IMMUNOMODULATORY EFFECTS OF DIESEL EXHAUST PARTICLES

by

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DEDICATION

To my family and friends, without whom I would be lost; thank you. Without your continual support and unfailing optimism, I could not have accomplished this incredible task. A special thank you to my fiancé, Dan, whose positive influence has kept me focused. Finally, to my advisor and mentor, Dr. Denise Wingett, who has continually guided and encouraged me throughout this process. Thank you!

AUTOBIOGRAPHY

A Treasure Valley native, I was born April 26, 1982 to David and Jolene Masterson in Boise, Idaho and raised in the nearby town of Ontario, Oregon. My childhood consisted of horses, 4-H, hunting, fishing, school, or anything outdoor related with my parents, Grandma and Grandpa Williams, and my sister Cassie, until graduating as Valedictorian from Ontario High School in 2000. Following high school, I pursued higher education at Eastern Oregon University in La Grande, Oregon, where I graduated in 2000 with a B.S. in Biology with a minor in Chemistry. After completion of my undergraduate degree, I worked as a food microbiologist and a research and development associate for Dickinson Frozen Foods, Inc. and Heinz Frozen Foods Inc, Ore-Ida Division, respectively. During this time, I became actively involved in the Oregon 4-H Horse Program as a leader and continue to work with this program today. Following my employment in the food industry, I began my Master's thesis in August 2006 at Boise State University. During my graduate tenure, I met my fiancé, Daniel Armichardy. Following the completion of my degree, I will be accepting a position as a Research Associate in the Wingett Immunology Lab in addition to teaching general biology lab courses as an adjunct instructor.

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ABSTRACT

Diesel exhaust particles (DEP) represent a key component of particulate matter pollution and a serious cardiopulmonary health risk, as these particles have been associated with increased morbidity and mortality following exposure. In this study, we investigated the immunomodulatory properties of DEP on helper T cells by measuring changes in activation, cytokine production, and viability. The expression of CD40L, a key regulatory protein, was increased by DEP in the absence of physiologic stimuli without parallel increases in the expression of CD25 and CD69 activation markers. Additional studies utilizing a variety of T cell stimuli, including T cell receptor signaling and CD28 costimulation, showed consistent and reproducible increases in the expression of CD40L with negligible effects on other activation markers. Further studies demonstrated that the ability of DEP to augment CD40L production was restricted to the induction of the membrane-bound form of this protein, as soluble CD40L (sCD40L) was generally decreased. However, an increase in both membrane and sCD40L was observed in the context of cAMP signaling, which may have implications for at-risk populations utilizing the therapeutic effects of this important second messenger, including beta (β)-adrenergic agonists bronchodilators for the treatment of respiratory disease. Additional studies were performed to evaluate changes in the production of IL-8 and IL-17

cytokines. DEP produced no appreciable effect on IL-8 generation, but inhibited the production of IL-17. Evaluation of cytotoxicity indicated DEP had no measurable effect on T cell viability in resting cells. Collectively, these findings demonstrate an immunomodulatory capacity of DEP and may, in part, provide a mechanism explaining the contributions of DEP to the observed changes in morbidity and mortality. Given the importance of CD40L signaling in normal immune function, the findings that CD40L expression was increased regardless of the cellular activation status may be very relevant to diseases where the immune system contributes to pathogenic processes.

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LIST OF ABBREVIATIONS

Ab	Antibody
ADAM	A Disintegrin and Metalloprotease
Ag	Antigen
AIDS	Acquired Immunodeficiency Disease
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
β-Adrenergic	Beta-Adrenergic
β-Agonist	Beta-Adrenergic Agonist
BLAST	Basic Local Alignment Search Tool
Ca^{2+}	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate, Cyclic AMP
$CD4^+$	T cell expressing Cluster of Determination 4, T_H cell, Helper T cell
CD40L	CD40 Ligand, CD154, Trap1, GP39
$CD8^+$	T cell expressing Cluster of Determination 8, T_C cell, Cytotoxic T cell
DAG	Diacylglycerol
DEP	Diesel Exhaust Particles

EPA	Environmental Protection Agency
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
IL	Interleukin
IP_3	Inositol 1,4,5-Triphosphate
kDa	Kilodalton
MFI	Mean Fluorescent Intensity
µg	Microgram
MHC II	Major Histocompatibility Complex
µl	Microliter
NFAT	Nuclear Factor of Activated T Cells
NK	Natural Killer
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PIP_2	Phosphoinositol Biphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PM	Particulate Matter
$PM_{1.0}$	Particulate Matter ≤ 0.1 µm
PM_{10}	Particulate Matter 2.5 – 10 µm
$PM_{2.5}$	Particulate Matter ≤ 2.5 µm
PMA	Phorbol 12-Myristate 13-Acetate

RT	Room Temperature
sCD40L	Soluble CD40L
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SE	Standard Error
SLE	Systemic Lupus Erythmatosus
T_C	Cytotoxic T cell
TCR	T Cell Receptor
T_H	Helper T cell
T_H1	Helper T cell Type 1
T_H2	Helper T cell Type 2
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
U.S.	United States
WBC	White Blood Cell
Zn	Zinc

INTRODUCTION

As stated by the World Health Organization, unlimited and free access to clean air of acceptable quality is a fundamental human necessity and right (World Health Organization, 2004). In an attempt to achieve this for all Americans, the United States Department of Environmental Quality issued the first National Ambient Air Quality Standards in 1971 to address the major environmental and health challenges of particulate matter (PM) pollution facing our society. Since then, as scientific and epidemiologic data has accumulated, the initial standards set forth have been improved to address the serious effects of respirable particulates on human health with the latest version in final review as of December 2008 (United States Environmental Protection Agency, 2008). PM is a heterogeneous substance, composed of extremely small particles and liquid droplets, known to negatively affect overall health as measured by the observed increases in PM-related morbidity and mortality. The largest contributing source of PM pollution, diesel exhaust particles (DEP), represents a complex, anthropogenic pollutant produced during the process of diesel fuel combustion that has been directly linked to adverse cardiovascular, neurologic, and respiratory effects (Krivoshto, Richards, Albertson, & Derlet, 2008; Pope, III & Dockery, 2006; Ris, 2007). Extensive evidence exists that assigns a causal role for PM and DEP in the development and exacerbation of asthma and

other allergic diseases (Koren, 1995; Holguin, 2008; Pandya, Solomon, Kinner, & Balmes, 2002). Due to the fact that asthma involves an inflammatory immune response, many studies have implicated DEP as an adjuvant for allergic diseases by altering normal immune system activity potentially contributing to the increased prevalence and severity of this disease. Of the multitude of cells comprising the human immune system, helper T cells are central for proper immune function as this cell type communicates with other cells to mount a rapid and efficient immune response. However, improper function of helper T cells has been assigned a causal role in the development of certain allergic and autoimmune diseases. Therefore, it is important to understand how DEP exposure may affect the function of this cell and thereby contribute to the pathogenesis of diseases that are complicated by exposure to air pollution. The consequence of this line of research will hopefully achieve important short and long term goals: to properly diagnose and treat life threatening events, reduce PM-associated morbidity, and reinforce the need for higher emission standards, resulting in an improved quality of life for individuals living in industrialized societies.

Air Pollution

The linkage between air pollution and negative health effects is not a novel concept. Reports documenting the negative effects of air pollution exist from as early as the 1930's in Belgium, where a dense fog of industrial pollutants covered the Meuse Valley and resulted in 60 deaths attributed to severe respiratory distress (Nemery, Hoet, & Nemmar, 2001). When the fog lifted, rapid improvement of respiratory symptoms was achieved. Similar events occurred in the next decades in Donora, PA and in London.

The London fog of 1952 has received notoriety as over 4000 individuals succumbed to combined cardiovascular and respiratory complications as a result of air pollution exposure (Logan, 1953). A subsequent fog event in London in 1956 reported 1000 deaths with a 94% increase in bronchitis-related deaths (Logan, 1956). Therefore, extensive research has been conducted to define air pollution, understand the mechanisms by which it alters human health, and implement stringent air quality standards.

Air pollution comes from a variety of sources, which results in a complex mixture of particle and gas phase pollutants. Most epidemiologic studies on air pollution have focused on understanding the health effects of six principal or criteria air pollutants, as defined by the Clean Air Act established in 1963, routinely monitored by the Environmental Protection Agency (EPA) and World Health Organization (Holguin, 2008). Guidelines for exposure to these pollutants; ozone, nitrogen dioxide, sulfur dioxide, lead, carbon monoxide, and particulate matter, has been established by the National Ambient Air Quality Standards. Each criteria pollutant is derived from different emission sources, which yield pollutants that exhibit variations in chemical composition. This results in different toxicity profiles and unfavorable health effects dependent on the circumstances surrounding the combustion process such as season, fuel source, and geographic location (McCreanor et al., 2007). Of these identified pollutants, the EPA identifies both ozone and particulate matter pollution to be the most concerning health threats (United States Environmental Protection Agency, 2009).

Particulate matter (PM) is currently classified based on size of the inhalable particles, with a direct relationship between particle size and detrimental health effects (Siegel et al., 2004). Coarse PM (PM_{10}) includes particles that range from 2.5 – 10

micrometers (μm) in diameter with fine PM ($PM_{2.5}$) being 2.5 μm in diameter and smaller. Another recognized category is ultrafine particles ($PM_{1.0}$), with diameters less than 1.0 μm, which rapidly aggregate to become components of $PM_{2.5}$ (Pope, III & Dockery, 2006). Coarse and fine particulates differ not only in their size and physical properties, but also in their chemical composition. PM_{10} is largely composed of geological materials, in contrast to $PM_{2.5}$ and $PM_{1.0}$, which have larger fractions of elemental and organic carbon (Hetland et al., 2005). Coarse particulate matter is primarily derived from a suspension of dispersed ground dust in addition to soil, pollen, spores, and other materials (Pope, III & Dockery, 2006; Holgate et al., 2003). Fine and ultrafine particulate matter is produced as a result of different combustion processes such as wood burning, smelting, and cement processing. However, the major contributor to fine and ultrafine particulate matter is vehicle fuel exhaust (Wichmann, 2007). In general, all classes of PM pollution represent a major environmental health concern and epidemiological studies have identified a positive correlation between levels of PM and PM-related morbidity and mortality highlighted by increasing hospital emergency room visits and admissions (Hesterberg et al., 2009; Bunn, III et al., 2004; Laden, Schwartz, Speizer & Dockery, 2006). While all categories of inhalable PM are linked with negative health effects; the smaller, inhalable particles play a larger role in cardiovascular, neurologic, and respiratory disease as these particles easily pass through the nasal passage, into the lungs, and out into circulation allowing for penetration of these particles throughout the entire body. One specific pollutant that has garnered extensive attention for its association with negative health effects are DEP.

Diesel Exhaust

Diesel exhaust is a highly complex mixture formed during the combustion process resulting in the production of hundreds of different components, either in the gas or solid form with solid particulates in the fine and ultra-fine size PM categories (Krivoshto, Richards, Albertson, & Derlet, 2008; Wichmann, 2007). This vehicle emission source represents the largest single source of airborne PM with diesel engines emitting higher levels of gaseous emissions and up to 100 times more particles compared with modern gasoline engines (Riedl & Diaz-Sanchez, 2005). Despite the unfavorable impacts to our environment and human health, diesel is the primary source of fuel for mass transportation in the United States based on increased efficiency and endurance of diesel engines in comparison to gasoline engines (Krivoshto, Richards, Albertson, & Derlet, 2008). Other industrialized countries are even further dependent upon diesel fuel. In 1998, it was reported that 18% of Japanese motor vehicles relied upon diesel fuel with 98% of the nation's buses running on diesel fuel. While 18% may not be viewed as a substantial value, the emissions resulting from these vehicles accounted for almost 100% of emission-related PM pollution (Kagawa, 2002).

Recognized as a heterogeneous compound; the components of diesel exhaust may include elemental carbon, polycyclic aromatic hydrocarbons, carbon monoxide, nitrogen and sulfur compounds. To date, over 450 different compounds have been identified in diesel exhaust with 40 recognized hazardous pollutants (Inadera, 2006; Annesi-Maesano et al., 2007). Based on the size of DEP, with 80-95% of the particles in diesel exhaust measuring less than 2.5 μm in diameter, these particles bypass the protective barriers of our lungs and penetrate deep into the respiratory system (Ris, 2007). The complexity of

diesel exhaust is further enhanced by its elemental carbon core, which has the ability to adsorb hundreds of chemicals and transition metals (Riedl & Diaz-Sanchez, 2005). The large surface area of the smaller particles allows for greater chemical adsorption of compounds from the surrounding environment, which translates into increased biological effect (United States Environmental Protection Agency, 2002). This idea is further supported by studies which have identified the adverse effects of DEP is not necessarily due to the carbon core but the chemical constituents found in DEP samples (Siegel et al., 2004). The inhalation of particles with adsorbed compounds transports novel or foreign materials deep into our circulation, which may be recognized by the organ system responsible for eliminating foreign invaders, the immune system.

As previously mentioned, the severe fog incidents occurring in Europe were linked to a substantial increase in cardiovascular and respiratory mortality (Pope, III & Dockery, 2006). In the context of DEP, the ability of the nanoparticle component to migrate through the lung and into the circulatory system has been directly linked to cardiovascular mortality as viewed by alterations in atherosclerosis and acute myocardial infarction and physical changes that occur in the heart and blood vessels following DEP exposure (Mills et al., 2007; Wichmann, 2007). As understanding of cardiovascular events grows, altered immune cell activity is now recognized as a contributor to disease progression. Respiratory mortality is also thought to involve the immune system as diseases such as chronic obstructive pulmonary disease and asthma are exacerbated by DEP exposure and clearly involve an inflammatory component (Inadera, 2006). However, the exact mechanisms by which DEP alter immune system function has yet to be fully elucidated and are a goal of the present study.

The Immune System

The human immune system is a highly complex organ system which has evolved to prevent illness from a variety of pathogens and foreign invaders. This protection is achieved through the action of two distinct, yet intertwined branches: innate and adaptive immunity (Huston, 1997). Innate immunity provides rapid, yet non-specific, protection from foreign invaders, as the cell types of this branch are present prior to exposure to infectious agents. In contrast, the adaptive branch provides an antigen (Ag) specific response, where Ags are defined as molecules representing small fragments of a pathogen or foreign material, recognized by the immune system with immunological memory resulting following Ag encounter (Delves & Roitt, 2000). The ability to recognize self from non-self is a key feature of the adaptive immune system, which can be further divided into the cell-mediated and humoral systems, with white blood cells central to proper functioning. Humoral immunity involves the action of B cells to encounter, process, and produce Ag-specific antibodies following Ag encounter. The cell-mediated arm of the immune system provides a highly coordinated defense system through the action of T cells, which recognize the processed Ag and in turn direct the immune response by providing proper signals to multiple other immune cells (Delves & Roitt, 2000). Therefore, the crucial interactions occurring between B and T cells must be tightly regulated to provide adequate protection, and this high level of control is achieved through the activity of a specific T cell, the T helper lymphocyte.

Helper T (T_H) Cells

T lymphocytes exist as two subpopulations, helper T (T_H) cells and cytotoxic T (T_C) cells, and are identified based on the expression of distinct cell surface receptors. T_H cells express a receptor known as CD4 (Cluster of Determination #4) and T_C cells express the CD8 receptor. While T_C cells are known for their ability to eliminate virally infected cells or cancer cell, T_H cells are recognized as crucial immune system directors (Behrens et al., 2004). T_H cells exert this level of regulation via involvement in multiple signaling pathways which control the action of other immune cells following Ag encounter. Helper T cells are able to recognize Ag through interactions with Ag in the context of the major histocompatibility complex II (MHC II), a protein expressed by antigen presenting cells (APC) such as B cells, dendritic cells, and macrophages (Davis & Bjorkman, 1988). The importance of T_H cells is highlighted by the human immunodeficiency virus, which may develop into human acquired immunodeficiency syndrome (AIDS) as the virus invades and eliminates T_H cells, resulting in an immunocomprimised state with reduced ability to prevent infection as a result of foreign pathogens. The fatality of AIDS is directly related to secondary infections, where the immune system cannot respond properly due to the decreased number of circulating T_H cells (Varbanov, Espert, & Biard-Piechaczyk, 2006). Based on the integral role of T_H in proper immune function, the activity of this cell type must be tightly regulated as improper T_H cell activation is a key hallmark of a multitude of disorders including asthma, multiple sclerosis, and cardiopulmonary disease.

T Cell Receptor and T Cell Activation

Central to the function of T_H cells is the presence of a T cell receptor (TCR), a multi subunit protein required to recognize Ag presented via MHCII (Rojo, Bello, & Portoles, 2008). The TCR is composed of two transmembrane polypeptides, α/β or γ/δ, with one variable and one constant domain (Delves & Roitt, 2000). The α/β configuration is the most prominent TCR combination and is responsible for foreign Ag recognition. The ability to recognize Ag is accomplished by the TCR in conjunction with the CD3 signaling complex, composed of five transmembrane polypeptides (γ,ε,δ,ξ, and η) that assemble to form three dimers (γε, δε, and ξξ or ηη). CD3 derived signal transduction, which results in T cell activation, proliferation, and differentiation cannot occur unless the TCR-CD3 complex is accurately assembled (Feito et al., 2002; Davis & Bjorkman, 1988).

While the presence of the TCR-CD3 complex is essential for T cell function, additional cell surface proteins are stimulated during Ag encounter to provide costimulatory signals, which augment the TCR derived signal. The most prominent protein in this category, CD28, is displayed constitutively from all T_H cells and interacts with B cells via the B7 family of proteins (Lenschow, Walunas, & Bluestone, 1996). Following TCR encounter with Ag, the CD28/B7 signaling pathway is activated, further inducing T cell activation via increased signal transduction and lowered T cell activation threshold.

T_H Cell Signal Transduction

Activation of T_H cells is tightly regulated via interactions between the TCR and Ag-MHCII in addition to the role of costimulatory pathways. Upon TCR-Ag encounter, T cell activation is achieved via increased gene transcription. This transcription results in the production of numerous proteins responsible for foreign Ag recognition and removal. In order to achieve this activation state, signal transduction must occur within the T_H cell involving two distinct signaling pathways: calcium and protein kinase C (PKC).

Calcium (Ca^{2+}) Signaling

Intracellular calcium signaling is achieved following TCR/CD3 stimulation, which results in the mobilization of internal calcium stores (Rao, Luo, & Hogan, 1997). This stepwise process begins with the phosphorylation of phospholipase C, which cleaves phosphoinositol biphosphate (PIP_2) into two important second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglyceral (DAG). Of these two second messengers, IP_3 binds to Ca^{2+} receptors in the surface of the cell's endoplasmic reticulum, which opens Ca^{2+} channels resulting in an increase in cytoplasmic Ca^{2+}. Additionally, calcium channels in the plasma membrane are opened, allowing an influx of Ca^{2+} further increasing intracellular Ca^{2+} (Kindt, Goldsby, & Osborne, 2007). Once Ca^{2+} has entered the cytoplasm, it acts upon a group of calcium-sensitive proteins including calmodulin, calcineurin, and the family of nuclear factor of activated T cells (NFAT) transcription factors. The increased intracellular Ca^{2+} combined with the activity of calcium-sensitive proteins causes a synergistic increase in gene transcription, which results in the translation of key immunoregulatory proteins (Rao, Luo, & Hogan, 1997).

Protein Kinase C (PKC) Signaling

Following the phosphorylation of phospholipase C, cleavage of PIP_2 into second messengers IP_3 and DAG occurs with IP_3 influencing Ca^{2+} signaling and DAG directly activating protein kinase C (PKC)-dependent signaling processes (Kindt, Goldsby, & Osborne, 2007). The PKC family is a large family of serine/threonine kinases with ten identified isoforms that are widely distributed in mammalian tissues and are categorized into three groups based on their mode of action (Tan & Parker, 2003). Upon activation, PKC moves from the cytosol to the cell membrane, where it phosphorylates serine and threonine amino acids on transcription factors important to T cell activation, such as the IκB/NFκB complex. Upon phosphorylation, NFκB is released from IκB and translocates to the nucleus, resulting in an increase in gene transcription and translation of additional immunoregulatory proteins (Kindt, Goldsby, & Osborne, 2007). The combined action of Ca^{2+} and PKC signaling provides the proper stimuli required for T cell activation.

T Cell Activation: Membrane Bound Markers

Following ligation of the T cell receptor, T cell activation is achieved and key immunomodulatory proteins are produced, including both membrane-bound and secreted soluble proteins that are crucial for mounting an immune response (Kindt, Goldsby, & Osborne, 2007). Membrane-bound proteins are frequently classified as "activation markers" as these proteins are not constitutively expressed and their level of expression is frequently correlated with the degree of activation stimulus the T cell received. In addition, membrane-bound proteins can have profound effects on the immune system as

they can act as immune system activators themselves. Examples of such membrane-bound proteins are CD40 ligand (CD40L), CD69, and CD25.

CD40 Ligand

Helper T cells exert a high level of control over immune system function partially through the tightly regulated expression of a key protein, CD40 ligand (CD40L) (Schonbeck, Mach, & Libby, 2000). CD40L (CD154, gp39, TRAP) is a type II transmembrane protein, found on activated T_H cells in addition to other immune and non-immune cells such as platelets, basophils, eosinophils, natural killer (NK) cells, B cells, mast cells, dendritic cells, smooth muscle, and epithelial cells , and belongs to the tumor necrosis family (TNF) superfamily (Schonbeck & Libby, 2001). This family of proteins is integral in the control of immune cellular functions such as regulation of proliferation, differentiation, and apoptosis (programmed cell death). Specifically, CD40L expression is essential for proper function of the adaptive immune system where this protein has the ability to regulate the humoral and cell-mediated branches via the aforementioned control of cellular functions (Banchereau et al., 1994). Additionally, the expression of CD40L is considered to be a rate-limiting step in immune system function as small changes in CD40L can have significant biological effects given that a 1.1 to 2.0 fold increase in CD40L can result in a 4- to 5 fold increase in B cell antibody (Ab) production and a similar increase in B cell proliferation and monocyte matrix metalloproteinase production (Perez-Melgosa, Hollenbaugh, & Wilson, 1999; Mach et al., 1999; Hermann, Van Kooten, Gaillard, Banchereau, & Blanchard, 1995). This key protein is involved in the regulation of both humoral and cell-mediated immunity via interactions with its receptor,

CD40 yielding enhanced B cell survival, growth, differentiation, Ab isotype switching, and IgE production in addition to providing signals to increase T cell cytotoxicity, macropharge tumoricidal activity, and enhanced expression of pro-inflammatory cytokines (Schonbeck, Mach, & Libby, 2000; Banchereau et al., 1994). CD40L is essential to proper immune function, as inappropriate expression of this protein has been linked to human diseases such as systemic lupus erythematosus (SLE), atherosclerosis, autoimmune diabetes, and multiple sclerosis (MS) (Schonbeck & Libby, 2001; Xu & Song, 2004). The importance of CD40L is further highlighted by X-linked Hyper IgM syndrome, an immunodeficiency where individuals experience repeated infections, possess defects in cell-mediated immunity and germinal center formation, and the inability of B cells to express IgG, IgA, and IgE Ab isoforms as a result of non-functional CD40L expression (Schneider, 2000).

CD69

The expression of CD69 appears to be the earliest inducible cell surface glycoprotein appearing after immune system activation begins (Cambiaggi et al., 1992). Also known as the activation-inducer molecule or very early activation marker, CD69 is rapidly and transiently expressed by a host of immune cells such as B cells, NK cells, neutrophils, and T cells (Kindt, Goldsby, & Osborne, 2007). The exact function of CD69 has yet to be determined as the ligand for CD69 has not been identified, however it is theorized that CD69 may be an immunomodulatory protein with activation and regulatory properties CD69 expression is also associated with high levels of inflammation and RA, asthma, and acquired immunodeficiency syndrome disease states

(Marzio, Mauel, & Betz-Corradin, 1999). Although the precise biological function of CD69 has yet to be completely elucidated, it is very commonly used as a marker of immune cell activation as described in this study (Marzio, Mauel, & Betz-Corradin, 1999; Sancho, Gomez, & Sanchez-Madrid, 2005).

CD25

Also known as the low-affinity IL-2 receptor or T-cell activation antigen, CD25 is expressed by activated T cells, B cells, macrophages, and other immune cell types. Additionally, $CD4^+$ T cells bearing CD25 surface proteins have recently been identified as a unique T cell subset known as the regulatory T cells, playing a large role in the development of immune system tolerance, although these cells represent a distinct cellular phenotype from stimulated T_H cells displaying CD25 (Nelson, 2004). Similar to CD40L and CD69, this protein is expressed at low levels in the absence of stimuli and is rapidly and transiently expressed following TCR signaling. This membrane-bound protein is a fundamental component of the IL-2/IL-2 receptor signaling cascade resulting in increased T cell activation and proliferation. Additionally, this receptor signaling complex is involved in the growth and differentiation of other immune cells such as B cells, NK cells, and macrophages (Kindt, Goldsby, & Osborne, 2007).

T Cell Activation: Soluble Markers

In addition to the production of membrane-bound proteins, soluble immunomodulatory factors may be produced and secreted by activated T cells. These soluble factors may be truncated versions of the membrane-bound protein produced by

cleavage events, i.e. soluble CD40L, or directly secreted proteins such as cytokines and chemokines, i.e. interleukins (IL). These factors utilize the circulatory system to exert a high level of control over immune system function at distant sites in the body, allowing for removal of pathogens with minimal delay (Delves & Roitt, 2000). T_H cells are classified in two established categories in regards to the soluble cytokines in which they produce, type 1 (T_H1) or type 2 (T_H2) cytokines. Essential for cell-mediated functions, T_H1 cytokines are typically associated with inflammatory-based immune responses, focusing the immune system towards removal of viral and intracellular pathogens. In contrast, T_H2 cytokines function to remove extracellular pathogens by stimulating eosinophils and B cells, resulting in increased Ab production (Kindt, Goldsby, & Osborne, 2007; Zhu & Paul, 2008). The tightly controlled regulation of these cytokines is essential as they may be both pro-inflammatory and anti-inflammatory in nature and altered cytokine production may be associated with different disease states, including asthma where a T_H2 cytokine environment is favored and contributes to disease pathogenesis (Delves & Roitt, 2000; Holgate, 2008).

Soluble CD40 Ligand

As mentioned above, T_H cells produce a key immunoregulatory membrane-bound protein called CD40L. However, a soluble form of this protein, soluble CD40L (sCD40L), also exists and represents a truncated version of the full length protein created by proteolytic cleavage of the transmembrane domain of membrane CD40L (Hirohata, 1999). Despite its truncated state, sCD40L possesses significant biological activity and can induce the CD40 signaling cascade at distant sites via circulatory system distribution,

albeit to a lesser extent than membrane CD40L (Ludewig, Henn, Schroder, Graf, & Kroczek, 1996). Additionally, our lab has shown that membrane CD40L and sCD40L are differentially regulated in response to the various TCR and costimulatory signaling pathways (Matthies, Newman, Hodzic, & Wingett, 2006). Similar to membrane CD40L, sCD40L expression must be tightly regulated as elevated serum levels are associated with diseases such as atherosclerosis, hypercholesterolemia, and SLE (Aukrust et al., 1999; Kato et al., 1999; Vishnevetsky, Kiyanista, & Gandhi, 2004).

While the exact proteolytic cleavage mechanisms responsible for sCD40L production are still being investigated, the process of ectodomain shedding is becoming recognized for its role in producing soluble factors. The process of ectodomain shedding is thought to involve a family of proteins, adamlysins, which include matrix metalloproteinases (Black & White, 1998). Specifically, ADAM (a disintegrin and metalloprotease domain) proteins have been implicated in ectodomain shedding and research conducted by our lab implicates ADAM-10 as a key proteinase involved in the production of sCD40L derived from T cells (Matthies, Newman, Hodzic, & Wingett, 2006; Schlondorff & Blobel, 1999).

Interleukin 8

Interleukin 8 (IL-8) is a prototypic member of the CXC family of chemokines produced by many different cells types, including T cells, in response to pro-inflammatory signals (Kindt, Goldsby, & Osborne, 2007). Also known as the neutrophil activating protein, IL-8 is a chemoattractant cytokine involved in the chemotaxis and activation of neutrophils, in addition to basophils and some lymphocytes (Rajarathnam et

al., 1994). IL-8 is produced in response to a wide variety of stimuli and is recognized as a pro-inflammatory cytokine that is linked with chronic inflammatory diseases such as inflammatory bowel disease and atherosclerosis, as increased IL-8 level expression is positively correlated with progression of these diseases (Grimm, Elsbury, Pavli, & Doe, 1996; Apostolakis, Vogiatzi, Amanatidou, & Spandidos, 2009). In terms of asthma and other chronic respiratory disorders, the activation and recruitment of neutrophils contributes to disease progression by inducing inflammation, which leads to oxidative stress and subsequent cell damage (Pease & Sabroe, 2002; Pease, 2006). Following acute exposure to DEP, bronchial epithelial cells produced more IL-8 than control providing a possible mechanism for the alteration of immune cell subsets in tissues exposed to environmental toxins (Salvi et al., 2000).

Interleukin 17

IL-17 is a pro-inflammatory cytokine, produced primarily from $CD4^+$ T cells, recognized to participate in certain inflammatory diseases. Specifically, IL-17 is central to the initiation and pathogenesis of diseases such as asthma, psoriasis, RA, Crohn's disease, and MS. This cytokine contributes to these disease both directly and indirectly, as IL-17 may induce the production of other pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α (Wang & Liu, 2008). Additionally, IL-17 may contribute in the process of angiogenesis, although through secondary mechanisms (Kindt, Goldsby, & Osborne, 2007). Despite the previously mentioned role as a pro-inflammatory mediator, IL-17 may have dual functions. Initial studies in IL-17 knockout mice showed that IL-17 was required for the development of asthma in a murine model. However, experiments where

IL-17 was neutralized during the effector phase of asthma, where an asthma model system was already established, resulted in increased disease progression (Schnyder-Candrian et al., 2006). Therefore the role of IL-17 in inflammatory diseases needs to be further investigated, especially in the context of asthma where this cytokine exhibits pleiotropic effects.

Asthma

Asthma is a multifaceted disorder, with a recognized immunopathogological component, represents a significant obstacle facing individuals, researchers, and healthcare professionals in our contemporary society. While current research has yielded an increased understanding of asthma with improved medicinal efficacy, the incidence and severity of asthma continues to rise in both adults and children worldwide at an alarming rate (Clement, Jones, & Cole, 2008; Dietert & Zelikoff, 2008). Despite this increased body of knowledge, the exact factors responsible for the observed increase in asthma prevalence, morbidity, and mortality remain unknown. According to the Center for Disease Control Asthma Surveillance Summary, the estimated number of U.S. citizens reporting an asthma episode in the previous 12 months increased from 6.8 million in 1980 (3.1%) to 14.9 million (5.6%) in 1995 (Martinez, 2008). It is estimated that more than 300 million individuals and greater than 20% of the developed world suffer from asthma-related symptoms with an estimated American 5,000 deaths per year (Takhar et al., 2007). Recent data analysis suggests that the rise in asthma may be stabilizing; however disparities are evident when looking at factors such as age, race, sex, and socio-economic class (Moorman et al., 2007). In the context of childhood diseases,

asthma is the most common chronic disease affecting children in the U.S. today (Center for Disease Control, 2003). In terms of economic burden, it is estimated that the annual cost of asthma is greater than 16 billion dollars, which factors in both cost of health care and loss in productivity (Gergen, 2001).

Asthma is a complex inflammatory disease characterized by physiological changes in the airway. These changes include an increase in smooth muscle mass, airway hyperresponsiveness, decreased air flow, and an increase in size and number of mucosal glands resulting in a thickened basement membrane (Ramos-Barbon, Presley, Hamid, Fixman, & Martin, 2005; Munakata, 2006). Key hallmarks of asthma include eosinophil infiltration, activation of T cells, inflammatory cytokine production, and local IgE synthesis in bronchial mucosa (Kay, 2006). Current understanding of disease pathophysiology recognizes that asthma is a chronic condition with activated helper T (T_H) cells facilitating disease progression. As allergens are inhaled and encountered in the airways, APCs process and present Ag to other immune cells (Smit & Lukacs, 2006). This process provides key signals that activate T_H cells, which begin to produce cytokines that drive T_H2 type responses such as IL-3, IL-4, IL-5, and IL-13 (Kay, 2006). The increase in T_H2 cytokine production is paralleled by a decrease in T_H1 cytokines (Ngoc, Gold, Tzianabos, Weiss, & Celedon, 2005). Specifically, activated T cells are thought to be the prominent cell type in chronic asthma and disease severity is positively correlated with activated T_H2 cell count (Kay, 2006; Holgate, 2008). These cytokines affect other cells types such as B cells and eosinophils, which are integral in asthma pathogenesis by production of IgE antibody and release of granular proteins, respectively (Holgate, 2008).

Other cell types such as mast cells, monocytes, macrophages, and basophils are also affected by T_H2 cytokines further enhancing disease processes.

Beta-Adrenergic Receptor Agonists and Adverse Effects in Asthma

As the understanding of asthma pathogenesis continues to increase, so has the usage of beta (β)-adrenergic agonist medications. The mechanism of action of this very commonly used class of medication begins by binding to β-adrenergic receptors, a protype member of the "seven pass membrane" family of proteins that is widely distributed in the respiratory tract and to a lesser extent in cardiac and adipose tissue (Johnson, 2006). This family of receptors, known as the G-protein-linked receptors, may exist in two conformational states; active and inactive. While in the inactive conformation, the β-adrenergic receptor is bound to the three subunits of the G protein (α, β, and γ) in a complex bound to guanosine diphosphate (118). When β-adrenergic agonists (β-agonists), or associated ligands such as glucagon or epinephrine, bind to the receptor, a conformational change is induced producing an activated α subunit through the process of exchanging guanosine diphosphate for guanosine triphosphate (Benovic, 2002). This allows the activated α subunit to disassociate from the βγ complex and initiate a signaling cascade, producing dramatic changes within the cell due to the action of second messengers (Benovic, 2002; Houslay & Kolch, 2000). While IP_3, Ca^{2+}, G proteins, and adenylyl cyclase are all activated through this pathway; the mechanism of β-agonist medications is thought to involve the conversion of adenosine triphosphate to cyclic adenosine monophosphate (cyclic AMP, cAMP) by adenylyl cyclase (Kindt, Goldsby, & Osborne, 2007; Rot & von Andrian, 2004). While the exact mechanism of

cAMP signaling has not been completely elucidated, it is thought to involve the action of protein kinase A (PKA), a tetramer consisting of two regulatory and two catalytic units (Tasken & Aandahl, 2004). The activated catalytic subunits act on target proteins distributed throughout almost all mammalian tissues. In the context of asthma, the end result is relaxation of airway musculature, alleviating asthma symptoms through airway muscle relaxation producing bronchodilation (Newhouse & Dolovich, 1986).

The utilization of β-adrenergic agonist therapeutic properties to alleviate airway constriction is not a new concept. Earliest historical record finds that these medications were used in ancient Chinese herbal medicines to relieve breathing difficulties as early as 3000 BC. The active material, known as Ma Huang, was a plant derived alkaloid now identified as ephedrine, which was not introduced into Western medicine until the 1920's (Sakula, 1986; Sears & Lotvall, 2005). As an understanding of β-adrenergic agonists has advanced, improved second generation medications have been developed that are currently divided into short lasting (4 – 6 h) and long lasting (10 – 12 h) bronchodilators, which represent the most commonly prescribed types of asthma medication utilized today (Johnson, 2006; Salpeter, Buckley, Ormiston, & Salpeter, 2006). While these medications are popular among physicians and patients, the use of long acting β-agonists has been controversial for more than 50 years (Fahy & Boushey, 1995). As evidence accumulated on the potentially life-threatening and deadly β-agonist related events, it was concluded that while the initial result of β-agonist therapy is beneficial for relieving asthma symptoms, usage of these medications was possibly contributing to a worsening of disease and lack of disease control. Therefore, the FDA launched the Salmeterol® Multicenter Asthma Research Trial in order to delineate a direct relationship between

long-acting β-agonists and severe asthma exacerbations and death (Salpeter, Buckley, Ormiston, & Salpeter, 2006). This large scale study found a significant increase in the rate of asthma episodes requiring medical intervention and asthma-related deaths in the cohort receiving long acting β-agonist therapy, as compared to other asthma medications (Nelson, Weiss, Bleecker, Yancey, & Dorinsky, 2006). Based on these findings, a "black box" warning label has been implemented for this medication and is now recommended that β-agonists be restricted for individuals whose asthma is not controlled via other medicinal intervention (Salpeter, Buckley, Ormiston, & Salpeter, 2006). How β-agonists therapy contributes to asthma pathogenesis remains to be determined but several theories exist: tolerance of β-adrenergic agonist receptors to the medication, genetic variability, and that this class of medication may mask the underlying inflammatory processes (Wechsler et al., 2006; Salpeter, Buckley, Ormiston, & Salpeter, 2006). One finding from our lab indicates that the increase in asthma severity, morbidity, and mortality may involve the effect of cAMP modulating β-agonists on the expression of CD40L. Specifically, increases in intracellular cAMP in T cell receptor (TCR) activated T cells can cause a further increase the level of CD40L expression in asthmatics while downregulating expression in similarly activated cells from control subjects, (Wingett & Nielson, 2002; Wingett & Nielson, 2003; Wingett, Forcier, & Nielson, 1999). As the mechanisms of long-acting β-agonists are elucidated, therapies are being developed to maximize the therapeutic effects while minimizing the associated side effects.

Negative Health Effects of Diesel Exhaust Particles

While the biological effects of DEP exposure on the respiratory system can be manifested by coughing, wheezing, and bronchitis, life-threatening respiratory events involving decreased lung function, chronic obstructive pulmonary disease, inflammation, and asthma attacks can occur resulting in an elevated rate of hospital admissions for respiratory distress (Eder, Ege, & von Mutius, 2006; Tatum & Shapiro, 2005; Pope, III & Dockery, 2006; Hesterberg et al., 2009; Maier et al., 2008). In addition to the respiratory system, DEP can affect a multitude of organ systems. Epidemiological, *in vitro,* and *in vivo* studies demonstrate DEP to be a potential carcinogen with lung cancer predominantly manifested, with bladder and lymphatic tissues cancers occurring less frequently following occupational exposure (Ris, 2007; Lewtas, 2007). Acute and chronic exposure to DEP has also been linked to increased cardiac-related morbidity and mortality with recent attention focused on the contribution of DEP in neurodegenerative and other neurologic pathologies (Pope, III & Dockery, 2006; Ris, 2007; Hartz, Bauer, Block, Hong, & Miller, 2008).

Diesel Exhaust and Asthma

Epidemiological studies demonstrate a positive relationship between decreasing air quality and the rate of asthma exacerbations, especially those requiring medical intervention (Holguin, 2008). In addition to the change in exacerbation rate, the severity of these events is increased, resulting in an amplified asthma related morbidity and mortality, especially in children (Pandya, Solomon, Kinner, & Balmes, 2002; Riedl & Diaz-Sanchez, 2005; Parker, Akinbami, & Woodruff, 2009; Kim et al., 2008; Samuelsen,

Nygaard, & Lovik, 2008). The underlying mechanism of these events is unknown but studies have shown associations between the proximity to high traffic roadways, especially mass transit routes such as freeways, and respiratory distress (Lwebuga-Mukasa, Oyana, Thenappan, & Ayirookuzhi, 2004). Recent studies have linked traffic-related PM with negative health effects by utilizing geographic information systems technology to establish associations between major traffic ways and adverse health events (Samuelsen, Nygaard, & Lovik, 2008; Annesi-Maesano et al., 2007; McEntee & Ogneva-Himmelberger, 2008). Additionally, the increased medical intervention in children has been linked to living near major roadways versus suburban locations (Morgenstern et al., 2007). A recent large scale national study on respiratory stress in children confirmed this relationship as individuals living within 20 miles from EPA pollution monitoring stations that had increased levels of $PM_{2.5}$ were more likely to experience adverse respiratory events (Parker, Akinbami, & Woodruff, 2009).

In addition to the epidemiological studies, *in vivo* and *in vitro* analyses confirm a role for air pollution, specifically diesel exhaust, in asthma exacerbations. Some of these studies implicate that the deleterious effects observed for respiratory health may involve an inflammatory component as DEP exposure is linked to asthma exacerbations and increased asthma-related morbidity and mortality, known to be partially immune-related (Holgate, 2008; Mitschik, Schierl, Nowak, & Jorres, 2008). While the inhalation of PM can act as a direct respiratory irritant, the carbon core of DEP can bind allergens and toxins from the environment further enhancing DEP-related toxicity (Hesterberg et al., 2009). *In vitro* and *in vivo* studies have shown DEP exposure to contribute to asthma pathogenesis via increased allergen-specific IgE, production of asthma-associated

cytokines, and increased airway hyperresponsiveness (Inadera, 2006; Nel, Diaz-Sanchez, Ng, Hiura, & Saxon, 1998). Specifically, allergen sensitive individuals exposed to ragweed pollen plus DEP produced significantly more allergen-specific IgE (16-fold greater) in comparison to DEP alone, identifying DEP as a possible immune system adjuvant (Diaz-Sanchez, 1997). Animal studies further support the adjuvant capacity of DEP as allergen-associated inflammation was further heightened when DEP treatment was combined with model allergens such as ovalbumin or lipopolysaccharide (Ris, 2007; Inadera, 2006). Human controlled-exposure studies using short term exposures to DEP show that exposure can alter immune cell distribution in bronchalveolar lavage fluid and bronchial biopsies such as neutrophils, mast cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, and platelets, in addition to increasing neutrophils in sputum samples (Salvi et al., 1999). The alteration of immune cell subsets and the adjuvant role of DEP implicates this environmental pollutant as a key contributor to the pathogenesis of asthma and other allergic diseases. Despite these advances in knowledge, it still unclear how DEP modulate T cell function by altering activation, distribution, and cytokine production in T_H cells. One of the goals of the present study is to further advance the field of DEP research by addressing this gap in knowledge.

Diesel Exhaust, Immune Cells, & Inflammatory Cytokines

While it is accepted that DEP are a health hazard, the exact mechanisms of action for these particles in the living organism remain unclear. Studies conducted on airway epithelial cells *in vitro* demonstrate many different affects. While these studies are hard to compare as experimental conditions vary, the main point of these studies is that

inhalation of diesel exhaust particles can alter normal epithelial cell function (Mitschik, Schierl, Nowak, & Jorres, 2008). Other studies have focused on the ability of DEP to alter cytokines, which contribute to asthma pathogenesis. Specifically, the T_H2 family of cytokines is of interest as these cytokines are associated with asthma pathogenesis. DEP exposure to both human and animal immune cells results in an increase in the expression of IL-4, IL-5, IL-13 and decrease the production of IL-2 and IFN-γ, which favors a T_H2 cytokine environment (Mamessier, Nieves, Vervloet, & Magnan, 2006; Riedl & Diaz-Sanchez, 2005; Ohtani et al., 2005; Pourazar et al., 2004; Takano, Yanagisawa, & Inoue, 2007; Samuelsen, Nygaard, & Lovik, 2008; Stevens, Cho, Linak, & Gilmour, 2009; Gowdy et al., 2008). *In vitro* studies further confirmed the role of T_H2 cells by polarizing peripheral blood mononuclear cells from healthy individuals towards either T_H1 or T_H2 phenotype and exposing the cells to DEP. Only the T_H2 polarized cells had the ability to undergo chemotaxis, allowing these cells to migrate to the site of DEP exposure (Chang et al., 2006). Considering the role of T_H2 cells in asthma pathogenesis and the ability of DEP to alter this balance, it is plausible that DEP can alter production of these cytokines to favor T_H2 type cell development and further promote asthma disease progression providing a possible link to the epidemiological studies that demonstrate an association between particulate matter levels and adverse respiratory events.

Based on the integral role of T_H cells in the development and pathogenesis of allergic disease, it is surprising to find a lack of research on the direct effects of DEP exposure on T_H cell activity. The research presented in this study contributes to the body of knowledge identifying DEP as a major health concern by showing *in vitro* evidence of an immunomodulatory effect of DEP on T_H cells. Specifically, DEP exposure alters the

tightly regulated expression of CD40L with a consistent and preferential induction of the membrane-bound isoform versus the less biologically active soluble form of the CD40L protein. The ability of DEP to augment CD40L expression, both soluble and membrane forms, in the context of an *in vitro* asthma model is also reported, providing a mechanism for the negative effects of DEP on at-risk individuals. Additionally, DEP exposure alters production of an important immunomodulatory cytokine factor, IL-17, without having any appreciable effect on T_H cell viability. Collectively, these findings elucidate a potential molecular mechanism for the deleterious effects of DEP exposure on T_H cells, as measured by changes in key parameters of immune system function.

EXPERIMENTAL PROCEDURES

Diesel Particulate Matter

Diesel exhaust particles (DEP) SRM 2975 was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). These particles were generated by a heavy-duty forklift diesel engine and collected using a filtering system designed for diesel forklifts under "hot" conditions, without a dilution tunnel (Singh et al., 2004). Certified analyses for SRM 2975 are accessible online (National Institute of Technology, 2000). DEP solutions were prepared in phosphate buffered saline (PBS) and sonicated in a water bath for 15 min. Particles were vortexed immediately prior to dispensing into each individual cell culture well.

Human Subjects and Isolation of $CD4^+$ T Cells

Written, informed consent was obtained from all blood donors and the University Institutional Review Board approved this study. For isolation of $CD4^+$ T cells, peripheral blood mononuclear cells (PBMC) were obtained via Ficoll-Hypaque (Histopaque-1077, Sigma, St Louis, MO) gradient centrifugation using heparinized phlebotomy samples as previously described (Coligan, 1995). After removal of the leukocyte layer, cells were washed three times with Hank's buffer (Sigma, St. Louis, MO) and once in PBS. After

isolation of human PBMC, $CD4^+$ T cells were purified using negative immunomagnetic selection per manufacturer's instructions using a cocktail of antibodies against CD8, CD14, CD16, CD19, CD56, and glycophorin A (StemCell Technologies, Vancouver, BC) with collection of unlabeled T cells (typically >96% $CD4^+$ and >98% viable as assessed by flow cytometry). Purified $CD4^+$ cells were subsequently cultured in RPMI-1640 media (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C and 5% CO_2.

T Cell Activation and Culture Conditions

Purified $CD4^+$ cells were cultured in RPMI/10% FCS at 1 x 10^6 cells/ml in 200 μl total volume and activated using immobilized anti-CD3 Ab (OKT3, ATCC, Rockville, MD) at 0.24 μg/well or anti-CD3/anti-CD28 beads (Dynabeads® CD3/CD28 T Cell Expander, Invitrogen, Oslo, Norway) at a 1:1 bead to cell ratio in 96-well tissue culture plates (Fisher Scientific, Pittsburg, PA). Alternate activation schemes involved treating purified $CD4^+$ T cells with various combinations of ionomycin (Calbiochem, San Diego, CA), phorbol 12-myristate 13-Acetate (PMA, Sigma), and/or 100 μM dibutyryl adenosine monophosphate (dbcAMP, Sigma) was utilized in combination with ionomycin. Cultures were concurrently treated with varying concentrations of freshly prepared and sonicated DEP for 24 h of culture.

Antibodies and Flow Cytometry

Methods of immunofluorescent staining and flow cytometry were performed as previously described (Coligan, 1995). Briefly, cells were harvested from 96-well culture

plates into FACS buffer and pelleted by centrifuged at 2000 rpm for 7 min. The cell pellet was resuspended in FACS buffer and stained with fluorescently labeled antibodies for 30 min at 4°C, washed two times, fixed in 2% paraformaldehyde, and analyzed on a 4-color Epics flow cytometer (Beckman Coulter, Miami, FL). Ten thousand events gated on size and side scatter were analyzed and the expression of the percentage of positively staining cells or the mean fluorescence intensity (MFI) determined by comparisons to appropriate isotype controls. Phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated anti-CD40L, anti-CD69, anti-CD25, or isotype control antibodies were purchased from Beckman Coulter or BD Biosciences (San Jose, CA). Appropriate concentrations of each Ab were determined by titration for optimal staining prior to experimental use.

Cell Viability Assay

To assess the effect of DEP on $CD4^+$ T cell viability, propidium iodide (PI) staining was employed. Purified T cells were treated with increasing concentrations of DEP (0-800 μg/ml) for 24 h. Cell viability was determined by staining with 50 μg/ml of PI, a fluorogenic dye used to monitor losses in cell membrane integrity, for 10 min prior to flow cytometric analysis.

Enzyme-Linked Immunosorbent Assay

Levels of soluble CD40L (sCD40L) in culture supernatants was quantified using an enzyme-linked immunosorbent assay (ELISA). $CD4^+$ T cells were treated with various cell specific stimuli, as described above, for 24 h. Following incubation, cell-free

supernatant was harvested via successive 10 min centrifugations (2,000 rpm, 7,000 rpm and 13,000 rpm) and cell supernatants stored at -80°C until analysis. A 96-well plate (Nunc-Immuno Module, Nunc, Rochester, NY) was coated with 50 µl of TRAP-1 monoclonal Ab (R & D Systems, Minneapolis, MN) at 12 µg/ml in 0.05 M carbonate buffer (pH 9.4) and incubated for 2 h at 37°C. Wells were blocked with 10% FCS/2% azide in PBS and incubated at room temperature (RT) for 2 h. Samples (50 µl/well) were added to the plate and incubated overnight at 4°C. Serial dilutions of recombinant human sCD40L protein standard (Bender Medsystems, Burlingame, CA) were used for standardization. Bound sCD40L was detected using 50 µl/well HRP-conjugated M90 mAb (ATCC, Manassas, VA) at 12 µg/ml in 10% FCS in PBS and incubated at RT for 2 h. The assay was developed with 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma) and incubated at RT protected from light for 30 min. The reaction was stopped with 0.5 M H_2SO_4 and optical density determined at 450 nm and reference wavelength at 630 nm, on a microplate reader (Bio-Rad, Hercules, CA).

ELISA assays were also used to investigate the effect of DEP treatment on IL-8 and IL-17 cytokine production using primary $CD4^+$ T cells (5×10^6 cells/ml) treated with anti-CD3/anti-CD28 Dynabeads® (200 beads/ml) for 24 h ± DEP. Following DEP treatment, cell-free supernatant was harvested via successive 10 min centrifugations (2,000 rpm, 7,000 rpm and 13,000 rpm) and cell supernatants stored at -80°C until analysis. ELISA assay was performed by the UMAB Cytokine Core Laboratory (Baltimore, MD) with all samples analyzed in triplicate and cytokine production expressed in pg/ml.

In Vitro Cleavage of CD40 Ligand by Recombinant ADAM-10

For *in vitro* CD40L cleavage assays, the murine CD40L/CD33 fusion protein was used (R&D) which contains amino acids 61-260 of the extracellular CD40L domain joined to the signal peptide of human CD33 followed by 10 histidine residues and a linker peptide. The cleavage of 0.5 μg of murine CD40L/CD33 fusion protein was accomplished using 20 μg/ml recombinant mouse ADAM-10 (R&D) for 5 h at 37°C in a total reaction volume of 25 μl. The cleavage products were separated by 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) under reducing conditions and transferred to a polyvinylidene difluoride membrane utilizing a wet transfer system (Bio-Rad Life Sciences Research, Hercules, CA). Detection was performed using an anti-CD154 C-20 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a secondary goat anti-rabbit horseradish peroxidase-conjugated Ab (Santa Cruz) using an enhanced chemiluminsence system (PerkinElmer Life Sciences, Boston, MA).

Data Analysis

Statistical significance was determined using either a two-way paired Student's T-test or repeated measures analysis of variance (ANOVA) with post hoc comparisons. A Student's T-test was utilized to determine the effect of DEP treatment between treatments in paired samples. A repeated measures ANOVA with post hoc comparisons was used when two or more measurements of the same type were made on the same subject to determine statistical differences between the means and to allow separation of within-subject variation from between subject variation. ANOVA analysis was performed using

SAS (Statistical Analysis Systems Incorporated) software (Cary, NC). Significance levels were defined as $p < 0.05$.

RESULTS

Effects of DEP on Resting T Cells

Although limited studies have demonstrated an immunomodulatory effect of diesel exhaust particles (DEP) on select cell types and biological systems, additional studies are needed to assess the role of DEP on normal T_H cell function. To help address this gap in knowledge, experiments were conducted to assess the effects of DEP on the expression of T cell activation markers in the absence of external stimuli. Purified human peripheral blood $CD4^+$ T cells were treated with 800 μg/ml DEP for 24 h and expression of CD40L, CD69, and CD25 membrane proteins evaluated using flow cytometry. As shown in Fig. 1, CD40L expression was markedly upregulated following treatment with DEP with a 3.5-fold increase observed compared to control samples ($p < 0.0001$). A slight yet significant increase (1.5-fold) in CD69 expression was also observed ($p = 0.037$), although to a considerably lesser extent than CD40L. In addition, an increasing trend for CD25 was also observed (1.1-fold), although this did not achieve statistical significance ($p = 0.068$). These results indicate that DEP can alter helper T cell activation in resting cells, with the most pronounced effects observed for CD40L expression.

Additional studies were performed to verify that DEP-induced CD40L expression occurred in a concentration-dependent manner. Purified T cells were treated with

increasing concentrations of DEP (200 – 800 μg/ml) for 24 h and evaluated using flow cytometry. As shown in Fig. 2, a reproducible 1.7-fold increase in membrane CD40L expression was observed at the lowest concentration of DEP (200 μg/ml), although statistical significance was not achieved in these samples ($p = 0.11$). In contrast, DEP induced significant increases in CD40L expression at both 400 μg/ml of DEP (2.5-fold increase, $p = 0.03$) and at 800 μg/ml (3.5-fold increase, $p = <0.0001$). Higher concentrations of DEP (i.e. 1600 μg/ml) induced similar levels of CD40L compared to that observed with 800 μg/ml DEP (data not shown).

DEP Augments Physiological Activation of T Cells

Additional studies were performed to evaluate the effects of DEP on activated T cells using a variety of physiologically relevant activation schemes. The first set of experiments were designed to assess the immunomodulatory effects of DEP on cells previously activated through the T cell receptor (TCR) signaling pathway using antibodies directed against the CD3 component of the TCR to mimic the potential effects of DEP exposure in patients with low-level immune cell activation that can accompany chronic inflammatory disease. Purified $CD4^+$ T cells were pre-treated with immobilized anti-CD3 Ab for 6 h, removed from activation stimuli and subsequently treated with DEP for 18 h. Cells were then evaluated for changes in T cell activation markers using flow cytometry. Notably, a modest, yet reproducible and statistically significant increase in DEP-induced CD40L expression (1.5-fold, $p = 0.03$) was observed, Fig. 3A. In contrast, neither CD69 nor CD25 expression (Figs. 3B and 3C) was induced by DEP exposure using this activation scheme ($p = 0.81$ and 0.11, respectively).

The effects of DEP were next investigated using T cell stimuli consisting of combined activation through the TCR signaling pathway and CD28 costimulation. The CD28 costimulatory pathway plays a major role in T cell activation by lowering the activation threshold, or the number of bound TCRs required to achieve activation, by ~8-fold (Salomon & Bluestone, 2001; Lanzavecchia, Lezzi, & Viola, 1999). To simultaneously provide both signals important for T cell activation, polystyrene beads with covalently linked anti-CD3 and anti-CD28 antibodies (Dynabeads®) were used to optimally activate $CD4^+$ T cells. This activation scenario utilizing beads of similar size to lymphocytes is recognized to better mimic the physiological conditions occurring during antigen presentation between T cells and APC than activation using plate-immobilized antibodies (Trickett & Kwan, 2003). Following 24 h of culture, T cell activation markers were evaluated using flow cytometry. DEP was found to significantly augment (1.2-fold, $p = 0.03$) the expression of CD40L protein (Fig. 4). In contrast, a decreasing trend in CD69 expression was observed following DEP treatment (1.2-fold, $p = 0.68$) and a slight, yet insignificant, increase in CD25 was observed (1.1-fold, $p = 0.18$). Collectively, these findings suggest potentially unique actions of DEP to consistently enhance CD40L expression in both resting T cells, as well as in the presence of different activation stimuli.

DEP Plus PKC Signaling Results in Increased T Cell CD40L

Based on the observation that CD40L expression is increased in response to DEP exposure in TCR activated cells, experiments were performed to dissect TCR signal transduction events into the component calcium and protein kinase C (PKC) dependent

pathways. The PKC pathway was isolated using the phorbol ester, PMA, which is well recognized as a chemical initiator of PKC-dependent signal transduction in T cells (Ding, Green, Thompson, & Shevach, 1995). For these studies, primary $CD4^+$ T cells were concurrently treated with PMA and DEP for 24 h, and changes in expression of CD40L, CD69, and CD25 measured using flow cytometry. As shown in Fig. 5A, DEP was found to induce CD40L expression (3.5-fold, $p < 0.0001$). In contrast, no effects on CD25 or CD69 expression were observed at this particular concentration of PMA, which induced near maximal expression of these proteins (data not shown). In order to reveal any possible actions of DEP to modulate CD25 and CD69 expression, a lower concentration of PMA was used. As shown in Fig. 5B and 5C, DEP caused a modest reduction in CD25 expression (1.3-fold, $p = 0.05$), while no effects on the percentage of CD69 positive cells were discernable, which could be related to the fact that the very low concentration of PMA used in these experiments still induced near maximal levels of CD69 expression. However, the mean fluorescence intensity (MFI) of CD69 was decreased by DEP (1.3-fold; $p = 0.008$; MFI values of 299.2 ± 1.7 vs. 230.2 ± 7.8). Therefore, the ability of DEP to augment PKC–dependent signaling may be relatively unique to CD40L as either the percentage of positively staining cells or the MFI of CD25 and CD69 was reduced by DEP.

Further dissection of the TCR signaling pathway was achieved using the calcium ionophore, ionomycin, to activate calcium-dependent signaling as commonly described (Nusslein et al., 1996; Chatila, Silverman, Miller, & Geha, 1989). Primary $CD4^+$ T cells were treated with ionomycin in the presence of DEP for 24 h and changes in membrane expression of CD40L, CD69, and CD25 evaluated. As shown in Fig. 6A, DEP plus

calcium ionophore produced modest but consistent increases in CD40L expression (1.3-fold, $p = 0.03$), although not to the extent of PMA activated samples (Fig. 5A). In contrast, a slight, yet significant, DEP-induced decrease (1.1-fold, $p = 0.004$) in CD25 expression was observed (Fig. 6B). As shown in Fig. 6C, no effects of DEP on the percentage of CD69 positive cells were discernable, but again this could be related to the fact that near maximal expression of this protein was induced by ionomycin. Nevertheless, a significant 2.1-fold decrease in the MFI of CD69 (144.8 ± 2.6 to 70.7 ± 7.3) was again observed following treatment with DEP, $p = 0.011$, indicating yet another circumstance where CD40L expression appears to be preferentially induced by DEP relative to the other activation markers studied.

Additional studies were performed to evaluate the effects of DEP in cells activated by both calcium- and PKC-dependent signaling pathways. As the combined treatment of PMA and Ca^{2+} can frequently result in the synergistic and maximal expression of CD40L (Chatila, Silverman, Miller, & Geha, 1989), lower concentrations of these physiological agents were utilized for these experiments. As shown in Fig. 7, at both of the lower concentrations of PMA and ionomycin tested, a slight yet significant induction of CD40L was observed following DEP exposure (1.5-fold in 0.125 μM ion/0.30 ng/ml PMA and a 1.1-fold in 0.25 μM ion/0.67 ng/ml PMA activated cells, $p < 0.0001$ and $p = 0.017$, respectively). No measurable induction of CD40L was noted at the highest concentrations of PMA/Ionomycin used, but this is not unexpected as these concentrations of mitogens induced near maximal levels of CD40L on their own. As both PKC and calcium pathways are involved in TCR signaling, the results obtained from

these studies are consistent with those presented in Fig. 3, where DEP also induced CD40L expression in CD3/TCR activated cells.

DEP Differentially Regulates the Expression of Membrane and Soluble CD40L

Based on the observation that membrane CD40L expression is induced by DEP, experiments were performed to evaluate the effects of DEP on the expression of the soluble form of the CD40L protein (sCD40L), which also has biological activity and can function in a cytokine-context by distribution throughout the body. As previously shown by our lab, the membrane and soluble forms of the CD40L protein can be differentially regulated by the PKC and Ca^{2+} pathways, with sCD40L being preferentially produced following PKC signaling (Matthies, Newman, Hodzic, & Wingett, 2006). Following 24 h of DEP/PMA exposure, cells were concurrently evaluated for membrane CD40L and sCD40L by flow cytometry and ELISA as shown in Fig. 8A & B. In contrast to the DEP-induced increase in membrane CD40L (2.7-fold, $p = 0.005$), the production of sCD40L was not altered following DEP exposure in the absence of external stimuli ($p = 0.11$). In samples activated with PMA and DEP, and consistent with our previous experiments (Fig. 5), membrane CD40L expression was increased (3.5-fold, $p < 0.0001$) by DEP as shown in Fig. 8A. In marked contrast, sCD40L levels were reduced by 2.4-fold following DEP treatment, (Fig. 8B, $p = 0.04$).

In parallel experiments, the effects of DEP to alter sCD40L/membrane CD40L expression in ionomycin activated cells were evaluated. As shown in Fig. 8C, DEP induced the expected increase (1.3-fold, $p = 0.004$) in membrane CD40L expression in ionomycin activated cells. In contrast, DEP failed to appreciably affect sCD40L

expression in ionomycin-activated cultures (Fig. 8D, $p = 0.48$). Collectively, the results presented in Fig. 8 indicate that the membrane and soluble forms of the CD40L protein can be differentially regulated by DEP.

To further investigate the ability of DEP to polarize CD40L expression towards the membrane-bound form, experimental conditions combining ionomycin and PMA to mimic the calcium and PKC signaling pathways accompanying T cell receptor ligation (Chatila, Silverman, Miller, & Geha, 1989), as well as direct TCR activation using anti-CD3 and CD28 antibodies were used. As shown in Fig. 9A, following 24 h of PMA/ionomycin activation, sCD40L expression was decreased by an ~1.3-fold, $p = 0.018$. In parallel experiments involving CD3 (TCR)/CD28 antibodies linked to polystyrene Dynabeads®, DEP was also shown to produce a significant decrease (1.6-fold, $p = 0.04$) in sCD40L expression (Fig. 9B). The effects of DEP to enhance the production of membrane CD40L in both PMA/ionomycin and anti-CD3/CD28 activated cells was shown previously (see Fig. 4 and 7). These finding indicate the ability of DEP to preferentially induce the membrane form of the CD40L protein relative to the soluble protein.

Effects of DEP on ADAM-10 Mediated Proteolytic Cleavage of CD40L

Given that DEP has different regulatory effects on the production of membrane and soluble CD40L protein forms, experiments were performed to gain insights into the mechanisms of DEP action. It has been recently been reported that stimuli leading to the differential production of membrane or soluble CD40L proteins can involve the action of matrix metalloproteinases, including ADAM-10 (Matthies, Newman, Hodzic, & Wingett,

2006; Jin et al., 2001). This protease has been implicated in the process of ectodomain shedding, a process integral for the production of numerous soluble factors including Beta-amyloid precursor protein, Notch, CXCL13, and Type IV collagen (Parkin & Harris, 2009; Abel et al., 2004; Millichip, Dallas, Wu, Dale, & McKie, 1998; Tian et al., 2008). Most relevant to this study, ADAM-10 has been shown to contribute to the production of T cell-derived sCD40L by generating this smaller protein product via cleavage from the full-length membrane-bound CD40L precursor molecule (Matthies, Newman, Hodzic, & Wingett, 2006). In the present study, experiments were performed to determine whether the preferential production of membrane CD40L relative to sCD40L occurred by actions of DEP to inhibit ADAM-10-mediated cleavage of the full-length protein. Recombinant ADAM-10 enzyme was incubated with a murine CD40L fusion protein (32 kDa), the mixture fractionated by SDS–PAGE, and Western blotting performed to detect the sCD40L product. As shown in Fig. 10, in a control sample containing the purified CD40L fusion protein, only a single band of ~32 kDa corresponding to the full-length membrane form of CD40L was observed (Lane 1). Likewise, in a control containing the murine CD40L fusion protein and DEP, only the 32 kDa band was detected (Lane 2). The addition of the ADAM-10 protease to the CD40L fusion protein resulted in the generation of an ~18 kDa fragment corresponding to sCD40L, and another band of ~ 9 kDa, as shown in Lane 3. The 18 kDa band has been previously described by multiple labs (Graf et al., 1995; Ludewig, Henn, Schroder, Graf, & Kroczek, 1996; Matthies, Newman, Hodzic, & Wingett, 2006; Mazzei et al., 1995), while the 9 kDa cleavage product likely represents an additional cleavage event by ADAM-10 that is detected by the polyclonal antibody used in our studies. Of interest,

DEP increased the abundance of the 18 kDa band, while markedly decreasing the intensity of 9 kDa band (Lane 4). Although somewhat contrary to the initial hypothesis, these results indicate that DEP can alter the activity of ADAM-10 to proteolytically process the CD40L protein. This is indicated by the ability of DEP to block the production of the 9 kDa fragment and concurrently increase the 18 kDa product, leading to a slight overall increase in the level of sCD40L in this cell-free system. It should also be noted that slightly lower levels of full-length 32 kDa CD40L protein appear in both samples containing DEP. This is likely due to the presence of very high molecular weight aggregates containing DEP-absorbed CD40L fusion protein, interfering with some of the loaded protein's ability to migrate into the gel matrix. Nevertheless, our results indicate that the presence of DEP can alter the proteolytic activity of ADAM-10 cleavage of CD40L, leading to an increase in the 18 kDa product, known as sCD40L, while simulataneously preventing the formation of a smaller 9 kDa CD40L-derived fragment.

IL-17, Not IL-8, Production Is Decreased Following DEP Exposure

To further investigate the effects of DEP on T cell activation processes, changes in the expression of two T cell cytokines, IL-8 and IL-17, were evaluated. Both of these cytokines are considered to be proinflammatory factors, where their altered expression has been implicated in the pathogenesis of various inflammatory disease, including asthma (Hashimoto, Akiyama, Kobayashi, & Mori, 2005; Pease & Sabroe, 2002). $CD4^+$ T cells were activated using soluble anti-CD3/CD28 Dynabeads® and concurrently treated with DEP. Following 24 h, cell free supernatants were harvested and evaluated by ELISA. As shown in Fig. 11, DEP was found to inhibit the production of IL-17 by

2.9-fold in comparison to control cells, $p = 0.04$. In contrast, DEP had no significant effect on IL-8 production following treatment with DEP ($p = 0.28$). The DEP-induced decrease in IL-17 generation may be relevant to allergic diseases, or other conditions involving an immune component as this cytokine, while considered a proinflammatory cytokine, exhibits pleiotropic effects and may actually be beneficial in the amelioration of certain diseases (Schnyder-Candrian et al., 2006; Miossec, 2009). Further research is needed to elucidate the implications of IL-17 inhibition in the context of asthma and other allergic diseases.

T Cell Viability Is Not Altered Following DEP Exposure

Based on our findings that DEP may have immunomodulatory effects capable of altering cytokine production and the expression of certain T cell activation markers, experiments were performed to evaluate the extent to which DEP potentially affects T cell viability. Resting purified $CD4^+$ cells were treated with increasing concentrations of DEP (0-800 μg/ml) for 24 h and effects on viability determined using propidium iodine (PI) staining and flow cytometry. PI is a red fluorescent nuclear stain that enters only cells with disrupted plasma membranes, a characteristic utilized as an indicator of cell death (Riccardi & Nicoletti, 2006). As shown in Fig. 12, no measurable effects of DEP on T cell viability were observed using identical experimental conditions in which significant enhancement of CD40L was detected.

DEP Augments Cyclic AMP-induced Membrane and Soluble CD40L Expression

As mentioned previously, the ability of DEP to augment immune cell function has been implicated in the increase in asthma prevalence in developed countries (Kagawa, 2002). Our observation that DEP can induce T cell CD40L expression under a variety of relevant activation stimuli (Figs. 1-7) led us to examine the contributions that alterations in cyclic AMP (cAMP) may exert, given that actions of the most commonly prescribed class of asthma medications, β-adrenergic agonists, function to increase intracellular cAMP. Despite its well recognized immunosuppressive role, cAMP has been recently recognized to have certain pro-inflammatory properties. For example, dbcAMP, a cell permeable analog of cAMP, has been shown to markedly increase T cell CD40L expression, especially in cells activated with calcium ionophore or costimulated to increase their intracellular calcium levels (Wingett, Forcier, & Nielson, 1999). Therefore, we investigated whether the combined treatment of cAMP and DEP leads to a greater, or possibly even synergistic, induction of T cell CD40L expression. For these experiments, purified cells were treated with ionomycin or ionomycin and dbcAMP, and concurrently treated with DEP or vehicle control. After 24 h of culture, CD40L was measured using flow cytometry. As previously reported, treatment with dbcAMP in the absence of external physiological stimuli failed to appreciably affect CD40L expression (Nielson & Wingett, 2002). DEP in the absence of external stimuli resulted in a significant increase in membrane CD40L (3.1-fold, $p = 0.005$), further validating the immunomodulatory effect shown in Figs. 1A and 2. Also as expected (Wingett, Forcier, & Nielson, 1999), calcium ionophore alone was sufficient to induce moderate levels of CD40L, while the addition of dbcAMP resulted in even greater CD40L expression (1.7-

fold). Of interest, the combination of DEP plus dbcAMP plus ionomycin lead to the greatest induction of membrane CD40L that was 1.2-fold higher than cells treated with ionomycin and dbcAMP alone, $p = 0.002$.

With regards to sCD40L expression, treatment with DEP alone had no effect on sCD40L. However, the 3-way combination of DEP plus dbcAMP plus ionomycin lead to a marked increase (1.9-fold) in sCD40L compared to ionomycin plus dbcAMP controls (Fig. 13B, $p = 0.0004$). This finding is notable as only in this context has DEP been shown to increase sCD40L production, which could be very relevant to asthmatics who are commonly prescribed cAMP-inducing asthma medications. However, in contrast to membrane expression, DEP in the absence of stimuli was insufficient to induce sCD40L production. In the instance where β-agonists, DEP or other emission-based environmental pollutants, and low levels of chronically activated T cells exist (i.e. chronic asthmatics using beta-agonists in industrialized areas), this trifecta could lead to elevated circulating levels of membrane and biologically active sCD40L, which could mechanistically contribute towards asthma exacerbations associated with DEP exposure.

DISCUSSION

Diesel exhaust particles (DEP) represent a noteworthy and complex environmental health concern with extensive evidence accumulating to support a causal relationship between DEP and deleterious health impacts. Epidemiologic studies have linked high levels of ambient PM pollution to increased hospital admissions, morbidity and mortality for cardiovascular, neurological and pulmonary events (Pope, III & Dockery, 2006; Ris, 2007; Hesterberg et al., 2009; Maier et al., 2008). Current understanding of DEP suggests it possesses immunoregulatory properties altering immune system function, potentially contributing to the observed increase in life-threatening events following PM exposure. As activated T cells are important immune cells involved in the pathogenesis of the aforementioned conditions, experiments were performed to clarify the role of DEP on healthy T cell function, specifically the helper T cell subset.

Helper T (T_H) cells are integral regulators of immune system function and activation is tightly regulated. The data presented in this study provides evidence of an immunomodulatory effect of DEP as measured by the alteration of T_H cell activation by increasing the expression of T cell activation marker, CD40L (Fig. 1). The ability of DEP to induce CD40L expression may be very relevant to allergic disorders as

expression of this protein, primarily from activated T cells, is transiently expressed with peak expression occurring 3-6 h after activation (Roy, Waldschmidt, Aruffo, Ledbetter, & Noelle, 1993). While a small enhancement of CD69 and CD25 expression was also observed, it was to a lesser extent (Fig. 1), accounting for a potential ability of DEP to exacerbate allergic and inflammatory-based diseases, especially those involving altered CD40L expression (Pandya, Solomon, Kinner, & Balmes, 2002). In addition, CD40L is essential to proper immune function, as inappropriate expression may contribute to the pathogenesis of disorders such as SLE, diabetes, and MS (Schonbeck & Libby, 2001; Xu & Song, 2004). To further validate the effects of DEP on CD40L expression, a scheme was utilized to mimic the effects of DEP exposure where individuals may be exposed to greater levels of DEP as a result of occupational exposure or housing location. A modest, yet dose-dependent induction of CD40L following DEP exposure was observed (Fig. 2), suggesting that individuals living in proximity to emission sources or those with an occupational exposure may be at higher risk for the deleterious effects of DEP (Samuelsen, Nygaard, & Lovik, 2008; Annesi-Maesano et al., 2007; McEntee & Ogneva-Himmelberger, 2008). The ability of DEP to alter CD40L expression in the absence of T cell specific stimuli is concerning as even small changes can have significant biological effects given that a 1.1 to 2.0 fold increase in CD40L can result in a 4-5 fold increase in B cell antibody (Ab) production and a similar increase in B cell proliferation and monocyte matrix metalloproteinase production (Perez-Melgosa, Hollenbaugh, & Wilson, 1999; Mach et al., 1999; Hermann, Van Kooten, Gaillard, Banchereau, & Blanchard, 1995). Therefore, CD40L is recognized as a rate-limiting step in proper immune system function and overall human health.

Experiments were performed to assess the impact of DEP on activated cells, as individuals living with existing health conditions including asthma and other respiratory diseases, are considered to be at higher risk to the deleterious effects of DEP (Holguin, 2008). By delaying the introduction of DEP 6 h following TCR activation, the effect of DEP on previously activated cells was evaluated. Following DEP treatment, CD40L expression was significantly greater than in cells with TCR activation alone (Fig. 3). In comparison, DEP treatment elicited no effect on TCR-induced CD69 or CD25 expression further supporting CD40L as a novel target for negative effects of DEP exposure (Fig. 3). These findings have relevance to asthma and certain autoimmune disorders, as these diseases are characterized by the presence of underlying low level T cell activation (Schonbeck, Mach, & Libby, 2000). To further evaluate the effect of DEP on CD40L, cells were concurrently provided DEP in the context of optimal T cell activation involving TCR and CD28 costimulation, mimicking DEP exposure in the context of antigen-induced T cell activation. Consistent with previous findings, only CD40L expression was augmented in cells provided optimal activation (Fig. 4). Again, these results indicate that DEP may uniquely affect CD40L expression, and may provide further support for DEP as an adjuvant in immune system activation as described by others (Samuelsen, Nygaard, & Lovik, 2008; van Zijverden et al., 2000; Finkelman et al., 2004). Given that CD40L is an essential immune component and individuals with chronic low-level expression of this protein are considered to be an at-risk population, the ability of DEP to alter signaling pathways responsible for immune system activation needs to be further addressed as this may provide a pivotal piece of information as to how DEP elicits its effects on human health.

CD40L-mediated immune responses are not solely based upon the action of the membrane-bound protein but also via a soluble secreted factor. Soluble CD40L (sCD40L) exists as a truncated 18 kDa protein produced by the proteolytic cleavage of the full length 28-33 kDa membrane-bound form (Graf et al., 1995). Despite this truncation, sCD40L possesses appreciable biological activity, although to a lesser extent than membrane CD40L, and elevated sCD40L has been associated with a variety of human diseases including atherosclerosis, inflammatory bowel disease, and SLE (Aukrust et al., 1999; Kato et al., 1999; Ludewig, Henn, Schroder, Graf, & Kroczek, 1996). Based on the observation that expression of membrane and soluble CD40L appear to be differentially regulated (Matthies, Newman, Hodzic, & Wingett, 2006), experiments were conducted to evaluate the effects of DEP on both protein isoforms. Our experiments showed that in contrast to the increased expression of membrane CD40L (Figs. 1-2), DEP was insufficient to induce sCD40L in the absence of stimuli (Figs. 8-9). Studies combining DEP treatment with optimal T cell signaling further magnified this differential production of membrane CD40L. Specifically, evaluation of both isoforms showed a significant change in the expression of both protein forms, albeit in an inverse relationship, with an increase in membrane CD40L (Fig. 4) and a decrease in the production of sCD40L (Fig. 9). The preferential expression of membrane CD40L is significant as this isoform possesses superior biological activity in comparison to the soluble form and would be expected to elicit a stronger immune response (Ludewig, Henn, Schroder, Graf, & Kroczek, 1996). To further investigate this effect, experiments were performed to isolate the different signaling components of T cell activation to

determine if a specific pathway was affected by DEP, or if the effect was a global response.

Dissection of the TCR generated signals into the PKC and calcium pathways highlighted the larger contribution of DEP to induce PKC-dependent membrane CD40L induction. While PKC-induced membrane CD40L (Figs. 5 and 8) was markedly increased following DEP exposure, sCD40L levels were decreased (Fig. 8). Calcium signaling also clearly increased membrane CD40L expression (Fig. 6 and 8), although not to the extent of PKC, and had no appreciable effect on DEP regulated sCD40L expression (Fig. 8). Further studies to evaluate sCD40L regulation by DEP were employed to mimic physiological activation of T cells by TCR and CD28 costimulation. Membrane CD40L (Fig. 7) was augmented by DEP while sCD40L was again down-regulated (Fig. 9A) further suggesting a preferential production of membrane CD40L.

Interestingly, the only activation scenario that resulted in an induction of both membrane and soluble CD40L following DEP exposure was in experiments utilizing dbcAMP to mimic β-adrenergic agonists, medications commonly utilized in asthma treatment. In the context of asthma, aberrant CD40L expression may be one of many factors contributing to disease pathogenesis as supported by animal model studies (Mehlhop et al., 2000). Along these lines, research indicates that β-agonists can alter the expression of CD40L. Specifically, in a population of asthmatic patients following treatment with β–adrenergic agonist medications, and other agents that increase cAMP, an increase in CD40L expression was observed (Wingett, Forcier, & Nielson, 1999). Acting as a second messenger, cAMP mediates the signals that regulate a host of T cell functions such as proliferation and cytokine production via both inhibitory and

stimulatory activities (Lacour et al., 1994; Desai-Mehta, Lu, Ramsey-Goldman, & Datta, 1996). As cAMP further amplifies Ca^{2+}-induced CD40L expression (Wingett, Forcier, & Nielson, 1999), we evaluated the effect of DEP exposure on cells costimulated with ionomycin and dbcAMP. The stimulatory property of cAMP to increase CD40L was enhanced in T cells exposed to DEP as measured by a modest, yet significant increase in the production of both membrane and soluble CD40L (Fig. 13). While the previously shown increase in membrane CD40L is a considerable finding, the increase in both isoforms of CD40L in cells treated with cAMP/β-agonists is noteworthy as individuals with chronic respiratory disease are far more susceptible to the injurious health effects of DEP. The ability of DEP to increase CD40L, when combined with cAMP-inducing agents, may in some way contribute to the increased severity of asthma symptoms occurring in recent years, and illustrates the importance of appropriate warning systems to alert at-risk individuals when environmental PM levels exceed acceptable ranges.

As the production of sCD40L is thought to involve the process of proteolytic cleavage, experiments were conducted to evaluate the effects of DEP on this mechanism, also known as shedding. While not completely elucidated, shedding is understood to be an important mechanism for regulating immune cell signaling by either reducing the amount of receptor proteins on the cell surface or by releasing soluble domains of proteins, which retain similar biological ability to their membrane-bound counterparts (Schlondorff & Blobel, 1999). The proteolytic cleavage mechanism implicated in the production of sCD40L, and other TNF family members, involves the action of the ADAM (a disintegrin and metalloproteinase) family proteins, including the ADAM-10 protease (Matthies, Newman, Hodzic, & Wingett, 2006). ADAM-10 mediated

proteolytic cleavage results in the production of a truncated, yet biologically active, CD40L protein with a molecular weight at ~18 kDa, in contrast to full length 28-33 kDa membrane-bound CD40L (Mazzei et al., 1995). As the production of sCD40L was down-regulated following DEP exposure in almost every activation scenario utilized in our studies, it was hypothesized that DEP was inhibiting the process of shedding by acting upon ADAM-10. Therefore, a recombinant chimeric fusion protein system was employed to assess whether ADAM-10 function was affected by DEP. Specifically, a murine recombinant protein was utilized that contained the putative cleavage site (methionine at position 113 in the extracellular region) that results in the production of the 18 kDa native sCD40L. A schematic representation of the murine CD40L-CD33 fusion protein, which contains the extracellular domain of CD40L, is shown in Fig. 14. Consistent with previous reports (Matthies, Newman, Hodzic, & Wingett, 2006) and as shown in Fig. 10, a distinct 32 kDa band corresponding to the membrane CD40L protein was visualized with the cleaved 18 kDa band corresponding to sCD40L present only in ADAM-10 treated samples. Of interest, the appearance of a second, distinct cleavage product was observed in ADAM-10 samples (~9 kDa) and may represent a secondary cleavage event. As expected, the appearance of the ~9 and 18 kDa cleavage products was also correlated with the decreasing intensity of the full length protein. In order to identify a potential amino acid sequence for the secondary cleavage event, the Biology WorkBench Pattern Match program was utilized. This program is provided by the University of California, San Diego Supercomputer Center. The fusion protein construct was analyzed for the presence of the putative ADAM-10 cleavage sequence responsible for the production of soluble tumor necrosis factor (TNF-α) (Rosendahl et al., 1997) since

CD40L and TNF-α are structurally related and belong to the same super family of proteins (Bossen et al., 2006). While the exact sequence for TNF-α was not found, it is important to note that a detailed series of studies have been performed where amino acid substitutions in the putative TNF-α cleavage site were made and found to have varying effects on ADAM-10 proteolytic activity. Therefore, our analyses were extended to include these amino acid substitutions, especially given the increasingly recognized lack of ADAM-10 consensus cleavage sequences among characterized substrates to date (Rosendahl et al., 1997). Using this approach, a putative core cleavage sequence starting at amino acid 184 (SSQR) of the CD40L protein was identified, which may represent a second cleavage site. As described for various TNF-α amino acid substituted peptides, only the substitution of amino acid 77 from an alanine to serine was found to reduce the ability of ADAM-10 to cleave TNF-α (~39%), while the other three amino acids conferred 100% ADAM-10 mediated cleavage relative to the wild-type substrate (Rosendahl et al., 1997).

The known cleavage sites for ADAM-10 substrates are listed in Table 1, emphasizing the variability between amino acid sequences which makes the determination of a well-defined putative cleavage site difficult given the current state of knowledge regarding ADAM-10. Given that the amino acid sequences at substrate cleavage sites are highly variable, it is thought that structural and kinetic characteristics are more important than minimal amino acid sequence (Amour et al., 2000). In this regard, it is important to note that a region of significant homology (~86%) surrounds the putative second cleavage site between CD40L and TNF-α as determined using the Basic Local Alignment Search Tool (BLAST). This protein sequence analysis found an

alignment (12 out of 14 amino acids) between the two proteins from amino acids 165 – 180 in the CD40L extracellular domain. This TNF-like structural domain could potentially play a larger role in directing ADAM-10 to the second cleavage site in the CD40L protein than the individual amino acids themselves. The observation that DEP increased the abundance of the 18 kDa band, while markedly decreasing the intensity of 9 kDa band suggests that DEP can alter the activity of ADAM-10 to proteolytically process CD40L.

To fully understand the role of DEP on ADAM-10 function, further experiments are needed to investigate the cleavage events occurring within the CD40L fusion construct. Specifically, various antibodies directed against different regions of the CD40L extracellular domain could be utilized in Western blot experiments to conclusively demonstrate the occurrence of a secondary cleavage event, as the current polyclonal antibody detects both the ~18 kDa and 9 kDa protein cleavage products. Additional supporting experiments to evaluate the effects of ADAM-10 mediated cleavage on other known cleavage sites such as TNF-α or β-amyloid precursor protein (Table 1) are needed and would further elucidate the role of DEP on this matrix metalloproteinase.

An alternative explanation of the ADAM-10 Western blot results may be considered based on observations that the intensity of the ~32 kDa band corresponding to membrane CD40L appeared to decrease in samples containing DEP, without the appearance of the cleaved sCD40L product. In this cell-free system, the carbon core could bind to the CD40L fusion protein, resulting in the formation of high molecular weight aggregates. The presence of its carbonaceous core, an instrumental component of

DEP, can bind indiscriminately to various proteins and could lead to the formation of these aggregates, which would subsequently interfere with protein migration as the large molecular weight aggregates would fail to enter the acrylamide matrix. While it is possible that the 9 kDa product could be preferentially adsorbed to the carbon core relative to the 18 kDa product, the basis for this preferential adherence remains presently unclear. Nevertheless, ADAM-10 function may be altered by DEP and additional studies are needed to further address the impact of DEP exposure on ADAM-10 function.

While the results of our cell-free recombinant protein assay indicate that DEP may alter the function of ADAM-10 with respect to proteolytic cleavage of CD40L, these results are not readily resolvable with our cell-based sCD40L studies, which demonstrate a DEP-induced inhibition of sCD40L. We believe that the results of our cell-based assay represents a more physiological, and consequently more meaningful result as this system allows for evaluation of sCD40L as a combination of multiple mechanisms. Our cell-free system suggests that the down-regulation of sCD40L is not mediated by extracellular ADAM-10 but does not allow for the evaluation of other potential mechanisms responsible for the production of sCD40L. Recent observations do suggest that ADAM-10 proteolysis can also occur within intracellular compartments. A close link was reported between the transport of TNF-α to the cell surface and its cleavage (Arribas et al., 1996) and ADAM-10 appears responsible for the shedding of at least two proteins, CD44 and L1, in vesicles (Stoeck et al., 2006). Alternatively, sCD40L may be generated via an intracellular proteolytic event directly following protein synthesis since low levels have been detected in microsomes (Pietravalle et al., 1996). Alternate mechanisms for the production of sCD40L have been suggested such as alternative splicing to eliminate

transmembrane regions or the usage of alternate transcription initiation start sites, although no direct evidence has implicated these mechanisms in the production of sCD40L (Graf et al., 1995). Additionally, no Kozak consensus sequence in the corresponding nucleotide sequence is present allowing for alternative translation initiation. Another form of the soluble CD40L protein has been reported and begins at a slightly different amino acid position (Gln-114 instead of Met-113) and transfectants lacking nucleotides corresponding to the first 12 amino acids of full-length CD40L fail to express either membrane or soluble protein (Hsu et al., 1997). It is probably most likely that sCD40L production relies on a combination of intracellular processing and membrane cleavage events and further studies are needed to resolve the effect of DEP on these processes as the recombinant cell free system utilized in the ADAM-10 studies is unable to evaluate the contributions of different cleavage mechanisms to the *in vitro* production of sCD40L.

As DEP was found to alter the expression of membrane and soluble T_H cell activation markers, evaluation of other soluble immune mediators was conducted. These factors in general, known as cytokines, exert immune system control at distant sites in the body via the circulatory system. Specifically, the expression of IL-8 and IL-17 were evaluated following DEP exposure. Both IL-8 and IL-17 are considered to be pro-inflammatory cytokines as these factors have been linked with allergic and autoimmune diseases such as asthma, atherosclerosis, MS, and Crohn's disease (Grimm, Elsbury, Pavli, & Doe, 1996; Apostolakis, Vogiatzi, Amanatidou, & Spandidos, 2009; Miossec, 2009; Hashimoto, Akiyama, Kobayashi, & Mori, 2005). Samples from individuals with asthma showed an increase in IL-8 and IL-17 expression with animal models

demonstrating the importance of these two cytokines in asthma pathogenesis (Hashimoto, Akiyama, Kobayashi, & Mori, 2005; Pease & Sabroe, 2002; Wang & Liu, 2008). T_H cells are known to produce both IL-8 and IL-17 with IL-17 being a T_H cell specific cytokine. While no appreciable effect on IL-8 was observed following DEP exposure, a 3-fold decrease in IL-17 production occurred (Fig. 11). While it is accepted that environmental toxins such as DEP can suppress immune system function (Siegel et al., 2004), IL-8 expression was not affected suggesting that the immunosuppressant capacity of DEP is not widespread to all cytokines. The biological relevance of decreased IL-17 may have multiple, and even complex consequences, as the effect of this cytokine varies according to disease state and the context of other cytokines present. For example, IL-17 appears to be important for initiating asthma pathogenesis, but later can act as a negative regulator of asthma as blocking of IL-17 function in a mouse model exacerbates disease state (Schnyder-Candrian et al., 2006; Wang & Liu, 2008). Therefore, further investigation into the effects of DEP on IL-17 production, in the context of healthy and at-risk populations, needs to be addressed to evaluate whether or not a decrease in this cytokine in IL-17 contributes to or alleviates the impacts of DEP.

Based on the observed immunoregulatory properties, the effect of DEP on T cell cytotoxicity was evaluated. As shown in Fig. 12, no evidence of cytoxicity was observed as measured by propidium iodide (PI) staining, which measures changes in cell membrane permeability and is a classical indicator of cell death (Riccardi & Nicoletti, 2006). The failure of DEP to affect cytotoxicity in resting cells is interesting given that *in vitro* studies demonstrate death in other cell types, including bronchial epithelial cells, lung cells, and alveolar macrophages as a result of DEP (Nel, Diaz-Sanchez, & Li, 2001;

Wan & Diaz-Sanchez, 2007). Further studies are needed to assess the affect of DEP on viability in activated cells as they exhibit greater metabolic activity and may respond differently than resting cells.

In conclusion, the results presented here provide evidence for an immunomodulatory effect of DEP on T_H cell with changes in the expression of key activation markers and cytokines. Of the activation markers evaluated, the expression of CD40L appeared to be differentially affected by DEP exposure with an increase in the expression of membrane CD40L in the context of every *in vitro* activation model utilized, without parallel increases in the other membrane-bound activation markers. Concurrent evaluation of the effects of DEP on both naturally occurring forms of CD40L highlighted the preferential induction of membrane CD40L expression, with a parallel decrease in the soluble version of this protein, with cAMP induction being the only exception. Therefore, the increased expression of the membrane-bound CD40L isoforms, which possesses greater inherent biological activity, is significant as the deleterious effects would be proportionally larger in individuals with allergic disorders including asthma. An alteration in the processes responsible for the production of sCD40L was also reported. In the context of cAMP signaling, the increased expression of both membrane and soluble CD40L following DEP would be expected to have more biological significance for the individual as both isoforms can induce the same down-stream signaling events. Also, as many medications act through the important cAMP second messenger, the impact of DEP would be very relevant to individuals with existing health conditions, who are predisposed to the harmful health effects of this common pollutant. DEP exposure also suppressed the production of IL-17, an important cytokine with

pleiotropic effects involved in the pathogenesis of asthma and other allergic diseases. Importantly, all of these immunomodulatory effects occurred in the absence of cytotoxicity. As diesel fuel continues to be a vital component of daily activity for all individuals living in industrialized nations, further research is needed to understand the molecular mechanisms occurring after DEP inhalation. Based on the research presented, which further supports an already existing body of knowledge, it is clear that steps need to be taken to minimize these effects and improve overall air quality. Future directions need to focus on understanding the mechanisms by which DEP affect immune cells, in addition to other cell types, to fully understand the implications of exposure. As standards and filtration systems improve, research will need to evaluate whether or not these improvements result in an increased quality of life. In addition, as alternative fuel usage increases, studies will need to be conducted to verify that the combustion by-products of these fuels lack the same risks as current fuels. The end result of this research will hopefully result in the creation and implementation of more stringent air quality standards to protect individuals exposed to common air particulate matter pollutants such as diesel exhaust particles.

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APPENDIX A

Tables

Protein	Sequence	Reference
CD40L	SFEM*QRGD	(Ludewig, Henn, Schroder, Graf, & Kroczek, 1996)
Pro-TNF	LAQA*VRSS	(Rosendahl et al., 1997; Black et al., 1997)
IL-6 Receptor	LPVQ*DSSS	(Mullberg et al., 1995)
TNFR-55	QIEN*VKGT	(Mullberg et al., 1995)
TNFR-75	APGA*VHLP	(Crowe et al., 1995)
Pro-TFG-α	DLLA*VVAA	(Black et al., 1997)
c-*kit* ligand-1	PVAA*SSLR	(Pandiella, Bosenberg, Huang, Besmer, & Massague, 1992)
β-Amyloid precursor protein	HHQK*LVFF DSGY*EVHH	(Allinson et al., 2004; Asai et al., 2003)
Angiotensin-converting enzyme	AGQR*LATA	(Oppong, Turner, & Hooper, 1993)
L-selectin	KLDK*SFSM	(Rosendahl et al., 1997)
Myelin Basic Protein-1	GSLP*QKSQ	(Naus et al., 2006)
Delta-Like 1	SERH*MESQ	(Six et al., 2003; Dyczynska et al., 2007)
Heparin-binding EGF-like growth factor	PSKE*RNGK	(Hinkle et al., 2004)
Transforming growth factor α	VAAA*VVSH	(Hinkle et al., 2003)
Prion protein preproprotein	NMKH*MAGA	(Laffont-Proust et al., 2005)
Fc fragment of IgE, CD23A low affinity II	RAEQ*QRLK DQMA*QKSQ	(Lemieux et al., 2007)
Eph receptor B2	EYQT*SIKE	(Litterst et al., 2007)

Table 1. ADAM-10 proposed proteolytic cleavage sites. The experimentally determined cleavage sites for known ADAM-10 substrates. Cleavage sites are indicated by an * in the given amino acid sequence.

APPENDIX B

Figures

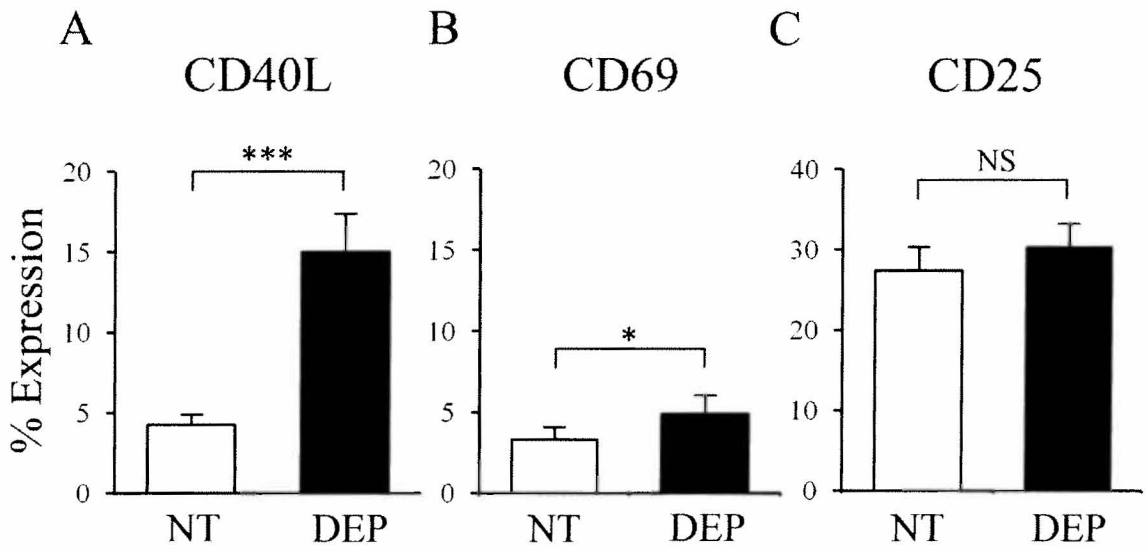

Figure 1. Diesel exhaust particles increase CD40L expression on unactivated T helper cells. Purified $CD4^+$ T cells were incubated for 24 h in the presence of DEP, stained with anti-CD40L, CD69, and CD25 Abs, and subsequently analyzed via flow cytometry. Results depict the percentage of positively staining cells and error bars depict standard error (SE). **A)** CD40L expression, n= 6. **B)** CD69 expression, n=6. **C)** CD25 expression, n=6. Statistical significance was determined using a paired T test, * = $p < 0.05$ and *** = $p < 0.0001$.

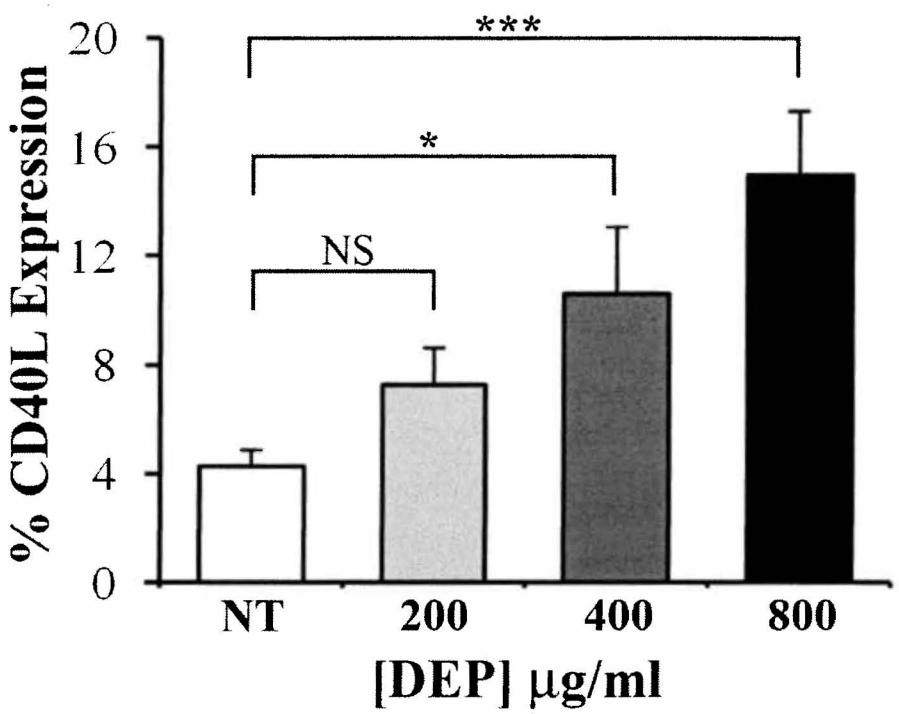

Figure 2. Concentration dependent increase in membrane CD40L by DEP. Freshly purified $CD4^+$ T cells were treated with different concentrations of DEP and CD40L expression was analyzed via flow cytometry. Results depict the percentage of CD40L positive cells and error bars depict SE, n=6. Statistical significance was determined using a repeated measures ANOVA, * = $p < 0.05$, and *** = $p < 0.0001$.

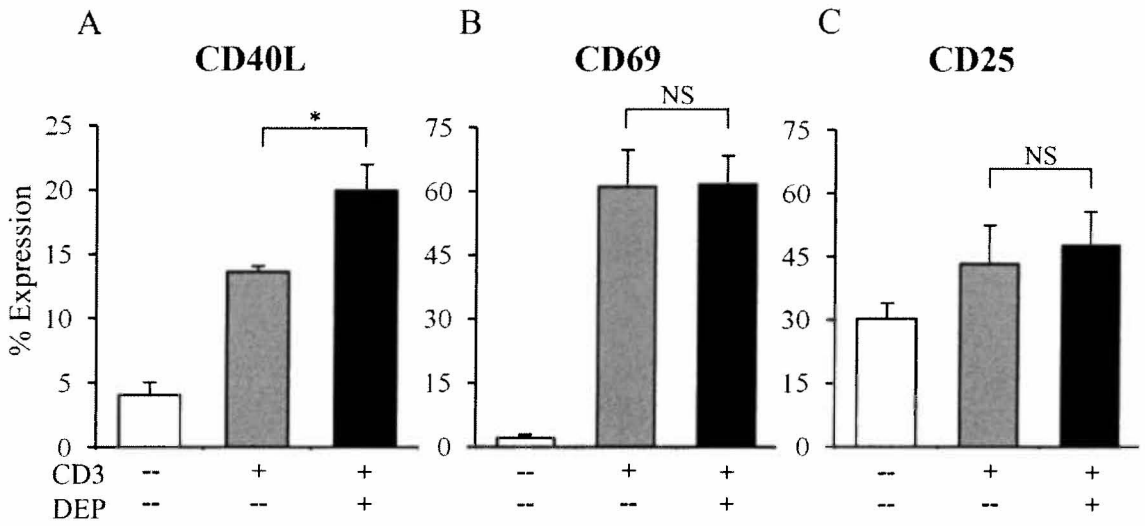

Figure 3. Effects of DEP on CD3 activated T cells. $CD4^+$ T cells were cultured with anti-CD3 mAb for 6 h and transferred to fresh culture wells. Cells were then treated with DEP (800 μg/ml) for an additional 18 h and CD40L expression evaluated using flow cytometry. Data is presented as percentage of CD40L positive cells, and error bars depict SE. **A)** CD40L expression, n=4 **B)** CD69 expression, n=3 **C)** CD25 expression, n=3. Significance was determined using a paired T test, * = $p < 0.05$.

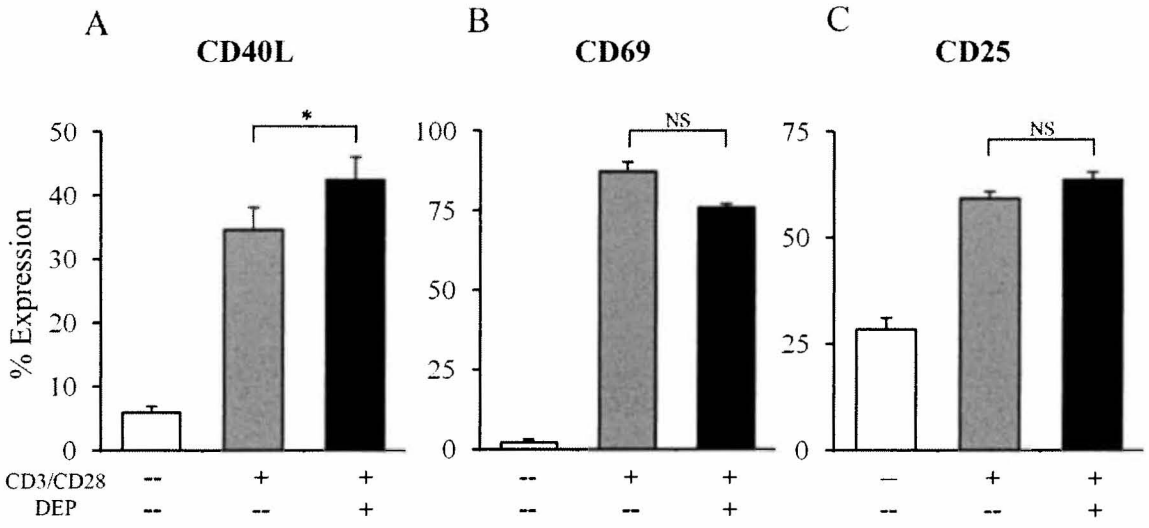

Figure 4. Effects of DEP on CD28 costimulated T cells. $CD4^+$ T cells were stimulated with Dynabeads® ± DEP (800 μg/ml) for 24 h. Surface expression of each respective T cell activation marker was analyzed using flow cytometry, n=3, with error bars depicting SE. Panel **A)** CD40L expression, **B)** CD69 expression, and **C)** CD25 expression. Significance was determined using a repeated measures ANOVA, * = $p < 0.05$.

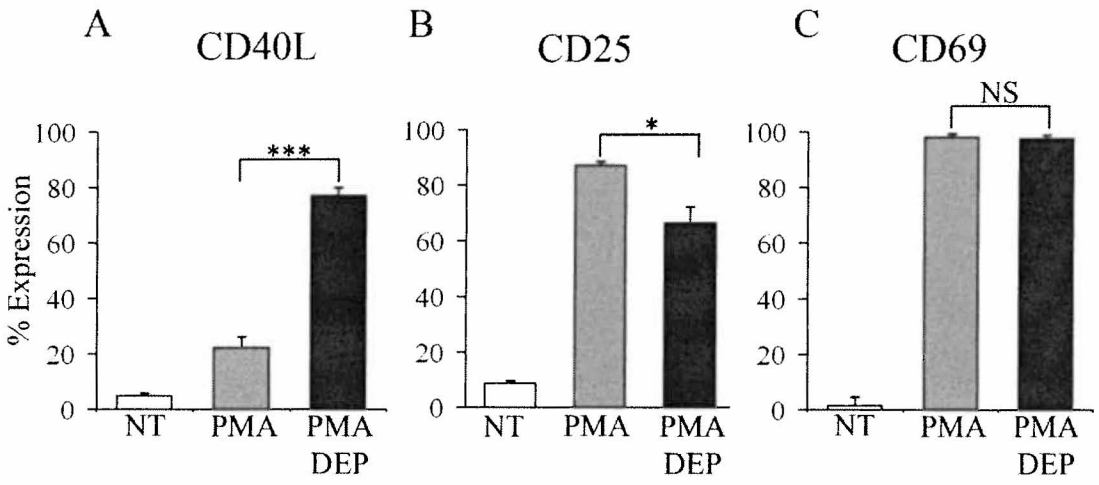

Figure 5. DEP augments PKC–dependent CD40L protein expression. $CD4^+$ T cells were stimulated with PMA ± DEP (800 μg/ml) for 24 h and expression of activation markers evaluated by flow cytometry. **A)** CD40L expression in PMA (2 ng/ml) ± DEP treated cells, n=7. **B)** CD25 expression in PMA (0.5 ng/ml) ± DEP treated cells, n=3. **C)** CD69 expression in PMA (0.5 ng/ml) ± DEP treated cells, n=3. Error bars depict SE with asterisks indicating statistical significance, * = $p < 0.05$ and *** = $p < 0.0001$.

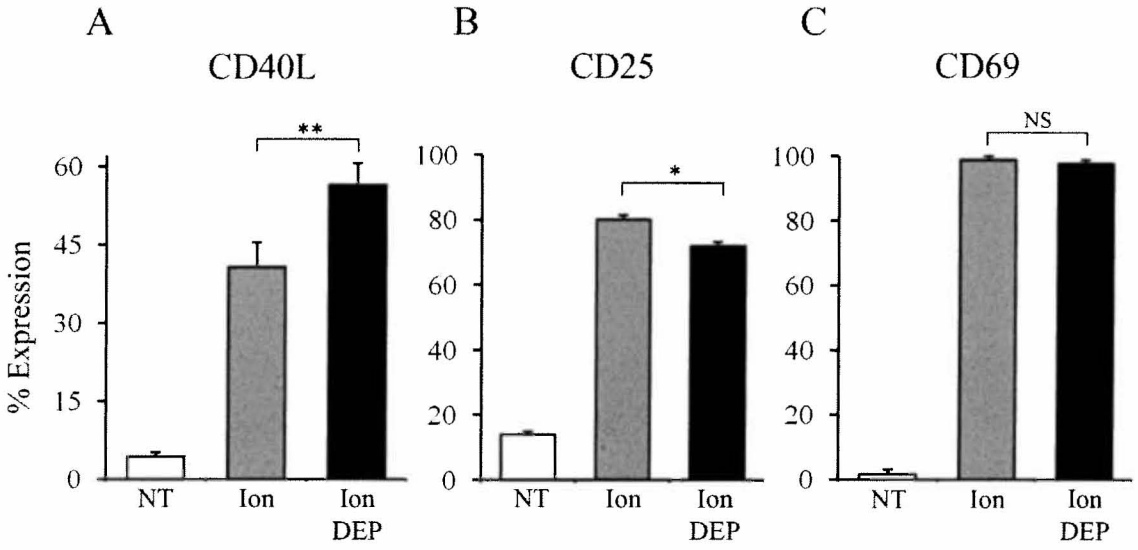

Figure 6. DEP augments calcium-dependent CD40L expression. $CD4^+$ T cells were stimulated with ionomycin 0.75 μM ± DEP (800 μg/ml) for 24 h and changes in protein expression evaluated using flow cytometry. Panel **A)** CD40L expression, panel **B)** CD69 expression triplicate, and panel **C)** CD25 expression. CD40L data, n=5, and CD25 and CD69 data presented are from a representative experiment performed in triplicate. Error bars depict SE with asterisks indicating statistical significance, * = $p < 0.05$ and ** = $p < 0.001$.

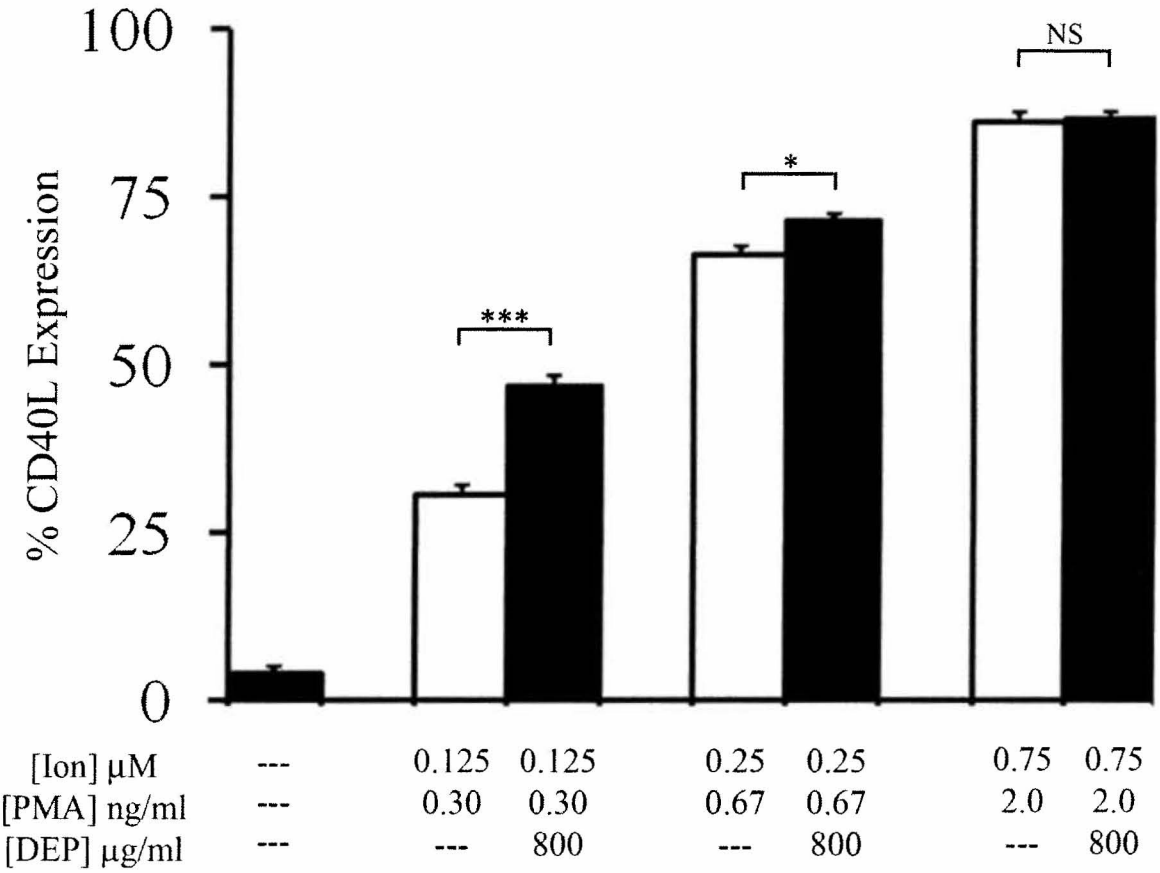

Figure 7. DEP induces CD40L in PMA and ionomycin activated cells. $CD4^+$ T cells were stimulated with varying amounts of ionomycin and PMA ± DEP (800 μg/ml) for 24 h and changes in CD40L expression evaluated using flow cytometry. The percentage of CD40L positive staining cells from a representative experiment performed in triplicate is shown. Error bars depict SE with asterisks indicating statistical significance, * = $p <$ 0.05 and *** = $p < 0.0001$.

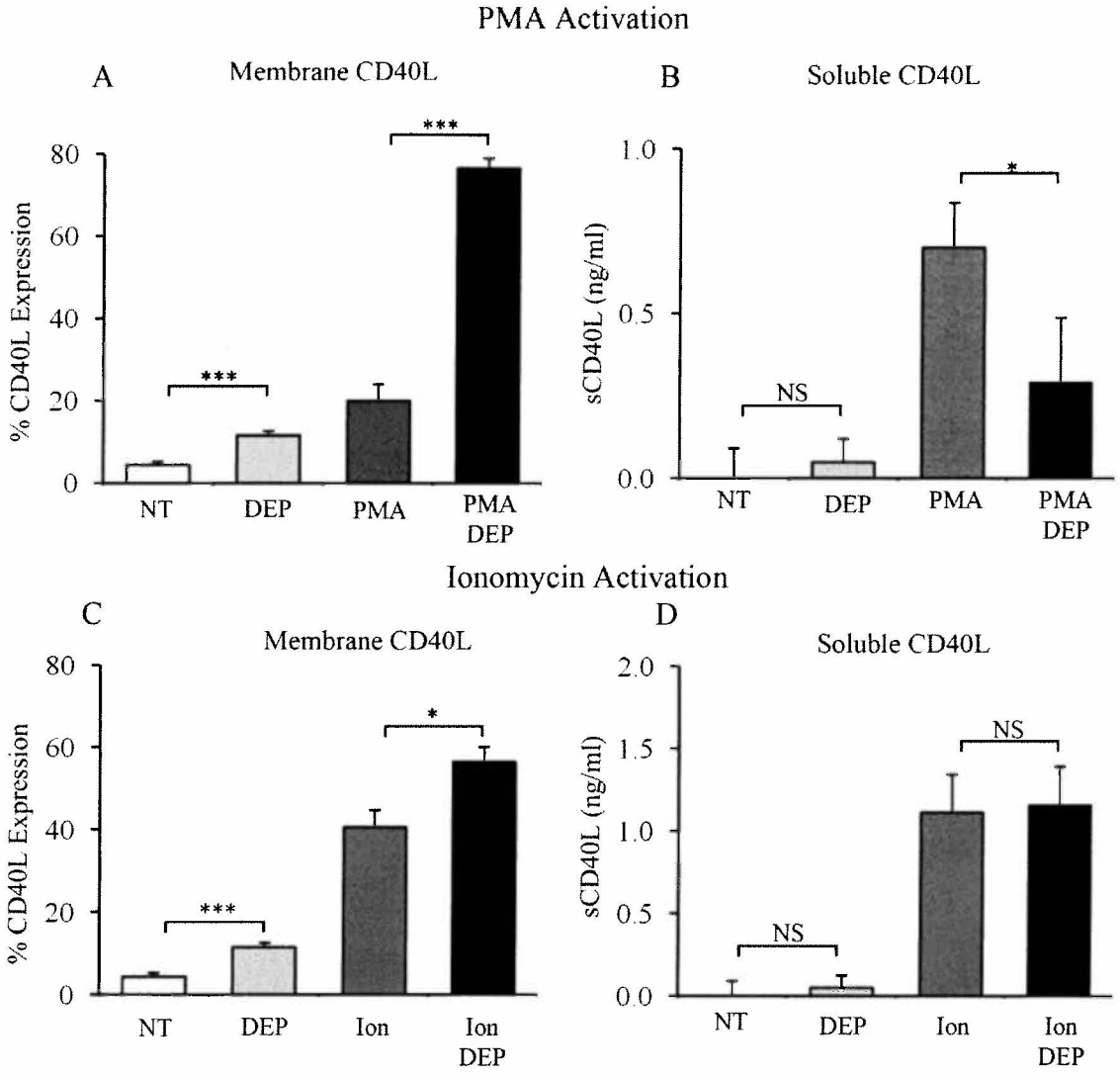

Figure 8. Membrane and soluble CD40L expression are differentially regulated by DEP. $CD4^+$ T cells were stimulated with ionomycin (0.75 μM) or PMA (2.0 ng/ml) for 24 h. Cells were dual harvested and concurrently analyzed for soluble and membrane CD40L expression by ELISA and flow cytometry, respectively. Panel **A)** PMA-induced membrane CD40L **B)** PMA-induced soluble CD40L **C)** Ionomycin-induced membrane CD40L **D)** Ionomycin-induced soluble CD40L. Data above represents n=4 and error bars depict SE. Significance was determined using a repeated measures ANOVA, * = $p< 0.05$ and *** = $p< 0.0001$.

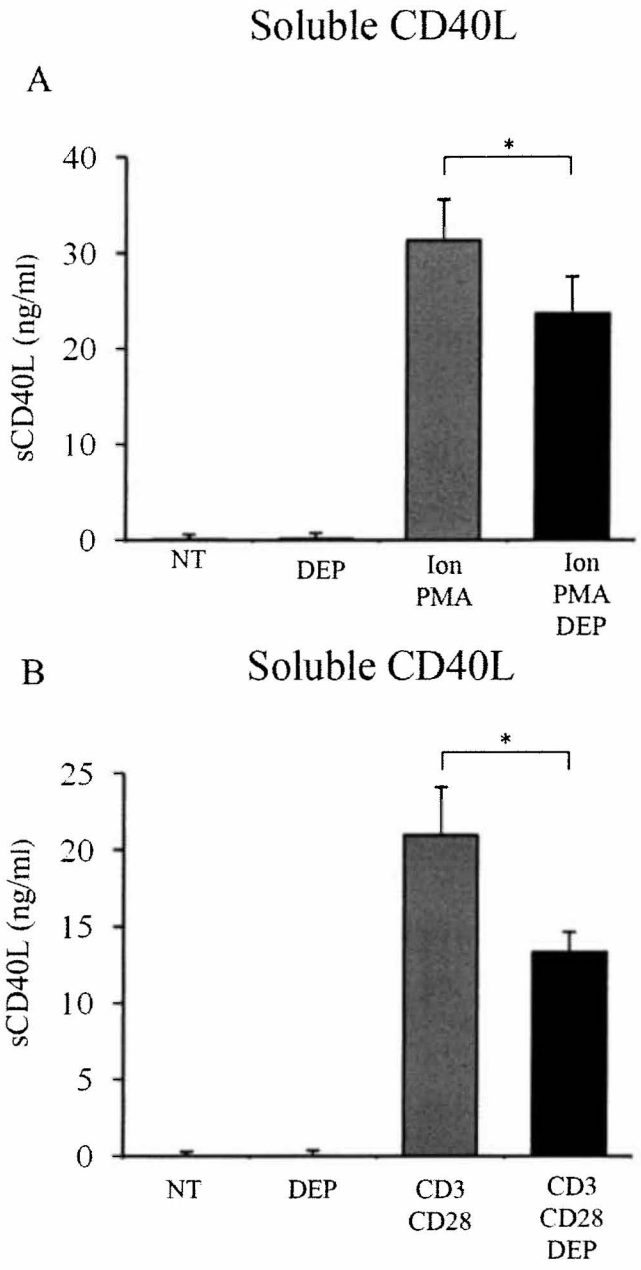

Figure 9. T cell receptor induced soluble CD40L expression is inhibited by DEP. $CD4^+$ T cells were stimulated through the TCR via ionomycin (0.75 μM) + PMA (2.0 ng/ml) or CD3/CD28 Dynabeads® for 24 h. Cells were dual harvested and concurrently analyzed for soluble and membrane CD40L expression by ELISA and flow cytometry, respectively. Panel **A)** Ion/PMA induced sCD40L n=3 **B)** CD3/CD28 induced sCD40L n=4. Error bars depict SE and significance was determined by paired T test, * = $p< 0.05$.

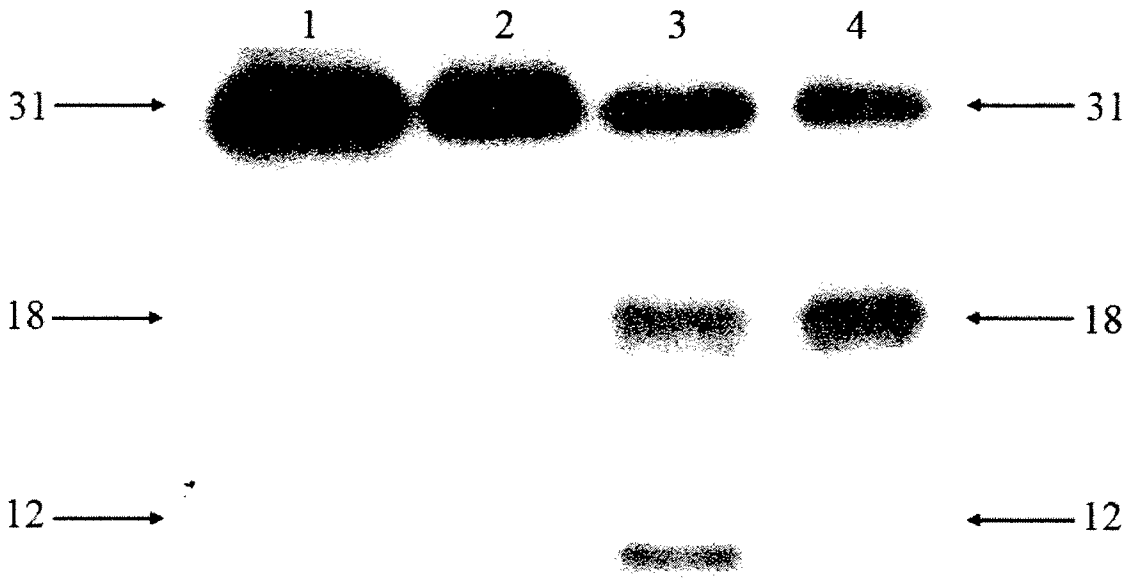

Figure 10. Effects of DEP on ADAM-10 mediated proteolytic cleavage of CD40L. Murine CD40L/CD33 fusion protein (0.5 μg) was incubated with 20 μg/ml recombinant human ADAM-10, with (lane 4) or without 800 μg DEP (lane 3), for 5 h at 37 °C in 25 μl of total reaction mixture. The mixture was then fractionated by 10% SDS – polyacrylamide gel electrophoresis under reducing conditions. Processed and full-length CD40L fusion proteins were detected by Western blotting using a goat-polyclonal antibody for the extracellular domain of CD40L. Lane 1, CD40L fusion protein; lane 2, CD40L fusion protein and DEP; lane 3, CD40L fusion protein and rADAM-10; lane 4, CD40L fusion protein, rADAM-10, and DEP.

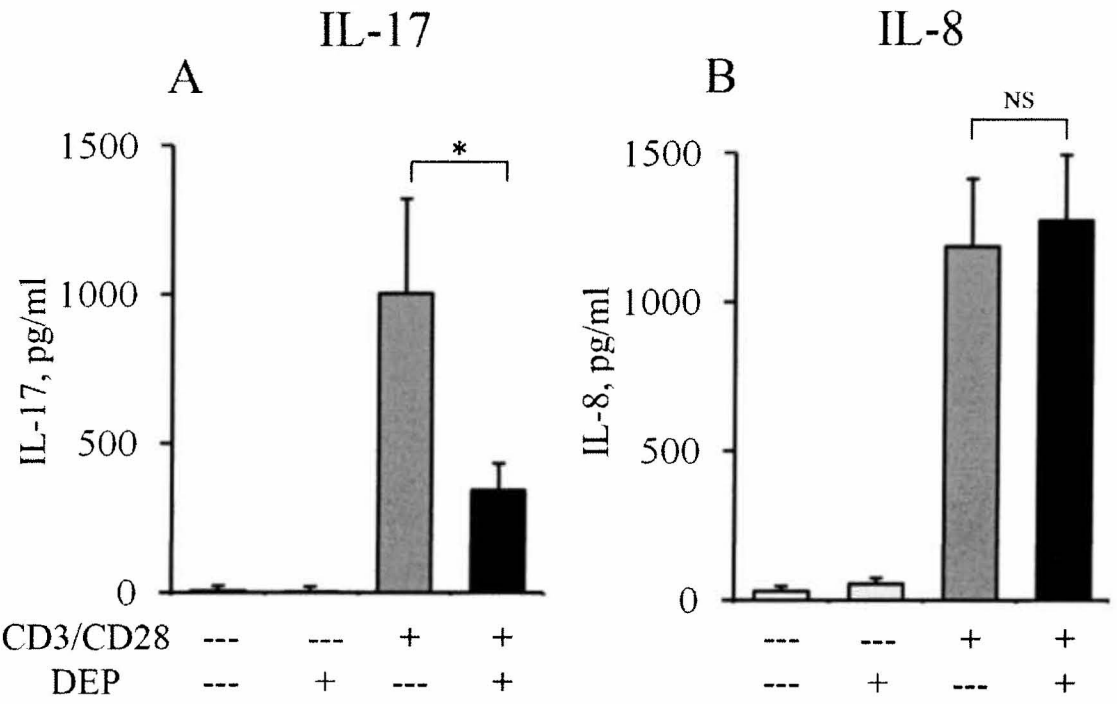

Figure 11. Production of IL-17 is down regulated following DEP exposure. $CD4^+$ T cells were stimulated with CD3/CD28 Dynabeads® ± DEP (800 µg/ml) for 24 h and cell free supernatants were analyzed for IL-8 and IL-17 cytokine production. Panel **A)** IL-17 n=6 **B)** IL-8 n=5. Error bars depict SE and significance was determined using a paired T test, * = p <0.05.

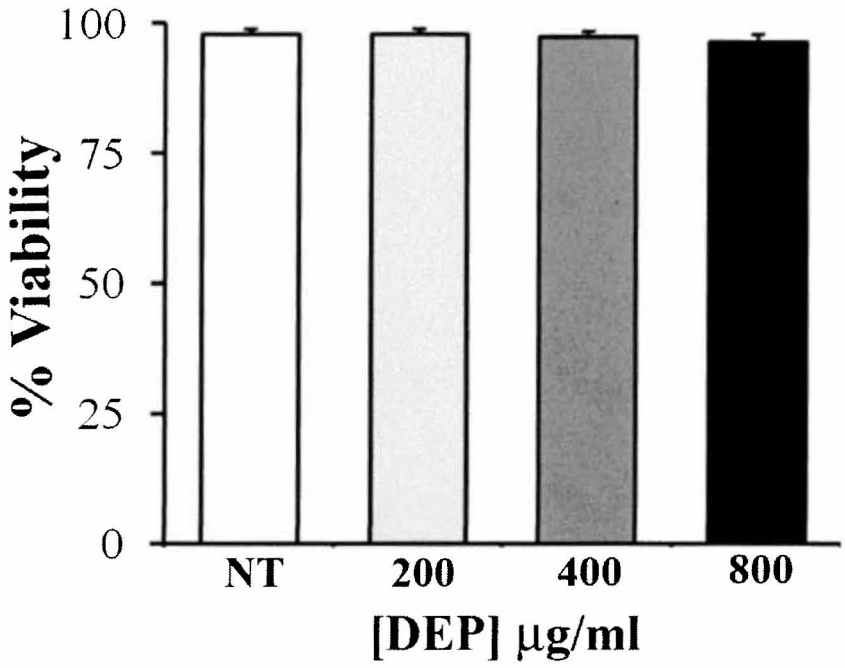

Figure 12. DEP fails to appreciably affect T cell viability. Purified $CD4^+$ T cells (>95%) were treated with increasing concentrations of DEP for 24 h and cell viability evaluated using propidium iodide staining and flow cytometry. Results are depicted in percent viability from three independent experiments and error bars depict SE.

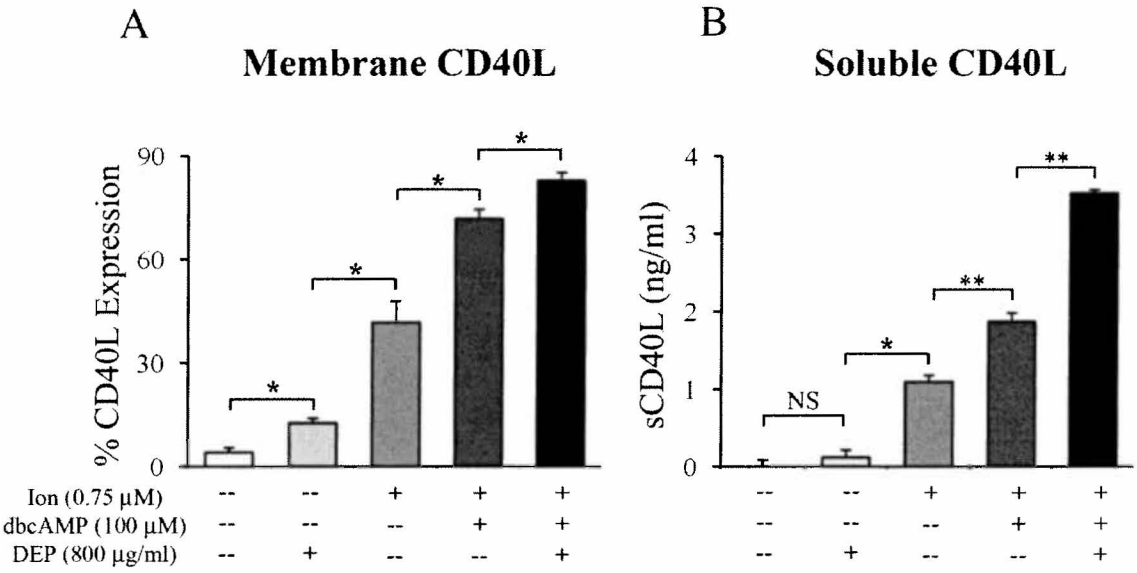

Figure 13. β-agonist induced membrane and soluble CD40L expression is heightened after DEP exposure. $CD4^+$ T cells were stimulated with ionomycin (0.75 µM) ± dbcAMP (100µm) ± DEP for 24 h. Cells were harvested and analyzed concurrently for membrane and soluble CD40L expression by ELISA. Panel **A)** Membrane CD40L, n=4; **B)** Soluble CD40L one representative experiment performed in triplicate. Error bars depict SE. * = $p < 0.05$ and *** = $p < 0.0001$.

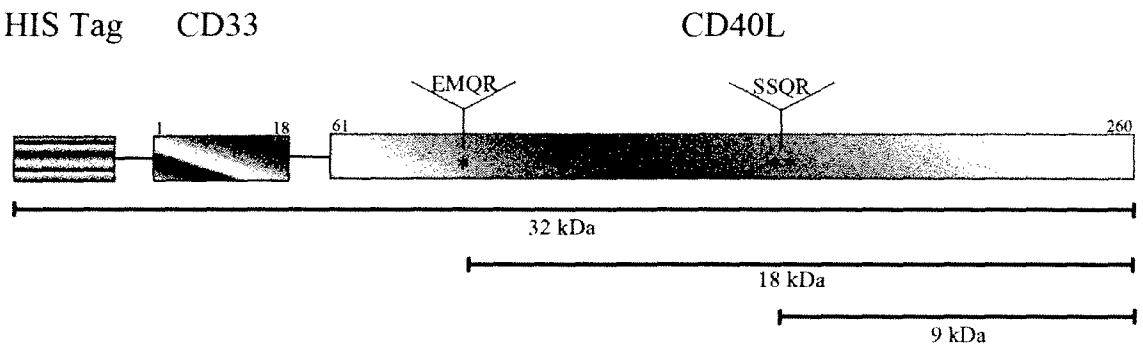

Figure 14. A schematic representation of the ADAM-10 cleavage sites located in the murine CD33-CD40L fusion protein. The proposed cleavage site at position 112-116, (*, EMQR) results in the production of an ~18 kDa band, representing sCD40L. A second cleavage site was identified using the Biology WorkBench Pattern Recognition Program created by the University of California, San Diego Supercomputer Center. The site at position 184-188 (**, SSQR), related to the known TNF-α cleavage sequence, may result in the production of the secondary cleavage product of 9 kDa.