epicutaneous Chemical Sensitization. *Toxicologist*. Society of Toxicology Annual Meeting, New Orleans, LA. Poster. 2016
- Long, CM**, Marshall NB, Lukomska E, Kashon M, Anderson S. The Immunomodulatory Potential of Regulatory T Cells in a Murine Model of Epicutaneous Chemical Sensitization. Van Lier Convocation, Morgantown, WV. Oral Presentation and Poster. 2016

- Long CM**, Marshall NB, Lukomska E, Nayak A, Siegel P, Meade B, Anderson S. Immunoregulatory potential of microRNA 210 in a murine model of chemical sensitization. AESOT Annual Meeting, Morgantown, WV. Oral Presentation. 2015
- Marshall NB, Lukomska E, **Long CM**, Anderson SE. Elucidating the mechanisms of augmented allergic responses with dermal triclosan exposure. AESOT Annual Meeting, Morgantown, WV. Oral Presentation. 2015
- Anderson SE, Franko J, Lukomska E, Meade BJ, **Long CM**, Marshall NB. The Influence of Innate Immune Signaling and the Microbiome on Adaptive Immune Responses following Exposure to the Antimicrobial Triclosan. Keystone Symposia on Molecular and Cellular Biology, "Innate Immunity and Determinants of Microbial Pathogenesis", Olympic Valley, CA. Poster. 2015
- Marshall NB, Lukomska E, **Long CM**, Nayak AP, Meade BJ, Anderson SE. Dermal exposure to the antimicrobial chemical triclosan augments allergic responses by inducing expression of thymic stromal lymphopoietin. *Toxicologist*. 144(1):143-144 #671. Society of Toxicology 2015 Annual Meeting, San Diego, CA. Poster. 2015
- Long CM**, Marshall NB, Lukomska E, Nayak AP, Siegel PD, Meade BJ, Anderson SE. Increased Expression and Immunoregulatory Potential of MicroRNA 210 in a Murine Model of TDI Sensitization. *Toxicologist*. 144(1):144-145 #676. Society of Toxicology 2015 Annual Meeting, San Diego, CA. Poster. 2015
- Nayak, AP, Green, B, Croston, T, Lemons, AR, Marshall, NB, Goldsmith, WT, Kashon, ML, Law, BF, Wagner, LM, **Long, CM**, Germolec, DM, Beezhold, D. Characterization of antigen presenting cells in a murine subchronic fungal exposure model. *J Allergy Clin Immuno*. American Academy of Allergy, Asthma, and Immunology 2015 Annual Meeting. Houston, TX. Poster. 2015
- Green, B, Nayak, AP, Croston, T, Lemons AR, Marshall NB, Goldsmith WT, Kashon ML, Law BF, Wagner, LM, **Long, CM**, Germolec DM, Beezhold, D. Fungal viability is essential in modulating of adaptive immune responses in mice. *J Allergy Clin Immuno*. American Academy of Allergy, Asthma, and Immunology 2015 Annual Meeting, Houston, TX. Poster. 2015
- Long, CM**, Marshall, NB, Siegel, PD, Meade, BJ, Lukomska, E, Anderson, KL, Beezhold, D, and Anderson, SE. Exploring the role of miRNA-210 and regulatory T cells during TDI sensitization. *Journal of Immunology*.

American Association of Immunologists Annual Meeting. Pittsburgh, PA. Poster. 2014

Marshall, NB, **Long, CM**, Anderson, KL, Lukomska, E, Anderson, SE. Dermal exposure to the commonly used antimicrobial chemical triclosan promotes allergic responses in skin and lung. *Journal of Immunology*. American Association of Immunologists Annual Meeting. Pittsburgh, PA. Poster. 2014

Hettick, J, Nayak, AP, **Long, CM**, Anderson, SE, Siegel, PD. [2014]. Identification of toluene diisocyanate-conjugated murine protein targets following dermal exposures. Baltimore, MD. Poster. 2014

Long, CM, Marshall, NB, Siegel, PD, Meade, BJ, Lukomska, E, Anderson, KL, Beezhold, D, Anderson, SE. An immunologic role of mirR-210 in a murine model of dermal toluene 2,4-diisocyanate sensitization. *Toxicologist*. Society of Toxicology Annual Meeting. Phoenix, AZ. Poster. 2014

Anderson, SE, Marshall, NB, **Long, CM**, Anderson, KL, Lukomska, E, and Meade, BJ. Dermal exposure to triclosan induces changes in expression of innate and adaptive immune genes in a mouse model. *Toxicologist*. Society of Toxicology Annual Meeting. Phoenix, AZ. Poster. 2014

Marshall, NB, Anderson, SE, Anderson, KL, **Long, CM**, Lukomska, E, and Meade, BJ. Early immunomodulatory effects of dermal triclosan exposure in mice. *Toxicologist*. Society of Toxicology Annual Meeting. Phoenix, AZ. Poster. 2014

Long, CM, Marshall, NB, Siegel, PD, Meade, BJ, Lukomska, E, Anderson, KL, Beezhold, DH, and Anderson, SE. Exploring the Role of miRNA 210 and Regulatory T Cells in a Murine Model of Toluene 2,4-diisocyanate Sensitization. Van Lier Research Convocation, Morgantown, WV. Oral Presentation. 2014

Long, CM, Beezhold K, Lukomska E, Anderson KL, Meade BJ, Beezhold D, Anderson SE. Expression Kinetics of miRNA Involved in Dermal Toluene 2,4-diisocyanate Sensitization. Allegheny-Erie Society of Toxicology Annual Meeting. Morgantown, WV. Poster. 2013

Anderson, SE, Beezhold, K, Lukomska, E, Richardson, J, **Long, CM**, Anderson, K, Franko, J, Meade, BJ, and Beezhold, DH. Differential Expression Kinetics of miRNA involved in Allergic Chemical Sensitization following Dermal exposure in a Murine Model. *Toxicologist*. Society of Toxicology Annual Meeting. San Antonio, TX. Poster. 2013

- Long, CM**, Franko, J, Kashon, ML, Anderson, KL, Hubbs, AF, Lukomska, E, Meade, BJ, and Anderson, SE. Exposure to Triclosan Augments the Allergic Response to Ovalbumin in a Mouse Model of Asthma. *Toxicologist*. Society of Toxicology Annual Meeting. San Antonio, TX. Poster. 2013
- Meade, BJ, Lukomska, E, Brock, K, **Long, CM**, Anderson, K, and Anderson, SE. Potential for Immune Sensitization following Dermal Exposure to Indium Tin Oxide. *Toxicologist*. Society of Toxicology Annual Meeting. San Antonio, TX. Poster. 2013
- Long, CM**, Anderson, SE, Beezhold, K, Lukomska, E, Anderson, KL, Meade, BJ, and Beezhold, D. Expression Kinetics of miRNAs involved in Chemical Sensitization. Van Liere Research Convocation. West Virginia University, Morgantown, WV. Poster. 2013
- Long, CM**. Darwinian Aspects of the Evolution of Methicillin-Resistant *Staphylococcus aureus*. Darwin's Legacy Conference. University of North Carolina-Wilmington. Wilmington, NC. Poster. 2009

PROFESSIONAL MEMBERSHIPS

American Association of Immunologists	2014-present
American Association for the Advancement of Science	2014-present
Society of Toxicology Dermal Toxicology, Immunotoxicology, Women in Toxicology	2012-present
Allegheny-Erie Society of Toxicology	2012-present
Beta Beta Beta National Biological Honors Society	2010
Sigma Zeta National Science and Mathematics Honor Society	2010
Alpha Chi National Honor Society	2009

RELEVANT SKILLS AND EXPERIENCE

- **Animal Models**
Small rodent exposure (dermal exposure, pulmonary aspiration, nasal instillation) and necropsy, Blood and tissue collection (mouse and rat), Bronchoalveolar lavage (mouse and rat), Intravenous tail vein and

intraperitoneal injection (mouse), Peritoneal lavage (mouse), Tail bleeding (mouse)

- **Cellular and Molecular Biology**

Bacterial Culture, Bacterial Staining, Mammalian Cell Culture, Cellometer, Coulter Counter, Cytometric Bead Array, Cytospin, ELISA, *Ex vivo* cellular stimulation, Local Lymph Node Assay, Magnetic Cell Separation, miRNA Detection (mature and primary), miRNA mimic and inhibitor transfection, Gene and miRNA expression microarray, Nanodrop, Sheep Red Blood Cell Plaque-forming Cell Assay, Real Time PCR (SYBR green and TaqMan), Regulatory T Cell Suppression Assay, Reverse Transcription, RNA/DNA isolation, Serum Chemistry/Hematology (IDEXX ProCyte and Catalyst), Thymidine-Uptake Lymphocyte Proliferation Assay, Tissue homogenization and lysis, Western Blot

- **Computing**

Adobe Photoshop, Applied Biosystems 7500 PCR Software, Cmap, Endnote, FACSDiva, FlowJo, GraphPad Prism, Microsoft Office, Softmax Pro

- **Flow Cytometry**

Cell Surface Staining, CFSE proliferation assay, Intracellular Staining, LSR II, Multicolor cellular phenotyping, viability dyes