Investigating the Role of TIR Domain-Containing Adaptor Protein (Mal) in Cancer Progression

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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Investigating the Role of TIR Domain-Containing Adaptor Protein (Mal) in Cancer Progression

A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree
of
Master of Science

by
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INDIAN INSTITUTE OF TECHNOLOGY INDORE

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Investigating the Role of TIR Domain-Containing Adaptor Protein (MAL) in Cancer Progression"** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, IIT Indore**, is an authentic record of my own work carried out during the period from July 2024 to May 2025 under the supervision of Dr Mirza S. Baig, Professor, Department of Biosciences and Biomedical Engineering.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Rigzin Yangdol 23/05/25

Signature of the student with date

RIGZIN YANGDOL

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Signature of the Supervisor of M.Sc. thesis

Dr. Mirza S. Baig

RIGZIN YANGDOL has successfully given his/her M.Sc. Oral Examination held on **5th May 2025.**

Signature of Supervisor of MSc thesis
Date: 05/05/2025

Convener, DPGC
23-05-2025

I

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Rigzin Yangdol

Dedicated to My Parents & My Family

ABSTRACT

TIRAP (Toll/Interleukin-1 receptor domain-containing adaptor protein) is a key adaptor in the immune system that is associated with the activation of inflammatory responses through Toll-like receptors (TLRs). Recent research indicates that TIRAP plays a dual role—supporting immune defense while also promoting tumor growth and metastasis. Its role in cancer progression varies widely depending on its level of expression. Wherein either ends of under and overexpression is implicated to hold some relation to cancer. A multitude of factors can influence its level of expression and gaining better understanding of these factors and signaling pathways affecting TIRAP expression can contribute to advancements in terms of finding new drug targets and in designing new diagnostic and prognostics.

LIST OF PUBLICATIONS

Khandu Wadhonkar[#,1,] Yashi Singh[#,1], Aurelia Rughetti[2,] Soumalya Das[1], **Rigzin Yangdol 1**, M Hassan Sk[*,3,4], Mirza S Baig[*,1.] **Role of Cancer Cell-Derived Exosomal Glycoproteins in Macrophage Phenotypic Change. Molecular Biology Reports (2025)**

Sk Rameej Raja [1], Mobassar Hassan Sk [2,3], Syed Wajeed [4], **Rigzin Yangdol** [1], Ayushi Yadav [1], Himanshi Jindal [1], Arif Siddiquie [5], Ramachandran Subramanian [6,*], Mirza S Baig [1,*]**Macrophage Transcriptional Dynamics During Acute and Chronic Wound Repair. (Submitted)**

Rigzin Yangdol [1], Sk Rameej Raja, Faaiza Siddiqi, Ayushi, Shreya Bharti and Mirza S Baig [1,*] **TIRAP in Cancer**: A New Frontier**. (Under preparation)**

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NOMENCLATURE

ng	nanogram
ml	milliliter
µl	microliter
°C	degree centigrade
µM	micro-molar
mM	millimolar

ACRONYMS

DAMP	Damage-associated Molecular Pattern Molecules
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL-1β	Interleukin 1 β
IL-6	Interleukin 6
LPS	Lipopolysaccharide
NCCS	National Centre for Cell Science
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate buffer saline
TLR4	Toll-like Receptor
RMPI 1640	Roswell Park Memorial Institute 1640
TIRAP	TIR Domain-Containing Adaptor Protein
Mal	Myd88 Adaptor Like
PMA	Phorbol 12-myristate 13-acetate
TGF-β	Transforming Growth Factor

Chapter 1

Introduction

Inflammation is a response activated to restore an injury as well as a pathogenic response (Balkwill et al., 2005), but if inflammation is not regulated then it can become Chronic, ultimately manifesting into various ailments (Mantovani et al., 2008). As far back in 1863 Rudolf Virchow described the role of inflammation in the development of cancer (Antonucci & Karin, 2024; Korniluk et al., 2017). Various molecular targets as well as signaling pathways are common to both inflammatory response and carcinogenic process. (Akkız et al., 2025; Nakagawa, 2012; Ramakrishnan, 2024) This includes apoptosis angiogenesis and excessive proliferation rate. Apart from this, the use of NSAIDs has been shown to lower the incidence and mortality in patients of various cancers. (Walker et al., 2012; Wong, 2019)

TIR Domain-Containing Adaptor Protein (TIRAP) also known as the MyD88 Adaptor Like (Mal) being a mediator of the inflammatory pathway can directly influence tumorigenesis. (Liu et al., 2025; Rajpoot et al., 2021) Its role in cancer progression varies widely depending on its level of expression (Burkhard et al., 2019). Wherein either ends of under and overexpression is implicated to hold some relation to cancer. Inflammation-driven cancers (e.g., gastric, colorectal, liver) often have dysregulated TIRAP expression. As TIRAP can interact with multiple proteins making it an important target of study to find various interacting partners thus signalling pathways with possible roles in cancers.

Inflammatory bowel disease such as Chronic ulcerative colitis and Crohn's disease progression leading to colon cancer, Hepatitis C infections associated with liver carcinoma risk and Chronic H. Pylori infection being

the leading cause of gastric cancer are all cases of this dysregulation resulting in cancer. (Li, 2004). In all the three cases quoted, TIRAP is implicated in one way or the other.

TIRAP being a mediator of the inflammatory pathway can directly influence tumorigenesis. Its role in cancer progression varies widely depending on its level of expression.

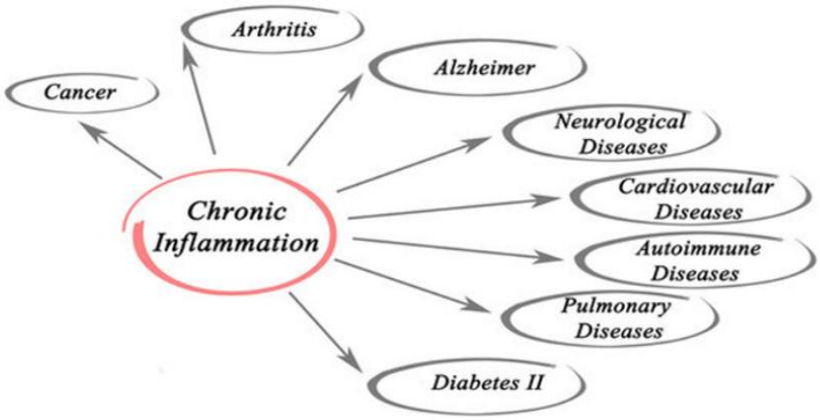

Figure 1.1: *Diagrammatic representation of various ailments resulting from Chronic Inflammation.* (*https://stock.adobe.com/*)

Generally, factors like epigenetics, genetic alterations and complex gene-gene interactions are responsible for its aberrant expression. Essentially in epithelial cells, TIRAP seems to be crucial in preserving barrier defenses as well as the initiation of inflammation, potentially protective. TIRAP deficiency results in a compromised intestinal epithelial barrier integrity increasing the susceptibility to bacterial infection via oral route (Corr et al., 2014) an increased mucosal and submucosal cell infiltration in gut lining suggesting that chronic inflammation under TIRAP-deficiency could be a predisposing factor for tumor development (Aviello et al., 2014). In a study done by Aviello et al. (2013) it was found that TIRAP deficiency led to mice

becoming susceptible to both spontaneous and inducible colon inflammation and cancer development.(Aviello et al., 2014) whereas in some cancers like breasts cancer (Zhang et al., 2024), non-small cell lung cancer (NSCLC) (X. Wang et al., 2020), pituitary neuroendocrine tumor (PitNET) (Vela-Patiño et al., 2024), TIRAP is upregulated. Thus, understanding the differential roles of TIRAP holds promise for developping targeted anticancer therapies. Modulation of TIRAP -associated pathways specifically in epithelial or immune cells may have therapeutic benefits where tumor growth could be abolished, but protective immunity is preserved.

1.1 Structural overview of (TIRAP)

TIRAP is constituted by 221 amino acids. Two of its key functional regions are phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain (PBD) (15-35 residues) at the N terminal and the TIR domain (84-213 residues) is located at the C-terminal (George et al., 2010; Lannoy et al., 2023; Rajpoot et al., 2021).

The former domain enables the recruitment of TIRAP to the plasma membrane upon production of $PIP_{2,}$ mediated by Phosphatidylinositol 4-Phosphate 5-Kinase (PIP5Kα) (Kagan & Medzhitov, 2006; Rajpoot et al., 2021) while the latter domain is responsible for interactions of TIRAP with other proteins (Lannoy et al., 2023; Rajpoot et al., 2021).

Toll/ interleukin (IL)-1 receptor (TIR) domain containing adaptor protein (TIRAP) is responsible for mediating intracellular signaling downstream of TLR 2 and 4 of the inflammatory pathway. TLR2/TLR4 and MyD88, being electropositively charged, fail to communicate directly with one another, making the presence of an electronegatively charged mediator a necessity. This is where TIRAP comes in, the TIR domain of TIRAP being highly electronegative acts as an effective channel of communication between the two (Antosz et al., 2013).

TIRAP is predominantly in its inactive form. It gets activated when tyrosine at position 86, 106, 159 and 187 get phosphorylated. Wherein phosphorylation at position 106 can be carried out either by Protein Kinase C-δ (PKCδ) or Bruton's Tyrosine Kinase (BTK). While the remaining three residues are phosphorylated by Bruton's Tyrosine Kinase (Rajpoot et al., 2021). Usually in the presence of a stimulus, for instance LPS TIRAP phosphorylation is induced (Antosz et al., 2013) TIRAP impacts innate immune responses, making it an important adaptor with roles in various diseases like cancer.

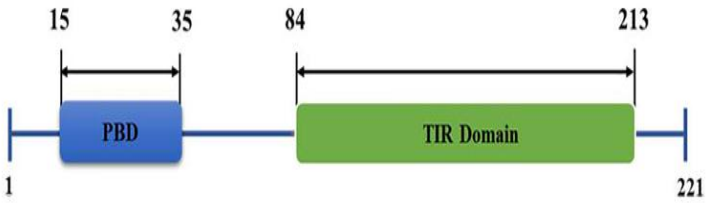

Figure 1.2: *Structural organization of Toll/interleukin 1 receptor (TIR) domain- containing adaptor protein (TIRAP) domains. The amino acid position of an N-terminal phosphatidylinositol (PI) binding domain (PBD) and a C-terminal Toll-like receptor (TIR) domain. Rajpoot et.al (2021)*

1.2 TIRAP Interactions with Other Proteins and involvement in cancer

TIRAP (Toll/Interleukin-1 receptor domain containing adaptor protein) interacts with several key signaling proteins that orchestrate innate immune responses and has emerging significance in cancer-related pathways. It primarily functions in the TLR2 and TLR4 signaling cascades by binding to the TIR domains of these receptors upon activation. This interaction facilitates the recruitment of MYD88 through TIR-TIR domain binding, forming a critical adaptor complex. The TIRAP-MyD88 complex then promotes the recruitment of and activation of IRAK family kinases,

including IRAK1 and IRAK4, which phosphorylate downstream targets and engage TRAF6, an E3 ubiquitin ligase. TRAF6 mediates polyubiquitination events that activate TAK1 and subsequently the IKK complex, leading to the leading to the activation of transcription factors such as NF-κB and AP1. These transcription factors induce the expression of pro-inflammatory cytokines, survival proteins, and factors involved in cell proliferation. TIRAP also binds phosphatidylinositol 4,5-bisphosphate (PIP2) through a phosphoinositide- binding motif, anchoring the adaptor complex to the plasma membrane and enabling signal propagation.

In the context of cancer, these protein interactions play a critical role in shaping the tumor microenvironment and promoting tumor progression. Persistent activation of the TIRAP-MyD88-IRAK-TRAF6 axis leads to chronic inflammation, a well-established driver of oncogenesis. The NF-κB and MAPK signaling pathways activated through these interactions enhance the expression of anti-apoptotic proteins (e.g., Bcl-2), pro-survival cytokines (e.g., IL-6, TNF-α), and cell cycle regulators, which collectively support cancer cell survival and proliferation.

Additionally, TIRAP-mediated signaling has been implicated in epithelial-mesenchymal transition (EMT), contributing to enhanced invasiveness and metastatic potential of cancer cells. These effects are further amplified through modulation of immune cell behaviour in the tumor microenvironment, particularly by influencing macrophage polarization toward a tumor-promoting phenotype. Elevated TIRAP expression or hyperactivation of its associated pathways has been observed in cancers such as breast, gastric, and liver cancer, where it often correlates with poor prognosis. Therefore, TIRAP’s interactions with proteins like TLR2/4, MyD88, IRAKs, and TRAF6 not only drive innate immunity but also play a significant role in cancer initiation and progression through sustained inflammatory signaling and immune modulation.

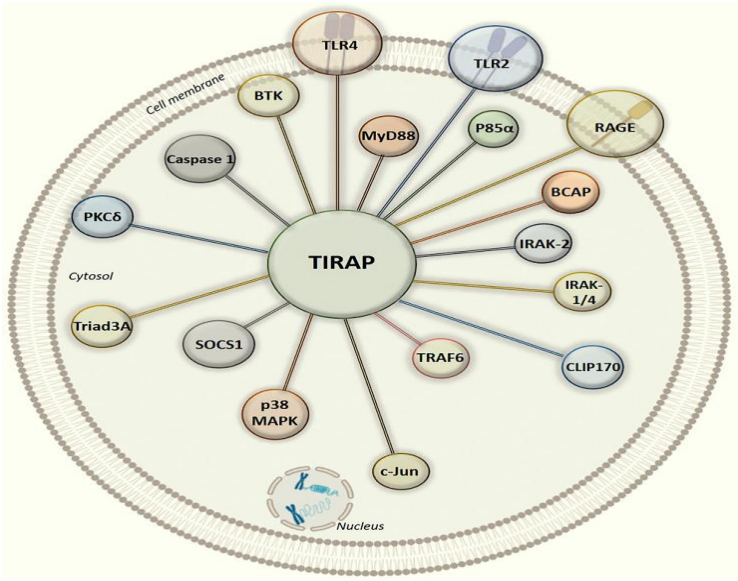

Figure 1.3: *Structural organization of Toll/interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP) domains. Rajpoot et.al (2021)*

1.3 Objective of the thesis

The main objective of this research is to explore the role of the adaptor protein TIRAP in cancers. As the role of this protein is well established in inflammation, its activity is responsible for causing a proinflammatory immune response. This study delves into the mechanism of as to how differential expressions in different cancers aids in cancer progression.

1. To assess the basal expression of TIRAP in different cancer cell lines based on availability and prominence of that cancer type, qRT-PCR was carried out.
2. To assess its expression in response to the secretome from the macrophages, an indirect co-culture method was employed.
3. To check the macrophage activation and downstream cytokines in whose response the TIRAP in cancer cells gets altered.

4. To find out the interacting partners of TIRAP which in response to the secretome from macrophages which could possibly aid in cancer progression.

1.4 Thesis Organization

The present work has been summarized in 5 chapters to gain insight into the current thesis:

1. **Chapter 1** serves as the inception of the thesis, delving into highlighting the relationship between inflammation and cancer. The commonality in terms of the mechanism and signaling pathways and molecules.
2. **Chapter 2** undertakes an exhaustive review of literature, meticulously examining studies aligned with the primary objectives of the research.
3. **Chapter 3** delineates the materials and methods employed in the present study, elucidating the diverse array of techniques utilized to address the research objectives. This chapter encompasses a comprehensive discussion on various methodologies, ranging from primary cell culture of macrophages and various cancer cells to experimental techniques such as real-time PCR, western blotting and immunofluorescence assays.
4. **Chapter 4** serves to present the outcomes of the experimental approaches employed in this study.
5. **Chapter 5** concludes the thesis, emphasizing the potential of the adapter protein TIRAP as a molecular target in the treatment of cancer.

Chapter 2

LITERATURE REVIEW

The aberrant expression of TIRAP in cancers highlights its role in the immune response associated with these tumors potentially influence their behavior and interaction with the immune microenvironment. For instance, TIRAP is upregulated in pituitary neuroendocrine tumor (PitNET) (Vela-Patiño et al., 2024), and non-small cell lung cancer (X. Wang et al., 2020). There exists a multitude of reasons for altered TIRAP expression in tumors.

Epigenetic modifications (including DNA methylation of TIRAP and histone modifications), chromosomal aberrations majorly resulting due to deletions and rearrangement of TIRAP or its regulatory machinery and lastly based on different polymorphisms of TIRAP determines the susceptibility of an individual, disease progression and patient survival rates. For instance, variants pR81C and D96N are inherited lymphoma risk variants whereas variants rs611953 and rs625413 show worse clinical outcome in case of colorectal cancer (Klimosch et al., 2013). Carcinogenesis is regulated by different genes along with various signaling pathways and their crosstalk and abnormal epigenetic modification therefore is an important factor impacting the pathogenesis. In cryptogenic hepatocellular carcinoma (CR-HCC) which is a subset of hepatocellular carcinoma (HCC) TIRAP is among one of the genes that is hypomethylated in CR-HCC tissues compared to the adjacent peritumor tissues (APTs) (X. Wang et al., 2020). This dysregulation could contribute to tumor progression by promoting pro-inflammatory tumor microenvironment or enhancing oncogenic signaling. In lung Cancer during the early stages of the transformation there is an epigenetic silencing of the TIRAP gene due to chromatin remodeling by the addition of methyl group at the 27^{th} lysine residue (H3K27me3) followed by subsequent DNA methylation by DNMT3b (a de novo methyltransferase) and upon re-expression of TIRAP

a significant reduction in the tumor growth xenograft models can be observed (Teneng et al., 2015; Yamagishi et al., 2024). Henceforth, the abnormal expression of TIRAP in cancers highlights its role in tumor-associated immune responses, potentially influencing tumor behavior and interactions within the immune microenvironment.

Various biomolecules can directly or indirectly impact their expression. For instance, in inflammation specially associated with cancer, the extracellular S100A8/A9 (Calcium binding proteins) (S. Wang et al., 2018) produced by infiltrating neutrophils are said to promote increased cancer cell survival along with its invasive motility in bladder cancer upon interaction with the TLR4 receptor. This interaction results in enhanced tumor progression locus 2 (TPL2) activation via TIRAP. Activated tubby like protein 2 (TLP2) facilitates the activation of mitogen activated protein kinases (MAPK) signaling pathway of TPL2 promoting tumor growth, migration and invasion (Herik Rodrigo et al., 2022).

Other than that carotenoid pigment from brown algae and phycobiliprotein pigments from cyanobacteria and red algae are reported to downregulate the expression of TIRAP in triple negative breast cancer and NSCLC cells respectively (Hao et al., 2019). Apart from biomolecules various other factors like epigenetics, genetic alterations and complex gene-gene interactions also stand responsible for aberrant expression of TIRAP. (Lannoy et al., 2023). Cancer progression is reported to be impacted in the patients of gastric cancer (Castaño-Rodríguez et al., 2014), lymphoma (Burkhard et al., 2019), cervical cancer (Bekampytė et al., 2022) myeloma, upon being carriers of variants of TIRAP. The TIRAP mediated NF κB activation is well established in macrophages (Rajpoot et al., 2021) where the extracellular domains of the TLRs present on the membrane recognize their respective stimulant for instance peptidoglycan (TLR2), flagella (TLR5), lipopolysaccharide (LPS) (TLR4) respectively, (Castaño-Rodríguez et al., 2014) while their cytoplasmic domains via its TIR

domains, recruit several adaptor molecules including MyD88, TIRAP/TIRAP, and TRIF/TICAM, which subsequently lead to diverse downstream signaling. The specificities of downstream signaling activations by distinct TLRs may be determined by differential recruitment of these adaptors (Li, 2004).

Table 2: *Various reported cases of TIRAP gene expression across different cancers from patient samples.*

Upregulation	Downregulation
Breast Cancer	Acute Leukemia
Cutaneous T Cell Lymphoma	Pancreatic Cancer
Pituitary Neuroendocrine Tumor	Endometrial Cancer
Lung Cancer	Diffused Large B Cell Lymphoma
Myeloid Leukemia	Colitis Derived Colorectal Cancer
Gastric Cancer	Clear Cell Renal Cell Carcinoma
Hepatocellular Carcinoma	

The ligand receptor interaction between recruit's adaptor kinases and transduce downstream signaling leading to the activation of the transcription factor NF-kB, AP1(activator protein 1), p38 and ERK (extracellular signal-regulated kinase) MAPK (mitogen-activated protein kinase) pathways. These pathways work in concert to regulate the expression of a wide array of genes, including those involved in the production of cytokines and other immune mediators essential for orchestrating immune responses (Antosz et al., 2013). Following the activation and engagement of TLR 4 at plasma membrane it is endocytosed causing depletion of PIP2 from the membrane. This results in dissociation

of TLR4 from TIRAP-MyD88 complex marking the completion of this signaling (Lannoy et al., 2023). NF-kB signaling via TLR4-TIRAP axis has been implicated as a link connecting inflammation with the progression of cancer.

Despite growing evidence that TIRAP plays a critical role in cancer-related immune signaling, several key gaps remain. Incomplete Understanding of TIRAP Regulation. While studies have shown that biomolecules (e.g., S100A8/A9, carotenoid pigments) and factors like epigenetic changes influence TIRAP expression, the full range of upstream regulatory mechanisms remains poorly characterized. The contribution of gene-gene and gene-environment interactions to aberrant TIRAP expression in cancer is underexplored. Limited Functional Studies in Diverse Tumor Types: Although TIRAP overexpression has been observed in cancers like PitNET and NSCLC, functional studies delineating its tumor-promoting versus tumor-suppressive roles across different tumor types are scarce. Insufficient In Vivo Evidence, most findings are derived from in vitro or correlative studies. There is a lack of robust in vivo models assessing the causal role of TIRAP in tumor initiation, progression, metastasis, or immune evasion. Poorly Defined Mechanisms Linking TIRAP to EMT and Metastasis: While TIRAP activation via TLR4 has been associated with MAPK pathway engagement and tumor invasion, the downstream effectors that drive processes like EMT remain largely undefined. Unexplored Therapeutic Potential: Compounds like carotenoids and phycobiliproteins can downregulate TIRAP, but the translational significance of targeting TIRAP for therapeutic purposes remains speculative and unvalidated.

Overall, TIRAP stands at the intersection of inflammation and cancer, yet critical gaps remain in understanding its precise molecular functions, regulatory mechanisms, and therapeutic potential in oncology.

Chapter 3

MATERIALS AND METHODS

3.1 Materials

Cell lines A549 (Lung cancer), DU145 (Prostrate cancer cell line), MCF-7 (Breast cancer cell line), A375 (Melanoma cell line), Colo205 (Colorectal cancer cell line), THP1 (macrophage) and HEK (Kidney cell line) were obtained from National Centre for Cell Science (NCCS).

Dulbecco's modified Eagle's medium (MP Biomedicals), Roswell Park Memorial Institute medium (Gibco), Ref- 11875-119, Trypsin- EDTA solution 1X (Himedia), Phosphate Buffered Saline, Sodium chloride (Himedia), Tri-Buffer (Himedia), Sodium dodecyl sulfate (MP Biomedicals), Bromophenol blue (Himedia), Glycine (Himedia), Protease Inhibitor, β-mercaptoethanol, Tetramethylethylenediamine, (SRL), Triton-X-100 (SRL). Primers (IDT), Antibodies – anti-TIRAP (sc-166149; Santa Cruz Biotechnology, CA, USA) and anti-phospho-TIRAP (Santa Cruz), tris-buffered saline with tween (TBST), Alexa Fluor 594 Chicken anti-rabbit (A21441; Invitrogen, CA, USA) and Alexa Fluor 488 Goat anti-mouse (A11001; Invitrogen), DAPI (F6057; Sigma), RNAiso Plus reagent (9109; Takara Bio Inc., Shiga, Japan), Prime Script Takara kit (catalog no - 6110A). Pen Strep (Gibco by Life Technologies), Ref- 15140-122, Fetal Bovine Serum, Cat. No: A5256701 HSN No: 30021290 (Thermo Fisher), Power Up™ SYBR™ Green Master Mix for qPCR, Catalog number A25777.

Biosafety cabinet (CESCO), CO_2 incubator (CESCO), Inverted microscope, Centrifuge (Thermo Fisher Scientific), Hemocytometer, Quantitative Real-Time PCR (Thermo Fisher Scientific), Nanodrop, Microplate reader, Micropipette, T-75 flask, 100 mm flask, 60 mm plate, 35 mm plate, 96 well plate, Multichannel pipette, Scrapper, Gel casting unit

(Invitrogen), Electrophoresis system (Invitrogen), Vortex, Gel documentation system, Refrigerator, Autoclave, Ice Flaker, Oven, Ultrasonicator, Water bath.

Table 3.1: *List of Primers set used for RT-PCR.*

S.No.	Gene target	Sequence (5'-3')
1.	GAPDH (F)	AATCCCATCACCATCTTCCA
2.	GAPDH (R)	TGGACTCCACGACGTACTCA
3.	IL-1β (F)	ACCACTACAGCAAGGGCTTC
4.	IL-1β (R)	TCGTGCACATAAGCCTCGTT
5.	IL-6 (F)	TGCAATAACCACCCCTGACC
6.	IL-6 (R)	GTGCCCATGCTACATTTGCC
7.	TGF β (F)	CAAGCAGAGTACACACAGCAT
8.	TGFβ (R)	TGCTCCACTTTTAACTTGAGCC
9.	TIRAP (F)	GCGCAGGCCTTACATAGGAA
10.	TIRAP (R)	AGACCAGGCATTGGTGAAGG
11.	FIBRONECTIN (F)	ACAACGTCATAGTGGAGGCA
12.	FIBRONECTIN (R)	CATCCGTAGGTTGGTTCAAGT
13.	SNAIL 1 (F)	ACCCCAATCGGAAGCCTAAC
14.	SNAIL 1 (R)	TCCCAGATGAGCATTGGCAG
15.	VIMENTIN (F)	CGGGGTTATAAAAACAGCGC
16.	VIMENTIN (R)	GGTGGACGTAGTCAGGTAGC
17.	E-CADHERIN (F)	TCGAGGAGGAAATTCCAATG
18.	E-CADHERIN (R)	ACACACGTGCACCTCATCAT

Table 3.2: *List of Antibodies used for Immunofluorescence.*

S.No.	Antibody	Cat.No.	Dilution used for immunofluorescence
1.	*p-TIRAP*	*BS-756R*	*(1:200)*
2.	*TIRAP*	*13077S*	*(1:200)*
3.	*Anti-Mouse HRP*	*13077S*	*(1:1000)*
4.	*Anti-Rabbit-AF594*	*A11012*	*(1:1000)*

3.2 Methodology

3.2.1 Cell culture: Cell lines A549 (Lung cancer cell line), DU145 (Prostrate cancer cell line), MCF-7 (Breast cancer cell line), and A375 (Melanoma cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37ºC. Colo205 (Colorectal cancer cell line) and THP1 (macrophage) were cultured in RPMI media supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37ºC. The medium was renewed every 2-3 days during the experiment in all cases.

Differentiation of Human Monocytic Cell Line (THP1): THP1 Cells were seeded into 60 mm plate to it 20 ng/ml of PMA (Phorbol 12-myristate 13-acetate) was added and incubated at 37 ºC, 5% CO_2, and 95% humidity for 24 Hrs. Following which for the next 24 Hrs. the cells were kept in PMA free media. Following which respective treatments can be given.

Condition Media Generation and Treatment:

Condition media from Cells: Each of the cells were cultured respectively and the media from the cells were collected, centrifuged at 2900 g for a period of 10 minutes following which the media was filtered using a 0.2 µm filter. The collected media was stored at -80 ˚C until use.

Generation of Condition Media from Pre-Treated THP1: To the differentiated THP1 added Condition Media collected from the respective cancer cells. Ratio of Condition Media: Fresh Media taken was 1:1. The Incubation time was 24 Hrs. at 37 ºC in 95 % humidity and 5% CO_2. Following which the media from the THP1 cells were collected, centrifuged at 2900 g for a period of 10 minutes following which the media was filtered using a 0.2 µm filter. The collected media was stored at -80 ˚C until use.

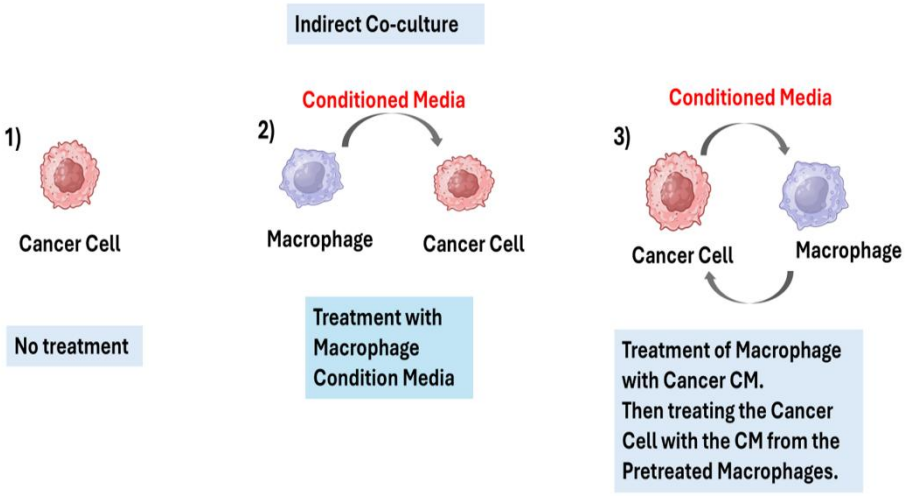

Figure 3.1: *Diagrammatic representation of indirect co-culture, generation of Condition Media followed by the treatment of the respective cells.*

3.2.2 RNA Isolation and cDNA synthesis: When the respective cells attained 80% confluency the supernatant media was removed from the culture plate. The cells were washed with phosphate-buffered saline (PBS,

pH 7.4) to remove the extra media. 400 µl of RNAiso Plus reagent (9109; Takara Bio Inc., Shiga, Japan) was added to the culture plate and stored at -80ºC. Following this, the cells were pulverized by pipetting after which the entire content was collected in an MCT. To it added equal volume of chloroform, and vortexed for 15 seconds and centrifuged at 13000 rpm for 30 minutes at 4ºC. The supernatant was collected and added 200 µl isopropanol. Mixed it gently and centrifuged at 13000 rpm for 30 minutes at 4 ºC. Discarded the pellet and the obtained pellet was washed with 75% ethanol at 13000 rpm for 30 minutes at 4 ºC. Decanted the ethanol, dried the pelleted, and resuspended it in nuclease-free water. RNA quantification was done using a nanodrop. cDNA was synthesized using Prime Script Takara kit (catalog no -6110A) according to the manufacturer's instructions.

Quantitative reverse transcriptase PCR: The total RNA isolation and cDNA synthesis was carried out according to the above-mentioned protocol. The real-time quantitative PCR (qPCR) was performed using SYBR green master mix (A25742; Applied Biosystems, MA, USA) in Step One Plus Real Time PCR Systems (Applied Biosystems). PCR amplification of two genes TIRAP and GAPDH was done. The cycle threshold value was analyzed using the 2-Ct method for the relative cytokine expression, wherein GAPDH was considered as a reference gene.

3.2.3 Immunofluorescence: 5 lakh THP1 Cells were seeded into 6 well plate containing sterile coverslips and 20 ng/ml of PMA (Phorbol 12-myristate 13-acetate) was added accordingly followed by incubation at 37 ºC, 5% CO_2, and 95% humidity for 24 Hrs. For the next 24 Hrs. the cell was kept in PMA free media. Then the cells were treated with different treatment conditions for 24 Hrs. Prior to fixation, cells were washed twice with phosphate-buffered saline (PBS; pH 7.4) at room temperature (RT), then washed three times with PBS under gentle shaking. The cells were then fixed with freshly prepared 4% paraformaldehyde (pH 7.2) in PBS for 15 minutes at RT. Following fixation, cells were permeabilized with 0.01%

Triton-X in 1X PBS for 10 minutes at RT and then washed three times with PBS under gentle shaking. Blocking was performed with freshly prepared 5% BSA (Bovine serum albumin) for 1 hour at RT with gentle shaking. After blocking, cells were washed three times in tris-buffered saline with tween (TBST), each wash lasting 5 minutes. For immunostaining, the primary antibodies-anti-TIRAP (sc-166149; Santa Cruz Biotechnology, CA, USA) and anti-phospho-TIRAP (Santa Cruz) were applied at a dilution of 1:200 in TBST buffer and incubated at 4°C overnight. After incubation, excess antibody was removed by washing three times in TBST buffer with gentle shaking. Fluorescently labeled secondary antibodies, Alexa Fluor 594 Chicken anti-rabbit (A21441; Invitrogen, CA, USA) and Alexa Fluor 488 Goat anti-mouse (A11001; Invitrogen), were applied at a 1:500 dilution in TBST buffer and incubated at RT for 1 hour in the dark. Excess secondary antibodies were removed by washing three times in TBST buffer for 5 minutes each. Cells were counterstained with DAPI (F6057; Sigma) for nuclear visualization and then mounted on coverslips. Imaging was performed using a confocal laser scanning microscope (Olympus, Tokyo, Japan), analysis was conducted using ImageJ and graphs plotted using Graph pad prism. In case of cancer cells each step remains the same except PMA treatment isn’t done.

Chapter 4

RESULTS AND DISCUSSION

1. **No significant change in basal level of TIRAP expression in different cancer cells**:

TIRAP is an adaptor protein essential for signaling through Toll-like receptors (TLRs), particularly TLR2 and TLR4. These receptors are involved in innate immune responses and have been implicated in cancer progression by promoting inflammation and cell proliferation. Although reported in literature to show an aberrant expression in different cancers initially to validate the literature based on availability of the cell line and prominence of occurrence upon comparing TIRAP expression levels in cancer and non-cancer cells, via RT-PCR, it was observed that compared with the non-cancerous cell line HEK, A375 (skin cancer), Du145 (prostate cancer) and Colo205 (colorectal cancer) showed a non-significant decrease. Whereas in cases of A549 and MCF7 there was a 0.5-fold decrease.

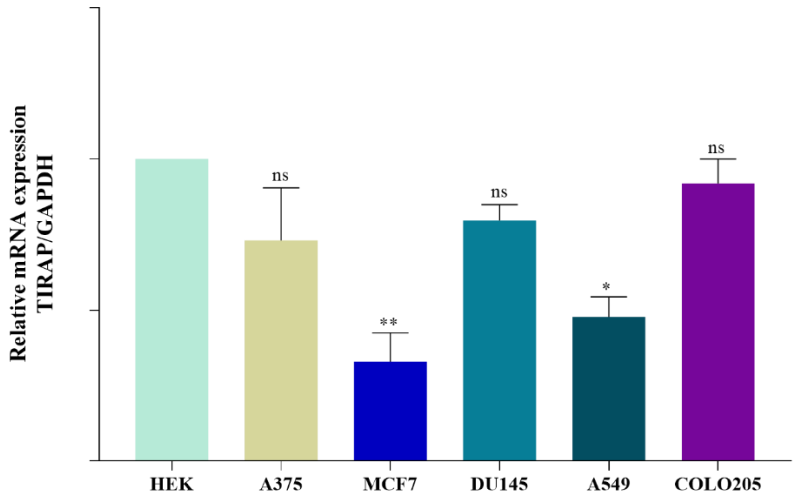

Figure 4.1: *The mRNA expression levels of TIRAP in Different Cancer Cell lines.*

The fold change was calculated with respect to HEK, a non-cancerous cell to draw a comparative difference in the level of TIRAP expression with respect to other cancer cell lines. Based on data obtained from two biological repeats, error bars were calculated. As significant difference in terms of expression of TIRAP wasn't observed and due to variance from the data in the literature we proceeded to check its expression in response to external stimulus.

2. **Varied TIRAP expression in different cancers in response to macrophage secretome from the macrophage preconditioned with cancer condition media:**

As macrophages are the major population of immune cells present in the tumor microenvironment, said to support the tumor at site, therefore considering a definite cell to cell communication occurring that might alter the status of TIRAP in the cancer cell thus, the TIRAP expression in cancer cells upon treatment with the secretome from the macrophages was observed. Considering the factors released by it could play a major role in stimulating signaling mechanisms affecting cancer progression. Upon treatment with the condition media from THP1 the TIRAP expression in the cancer cells increased in cancer cells but upon treatment with the condition media pretreated with the respective cancer cells the TIRAP expression decreased by nearly 1-fold, but in case of MCF7 no decrease was observed. Whereas no significant difference was observed in case of HEK cells. The RT-PCR data corresponded with the immunofluorescence data.

The increase in TIRAP expression in cancer cells treated with THP1 conditioned media indicates that factors secreted by THP1 cells can stimulate TIRAP expression. This suggests a potential role for immune cell-derived signals in modulating TIRAP levels in cancer cells.

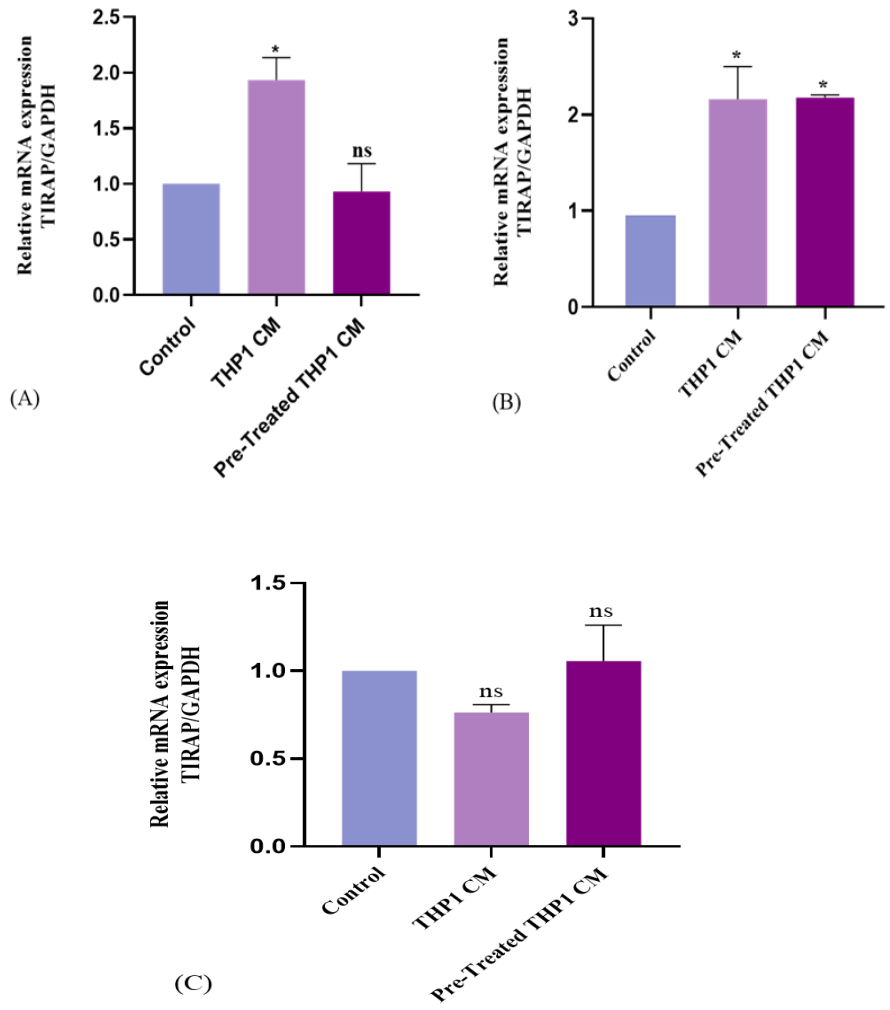

Figure 4.2A: *The mRNA expression level of TIRAP in Colo205 cell line in response to treatment with conditioned media from THP1 and pretreated THP1 conditioned media.* ***4.2B:*** *The mRNA expression level of TIRAP in MCF7 cell line in response to treatment with conditioned media from THP1 and pretreated THP1 conditioned media.* ***4.2C:*** *The mRNA expression level of TIRAP in HEK cell line in response to treatment with conditioned media from THP1 and pretreated THP1 conditioned media.*

The decrease in TIRAP expression in Colo205 upon treatment with conditioned media from the pretreated THP1 Cells implies that cancer cells

can modify the conditioned media in a way that suppresses TIRAP induction. This may either be through the depletion or modification of certain factors contained within the conditioned media that are responsible for TIRAP upregulation. Where the persistent upregulation in case of MCF7 upon treatment with conditioned media from the pretreated THP1 Cells implies that cancer cells can also modify the conditioned media in a way that upregulate TIRAP induction possibly through modification of factors that are responsible for TIRAP downregulation.

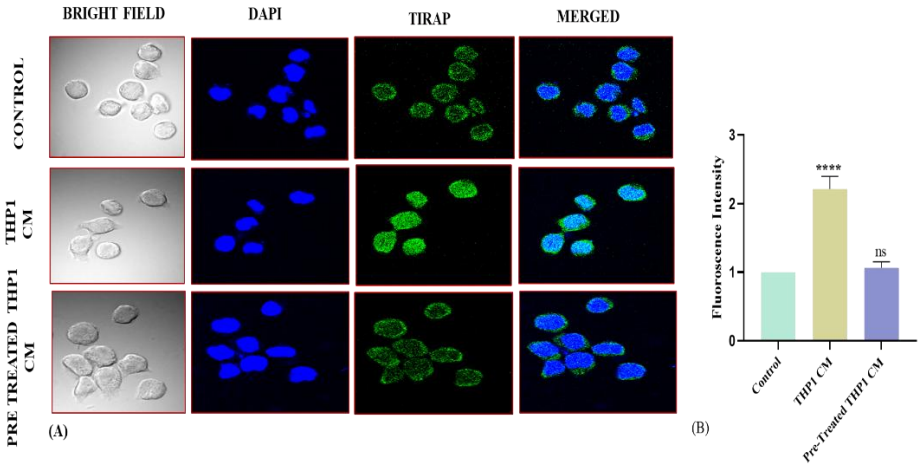

Figure 4.3A: *TIRAP Expression in Colo205 cell line treated with THP1 Conditioned media and pretreated conditioned media for 24 hours. The images were taken under confocal microscope at 100X magnification.* ***4.3B:*** *Quantification of figure* ***4.3A***.

The absence of differences in TIRAP expression in HEK under the same conditions indicates that the effects observed are cancer cells specifically. This can be attributed to variations in the signaling mechanisms or receptor presence in cancer and non-cancer cells. The observations point toward an active interaction between cancer and immune cells. This interaction can determine the expression of immune signaling proteins like TIRAP, which might impact the tumor microenvironment

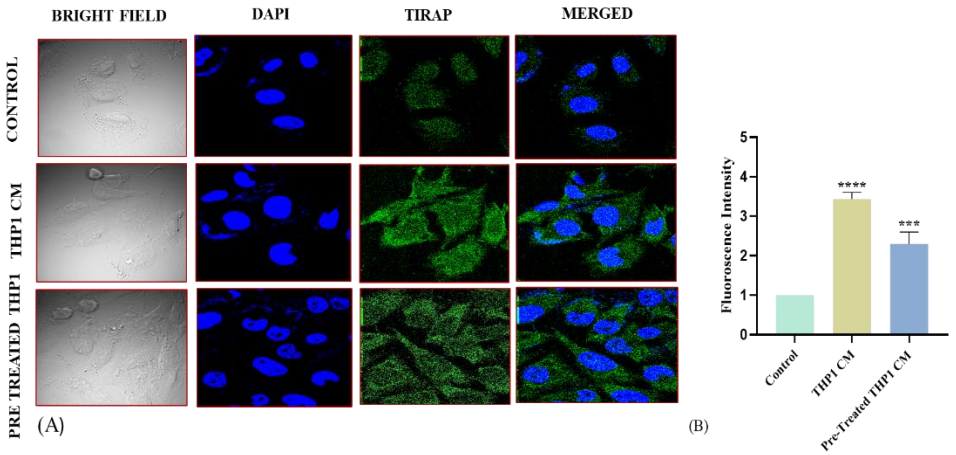

Figure 4.4A: *TIRAP Expression in MCF-7 cell line treated with THP1 Conditioned media and pretreated conditioned media for 24 hours. The images were taken under confocal microscope at 100 X magnification.* ***4.4B:*** *Quantification of figure* ***4.4A****.*

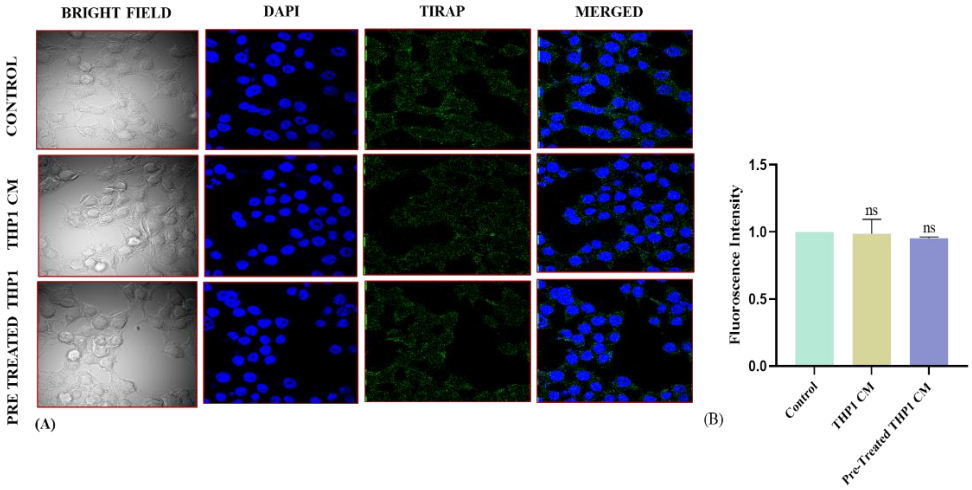

Figure 4.5A: *TIRAP Expression in HEK cell line treated with THP1 Conditioned media and pretreated conditioned media for 24 hours. The images were taken under confocal microscope at 100X magnification.* ***4.5B:*** *Quantification of figure* ***4.5A****.*

3. Cytokines Downstream to THP1 in Response to treatment with Colo205 and MCF7 Condition Media

To assess which are the possibly major cytokines impacting the TIRAP expression present in the condition media of the macrophages various cytokines expression were checked in the macrophages in response to treatment with the cancer condition media through qRT-PCR. Wherein it was observed that three cytokines, IL-1β, IL-6 and TGF-β showed marked differences.

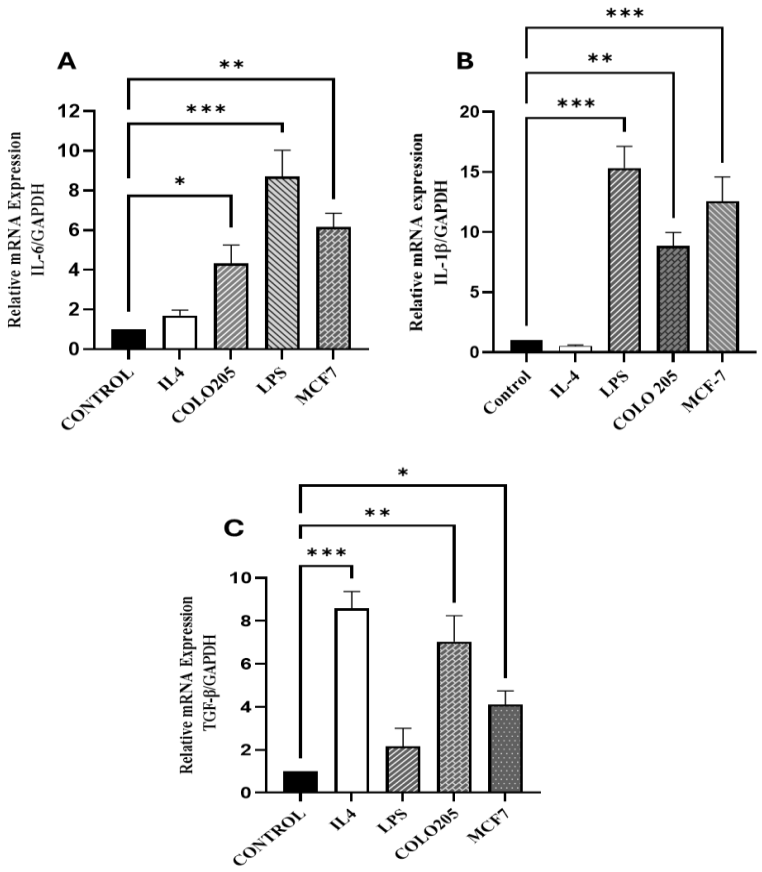

Figure 4.6: *Cytokines Downstream to THP1 in response to treatment with Colo205 and MCF7 Condition Media.* ***4.6A:*** *IL-6,* ***4.6B:*** *IL-1β and* ***4.6C:*** *TGF β.*

In response to MCF7 condition media proinflammatory markers like IL-6 and IL-1β were comparatively increased in THP1 indicating that these are

the major cytokine influencing TIRAP mediated signalling in MCF7. As ultimately this media from this MCF7 treated THP1 media will be rich in these cytokines as well. In case of colo205 cytokines like TGF-β seems to play a major role.

4. **No change in total TIRAP in THP1 cell line at protein level in response to conditioned media from respective cancer:**

Interested to see whether in the cancer condition media can alter the TIRAP expression in macrophages or not, as well to check the status of TIRAP that is an upstream molecule to the cytokines first qRT-PCR was done. This was followed by immunofluorescence for the same. There was an increase in case of MCF7 at mRNA level, but protein levels don't show any significant changes.

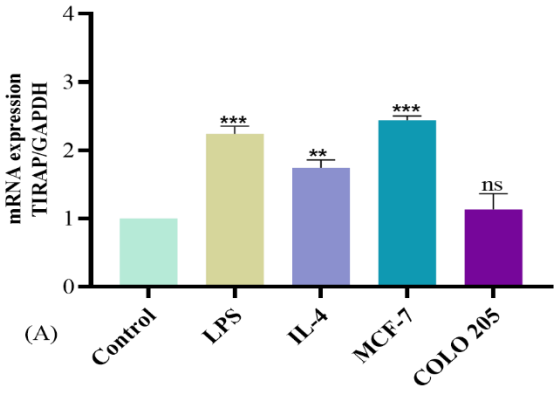

Figure 4.7A: *mRNA Expression of total TIRAP in THP1 in response to different condition media.*

The lack of change in TIRAP levels in macrophages suggests that TIRAP expression is differentially regulated in macrophages compared to cancer cells. This differential regulation could be due to differences in signaling pathways or the presence of specific transcriptional regulators in each cell type. The fact that macrophages can influence TIRAP expression in cancer cells without altering their own TIRAP levels suggests that macrophages

exert indirect effects. This could involve the secretion of soluble factors by macrophages that act on cancer cells to modulate TIRAP expression.

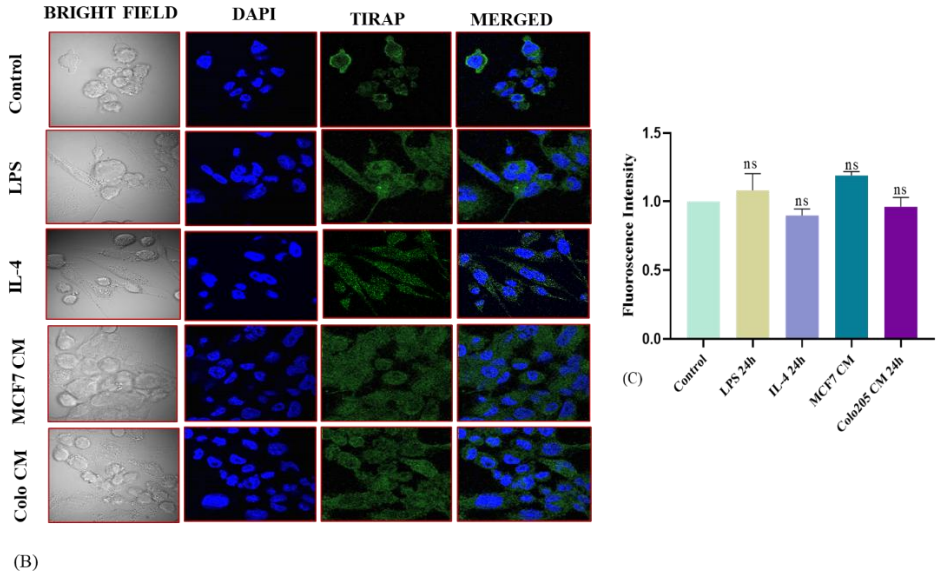

Figure 4.7B: *TIRAP Expression in THP1 cell line treated with LPS, IL4, COLO205 CM, and MCF7 CM for 24 hours. The images were taken under confocal microscope at 100X magnification.* ***4.7C:*** *Quantification of Figure* ***4.7B***

5. Alteration only at the phosphorylation level in THP1 Cell Line in Response to Conditioned Media from COLO205:

As no change was observed in case of macrophage total TIRAP expression at the protein level, in response to treatment with different stimuli. So, we decided to check the phosphorylation status of TIRAP instead. It was observed that there was difference in terms of activation of TIRAP. The fact that TIRAP activation varies despite no change in total expression levels suggests that phosphorylation plays a crucial role in regulating TIRAP activity.

Phosphorylation can modulate TIRAP'S ability to interact with other signaling partners or affect its localization and stability. This finding highlights that functional regulation of proteins like TIRAP can occur independently of changes in their expression's levels. Phosphorylation can activate or inhibit proteins, thereby influencing downstream signaling pathways without altering protein abundance.

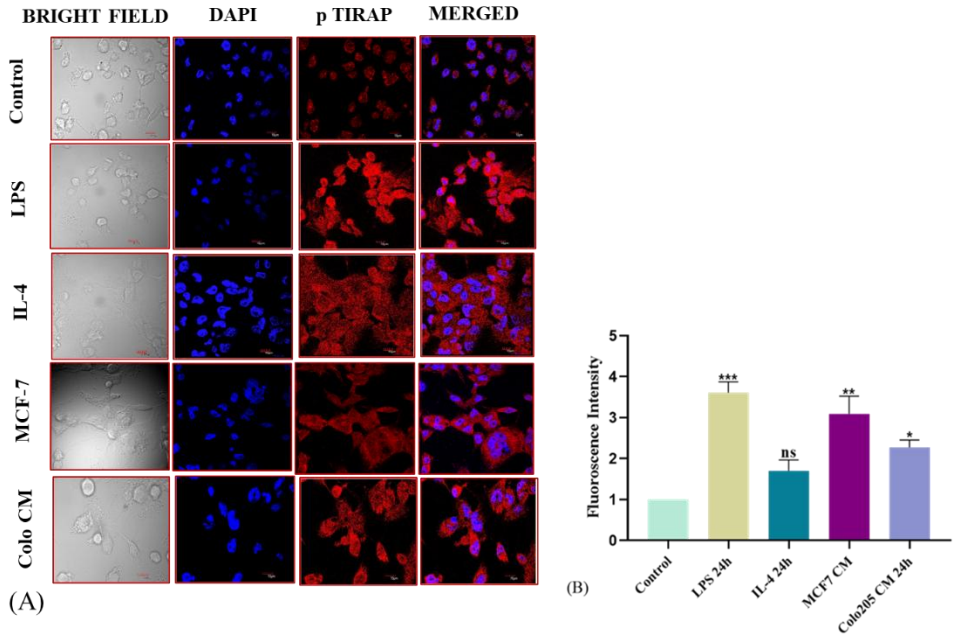

Figure 4.8A: *p-TIRAP Expression in THP1 cell line treated with LPS, IL4, COLO205 CM and MCF7 CM for 24 hours. The images were taken under confocal microscope at 100X magnification.* ***4.8B:*** *Quantification of figure* ***4.8A****.*

6. **Increase in the Epithelial to Mesenchymal Transition Marker in cancers in response to macrophage secretome from the macrophage preconditioned with cancer condition media:**

Simultaneously under the various treatment with the different condition media from the macrophages the expression of different epithelial to mesenchymal transition markers were checked.

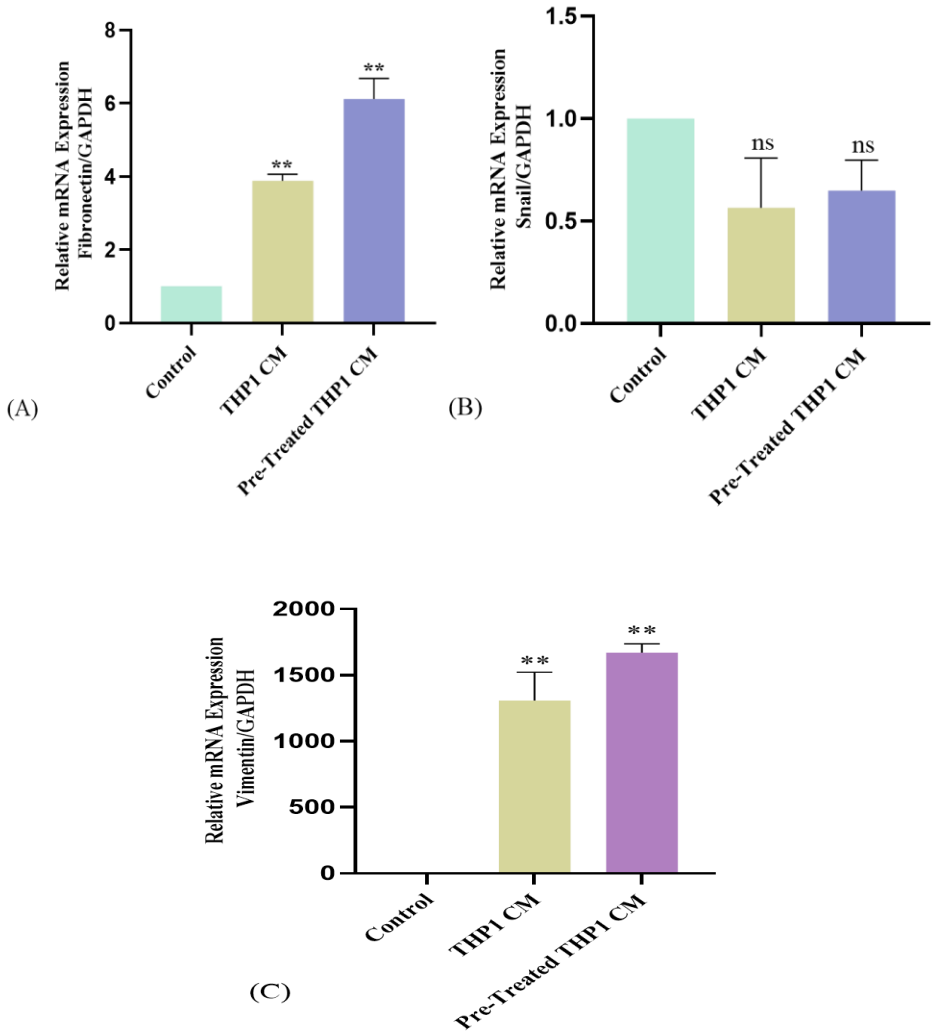

Figure 4.9: *The relative mRNA expression of* ***4.9A****: Fibronectin,* ***4.9B:*** *Snail and* ***4.9C:*** *Vimentin in MCF7 cell line in response to treatment with conditioned media from THP1 and Pretreated THP1.*

The major markers that were checked were Vimentin, E-cadherin, Snail 1 and Fibronectin in MCF7 and Colo205. Wherein MCF7 showed an increase in vimentin and fibronectin in response to the condition media from the pretreated THP1 cells. An increase in the EMT markers depicts that the cells

are undergoing the epithelial to mesenchymal transition, wherein the epithelial cells lose their polarity and adhesion properties and acquire a mesenchymal property characterized by a more migratory and invasive tendency. This Symbolizes a more aggressive phenotype. Vimentin and Fibronectin's increased expression in response to condition media from the pretreated THP1 leads to the inference that this condition media serves as a pro-EMT stimulus by providing soluble factors or exosomal contents that alter gene expression in the cancer cells. Although markers like snail1 don't change much with the 24 Hrs. exposure to the condition media and in case of colo205 at 24 Hrs. treatment not much of difference was observed in any of the above markers (data not included). This could be attributed to the duration of exposure, EMT is a gradual process and upregulation of transcriptional targets may require a longer exposure beyond 24 hrs. and the expression of these markers could also vary from cancer to cancer as well.

Chapter 5

CONCLUSION

TIRAP acts to play a bivalent role within immune cells, enhancing anti-tumor immunity but improperly regulated, thereby contributing to chronic inflammation that perpetuates cancer progression. In epithelial cells, TIRAP seems to be crucial in preserving barrier defenses as well as the initiation of inflammation, potentially protective. However, this protein's persistent activation in these cells can drive tumor initiation and progression by maintaining a proinflammatory and protumor genic environment. Thus, understanding the differential roles of TIRAP holds promise for developing targeted anticancer therapies. Modulation of TIRAP associated pathways, especially in epithelial or immune cells may have therapeutic benefits where tumor growth could be abolished, but protective immunity is preserved.

Betterment in the designing of therapeutic strategies needs a wholesome understanding of the systemics of tumor biology and gaining insights into the functional significance of the inflammatory adapter proteins in the development and progression of cancer forms one part of the ongoing studies. Deciphering the role of TIRAP and other inflammatory adaptors can contribute to advancement in therapeutics in terms of diagnostics, prognostics and provide newer target for drug development. In cancers with TIRAP upregulation, its knockdown using SiRNA or else targeting TIRAP using an inhibitor to prevent its interaction with other proteins can help subdue cancer proliferation. Blocking TIRAP could be used in combination with other therapeutic agents to enhance the overall effectiveness of cancer treatments. For instance, when used alongside (photodynamic therapy) PDT, TIRAP inhibitors could help in managing the immune response, inhibiting unnecessary complement gene activation, and enhancing the overall effectiveness of cancer therapies like PDT potentially leading to better tumor control and reduced side effects incurred from PDT. A study

by Hao et al., observed that the treatment with phycocyanin exerted its anti-proliferative effects by downregulating TIRAP/NF-kB pathway in NSCLC cells. Likewise, plant derived soy isoflavones which are a class of phytoestrogens in combination are reported to notably downregulate the TIRAP gene expression. This opens the possibility of using these molecules as a therapeutic molecule to modulate TIRAP expression. Certain agents currently being utilized in cancer immunotherapy such as Polysaccharopeptide (PSP), derived from the Coriolus versicolor COV-1strain, is an immunoadjuvant that exerts its effect by modulating the TLR4-TIRAP/MAL-MyD88 signaling pathway. Likewise further research can be done to discover newer therapeutic agents to modulate TIRAP.

Other than that, investigating mutations in immune pathways could enhance understanding of molecular mechanisms, potentially improving disease risk prediction and treatments by identifying impactful genetic variants that influence susceptibility and outcomes in immune-related diseases. More of functionally interesting variants with corresponding nonsynonymous SNPs need to be identified with respect to other cancers, this might be of further epidemiological interest. TIRAP polymorphisms might be employed in the future as a potential biomarker for determining the phenotype and prognosis of different cancers. In conclusion, understanding the differential roles of TIRAP holds promise for developing targeted anticancer therapies. Modulation of TIRAP -associated pathways specifically in epithelial or immune cells may have therapeutic benefits where tumor growth could be abolished

Chapter 6

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