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An Essential Oil Blend Modulates Important Inflammation- and Immune Response-Related Biomarkers in Human Cell Cocultures

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Short Title: Essential oil modulates inflammation biomarkers

Abstract: Despite growing scientific evidence that essential oils possess important therapeutic benefits, research on their biological activities in complex human disease models is scarce. To enhance understanding in this regard, we analyzed the biological activities of an essential oil blend (EOB) in validated human cocultures with or without tumor cells. These disease models allow for measurement of changes in protein biomarkers induced by EOB treatment. This EOB is primarily composed of essential oils from frankincense resin, sweet orange peel, litsea fruit,

thyme plant oil, clove bud, summer savory plant, and niaouli leaf. EOB showed significant effects on levels of important biomarkers related to inflammation, immune response, tissue remodeling, and tumor biology. In tumor cocultures, EOB treatment resulted in elevated inflammatory- and immune-related biomarkers, including soluble interleukin (sIL)-17A, sIL-2, sIL-6, vascular cell adhesion molecule-1 (VCAM-1), cluster of differentiation (CD)40, CD69, soluble granzyme B (sGranB), and soluble interferon-gamma (sIFN-γ). However, several of these same biomarkers were decreased in EOB-treated nontumor cell cocultures, suggesting that EOB exhibits tumor-specific immune enhancement. In conclusion, EOB may potentially impact human cells through anti-inflammatory activities, immune enhancing functions, and modulation of wound healing.

Subjects: Herbal Medicine; Cell Culture; Inflammatory Disease; Autoimmune Disease; Cancer

Keywords: essential oil; frankincense; sweet orange; litsea; thyme; clove; inflammation; cancer; immune response

1. Introduction

Many experimental studies on essential oils and their biological activities have examined only individual essential oils or their constituents in single cell lines or mouse models (Hong et al., 2014; Kathirvel & Ravi, 2012). However, cell lines alone do not model primary disease biology, and mouse models do not accurately reflect regulatory networks present in human disease (Chong, Alegre, Miller, & Fairchild, 2013; Mak, Evaniew, & Ghert, 2014). Human cell coculture systems can compensate for these limitations by combining healthy host cells, disease cells (e.g., tumor cells), and disease-relevant stimuli to mimic host-disease microenvironments (Bergamini et al., 2012). The combining of multiple essential oils into what is known as an essential oil blend (EOB) is a common practice among aromatherapists, alternative medicine practitioners, and mainstream essential oils companies. It has been assumed that such combining of essential oils can lead to greater therapeutic benefits as a result of the additive or potentially synergistic actions provided by the blended oils. However, this assumption still remains to be tested in systems that mimic human host-disease biology.

For these reasons, we chose to study the effects of an EOB in human cell coculture systems. The present study was designed to assess the biological activities of EOB in several well-validated human cell cocultures that have been successfully used to measure the effects of a variety of chemical compounds on inflammation and other immunomodulatory processes (Berg et al., 2010; Bergamini et al., 2012). We analyzed the effects of EOB on dozens of protein biomarkers in these cell coculture systems. This experimental approach allowed us to determine whether EOB can modulate a variety of intra- and extracellular regulatory pathways

in ways that are not predictable by looking at the individual EOB components and that can potentially benefit human health.

2. Materials and methods

All experiments were conducted in the BioMAP platform, a set of primary human cell systems designed to model disease biology in a robust and reproducible way. The systems consist of three components, a cell type or cell types (many systems involve cocultures), molecular stimuli to create the disease environment, and a set of biomarker (protein) readouts to examine how treatments impact that disease environment (Berg et al., 2010).

2.1. Cell cultures

Primary human (H) cells (i.e., neonatal dermal fibroblasts [HNDFs], umbilical venule endothelial cells [HUVECs], peripheral blood mononuclear cells [PBMCs], and $CD19^+$ B cells) were obtained as previously described (Bergamini et al., 2012). HNDFs were plated in low serum conditions 24 h before stimulation with cytokines. HUVECs were obtained from Cascade Biologics (Portland, OR, USA), cultured in endothelial cell growth medium-2 containing manufacturer-provided supplements and 2% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), and then subcultured with 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (Mediatech, Herndon, VA, USA) according to the manufacturer's instructions. PBMCs were prepared from buffy coats (Stanford Blood Bank, Stanford, CA, USA) by centrifugation over Hisopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) (Bergamini et al., 2012). HT-29, a colorectal cancer (CRC) cell line, was obtained from the American Type Culture Collection and maintained according to their recommended protocol.

Stimulatory molecules for these cell coculture systems were as follows: T-cell receptor (TCR) ligands (1×) for SAg, immunoglobulin M antigens and TCR ligands (0.001× each) for BT, TCR ligands (0.001×) for HDFSAg, IL-2 and TCR ligands (0.1× each) for /TH2, and TCR ligands (0.001×) for StroHT29 and VascHT29. Cell culture and stimulation conditions for the BT, SAg, and /TH2 coculture assays have been described in detail elsewhere and were performed in a 96-well format (Bergamini et al., 2012; "R," n.d.). For the StroHT29 system, PBMCs primed with a low level of superantigen were added to a coculture of HNDFs and HT-29 cells and then cultured for 48 h. For the VascHT29 system, PBMCs primed with a low level of superantigen were added to a coculture of HUVECs and HT-29 cells and cultured for 48 h.

2.2. Protein-based readouts

Direct ELISA was used to measure levels of various cell markers. Soluble factors in supernatants were quantified using either homogeneous time-resolved fluorescence detection, bead-based multiplex immunoassay, or capture ELISA. Overt adverse effects of test agents on cell proliferation and viability (cytotoxicity) were measured by sulforhodamine B (SRB) assay for adherent cells and alamarBlue staining for cells in suspension. For proliferation assays, individual cell types were cultured at subconfluence and measured at time points optimized for each system (48–96 h). Detailed information has been described elsewhere (Bergamini et al., 2012). Measurements were performed in triplicate wells.

2.3. Reagents

The essential oil blend (DDR Prime™, dōTERRA International LLC, Pleasant Grove, UT, USA) was diluted in dimethyl sulfoxide (DMSO) to 8× the specified concentrations. (Final DMSO concentration was no more than 0.1%.). Specifically, 25 µL of each 8× solution was added to the

cell culture to yield a final volume of 200 µL. DMSO alone (0.1%) served as the vehicle control. The composition of EOB is as follows: frankincense (a mix of *Boswellia carterii*, *B. frereana*, and *B. sacra*) resin oil, sweet orange (*Citrus sinensis*) peel oil, litsea (*Litsea Cubeba*) fruit oil, thyme (*Thymus vulgaris*) plant oil, clove (*Eugenia caryophyllata*) bud oil, summer savory (*Satureja hortensis*) plant oil, and niaouli (*Melaleuca quinquenervia*) leaf oil. GC-MS analysis of EOB showed that it contained about 23–27% limonene, 11–13% alpha-pinene, 6–8% eugenol, 6–8% thymol, 5–7% carvacrol, 5–7% eucalyptol, 4–6% gamma-terpinene, and smaller amounts of other aromatic compounds.

2.4. Statistical analysis

Quantitative biomarker data are presented as the mean log relative expression level (compared to the respective mean vehicle control value) ± standard deviation (SD) of triplicate measurements. Differences in biomarker levels between EOB- and vehicle-treated cocultures were tested for significance with the unpaired Student's *t* test. A p value < 0.05, with an effect size of at least 10% (more than 0.05 log ratio units), was regarded as statistically significant.

3. Results and discussion

See Tables S1 and S2 in **Supplementary material** for a glossary of cell cocultures and of biomarkers analyzed in the study.

3.1. Bioactivity profile of EOB in immune-oncology coculture systems

We first analyzed four different EOB concentrations (0.1, 0.033, 0.011, and 0.004%, v/v) in two different oncology (CRC) systems (StroHT29 and VascHT29) for biological activity. These concentrations were chosen in an attempt to find a concentration that would be viable for further *in vitro* studies. The three highest concentrations yielded > 50% reduction in cellular

protein levels (by SRB assay), and/or > 50% reduction in PBMC viability. These values indicate that EOB was overtly cytotoxic to these cells at these concentrations, and therefore they were excluded from further analysis. Only the 0.004% concentration was used for further analysis of key activities, the results of which are discussed below.

In the StroHT29 system (Figure 1A), EOB significantly increased levels of collagen III and matrix metalloproteinase-9 (MMP-9), two biomarkers related to matrix remodeling activities. Another tissue remodeling biomarker, tissue inhibitor of matrix metalloprotease-2 (TIMP-2), was only marginally increased. EOB increased levels of the following immune-related biomarkers: soluble interleukin (sIL)-17A, sIL-2, sIL-6, and soluble tumor necrosis factor-alpha (sTNF-α). Two tumor-related biomarkers, carcinoembryonic antigen-related cell adhesion molecule-5 (CEACAM5) and keratin 20, were elevated by EOB. In addition, EOB increased levels of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) but decreased the mean level of soluble vascular endothelial growth factor (sVEGF). No significant change in the levels of vascular cell adhesion molecule-1 (VCAM-1), the receptor for uPA (uPAR), collagen I, interferon gamma-induced protein-10 (IP-10), plasminogen activator inhibitor-1 (PAI-1), soluble granzyme B (sGranB), soluble interferon gamma (sIFN-γ), sIL-10, or SRB was observed.

In the VascHT29 system (Figure 1B), EOB significantly increased levels of monocyte chemo-attractant protein-1 (MCP-1), VCAM-1, and cluster of differentiation (CD) proteins (CD40 and CD69), all of which are biomarkers related to immunomodulatory activities. EOB also increased levels of many immune-related biomarkers, including sIFN-γ, sIL-17A, sIL-2, sIL-6, and sTNF-α. Another immune-related biomarker, IP-10, was slightly decreased. Collagen IV (a biomarker for tissue remodeling activity) and CEACAM5 were increased after exposure to EOB.

Unlike the StroHT29 system, mean sGranB level was selectively increased in VascHT29 after EOB treatment.

3.2. Bioactivity profile of EOB in autoimmune T cell coculture systems

In the BT system, EOB decreased levels of the immunomodulatory biomarkers, secreted IgG (sIgG), sIL-17A, and sIL-17F, but it slightly increased the level of another immunomodulatory biomarker, sIL-6 (Figure 2A). In the SAg system, EOB was both antiproliferative to T cells and overtly cytotoxic to PBMCs (Figure 2B). EOB also significantly decreased CD40 and slightly but significantly inhibited levels of CD38 (another immune modulatory biomarker) and CD62E/E-selectin (an inflammation biomarker).

In the HDFSAg system (Figure 2C), EOB inhibited several inflammation-related biomarkers (MCP-1, VCAM-1, IP-10, monokine induced by interferon gamma [MIG] and sTNF-α) as well as immunomodulatory biomarkers (macrophage colony stimulating factor [M-CSF], sIL-17A, sIL-17F, sIL-2, sIL-10, and sIL-6). No significant change in the levels of sIL-8, MMP-1, SRB, or soluble transforming growth factor-beta1 (sTGF-β1) was observed. In addition, EOB decreased the mean level of tissue remodeling biomarker, collagen I, but it slightly increased the mean sVEGF level. This effect of EOB on sVEGF is the opposite of that observed in the StroHT29 immune-oncology system (Figure 1A). In the /TH2 system, EOB significantly decreased levels of MCP-1, Eotaxin-3, VCAM-1, E-selectin, and P-selectin, all of which are important inflammation-related biomarkers (Figure 2D). Several immunomodulatory biomarkers, including CD38, CD40, and sIL-17A, were significantly decreased in response to EOB. EOB also decreased levels of collagen IV and was overtly cytotoxic to PBMCs.

3.3. Anti-inflammatory and immune-enhancing properties of EOB

The observed effects of EOB on biomarkers such as VCAM-1, Eotaxin-3, CD40, sIL-17A, and sIL-17F in these preinflamed cell cocultures suggest that EOB might reduce elevated inflammatory responses, such as those that occur in a disease environment. In either murine models or cell cultures, similar anti-inflammatory and immune-enhancing effects of the individual oils and/or major constituents included in the blend have been reported by other research groups (Chaudhary, Siddiqui, Athar, & Alam, 2012; Him, Ozbek, Turel, & Oner, 2008; Martin et al., 1993; Riella et al., 2012; Yoon, Lee, & Hyun, 2010). Taken together, the growing literature suggests that essential oils are pharmacologically active and generally inhibitory in multiple models of stimulated inflammatory and immunomodulatory responses, with which the current study is consistent. The finding that EOB significantly impacted these important biomarkers in both cancerous and noncancerous cell cocultures suggests that it may play important roles in both types of disease biology and therefore may provide potential therapeutic benefits to human health.

It is equally important to note that EOB exerted different effects in cancerous and noncancerous cell cultures. Generally, EOB elevated the inflammation- and immunity-related biomarkers (e.g., sIL-17A, sIL-2, sIL-6, VCAM-1, CD40, CD69, sGranB, sTNF-α, and sIFN-γ) in cancerous cell cocultures; however, in the noncancerous cocultures, several of these same biomarkers were inhibited in response to EOB. Specifically, EOB decreased the levels of sIL-17A, sIL-2 and sTNF-α in StroHT29 (HT-29 colon adenocarcinoma cell line + primary human fibroblasts + PBMCs), while it increased these levels in HDFSAg (primary human fibroblasts + PBMCs) that lacks the cancer cells. Whereas EOB inhibited CD40 production in VascHT29 (HT-29

colon adenocarcinoma cell line + primary human endothelial cells + PBMCs), it enhanced CD40 production in SAg (PBMCs + venular endothelial cells). These observations suggest that EOB possesses tumor-specific immune-enhancing potential. The opposite regulatory effects of EOB on these biomarkers in cancerous vs. noncancerous cell cocultures indicate that EOB exerts its effects via different pathways or mechanisms in different disease microenvironments. Further studies are warranted to determine its biological mechanism(s) of action.

4. Conclusions

In primary human cell models of disease, EOB significantly impacted critical biomarkers related to inflammation and immune function. EOB appears to possess tumor-specific immune-enhancing properties, and it may also impact human cells via anti-inflammatory activities and modulation of wound healing. To the best of our knowledge, this is the first study exploring the biological activities of an EOB in complex human cell cocultures. This study provides original and important knowledge of how an EOB affects inflammation- and immune-related biomarkers in validated human cocultures.

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Competing interest

X.H. and T.P. are employees of dōTERRA, where the study agent, EOB, was manufactured.

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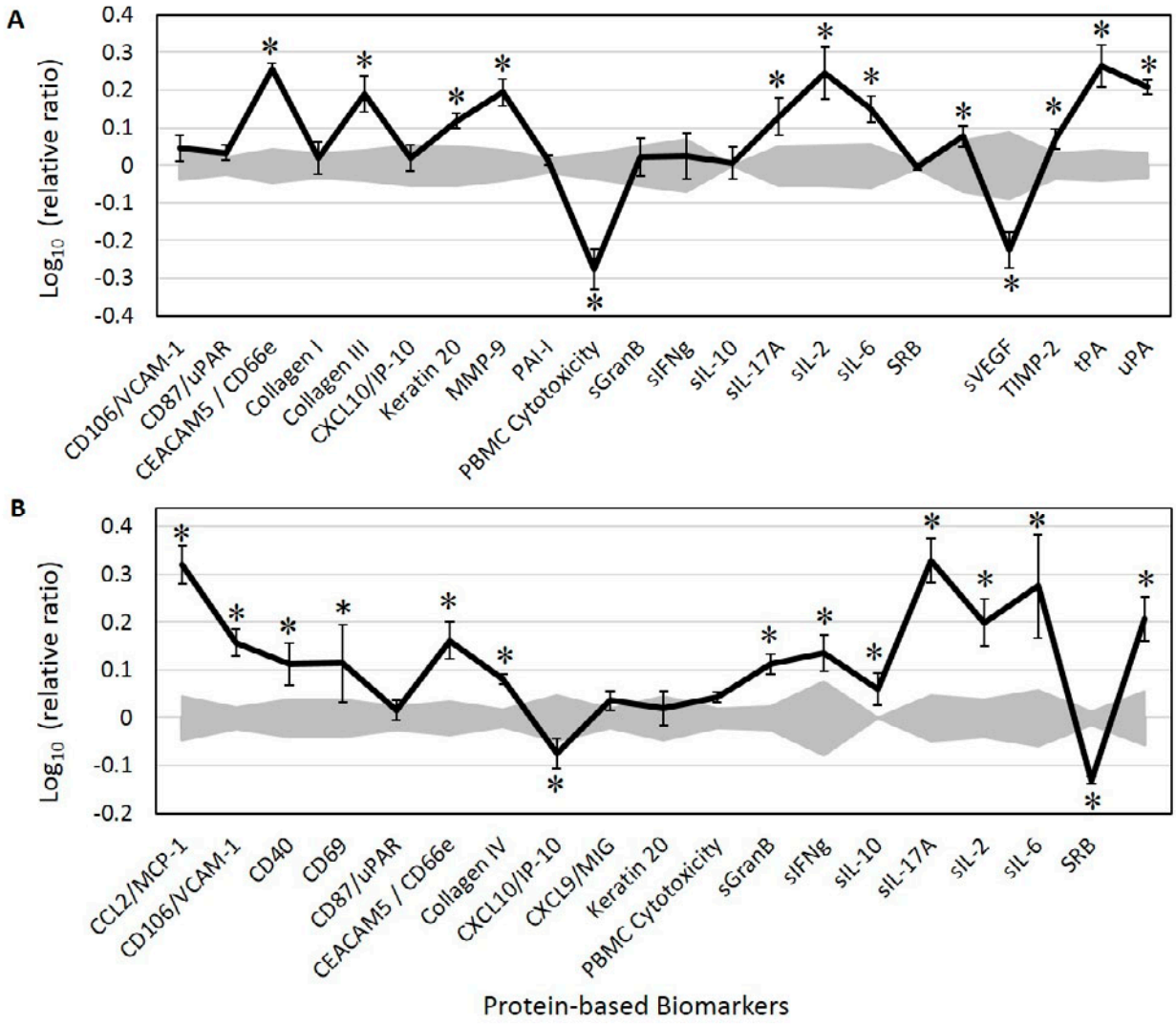
Figure Legends

Figure 1. Bioactivity profile of EOB (0.004%, v/v, in DMSO) in two immune-oncology coculture systems, StroHT29 (A) and VascHT29 (B). Each x-axis denotes protein-based biomarker readouts in the respective system. Each y-axis denotes the log relative expression levels of these biomarkers compared to respective vehicle control values. The 95% confidence interval of the mean vehicle control values are marked by the gray shaded area. Each point on each curve represents the mean ± SD of three measurements. * $p < 0.05$ vs. vehicle control, with an effect size of at least 10% (more than 0.05 log ratio units).

Figure 2. Bioactivity profile of EOB (0.004%, v/v, in DMSO) in the autoimmune T cell coculture systems, BT (A), SAg (B), HDFSAg (C), and /TH2 (D). Each x-axis denotes protein-based biomarker readouts in the respective system. Each y-axis denotes the log relative biomarker expression levels compared to respective vehicle control values. * $p < 0.05$ vs. vehicle control, with an effect size of at least 10%.


A
Log10 (relative ratio)
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
CD106/VCAM-1
CD87/uPAR
CEACAM5 / CD66e
Collagen I
Collagen III
CXCL10/IP-10
Keratin 20
MMP-9
PAI-I
PBMC Cytotoxicity
sGranB
sIFNg
sIL-10
sIL-17A
sIL-2
sIL-6
SRB
sVEGF
TIMP-2
tPA
uPA
B
Log10 (relative ratio)
0.4
0.3
0.2
0.1
0
-0.1
-0.2
CCL2/MCP-1
CD106/VCAM-1
CD40
CD69
CD87/uPAR
CEACAM5 / CD66e
Collagen IV
CXCL10/IP-10
CXCL9/MIG
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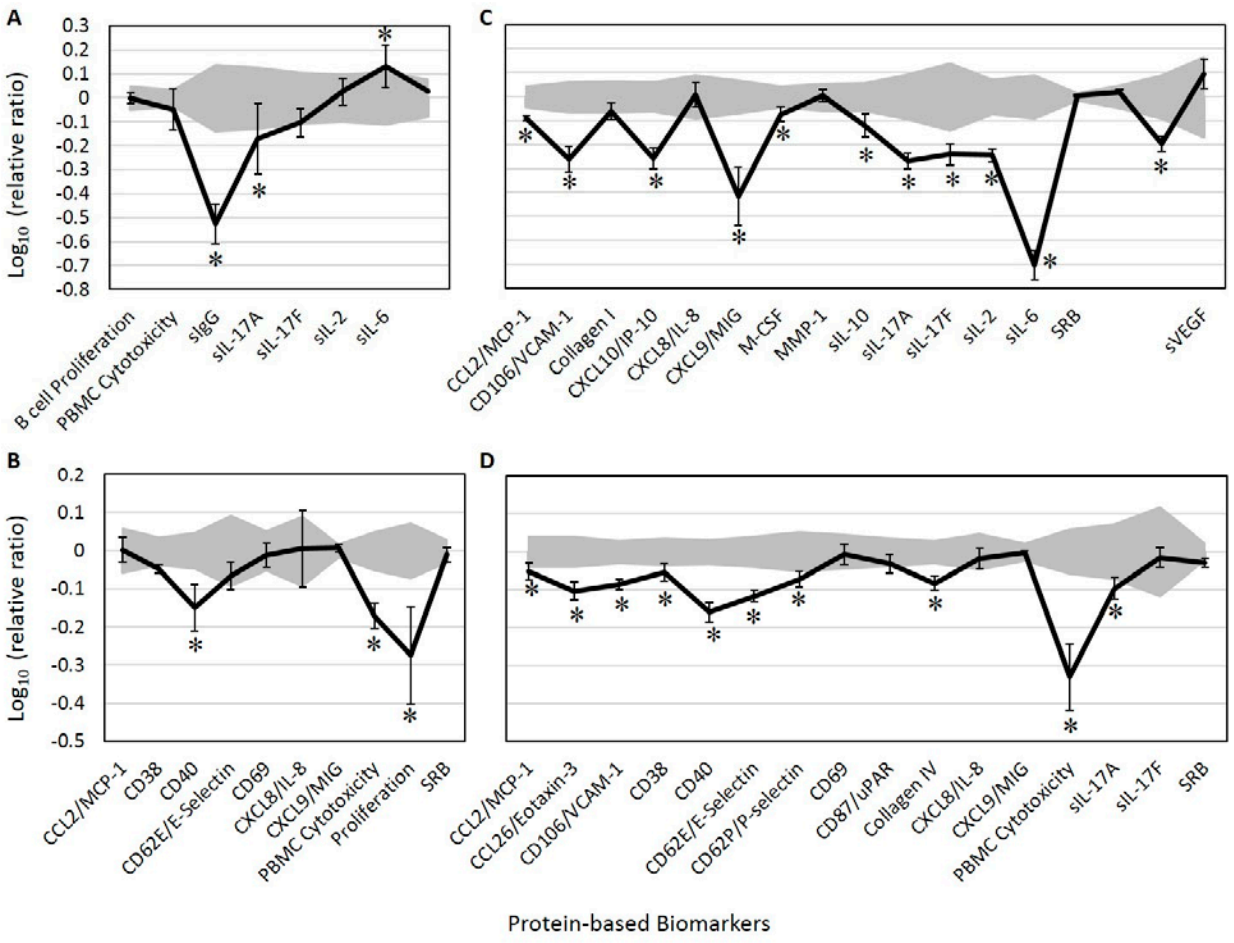
Protein-based Biomarkers


A
B
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Log10 (relative ratio)
B cell Proliferation
PBMC Cytotoxicity
sIgG
sIL-17A
sIL-17F
sIL-2
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CCL2/MCP-1
CD106/VCAM-1
Collagen I
CXCL10/IP-10
CXCL8/IL-8
CXCL9/MIG
M-CSF
MMP-1
sIL-10
sIL-17A
sIL-17F
sIL-2
sIL-6
SRB
sVEGF
CCL2/MCP-1
CD38
CD40
CD62E/E-Selectin
CD69
CXCL8/IL-8
CXCL9/MIG
PBMC Cytotoxicity
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CCL2/MCP-1
CCL26/Eotaxin-3
CD106/VCAM-1
CD38
CD40
CD62E/E-Selectin
CD62P/P-selectin
CD69
CD87/uPAR
Collagen IV
CXCL8/IL-8
CXCL9/MIG
PBMC Cytotoxicity
sIL-17A
sIL-17F
SRB
Protein-based Biomarkers

About the authors

At dōTERRA, our group primarily studies the health benefits of essential oils. We are specifically interested in the efficacy and safety of essential oils and their active components. Our studies of essential oils in both *in vitro* and clinical settings utilize a variety of experimental approaches, including analytical, biological, biochemical, and biomedical methodologies. We work closely with hospitals, clinics, and research institutes towards developing quality essential oils with therapeutic benefits. The research work discussed in this paper represents one part of a large research project, which was designed to extensively examine the impact of essential oils on human cells. This study, along with others, will further the understanding of the health benefits of essential oils for a wide research audience. We believe that a full understanding of these health benefits will ultimately lead to the evaluation and use of essential oils as an adjunctive therapy for a variety of diseases.

Public interest statement

Essential oils have become more popular globally for health reasons. Our study examined the effects of an essential oil blend (EOB) on several human cell systems that mimic different diseases. These effects of the EOB were determined by measuring levels of biomarkers that are linked to inflammation, immune function, wound healing, and cancer biology. We found that the EOB had strong anti-inflammatory, immune modulatory, and wound healing activities. More interestingly, this EOB showed tumor-specific immune boosting activity, a feature of immunotherapy that is a common treatment for many cancers. Findings from this study suggest that essential oils may be a good therapeutic candidate for inflammatory, immune, and cancerous diseases. Advanced exploration of the health benefits of essential oils may lead to viable options for fighting many of these diseases. Thus, this study provides an important stepping stone for further research on essential oils and their health benefits for human beings.

Supplementary material

Table S1. Glossary of BioMAP systems used in the study

System	Primary Human Cell Types	Disease/Tissue Relevance	Readout Parameters	System Description
StroHT29	HT-29 colon adenocarcinoma cell line + primary human fibroblasts + PBMCs	Oncology: Host stromal-tumor microenvironment	VCAM-1, uPAR, collagen I, collagen III, IP-10, MMP-9, PAI-1, PBMC cytotoxicity, sGranB, sIFN-γ, sIL-10, sIL-17A, sIL-2, sIL-6, SRB, sTNF-α, sVEGF, TIMP-2, tPA, uPA, CEACAM5, keratin 20	The StroHT29 system models the host stromal-tumor microenvironment by capturing the complex interactions between tumor cells, the host stromal network, and infiltrating immune cells recruited into the tumor mass.
VascHT29	HT-29 colon adenocarcinoma cell line + primary human endothelial cells + PBMCs	Oncology: Host vascular-tumor microenvironment	MCP-1, VCAM-1, CD40, CD69, uPAR, collagen IV, IP-10, MIG, PBMC cytotoxicity, sGranB, sIFN-γ, sIL-10, sIL-17A, sIL-2, sIL-6, SRB, sTNF-α, CEACAM5, keratin 20	The VascHT29 system models the host vascular-tumor microenvironment by capturing the complex interactions between tumor cells, the host vascular network, and infiltrating immune cells associated with angiogenesis.
SAg	PBMCs + venular endothelial cells	Autoimmune disease, chronic inflammation	MCP-1, CD38, CD40, E-selectin, CD69, IL-8, MIG, PBMC cytotoxicity, proliferation, SRB	The SAg system models T helper 1 (Th1) type chronic inflammation and T cell effector responses to T cell receptor (TCR) signaling with costimulation. This system is relevant to inflammatory conditions where T cells play a key role, including organ transplantation, rheumatoid arthritis, psoriasis, Crohn's disease, and multiple sclerosis.

BT	B cells + PBMCs	Asthma, allergy, oncology, autoimmunity	B cell proliferation, PBMC cytotoxicity, sIgG, sIL-17A, sIL-17F, sIL-2, sIL-6, sTNF-α	The BT system models T cell-dependent B cell activation and class switching as would occur in a germinal center. This system is relevant for diseases and conditions where B cell activation and antibody production are relevant. These include autoimmune disease, oncology, asthma, and allergy.
HDFSAg	Dermal fibroblasts + PBMCs	Autoimmune disease, chronic inflammation, rheumatoid arthritis	MCP-1, VCAM-1, collagen I, IP-10, MMP-1, sIL-10, sIL-17A, sIL-17F, sIL-2, sIL-6, SRB, sTGF-β1, sTNF-α, sVEGF, IL-8, MIG, M-CSF	The HDFSAg system models Th1 type chronic inflammation and T cell effector responses to TCR signaling with costimulation. This system is relevant to inflammatory conditions where T cells play a key role, including rheumatoid arthritis, psoriasis, Crohn's disease, fibrosis, and wound healing biology.
/TH2	Venular endothelial cells + Th2 blasts	Asthma, allergy, oncology	MCP-1, Eotaxin-3, VCAM-1, CD38, CD40, E-selectin, P-selectin, CD69, uPAR, collagen IV, IL-8, MIG, PBMC cytotoxicity, sIL-17A, sIL-17F, SRB	The /TH2 system models vascular inflammation (mixed Th1 and Th2 types), an environment that is proangiogenic and promotes recruitment of mast cells, basophils, eosinophils, and T and B cells as well as vascular permeability. This system is relevant to diseases where Th2 type inflammatory conditions play a role, such as allergy, asthma, and ulcerative colitis.

Table S2. Glossary of biomarkers used in the study

System	Readout	Description
HDFSAg	**CCL2/ MCP-1**	MCP-1 (CCL2) is a chemoattractant cytokine (i.e., chemokine) that regulates the recruitment of monocytes and T cells into sites of inflammation. Migration of monocytes from the blood stream across the vascular endothelium is a critical early event in the response to inflammation. MCP-1 is categorized as an *inflammation-related activity* in this HDFSAg system that models chronic Th1-driven inflammation.
	CD106/ VCAM-1	VCAM-1 (CD106) is a cell adhesion molecule that mediates the adhesion and trafficking of immune cells from the blood into the surrounding tissue. VCAM-1 is upregulated in inflamed tissue and is categorized as an *inflammation-related activity* in this HDFSAg system.
	Collagen I	Collagen I is a fibrillar collagen that is a major component of the extracellular matrix (ECM), and it is upregulated during inflammation-induced matrix remodeling. Collagen I is categorized as a *tissue-remodeling activity* in this HDFSAg system.
	CXCL10/ IP-10	IP-10 (CXCL10) is a proinflammatory C-X-C chemokine produced by monocytes and fibroblasts that mediates T cell chemotaxis through its receptor, CXCR3. Serum concentrations of IP-10 have been suggested as markers of disease activity in patients with rheumatoid arthritis. IP-10 is categorized as an *inflammation-related activity* in this HDFSAg system.
	CXCL8/ IL-8	IL-8 (CXCL8) is a chemokine produced by multiple cell types that mediates neutrophil recruitment into acute inflammatory sites. IL-8 is also known to be a potent promoter of angiogenesis. IL-8 is categorized as an *inflammation-related activity* in this HDFSAg system.
	CXCL9/ MIG	MIG (CXCL9) is a proinflammatory C-X-C chemokine produced by monocytes and fibroblasts that mediates T cell chemotaxis through interaction with its receptor, CXCR3. Serum concentrations of MIG have been suggested as markers of disease activity in patients with rheumatoid arthritis. MIG is categorized as an *inflammation-related activity* in this HDFSAg system.
	M-CSF	M-CSF (CSF1) is a cytokine growth factor that is required for monocyte differentiation into macrophages. M-CSF is categorized as an *immunomodulatory activity* in this HDFSAg system.
	MMP-1	MMP-1 is a zinc-dependent protease that degrades collagens. MMPs may play a role in joint destruction in rheumatoid arthritis. MMP-1 is categorized as a *tissue-remodeling activity* in this HDFSAg system.
	sIL-10	sIL-10 is a cytokine produced by many cell types, most notably macrophages, but also by T cells, B cells, monocytes, and dendritic cells. IL-10 acts as an immunomodulatory cytokine, suppressing Th1 responses. IL-10 is categorized as an *immunomodulatory activity* in this HDFSAg system.

	sIL-17A	sIL-17A is a proinflammatory cytokine, produced by a subset of activated T helper cells (Th17 cells), which acts primarily on nonhematopoietic cells. sIL-17A is categorized as an *immunomodulatory activity* in this HDFSAg system.
	sIL-17F	sIL-17F is another proinflammatory cytokine produced by Th17 cells that acts primarily on nonhematopoietic cells. sIL-17F is categorized as an *immunomodulatory activity* in this HDFSAg system.
	sIL-2	sIL-2 is a cytokine produced by activated T cells and acts in an autocrine manner to promote T cell proliferation and maturation. sIL-2 is categorized as an *immunomodulatory activity* in this HDFSAg system.
	sIL-6	sIL-6 is a cytokine produced by fibroblasts, endothelial cells, T cells, B cells, and monocytes. IL-6 acts as a proinflammatory cytokine (acute phase reactant), and it also influences T helper cell differentiation. Blocking IL-6 signaling has been shown clinically to reduce inflammation in rheumatoid arthritis patients. sIL-6 is categorized as an *immunomodulatory activity* in this HDFSAg system.
	sTGF-β1	sTGF-β1 is an immunomodulatory cytokine produced by multiple cell types. sTGF-β1 influences differentiation of T helper cells towards a Th17 or regulatory T cell (Treg) phenotype and also influences the activation of fibroblasts to produce ECM. sTGF-β1 is categorized as an *immunomodulatory activity* in this HDFSAg system.
	sTNF-α	sTNF-α is a proinflammatory cytokine, produced by macrophages, monocytes, neutrophils, T cells, and natural killer (NK) cells, which is important in systemic inflammation. Blocking TNF-α signaling has been shown clinically to reduce inflammation in arthritis patients. sTNF-α is categorized as an *inflammation-related activity* in this HDFSAg system.
	sVEGF	sVEGF is a widely expressed growth factor that induces vascular permeability, angiogenesis, and vasculogenesis and inhibits apoptosis. sVEGF is categorized as a *tissue-remodeling activity* in this HDFSAg system.
	SRB	SRB in the HDFSAg system is a *measure of the density* of the cocultured fibroblasts and PBMCs. SRB staining is used to determine the total protein content of the system. A decrease in SRB signal (i.e., total protein) of ≥ 50% indicates that the compound is overtly cytotoxic to the system at that concentration.
/TH2	**CCL2/ MCP-1**	MCP-1 (CCL2) is a chemoattractant cytokine (i.e., chemokine) that regulates the recruitment of monocytes and T cells into sites of inflammation. Migration of monocytes from the blood stream across the vascular endothelium is a critical early event in the response to inflammation. MCP-1 is categorized as an *inflammation-related activity* in this /TH2 system that models allergic inflammation.
	CCL26/ Eotaxin-3	Eotaxin-3 (CCL26) is a proinflammatory chemokine that mediates the trafficking of eosinophils, cytotoxic T lymphocytes, NK cells, and monocytes. Eotaxin-3 is categorized as an *inflammation-related activity* in this /TH2 system.

CD106/ VCAM-1	VCAM-1 (CD106) is a cell adhesion molecule that mediates the adhesion and trafficking of immune cells from the blood into the surrounding tissue. VCAM-1 is upregulated in inflamed tissue and is categorized as an *inflammation-related activity* in this /TH2 system.
CD38	CD38 is a membrane-associated bifunctional enzyme that regulates cyclic ADP ribose and is an activation marker expressed on hematopoietic and nonhematopoietic cells. CD38 is involved in diverse processes, such as T cell activation/costimulation and chemotaxis. CD38 is categorized as an *immunomodulatory-related activity* in this /TH2 system.
CD40	CD40 is a cell surface adhesion receptor essential for mediating a broad variety of immune and inflammatory responses. This costimulatory receptor is expressed on antigen presenting cells and is required for T cell activation. CD40 can also be expressed by endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. CD40 is categorized as an *immunomodulatory-related activity* in this /TH2 system.
CD62E/ E-selectin	E-selectin (CD62E) is a cell adhesion molecule expressed only on endothelial cells and, like other selectins, it plays an important part in inflammation by mediating leukocyte-endothelial cell interactions via binding sialylated carbohydrates. During inflammation, localized cytokines induce E-selectin overexpression on nearby blood vessels. Leukocytes in the blood expressing the correct ligand will bind with low affinity to E-selectin and "roll" along the internal surface of the blood vessel, becoming “recruited” at the inflammation site. E-selectin is categorized as an *inflammation-related activity* in this /TH2 system.
CD62P/ P-selectin	P-selectin (CD62P) is an adhesion molecule, expressed on activated endothelial cells and platelets, which mediates leukocyte rolling and adhesion by binding P-selectin glycoprotein ligand-1, heparan sulfate, and fucoidan. Selectins play an important role in early recruitment and inflammation. P-selectin is categorized as an *inflammation-related activity* in this /TH2 system.
CD69	CD69 is a human transmembrane C-type lectin protein induced by activation of T lymphocytes. CD69 appears to be the earliest inducible cell surface glycoprotein acquired during immune activation and is involved in lymphocyte proliferation and activation. CD69 is categorized as an *immunomodulatory activity* in this /TH2 system.
CD87/ uPAR	uPAR (CD87) is the cell surface receptor for uPA that localizes uPA and therefore plasmin to the cell surface, which is important for a localized tissue remodeling response. uPAR is categorized as a *tissue-remodeling activity* in this /TH2 system.

	Collagen IV	Collagen IV is an ECM protein that is a major component of the basement membrane and is primarily produced by endothelial cells. Increased collagen IV is associated with airway remodeling in asthma. Collagen IV is categorized as a *tissue-remodeling activity* in this /TH2 system.
	CXCL8/ IL-8	IL-8 (CXCL8) is a chemokine produced by multiple cell types that mediates neutrophil recruitment into acute inflammatory sites. IL-8 is also known to be a potent promoter of angiogenesis and is categorized as an *inflammation-related activity* in this /TH2 system.
	CXCL9/ MIG	MIG (CXCL9) is a proinflammatory C-X-C chemokine that mediates T cell chemotaxis through interaction with its receptor, CXCR3. MIG is categorized as an *inflammation-related activity* in this /TH2 system.
	sIL-17A	sIL-17A is a proinflammatory cytokine produced by Th17 cells that acts primarily on nonhematopoietic cells. sIL-17A is categorized as an *immunomodulatory activity* in this /TH2 system.
	sIL-17F	sIL-17F is another proinflammatory cytokine produced by Th17 cells that acts primarily on nonhematopoietic cells. sIL-17F is categorized as an *immunomodulatory activity* in this /TH2 system.
	PBMC cytotoxicity	PBMC cytotoxicity is a *measure of PBMC death*. Cell viability of nonadherent cells is measured by alamarBlue staining, a method based on a cell permeable compound that emits fluorescence after entering cells. The number of living cells is proportional to the amount of fluorescence produced.
	SRB	SRB in the /TH2 system is a *measure of the density* of cocultured venular endothelial cells and Th2 blasts. SRB staining is used to determine the total protein content of the system. A decrease in SRB signal (i.e., total protein) of ≥ 50% indicates that the compound is overtly cytotoxic to the system at that concentration.
SAg	**CCL2/ MCP-1**	MCP-1 (CCL2) is a chemoattractant cytokine (i.e., chemokine) that regulates the recruitment of monocytes and T cells into sites of inflammation. Migration of monocytes from the blood stream across the vascular endothelium is a critical early event in the response to inflammation. MCP-1 is categorized as an *inflammation-related activity* in this SAg system that models Th1 cell-driven vascular inflammation.
	CD38	CD38 is a membrane-associated bifunctional enzyme regulating cyclic ADP ribose and is an activation marker expressed on hematopoietic and nonhematopoietic cells. CD38 is involved in diverse processes, such as T cell activation/costimulation and chemotaxis. CD38 is categorized as an *immunomodulatory-related activity* in this SAg system.

CD40	CD40 is a cell surface adhesion receptor essential to mediating a broad variety of immune and inflammatory responses. This costimulatory receptor is expressed on antigen presenting cells and is required for T cell activation. CD40 can also be expressed by endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. CD40 is categorized as an *immunomodulatory-related activity* in this SAg system.
CD62E/ E-selectin	E-selectin (CD62E) is a cell adhesion molecule expressed only on endothelial cells and, like other selectins, it plays an important part in inflammation by mediating leukocyte-endothelial cell interactions via binding sialylated carbohydrates. During inflammation, localized cytokines induce E-selectin overexpression on nearby blood vessels. Leukocytes in the blood expressing the correct ligand will bind with low affinity to E-selectin and "roll" along the internal surface of the blood vessel, becoming “recruited” at the inflammation site. E-selectin is categorized as an *inflammation-related activity* in this SAg system.
CD69	CD69 is a human transmembrane C-type lectin protein induced by activation of T lymphocytes. CD69 appears to be the earliest inducible cell surface glycoprotein acquired during immune activation and is involved in lymphocyte proliferation and activation. CD69 is categorized as an *immunomodulatory-related activity* in this SAg system.
CXCL8/ IL-8	IL-8 (CXCL8) is a chemokine, produced by immune and other cell types such as epithelial cells, airway smooth muscle cells, and endothelial cells, which mediates neutrophil recruitment into acute inflammatory sites. IL-8 can be secreted by any cells with toll-like receptors that are involved in the innate immune response. IL-8 is also known to be a potent promoter of angiogenesis and is categorized as an *inflammation-related activity* in this SAg system.
CXCL9/ MIG	MIG (CXCL9) is a proinflammatory C-X-C chemokine that mediates T cell chemotaxis through interaction with its receptor, CXCR3. MIG is categorized as an *inflammation-related activity* in this SAg system.
PBMC cytotoxicity	PBMC cytotoxicity in the SAg system is a *measure of PBMC death*. Cell viability of nonadherent cells is measured by alamarBlue staining, a method based on a cell permeable compound that emits fluorescence after entering cells. The number of living cells is proportional to the amount of fluorescence produced.
Proliferation	Proliferation in the SAg system is a *measure of T cell proliferation* which is the critical event driving both adaptive immunity as well as many autoimmune diseases (rheumatoid arthritis, psoriasis, multiple sclerosis, inflammatory bowel disease, etc.). Inhibition of T cell proliferation is classified as an immune suppressive effect.

	SRB	SRB in the SAg system is a *measure of the density* of cocultured venular endothelial cells and PBMCs. SRB staining is used to determine the total protein content of the system. A decrease in SRB signal (i.e., total protein) of ≥ 50% indicates the compound is overtly cytotoxic to the system at that concentration.
BT	**B cell proliferation**	B cell proliferation is a critical event driving both adaptive immunity (antibody production) as well as autoimmune diseases where B cells are key disease players (lupus, multiple sclerosis, rheumatoid arthritis, etc.). Inhibition of B cell proliferation is classified as an immune suppressive effect.
	PBMC cytotoxicity	PBMC cytotoxicity is a *measure of PBMC death*. Cell viability of nonadherent cells is measured by alamarBlue staining, a method based on a cell permeable compound that emits fluorescence after entering cells. The number of living cells is proportional to the amount of fluorescence produced.
	sIgG	sIgG is produced by B cells and is the main type of antibody found in blood and extracellular fluid that mediates the immune response against pathogens. sIgG is categorized as an *immunomodulatory activity* in this BT system that models T cell-dependent B cell activation.
	sIL-17A	sIL-17A is a proinflammatory cytokine produced by T cells that induces cytokine production and mediates monocyte and neutrophil recruitment to sites of inflammation. sIL-17A is categorized as an *immunomodulatory activity* in this BT system.
	sIL-17F	sIL-17F is a proinflammatory cytokine produced by T cells that induces cytokine, chemokine, and adhesion molecule production and mediates neutrophil recruitment to sites of inflammation. sIL-17F is categorized as an *immunomodulatory activity* in this BT system.
	sIL-2	sIL-2 is a cytokine produced by activated T cells and acts in an autocrine manner to promote T cell proliferation and maturation. sIL-2 is categorized as an *immunomodulatory activity* in this BT system.
	sIL-6	sIL-6 is a secreted proinflammatory cytokine and acute phase reactant. sIL-6 is categorized as an *immunomodulatory activity* in this BT system.
	sTNF-α	sTNF-α is a secreted proinflammatory cytokine involved in Th1 inflammation. sTNF-α is categorized as an *inflammation-related activity* in this BT system.
CRC Systems—StroHT29 & VascHT-29	**CD40**	Cell surface adhesion receptor found on antigen presenting cells, endothelial cells, and fibroblasts. CD40 is a costimulatory molecule important for B-T cell interaction and macrophage-dependent immune surveillance activities. CD40-activated macrophages infiltrate tumors, become tumoricidal, and deplete the tumor stroma in pancreatic ductal adenocarcinoma.
	CD69	Very early activation marker transiently expressed on activated T cells, B cells, NK cells, and other leukocytes.

sGranB	Serine protease found in granules of effector cytotoxic T cells and NK cells that mediates apoptosis of pathogen-infected or malignant target cells. sGranB expression can be significantly reduced in $CD8^+$ T cells and $CD8^+$ NK T-like cells in cancer.
sIFN-γ	Produced by activated T cells and has antiviral activity. Exhibits both antitumorigenic and protumorigenic activities, depending on the cellular, microenvironmental, and/or molecular context.
sIL-2	Produced by activated T cells and promotes T cell proliferation and maturation. Th1 type cytokine favoring the antitumor immune response. In cancer, Th cells augment NK cell cytolytic activity and macrophage phagocytosis, and they amplify antigen-specific immunity via local secretion of IL-2.
sIL-6	Produced by fibroblasts, endothelial cells, T cells, B cells, and monocytes. Acts as a proinflammatory cytokine and acute phase reactant. However, IL-6 also acts to promotes proliferation and suppresses apoptosis of tumor cells in CRC.
sIL-10	Produced by many cell types, most notably macrophages, but also by T cells, B cells, monocytes, and dendritic cells. IL-10 acts as an immunomodulatory cytokine by suppressing Th1 responses. Although commonly regarded as immunosuppressive, high levels of IL-10 within the tumor microenvironment may favor immune-mediated tumor rejection by enhancing NK cell activity, increasing the tumor-associated antigen (TAA) upload capability of dendritic cells, and by enhancing cytotoxicity and migration of cytotoxic T lymphocytes.
sIL-17A	A proinflammatory cytokine secreted by Th17 cells as well as by gamma delta T cells and neutrophils. Exhibits both protumor functions, such as angiogenesis and metastasis, and antitumor functions, such as induction of cytolytic T cell responses.
MCP-1	MCP-1 is a chemokine that mediates recruitment of monocytes and T cells into sites of inflammation. Recruits endothelial progenitors, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and Tregs to the tumor microenvironment. TAMs and MDSCs produce VEGF, which promotes angiogenesis.
VCAM-1	VCAM-1 is a cell adhesion molecule upregulated by TNF-α and IL-1 that mediates adhesion of monocytes and T cells to endothelial cells. Regulates trafficking of tumor cells and adhesion of lymphocytes in the tumor microenvironment.
IP-10	IP-10 is a chemokine produced by monocytes, endothelial cells, and fibroblasts that mediates T cell, monocyte, and dendritic cell chemotaxis. IP-10 expression in human lung cancers is inversely related to angiostatic and metastatic potential, and it positively correlates with patient survival.
MIG	Produced by monocytes, macrophages, B cells, endothelial cells, and fibroblasts. MIG is induced by INF-γ and mediates T cell recruitment. Expression is downregulated in metastatic CRC.

sTNF-α	sTNF-α is a proinflammatory cytokine produced by macrophages, monocytes, neutrophils, T-cells, and NK cells. TNF-α plays a major role in diseases involving systemic inflammation. In cancer, TNF-α has pleotropic effects that are context dependent. These include promotion of growth, survival, proliferation, and angiogenesis, but also death of tumor cells.
Collagen I	ECM protein and fibrillar collagen found in extensible connective tissues (bone, skin, tendons, ligaments, etc.). Most abundant collagen. Collagen I upregulates epithelial-mesenchymal transition marker N-cadherin and increases mobility of pancreatic cancer cells.
Collagen III	ECM protein and fibrillar collagen found in extensible connective tissues (skin, lung, liver, vascular system, etc.). Expressed in stromal fibroblasts, not epithelial cells. Increased expression of types I and III procollagen and aberrant collagen bundles are found at the invasive front of malignant breast tumors.
Collagen IV	ECM protein forming basal lamina of basement membranes. Downregulation of the α5(IV) and α6(IV) chains of type IV collagen and remodeling of the basement membrane correlates with early invasive stage CRC.
MMP-9	MMP-9 is a gelatinase MMP that degrades collagen IV and gelatin. MMP-9 cleavage/activation of ECM, adhesion molecules, and chemokines in the tumor microenvironment play a major role in tumor growth, angiogenesis, metastasis, and pro- and antitumor immune responses.
TIMP-2	TIMP-2 maintains tissue homeostasis by inhibiting MMP-induced degradation of ECM and suppressing angiogenic factor-induced endothelial cell proliferation. Conversely, TIMP-2 also induces IL-8 expression and suppresses apoptosis by direct activation of nuclear factor kappa B.
uPAR	uPAR is the cell surface receptor for uPA that localizes uPA and therefore plasmin to the cell surface. Directs migration of endothelial cells in the tumor microenvironment.
PAI-1	PAI-1 is a serine proteinase inhibitor and inhibitor of tPA and uPA. Regulates tumor invasion/metastasis and angiogenesis. High levels of PAI-1 in many solid tumors has been associated with poor prognosis.
tPA	tPA is a serine protease that catalyzes the cleavage of plasminogen to plasmin and regulates clot degradation. May play a role in tumor invasion and metastasis.
uPA	uPA is a serine protease with thrombolytic activity. Triggers fibrinolysis and ECM degradation. Plays multifunctional roles in the tumor microenvironment, such as proliferation, angiogenesis, invasion, and migration. Several therapeutic strategies targeting uPA in order to slow tumor progression are currently being investigated.

sVEGF	sVEGF induces vascular permeability, angiogenesis, vasculogenesis, and endothelial cell growth and inhibits apoptosis. Expressed in a wide variety tissues and organs, including malignant cells (HT-29, H1299). Anti-VEGF antibody treatment of colorectal tumors in mice resulted in 90% tumor growth inhibition.
CEACAM5	CEACAM5 is found on HT-29 cells and is used as a clinical biomarker for gastrointestinal cancers. A cell surface glycoprotein that plays a role in cell adhesion and intracellular signaling. Protects tumor cells from apoptosis and enhances metastatic seeding.
Keratin 20	Major cellular protein of enterocytes and goblet cells. Specifically found in gastric and intestinal mucosa. Colorectal adenocarcinomas are largely and uniquely keratin 20 (a.k.a. cytokeratin 20 [CK20]) positive; thus, the expression of CK20, along with CK7, is used for determining the site of origin of metastatic adenocarcinomas.