Immune-Associated Chronic Inflammation: Biological Responses Between Endothelial Cells and T Cells Following Treatment with *Thymus vulgaris* and *Eugenia caryophyllata* Essential Oils

By

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Abstract

Chronic inflammation is associated with autoimmune disorders and chronic diseases such as diabetes and cardiovascular disease. Prolonged interaction between circulating leukocytes (CD4+ T cells) and microvasculature endothelial cells (ECs) triggers excessive cytokine production, exposing adhesion and coagulation molecules. Consequences include altered T cell phenotype and EC physiology such as disrupted vascular integrity, immune cell transmigration, and platelet aggregation. Although excess inflammatory cytokines and coagulation factors can be detrimental, these otherwise protective molecules cannot be eradicated entirely. Medicinal plant extracts, including essential oils (EOs), have various constituents resulting in biological effects that are anti-inflammatory. Furthermore, phenolic EOs such as clove and thyme demonstrate immunomodulatory and anti-platelet activity. EOs naturally contain compounds with diverse physiological benefits, suggesting a multi-targeted therapeutic approach for prevention and treatment of immune-mediated inflammatory diseases. Thus, the present study was conducted to assess potential therapeutic effects of clove and thyme EOs towards markers of immune-associated vascular inflammation including E-selectin, intracellular adhesion molecule 1 (ICAM-1), and interleukin 6 (IL-6). Additionally, we investigated the effects of these oils towards the initiator of the coagulation cascade, tissue factor (TF). Tumor necrosis factor alpha (TNF-α) activated human umbilical vein endothelial cells (HUVECs) were incubated with clove (0.01%) and thyme EOs (0.01%) for 3 hr and 6 hr. These data show significant TF protein expression in all HUVEC conditions activated with TNF-α ($p < 0.001$), yet no differences were observed with the addition of either EO as compared HUVECs treated with TNF-α alone. Other markers did not reveal significant differences statistically. As a secondary aim, activated and inactivated HUVECs were coincubated with CD4+ T cells prior to treatment with clove (0.01%) and thyme (0.01%) EO for

6 hr. These preliminary data suggest there may be a mild preventative and treatment effect with clove EO, while thyme EO increased protein expression of immune-associated vascular inflammation markers. We conclude that TF expression may contribute to TNF-α mediated inflammation, while ICAM-1, E-selectin, and IL-6 did not reveal significant modulation under TNF-α stimulation or treatment with EOs.

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Abbreviations

CVD	cardiovascular disease
SI	systemic inflammation
APC	antigen presenting cell
MHC	major histocompatibility complex
ECs	endothelial cells
VCAM-1	vascular cell adhesion molecule 1
ICAM-1	intracellular adhesion molecule 1
E selectin	CD62E or ELAM-1 endothelial leukocyte adhesion molecule 1
ED	endothelial dysfunction
TF	tissue factor or thromboplastin or CD142
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
MS	multiple sclerosis
MG	myasthenia gravis
EOs	essential oils
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
TNF-α	tumor necrosis factor alpha
ERK	extracellular signal regulated kinase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
NF-κB	nuclear factor kappa B
COX	cyclooxygenase
iNOS	inducible nitric oxide synthase

ECA	endothelial cell activation
INF-γ	interferon gamma
ICOS-L	inducible costimulatory ligand 1
IL-2	Interleukin-2
Th17	T-helper 17
EMP	endothelial microparticle
Th1	T-helper 1
CNS	central nervous system
Tregs	T regulatory cells
RV	rheumatoid vasculitis
VIIa	activated factor VIIa
PMA	phorbol ester
NSAIDs	non-steroidal anti-inflammatory drugs
DMARDs	disease modifying anti-rheumatic drugs
PTM	post translational modification
MAPK	mitogen activated protein kinase
PI3K	Phosphoinositide 3-kinase
AkT	protein kinase B
mTOR	mammalian target of rapamycin
JAK	Janus kinase
STAT	signal transducer and activator of transcription
GC/MS	gas chromatography mass spectrometry
LDL	low density lipoprotein
AA	arachidonic acid
PAF	platelet activated factor
HNDFs	human neonatal dermal fibroblasts
PBMCs	peripheral blood mononuclear cells
bFGF	basic fibroblast growth factor

PDGF	platelet derived growth factor
IC50	half maximal inhibitory concentration
MTT	3,-4,5 dimethylimidazole-2,5 diphenyl tetrazolium bromide
NFAT	nuclear factor of activated T cells
AP-1	activator protein 1
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
WB	western blot

1.0 Introduction

At a surface level, prevalent diseases seem unrelated; yet many diseases express similar biomarkers, inflammation perhaps being the most common. Often referred to as "inflammatory diseases," hypertension, cancer, autoimmune disorders, a variety of motor neuron diseases, atherosclerosis, and cardiovascular diseases (CVD) all share the trait of chronic (often systemic) inflammation (Edris, A. et al. 2007, Querio, G. et al. 2018, Tao, L. et al. 2013, Huang, N. et al. 2017, Hunter, P. 2012, de Lavor, E. et al., 2018, Vasto, S. et al. 2006, Kaur, S. et al. 2012, Aoe, M. et al. 2017). Unlike acute localized inflammation that occurs in wound healing (specific to the site of injury), on-going systemic inflammation has been linked to an overactive immune system (Castellheim, A. et al. 2011). The immune system, designed to maintain balance between endogenous and exogenous molecules, pathogens, and various environmental threats, becomes progressively unbalanced when the ability to maintain homeostasis is chronically dysregulated (Horwitz, D. et al. 2019). Immune tolerance is a term used to describe the lack of pathologic response to infection, tissue damage, vaccination, and environmental toxins (Abbas, A. K. et al. 2004; Horwitz, D. et al. 2019).

For example, during normal immune response to a threat, the immune system is acutely initiated to upregulate immune complexes and inflammatory mediators but returns to steady state after the threat has cleared (Horwitz, D. et al. 2019). When homeostasis is challenged for long periods of time, various imbalances can transpire due to persistent release of inflammatory mediators, uncleared immunogens, downregulated macrophage activation due to acquired allergic responses, as well as activation of self-reactive T cells, altogether provoking a loss of immunogenic tolerance (Horwitz, D. et al. 2019; Skapenko, A. et al. 2005). Autoimmune diseases occur when immune responses attack self (harmless) antigens, which can be triggered by faulty

signaling within several mechanisms of immune tolerance. Pathogens can be eliminated, but when a self-antigen becomes immunogenic, it cannot. Immune cells respond towards self-antigens by secreting proinflammatory molecules (such as chemokines and cytokines) that perpetuate tissue damage, a process referred to as autoimmune inflammation (Mackay, I. 2001).

Because immune-mediated mechanisms of protective (acute) inflammation and chronic inflammation are similar, there are multiple mechanisms at work to maintain tolerance. Central tolerance for lymphocyte cells takes place in central lymphoid organs, such as the thymus or bone marrow; either weak antigen interactions lead to stimulation (positive selection), or strong interactions lead to a self-reactive phenotype, thus removing the lymphocyte before it is released to circulation via apoptosis (negative selection) (Mackay, I. 2001). However, central tolerance "leaks" autoreactive (anti-self) lymphocytes to the periphery, exposing vascular barriers where secondary processes are utilized when needed. Peripheral tolerance, therefore, works to prevent the activation of self-reactive lymphocytes by ignorance, anergy, homeostatic control, and regulation, which are the various mechanisms involved to inhibit T cell function/promote cell death (Mackay, I. 2001). Autoimmune diseases involve dysregulation of more than one mechanism of tolerance and can thus be classified by the mechanism of tissue damage (Abbas, A. K. et al. 2004). Chronic inflammation in the peripheral microvasculature is often the result of defective systems of immune tolerance and associated with many autoimmune diseases (Abbas, A. K. et al. 2004; Horwitz, D. et al. 2019; Mackay, I. 2001).

Due to the complexity of the immune system, two complimentary divisions of functionality are specified and include innate immunity and adaptive immunity. Immediate reactions to infection (viruses, bacteria, and fungi) are regulated by the primary defense mechanisms of the innate immune system, which utilizes both chemical and physical barriers to protect the body against

invaders within minutes of exposure (Punt, p.113). Cellular responses initiated by pathogen recognition serve as secondary defense mechanisms within innate immunity, which are followed by an upregulation of receptors and release of soluble mediators that consequently activate adaptive immune responses. Adaptive immunity is elicited by exposure to various pathogens and environmental factors, which cause the development of specific immune responses to a multitude of potential invaders. Adaptive immunity facilitates natural host defenses towards evolving microorganisms by initiating B and T lymphocytes to infiltrate areas of infection and/or tissue injury (Punt, p.17-18). Hence, the innate and adaptive immune systems work in tandem for proper modulation and activation of regulatory systems in the body that react to cell signaling between various cell types (Raphael, I. et al. 2020).

Undifferentiated (naïve) T cells are T cells that have entered circulation and have not encountered antigen (Punt, p.354). T cells circulate between blood, lymph, and secondary lymphoid tissues in search for antigens, wherein recirculation of T cells occurs every 12-24 hours to ensure antigen recognition between surface receptors on both T cell and antigen presenting cell (APC) (Punt, p.354; Luckheeram, R. et al. 2011). Via positive selection, naïve T cells may either develop into the cytotoxic (CD8+) or helper (CD4+) T cell lineages, depending on the class of major histocompatibility complex (MHC) they bind to (Punt, p.354; Luckheeram, R. et al. 2012). CD8+ T cells (cytotoxic T cells) and CD4+ T cells (T helper cells) are associated with classes MHC-I and MHC-II, respectively. While CD8+ T cells directly kill infectious cells, the CD4+ T cell lineage regulates immune responses by addressing their specific antigens, mediating tolerance, and controlling inflammation. Mature CD4+ T cells require antigen presentation to initiate activation, followed by downstream activation pathways that are comprised of various costimulatory molecules and cytokines that further differentiate CD4+ T cells into subsets.

Remarkably, activated CD4+ T cells are capable of regulating both innate and adaptive immune responses and are used as a diagnostic tool in many infectious and autoimmune disorders (Luckheeram, R. et al. 2012; Battistini, G. et al. 2021).

Of particular interest is the response of circulating CD4+ T cells during vascular inflammation, where cells lining the vessel wall can provide costimulatory signals or cause T-lymphocyte adhesion (Razakandrainible, R. et al. 2012). Whereas antigen presenting cells (APCs) are typically responsible for providing antigen stimulation to CD4+ T cells, activated vascular cells act as non-professional APCs by upregulating expression of MHC II and other costimulatory molecules (Razakandrainible, R. et al. 2012; Neefies, J. et al. 2011; Mai, J. et al. 2013; Mestas, J. et al. 2005). Inflamed vascular tissue also upregulates adhesion molecules that allow interactions between these two cell types to be prolonged. Consequences include sustained cell activation, transmigration of T cells, and a positive feedback loop between coagulation and inflammatory mediators. Overall, chronic inflammatory threat in the microvascular compartment disrupts the endothelial barrier, exposing adhesion molecules, inflammatory cytokines, and procoagulant factors.

The microvasculature is a semi-permeable single layer of endothelial cells (ECs) that separates blood and blood components from lymph (Mai, J. et al. 2013). The endothelium is considered an organ with various subtypes (i.e., heterogeneity) across the vascular tree and in major organs (Mai, J. et al. 2013). Regardless of location, the endothelium is a protective organ for both blood components and molecules within the subendothelial compartment in which three major categories of function have been discussed. These categories include trophic (metabolic), tonic (vascular hemodynamics), and trafficking (permeability, coagulation, and extravasation) (Mai, J. et al. 2013). ECs release molecules to regulate blood pressure and flow as well as

coagulation. In the basal state, very few molecules cross the endothelium; however, a compromised endothelium increases permeability which facilitates transmigration of activated immune cells. Interestingly, there is little interaction between ECs and leukocytes (white blood cells) under homeostatic conditions. It is during activation that both cell types upregulate adhesion molecules, recruiting leukocytes to damaged sites along the endothelium. Activated ECs express adhesion molecules that tether and roll the circulating leukocyte to a site of entry where the rolling stalls, eventually permitting leukocyte transmigration to the underlying tissues (Mai, J. et al. 2013). Although adhesion molecules are expressed in arterioles and capillaries, venules have a distinctly higher concentration. This may be due to the small diameter of venules or location (thus lower pressure of blood flow) that allow for more leukocyte adhesion responses, making post-capillary venules an important site for leukocyte transmigration and coagulation via adhesion of platelets to trapped/stationary leukocytes (Granger, D. and Senchenkova, E. 2010 p.40). Leukocyte-endothelial interactions are typically weak, yet when both cell types are activated their receptor-ligand affinity is greatly increased. Harmful byproducts produced during firm adhesion between activated ECs and other activated cells can result in oxidative and enzymatic degradation of the microvascular compartment (Granger, D. and Senchenkova, E. 2010 p.40).

Several adhesion molecules of interest sequentially facilitate T cell rolling, and the expression patterns closely correlate with the stage of inflammation (Muller, W. 2002). For example, tethering is mediated by vascular adhesion molecule 1 (VCAM-1), rolling is mediated by E-selectin (also referred to as CD62E), and firm adhesion by intracellular adhesion molecule 1 (ICAM-1). Unperturbed endothelial cells do not express high levels of ICAM-1, VCAM-1, or E-selectin, yet during the inflammatory process these molecules are markedly increased (Muller, W. 2002). All three of these EC adhesion molecules are upregulated in the presence of

proinflammatory cytokines, thus maintaining cell-to-cell contact and resulting in alterations of T cell phenotype and EC physiology (Brezinschek, R. et al. 1998).

As mentioned, proinflammatory cytokines released by activated immune cells stimulate ECs. Cytokines are proteins utilized by the immune system to accelerate proinflammatory or anti-inflammatory signaling between immune cells (Punt, J. 2019, p.91). Additionally, cytokines play a modulatory role towards effector functions in immune cells, consequently influencing adhesion molecule expression in the target cell. Only a small concentration of cytokine secretion is needed to induce significant physiological responses due to the close location and high affinity to their receptors (Punt, J. 2019, p.91). When initiated as a protective physiological response, the close proximity and high affinity of cytokines are beneficial, but when the immune response is propelled by endothelial dysfunction, over-stimulated cells within the vasculature contribute to excessive production and/or effector responses to inflammatory cytokines. Although cytokines are often noted for contributing to endothelial dysfunction, some cytokines are vital for CD4+ T cell development, enabling them to perform regulatory roles. In fact, differentiation of CD4+ T cell subsets are determined by cytokines in the microenvironment in addition to interaction with specific antigen (Luckheeram, R. et al., 2011). Therefore, the regulation of various cytokines must be maintained by proper communication between various cell types that are responsible for cytokine synthesis and release. Although chronic inflammation concerning excessive cytokine production permits detrimental trapping and transmigration of leukocytes, these otherwise protective molecules cannot be eradicated entirely. The balance of mechanisms that support either normalizing cytokine production or inhibiting unfavorable interactions with target cells could be advantageous towards regaining physiological harmony within the microvasculature.

The impact of disproportionate interactions within the vasculature itself presents a wide range of physiological outcomes. Unrelenting proinflammatory signaling can be damaging to the endothelium, leading to endothelial dysfunction (ED) and loss of adherent junctions between ECs. Disruption of vascular integrity increases EC permeability, which promotes local hemoconcentration and reduces shear rate of blood flow, allowing prolonged interactions between CD4+ T cells and ECs to favor adhesion/transmigration in postcapillary venules (Muller, W. 2002). As adhesion molecules slow CD4+ T cell movement, the over-stimulated microvascular environment creates a layered series of events that altogether decrease vascular tone and support coagulation. Loss of endothelial integrity permits exposure of other small molecules to the luminal wall, allowing for interaction with platelets in circulation. Cross-talk between endothelial cells, T cells, and platelets directly initiate coagulation pathways. Coagulation factors that are typically not expressed on the EC surface are able to gain exposure through endothelial cell gaps, exposing tissue factor (TF), the initiator of the inflammatory cascade. The interactions between these three cell types have been associated with the pathogenesis of atherosclerosis and cardiovascular disease (CVD) (Gonchovo, et al. 2017; Gimbrone M. et al. 2016).

As well, interactions between CD4+ T cells and microvasculature ECs is a current area of focus for research towards the elucidation of complex relationships between chronic inflammation, immune deficiency, neurodegenerative diseases, skin disorders, and infectious disease pathology (Danikowski, K., et al. 2017; Raphael, I. et al. 2020; Hirahara, K., Nakayama, T. 2016). Systemic inflammation is typically a silent contributor of unknown etiology to many chronic diseases; however, the addition of unfavorable genetic and environmental factors also contributes to the latent untoward consequences of inflammation. Exogenous molecules may turn on specific genes that favor an inflammatory response (Hunter, P. 2012). Environmental factors (diet, lifestyle,

living conditions) provide a host of externally derived risks to one's health and are the reason diseases like CVD, diabetes, and atherosclerosis have been classified as lifestyle diseases (also referred to as "modifiable diseases").

Aside from vascular dysfunction and coagulation as a result of inflammation in lifestyle diseases, many other chronic health conditions have a vascular component. As discussed previously, overactivity of the body's immune response, termed "autoimmunity," is characterized by loss of tolerance or self-discrimination (Danikowski, K. et al. 2017). Autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), and myasthenia gravis (MG) involve chronic inflammation and other complications associated with ED as classic symptomology (Horwitz, D. et al. 2019; De Lavor, E., et al. 2018; Wu, Y. et al. 2016; Atehortúa, L. et al. 2017; Murdaca, G., et al. 2012; Uzawa, A. et al. 2016). As a result of the complexity of autoimmune pathologies, there are many challenges for pharmacological interventions. Drug-related therapies attempt to slow disease progression but involve side effects and often result in debilitating flare-up of symptoms if medication is discontinued. (Horwitz, D. et al. 2019).

Direct inhibition of inflammation to a specific site of damage (targeted delivery of therapeutics, also referred to as "vascular immunotargeting") with the use of highly selective therapeutics may provide effective treatment while protecting the well-designed inflammatory response and overall function of the immune system. Avoiding unnecessary interruption of inflammatory/coagulation pathways under appropriate conditions while allowing the therapeutic effect to stabilize excessive leukocyte/endothelial cell interactions could have primary and secondary treatment efficacy. Likewise, a balance of target-independent and target-dependent

delivery of therapeutics may offer both passive and specific distribution of anti-inflammatory agents (Muller, W. 2002, Kiselva, R., et al 2017).

Current research is aimed towards identifying specific constituents and biological effects of volatile compounds derived from plants, otherwise known as essential oils (EOs). Several independent laboratories have proposed EOs promote anti-angiogenesis, anti-inflammatory, and immunomodulatory properties in both *in vitro* and *in vivo* studies (Querio et al., 2018, Tao et al., 2013, Huang, C. et al. 2017, Aoe et al. 2017, Woronuk et al. 2011, Siddqui et al., 2011, Han et al. 2017, de Lavor et al. 2018, AL-Okbi et al. 2018, Tomoyuki et al.2003, Theurer et al. 2013, Edris et al. 2007, Figueiredo et al. 2008, Kaur et al. 2012, Singh et al. 2009, Bostancioğlu et al. 2012). EOs possess immunomodulatory properties that affect cellular and molecular functions of the immune system. While some EOs are immunostimulatory, others act as immunosuppressants. However, because EOs contain many biologically active compounds, a naturally sourced pure EO can have both immune suppressive and stimulatory abilities. Furthermore, EOs can have immunomodulatory abilities via secondary and tertiary active metabolites, promoting both longer duration and range of therapeutic effects. Yet it is important to note that the synergy of all constituents within a plant's volatile component work together and collectively contribute to their therapeutic effects (Han, X. et al. 2017; Huang, N. et al. 2008).

One class of EOs that have powerful medicinal properties are the phenolic EOs. Many phenols are antibacterial, anti-inflammatory, antimicrobial, and can increase permeability of the cytoplasmic membrane, which allows binding of EO constituents to intracellular proteins. Further, phenolic EOs contain secondary metabolites that have double-conjugated bonds and provide an array of antioxidant properties. Both clove bud and thyme are considered phenolic EOs. The chemical composition of clove bud EO has demonstrated antidiabetic activity (Oboh, G. et al.

2015) as well as potent anti-thrombotic activity via inhibition towards thromboxane synthesis and platelet aggregation (Saeed, S. et al. 1994). Furthermore, clove EO inhibits inflammatory cytokine production in macrophages, specifically interleukin 1-beta (IL-1β) and interleukin 6 (IL-6) *in vitro* and *in vivo* (Rodrigues, T. et al. 2009). Likewise, the phytotherapeutic qualities of thyme EO and its dominant constituents have been observed in many studies. Previous studies have demonstrated enhanced endothelial function after exposure to thyme EO, such as significant reductions in interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), ICAM-1, and VCAM-1. One of the major constituents of thyme, thymol, showed significant suppression in the phosphorylation of ERK and JNK pathway proteins when administered to mouse epithelial cells that were stimulated with lipopolysaccharide, thereby decreasing inflammation (Liang, D. et al. 2014). This study suggests the inhibitory effects towards NF-κB and COX-2/iNOS are the "therapeutic qualities" of thymol that may be beneficial for treating inflammatory diseases (Liang D. et al. 2014). The collective narrative of these studies assessing clove and thyme EOs and their respective effects towards mitigating inflammation and coagulation conveys the potent and protective properties of EOs, specifically those within the phenol classification.

The objective of the proposed research is to simulate an inflammatory environment between cultured ECs/CD4+ T cells and analyze the expression of adhesion molecules and pro-coagulant activity before and after treatment of clove bud and red thyme EOs. To accomplish this objective, the following aims will be addressed:

Aim 1

Aim 1 assessed the anti-inflammatory/anti-coagulation abilities of *Thymus vulgaris* (red thyme) and *Eugenia caryophyllata* (clove bud) EOs. Human umbilical vein endothelial cells (HUVECs) were grown to confluency, activated with TNF-α, and treated with either thyme or

clove bud EOs for 3 hr and 6 hr using a non-toxic concentration (0.01% or 0.1µL/mL) identified with a cytotoxicity assay. Inflammatory markers of interest (ICAM-1, E-selectin, IL-6) and a coagulation marker (TF) were analyzed from cell lysates. It was hypothesized that clove and thyme EO would mitigate markers of inflammation as well as lower the expression of TF that typically follows endothelial dysfunction characterized by excessive inflammation.

Aim 2

The second aim of this project was to assess the effects of clove and thyme EOs towards the same markers of protein expression in activated HUVECs that also included coincubation with CD4+ T cells. Reducing the affinity of EC/T cell interactions by way of interrupting oxidant-sensitive inflammatory pathways common to the microvascular, such as NF-κB and MAPK, may be a mechanism through which clove bud and thyme regulate inflammatory molecules associated with endothelial dysfunction. Due to a robust amount of literature revealing antioxidant and immunomodulatory properties of thymol, p-cymene, eugenol, and β-caryophyllene, the most prominent constituents of the EOs used in this study, we expect prominent changes in expression of cell-to-cell adhesion chemical mediators and downstream inflammatory markers. It is our hypothesis that both clove bud and thyme EOs will perform similar and meaningful anti-inflammatory effects at modest concentrations, proposing these EOs to be a cost and resource effective options for future experimental studies in relation to mitigating inflammation.

2.0 Literature Review

2.1 Immune-mediated Inflammation

The vascular endothelium responds to both mechanical and chemical stimuli by releasing factors that regulate the underlying smooth muscle. Because microvascular inflammation plays a major role in the pathology of multiple diseases, it is imperative to better understand underlying mechanisms of systemic inflammation as it relates to local tissue damage and in relationship to secondary undesirable responses in the affected tissues. As inflammation evolves, the endothelium responds by exposing procoagulant factors in addition to a continual synthesis of cytokines and adhesion molecules, a process previously defined as endothelial cell activation. The environment created by these orchestrating inflammatory factors within the microvascular compartment affords various communication signals for circulating immune cells. Because chronic inflammation lacks a resolution phase, both anti-inflammatory and proinflammatory cytokines are released incessantly, leading to a process known as immunosuppression (Rogovskii, V. 2020). Suppression of the immune response may cause immune cells to lose their function or suspend development of CD4+ T cell differentiation towards regulatory/anti-inflammatory T cell subsets. As mentioned previously, cytokine concentration does not need to be high to induce an effect on nearby cells. Chronic low-grade inflammation has been linked to immune tolerance wherein very low levels of cytokines can significantly impact cellular responses (Rogovskii, V. 2020). The initiation of inflammation via endothelial activation can be provoked by factors present in circulation as well as factors released by the ECs themselves, as some proinflammatory molecules released by ECs act in an autocrine fashion, sustaining endothelial cell activation or initiating apoptosis. Examples of proinflammatory factors that activate ECs are TNF-α, IFN-γ, and other members of the interleukin family that regulate immune and inflammatory responses to infection (Brown, M. et

al. 2011; Brezinschek, R. et al. 1998; Mestas, J. et al. 2005; Rogovskii, V. 2020). During the early stages of ED, activated ECs produce high levels of interleukin-6 (IL-6). TNF-α and IL-6 both utilize the NF-κB pathway, in which the initiation from TNF-α during ECA consequently upregulates IL-6 expression while also regulating the expression of inflammatory genes (Brown, M. et al. 2011; Madge, L. et al. 2001). Although many markers of inflammation are associated with CVD and autoimmunity, IL-6 is a common contributor to many lifestyle and immune-mediated diseases during processes involved across all stages of diseases progression (Brown, M. et al. 2011; Ataie-Kachoie, P. 2013). Furthermore, IL-6 can be derived from T cells, provide T cell stimulation, regulate antigen-specific immune responses, and control hematopoiesis (Ataie-Kachoie, P. 2013).

Though indirect, ECs have the ability to act as APCs in which endothelial expression of MHC class-II gene has been demonstrated under inflammatory conditions, indicating ECs as non-professional APCs (Neefjes, J. et al. 2011; Geppert, T. D., Lipsky. 1985, Certo, M. et al. 2020). ECs do not express CD80 and CD82 (B7.1 and B7.2, respectively), which are traditional co-stimulatory molecules that typically bind to the CD28 receptor. Instead, ECs use alternative ligands such as inducible costimulatory ligand (ICOS-L), OX40L, and members of the TNF-receptor families to provide adequate stimulus for T cell activation. Furthermore, because ECs do not express CD80 or CD86, they are unable to provide activation for naïve T cells yet are able to activate effector/memory T cells as well as provide differentiation machinery (Certo, M. et al. 2020). While antigen presentation to CD4+ T cells is a critical part of T cell activation, the addition of proinflammatory molecules or other co-stimulatory ligand/receptor interactions may provide more assistance in the activation and differentiation of T cells in the periphery (Hirahara, K., Nakayama, T. 2016, Lee, W. et al. 2010). Interleukin 2 (IL-2) is an example of a cytokine crucial

for T cell growth, differentiation, as well as protecting T cells from entering an anergic (abnormal or tolerant) state (Boulougouris, G. et al. 1999; Fathman, C. F. 2007). Another example of a proinflammatory cytokine that interacts with T cells and is a potent activator of ECs is IL-1β, produced by activated macrophages, monocytes, and dendritic cells. IL-1β plays an important role towards differentiation of CD4+ T cells to the anti-microbial/anti-inflammatory T-helper 17 (Th17) subset, and interactions between EC/T cells are sustained in the presence of IL-1 β, indicating a link between innate and adaptive immunity during peripheral inflammatory responses (Velázquez, F. et al. 2016, Volpe, E. et al. 2008, Hirahara, K., Nakayama, T. 2016, Lee, W. et al. 2010). Former work has shown ECs to have immunostimulatory mechanisms that prolong cytokine synthesis in resting memory CD4+ T cells (Mestas, J. et al. 2005). Overall, the increased expression of MHC class-II molecules on both ECs and T cells along with various cytokines in the microenvironment are important markers of systemic inflammation and ED, especially in autoimmune disease populations (Certo, M. et al. 2020; Turesson, C. 2004). Altogether, T cell proliferation is often driven by cytokines released by circulating immune cells or provided by activated ECs with an adhesive surface, while the co-stimulatory mechanisms between EC/T cells provide additional synthesis of cytokines that up-regulate MHC class-II signaling pathways, thus supporting inflammation, proliferation, differentiation, and migration at sites of EC/T cell interactions (Mestas, J. et al. 2005).

While the cellular mechanism of downregulating cell surface adhesion molecules is unknown, a previous study showed an ability of adhesion molecules to be partially detached from cell membranes during a process referred to as internalization or shedding, in which the extracellular domain of adhesion molecules E-selectin and ICAM-1 become “soluble” forms, detached from the EC surface (Leeuwenberg, J. F. M., et al. 1992). This study also discussed that

soluble (disassociated) adhesion molecule domains demonstrate ligand-binding activity, in which inhibition of leukocyte-endothelial adhesion may be an advantage of soluble adhesion molecules by way of occupying leukocyte receptor sites in the plasma, mitigating leukocyte adhesion and migration through the endothelium (Leeuwenberg, J. F. M., et al. 1992). Nonetheless, intact endothelial-leukocyte interactions that are sustained typically produce unwanted cellular modifications in both cell types that either lead to or progress ED. Interestingly, genetic modifications such as knocking out adhesion molecules on either ECs or leukocytes produced less damage to the endothelial wall during inflammation, suggesting the role of adhesion molecules to be paramount in understanding endothelial-leukocyte interactions in light of vascular inflammation (Granger, N. 2010, p. 58).

Chronic inflammation involves an overreactive immune response that disrupts tightly regulated endogenous production of endothelial-derived molecules and initiates co-stimulatory mechanisms between lymphatic and connective tissue cells which damage peripheral vascular beds when regulatory mechanisms are not responding properly (Muller, W. 2002). Untreated inflammation can lead to changes in the affinity and valency of the endothelium, such as surface density and phenotypic characteristics of ECs, which may impact molecular recognition of ligand binding sites (Chavakis, E. et al. 2009; Kiseleva, R. et al. 2017). An activated endothelium favors T cell migration, in which T cells migrate more efficiently when in an activated state (Brezinschek, R., et al. 1998). Extravasation of lymphocytes to perivascular tissue is connected to immune-mediated inflammation, and it has been demonstrated that T cells acquire endothelial membrane components during transmigration. In a former study, ECs were activated with TNF-α for 4 hours to upregulate E-selectin, ICAM-1, and VCAM-1. (Brezinschek, R., et al. 1998). Activated CD4+ T cells acquired E-selectin during transmigration, changing the phenotype of the T cell. The

authors also point out that activated CD4+ T cells upregulate expression of E-selectin on ECs, suggesting a critical role during chronic inflammation through both the synthesis and delivery of endothelial factors to perivascular tissue. This study also demonstrated that activated CD4+ T cells initiated apoptosis in TNF-α activated ECs, whereas resting CD4+ T cells did not (Brezinschek, R., et al. 1998). Correspondingly, another study explored a role of endothelial microparticles (EMP), extracellular vesicles that are shed from the plasma membrane during ECA, to elicit an immunomodulatory response. Just as ECs can present MHC and co-stimulatory molecules to T cells, EMP promoted binding of human brain microvascular endothelial cells to both CD4+ and CD8+ T cells, which led to T cell proliferation and conjugate-like interactions that are necessary for T cell activation and differentiation. Interestingly, cytokine stimulation (TNF-α alone or combined with IFN-γ) from EMP significantly augmented T cell proliferation, yet proliferation was unresponsive to EMP in resting CD4+ T cell conditions. EMP binding has been shown to be preferential with effector T cell populations over naïve or terminally differentiated memory T cells. Despite these impressive immunomodulatory abilities, it has been shown that EMP induced proliferation of T cells to be inferior to proliferation levels induced by parent ECs or APCs (Wheway, J. et al. 2014).

2.2 Pathogenesis of Endothelial Dysfunction

Loss of endothelial integrity inhibits healthy immune responses such as fighting off infection by releasing harmful microparticles and pro-inflammatory molecules into circulation, contributing to pathology for several systemic autoimmune diseases including SLE, RA, MS, atherosclerosis, and psoriasis (Saadi, S. et al. 1995, Miller, D. et al. 1998, Atehortúa L. et al. 2017, McInnes, I. et al. 2017, Skapenko, A. et al. 2005). In fact, substantial loss of endothelial integrity can result from direct injury from cytotoxic CD4+ T cells. Perforin, a protein released by killer

cells, has been identified on the surface of CD4+ T cells in patients with plaque instability associated with unstable angina. This study demonstrates that ECs, specifically within the microvasculature, is a target tissue for CD4+ T cells with cytotoxic capabilities, and more importantly that EC death induces plaque rupture (Nakajima, T. et al. 2002).

Many autoimmune diseases complicated by ED reveal harmful effects of transendothelial migration. For example, a key concept of MS is the transmigration of autoreactive T cells across the endothelial barrier, which exposes certain organs to excessive levels of inflammatory foci that damage nerve fibers in the brain and spinal cord (Punt, J. 2019 pg. 609; Danese, S. et al. 2007; Danikowski, K. et al. 2017). Myasthenia gravis (MG) is another example of an autoimmune disease mediated by CD4+ T cells, where patients have elevated cytokines that are specifically associated to CD4+ T cell subtypes, in which both MG and MS pathologies correlate to Th17 and T-helper 1 (Th1) subsets. Although MS and MG affect different tissues (CNS and neuromuscular junction, respectively), both diseases are characterized by dysregulation of T cell suppressive and migratory markers as well as immune overactivity (Danikowski, K., et al. 2017). Specifically, T regulatory cells (Tregs) are a subset of CD4+ T cells with anti-inflammatory function, yet Tregs have less suppressive ability in patients with either disease, which can lead to an increased production of proinflammatory molecules (Danikowski, K., et al. 2017). Because the microvascular compartment is stimulated by inflammatory molecules, disease pathology and severity may be related to a loss of self-tolerance in the periphery in conjunction with microvascular ED. It is for this reason that both systemic and organ specific autoimmune diseases rely on peripheral immune responses, in which manifestations of ED should be addressed to reduce risk of disease progression due to an ongoing inflammatory threat and increased risk of secondary concerns such as thrombosis and cardiovascular events.

The ability for peripheral endothelium to maintain an environment that allows for the proper function of T cells is imperative to immune function; in fact, correspondence and activation between these cell types rely upon inflammatory cytokines. Because T cell differentiation is cytokine dependent, the presence of various cytokines can be useful under homeostatic conditions. For instance, immune cell facilitation of peripheral tolerance is largely controlled by Tregs. As mentioned above, Tregs are a subset of differentiated $CD4^+$ T cells that have anti-inflammatory function. TNF-α is a proinflammatory cytokine involved in ECA and can inhibit the function of Tregs, which suggests that activated ECs are capable of supporting both immune associated inflammation and loss of immune tolerance via their ability to modulate T cell function (Skapenko, A. et al. 2005). Inflammatory immune responses cause various cell types (including innate immune cells, adaptive T cell subtypes, and cytotoxic CD8+ T cells) to release TNF-α, arresting EC growth and upregulating adhesion molecules that begin to traffic circulating CD4+ T cells (Certo, M. et al. 2020). Alternatively stated, TNF-α synthesized by T cells activates ECs, thus enhancing T cell adhesion while disrupting EC homeostasis and confusing immune-driven pathways (Certo, M. et al. 2020).

Yet, just as peripheral ED impacts the immune system at large, localized endothelial damage resulting from chronic inflammation in a specific site provides another reciprocal example between chronic inflammation and autoimmunity. Pathogenesis of rheumatoid arthritis begins as inflammation at the site of a joint and is closely related to patterns observed in common ED in which small and medium vessels are affected by chronic inflammation. Untreated RA may progress to a secondary disease known as rheumatoid vasculitis, or “complicated RA,” which closely resembles other types of vasculitis and atherosclerosis (Atehortúa L. et al., 2017; Witkowski et al. 2015). Rheumatoid vasculitis is a form RA that has become widespread and

altered homeostasis in multiple blood vessels. Importantly, this provides an example of how localized chronic inflammatory threat causes systemic loss of homeostatic control, either contributing to initial disease pathology or introducing new manifestations of the existing condition. Altogether, hemostatic components, activated leukocytes, and pro-inflammatory mediators are used clinically to diagnose many chronic illnesses of both lifestyle and autoimmune disease classifications. Therapeutic targets that act to inhibit endothelial-leukocyte interactions by way of interrupting proinflammatory cytokine release while protecting immune tolerance may be useful in the treatment of such conditions (Skapenko, A. et al. 2005).

2.3 Immune-mediated Coagulation

As ED precedes coagulation and cardiovascular events, elucidating the mechanisms by which endothelial-leukocyte interactions contribute to thrombus formation is critical. Inflammatory cytokines promptly upregulate adhesion molecule expression on both T cells and ECs, followed by a slow decline (Leeuwenberg, J. F. M., et al. 1992). Cell adhesion molecules are involved in over-recruitment of leukocytes, leading to aggregation of inflammatory responses, including those seen in asthmatic patients and autoimmune disorders (Aoe M. et al., 2017, Hunter et al., 2012, Theofilopoulos A. et al., 2017, Shimizu Y. et al., 1991). Consequential formation of intracellular gaps between otherwise adjacent endothelial cells exposes endothelial-derived clotting factors as well as inhibiting intercellular communication (Okamoto, T., Suzuki, K. 2017). Interactions between clotting factors and adhesion molecules on circulating leukocytes and platelets promotes procoagulant activity along the microvascular endothelium, further assaulting regulatory pathways involved with vascular function (Saadi, et al. 1995; Muller, W. 2002; Okamoto, T., Suzuki, K. 2017).

As mentioned previously, excessive damage and infiltration to the arterial wall exposes intercellular components that further contribute to the inflammatory process and thrombosis (Gimbrone, M. et al. 2016; Muller, W. 2002 review; Ryan, J. et al. 1992). Various inflammatory cytokines have been shown to activate ECs (IL-1β, IFN-γ, and IL-6, and TNF-α) (Doshi, S. et al. 2002; Ataie-Kachoie, P. 2013; Ryan, J. et al. 1994). Activated platelets in circulation can interact with these adhesion molecules and TF on the vessel wall, forming a bridge with the endothelium via P-selectin which is expressed on both platelets and ECs and mediates adhesion. This bridge creates an endothelial-platelet-leukocyte interaction that allows leukocytes to roll with firm adhesion, fueling a positive feedback loop for coagulation and inflammatory pathways (Danese, S. et al. 2007; Rao, V. *review.* 2004). It is for this reason the endothelium is considered the master regulator of homeostatic control of various cell types in circulation (Danese, S. et a. 2007). The involvement of activated platelets has been proposed as the link between innate and adaptive immunity, in which activated platelets are capable of facilitating leukocyte adhesion/transmigration and can also emulate the adhesive ability of activated T cells. Activated platelets mediate endothelial-leukocyte adhesion as well as platelet-leukocyte adhesion, via a number of platelet ligands that are specific for CD4+ T cell receptors (Li, N. 2013). Furthermore, the activation of leukocytes directly increases vascular permeability by disrupting EC wall barrier during transmigration, exposing procoagulant molecules and further perpetuating inflammation, edema, and thrombus formation (Guven, G. et al. 2020; Li, N. 2013).

Common features of autoimmune diseases may include loss of immunological tolerance or delayed reactions to immune response termination. Balance of the immune system thus relies on a highly regulated interplay of T cells which govern these functions (Rosenblum, M. et al. 2015). ED combined with overactive T cell responses are distinguishing pathologies of atherosclerosis,

an autoimmune disease characterized by platelet formation and potential rupture leading to cardiac events, deep vein thrombosis, and altered cellular function (Li, N. 2013; Koltsova, E. et al. 2012; Okamoto, T., Suzuki, K. 2017). Atherosclerosis, like many other autoimmune disease classifications, can also present as a secondary/acquired disease for another primary/preexisting autoimmune disease such as MS, MG, or asthma (Danikowski, K. M. et al. 2017; Muller, W. 2002). Interestingly, unstable atherosclerotic plaques have been found to contain cells of the immune system, including CD4+ T cells, dendritic cells, and macrophages, with CD4+ T cells being the most abundant (Koltsova, E. et al. 2012; Li, N. 2013). During the development of atherosclerosis, APCs and T cells are upregulated in number, in which the interaction between dendritic cells and CD4+ T cells promote the proliferation of T cells with production of proatherogenic cytokines IFN-γ and TNF-α (Koltsova, E. et al. 2012). Moreover, previously activated T cells can be restimulated (expressing IL-2 and MHC class II receptors), supporting continual plaque formation in the arterial wall (Koltsova, E. et al. 2012; Li, N. 2013). All CD4+ T cell lineages (characterized by their respective cytokine secretion) have been located within atherosclerotic plaques; however, the Th1 lineage demonstrates pro-atherosclerotic activity while the Treg lineage is athero-protective, suggesting that T cell directed therapy may be implicated by T cell differentiation (Li, N. 2013).

2.4 Exposure of Tissue Factor

Tissue factor (thromboplastin/CD142) (TF) is expressed on many cell types as a cell surface glycoprotein important for homeostasis and thrombosis. (Lopes-Bezerra, L. et al 2003, Bogdanov, V. et al 2015). As a primary initiator in the coagulation cascade, it serves as the only known receptor for coagulation factor VIIa (activated factor VII). TF mediates a complex formation with factor VIIa, which activates factor IX and X, altogether forming a localized blood

thrombus (via thrombin and fibrin) (Lopes-Bezerra, L. et al. 2003; Witkowski M. et al. 2016). As a transmembrane receptor, TF is a member of the cytokine II receptor family and is typically only expressed by vascular cells when homeostasis is interrupted by immune and/or inflammatory mediators that damages the vessel wall (Ruf W., Reiwald, M. 2013; Lopes-Bezerra, L., et al. 2003). Expression of TF is upregulated in the presence of certain inflammatory cytokines and adhesion molecules, which also downregulates thrombomodulin, thus contributing to both endothelial damage and fibrin formation when endothelial activation is persistent (Miller, D. et al. 1998). Homeostasis can be threatened by an overly-adhesive endothelial layer (inflamed microvasculature), creating paracrine-like cell signaling between circulating leukocytes that leads to their accumulation on the endothelium. Red blood cells may then accumulate behind the leukocyte-endothelial complex and push towards the venular wall. In vitro research has demonstrated TF regulation of monocyte diapedesis, suggesting a role in regulating tissue extravasation in addition to its role in initiating vascular coagulation (Doshi, S. et al. 2002).

Additionally, ED impacts hemostasis and immunity by interfering with tissue transport of circulating nutrients, hormones, and gas exchange, as well as interrupting electrical communication along the endothelium that typically flows through intact gap junctions (Goven, G. et al. 2020). Endothelial gap junctions have been identified as a location for TF to gain access to the cell surface, allowing excessive interaction with other coagulation molecules in the passing blood compartment. Former research has found TF to be exposed on EC surface following damage to the endothelial cell wall, suggesting that ED primes coagulation via exposure of otherwise intercellular molecules (Ryan, J. et al. 1992; Doshi, S. et al 2002; Okamoto, T., Suzuki, K. 2017; Rao, V. 2004). Furthermore, previous research observed that apical EC surface exposure of TF to corresponding clotting substrates in the blood was not achieved until EC surface was disrupted

with both TNF-α stimulation and permeabilization with a detergent, indicating that TF is sequestered in subendothelial vesicles until cell wall integrity is altered (Ryan, J. et al. 1992). Thus, injury of the vessel wall consequently supports coagulation.

In a study that analyzed four molecular pathways of T cell adhesion in HUVECs, activation (via IL-1β) showed a significant increase in adhesion between the two cell types (Shimizu, Y. et al 1991). Furthermore, this study also demonstrated that the activation of T cell (via PMA, phorbol ester) allowed for even greater adhesion of T cell to activated HUVECs, suggesting activation of both cell types to be advantageous towards understanding leukocyte adhesion in autoinflammatory diseases (Shimizu, Y. et al 1991). However, this study also showed endothelial leukocyte adhesion molecule (ELAM-1, also referred to as E-selectin) plays a significant role in basal $CD4^+$ T cell adhesion to activated HUVECs (Shimizu, Y. et al 1991), suggesting that activation of both cell types is not always necessary to elicit adhesion in-vitro, which may be more representative of a biological model. Cell adhesion differences based upon activation type has also been demonstrated in vitro with a coincubation model (Jurkat T cell/HUVECs) by activating HUVECs with TNF-α. Adhesion was assessed via atomic force microscopy, representing changes in interaction with compression force and contact time, revealing intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule (VCAM-1) to have better adhesion strength than E-selectin (Zhang, X. et al. 2006). However, E-selectin is induced by several inflammatory cytokines in most tissues and has several known ligands on specific T cell lineages and is a diagnostic measure of auto-inflammatory diseases such as psoriasis, arthritis, or contact dermatitis (Matsumoto, M. et al. 2005).

Prior experiments like these provide compelling evidence about cytokine involvement in the interaction between cells of the immune system and microvasculature. For example,

inflammatory cytokines circulating in the plasma, such as TNF-α and IL-6, are significantly higher in patients with coronary artery disease and may provoke endothelial expression of TF (Szotowski, B. et al. 2005). Procoagulation induced by TF activation can also be stimulated by membrane microparticles and microorganisms, though pathogenic stimulation is a tightly regulated process and responds to only specific microbial pathogens (Lopes-Bezerra, L. et al 2003). TF plays an important role in both homeostasis and procoagulant activity in the vasculature (Witkowski M. et al., 2016, Miller D. et al. 1998). Endothelial damage that progresses as a result of chronic inflammation causes procoagulant factors on the membrane to be increased over time, potentially altering the phenotype towards sustained procoagulation (Martin, D. et al. 1995). Therefore, a two target approach that promotes antithrombotic and anti-inflammatory effects should be the focus for treating autoimmune conditions where vascular dysfunction is involved.

2.5 Current Implications in Prevention/Treatment for Autoinflammatory Diseases

Patients with inflammatory autoimmune conditions are not only susceptible to intolerance of humoral and cell-mediated immunity but also an inability to maintain vascular homeostasis, increasing risk for cardiovascular events (McInnes, I. et al. 2017; Guo, Q. et al. 2017; England, B. et al., 2018; Atehortũa L. et al., 2017). Current approaches for treatment/prevention of autoinflammatory diseases include reduction of antigen expression and prevention of immune complex formation, cytokine inhibitors, and oral corticosteroids (McInnes, I. et al. 2017; Guo Q.et al. 2017; Rosenblum, M. et al. 2015). To decrease pain associated with inflammation, clinicians are traditionally taught to prescribe non-steroidal anti-inflammatory drugs (NSAIDs). These medications are effective in their interaction with targeted proinflammatory cytokines as well as selective inhibition of prostaglandins and cyclooxygenase-2 (COX-2 enzymes). However, negative effects have been observed from prolonged use of NSAIDs. Inhibition of COX-2 blocks

production of prostaglandins, and because downstream transformation of prostaglandins and thromboxanes are dependent on the COX pathway, sustained inhibition of COX-2 can increase the risk of platelet aggregation and cardiovascular events, especially for patients already at risk (Graham, D. et al. 2006; Fitzgerald, G. et al. 2004). NSAIDs also delay muscle generation, impair regeneration of ligaments, tendons, and cartilage, indicating these drugs to be unsuitable for patients with RA and associated conditions (Maroon, J. et al. 2010).

Another class of drugs used more specifically to treat autoimmune rheumatic diseases are called disease-modifying anti-rheumatic drugs (DMARDs) (Schrezenmeier, E., Dörner, T. 2020). Hydroxychloroquine, also classified as an antimalarial drug, is one of the most commonly prescribed DMARDs for various rheumatic diseases such as SLE, RA, APS, and Sjögren syndrome (Schrezenmeier, E., Dörner, T. 2020). Hydroxychloroquine is immunomodulatory via its multi-targeted mechanism of action. Prevention of disease flares/symptoms, delaying organ damage, protection against infections, and reducing risk of atherosclerosis are the major mechanisms related to immune modulation (Schrezenmeier, E., Dörner, T. 2020). One particularly interesting mechanism of action has been identified, in which impairment of antigen presentation through the lysosomal pathway inhibits both cytokine synthesis and MHC class II presentation to T cells. Without proper T cell activation through antigen presentation or co-stimulatory molecules, differentiation of T cells is inaccessible (Schrezenmeier, E., Dörner, T. 2020). Although not prescribed as an anticoagulant, hydroxychloroquine is believed to have vasoprotective effects, thus preventing thrombotic complications. The anticoagulant properties include inhibition of antiphospholipid antibody binding and platelet aggregation, thus improving endothelial function (Schrezenmeier, E., Dörner, T. 2020). Although hydroxychloroquine continues to serve as one of the leading DMARDs, it does not come without side effects. Hydroxychloroquine has a long half-

life of ~50days which produces prolonged effects after discontinuing use. This increases potential toxicity or risk of adverse events. Furthermore, there is an increased risk of retinopathy in high-dose and/or long-term users, due to the drug binding to melanin in the retinal pigment epithelium which can cause irreversible blindness (Wolfe, F., Marmor, M. 2010). More common effects associated with short term use are gastrointestinal discomforts including nausea, vomiting, and diarrhea (Schrezenmeier, E., Dörner, T. 2020).

An additional clinical practice for controlling mild autoinflammatory disease states is the use of medications that suppress the immune system (immunosuppressants). Inducing immunological reset while keeping the immune system appropriately active could be the future direction of therapy for autoimmunity characterized by ED. Lastly, anti-TNF biologics, which are designed to block the biological function of TNF, have also been developed to treat autoimmune inflammatory diseases (Meier et al., 2013). Anti-TNF biologics have been reported to have side effects on the neurological system. For example, anti-TNF treatment exacerbated diseases in almost all multiple sclerosis (MS), which may be explained by one possible mechanism by which TNF-α antagonists increase the number and activity of autoreactive T cells (Robinson, W. et al., 2001). Furthermore, TNF-α antagonists cannot cross the blood brain barrier, in which presence of TNF-α in the cerebrospinal space and brain is a major implication for demyelination in MS. Ironically, an addition of secondary autoimmune diseases can be presented due to anti-TNF therapy. One example of this is the onset of psoriasis in patients being treated with TNF inhibitors for irritable bowel disease (Robinson W. et al., 2001). The all-inclusive antagonism of all TNF activity, which include both protective and pathogenic functions, may indicate that anti-TNF therapy is inappropriate and contradictory for treating autoimmune diseases.

Because autoimmunity is often developed years before a patient becomes symptomatic, there is a therapeutic window that exists during which clinical signs of vascular dysfunction (such as excessive production of autoantibodies, procoagulant factors, and superfluous inflammation) can be detected in the periphery via basic hematology practices. Combination therapy that focuses on reducing environmental factors such as smoking and obesity as well as administering immunomodulating substances may be of use for therapy in the prevention of inflammatory autoimmune and cardiovascular diseases. Natural anti-inflammatory agents, such as the use of organic antioxidants, may be a valid way to supplement treatment for individuals in progressed disease stages as well as offer a safe practice of disease prevention for at risk populations (McInnes, I. et al. 2017).

2.6 Integrative Practices for Autoinflammatory Diseases

Though the modern approach for treatment (pharmaceuticals prescribed to treat symptoms) can effectively target inflammation, patient responses to drugs as well as prescription adherence vary and often complicates side effects and drug tolerance. Many natural compounds found in plants have been researched with the intent of identifying the biological effects of EOs. While some EOs are immunostimulatory, others act as immunosuppressants; however, some EOs possess both suppressant and stimulatory abilities due to to the wide array of chemical constituents found in the EO from a single plant. For example, lavender EO stimulates phagocytosis while also circumventing the inflammatory response in macrophages as a response to infection (Giovannini D. et al. 2016). As mentioned above, it is important to understand the concept of synergy, in which many constituents within a single EO or a blend of several EOs contributes to their ability to deliver multiple therapeutic effects (Han, X. et al. 2017; Huang, C. et al. 2008). Furthermore, synthesis of secondary metabolites in aromatic plants are impacted by factors that are present in the growing

conditions/environment, such as type of soil, temperature, salinity, humidity, light sensitivity, and microorganisms specific to the region (Alves, N. et al. 2022; Ramakrishna, A. et al. 2011). Plant sensitivity to its environment and abiotic signals emphasizes the consequence of aromatic plants grown for their medicinal extractions in green houses/labs to be inhibitory to their medicinal qualities, unless these chemistry-contributing conditions are known and can be duplicated in a lab.

2.7 Essential Oil Classifications

EOs are comprised of many diverse compounds that vary from plant to plant and between plant species (Figueiredo, A. et al., 2008). These bioactive compounds are categorized by their terpene content (Dhifi W. et al. 2016). Terpenes are also referred to as hydrocarbons and classified as either mono-, sesqui-, or di- terpenes depending on their structure and placement of functional groups around the main carbon atom (Figueiredo, A. et al. 2008; Alves, N. et al. 2022). EOs are also categorized by oxygenated derivatives in the terpene family (such as alcohols, aldehydes, ketones, and phenols) as well as non-terpenic compounds such as phenylpropanoids (namely eugenol, cinnamaldehyde, and safrole) (Dhifi. W. et al. 2016). Due to their vastly differing chemistry, EOs contain many structurally differing compounds and can produce a wide variety of physiological effects when introduced to the human body. Structural features like lipid solubility and small molecular size allow binding of EO constituents to intracellular proteins including degradation of genetic material in bacterial cells (Leja, K. et al. 2019). EOs that contain high levels of phenol constituents are antibacterial, anti-inflammatory, antimicrobial, and have the ability to increase permeability of the cytoplasmic membrane (Michel J. et al., 2020; Leja, K. et al. 2019). Further, phenolic oils contain secondary metabolites that have double-conjugated bonds and deliver profound antioxidant properties. The oxidizing properties in phenolic compounds bond readily with free radicals. These antioxidant properties contribute to their anti-inflammatory

potential. The endogenous antioxidant system is enhanced by these medicinal characteristics of phenols, specifically from their ability to chelate metal ions (Michel J. et al., 2020). Because chronic inflammatory diseases inherently disrupt normal processes of cellular function, including eradication of free radicals, the anti-inflammatory and immune-regulatory potentials of EOs is an expanding area of aromatic plant research.

Synergy of various plant constituents that comprise an EO and contribute to the bioactivity when introduced to the human body, which provides a plausible spectrum of treatment for various illnesses derived from bacterial or fungal infections, chronic inflammatory diseases, and immune dysregulation (Bostancioğlu et al., 2012). One study observed therapeutically-relevant cellular effects with EOs that were not induced by their isolated compounds (Urasaki, Y., Le, T. T. 2019). As mentioned, the synergistic effects orchestrated by the whole EO demonstrate the most promising physiological benefits. In the study by Urasaki and colleagues, four essential oils (copaiba, mandarin, melissa, and turmeric) were profiled using nanofluidic protein post-translational modification (PTM) assay to assess regulatory effects on various cell signaling pathways. This study found isolation of EO components as well as adulteration of EOs produced opposite, or negative, effects on three signaling pathways (MAPK, PI3k/AkT/mTOR, JAK/STAT). Interestingly, even when identical chemical composition was confirmed through gas chromatography mass spectrometry (GC/MS), the biochemical effects of synthetic oils did not produce therapeutic outcomes compared to pure EOs. In addition to the finding that adulteration of an EO profile may be undetectable with only basic GC/MS, thus the biochemical effect of EOs cannot be replicated with adulterated EOs, this study also found that the synergistic effect of all the compounds within a whole EO are vital to the therapeutic outcome (Urasaki, Y., Le, T. 2019).

Equally important to appreciating the synergy of EOs is understanding that an EO chemical profile may be different in various parts of the plant. For example, oil extracted from a plant stem may have the same constituents as the leaf, but in different amounts. In other words, the percentage of a particular constituent is often higher in one plant part as opposed to another (i.e., chemical profile of an EO derived from a seed vs. leaf, or leaf vs. stem, of the same plant will differ). Interestingly, the time of day also influences the placement of these secondary metabolites in living plants. The exact profile of an EO can be shown with GC/MS, though, as mentioned previously, GC/MS does not detect adulteration (Urasaki, Y., Le, T. 2019). Many practices use all or most plant parts to produce a higher yield (or overall percentage) of EO content, yet the quality is compromised due to the chemical profile being influenced by constituents found in the entire plant. For instance, some plant parts may possess less therapeutic constituents than other plant parts, especially in light of the preferred therapeutic quality/biological outcome desired. Furthermore, the distillation method used also has a major impact on the outcome of an EOs chemical profile. For example, one study compared water-steam distillation to hydrodistillation (Wesolowska, A. et al. 2016). The EO analyzed in this study, *Thymus vulgaris* L. (garden thyme), produced significantly higher percentages of phenolic constituents in the EO produced from water-steam distillation, though the overall content/yield of EO was the same between both methods. The importance of expertise for the delicate process of distillation is evident when seeking an EO for medicinal purposes.

2.8 Therapeutic Potentials of Essential Oils

The pharmaceutical model is committed to a biologically targeted approach and locating the active ingredient in a drug in which a cascade of physiological events may be explained; however, these pharmaceutical therapies often result in unwanted side effects for the patient as

well as complicated dosing regimen for rapid metabolizers/acquired tolerance to the drug. In contrast, the unique combination of a plant's volatile compounds suggests that it is the synergy between each molecule that interacts in multiple biological processes, yielding multiple benefits, otherwise known as pleiotropic effects (Schnaubelt, K. 2011). Lipophilic interactions of EOs allow them to insert themselves into the lipid membrane, thus gaining access into the cell, changing the membrane function/permeability, as well as affecting genetic expression by attaching to the receptor proteins/transcription factors that influence genetic machinery (Shin, S. et al. 2018; Gholijani, N. et al. 2015; Schnaubelt, K. 2011). The mechanistic advantage of such lipophilicity and receptor recognition also gives EOs exceptional effectiveness towards intracellular viruses, unlike many conventional drugs (Schnaubelt, K. 2011). EOs readily pass through lipophilic cellular membranes, making their volume of distribution to various body tissues broader than their synthetic counterparts/insoluble molecules used in many medications (Dhifi, W. et al., 2016). The wide distribution of EOs is why establishing dosing guidelines and application methods is necessary for EOs to be acknowledged and safely practiced.

Many pure EOs can safely be administered orally, topically, and aromatically. Upon further evaluation of safety and efficacy, establishing dosing and application regimens specific to each EO may offer a variety of therapeutic replacements for medications. EOs have demonstrated capabilities similar to or better than pharmaceutical drugs regarding improving markers of oxidative stress, such as premature ageing, atherosclerosis, and lipid peroxidation that may result in consequential autoimmune disorders or cardiovascular complications (Lee, S. et al 2005; Michel, J. et al., 2020). For example, basil, cinnamon, clove, nutmeg, and oregano EOs have been noted as highly effective free radical scavengers through their antioxidant activity (Peterflavi, A. et al. 2019; Han, X. et al. 2017). Interestingly, EO compounds in the phenol classification (eugenol

and thymol) were found to not only have higher antioxidizing effects but also increased affinity of low-density lipoprotein (LDL) particles towards the LDL receptor in comparison to other volatile compounds that are not considered phenolic (linalool, geranial, anethol, pulegone, limonene, and *p*-cymol) (Naderi, G. et al 2004). The lipophilic properties of eugenol and thymol allows them to penetrate the LDL particles, thus leading to increased LDL affinity. These results were linked to LDL oxidation in vessel walls in the development of atherosclerosis (Naderi, G. et al 2004).

2.9 Essential Oils Clove and Thyme

2.9.1 Clove EO

In particular, clove EO demonstrated a similar mechanism of action as NSAIDs in the inhibition of platelet aggregation and thromboxane synthase (Saeed, S. et al. 1994). In a study that analyzed in vitro effects of clove EO against platelet aggregation induced by arachidonic acid (AA), collagen, and platelet-activating factor (PAF), clove EO was 20-40x more of an effective antiplatelet than aspirin against all stimulus factors, in a dose-dependent manor. In this same study, clove EO was analyzed in vivo (rabbit) against the lethal effect of injected AA to induce pulmonary thrombosis, in which animals received either clove EO or aspirin 2 hours prior to stimulation of AA or PAF. Clove EO provided protection against sudden shock/death in the rabbits (100% protection against PAF and 70% against AA). This comprehensive study suggests clove EO to be a potent anti-thrombotic agent both in-vitro and in-vivo (Saeed, S. et al. 1994).

Another study attributed the favorable chemical composition of clove bud oil, rich in a particular constituent eugenol, to be responsible for enhanced antidiabetic activity. Clove bud EO inhibited type-2 diabetic enzymes, which contribute to hyperglycemia, oxidative damage to β-cells, and early onset of type-2 diabetes (Oboh, G. et al., 2015). The specific enzymes in this study,

α-amylase and α-glucosidase, support the release of glucose in the blood, which is an issue in diabetics who do not secrete sufficient amounts of insulin. This study demonstrated clove bud EO inhibitory effect towards α-amylase and α-glucosidase, while also demonstrating antioxidant activity in a dose dependent manner, suggesting clove bud EO to be a prospective intervention for oxidative stress induced type 2 diabetes. Another study administered several EOs (clove, ginger, and cinnamon) in rats exposed to diabetes (via injection). This study, too, found anti-diabetic effects of these EOs to significantly decrease circulating levels of blood glucose and increase insulin while improving lipid profile, liver, and kidney function (Hassanen, N. et al., 2010).

Furthermore, clove EO inhibits cytokine production in macrophages, specifically IL-1β and IL-6 in vitro and in vivo (Rodrigues, T. et al., 2009). This study verifies via GC/MS that eugenol is the dominant component of clove EO used in this study, in which the author attributes the reduced cytokine expression to the active component eugenol. Another study conducted dose-response experiments with clove EO and isolated eugenol extract in marine macrophages towards immune-associated inflammatory markers, both before and after macrophage stimulation of LPS (Bachiega, T. et al. 2012). IL-1β, IL-6, and IL-10 were all significantly inhibited before and after LPS stimulation at 100 µg/well. The effects of eugenol alone were significant inhibition of IL-6 both before and after LPS stimulation at 50 µl and 100 µl, inhibition of IL-10 only after cells were treated with LPS but did not affect production of IL-1β at either time point. The authors proposed that the anti-inflammatory mechanism of action of clove is through suppression of NF-κB signaling (Bachiega, T. et al. 2012).

Clove EO has also previously been evaluated within a blend of EOs high in terpene content for an enhanced synergy on several autoimmune systems, including skin cell gene expression and markers of inflammation in vitro (Han, X. et al. 2017). In this study, the oil blend consisted of wild

orange, clove, cinnamon, eucalyptus, and rosemary. The autoimmune systems resembled that of 4 different T-cell stimulated autoimmune complexes with the following cell lines: HUVECs, human neonatal dermal fibroblasts (HNDFs), B cells, and peripheral blood mononuclear cells (PBMCs). Using multiple cell lines to represent different immune system interactions followed by incubation with the EO blend allowed for insight as to how these body systems may respond to the EO blend. Stimulatory factors used to induce inflammatory and genetic responses in cultured cells included IL-1β, TNF-α, IFN-γ, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF). The results of this study revealed the bioactivity of the EO blend to resemble that of immunomodulation; in particular, the EO blend upregulated genes in unhealthy cells (immunostimulatory) while also downregulating genes that were producing negative immunosuppressive responses. The genome-wide gene expression readout additionally showed the EO blend affected signaling pathways corresponding with cell cycle control and inflammation, while others altered mRNA levels in pathways related to DNA damage response (Han, X. et al. 2017). Altogether, this study delicately demonstrates of the potential therapeutic effects for a variety of whole EOs combined for an enhanced overall chemical profile reflected in the EO blend. These prior experiments underline a relevance towards investigating anti-inflammatory and anti-coagulation potentials of pure clove bud EO as it relates to ECD and immune function in the microvascular compartment.

2.9.2 Thyme EO

The phytotherapeutic qualities of thyme EO and its dominant constituents have been observed in many studies. Thyme EO, across many species, is typically comprised of carvacrol, p-cymene, and thymol in high percentages compared to its other constituents. However, the concentration of constituents relies heavily on species and geographic origin. One study assessed

the chemical diversity of 11 Moroccan thyme species and chemotypes towards tumor survival in a mastocytoma cell line (P815 tumor cells) as well as an effect on proliferation in PBMCs (Jaafari, A. et al. 2007). This study found the overarching constituents in all Moroccan thyme species to be carvacrol and thymol (phenols) along with p-cymene and γ-terpene (monoterpene hydrocarbon precursors), and borneol and linalool (oxygenated monoterpenes). When comparing isolated compounds, carvacrol and thymol, carvacrol demonstrated cytotoxic activity at the half maximal inhibitory concentration (IC50) of less than 0.004% v/v, whereas thymol was less cytotoxic at an IC50 of 0.015%. Although the authors suggest thymol to have a preventative effect towards cancer via its antioxidant properties, and carvacrol to have better antitumor properties, it is important to note that pure thyme EO contains both of these constituents, and demonstrated antitumor effects at 0.01%, 0.016%, 0.22%, 0.225%, and 0.24% IC50 values, regardless of species/chemotype. Because chemotherapy can be detrimental to the body, this study also analyzed the effect of thyme EO on PBMC viability. Thyme EO at the same concentrations used to produce a cytotoxic effect in tumor cells, showed no toxicity in PBMCs, but rather a proliferative effect, while the isolated extract thymol was 100% cytotoxic to PBMCs at the higher concentration 0.5% v/v (Jaafari, A. et al. 2007). These findings allude to the safety of using thyme EO and suggest the effectiveness of a whole EO vs. its isolated extracts.

Another study analyzed the same two components of thyme, thymol and carvacrol, in relation to activated T-cell mediated inflammatory diseases (Gholijani, N. et al. 2015). This study utilized a T cell leukemia cell line (Jurkat T cells) to investigate several transcription factors that control the activation and secretion of specific cytokines released by T cells during an inflammatory response. Given that a MTT cell viability assay revealed concentrations of 50 µg/ml and higher to be cytotoxic for the Jurkat T cell line, 10 µg/ml and 25 µg/ml were used for

experiments. A dose dependent response towards IL-2 and IFN-γ in PMA/calcium ionophore-activated T cells was observed for both thymol and carvacrol, in which there were significant decreases in both cytokines at both concentrations 10 µg/ml and 25 µg/ml. For transcription factor analysis, cells were pretreated with thymol and carvacrol before PMA/calcium ionophore stimulation, which revealed no effect for NFAT-1, nuclear c Jun, or phosphor-NFκB p65 levels in either thymol nor carvacrol, yet both significantly decreased NFAT-2 (which induces the expression of genes in immune cells involved with cell growth and survival) and c-Fos (a heterodimer protein required for AP-1 activation). Hence, the inhibition of NFAT-2 and AP-1 activity by these compounds may, in part, be a mechanism of action responsible for the anti-inflammatory and immunomodulatory qualities of thymol and carvacrol in activated T cell mediated diseases (Gholijani, N. et al. 2015). Likewise, another study analyzed the suppressive effects of several EOs (thyme, clove, rose, eucalyptus, fennel, and bergamot) towards LPS-induced COX-2 promoter activity and PPARα/γ activation in bovine arterial ECs (Hotta, M. et al. 2010). Thyme had a dose-dependent effect on the suppression of COX-2 promoter activity, and though other EOs also demonstrated similar effects, thyme EO had the strongest influence. Two of the main individual constituents of thyme, carvacrol and thymol, also showed dose-dependent suppressive effects towards COX-2 promoter activity while at the same concentrations activated PPARα/γ; however, another main constituent of thyme, p-cymene, did not demonstrate similar activity towards either marker (Hotta, M. et al. 2010). Another study evaluated in-vitro modulation of cytokine secretion in LPS-stimulated murine macrophage-like cells (RAW 264.7 cells) treated with several concentrations of p-cymene (Zhong, W. et al. 2013). Cytokine secretion in vitro revealed a significant reduction of IL-1β and IL-6 at the two higher concentrations, while suppression of TNF-α was observed at all three concentrations (53.58 µg/ml, 107.16 µg/ml, 214.32

μg/ml). In vivo, no effect was observed for IL-6; however, TNF-α and IL-1β were significantly decreased for groups pre-treated with p-cymene, and IL-10 increased significantly. The upregulation of anti-inflammatory IL-10 suggest that p-cymene may regulate inflammation by multiple mechanisms, via increasing anti-inflammatory factors while inhibiting pro-inflamamtory cytokines. mRNA levels of the cytokine genes corelated with the in vitro data, in which a significant decrease in phosphorylated ERK 1/2, JNK, and p38 trended in a dose dependent manner. The authors suggested that the suppression of inflammatory genes to inhibition of MAPK and NF-κB pathways, concluding that p-cymene has regulatory functions towards immune and inflammatory pathways (Zhong, W. et al. 2013).

While each aromatic-producing plant has its own unique chemical profile of EO composition, there is often overlap of many constituents in various proportions. Further, while the percentages of the similar constituents differ, the synergistic quality of the whole, pure EO from each plant is worth considering as their components may yield similar therapeutic outcomes. One example is hinoki cypress, a needled evergreen, that shares several constituents as thyme. Past research has shown that Chamaecyparis obtusa (hinoki cypress) has immune-modulating and anti-inflammatory properties in isolated dendritic cells and CD4+ T cells (Shin, S. et al. 2018). Specifically, pretreatment for 1 hour with 0.05% of the EO significantly inhibited cytokine production and inhibited costimulatory molecules in activated dendritic cells. In coculture experiments with activated, pretreated dendritic cells and CD4+ T cells, the oil significantly inhibited IFN-γ production from the CD4+ T cells in a dose dependent manner, yet IL-10 production was not affected (Shin, S. et al. 2018). Undoubtedly, this study shows another example of an EO with medicinal properties that include side benefits, in which the synergy of various constituents assists the immune system in the direction of homeostasis. Critical to the therapeutic

effect is the chemical profile of the EO, in which more than half of the constituents of hinoki cypress EO used in this study are also identified in the thyme EO used in the present study. For this reason, the pharmacological potential of thyme EO will likely be reproduced in other immune cell interactions as it relates to inflammation and T cell polarization.

3.0 Methodology

3.1 Materials

Plant Materials/Reagents

Essential oils *Thymus vulgaris* (red thyme) (lot# E7727) and *Eugenia caryophyllata* (clove bud) (lot# 55532) were used for experiments (doTERRA Intl., Pleasant Grove, UT). Chemical analysis of each EO verified by gas chromatography mass spectroscopy (GC/MS) (provided by doTERRA Intl.) indicated individual EO components expressed in percentages. Purity of each EO was verified through optical rotation, specific gravity, refractive index, and colorimetry to ensure the absence of pesticides, allergens, or carrier oils to ensure quality control.

Antibodies and Reagents

Recombinant human IL-2 protein was purchased from R&D Systems (cat. No. 202-IL-010/CF). Recombinant human TNF was purchased from R&D Systems (cat. No. 210-TA-020/CF, Minneapolis, MN). Anti-CD28 was purchased from VWR (cat. No. 302904-BL/302903-BL). Anti-CD3 was purchased from VWR (cat. No. 317325-BL).

3.2 Cell Culture

3.2.1 HUVECs Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Morristown, NJ) and cultured in endothelial medium-2-medium containing 2% fetal bovine serum and growth supplements VEGF, FGF, EGF, IGF, ascorbic acid, GA 1000, 1% penicillin-streptomycin, 1% gentamycin. Caucasian male umbilical endothelial cells from two donors were grown in parallel and repeated five times (N=5). HUVECs were seeded and subcultured in T-75 flasks and

maintained at 37°C in a 5% CO2 atmosphere. HUVECs were seeded into 100mm dishes (2.0 x 10^5) at passage 6 for aim 1 experiments and seeded into 6-well plates (1.0 x 10^5) at passage 6 for aim 2 experiments.

3.2.2 CD4+ T Cell Culture

Human peripheral blood CD4+ T cells (Lonza, cat. No. 2W-200) were seeded into a 96-well plate at 0.01 x 10^6 with serum-free, xeno-free LGM-3™ lymphocyte growth medium-3 (Lonza, cat. No. CC-3211) which was supplemented daily with IL-2 (100 IU/mL) (R & D Systems, cat. No. 202-IL-010/CF). For optimal stimulation, CD4+ T cells were activated with 50µl of a final solution of anti-CD3 (final concentration 1ng/µl) and anti-CD28 (final concentration 2ng/µl). Cells were subcultured in T-25 flasks and supplemented with fresh IL-2 every day (100 IU/mL) until they reached a quiescent stage and preserved in liquid nitrogen until aim 2 experiments ensued. Once thawed, CD4+ T cells were centrifuged at 300g 21°C for 10min, resuspended, and counted using a hemocytometer. Cells were then used in coincubation experiments at a seeding density of 2.0 x 10^5 with confluent HUVEC monolayer (1.0 x 10^5) for 24 hours.

3.2.3 Cytotoxicity Assays

Preliminary data to assess potential toxicity of clove bud and thyme EOs were applied towards both cell lines using Trypan Blue viability test (Lonza BioResearch, USA). Trypan blue dye measures viability via cell membrane structure, in which dead cells with ruptured membranes obtain dye and live cells do not. Samples were diluted in Trypton Blue dye of an acid azo exclusion medium by preparing a 7-fold dilution of the cell suspension and 0.4% Trypan Blue solution. Cell viability is determined with an automated cell counter (Thermo Fisher Countess 3 Automated Cell Counter, cat. No. A49891). Total count as well as live and dead cell count with percentages were

obtained and averaged from a double sided slide (Thermo Fisher Countess™ Cell Counter Chamber Slides, cat. No. C10228). Four concentrations (0.0025%, 0.005%, 0.01%, 0.02%) of clove and thyme EOs were added to confluent HUVECs and CD4+ T cell cultures to obtain cytotoxicity data. Each cell line was maintained in their respective cell culture medium mentioned in sections above (3.2.1 and 3.2.2, respectively).

3.2.4 Coculture Experiments

Once HUVECs reached ~95% confluency in 6-well plates, serum-free media was added to control plates, while select plates were incubated with 30ng/ml TNF-α in serum-free media for 4 hours to stimulate cells. Activated and inactivated HUVECs were then cocultured with CD4+ T cells for 24 hours using a HUVEC/CD4+ T cell ratio of 1:2. Activated cells were treated with either clove bud or thyme EO (0.01%) for 6 hours. EO mixtures also contained 8ul/mL (0.04%) DMSO as a vehicle control. For comparative purposes, control conditions included confluent inactivated HUVECs monolayer, inactivated HUVECs with DMSO, TNF- α activated HUVECs, as well as a condition of activated HUVEC + CD4+ T cell with no addition of either EO. For cell harvest, the HUVEC monolayer washed with HBSS for removal of non-adherent T cells and culture media. Cells were then lysed with 1x cell lysis buffer (Cell Signaling, cat. No. 9803). Cell supernatants were frozen down and later assayed for expression of ICAM-1, E-selectin, IL-6, and TF.

3.3 Western Blot

Proteins of interest from cell lysates (ICAM-1, E-selectin, IL-6, TF) were detected using Western Blot (WB) analysis. First, total cellular protein concentrations were determined with BCA assay kits (BioRad, Hercules, CA, USA). Supernatants were then prepped with 4x Laemmli buffer at 1 µg/µL for WB analysis and 15 µl per lane were loaded on pre-casted 4-15% SDS-PAGE gels (Bio-

Rad Laboratories; Hercules, CA, USA). Electrophoresis was performed for ~50 minutes at 150 V using 1x SDS-PAGE run buffer (VWR Laboratories; Randor, PA, USA). Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad; Hercules, CA, USA) for 120 minutes at 200 mA. Membranes were stained with Ponceau S for protein visualization and equal loading of samples and imaged on a gel documentation system (UVP ChemiDoc-It2 Touch imaging system, Bio-Rad). Membranes were then blocked for 1 hr with 5% non-fat milk powder and Tris-buffered saline with 0.1% Tween-20 (TBST). Primary antibodies were prepared in TBST containing 5% bovine serum albumin (BSA, Ameresco) and incubated with gentle agitation for 72 hr at 4°C. Primary antibodies included human tissue factor/CD142 (E9M6T) XP® rabbit mAb (1:1000, Cell Signaling, cat. No. 97438S), human E-selectin/CD62E (ELAM-1) mouse mAb (1:1000, Thermo Fisher Scientific, cat. No. MA1-22165), ICAM-1 mouse mAb (1:1000, Thermo Fisher Scientific, cat. No. MA5-13021), IL-6 mouse mAb (1:1000, Thermo Fisher Scientific, cat. No. MA5-23698). Following primary antibody incubations, membranes were washed 3x TBST and subsequently incubated with HRP-linked species-specific secondary antibodies at room temperature for 1 hour. Secondary antibodies included anti-rabbit IgG (1:2000, Cell Signaling, cat. No. 7074) and anti-mouse IgG (1:2000, Cell Signaling, cat. No. 7076). Total protein expression was detected by chemiluminescence using enhanced chemiluminescent reagent (EMD, Millipore, Billerica, MA, USA). Images were captured using a band densitometry analyses (UVP ChemiDoc-It2 Touch imaging system, Bio-Rad). The densities of the selected protein bands were quantified with Vision Works software, in which protein band densities were quantified relative to the densities of the internal control gene, β-Actin, for total protein normalization (1:2000 anti-β-Actin, Cell Signaling, cat No. 3700S).

3.4 Statistical Analysis

Between-treatment comparisons were performed using SPSS (IBM Corp., Armonk, NY USA) as well as independent t-tests through Excel. All values are expressed as mean ± standard deviation (SD) values. P values of 0.05 or less were considered statistically significant. Sample size for aim 1 (n=5) sample size for aim 2 (n=1).

3.5 Ethical Considerations

This study was carried out on one cell line which includes only one race (Caucasian). This study is an exploratory study, and future studies may want to implement a racial component.

Chapter 4.0

COMPLETED MANUSCRIPT

Immune-Associated Chronic Inflammation: Biological Responses Between Endothelial Cells and T Cells Following Treatment of *Thymus vulgaris* and *Eugenia caryophyllata* Essential Oils

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ABSTRACT

Chronic inflammation is associated with autoimmune disorders and lifestyle diseases such as hypertension, cardiovascular disease, and atherosclerosis. Prolonged interaction between circulating leukocytes (CD4+ T cells) and microvasculature endothelial cells (ECs) triggers excessive cytokine production, exposing adhesion and coagulation molecules. Consequences include disrupted vascular integrity, transmigration, platelet aggregation, altered T cell phenotype and EC physiology. Although inflammatory cytokines and coagulation factors can be detrimental, these otherwise protective molecules cannot be eradicated entirely. Mechanisms that support balanced cytokine production and appropriate adaptive immune responses could facilitate microvascular homeostasis. Medicinal plant extracts, essential oils (EOs), have biological effects that are multiplied by the synergistic nature of their various constituents. Phenolic EOs such as clove and thyme demonstrate immunomodulatory, anti-inflammatory, and anti-platelet activity. EOs naturally contain compounds with diverse physiological benefits, suggesting a multi-targeted therapeutic approach for prevention and treatment of immune-mediated inflammatory diseases. Thus, the present study was conducted to assess potential therapeutic effects of clove and thyme EOs towards markers of immune-associated vascular inflammation (E-selectin, ICAM-1, IL-6). Additionally, we investigated the effects of these oils towards the initiator of the coagulation cascade, tissue factor (TF). TNF-α activated human umbilical vein endothelial cells (HUVECs) were incubated with CD4+ T cells. These data show significant TF protein expression in all HUVEC conditions activated with TNF-α ($p = <0.001$), yet no differences were observed with the addition of either EO as compared to untreated TNF-α activated HUVECs. Other markers did not reveal significant differences statistically, although visual differences observed may suggest a trend of anti-inflammatory effects by both oils in some conditions. For HUVECs coincubated with

CD4+ T cells, there seems to be a mild preventative and treatment effect with clove EO while thyme EO increased protein expression, yet this data is preliminary. We conclude that TF expression may contribute to cytokine-mediated inflammation, while ICAM-1, E-selectin, and IL-6 did not reveal significant modulation under cytokine stimulation or treatment with EOs.

Keywords: Endothelial Dysfunction, Autoimmune, Chronic Inflammation, Essential Oils, CD4+ T Cells

INTRODUCTION

At a surface level, prevalent diseases seem unrelated; yet many diseases express similar biomarkers, inflammation perhaps being the most common. Often referred to as "inflammatory diseases," hypertension, cancer, autoimmune disorders, a variety of motor neuron diseases, atherosclerosis, and cardiovascular diseases (CVD) all share the trait of chronic (often systemic) inflammation (Edris, A. et al. 2007, Querio, G. et al. 2018, Tao, L. et al. 2013, Huang, N. et al. 2017, Hunter, P. 2012, de Lavor, E. et al., 2018, Vasto, S. et al. 2006, Kaur, S. et al. 2012, Aoe, M. et al. 2017). Unlike acute localized inflammation that occurs in wound healing (specific to the site of injury), on-going systemic inflammation has been linked to an overactive immune system (Castellheim, A. et al. 2011).

Autoimmune diseases involve dysregulation of more than one mechanism of tolerance and can thus be classified by the mechanism of tissue damage (Abbas, A. K. et al. 2004). Chronic inflammation in the peripheral microvasculature is often the result of defective systems of immune tolerance and associated with many autoimmune diseases (Abbas, A. K. et al. 2004; Horwitz, D. et al. 2019; Mackay, I. 2001). Of particular interest is the response of circulating CD4+ T cells during vascular inflammation, where cells lining the vessel wall can provide costimulatory signals or cause T-lymphocyte adhesion. (Razakandrainible, R. et al. 2012). Whereas antigen presenting cells (APCs) are typically responsible for providing antigen stimulation to CD4+ T cells, activated vascular cells act as non-professional APCs by upregulating expression of MHC II and other costimulatory molecules (Razakandrainible, R. et al. 2012; Neefies, J. et al. 2011; Mai, J. et al. 2013; Mestas, J. et al. 2005). Beyond this, inflamed vascular tissue upregulates adhesion molecules that allow interactions between these two cell types to be prolonged. Consequences include sustained cell activation, transmigration of T cells, and a positive feedback loop between

coagulation and inflammatory mediators. Overall, chronic inflammatory threat in the microvascular compartment disrupts the endothelial barrier, exposing adhesion molecules, inflammatory cytokines, and procoagulant factors.

The microvascular compartment is a semi-permeable single layer of endothelial cells (ECs) that separates blood and blood components from lymph (Mai, J. et al. 2013). Due to its size (~ 6 x 10^{13} cells), the endothelium is considered an organ with various subtypes in other major organs (Mai, J. et al. 2013). Regardless of location, the endothelium is a protective organ for both blood components and molecules within the subendothelial compartment in which three major categories of function have been discussed. These categories include trophic (metabolic), tonic (vascular hemodynamics), and trafficking (permeability, coagulation, and extravasation) (Mai, J. et al. 2013). ECs release molecules to regulate blood pressure and flow as well as coagulation. At rest, very few molecules cross the endothelium; however, a compromised endothelium increases permeability which facilitates transmigration of activated immune cells. Interestingly, there is little interaction between ECs and leukocytes under homeostatic conditions. It is during activation that both cell types upregulate adhesion molecules, recruiting leukocytes to damaged sites along the endothelium. Activated ECs express adhesion molecules that tether and roll the circulating leukocyte to a site of entry where the rolling stalls, eventually permitting leukocyte transmigration to the underlying tissues (Mai, J. et al. 2013).

Several adhesion molecules of interest sequentially facilitate leukocyte rolling, and the expression patterns closely correlate with the stage of inflammation (Muller, W. 2002). For example, tethering is mediated by vascular adhesion molecule 1 (VCAM-1), rolling is mediated by E-selectin (also referred to as CD62E), and firm adhesion by intracellular adhesion molecule 1 (ICAM-1). Basal endothelial cells do not express high levels of ICAM-1, VCAM-1, or E-selectin,

yet during the inflammatory process these molecules are markedly increased (Muller, W. 2002). All three of these EC adhesion molecules are upregulated in the presence of proinflammatory cytokines, thus maintaining leukocyte contact and resulting in alterations of T cell phenotype and EC physiology (Brezinschek, R. et al. 1998). Although cytokines are often noted for contributing to endothelial dysfunction, some cytokines are vital for CD4+ T cell development, enabling them to perform regulatory roles. In fact, differentiation of CD4+ T cell subsets are determined by cytokines in the microenvironment in addition to interaction with specific antigen (Luckheeram, R. et al., 2011). Therefore, the regulation of various cytokines must be maintained by proper communication between various cell types that are responsible for cytokine synthesis and release. Although chronic inflammation concerning excessive cytokine production permits detrimental trapping and transmigration of leukocytes, these otherwise protective molecules cannot be eradicated entirely. Suggestive of the term "immune balance," mechanisms that support either regaining balance of cytokine production or inhibit unfavorable interaction with target cells could be advantageous towards regaining physiological harmony within the microvasculature.

Autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), and myasthenia gravis (MG) refer to chronic inflammation and other complications associated with ED as classic symptomology (Horwitz, D. et al. 2019; De Lavor, E., et al. 2018; Wu, Y. et al. 2016; Atehortúa, L. et al. 2017; Murdaca, G., et al. 2012; Uzawa, A. et al. 2016). As a result of the complexity of autoimmune pathologies, there are many challenges for pharmacological interventions. Drug-related therapies attempt to slow disease progression but involve side effects and often result in debilitating flare-up of symptoms if medication is discontinued. (Horwitz, D. et al. 2019). For this purpose, therapies that reestablish order within innate and adaptive immune responses while restoring a healthy inflammatory

response is a promising opportunity for the treatment of immune-mediated inflammatory conditions.

Current research is aimed towards identifying specific constituents and biological effects of volatile compounds derived from plants, otherwise known as essential oils (EOs). EOs possess immunomodulatory properties that affect cellular and molecular functions of the immune system. Because EOs contain many biologically active compounds, a naturally sourced pure EO can have both immune suppressive and stimulatory abilities. Furthermore, EOs can have immunomodulatory abilities via secondary and tertiary active metabolites, promoting both longer duration and range of therapeutic effect. Yet it is important to note that the synergy of all constituents within a plant's volatile component work together and collectively contribute to their therapeutic effects (Han, X. et al. 2017; Huang, N. et al. 2008).

One class of EOs that have powerful medicinal properties are the phenolic EOs. Phenols are antibacterial, anti-inflammatory, antimicrobial, and can increase permeability of the cytoplasmic membrane, which allows binding of EO constituents to intracellular proteins (Leja, K. et al. 2019). Both clove bud and thyme are considered phenolic EOs. Clove bud EO has demonstrated antidiabetic activity (Oboh, G. et al. 2015) as well as potent anti-thrombotic activity via inhibition towards thromboxane synthesis and platelet aggregation (Saeed, S. et al. 1994). Furthermore, clove EO inhibits inflammatory cytokine production in macrophages, specifically interleukin 1-beta (IL-1β) and interleukin 6 (IL-6) in vitro and in vivo (Rodrigues, T. et al. 2009). Likewise, the phytotherapeutic qualities of thyme EO and its dominant constituents have been observed in many studies. Previous research demonstrated enhanced endothelial function after exposure to thyme EO, such as significant reductions in interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), ICAM-1, and VCAM-1. This study suggests the inhibitory effects towards NF-κB

and COX-2/iNOS are the “therapeutic qualities” of thymol that may be beneficial for treating inflammatory diseases (Liang D. et al. 2014). The collective narrative of these studies assessing clove and thyme EOs and their respective chemical compound towards inflammation and coagulation conveys the potent and protective properties of EOs, specifically those within the phenol classification. The objective of the proposed research is to simulate an inflammatory environment between cultured ECs/CD4+ T cells and analyze the expression of adhesion molecules and pro-coagulant activity before and after treatment of clove bud and red thyme EOs. To accomplish this objective, the following aims will be addressed:

Aim 1

Aim 1 will assess the anti-inflammatory/anti-coagulation abilities of *Thymus vulgaris* (red thyme) and *Eugenia caryophyllata* (clove bud) EOs. Human umbilical vein endothelial cells (HUVECs) were activated with TNF-α and treated with either thyme or clove bud EOs for 3 hr and 6 hr using a non-toxic concentration (0.01% or 0.1µL/mL) identified with a cytotoxicity assay using Trypan Blue. Inflammatory markers of interest (ICAM-1, E-selectin, IL-6) and coagulation marker (TF) were analyzed from cell lysates. It is hypothesized that clove and thyme EO will mitigate markers of inflammation as well as lower the expression of TF that typically follows endothelial dysfunction characterized by excessive inflammation.

Aim 2

The second aim of this project is to assess the effects of clove and thyme EOs towards the same markers of protein expression in activated HUVECs that also includes coincubation with CD4+ T cells. Because endothelial-leukocyte adhesion is prolonged during oxidative stress, is it proposed that the antioxidant abilities of each EO will reduce the capacity of leukocyte adhesion

via downregulation of adhesion molecules, thus limiting the downstream upregulation of IL-6 and TF that follows endothelial-leukocyte interactions. It is our hypothesis that both clove bud and thyme EOs will perform similar and meaningful anti-inflammatory effects at a rather modest yet potent concentrations, proposing these EOs to be a cost and resource effective option for future experimental studies in relation to pharmacological purposes.

METHODS

Materials

Plant Materials/Reagents

Essential oils *Thymus vulgaris* (red thyme) (lot# E7727) and *Eugenia caryophyllata* (clove bud) (lot# 55532) were used for experiments (doTERRA Intl., Pleasant Grove, UT). Chemical analysis of each EO verified by gas chromatography mass spectroscopy (GC/MS) (provided by doTERRA Intl.) indicates individual EO components expressed in percentages. Purity of each EO is verified through optical rotation, specific gravity, refractive index, and colorimeter to ensure the absence of pesticides, allergens, or carrier oils to ensure quality control.

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Recombinant human IL-2 protein was purchased from R&D Systems (cat. No. 202-IL-010/CF). Recombinant human TNF was purchased from R&D Systems (cat. No. 210-TA-020/CF, Minneapolis, MN). Anti-CD28 was purchased from VWR (cat. No. 302904-BL/302903-BL). Anti-CD3 was purchased from VWR (cat. No. 317325-BL).

Cell Culture

HUVECs Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Morristown, NJ) and cultured in endothelial medium-2-medium containing 2% fetal bovine serum and growth supplements VEGF, FGF, EGF, IGF, ascorbic acid, GA 1000, 1% penicillin-streptomycin, 1% gentamycin. Cells from 2 Caucasian male doners were grown in parallel and repeated five times (N=5). HUVECs were seeded and subcultured in T-75 flasks and maintained at 37°C in a 5% CO2 atmosphere. HUVECs were seeded into 100mm dishes (2.0×10^5) at passage 6 for aim 1 experiments and seeded into 6-well plates (1.0×10^5) at passage 6 for aim 2 experiments.

CD4+ T Cell Culture

Human peripheral blood CD4+ T cells (Lonza, cat. No. 2W-200) were seeded into a 96-well plate at 0.01×10^6 with serum-free, xeno-free LGM-3™ lymphocyte growth medium-3 (Lonza, cat. No. CC-3211) which was supplemented daily with IL-2 (100 IU/mL) (R & D Systems, cat. No. 202-IL-010/CF). For optimal stimulation, CD4+ T cells were activated with 50µl of a final solution of anti-CD3 (final concentration 1ng/µl) and anti-CD28 (final concentration 2ng/µl). Cells were subcultured in T-25 flasks and supplemented with fresh IL-2 every day (100 IU/mL) until they reached a quiescent stage and preserved in liquid nitrogen until aim 2 experiments ensued. Once thawed, CD4+ T cells were centrifuged at 300g 21°C for 10min, resuspended, and counted using a hemocytometer. Cells were then used in coincubation experiments at a seeding density of 2.0×10^5 with confluent HUVEC monolayer (1.0×10^5) for 24 hours.

Cytotoxicity Assays

Preliminary data to assess potential toxicity of clove bud and thyme EOs were applied towards both cell lines using Trypan Blue viability test (Lonza BioResearch, USA). Trypan blue dye

measures viability via cell membrane structure, in which dead cells with ruptured membranes obtain dye and live cells do not. Samples were diluted in Trypton Blue dye of an acid azo exclusion medium by preparing a 7-fold dilution of the cell suspension and 0.4% Trypan Blue solution. Cell viability is determined with an automated cell counter (Thermo Fisher Countess 3 Automated Cell Counter, cat. No. A49891). Total count as well as live and dead cell count with percentages were obtained and averaged from a double sided slide (Thermo Fisher Countess™ Cell Counter Chamber Slides, cat. No. C10228). Four concentrations (0.0025%, 0.005%, 0.01%, 0.02%) of clove and thyme EOs were added to confluent HUVECs and CD4+ T cell cultures to obtain cytotoxicity data. Each cell line was maintained in their respective cell culture medium, as mentioned above in sections 3.2.1 and 3.2.2., respectively.

Coculture Experiments

Once HUVECs reached ~95% confluency in 6-well plates, SFM was added to control plates, while select plates were incubated with 30ng/ml TNF-α in SFM for 4 hours to stimulate cells. Activated and inactivated HUVECs were then cocultured with CD4+ T cells for 24 hours using a HUVEC/CD4+ T cell ratio of 1:2. Activated cells were treated with either clove bud or thyme EO (0.01%) for 6 hour. EO mixtures also contained 8ul/mL (0.04%) DMSO as a vehicle control. For comparative purposes, control conditions included confluent inactivated HUVECs monolayer, inactivated HUVECs with DMSO, TNF- α activated HUVECs, as well as a condition of activated HUVEC + CD4+ T cell with no addition of either EO. For cell harvest, the HUVEC monolayer washed with HBSS for removal of non-adherent T cells and culture media. Cells were then lysed with 1x cell lysis buffer (Cell Signaling, cat. No. 9803). Cell supernatants were frozen down and later assayed for expression of ICAM-1, E-selectin, IL-6, and TF.

Western Blot

Insoluble factors from supernatants (ICAM-1, E-selectin, IL-6, TF) were detected using Western Blot (WB) analysis. Total cellular proteins were extracted, and concentrations determined with BCA assay kits (BioRad, Hercules, CA, USA). Supernatants were prepped with 4x Laemmli buffer at 1 µg/µL for WB analysis; 15 µl per lane were loaded on a 4-15% SDS-PAGE (Bio-Rad Laboratories; Hercules, CA, USA). Electrophoresis ran for ~50 minutes at 150 V using 1x SDS-PAGE run buffer (VWR Laboratories; Randor, PA, USA). Following, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad; Hercules, CA, USA) for 120 minutes at 200 amps. Membranes were stained with Ponceau S for protein visualization and equal loading of samples and imaged on a gel documentation system (UVP ChemiDoc-It2 Touch imaging system, Bio-Rad). Membranes were then blocked for 1 hr with 5% non-fat milk powder and Tris-buffered saline with 0.1% Tween-20 (TBST). Primary antibodies were prepared in TBST containing 5% bovine serum albumin (BSA, Ameresco) and incubated with gentle agitation for 72 hr at 4°C. Primary antibodies: human tissue factor/CD142 (E9M6T) XP® rabbit mAb (1:1000, Cell Signaling, cat. No. 97438S), human E-selectin/CD62E (ELAM-1) mouse mAb (1:1000, Thermo Fisher Scientific, cat. No. MA1-22165), ICAM-1 mouse mAb (1:1000, Thermo Fisher Scientific, cat. No. MA5-13021), IL-6 mouse mAb (1:1000, Thermo Fisher Scientific, cat. No. MA5-23698). Following primary antibody incubation, membranes were washed 3x TBST and subsequently incubated with HPR-linked secondary antibodies at room temperature. Secondary antibodies: anti-rabbit IgG (1:2000, Cell Signaling, cat. No. 7074) and anti-mouse IgG (1:2000, Cell Signaling, cat. No. 7076). Total protein expression was detected by chemiluminescence using HPR illumination substrate Luminate Forte (EMD, Millipore, Billerica, MA, USA). Images were captured using a band densitometry analyses (UVP ChemiDoc-It2 Touch imaging system, Bio-Rad). The densities of the selected protein bands were quantified relative to the densities of the

internal control gene, β-Actin, for total protein normalization (1:2000 anti-β-Actin, Cell Signaling, cat No. 3700S).

Statistical Analysis

Data collections were obtained by values provided by imaging software and translated into statistical analysis software, statistical package for the social sciences (SPSS) (IBM Corp., Armonk, NY, USA), as well as comparative t-test through Excel. One-way ANOVA was used for aim 1 data (n=5). Aim 2 data was underpowered to run statistics (n=1). All values are expressed as the mean ± SD. Differences between mean values and normally distributed data were assessed with two-tailed t-tests. P values of 0.05 or less were considered statistically significant, with an effect size of at least 10% (0.05 log units).

Ethical Considerations

This study is being carried out on one cell line which includes only one race (Caucasian) which excludes other ethnicities. This study is an exploratory study, and future studies may want to implement a racial component.

RESULTS

Aim 1

Markers of inflammation in activated HUVECs following treatment with clove and thyme EOs

Adhesion molecules (ICAM-1, E-selectin) and an inflammatory cytokine (IL-6) were selected for this study based on previous literature that supports the concept of these molecules being

upregulated in chronic vascular inflammation. Of the proteins analyzed, none of results obtained via one-way ANOVA were significantly expressed respective to controls (media only) nor did treatment with EOs modulate protein expression significantly (Fig. 1-3). All EO treatment conditions were first activated with TNF-α. Unexpectedly, TNF-α activation did not induce a significant increase in the studied markers in HUVECs.

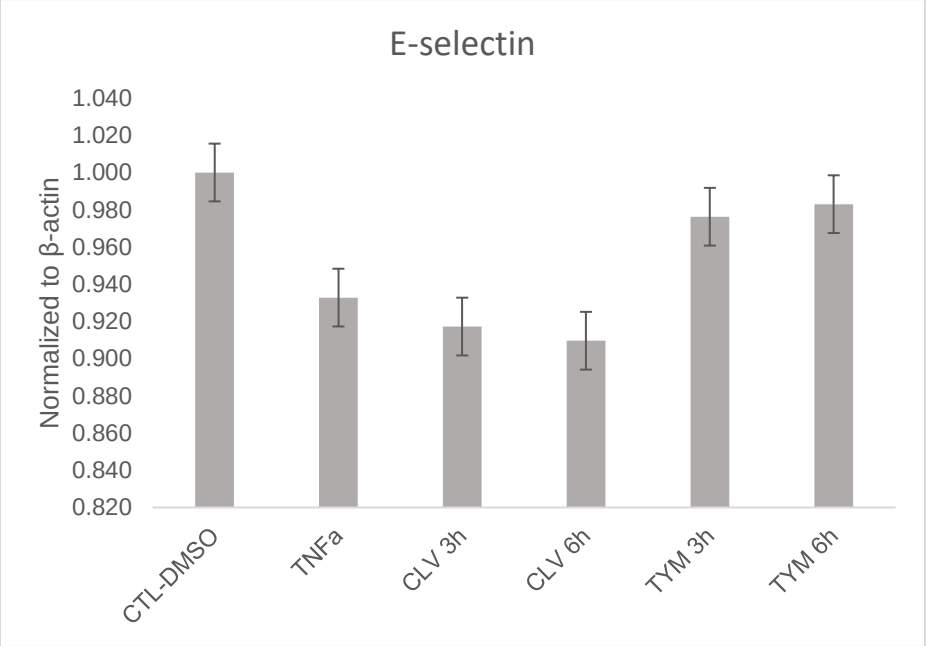

Fig. 1. Effects of TNF-α-activated HUVECs treated with clove EO (0.01%) and thyme EO (0.01%) at 3 and 6 hrs on E-selectin protein expression (n=5 replicates per treatment, no treatment differed from CTL DMSO, $p > 0.050$).

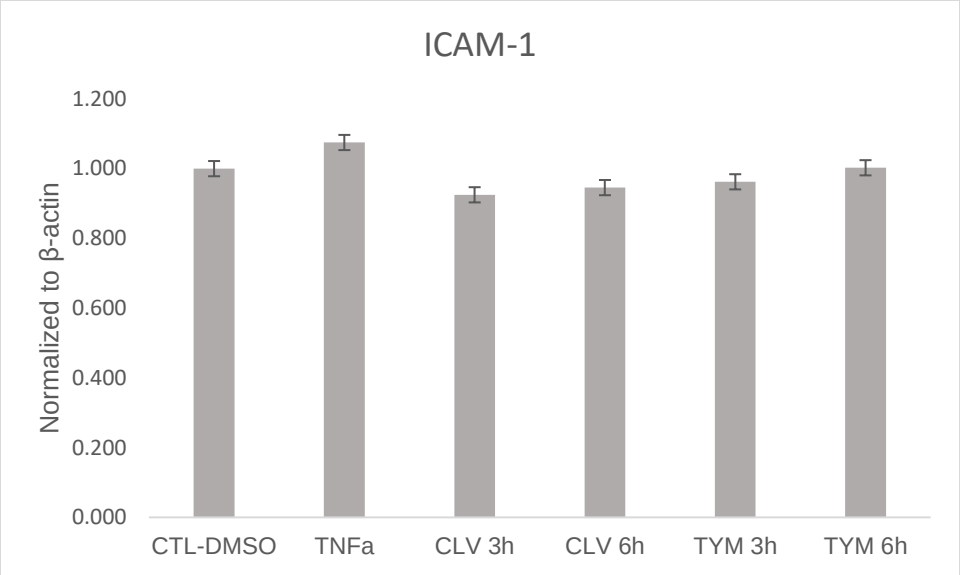

Fig. 2. Effects of TNF-α-activated HUVECs treated with clove EO (0.01%) and thyme EO (0.01%) at 3 and 6 hrs on ICAM-1 protein expression (n=5 replicates per treatment, no treatment differed from CTL DMSO, $p > 0.050$).

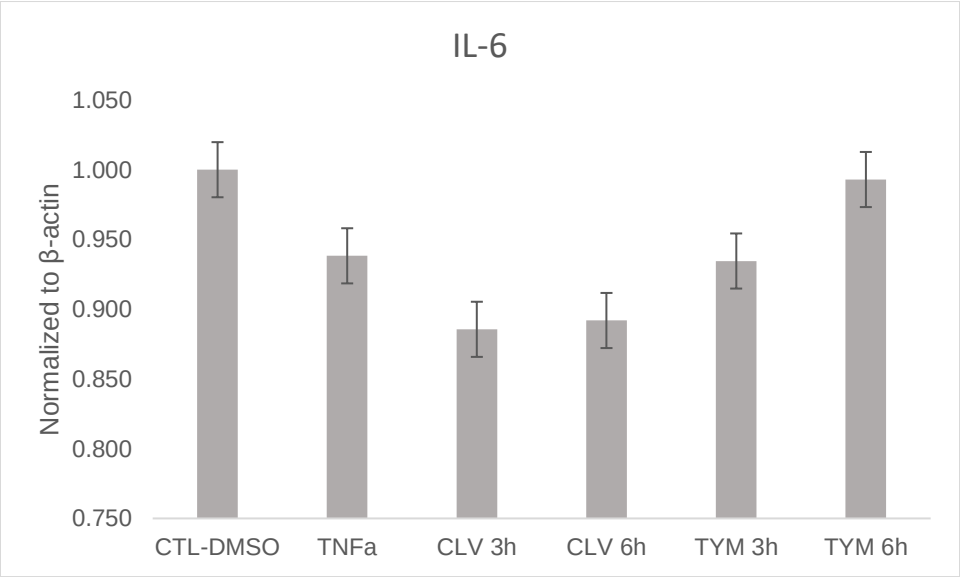

Fig. 3. Effects of TNF-α-activated HUVECs treated with clove EO (0.01%) and thyme EO (0.01%) at 3 and 6 hrs on IL-6 protein expression (n=5 replicates per treatment, no treatment differed from CTL DMSO, $p > 0.050$)

Marker of coagulation in activated HUVECs following treatment with clove and thyme EOs

Coagulation molecule (TF) was selected for this study due to its involvement in endothelial dysfunction and immune cell migration during excessive inflammation. One-way ANOVA revealed TF expression significant ($p<0.001$) for each condition involving stimulation with TNF-α (TNF-α alone, TNF-α + EOs at both time points) (Fig. 4). All EO treatment conditions were activated with TNF-α prior to EO incubation. These data suggest that TF protein expression is strongly upregulated under cytokine stimulation in vitro, yet the hypothesized downregulation of TF for treatment conditions was not observed.

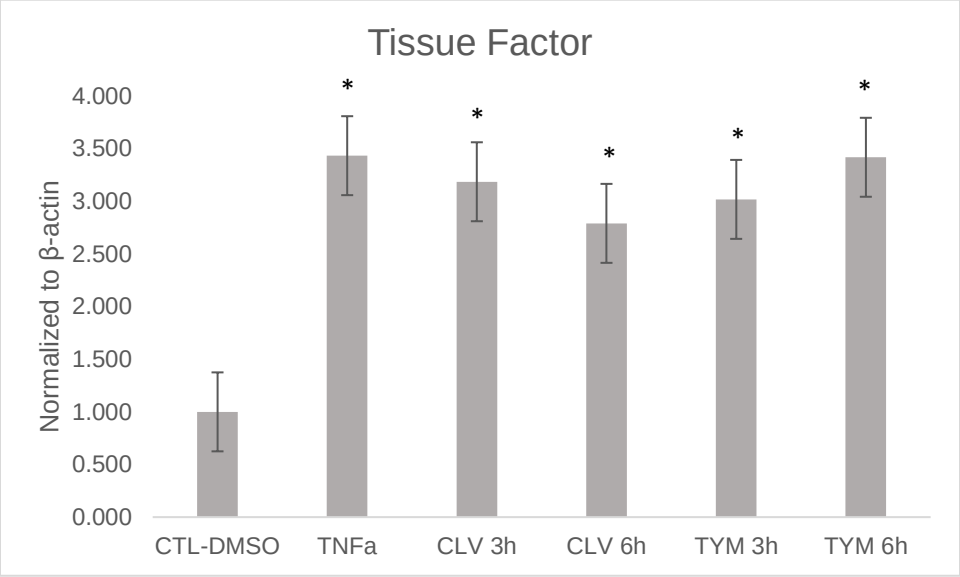

Fig. 4. Effects of TNF-α-activated HUVECs treated with clove EO (0.01%) and thyme EO (0.01%) at 3 and 6 hrs on TF protein expression (n=5 replicates per treatment, *$p < 0.001$ relative to CTL DMSO).

Aim 2

Markers of inflammation and coagulation in activated HUVECs and activated HUVECs coincubated with CD4+ T cells following treatment with Clove EO

Adhesion molecules (ICAM-1 and E-selectin) as well as an inflammatory cytokine (IL-6) were analyzed for protein expression in a coculture model with treatment of clove EO. As these data were preliminary and underpowered for statistical analysis, visual differences were observed from western blot images. Overall, clove EO in unstimulated HUVECs alone showed the lowest expression of all four markers (E-selectin, ICAM-1, IL-6, and TF), which could be worth investigating for preventative therapeutic effects for vascular inflammation and coagulation. The addition of CD4+ T cells did not reveal substantial visual or numerical differences when compared to the control in any of the four protein markers (Fig. 5-8).

Markers of inflammation and coagulation in activated HUVECs and activated HUVECs coincubated with CD4+ T cells following treatment with Thyme EO

The addition of thyme EO had opposite effects from that of clove on all four protein markers (E-selectin, ICAM-1, IL-6, TF) as observed in western blot images. For E-selectin, thyme EO alone (with inactivated HUVECs) had a similar effect as TNF-α activated HUVECs in terms of upregulating E-selectin (Fig. 5). For ICAM-1, expression was very mildly modulated by any of the conditions including thyme EO treatment with inactivated HUVECs alone. However, in both TNF-α activated HUVECs and TNF-α HUVECs coincubated with T cells treated with thyme EO, ICAM-1 expression was considerably upregulated (Fig. 6). IL-6 expression was most highly expressed in the TNF-α activated HUVECs treated with thyme EO. Following was the similar condition that included coincubation of T cells (Fig. 7). Lastly, the effects of thyme EO towards TF expression, the addition of thyme EO and T cells mildly downregulated TF when compared to TNF-α activation alone; however, the addition of thyme EO to TNF-α activated HUVECs slightly increased expression when compared to TNF-α HUVECs alone (Fig. 8).

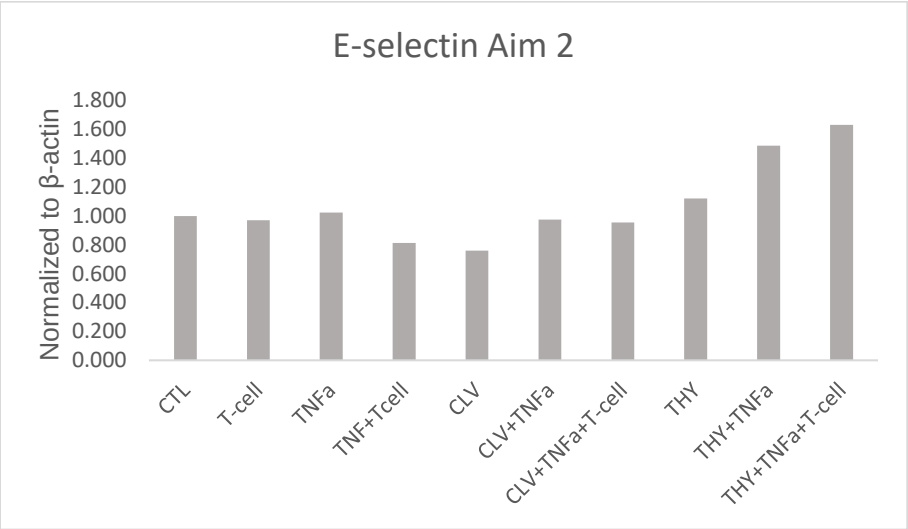

Fig. 5. Effects of coincubation with CD4+ T cells, TNF-α, clove EO, and thyme EO on E-selectin protein expression in HUVECs.

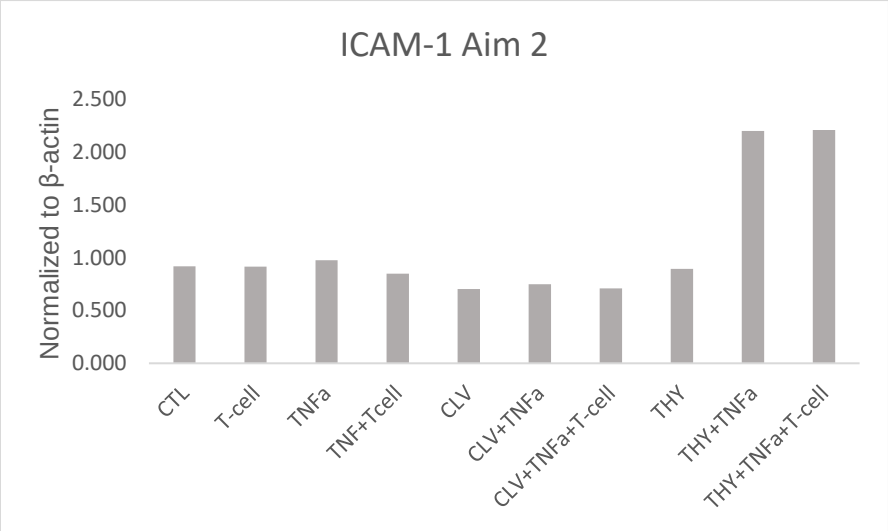

Fig. 6. Effects of coincubation with CD4+ T cells, TNF-α, clove EO, and thyme EO on ICAM-1 protein expression in HUVECs.

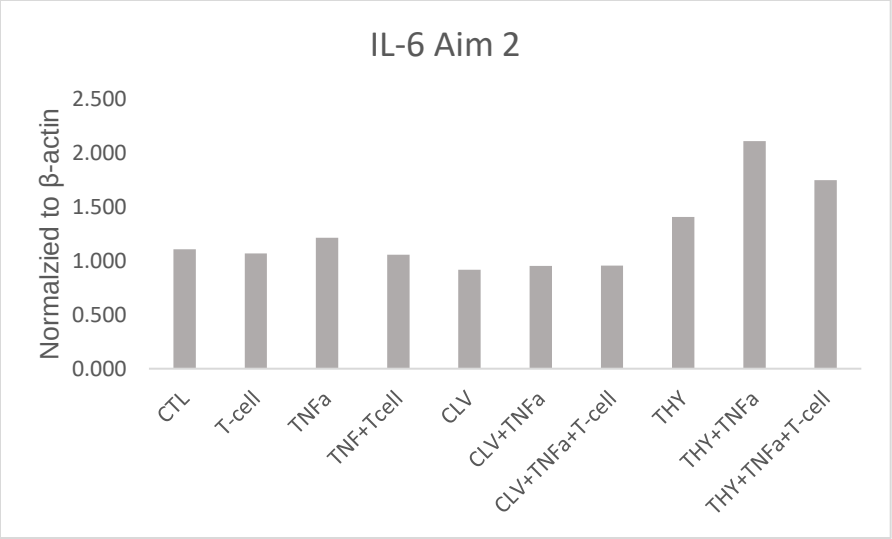

Fig. 7. Effects of coincubation with CD4+ T cells, TNF-α, clove EO, and thyme EO on IL-6 protein expression in HUVECs.

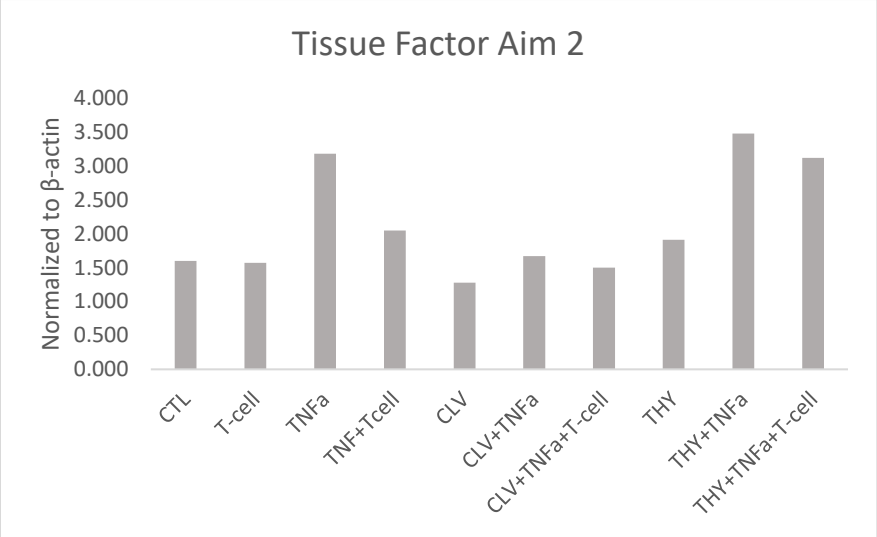

Fig. 8. Effects of coincubation with CD4+ T cells, TNF-α, clove EO, and thyme EO on IL-6 protein expression in HUVECs.

DISCUSSION

The heart and cardiovascular system are essentially supported by the vascular endothelium. While it is unlikely that any one stimulus accounts for ECA, inflammatory cytokines play a large role in both initiating and sustaining ECA and perpetuating vascular inflammation. Structural changes associated with vascular inflammation are arterial stiffness, plaque buildup, blood clotting, and unnecessary immune responses such as adhesion of leukocytes to sites of injury and increased adhesiveness along the endothelium. Chronic inflammatory threat can lead to ED, in which loss of proper endothelial function results in dysregulated immune responses while weakening endothelial anticoagulant abilities (Huertas, A. et al. 2014). For these reasons, many autoimmune diseases have cardiovascular complications. CD4+ T cells play the largest role in autoimmune disease, as compared to other T cell types. TNF-α produced by activated T cells activates ECs, advancing T cell adhesion and endothelial dysfunction (Certo, M. et al. 2020). Although ECs do not express traditional co-stimulatory molecules CD80 and CD82 (B7.1 and B7.2, respectively) that typically bind to the CD28 receptor on CD4+ T cells, they are able to use alternative ligands including members of the TNF-receptor families to provide adequate stimulus for T cell activation. Because these two cell types sustain activation for one another for the duration of their interactions, it is important to investigate the effects of endothelial cell surface proteins that are involved in chronic inflammatory diseases as well as undesirable protein expression involved with CD4+ T cell interactions. Furthermore, early detection of coagulation factor TF has been proposed as a therapeutic strategy for inflammatory conditions such as sepsis, systemic inflammatory response syndrome, and disseminated intravascular coagulation (Egorina, E. M. et al. 2011). Because proinflammatory cytokines induce TF expression in ECs, one piece of this study was to investigate TF expression in activated HUVECs as well as HUVECs coincubated with

CD4+ T cells. Therefore, the current study explored the effects of inflammatory cytokine TNF-α towards several markers of ED (E-selectin, ICAM-1, IL-6, TF), and observed a significant increase in TF protein expression (p=<0.001) following cytokine activation.

EOs containing phenolic compounds are considered for their potent therapeutic qualities, in which similar mechanism of action of NSAIDs have been mentioned in former research. EOs have demonstrated inhibition of T cell migration to sites of injury (preventing edema), inducing endothelium-dependent relaxation, and modulating T cell activity in autoimmune diseases. Furthermore, EOs augment endogenous antioxidants as well as membrane stabilizing effects that contribute to homeostasis (Meeran, M. F. N. et al. 2017). Previous studies have demonstrated enhanced endothelial function after exposure to thyme EO, such as significant reductions in interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), ICAM-1, and VCAM-1. Clove bud EO was selected for this study due to potent anti-thrombotic activity (Saeed, S. et al. 1994) as well as its ability to inhibit inflammatory cytokine production, specifically interleukin 6 (IL-6) in vitro and in vivo (Rodrigues, T. et al. 2009). Clove also demonstrates control of inflammation and pain via the endocannabinoid system (Basu, S., Dittel, B. 2011; Eisenstein, T. 2015) which is involved with regulation of vascular function (Bondarenko, A. 2014). Therefore, the primary purpose of this study was to assess potential modulation of markers of ED (ICAM-1, E-selectin, IL-6, TF) with clove and thyme EOs.

Aim 1 Discussion

Data regarding TNF-α activated HUVECs did not align with previous research related to cytokine stimulated upregulation of adhesion molecules (E-selectin, ICAM-1) and an inflammatory cytokine (IL-6), though a coagulation molecule (TF) was significantly increased following TNF-α activation across all activated HUVEC conditions. While hyperactivation of TF

in damaged tissues has been previously reported, it is typically associated with increased levels of IL-6 in addition to increased levels of TNF- α (Swotowski, B. et al. 2005). However, previous work indicates that IL-6 has a lessened ability to upregulate TF compared to TNF- α, which may be a protective mechanism by ECs within the context of evading the overexpression of thrombogenic proteins that may otherwise be initiated by IL-6 (Swotowski, B. et al. 2005). It is interesting that the present study found TNF- α significantly induced TF expression without affecting other adhesion molecules. As mentioned previously, TF exposure is achieved by endothelial gaps that are a result of pro-inflammatory tissue damage involving EC expression of adhesion molecules (Ruf W., Reiwald, M. 2013; Lopes-Bezerra, L., et al. 2003, Mai, J. et al. 2013, Muller, W. 2002). Nonetheless, TF plays an important role in both homeostasis and procoagulant activity in the vasculature and the findings in this study merit reason to further analyze TF expression in ECs in response to proinflammatory molecules.

E-selectin was selected for this study as it is involved in early ECA and responsible for tethering of T cells along endothelial tissue (Luster, A. et al. 2005). E-selectin is induced by several inflammatory cytokines in most tissues and has several known ligands on specific T cell lineages and is a diagnostic measure of auto-inflammatory diseases such as psoriasis, arthritis, or contact dermatitis (Matsumoto, M. et al. 2005). The results for E-selectin in this study may be inconclusive due to multiple bands being expressed in the western blot. ICAM-1 is involved with ECA and T cell firm adhesion (Luster, A. et al. 2005). While the results indicated by ANOVA suggest no significant findings, visualization of ICAM-1 protein expression showed some treatment effect for both clove and thyme as compared to the untreated activated HUVECs.

Aim 2 Discussion

Investigative data including T cell coincubation revealed potential therapeutic effects for clove EO while thyme EO had the opposite effect, which seemed to be compounded by the addition of TNF-α activated HUVECs. One explanation for this could be due to the potency of thyme EO, as our cytotoxicity assay in HUVECs showed thyme to have cytotoxic effects in HUVECs at higher concentrations (0.02%) while the same concentrations of clove were tolerable (results of cytotoxicity not included). One study demonstrated antitumor effects of thyme EO at 0.01%, 0.016%, 0.22%, 0.225%, and 0.24% IC50 (Jaafari, A. et al. 2007). It could be possible that activated ECs are recognized as "tagged for cell death" wherein overexpression of adhesion molecules and cytokines may prompt thyme EO to further expand the progression to cell death, similar to anti-tumor abilities of thyme observed in previous research (Theofilis, P. et al. 2021; Jaafari, A. et al. 2007).

In TNF-α activated HUVECs, treatment with clove downregulated all four markers, which were similarly downregulated in HUVECs cocultured with CD4+ T cells (fig. 5-8). Interestingly, TF expression visually showed the largest treatment effect between TNF-α activated HUVECs alone and inactivated HUVECs treated with clove, as well as activated and coincubated conditions that were treated with clove EO. The present study's findings along with previous literature may indicate clove EO to be inhibitory towards vascular coagulation mediated by inflammation or immune cells and is worth further investigation. The addition of CD4+ T cells did not reveal substantial visual or numerical differences when compared to the control in any of the four protein markers, which may be interpreted as failure of both cell types to interact. However, conditions of TNF-α activated HUVECs coincubated with T cells consistently show a mild decrease in protein expression across all markers, in which the addition of clove and T cells further decreased protein

expression (Fig. 5-8). This may suggest endothelial protective effects of both CD4+ T cells and clove as they relate to cytokine activation.

For E-selectin, thyme EO alone (with inactivated HUVECs) had a similar effect as TNF-α activated HUVECs in terms of upregulating this protein. The condition that had activated HUVECs coincubated with T cells and subsequently treated with thyme EO surprisingly showed the highest expression of E-selectin as compared to all other conditions (Fig. 5). For ICAM-1, expression was very mildly modulated by any of the conditions including thyme EO treatment with inactivated HUVECs alone. However, in both TNF-α activated HUVECs and TNF-α HUVECs coincubated with T cells treated with thyme EO, ICAM-1 expression was considerably upregulated (Fig. 6). IL-6 expression was most highly expressed in the TNF-α activated HUVECs treated with thyme EO. Following was the similar condition that included coincubation of T cells (Fig. 7). Lastly, the effects of thyme EO towards TF expression, the addition of thyme EO and T cells mildly downregulated TF when compared to TNF-α activation alone; however, the addition of thyme EO to TNF-α activated HUVECs slightly increased expression when compared to TNF-α HUVECs alone (Fig. 8). Though these findings are contradictory to the literature regarding thyme EO and inflammation, the effects observed in this study may speak to the potency of thyme EO. Future studies should investigate lower incubation times as well as lower concentrations of thyme EO in cell culture for a better understanding of its potential effects on EC health. It also is worth mentioning that previous research may be based on EOs that have either been adulterated or were maintained in undocumented conditions, in which differences in growing, harvesting, handling, distillation methods and chemotype of aromatic plants has a major impact on the chemistry and quality of the EO. These undisclosed differences may greatly alter the therapeutic

outcome, introducing fairly indecipherable variation between studies in EO research, making their comparison indistinct.

LIMITATIONS

Limitations to this study include time and dose of essential oils. Expanding on the incubation time for the oils as well as using various concentrations may provide different outcomes and a better understanding of these EOs biological effects towards ECs. Using a lower concentrations for thyme EO due to its robust chemistry may offer different outcomes and render therapeutic qualities observed in other studies. On the other hand, a higher concentration of clove EO may reduce inflammatory protein expression to a much greater degree than did 0.01% concentration used in this study. Alternatively, clove and thyme may provide preventative effects towards TNF-α stimulation in HUVECs, such as reducing the magnitude of expression for the proteins analyzed in this study that followed TNF- α stimulation, if the EOs were incubated with HUVECs prior to TNF- α activation.

For coincubation experiments, a relatively low T cell seeding density was used (1:2) as compared to previous studies that use a 1:5 ratio of APC to T cells (Suryawanshi, A. et al. 2015; Manoharan, I. et al. 2014; Elgueta, R. et al. 2009). Furthermore, the additional freeze-thaw cycle for CD4+ T cells between CD3/CD28 activation and coincubation with ECs may have been a limitation as to possible reduction of T cell adhesion molecule expression, thus ability to interact with ECs. Finally, Aim 2 data only allocated singlet treatments, which precluded formal statistical analysis. Future studies may wish to re-evaluate these important aspects of study design that perhaps could better demonstrate an accurate representation of the analyzed proteins in a cell culture model.

CONCLUSIONS

In conclusion, the results of this study indicate that TNF-α activated HUVECs did not upregulate ICAM-1, E-selectin, or IL-6 but did significantly increase TF. These markers were not significantly influenced by the addition of EOs. Preliminary data involving coincubation may have showed a therapeutic trend of clove EO while thyme demonstrated an opposite effect at the concentration used, and this warrants future research consideration.

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