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Expression of SARS CoV2 Receptors Influenced Upon Cytokine Polarizations (IL-4 and IFNɣ) in Hemangioendothelioma Cells

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Expression of SARS CoV2 receptors influenced upon Cytokine polarizations (IL-4 and IFNγ) in Hemangioendothelioma cells

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by

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY CHANDRA LEKHA KOOPARI ENTITLED EXPRESSION OF SARS COV-2 RECEPTORS INFLUENCED UPON CYTOKINE POLARIZATIONS (IL-4 AND IFNγ) IN HEMANGIOENDOTHELIOMA CELLS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT

Koopari Chandra Lekha, M.S. Microbiology and Immunology Graduate Program, Wright State University, 2022. Expression of SARS CoV2 receptors influenced upon Cytokine polarizations (IL-4 and IFNγ) in Hemangioendothelioma cells.

Endothelial cells are distinct multifunctional cells with essential metabolic and synthetic roles along with their ability to function as selective permeability barrier. Endothelial cells (ECs), the major component of blood vessels, essentially interact directly yet differently with inflammatory cytokines. ECs are well recognized to be polarized cells, but little is known about the potential function of inflammatory mediators. Covid 19 may have long-term health effects on par with chronic illnesses. Vascular inflammatory disease and coagulopathy linked to COVID-19 are exacerbated by endothelial cell (EC) dysfunction. SARS-CoV-2 enters the host cell through the Angiotensin Converting Enzyme 2 (ACE2) receptor and primes the S protein using the serine protease Transmembrane Protease, Serine 2 (TMPRSS2). NRP1 (neuropilin-1) serves as a coreceptor. To deepen comprehension of the role of the cytokines on SARS CoV2 receptors of mouse Hemangioendothelioma endothelial cells is the goal of this research. In order to simulate an anti-inflammatory and pro-inflammatory microenvironment, ECs were treated with the cytokines IL-4 and IFNγ respectively. Immunofluorescence staining for ACE2, NRP1 and TMPRSS2 were then analyzed on the cytokine treated cells. The results indicate an increase in the expression of NRP1 and TMPRSS2 receptors when stimulated with Type I (IFNγ) cytokine ($p<0.03$) whereas Type II (IL4) induced an enhancement of the ACE2 and

TMPRSS2 ($p<0.0.3$) receptor expression. These findings identify distinct variations in the response of the cell receptors to cytokine stimulation by EOMA cells.

Table of contents

Page

I. INTRODUCTION……..……1

Hypothesis…….3

II. LITERATURE REVIEW……..……..……4

- Endothelial Cells…….…….4

Role of endothelial cells……4

- Hemangioendothelioma…….……....5

- Cytokines…….…….6

Pro-inflammatory cytokine- IFNɤ……...…….6

Anti-inflammatory cytokine- IL-4……...7

- SARS CoV2 and its receptors…….……7

Angiotensin Converting Enzyme (ACE2) ……....8

Neuropilin-1 (NRP-1) ……8

Transmembrane Protease, Serine 2 (TMPRSS2).……...……10

- SARS CoV2 cell entry.……...10

III. MATERIALS AND METHODS…….…..……13

- Cell culture...…….13

Culturing cells from frozen vials……...13

Cell counting…….14

Culturing cells in 8 well chamber slides & Cytokine polarization...14

- Immunofluorescence…….15

  Cell fixation and Permeabilization……15

  Cell Blocking……15

  Cell staining…….…….15

  Cell mounting……16

  Slide observation…….…….16

  Image processing……..….17

  Data interpretation……17

RESULTS…….……19

FIGURES AND TABLES……...…..…….21

DISCUSSION……...32

FUTURE RESEARCH……36

REFERENCES…….37

LIST OF FIGURES

1. Mechanism of SARS CoV-2 entry into cells……11
2. EOMA cells under light microscope at confluency…….21
3. Distinct morphology of EOMA cells under light microscope…….21
4. Detection of NRP1 in the nucleus and cytoplasm of EOMA cells by Fluorescence microscopy……..…22
5. Immunofluorescence staining of EOMA cells expressing ACE2 receptor upon IFNɤ treatment……....24
6. Immunofluorescence staining of EOMA cells expressing NRP1 receptor upon IFNɤ treatment……25
7. Immunofluorescence staining of EOMA cells expressing TMPRSS2 receptor upon IFNɤ treatment……...…26
8. Immunofluorescence staining of EOMA cells expressing ACE2 receptor upon IL4 treatment…….28
9. Immunofluorescence staining of EOMA cells expressing NRP1 receptor upon IL4 treatment……..…29
10. Immunofluorescence staining of EOMA cells expressing TMPRSS2 receptor upon IL4 treatment…….30

LIST OF TABLES

1. Experimental design- IFNɤ treatment……22
2. Experimental design- IL4 treatment……23
3. Statistical significance differences of ACE-2, NRP1 and TMPRSS2 immunofluorescence on EOMA cells- IFNɤ treatment…….27
4. Statistical significance differences of ACE-2, NRP1 and TMPRSS2 immunofluorescence on EOMA cells- IL4 treatment…….31

LIST OF ABBREVIATIONS

ACE2 = Angiotensin Converting Enzyme 2

AF488 = Alexa fluor 488

ATCC = American Type Culture Collection

BSA = Bovine Serum Albumin

CO2 = Carbon dioxide

CTCF = Corrected Total Cell Fluorescence

DAPI = 4’,6-diamidino-2-phenylindole (blue fluorescent DNA stain)

DC = Dendritic cells

DMEM = Dulbecco’s Modified Eagle Medium

DMSO = Dimethyl Sulfoxide

EC = Endothelial cells

EDHF = Endothelium-derived hyperpolarizing factor

ET-1 = Endothelin 1

Factor VIII = Antihemophilic factor

FBS = Fetal Bovine Serum

FGFR4 = Fibroblast growth factor receptor 4

FLT4 = fms-related tyrosine kinase-4

GFP = Green Fluorescence Protein

G αq = Guanine nucleotide-binding protein G(q) subunit alpha

IDH = Isocitrate Dehydrogenase

IFN-ɤ = Interféron gamma

IL-4 = Interleukin

NBCS = Newborn Calf Serum

NK cells = Natural Killer cells

NO = Nitric oxide

NRP-1 = Neuropilin 1

O2 = Oxygen

PBS = Phosphate Buffered Saline

PDGF-β = Platelet-derived growth factor receptor-β

PGI2 = Prostaglandin I2

ng/mL = Nanograms per Milliliter

T cells = Thymocytes

TMPRSS2 = Transmembrane Protease, Serine 2

TNF α = Tumor Necrosis Factor alpha

t-PA = Tissue-type Plasminogen Activator

TXA2 = Thromboxane A2

VEGF = Vascular endothelial growth factor

vW factor = Von Willebrand factor

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INTRODUCTION

Two significant coronavirus outbreaks—SARS-CoV (2002) and MERS—have occurred in the previous 20 years (2012). CoV-induced sickness in 2019 is caused by the most recent coronavirus outbreak that occurred in the Chinese city of Wuhan (COVID-19). While many SARS-CoV-2 infected people go asymptomatic or only experience a moderate illness, it may be fatal for some people, especially the elderly and those with comorbidities (Murgolo et al., 2021). The respiratory system is most frequently impacted in individuals who are susceptible to clinical sickness after contracting SARS-CoV-2. This further turns down the Vascular and the Circulatory systems consequentially. The respiratory tract serves as a prime site for viral infections. Ciliated epithelial cells are the main target cells for SARS-CoV-2 infection, multiplication, and release in the nose (Bridges et al., 2022). Under normal physiological conditions, macrophages are the most prevalent immune cell type in the lung. Alveolar macrophages (AM) can mount inflammatory responses in response to a lung infection. A pro-inflammatory program is switched on in AMs when the airway epithelium is destroyed (Knoll et al., 2021). In addition to infecting vascular endothelial cells, the majority of SARS-CoV-2 infections of ECs occur in septa at the pneumocyte-endothelial barrier. This may account for the elevated pulmonary vascular permeability frequently observed in infected patients (Liu et al., 2021). These viral particles were also noticed in the interstitial space or in

inflammatory cells emanating from the lungs in human cardiac biopsies. Accordingly, it is possible for the virus to spread from the coronary circulation to the cardiac interstitium via infecting pericytes (Abdi et al., 2022).

This current study is based on the observations of cytokine and receptor interactions on epithelial cells, macrophages, and cardiomyocytes. This research was conducted to investigate whether the cytokine mediators such as IL4 and IFNɣ induce the expression of ACE2, NRP1 and TMPRSS2 receptors on EOMA cells that facilitate the entry and replication of SARS CoV2.

HYPOTHESIS

Pro inflammatory- IFNg and Anti-inflammatory- IL4 cytokines alter the expression of SARS CoV 2 receptors on EOMA cells.

AIMS

- IFNɣ-Type I cytokine polarized cells increase the expression of NRP1 and TMPRSS2.
- Type II cytokines like IL4 decrease the expression of ACE2 and TMPRSS2.

LITERATURE REVIEW

ENDOTHELIAL CELLS

A permeable barrier in between the vessels and tissues, playing a vital role in the immunoinflammatory responses is Endothelium. It is made up of a single layer of squamous cells that line the inside of the heart and all blood and lymphatic vessels.

ROLE OF ENDOTHELIAL CELLS-

Endothelial cells selectively permeabilize the exchange of chemicals and O2/CO2 between the blood and tissues performing a barrier function. They regulate the blood flow by controlling the widening and narrowing of the blood vessels that are dependent on the Vasodilation factors- NO, PGI2, EDHF and Vasoconstriction factors- TXA2, ET-1 released by ECs (Sandoo et al., 2010). Alterations in the cell functioning lead to inflammatory conditions like Ischemia. Consequently, expression of certain leukocyte adhesion molecules and production of TNF α and IFN-ɤ increases in T cells, contributing to increased vascular permeability (Sharfuddin et al.,). ECs synthesize Factor VIII and secrete t-PA that dissolves the blood clots post wound healing. Inhibition of platelet aggregation by secreting Prostacyclin and release of vW factor that binds to Factor VIII in response to uncontrollable Thrombosis are some of the regulatory measures taken by ECs.

HEMANGIOENDOTHELIOMA

Hemangioendothelioma refers to both benign and malignant vascular neoplasms that exhibit 'borderline' behavior, which is in between completely benign hemangiomas and severely malignant angiosarcomas. Hemangioendotheliomas are of various types. Epithelioid Hemangioendothelioma (EHE) is caused by translocation of two genes. 90% of the cases have WWTR1-CAMTA1 translocations and the other 10% have YAP-TFE3 gene fusions (Lamar et al.,). The molecular genetic features recorded for Composite Hemangioendothelioma are translocations in PTBP1-MAML2 and YAP1-MAML2 genes (Sheena et al., 2021). Spindle cell hemangioma (SCH) is a rare benign tumor that often manifests as an extremity-afflicting deep dermal or subcutaneous lump (Gbolahan et al., 2015) IDH1 or IDH2 are the genes in charge of this illness (Cheraghlou et al., 2019). Retiform (or Hobnail) Hemangioendothelioma (RHE) is a low-grade angiosarcoma with a low risk of metastasizing (Ranga et al., 2014) and is caused due to abnormalities in YAP1 and MAML2 genes (Antonescu et al.,). The genes associated with Kaposiform (or Infantile Kaposiform) Hemangioendothelioma (KHE) belong to the family of (Gαq), especially the GNA14 (Cheraghlou et al., 2019). Papillary intralympahtic angioendothelioma (or Dabska tumor) is a sarcoma of the lymphatic channels presented in the skin, head, neck, testicles, tongues, bone, and spleen so far (Kaplan et al.,). During the first six months of life, Infantile Hemangioma (IH) is the most often identified symptomatic liver tumor in children. Multiple gene mutations including FGFR4, PDGF-β, FLT4, VEGF2 and VEGFR3 might be responsible (Cheraghlou et al., 2019).

CYTOKINES

Cytokines are small single polypeptides that regulate inflammation by communicating with the cells of both innate and adaptive immune systems. Foreign molecules of pathogens are recognized by Pattern Recognition Receptors (PRRs), especially Toll-like receptors (TLRs) in a virus infected cell. This then stimulates the production of a cascade of cytokines including IFN, GM-CSF, IL- 2, IL-6, IL-8, IL-12, IL-18, and IL-23 by the infected cells. Inflammatory signals further passed down activates and directs the Mast cells, NK cells, Neutrophils, Macrophages and DCs to the site of infection. Meanwhile, the anti-inflammatory cytokines such as IL-4, TGF-β, IL-10 activate the Th2/Th17 pathway, whereas the pro-inflammatory cytokines work on the Th1/TCL pathway to eliminate the exogenous pathogens, dead and infected cells (Munoz et al., 2018).

Interferon gamma-

Type I includes IFN-α and IFN-β that are produced by the DCs and the virus infected cells. Type II includes IFN-ɤ that is produced by activated T cells and NK cells. It is an immunomodulatory mediator. ECs are uniquely impacted in the presence of IFN-ɤ treated cells (IFN-ɤ treated ECs), where they upregulate the expression of MHC I & II, IL-1 & IL-6 production. They are well-known for the negative regulation of vW factor that plays a primary role in the platelet plug formation when induced by IL-1 (Alberto et al.,). Modification to the cell morphology by increasing the intercellular gaps that derange the barrier function of ECs in response to IFN-ɤ has also been reported (Fong et al., 2015). It

is known that Delta-like 4 protein regulates the survival of cancer cells which interact with the Notch pathway that controls gastrulation of the entire body. Intriguingly, IFN-ɤ treated ECs have a potential role in Angio stasis by inhibiting Angiogenesis and tumor growth, which is done by down regulating the DLL4/ Notch signaling pathway (Deng et al., 2014).

Interleukin-4-

IL-4 is the major inducer of Th2 cells. It functions by binding to certain ligands and acting as signal transduction molecules that ultimately activate the differentiation of B-cells and T-cells. Excess IL-4 produced function as positive feedback inhibitors for the differentiation of Th2 cells. Surprisingly, IL-4 both favors the amplified functioning of lymphocytes and monocytes in response to inflammation, but also turns off the activated monocyte binding on ECs. One of the major roles of ECs, the Anti-thrombotic property is also reported to be negatively regulated by IL-4 (Alberto et al.,).

SARS CoV 2 AND ITS RECEPTORS

The Severe Acute Respiratory Syndrome Coronavirus 2, SARS CoV-2 is a (+) sense ssRNA virus with approximately 30,000 nucleotides in the genome that causes COVID 19. Like all other Coronaviruses, SARS CoV 2 also has 4 structural proteins- the Nucleocapsid (N) protein, the Envelope protein, the S-glycoprotein, and the Matrix (M) protein and sixteen non-structural proteins. The S, E and M proteins make up the viral envelope protecting the genetic material, whereas the N protein encodes the RNA

genome. The Spike protein made up of glycoproteins upon proteolytic cleavage yields 2 subunits- S1 and S2 responsible for viral attachment. N-terminal domain (NTD) and Receptor binding domain (RBD) are the components of S1 while S2 is composed of Fusion peptide (FP), Central helix (CH), Heptad repeat 1 and 2 (HR1 and HR2), Connector domain (CD), transmembrane domain I, and cytoplasmic tail (CT) (12). CoV in general, has a high rate of mutation due to the presence of the RdRp- RNA dependent RNA polymerase that encodes its structural proteins (10). COVID-19 disease caused by SARS CoV 2 has various modes of transmission of which the Aerosol transmission is the most common route. It can also spread via Surface transmission and Animal vectors. Even though the mode and the extent of infection is different among different people, most of them have flu-like symptoms, whereas anosmia or ageusia, muscle sores, diarrhea, headaches, dyspnea, hypoxia are also observed. Total number of cases reported till date are approximately 605 million with about 6 million deaths. Virus entry to the body is aided by quite a few host molecules that function as receptors, coreceptors and cofactors. The most important host receptor that aids the entry of the virus is the Angiotensin-converting enzyme 2 (ACE2), whereas Basigin (CD147), Tyrosine-protein kinase receptor UFO (coded by AXL gene), Asialoglycoprotein receptor-1 (ASGR1) and Kringle containing transmembrane protein 1 (KREMEN1) are also identified as potential alternative receptors. The tissue tropism is influenced by some of the coreceptors, attachment receptors such as Sialic acid, Heparin sulfate, Neuropilin 1 (NRP1). Some of the cofactors responsible for the proteolytic cleavage of the Spike protein of SARS CoV2

are Transmembrane serine protease 2 (TMPRSS2), Trypsin, Cathepsins, Serine endoprotease proprotein convertase 1 (PC1), Furin (Peng et al., 2021).

Angiotensin Converting enzyme 2 (ACE2)

ACE2 is an enzyme belonging to the family of Dipeptidyl carboxypeptidases. Since ACE2 is known to express differently in different human organs and types of cells, it is possible that it regulates reproductive, cardiovascular, and renal function. Part of the reason why SARS-CoV-2 can damage so multiple organs is due to the widespread nature of this receptor. The normal role of ACE2 is to catalyze the cleavage reaction of Angiotensin I (1-10) into Angiotensin (1-9) and Angiotensin II (1-8) into Angiotensin (1-7). Angiotensin II further exerts Vasoconstrictive actions whereas Angiotensin (1-7) exerts Vasodilating actions. The full-length ACE2 (mACE2) is modified post translationally to its soluble form sACE2. mACE2 is present on the plasma membrane which contains a transmembrane domain projecting its N-terminal catalytic site onto the extracellular space. A receptor for the spike (S) protein of the SARS-CoV has been shown to exist in the extracellular domain (Daniel et al., 2020). When the S1 subunit of the SARS-CoV-2 Spike protein binds to the ACE2 receptor, it produces a soluble version of ACE2 (sACE2) that retains its catalytic function (Scialo et al., 2020). The sACE2 is proposed to competitively block the SARS CoV and other coronaviruses (Daniel et al., 2020).

Neuropilin 1 (NRP1)

NRP 1 is a membrane-bound, highly conservative, single-pass transmembrane, non-tyrosine kinase surface glycoprotein expressed in all vertebrates. There are numerous sNRP1 (soluble NRP1) isoforms, transmembrane domains, and cytoplasmic domains produced by alternative splicing of the NRP1 gene (Vedanta et al., 2018). In addition to interacting with the Vascular endothelial growth factor (VEGF), viruses like EBV, HTLV-1, and SARS-CoV-2 have also been reported to be interacting with the ligand binding region of the Transmembrane isoform of NRP1 (Bindu et al., 2021). The multifunctional receptor, Neuropilin 1 has been implicated in the vascularization and progression of cancers by serving as cell surface receptors for several endogenous cytokines including class III semaphorins, VEGF, heparin-binding protein, fibroblast growth factor, placental growth factor, platelet growth factor, fibronectin, and others (Jonathan et al., 2020).

Transmembrane protease, serine subtype 2 (TMPRSS2)

TMPRSS2 is an androgen-responsive transmembrane protein belonging to the serine protease family. In addition to its predominant presence the epithelial cells of the lung and prostate, the TMPRSS2 protein is also expressed in the cardiac endothelium, liver, kidney, gastrointestinal tract, and microvascular endothelial cells lining the blood vessels (Perry et al., 2017).

SARS CoV-2 cell entry

Viral membrane fusion takes place either at the cell surface or in the endosome. Upon first cleavage, the two subunits of the spike protein, S1 and S2, are exposed. The S1 head will gradually separate from the fusogenic S2 stalk as a result of interactions with ACE2 molecules, which allows for easier fusion activation by further proteolysis at the S2′ location. The Receptor Binding Domain (RBD) of the C-terminal domain binds to ACE2 in its open confirmation. A secondary cleavage is subsequently carried out by TMPRSS2 and other proteases to reveal the fusion peptide (FP) of the S2 that is shielded in the Upstream helix. As a result, S2 experiences permanent confirmational alterations. After the virion undergoes endocytosis as a result of ACE2 binding to S protein, the viral envelope fuses with the endosomal membrane to allow the release of the viral genome into the cytoplasm (Peng et al., 2021). NRP1 additionally acts as a host factor for SARS CoV-2. The S1 protein's C-end rule (CendR) motif directly binds to NRP1, acting as an entrance factor and enhancing the infection (Daly et al., 2020).

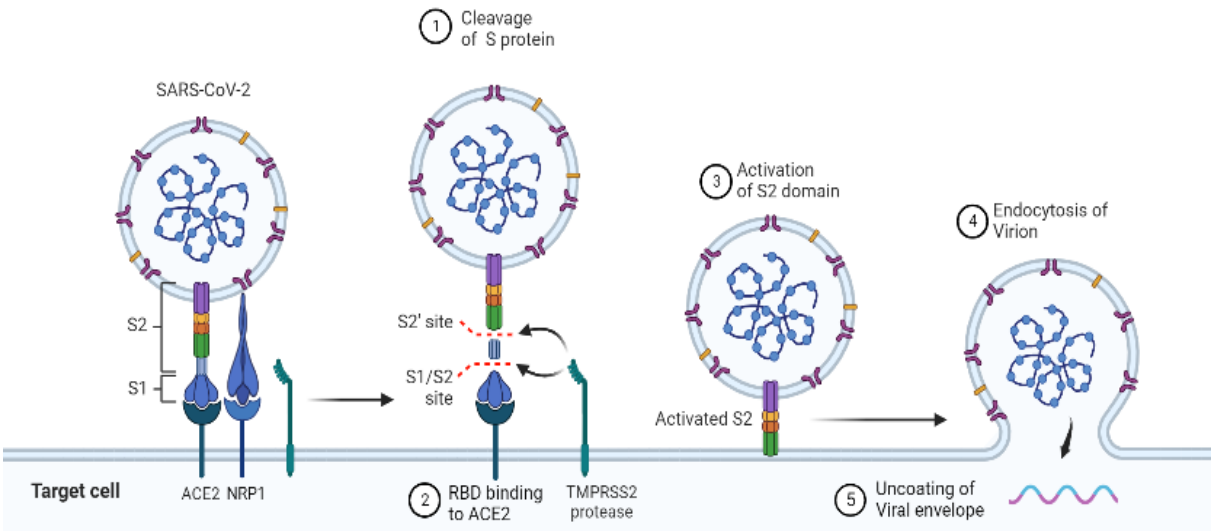


Figure 1- Mechanism of SARS CoV-2 entry into cells. SARS CoV-2 binds to the ACE2 receptor (majorly) or the NRP1 (alternatively) on the membrane. TMPRSS2 aids the cleavage of the Spike protein, initiating the endocytosis pathway. Viral RNA is shed into the nucleus and undergoes multiplication. Created with BioRender.com

MATERIALS AND METHODS

The following experiments were run at least three times and conducted twice.

CELL CULTURE

EOMA CRL 2586 Hemangioendothelioma endothelial cells were obtained from ATCC (American Type Culture Collection). This cell line was originally derived in 1980 from a mixed hemangioendothelioma arising in an adult Mus musculus.

Culturing cells from frozen vials-

The basal medium used is HyClone Dulbecco's Modified Eagle's Medium (DMEM) (Fisher Scientific) and to make it a complete medium 10% Fetal Bovine serum (FBS) (Fisher Scientific) was used. The frozen vial was initially thawed by gently agitating the vial in a water bath that was set at 37C for approximately 2 minutes. The contents (1ml) from the vial were then transferred to a tube containing 9ml of prewarmed complete culture medium (DMEM with 10%FBS). Then the cells were spun down at 250xg for 5 mins at room temperature. After discarding the supernatant, the cell pellet was then resuspended in 1ml growth medium. Complete cell suspension volume was then added to a T75 flask (Fisher Scientific) with 9ml of medium. The culture was incubated at 37C with 5% CO2.

Upon reaching 70% confluency, the cells were passed using 1X HyClone Dulbecco's Phosphate Buffer Saline (dPBS) without Ca, Mg and 0.25% (w/v) Trypsin-0.53mM

EDTA solution (Fisher Scientific), following ATCC protocol. Before being employed in subsequent research, the cells underwent several passages. The cells were used for experiments from passages between 5 and 15.

Cell counting-

Once the cells were confluent enough, the cells were trypsinized and dislodged. Before spinning the cells at 250xg for 5 mins, a volume of 20ul of the cell solution was taken out into a microcentrifuge tube. The Trypan blue staining method was used to evaluate the cell count. The cell solution was stained with trypan blue (Fisher Scientific) in a 1:1 ratio. A hemocytometer was then used to determine the cell count as well as the cell viability.

Total number of cells = (Number of cells counted) / (Number of squares counted (5))

$*10^{-4}$ *initial volume * dilution factor

Cell viability = [Live cells / Total number of cells (Dead and Live)] x 100

Culturing cells in Eight well chamber slides and Cytokine polarization-

8 well sterile removable chamber slides (Ibidi) were used for further experiments. $4x10^4$ cells/well suspension with a growth medium volume of 400ul were seeded in each well. Pro inflammatory cytokine IFN ɤ (Recombinant mouse protein-Sigma Aldrich) and Anti-inflammatory cytokine IL-4 (Recombinant mouse protein-R&D systems) were used in

the concentration of 10ng/ml. The slides were incubated at 37C and 5% CO2 for 2 days until a confluent layer of cells was achieved.

IMMUNOFLUORESCENCE

Cell fixation and Permeabilization-

The media was aspirated off and each well was washed with 300ul of chilled 1X PBS. 300ul/well of MeOH + 2% Glacial Acetic acid fixative was added slowly, and the slide was incubated for 2 mins at -20C. The fixative was then washed off thrice with 300ul 1X PBS. The last wash can be retained to store the slides at 4C for later applications.

Cell Blocking-

The blocking buffer was composed of 3% Bovine Serum Albumin with 1X PBS. 0.1% Saponin was also added to the cells that were to be stained with Anti NRP1 and Anti TMPRSS2 antibodies. The last PBS wash was discarded and 300ul/well of the Blocking buffer was added and the slides were incubated for 1h at room temperature.

Cell staining-

All the antibodies (Santa Cruz), normal mouse IgG1 AF488, TMPRSS2 (H-4), Neuropilin (A-12) and ACE2 (E-11) were diluted at a ratio of 1:100 with the Blocking buffer. 250ul/well Antibody solution was added to the slide and incubated overnight at 4C in dark (Tables 1 and 2). The slides were placed in a humidifying box to prevent any osmotic imbalances in the cells.

Cell mounting-

The antibody solution was aspirated off carefully and the slides were washed thrice with 300ul of chilled 1X PBS. The silicon gaskets were then removed using tweezers starting from one edge and the slides were air dried. For mounting of the slide samples, a few drops of Vectashield antifade mounting medium with DAPI (Vector laboratories) of approximately 25ul were added. The slides were cover slipped and allowed the mounting medium to spread across the section. The perimeter of the slides was sealed permanently with nail polish and were stored in dark boxes at 4C.

Slide observation-

The ACCU-SCOPE EXC-350 microscope was used to view the slides. Slides with fluorescent cells were positioned under a 10X objective lens to focus on the field of interest. The specimen was focused by using the coarse adjustment knob while using the left eye solely and the 10X objective. Turning the fine adjustment knob brought the image into great focus once it was in view. To achieve the finest focus, the diopter collar was then turned. Only the right diopter collar needed to be adjusted to provide the same sharp image in the right eye. The objective lens was modified to 50X for increased resolution and a better image when submerged in oil once the cells were clearly visible. Depending on the type of filter being used, the wavelengths were modified, the first one being DAPI and the second one GFP. A software application named Infinity Capture was used to capture images. The images were optimized by adjusting the Exposure, Gain,

Brightness, Contrast, Saturation, and other options. The adjusted selections were kept constant throughout the experiment.

Image processing-

Image-J, a Java-based image processing program was used to analyze the images. Each experiment was conducted in triplicates and the values were averaged. Initially, multiple areas of interest (at least 6) were selected using a rectangular selection tool. Upon clicking the shortcut “T” button, ROI (Region of Interest) dialogue box appeared. Multiple measurements were selected under the Analyze tab like the Area, Mean gray value, Integrated Density and Standard Deviation. Later, in the ROI Manager, Multi measure option was selected which gives all the selected values. These were calculated for both the fluorescent and background areas.

Corrected Total Cell Fluorescence was calculated as:

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

Data interpretation-

The software tool used for scientific graphing was Sigma plot. Each group's data points (mean values) were recorded in the notebook. Simple vertical error bars were plotted. Then a One-way Anova analysis was performed for the data sets. To evaluate if there is a statistically significant difference between the means of three or more independent

groups, a one-way ANOVA (Analysis of Variance) is utilized. This test is performed when the samples are distinct from one another and have roughly normal distributions, or when the sample size is large. Here, the one dependent variable is CTCF.

Null hypothesis- H0: $\mu 1 = \cdot \cdot \cdot = \mu k$

where μ= group mean, k= Number of groups

The Null hypothesis assumes that there is no difference among the group means.

Alternative hypothesis- HA: $\mu 1 \neq \cdot \cdot \cdot \neq \mu k$

This hypothesis assumes that all the group means are not equal.

The most typical criterion is $p<0.05$, which denotes that under the null hypothesis, the data are expected to occur fewer than 5% of the time. We define a test result as statistically significant when the p-value is less than the selected α value. If the test produces statistically significant findings, the alternative hypothesis (HA) is then accepted.

RESULTS

Observation of EOMA cells under inverted microscope

EOMA cells demonstrated Cobblestone morphology at confluence which is a characteristic property of ECs (Figure 2). Osteoclast like giant multinucleated cells, a distinct morphology of a typical EOMA cell was also noticed (Figure 3).

Expression of NRP1 in EOMA cells

EOMA cells expressed the NRP1 receptor both in the nucleus and the cytoplasm. This observation was made initially when the frozen cell lines were thawed out using serum free medium. It's possible that the stress brought on by serum starvation made it possible for the receptor to express in the nucleus.

IFN ɤ treated EOMA cells shift towards a significant increase in the expression of NRP1 and TMPRSS2 receptors but not ACE2

Treatment of EOMA cells with IFN ɤ for 48 hours followed by treatment with anti ACE2, anti NRP and anti TMPRSS2 antibodies showed an increasing trend in the expression of NRP1 and TMPRSS2 receptors but not ACE2 when compared to untreated EOMA cells stained with anti IgG1 Isotype control antibody. The increase was statistically significant ($p<0.03$) (Figures 5-7, Table 4).

IL-4 treated EOMA cells shift towards a significant increase in the expression of ACE2 and TMPRSS2 receptors but not NRP1

Treatment of EOMA cells with IL-4 for 48 hours followed by treatment with anti ACE2, anti NRP and anti TMPRSS2 antibodies showed an increasing trend in the immunofluorescence expression of ACE2, NRP1 and TMPRSS2 receptors when compared to untreated EOMA cells stained with anti IgG1 Isotype control antibody. The increase was statistically significant in terms of ACE2 and TMPRSS2 expression ($p<0.03$) but not NRP1 (Figures 8-10, Table 5).

FIGURES

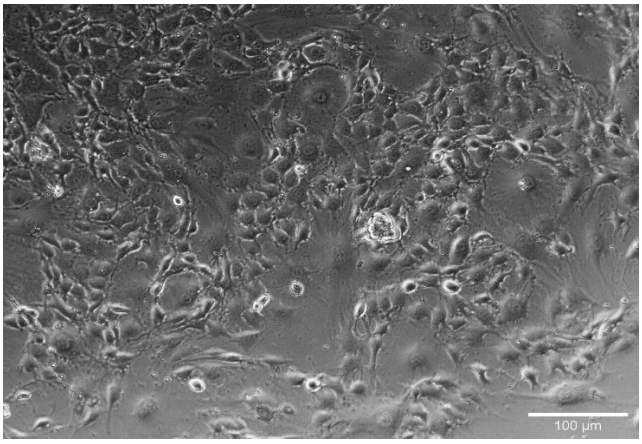


Figure 2- EOMA cells under inverted microscope at confluency. Cells demonstrating Cobblestone morphology at confluence which is a characteristic property of Endothelial cells (Lawley et al., 1989).

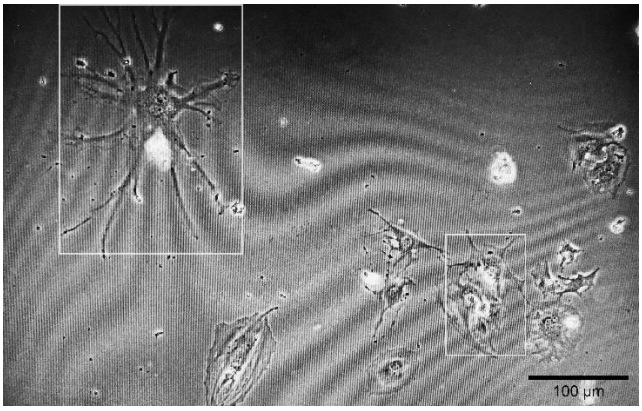


Figure 3- Distinct morphology of EOMA cells under inverted microscope. Osteoclast like giant multinucleated cells were observed, a typical morphology of Hemangioendothelioma cells (Adamane et al., 2016).

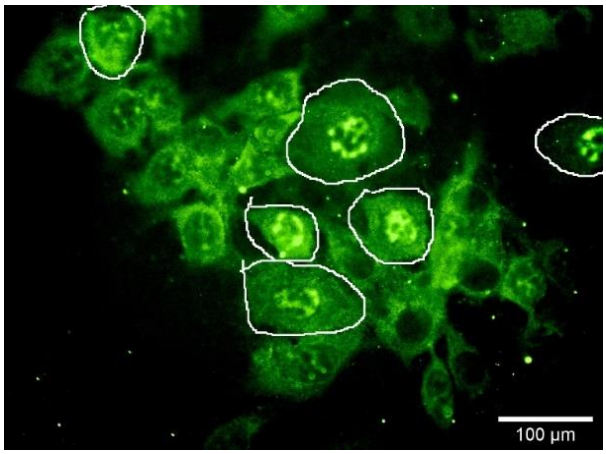


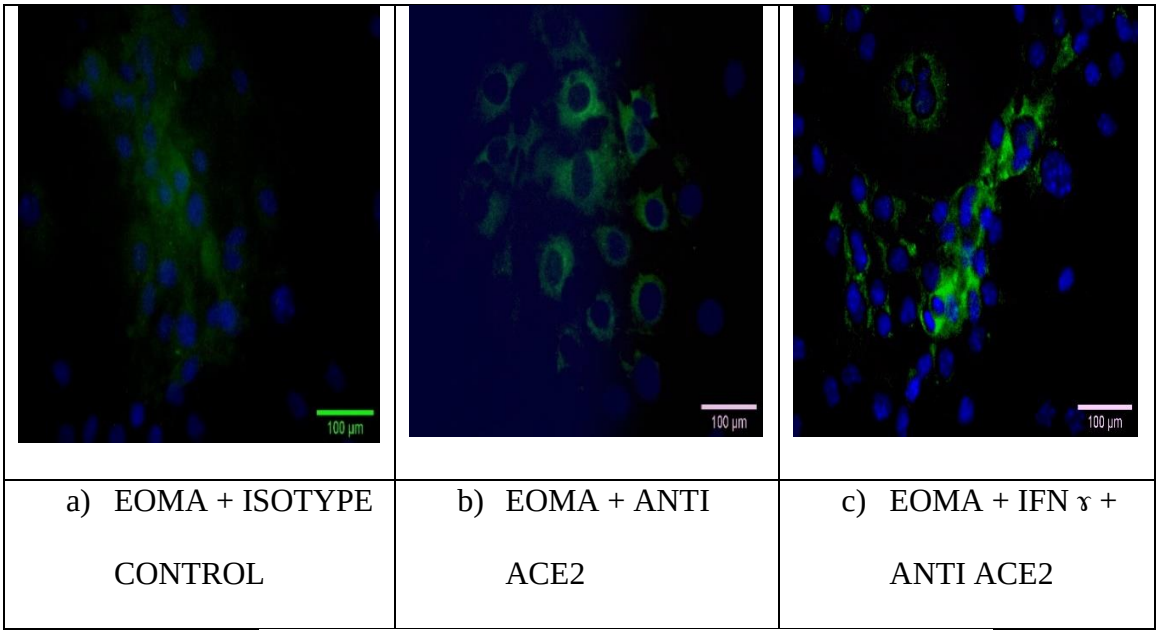
Figure 4- Detection of NRP1 in the nucleus and cytoplasm of EOMA cells by Fluorescence microscopy. EOMA cells stained with Anti NRP1 antibody exhibited fluorescence both in the cytoplasm and nuclei of the cells (Jiang et al., 2015).

TABLE 1: Experimental design- IFN ɤ treatment

EOMA + Anti IgG (1:100)	EOMA + Anti ACE2(1:100)	EOMA + Anti NRP1(1:100)	EOMA + Anti TMPRSS2(1:100)
-	EOMA + IFN ɤ (10ng/ml) + Anti ACE2(1:00)	EOMA + IFN ɤ (10ng/ml) + Anti NRP1(1:100)	EOMA +IFN ɤ (10ng/ml) + Anti TMPRSS2(1:100)

TABLE 2: **Experimental design- IL-4 treatment**

EOMA + Anti IgG (1:100)	EOMA + Anti ACE2(1:100)	EOMA + Anti NRP1(1:100)	EOMA + Anti TMPRSS2(1:100)
-	EOMA + IL-4 (10ng/ml) + Anti ACE2(1 :100)	EOMA + IL-4 (10ng/ml) + Anti NRP1(1 :100)	EOMA + IL-4 (10ng/ml) + Anti TMPRSS2(1:100)



a) EOMA + ISOTYPE CONTROL	b) EOMA + ANTI ACE2	c) EOMA + IFN ɤ + ANTI ACE2

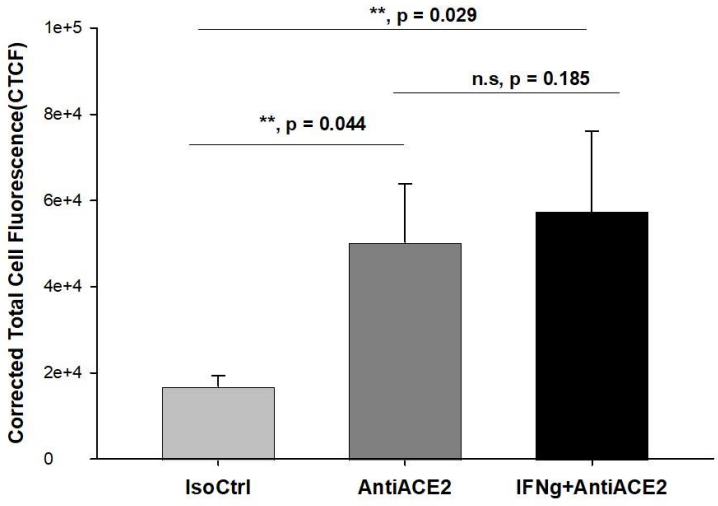


Figure 5- Immunofluorescence staining of EOMA cells expressing ACE2 receptor upon IFN ɤ treatment. Immunofluorescence images(500X) for EOMA cells untreated, stained with Isotype control (AntiIgG1) (a) stained with AntiACE2 (b) and IFN ɤ treated, stained with AntiACE2 (c) overlayed with DAPI. Simple error bars plotted using CTCF values.

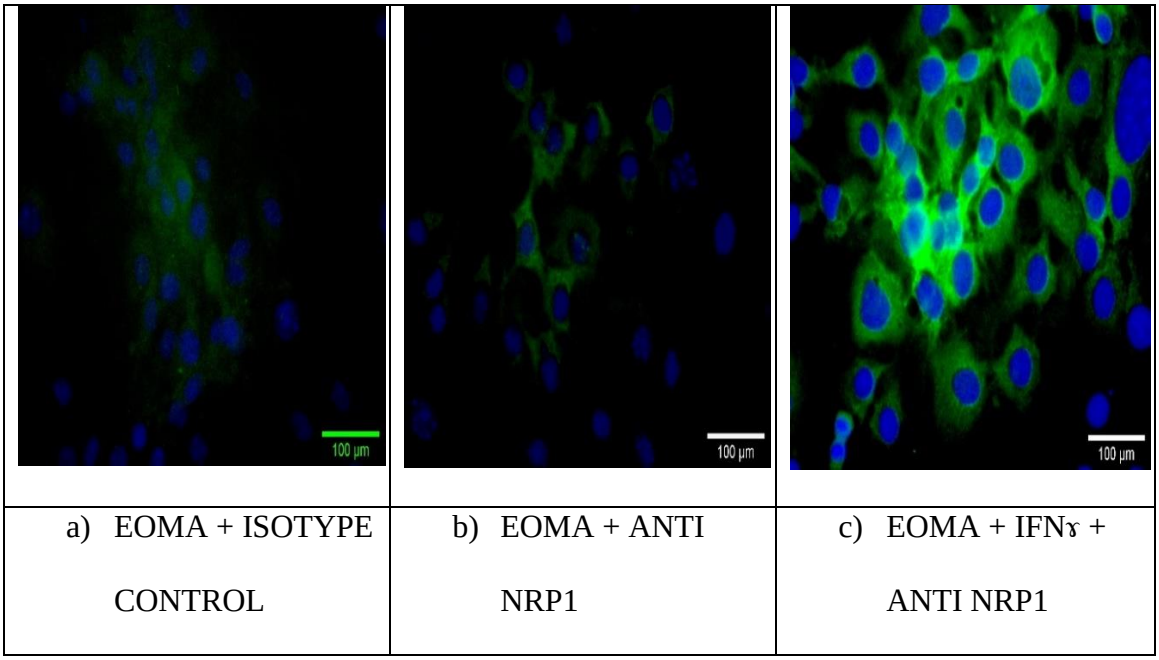
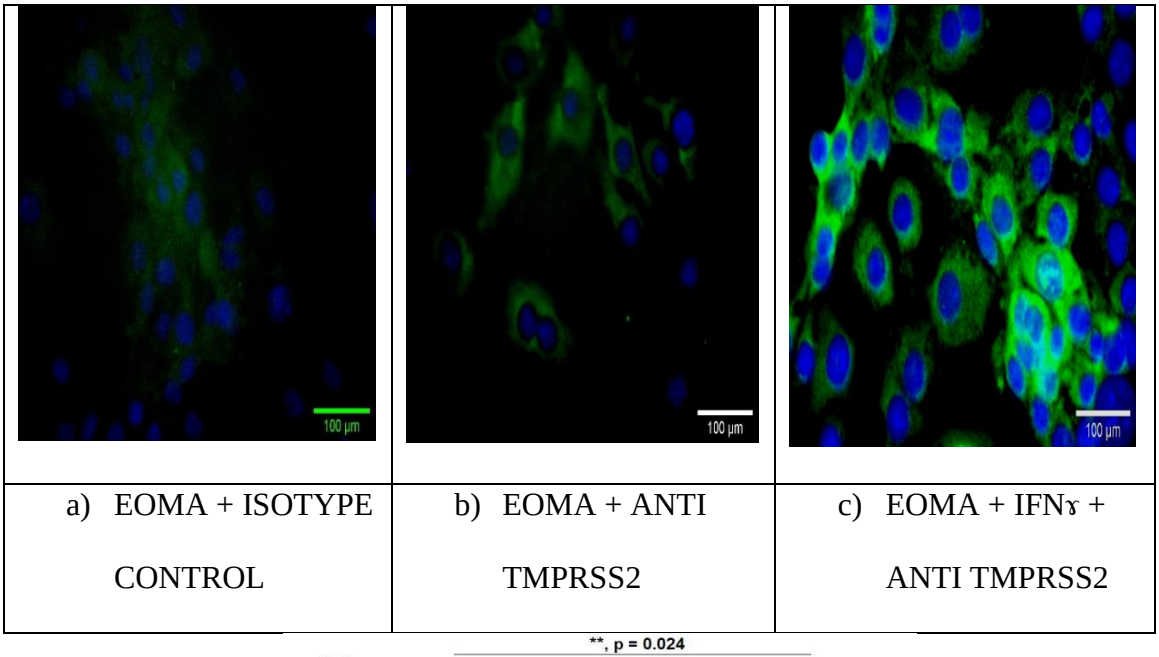


Figure-6: Immunofluorescence staining of EOMA cells expressing NRP1 receptor upon IFN ɤ treatment. Immunofluorescence images(500X) for EOMA cells untreated, stained with Isotype control (AntiIgG1) (a) stained with AntiNRP1 (b) and IFN ɤ treated, stained with AntiNRP1 (c) overlayed with DAPI. Simple error bars plotted using CTCF values.



a) EOMA + ISOTYPE CONTROL	b) EOMA + ANTI TMPRSS2	c) EOMA + IFNɤ + ANTI TMPRSS2

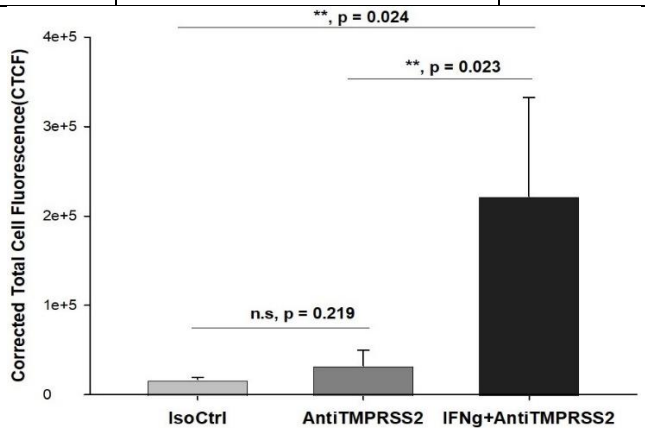
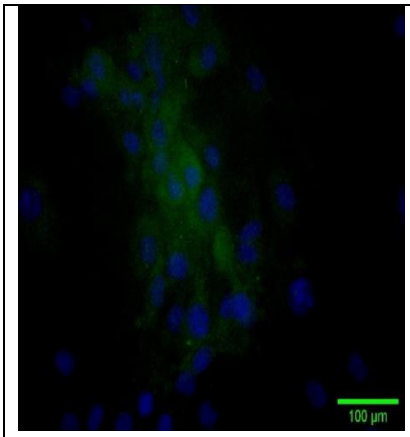


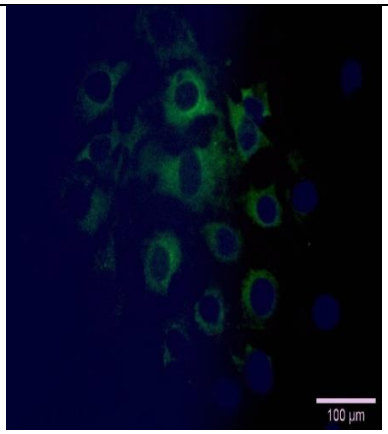
Figure-7: Immunofluorescence staining of EOMA cells expressing TMPRSS2 receptor upon IFN ɤ treatment. Immunofluorescence images(500X) for EOMA cells untreated, stained with Isotype control (AntiIgG1) (a) stained with AntiTMPRSS2 (b) and IFN ɤ treated, stained with AntiTMPRSS2 (c) overlayed with DAPI. Simple error bars plotted using CTCF values.

TABLE-3: Statistical significance difference of ACE2, NRP1 AND TMPRSS2 immunofluorescence on EOMA cells- IFN ɤ treatment.

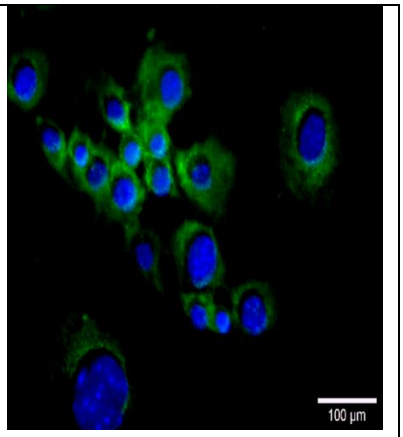
GROUPS	**p values**	**p< 0.05**
Iso Ctl vs Anti ACE2	0.044	YES
Anti ACE2 vs IFN ɤ + Anti ACE2	0.530	NO
Iso Ctl vs IFN ɤ + Anti ACE2	0.029	YES
Iso Ctl vs Anti NRP1	0.680	NO
Anti ACE2 vs IFN ɤ + Anti NRP1	0.0202	YES
Iso Ctl vs IFN ɤ + Anti NRP1	0.0190	YES
Iso Ctl vs Anti TMPRSS2	0.778	NO
Anti ACE2 vs IFN ɤ + Anti TMPRSS2	0.023	YES
Iso Ctl vs IFN ɤ + Anti TMPRSS2	0.024	YES



a) EOMA + ISOTYPE CONTROL



b) EOMA + ANTI ACE2



c) EOMA + IL4 + ANTI ACE2

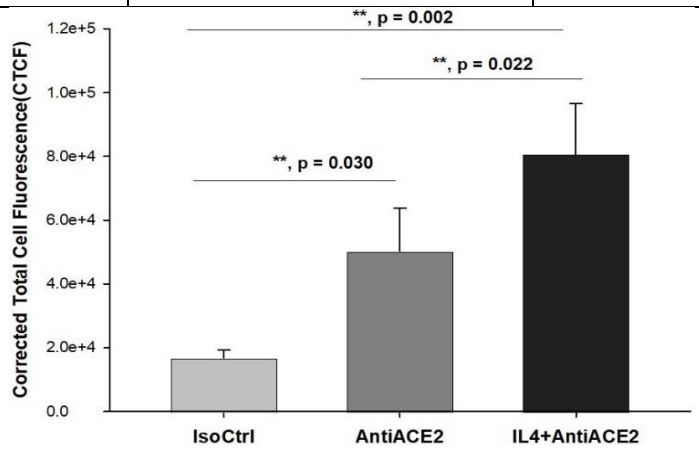


Figure-8: Immunofluorescence staining of EOMA cells expressing ACE2 receptor upon IL-4 treatment. Immunofluorescence images(500X) for EOMA cells untreated, stained with Isotype control (AntiIgG1) (a) stained with AntiACE2 (b) and IL4 treated, stained with AntiACE2 (c) overlayed with DAPI. Simple error bars plotted using CTCF values.

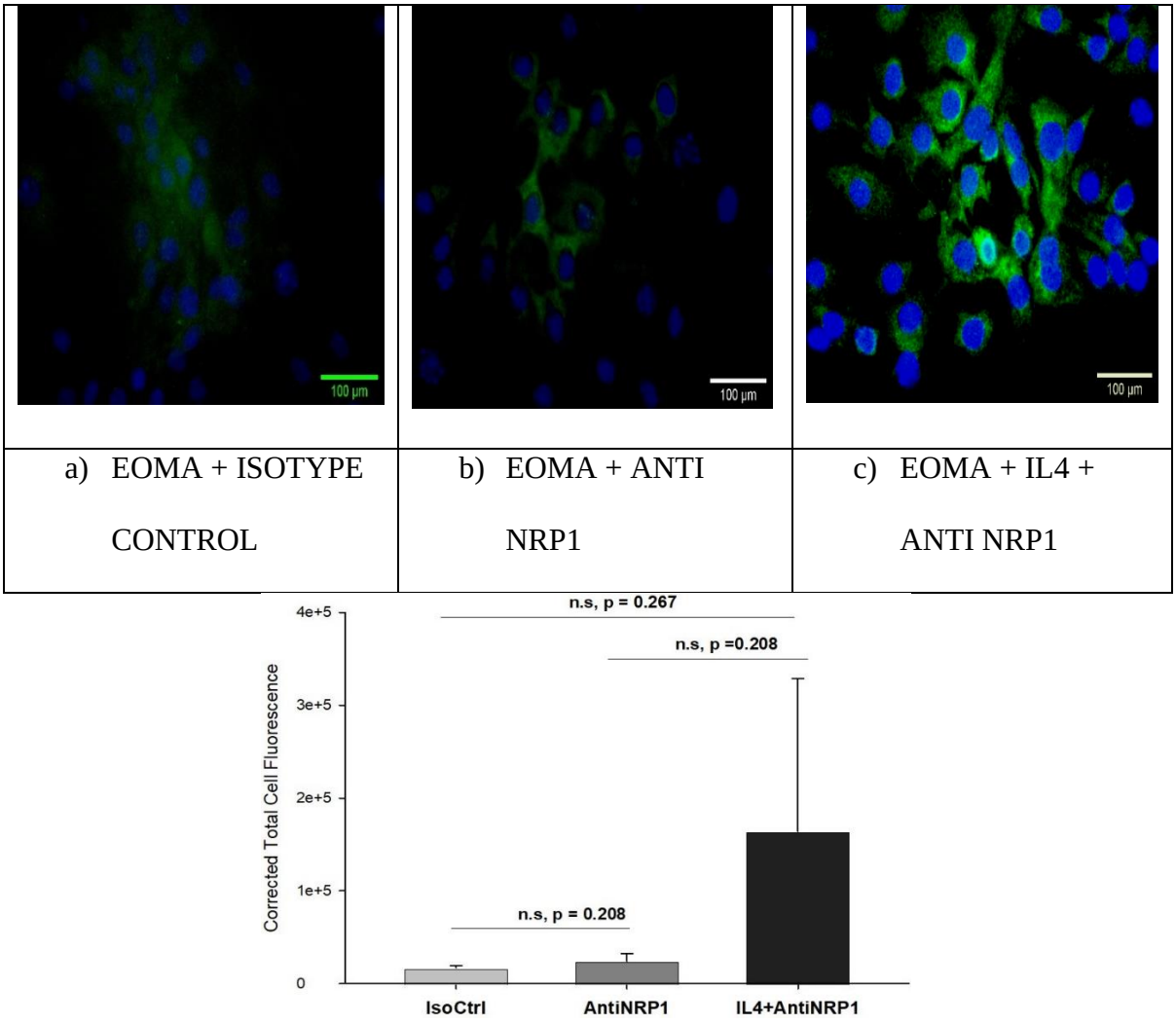
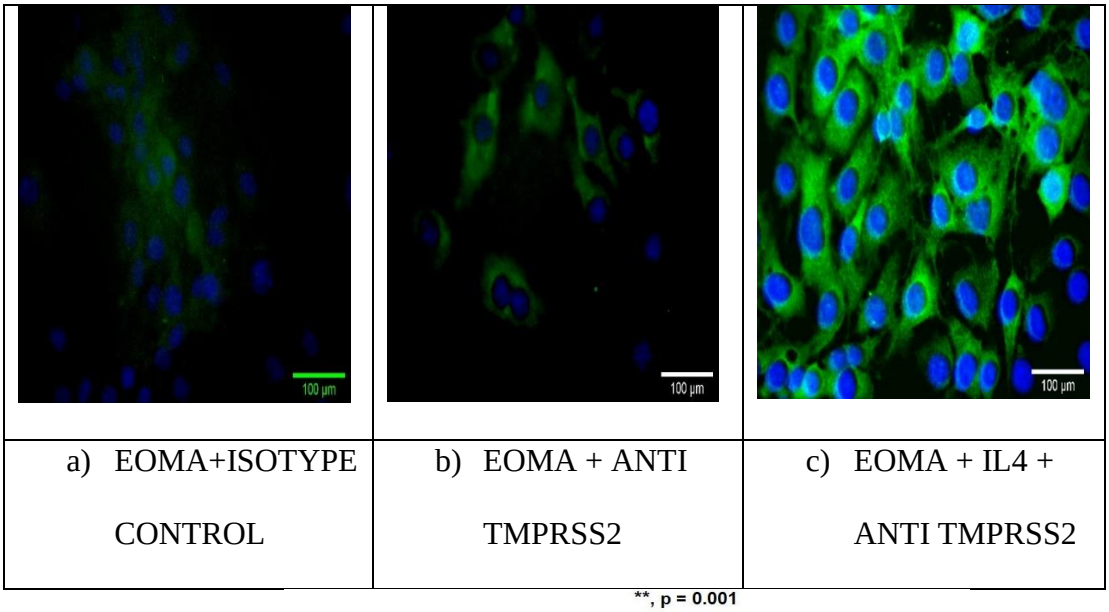


Figure-9: Immunofluorescence staining of EOMA cells expressing NRP1 receptor upon IL-4 treatment. Immunofluorescence images(500X) for EOMA cells untreated, stained with Isotype control (AntiIgG1) (a) stained with AntiNRP1 (b) and IL4 treated, stained with AntiNRP1 (c) overlayed with DAPI. Simple error bars plotted using CTCF values.



a) EOMA+ISOTYPE CONTROL	b) EOMA + ANTI TMPRSS2	c) EOMA + IL4 + ANTI TMPRSS2

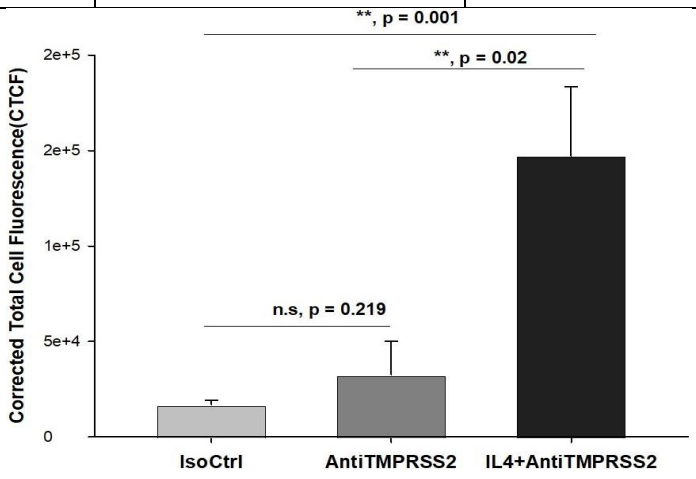


Figure- 10: Immunofluorescence staining of EOMA cells expressing NRP1 receptor upon IL-4 treatment. Immunofluorescence images(500X) for EOMA cells untreated, stained with Isotype control (AntiIgG1) (a) stained with AntiTMPRSS2 (b) and IL4 treated, stained with AntiTMPRSS2 (c) overlayed with DAPI. Simple error bars plotted using CTCF values.

TABLE-4: Statistical significance difference of ACE2, NRP1 AND TMPRSS2 immunofluorescence on EOMA cells- IL-4 treatment.

GROUPS	**p values**	**p< 0.05**
Iso Ctl vs Anti ACE2	0.030	YES
Anti ACE2 vs IL-4 + Anti ACE2	0.022	YES
Iso Ctl vs IL-4 + Anti ACE2	0.002	YES
Iso Ctl vs Anti NRP1	0.208	NO
Anti ACE2 vs IL-4 + Anti NRP1	0.208	NO
Iso Ctl vs IL-4 + Anti NRP1	0.267	NO
Iso Ctl vs Anti TMPRSS2	0.446	NO
Anti ACE2 vs IL-4 + Anti TMPRSS2	0.002	YES
Iso Ctl vs IL-4 + Anti TMPRSS2	0.001	YES

DISCUSSION

Through careful fine-tuning, various endothelial cells in the body keep coagulation, thrombus formation, and inflammation under check and adapted to the demands of the immediate environment. In COVID-19, there has been significant endothelial damage documented. We must comprehend how cytokines affect endothelial cells in relation to the virus given the rising infections, fatalities, and economical losses brought on by SARS-CoV-2. On the other hand, recent research has shown intriguing and even contradictory results about how cytokines may promote the expression of SARS-CoV-2 host receptors.

Despite the controversy on the expression of ACE2 receptor on human endothelial cells, the expression of ACE2 was detected on Hemangioendothelioma cells (EOMA) when stained with Anti ACE2 antibody in this study. As seen in Figure 5, EOMA cells exhibited an increase in the expression of ACE2 versus that seen in the isotype control ($p=0.040$). IFN-γ treatment of EOMA cells did not increase the expression of ACE 2 (0.185). This observation is similar to that of McCracken and associates who found low or no basal ACE2 expression in human endothelial cells compared with epithelial cells using single cell RNA sequencing. They examined endothelial cells from arterial, venous, and microvascular beds, and compared them with epithelial cells from respiratory, gastrointestinal, and skin sources. Out of the 100,579 ECs they analyzed, not even 1% (468 ECs) were $ACE2^{+}$ and only one ACE2 transcript was found in 424 of these samples.

Similar to the present study, they also failed to enhance expression of ACE2 on endothelial cells using in vitro exposure of endothelial cells to inflammatory cytokines reported as elevated in the plasma of patients with severe COVID-19. However, they unexpectedly found viral replication in pericytes, the cells that encircle the endothelial cells along the capillaries.

It is of interest that the anti-inflammatory cytokine, IL-4, induced a significant increase in ACE 2 expression of EOMA cells (Figure 8) compared with that seen for EOMA cells treated with the inflammatory IFN-γ. McCracken's group did not examine anti-inflammatory molecules on endothelial cells (McCracken et al., 2021).

In the present study, NRP1 and TMPRSS2 receptors were also expressed, albeit in lower concentrations, which was acceptable given their functions as a coreceptor and cofactor, respectively (Figures 6, 7, 9 and 10). Reports based on previous studies indicate that SARS-CoV-2 cannot replicate in primary human endothelial cells productively (McCracken et al., 2021 and Schimmel et al., 2021). Based on the tissue distribution of the SARS CoV 2 receptors, especially the ACE2 protein, the pathogenesis of the infection is self-explainable (Hamming et al., 2004). Even if the endothelial cells are not primarily attacked by the viral antigens, the balance between the homeostasis and inflammation is disturbed by the surrounding injured cells like the epithelial cells, macrophages, and others, which eventually affect the Th1/Th2 ratio. Studying the

expression of SARS CoV 2 receptors on EOMA cells treated with IFN ɤ and IL-4 is the main focus of the current investigation.

Upon treating the EOMA cells with the pro inflammatory cytokine, IFN ɤ, an increasing trend was observed in the expression of NRP1 and TMPRSS2 receptors (Figures 6 and 7) ($p<0.03$). But it failed to enhance the expression of ACE2 receptor (Figure 5), and this aligns with the study involving Human umbilical vein endothelial cells (HUVECs) failing to upregulate the expression of ACE2 when exposed to a combination of 4 proinflammatory cytokines (TNF-α, IL1-β, IL8, and IL6/IL6R chimeric protein) for 4 hours or 24 hours at 0, 0.01, 0.1, or 1.0 ng/mL (McCracken et al., 2021). These significant changes were assessed using the Sigma plot analysis (Table 4). The exposure of the anti-inflammatory cytokine, IL-4 did increase the expression of ACE2 and TMPRSS2 receptors on EOMA cells (Figures 8 and 10) ($p<0.03$). This significant enhancement was not observed on the NRP1 receptor (Figure 9 and Table 5).

SARS CoV 2 infection causes the release of proinflammatory cytokines, which are also responsible for platelet activation, leukocyte recruitment, and the secretion of other cytokines. Circulating endothelial cells (CECs) count, a biomarker of endothelial damage, was positively associated with an elevated concentration of inflammatory cytokines in the admitted patients in a study (Chioh et al., 2021). Varying to the hypothesis stated earlier, the current study with IFN ɤ enhancing the expression of NRP1 and TMPRSS2 receptors and IL 4 enhancing ACE2 and TMPRSS2 on EOMA cells

might explain why the EOMA cells are more susceptible to the SARS CoV 2 infection, leading to vascular complications (Vanessa et al., 2021). In addition to serving as a predictive biomarker for the course and severity of COVID-19, cytokine expression is known to have strong immunostimulatory properties. In another study, IFN ɤ was shown as a potential anti-tumor agent against Polyoma middle T (PmT)-transformed endothelial cells imitating vascular tumors (Dong et al., 1996). The difference between helpful and detrimental cytokines must be made, though.

Future research

With the different roles of endothelial cells, many factors, ligands, and receptors of the ECs are to be considered. From the foregoing discussion, it has become clear that endothelial activation is not only induced by cytokine production but also other factors like complement components (especially C4d and C5b-9) (Magro et al., 2020), vWF factor, factor VIII, Integrin αVβ3, VEGF and more. The underlying mechanism by which SARS-CoV-2 infects endothelium cells in vivo is yet unknown, however data show elevation of ACE2 with endothelial activation as a probable means of SARS-CoV-2 entry into endothelial cells (Liu et al., 2021). Furthermore, on NRP-1, VEGF-A and spike protein have a similar binding pocket (Moutal et al., 2021). Future studies should be directed to determine if the endothelial damage is worsened or relieved with these pro-inflammatory and anti-inflammatory cytokines in combination with other endothelial activating factors.

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