Functional Expression of TLRs and TRP Channels in T cells and their Role in Cell-Mediated Immunity

By

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Subhransu Sekhar Sahoo entitled "Functional expression of TLRs and TRP channels in T cells and their role in Cell-Mediated Immunity" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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Ph.D. Supervisor

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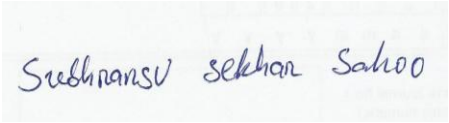

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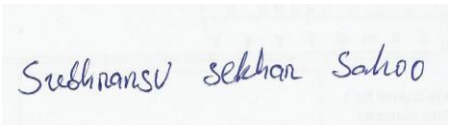
DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.



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LIST OF PUBLICATIONS

Journal Publications:

a. Published:

1. [#]**Sahoo S. S.**, Pratheek B. M., Meena V. S., Nayak T. K., Kumar P. S., Bandopadhyay S., Maiti P. K., Chattopadhyay S. (**2018**). VIPER regulates naive T cell activation and effector responses: Implication in TLR4 associated acute stage T cell responses. *Scientific Reports*, 8:7118.
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b. Communicated:

1. Majhi R.K., **Sahoo S.S.**, Acharya T.K., Chattopadhyay S., Goswami C. (**2018**). TRPM8 Channel is endogenously expressed in T cells and regulates T cell activation, differentiation and proliferation.

[#] Pertaining to thesis, *Contributed equally

Conference Proceedings and Workshops attended:

a. Conferences attended

1. **Subhransu S Sahoo**, P Sanjai Kumar, Dalai Jupiter Nanda Kishore, Tathagata Mukherjee, Chandan Mahish, Soma Chattopadhyay, Chandan Goswami and Subhasis Chattopadhyay; Functional Expression of sensory receptor channel(S) in cell-mediated immunity: Implication in Infection and Tumor Immunity. National seminar on science and technology for national development in India organized by Indian Science Congress Association, Bhubaneswar chapter, and KIIT University, Bhubaneswar, Odisha, India from 12^{th}-13^{th} Dec 2016.
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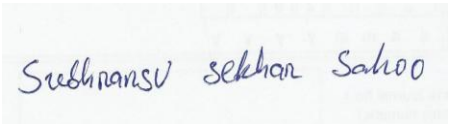

Subhransu Sekhar Sahoo

Dedicated to

MY FAMILY. . .

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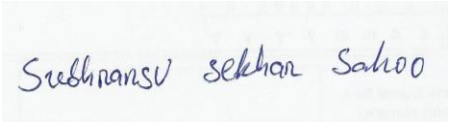

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1. Name of the Student:	**Subhransu Sekhar Sahoo**
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SYNOPSIS

(Limited to 10 pages in double spacing)

CHAPTER-1: INTRODUCTION AND REVIEW OF LITERATUR

1.1 Introduction:

T cell is a signature member of the adaptive immune system and an executioner of cell-mediated immunity (CMI). The established paradigm of T cell research is abundant with seminal observations involving T cell receptor (TCR) engagement, activation/effector function, and T immune-regulatory cell (Treg) responses with the altered state of adaptive immunity associated to basic and translational research. However recent evidence proposes that T cell function could be associated with newly emerging fields such as TLRs and TRPs. Immunological recognition of pathogens through innate immune receptors like Toll-like receptors (TLRs) and

involvement of transient receptor potential channels (TRP channels), which are relatively permeable to non-selective cations, towards immune responses have been proposed for innate immune responses. However, virtually nothing is clearly established for functional expression of TLR and TRP channel in T cell repertoire. Accordingly, there is a need for a critical understanding of such T cell responses in terms of adaptive and innate immune-regulatory pathways for the betterment of immune-therapeutic strategies.

1.2 Review of Literature:

TLR4 is one of the most investigated TLR which is expressed in cell surface and forms homodimer upon binding with Lipopolysaccharides (LPS) from gram-negative bacteria. This process is facilitated by CD14, Lipopolysaccharide Binding Protein (LBP) and Myeloid Differentiation Factor 2 (MD2) to activate downstream signaling (1). After binding with LPS, TLR4 signaling pathway follows Myeloid differentiation primary response gene (88) (MYD88) dependent pathway or TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathway in the cytoplasm which in turn results in activation of genes of pro-inflammatory cytokines. Typically TLRs are known to be one of the most competent modulators of innate immunity. However recent scientific studies propose an important role of TLRs in the modulation of adaptive immune responses. Moreover, recently there were several reports suggests that T cells also express TLR4 and there are some reports of contradiction about its role in the regulation of T cell function upon LPS treatment in different models(2-4). However, LPS sensitivity in T cell is a controversial topic since, T cell does not express CD14, which is an important co-receptor for TLR4. We will investigate the expression of TLR4 in primary mouse and human T cells and its functional role in T cell responses using TLR4 specific modulators.

Ca^{2+} works as an important second messenger in many cells including immune cells. When immune cells were stimulated with cell-specific receptors intracellular Ca^{2+} concentration increases(5). Ca^{2+}-signaling in immune cells is important for immune regulatory functions such as gene transcription, differentiation of immune cells, and effector functions(6). Different Ca^{2+}channels play important yet different regulatory functions in immune cells like T cells. However, the association of these different Ca^{2+}channels in the context of diverse immune

functions and their molecular identities are yet to be explored in details. Transient receptor potential (TRP) channels are a group of ion channels non-selectively permeable to cations comprising various members which are thermosensitive in nature. TRP channels are well studied and known to be involved in various physiological responses, such as neuronal responses associated with sensory functions (7). The thermosensitivity and Ca^{2+}-permeability property of TRPV channels seem to be appropriate in the perspective of immune cell function. Recent studies have suggested the expression and functional role of TRP channels in different immune cells, mainly in macrophages and dendritic cells (8). Expression of different TRP channels and their functional significance in T cells is not well studied and needs further investigation.

CHAPTER-2: HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis:

There could be an altered expression of TLRs and TRPs channels in T cell that might have important implications for T cell activation and effector responses

2.2 Objectives:

I. To study the differential expression of TLRs in T cells during resting and activation

II. To investigate the functional role of TLRs response in T cells by using specific TLR inhibitor during T cell activation

III. To study the differential expression of TRP channels during T cell activation

IV. To explore the functional role of TRP channels in T cell effector function by using TRP channel modulators

CHAPTER-3: MATERIALS AND METHODS

1. **Isolation of T cells:** Mouse splenocytes were isolated from mouse spleen as mentioned elsewhere (9,10). Mouse T cells were purified using T cell purification kit from Invitrogen. Human PBMC was isolated from healthy human blood using HiSep from Himedia. Human T cells were purified by using T cell purification kit from Invitrogen for human T cell(10).

2. **Cell Culture:** Isolated mouse T cells were cultured in complete RPMI-1640 media at 37^0C under humidified condition with 5% CO_2. Human T cells were cultured in complete IMDM media at 37^0C under humidified condition with 5% CO_2 (11,12).

3. **ELISA:** Sandwich ELISA was carried out for T cell effector cytokines according to manufacturer's instructions and as mentioned elsewhere (11,12).

4. **Flow Cytometry:** T cells were stained with specific antibodies and acquired by BD FACS Calibur. Data were analyzed using BD Cell Quest Pro software as mentioned elsewhere (9,12). To study cell viability, T cells were incubated with 7AAD for 15 minutes and then analyzed for the 7AAD negative population using BD FACS caliber and Cell Quest Pro software.

5. **Calcium Imaging:** Calcium imaging was carried out by loading the cells with Fluo-4 AM dye and then calcium influx was studied with a change in Fluo-4 AM intensity at different conditions as mentioned elsewhere (10).

6. **Immune fluorescence:** T cells were incubated with primary TRP channel specific antibodies and then washed with 1XPBS followed by incubation with fluorophore-tagged secondary antibody as mentioned elsewhere (10).

7. **Statistical Analysis:** Data are presented as means ± standard errors of the means (SEM) of three similar independent experiments. Differences between two groups were determined by One way ANOVA using GraphPad Prism 5.03 software. A *p*-value of <0.05 was considered significant between groups.

CHAPTER-4: RESULTS

4.1 Differential expression of TLR4 in T cells during resting and activation:

To study the differential expression of TLR4 in T cells during resting and T cell activation we activated the purified T cells with TCR mediated stimulation. We observed that in resting condition 22.68% cells were positive for TLR4 whereas during TCR mediated stimulation only 4.68% cells are positive for TLR4. This observation suggests TLR4 expression is differentially regulated in resting and activated T cells.

4.2 TLR4 specific signaling inhibitor VIPER differentially regulated TLR4 expression in T cells:

We used VIPER, an established TLR4 signaling inhibitor peptide derived from the A46 protein of Vaccinia virus(13) to study the functional significance of differential expression of TLR4 during T cell activation in naïve wild-type T cells. First, we studied the expression of TLR4 during VIPER treatment along with TCR mediated stimulation and found VIPER treated T cells showed similar level of TLR4 expression as we observed with resting cells.

4.3 VIPER suppressed ConA and TCR mediated T cell activation:

We pretreated Purified T cells with VIPER (VP) and Control Peptide (CP) for 2 hours and then stimulated the cells with ConA and TCR. After 36 hours cells were stained with T cell activation marker CD25 and CD69. We found that VP treated cells showed a significant reduction in expression of T cell activation marker CD25 and CD69 as compared to cells treated with only ConA, TCR or ConA+CP and TCR+CP.

4.4 VIPER suppressed T cell effector cytokine secretion:

ELISA was carried out with cell culture supernatant from the above experiment to study the secretion of T cell effector cytokines IL-2, IFN-γ and TNF. We found that VP treated cells showed significantly decreased cytokine secretion as compared to cells treated with only ConA, TCR or ConA+CP and TCR+CP.

4.5 VIPER down-regulated T cell proliferation:

T cell activation is followed by proliferation and it's facilitated by IL-2 secreted by T cells (14). We studied the effect of VP on T cell proliferation during both mitogenic and TCR mediated stimulation. We observed that VP treated cells showed significantly decreased T cell proliferation when compared with only ConA, TCR or ConA+CP and TCR+CP treated cells.

4.6 VIPER down-regulated Fas (CD95) and FasL (CD95L) expression:

T cell activation was followed by induction of apoptosis to maintain the T cell homeostasis (15). We found that after ConA or TCR mediated stimulation CD95 and CD95L expression was up regulated in purified T cells as compared with resting cells. However upon VP treatment CD95 and CD95L expression was significantly reduced as compared with only ConA, TCR or ConA+CP and TCR+CP treated cells.

4.7 TRP channels were expressed on T cells:

To study the expression of different TRP channels on T cells we stained purified T cells from both mice and humans with different TRP channel specific antibodies. The expression of TRP channels was studied by both microscopy and flow cytometry. We found that TRPV1, TRPV2, TRPV3, TRPV4, and TRPA1 channels were present on T cells from both humans and mouse. TRPV1, TRPV4, and TRPA1 channel expression were up-regulated during T cell activation.

4.8 TRP channels expressed on T cells were functional:

We carried out Calcium influx study to confirm that weather TRP channels expressed on T cells were functional. We activated the TRPV1, TRPV4, and TRPA1 with specific activators RTX, 4αPDD and AITC respectively. We observed that upon activation with channel-specific activators, intracellular Ca^{2+} concentration is significantly up-regulated. Further, when these channels were inhibited with specific TRP channel inhibitors IRTX, RN1734 and A96 for TRPV1, TRPV4 and TRPA1 respectively, intracellular Ca^{2+} concentration is decreased.

4.9 TRP channel inhibitors can downregulate the T cell activation:

To study the function of TRP channels during T cell activation and effector function, we inhibited the TRP channels during TCR mediated stimulation and found that increase in intracellular Ca^{2+} concentration is significantly reduced as compared to cells stimulated with only TCR. Further, we inhibited the TRP channels with channel-specific inhibitors and found that it decreases the expression of T cell activation markers CD25 and CD69 during TCR mediated stimulation.

TRP channel inhibitors downregulate T cell effector cytokine release:

We carried out sandwich ELISA for T cell effector cytokines IL-2, IFN-γ, and TNF with cell culture supernatant from T cells inhibited with TRP channel specific inhibitors followed by TCR mediated stimulation. We observed that T cells treated with TRP channel inhibitors showed a significant reduction in effector cytokine release as compared to cells treated with only TCR.

CHAPTER-5: DISCUSSION AND CONCLUSIONS

T cell is a major player in the adaptive immune system towards the cell-mediated immune response. TLR4 was shown to be mostly associated with innate immune cells like macrophages and Dendritic cells. However,

recent studies have suggested the presence of TLRs on adaptive immune cells and attributed their functional significance in different disease models which are differing in nature (3,16). Information on expression of TLR4 in naïve T cells and their functional role during T cell activation remains scanty and needs further investigation. In this study, we have observed that there is a differential expression of TLR4 during T cell activation as compared to resting naïve T cells. Moreover when TLR4 signaling was inhibited during mitogenic and TCR mediated T cell stimulation, it significantly downregulated T cell activation which is evident from the decreased expression of CD25, CD69. T cell effector cytokine secretion and T cell proliferation were also found to be regulated by VIPER. Expression of Fas (CD95) and FasL (CD95L) responsible for T cell Activation-Induced Cell Death (AICD) after mitogenic and TCR mediated stimulation were significantly decreased in VIPER treated cells. All these findings suggest an important role of TLR4 signaling pathway which might have implications for acute phase T cell activation.

Intracellular Ca^{2+} concentration plays a critical function during T cell activation and different effector functions(5). Apart from the classical Ca^{2+} channels non-selectively permeable thermo sensitive cation channels or TRP channels are found to be present in the immune cells. However, the expression and functional significance of these channels in T cells is less studied and needs further exploration. Accordingly, we studied the expression pattern of different TRP channels and found that TRPV1, TRPV4, and TRPA1 channel expression is differentially regulated during T cell activation. Ca^{2+} influx study with TRP channel specific activators and inhibitors confirmed the functionality of these channels in T cells. Moreover increase in intracellular Ca^{2+} level during TCR mediated stimulation of T cells was found to be down-regulated with cells treated with TRPV1, TRPV4, and TRPA1 inhibitors. Expression of T cell activation marker CD25, CD69, and effector cytokine release was also significantly reduced upon treatment with TRP channel specific inhibitors during mitogenic and TCR mediated stimulation. All these observations suggest an important role of different TRP channels during T cell activation and effector function.

In this work, we have investigated on the expression of TLR4 and different TRP channels in T cells and their role towards cell-mediated immunity which might have implication in designing therapeutic strategies for diseases associated with acute phase T cell activation and inflammation.

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Publications in Refereed Journal:

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5. #**Sahoo S. S.**, Pratheek B. M., Meena V. S., Nayak T. K., Kumar P. S., Bandopadhyay S., Maiti P. K., Chattopadhyay S. (2018). VIPER regulates naive T cell activation and effector responses: Implication in TLR4 associated acute stage T cell responses. *Scientific Reports,* 8:7118.
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Other Publications:

a. Conference/Symposium

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Abbreviations

2-APB: Aminoethoxydiphenyl borate

5'-IRTX: 5'-iodoresiniferatoxin

7-AAD: 7-Aminoactinomycin D

ACAID: Anterior Chamber Associated Immune Deviation

AF488: Alexa Fluor 488

AF647: Alexa Fluor 647

AM: Acetoxymethyl ester

AMP: Antimicrobial Peptide

ANOVA: Analysis of Variance

APC: Allophycocyanin

APC: Antigen Presenting Cells

APS: Ammonium Persulfate

ATP: Adenosine Triphosphate

BALT: Bronchus-Associated Lymphoid Tissues

CCR: CC Chemokine Receptor

CD: Cluster of Differentiation

CLIP: Class II-associated Invariant Chain Peptide

CMI: Cell Mediated Immunity

CS: Surface Staining

CTL: Cytotoxic T Lymphocytes

CTLA4: Cytotoxic T-Lymphocyte Associated Protein 4

Cx3CR: CX3C Chemokine Receptor

DAG: Di acyl glycerol

DC: Dendritic Cell

DDT: Dithiothreitol

DMSO: Dimethyl Sulfoxide

DN: Double Negative

DP: Double Positive

EDTA: Ethylenediaminetetraacetic Acid

EGTA: Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

ER: Endoplasmic Reticulum

ERAP: Endoplasmic Reticulum Amino-peptidase

Fab: Fragment Antigen Binding

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FC analysis: Flow Cytometry analysis

Fc: Fragment Crystallizable

FcR: Fragment Crystallizable Receptor

FOXP3: Forkhead box P3

FSC-H: Forward Scatter-height

GATA3: GATA Binding Protein 3

GITR: Glucocorticoid-Induced TNFR-Related protein

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HIV: Human Immunodeficiency Virus

HLA-DM: Human Leukocyte Antigen DM

HPLC: High-Performance Liquid Chromatography

HRP: Horseradish Peroxidase

HSCs: Hematopoietic Stem Cells

HSP: Heat Shock Protein

HSP90: Heat Shock Protein 90

IAEC: Institutional Animal Ethics Committee

ICS: Intracellular Staining

IFN: Interferon

IFN-γ: Interferon- γ

Ig: Immunoglobulin

IgG: Immunoglobulin G

IL: Interleukin

IL-2: Interleukin-2

IRF-3: Interferon Regulatory Factor 3

iTregs: induced Tregs

KO: Knock out

LPS: Lipopolysaccharide

mAb: monoclonal Antibodies

MAIT cells: Mucosal-Associated Invariant T Cells

MALT: Mucus Associated Lymphoid Tissues

MAPK: Mitogen-Activated Protein Kinase

MFI: Mean Fluorescence Intensity

MHC: Major Histocompatibility Complex

Min: Minutes (Time unit)

mM: millimolar

NALT: Nasal Associated Lymphoid Tissues

NK Cell: Natural Killer Cell

NKT cells: Natural Killer T cells

nTregs: natural Tregs

PAMP: Pathogen-Associated Molecular Patterns

pAPCs: Professional Antigen Presenting Cells

PBS: Phosphate-Buffered Saline

PE: Phycoerythrin

PFA: Paraformaldehyde

PHSC: Pluripotent Hematopoietic Stem Cell

PLOs: Primary Lymphoid Organs

PRR: Pathogen Recognition Receptors

PRR: Pattern Recognition Receptor

PVDF: Polyvinylidene Fluoride or Polyvinylidene Difluoride

RA: Rheumatoid Arthritis

RBCs: Red Blood Cells

RER: Rough Endoplasmic Reticulum

RIPA: Radio Immunoprecipitation Assay

R-M System: Restriction-Modification System

RORγt: RAR-related Orphan Receptor gamma t

ROS: Reactive Oxygen Species

RPMI-1640: Roswell Park Memorial Institute-1640

RT: Room Temperature

SDS: Sodium Dodecyl Sulphate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEM: Standard Error of the Mean

SLE: Systemic Lupus Erythematosus

SLOs: Secondary Lymphoid Organs

SOCE: Store Operated Calcium Entry

SP: Single Positive

SSC-H: Side Scatter-height

TAP: Transporters Associated with Antigen Processing

TBS: Tris-Buffered Saline

TBST: Tris-Buffered Saline Tween-20

Tc Cells: Cytotoxic T Cells

TCM: Central Memory T Cells

TCR: T Cell Receptor

TCRβ: T Cell Receptors β

TEF: Effector Memory T Cells

TEMED: Tetramethylethylenediamine

TGF-β: Transforming Growth Factor beta

TGS: Tris-Glycine-SDS

TH Cells: T helper Cells

TLR: Toll-like Receptor

TMB: 3,3‘‘,5,5‘‘-Tetramethylbenzidine

TNF: Tumor Necrosis Factor

Tregs: Regulatory T cells

v/v: Volume/Volume

w/v: Weight/Volume

WCL: Whole Cell Lysate

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CHAPTER # 1

Introduction

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1.1 Introduction:

1.1.1 Introduction to Immune System

The immune system works as a defense shield for our body which protects us from different harmful pathogens and maintains our health. It is a complex system where different cells and organs are continuously working in synchronization to maintain the homeostasis. The branch of science involved in the study of the immune system and its function is known as immunology. The immune system helps in discrimination between "self" and "non-self" components of the living system which helps in excluding the foreign agents that may cause harm to the organism. Our body is continually exposed to external elements like bacteria, viruses, pollens and other harmful materials during regular activities of inhalation, ingestion and occasionally injury and trauma. The immune system gives us protection against all these foreign substances by eliciting controlled and coordinated immune response which is mediated by different organs and cells (1). The single cell organisms like bacteria or lower multi-cellular organisms from invertebrates to higher organisms like mammals have some kind of protection mechanism which evolved according to the increasing complexity of the organism.

A balanced immune response is very critical for the host during defense against invading pathogens (2,3). An over-activated immune response may cause tissue damage by severe inflammation which sometimes proved to be lethal for the host. If immune system fails to discriminate between "self" and "non-self," then it may act against the host tissue resulting in causing several autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis and inflammatory bowel disease. Similar to the overactivation of the immune system, reduced activation of the immune system or immune deficiency is also harmful to the host. As a result of immune deficiency, the host becomes

vulnerable to many pathogens which can cause infection and disease. Moreover, immune deficiency leads to different disease conditions like severe combined immune deficiency (SCID), acquired combined immunodeficiency (AIDS) due to viral infection (HIV), etc.

Pathogens are continuously trying to evade the immune system by developing different immune evasion strategies which need to be recognized by our multi-layered immune system (4). The immune system comprises of different organs and cells which have been associated with various functional aspects. According to the function and mode of action, the immune system is divided into two distinct, but interdependent defense systems called as innate immune system and adaptive immune system which works in synchrony.

1.1.1.1 The Innate Immune system

The innate immune system is evolutionarily primitive and found in plants, insects, fungi, and animals. It is also known as non-specific or inborn immune system which has different cells and mechanisms to protect the host from infection. It is the first line of defense which gets activated once a pathogen attacks. It includes different physical barriers like skin, cilia and other body structures. Different secretions (mucous, sweat, gastric acid) and nonspecific cellular responses are also included under the innate immune system which aids in the protective mechanism. Highly specific conserved receptors (e.g., PRRs) are present on the cells of innate immune cells which help in recognition of foreign pathogens. Cells associated with the innate immune system are macrophages, mast cells, dendritic cells, natural killer cells, basophils which initiate a rapid response against invading pathogens through phagocytosis and secretion of inflammatory agents (3).

1.1.1.2 The Adaptive immune system

Adaptive immune system which is also known as acquired immune system or specific immune system, comprised of highly specialized cells that can initiate an immune response and eliminate the pathogen and can provide long-term protection against that specific pathogen. It takes more time for activation to specific pathogens as compared to innate immune system and can generate immunologic memory cells for a future encounter. Antigen-presenting cells (APC) from the innate immune system process the pathogen and present the antigen to adaptive immune cells so that it can be activated and carry out the necessary function. Mainly B cells and T cells are responsible for the function of the adaptive immune system. B cells and T cells are derived from the multipotent hematopoietic stem cells present in bone marrow. After they are made in bone marrow, B cells mature in bone marrow, and T cells mature in the thymus. According to the type of immune response they provide further B cell-mediated immunity is called as “humoral immunity” and T cell-mediated immunity is called as “cell-mediated immunity.” The specificity of the adaptive immune system is due to the specific receptors present on the adaptive immune cells for a different type of antigens. After antigen presentation by innate immune cells to adaptive immune cells, specific cells get activated which is followed by proliferation and clonal expansion for effective elimination of the pathogen. Some cells are converted to memory cells which provide long-term protection against repeated infection with the same pathogen. However, the lifespan of memory cells varies and depends upon the type of antigen. Some basic difference between innate and adaptive immunity are given below (5).

Table1. Comparison between Innate and Adaptive immune system

Table adapted from: (6)

S.N.	Characteristics	Innate Immunity	Adaptive immunity
1.	Presence	Innate immunity is something already present in the body.	Adaptive immunity is created in response to exposure to a foreign substance.
2.	Specificity	Non-Specific	Specific
3.	Response	Fights any foreign invader	Fight only specific infection
4.	Response	Rapid	Slow (1-2 weeks)
5.	Potency	Limited and Lower potency	High potency
6.	Time span	Once activated against a specific type of antigen, the immunity remains throughout the life.	The span of developed immunity can be lifelong or short.
7.	Inheritance	Innate type of immunity is generally inherited from parents and passed to offspring.	Adaptive immunity is not passed from the parents to offspring, hence it cannot be inherited.
8.	Memory	Cannot react with equal potency upon repeated exposure to the same pathogen.	Adaptive system can remember the specific pathogens which have encountered before.
9.	Presence	Present at birth	Develops during a person's lifetime and can be short-lived.
10.	Allergic Reaction	None	Immediate and Delay hypersensitivity
11.	Used Against	For microbes	Microbes and non-microbial substances called antigens
12.	Memory	No memory	Long term memory
13.	Diversity	Limited	High

14.	Speed	Faster response	Slower response
15.	Complement system activation	Alternative and lectin pathways	Classical pathway
16.	Anatomic and physiological barriers	Skin, Mucous membranes, Temp, pH, chemicals, etc.	Lymph nodes, spleen, mucosal associated lymphoid tissue.
17.	Composition	The innate immune system is composed of physical and chemical barriers, phagocytic leukocytes, dendritic cells, natural killer cells, and plasma proteins.	Adaptive immune system is composed of B cells and T cells.
18.	Development	Evolutionary, older and is found in both vertebrates and invertebrates.	Adaptive immunity system has been developed recently and is found only in the vertebrates.
19.	Example	White blood cells fighting bacteria, causing redness and swelling, when you have a cut.	Chickenpox vaccination so that we don't get chickenpox because adaptive immunity system has remembered the foreign body.

1.1.1.3 Components of the immune system

Immune system comprises of different organs and cells which helps in protecting us from different harmful agents in specific and nonspecific manner. The nonspecific protection offered by the innate immune system is mediated by physical barriers (skin, mucous) and cells (dendritic cells, macrophages) whereas adaptive immune system provides specific protection with the help of cells like B cells and T cells. Several organs are also involved in the process of generation and development of immune cells. These organs and cells generate a different layer of protection for

the host. However, at times these different layers of protection by the immune system fail to recognize and eliminate the pathogen due to several reasons which result in the occurrence of disease.

1.1.1.3.1 Physical and Chemical barriers

The physical and chemical barrier of the immune system works to prevent the entry of pathogens into the body (7). It provides the body's first line of defense to foreign invaders by restricting the passage inside through different mechanisms. The physical barriers are possessed naturally by the host such as the thick epithelial lining of our body. Pathogens are killed on the skin by skin's acidity or the beneficial bacteria growing on it. Where the skin is not present, specialized tissue layers encircle different systems such as mucus layers of the gastrointestinal tract, respiratory tract, ducts of exocrine secretory glands and urogenital tracts, etc. and prevent entry of pathogens by specialized structure and secretions. Specialized structures (such as cilia on respiratory tract) and body's secretions (urine, tears, saliva, mucus, and milk) help in sweeping out pathogens entered during different physiological functions like respiration, ingestion, etc. The chemical barriers include gastric pH, vaginal fluid pH, anti-microbial proteins and peptides secreted by different organ system also prevent microbial activity. However, at times pathogens cross the physical barrier due to tissue damage and can cause disease. At this point, different cells of the immune system come into action for the host protection.

1.1.1.3.2 Organs and Cells of the immune system

Different cells of the immune system work in an interdependent manner to elicit an effective immune response. For an effective immune response, proper growth and development of immune cells are required which is carried out at different organs of the immune system (8). According to the function organs of the immune system are divided into primary and secondary lymphoid organs. The primary lymphoid organs (PLOs) comprises of bone marrow and thymus, which

regulates the origin and development of the immune cells from their immature precursor cells. Immune cells were originated from multipotent hematopoietic stem cell present in bone marrow and then developed and experienced with antigens in different organs according to the cell type. For example, B cells mature in bone marrow, and T cells mature in the thymus. The secondary lymphoid organ's (SLO) antigen-specific responses are generated and maintained in order to activate the immune system against any pathogens and the development of immune tolerance for self and non-self antigenic detection. The SLOs includes lymph nodes, tonsils, appendix, Peyer's patches, MALT (mucus associated lymphoid tissues), GALT (gut-associated lymphoid tissues), NALT (nasal-associated lymphoid tissues), and BALT (bronchus-associated lymphoid tissues). All SLOs develops in the embryonic stages (pre-natal) except NALT and BALT which development is post-natal (9).

Hematopoietic stem cell in bone marrow generates two major cell types known as myeloid progenitor cells and lymphoid progenitor cells which differentiate into specialized immune cells (Fig. 1). The myeloid progenitor cells differentiated into dendritic cells (DC), monocytes/macrophages, neutrophils (through granulocyte monocyte progenitor), eosinophil (eosinophil progenitor), basophil/mast cells (basophil progenitor), platelets (from megakaryocytes) and finally RBCs (from erythroid progenitor). The lymphoid progenitor cells differentiated into DCs, natural killer cells (NK cells), T cells (from T cell progenitor) and B cells (B cell progenitor) (10-12).

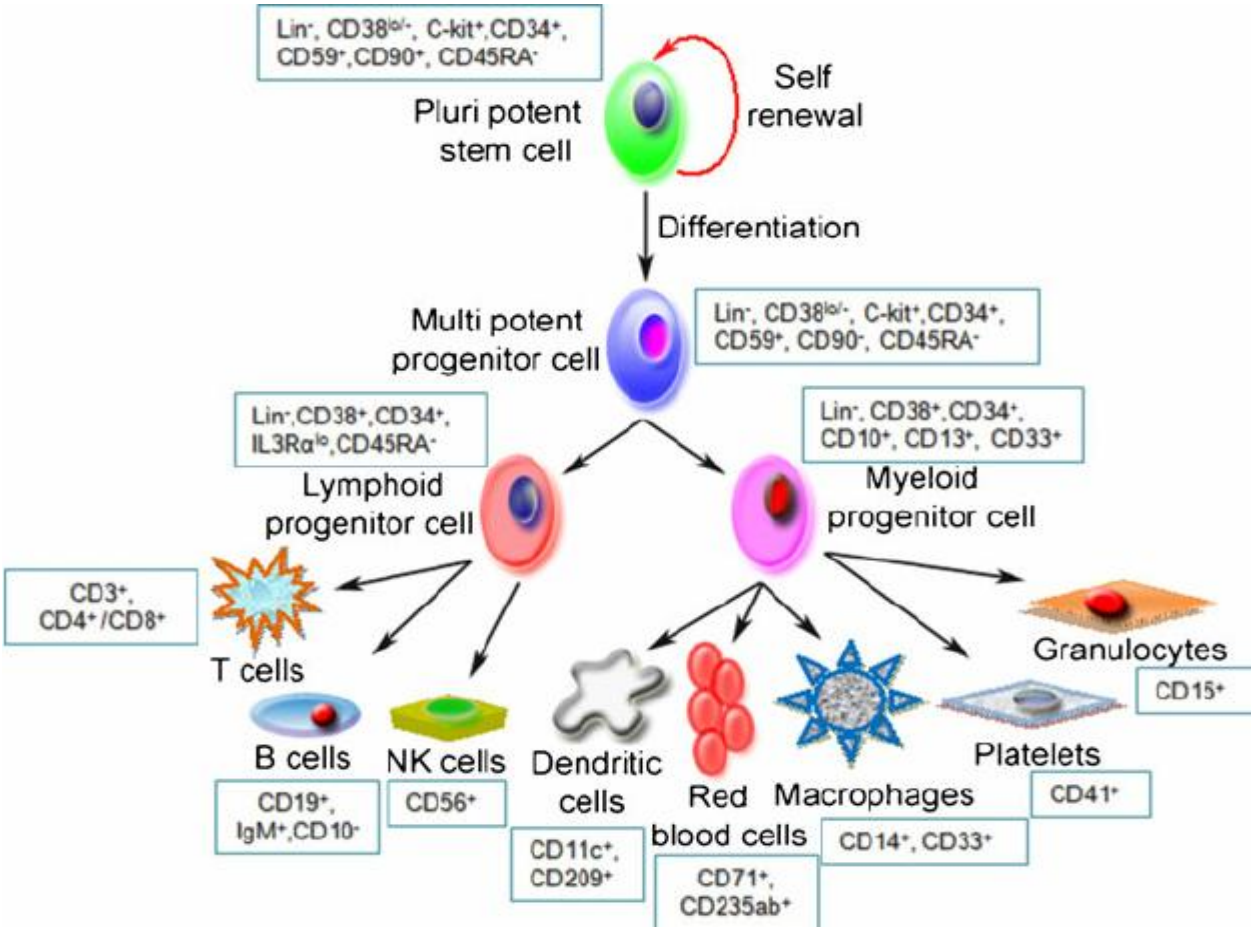


Figure 1. Pluripotent hematopoietic stem cell (PHSC) differentiation pathways: Pluripotent hematopoietic stem cell differentiates into lymphoid and myeloid progenitor cells. From lymphoid progenitor cells, the lymphocytes like T cells and B cells are originated. Myeloid progenitor cells differentiate into macrophages, Granulocytes, etc. Figure adapted from: (13)

1.1.1.3.3 Antigen Presenting Cells (APCs):

Activation of T cells requires the presentation of processed antigen through MHC molecules present on antigen presenting cells. All the nucleated cells can present the antigen to T cells. According to their origin and function, they are divided into professional antigen presenting cells (macrophages, DC and B cells) and nonprofessional antigen-presenting cells (all other cells). APCs are essential for mounting an effective adaptive immune response, as the functioning of both cytotoxic and helper T cells depend on antigen presentation via APCs. Antigen presentation

allows for the specificity of adaptive immunity and can contribute to immune responses against both intracellular and extracellular pathogens.

1.1.1.3.3.1 Professional APCs:

Professional APCs are specialized in exogenous antigen presentation to T cells. Dendritic cells, macrophages and B cells constitute professional APCs. They are efficient in internalizing antigens and present MHC-antigenic peptide complex. Dendritic cells and macrophages opt for phagocytosis whereas B cells opt for receptor-mediated endocytosis. They display processed antigen-MHC II complex to $CD4^+$ and $CD8^+$ T cells. Professional antigen presenting cells express both MHC class I and II molecule (14).

1.1.1.3.3.2 Non-professional APCs:

Non-professional APCs are specialized in endogenous antigen presentation to T cells. All nucleated cells constitute non-professional APCs. Non-professional APCs do not constitutively express the MHC class II proteins required for interaction with naïve T cells. These are expressed only when stimulated with certain cytokines like IFN-γ. Non-professional APCs include- fibroblasts, thymic epithelial cells, glial cells and endothelial cells (15). They mainly present the antigen to $CD8^+$ T cells.

1.1.1.3.4 Antigen processing and Presentation:

Antigen processing and presentation plays an important role to initiate a specific immune response against infectious agents and engagement of key elements from the adaptive immune system. The antigens are processed via two different pathways known as exogenous and cytosolic pathways according to the types of antigen. Endogenous antigens are processed through cytosolic or endogenous pathways and presented by MHC class I molecules, whereas, exogenous antigens processed through the exogenous or endocytic pathway by MHC class II molecule (16). Antigen

presentation via endogenous pathway tags different endogenous proteins by ubiquitination which is further subjected to proteasome-mediated degradation into small peptides. The small peptides are then transported to the ER lumen via transport protein TAP (transporters associated with antigen processing) which translocates the peptides from the cytoplasm into ER. Transport of peptides from the cytosol to ER lumen is facilitated by an ATP dependent manner via TAP which consists of a heterodimer of TAP1 and TAP2 protein with cytosolic ATP binding domain (17). Longer peptides transported by TAP1 and TAP2 are trimmed in the ER lumen by ERAP (endoplasmic reticulum amino-peptidase) into around 9 amino acids long which is the ideal peptide length for MHC class I molecule. The processed antigenic peptide binds to class I MHC molecule followed by the release of chaperone proteins which in turn proceeds to the plasma membrane via the trans-Golgi network for antigen presentation.

Exogenous antigens are internalized by phagocytosis (e.g., macrophages and DCs) or endocytosis (both receptor-mediated and pinocytosis) to follow the exogenous pathway of antigen processing and presentation to the T cells. After internalization, the antigens passes through different endosomal compartments named, early endosomes (pH 6.0-6.5), late endosomes or endolysomes (pH 4.5-5.0) and finally lysosomes (pH 4.5). The antigens are cleaved by different pH-dependent proteolytic enzymes into 13-18 residues for MHC class II compatibility during the sequential movement from different endosomal compartments. Invariant chain (li, CD74) in the RER prevents binding of peptides designed for MHC class I molecule with MHC class II molecules which are supposed to express exogenous antigens (18). Proper folding and transport of MHC class II molecule from RER to cis-Golgi to endosomal compartments through trans-Golgi networks requires li. The li is attached to antigen binding groove which is trimmed to short CLIP (Class II-associated invariant chain peptide), in the late endosome, MHC class II- compartments (MIIC). HLA-DM, a non-classical MHC class II molecule mediates the exchange of CLIP with

antigenic peptide in the MIIC, followed by transport of the resulting MHC class II antigenic peptide complex to the plasma membrane for presentation (19-21). Sometimes antigen cross-presentation happens when exogenous antigens are presented through MHC class I pathway instead of MHC class II pathway to $CD8^+$ T cells to activated CTL response (22-25). This kind of T cell activation is called cross-priming (26-28).

1.1.1.3.5 Antibodies and soluble Factors:

Antibody or immunoglobulin (Ig) is a γ-globular protein (so-called immunoglobulin) produced mainly by plasma cells (terminally differentiated B cells). B cells can recognize a wide diversity of antigens which is generated by the specific type of genetic rearrangements in the germline called V(D)J recombination. All antibodies belong to the immunoglobulin superfamily and glycosylated which is crucial for its structure and function (29). The basic structural unit of an antibody contains two heavy chains (approx. 55 kDa) and two light chains (approx. 22 kDa) which forms multiple immunoglobulin domains made up of anti-parallel β strands with loops. It has both variable domains (binds to antigens) and constant domains. The heavy chains and light chains are joined by disulfide bonds as well as non-covalent interactions. Five different types of heavy chains and two types of light chains are found in the placental mammals. These are referred as a class such as γ (G), α (A), μ (M), ε (E), and δ (D) with one of the light chain κ or λ. Each antibody recognizes a unique molecular pattern on antigen called as epitope which is derived from pathogens. Sometimes auto-reactive antibodies are produced against our own molecules due to the failure of the immune system in recognizing self from non-self, called autoimmunity (e.g., SLE, RA)(30). Antibodies work with different combinations such as monomeric form (IgG, IgE), dimeric form (IgA) or pentameric form (IgM). The region which binds to the epitope is called as Fab (fragment antigen binding), and it is variable in nature whereas the constant region is called Fc (fragment crystallizable) responsible for communication

and signal transduction with other components of the immune system. Secreted immunoglobulins (IgG, IgA, and IgE) are secreted into the blood or body fluids, whereas those bound to the plasma membrane of B cells or memory cells are called membrane or surface immunoglobulins (IgM and IgD). Humoral immunity is mediated by antibodies, which includes agglutination, complement activation, neutralization, precipitation and lysis of foreign cells (31) (20).

1.1.1.3.6 Cytokines and Chemokines:

Cytokines and chemokines are small (5 to 20 kDa) signaling proteins released by both immune and non-immune cells. Majority of them acts by either autocrine or paracrine manner whereas some are reported to have endocrine functions. These soluble proteins regulate several physiological processes which include cell activation, proliferation, differentiation, apoptosis, cell migration and neuro-immune interaction (32). Cytokine plays an important role in cross-talk between innate and adaptive immune systems. Moreover, cytokines like IFN are known to induce antimicrobial effector functions to reduce infections (33). Upon binding with the specific receptor on the target cells, cytokines induce intracellular signaling cascades which change the cell behavior and effector functions. However, some cytokines can interact with multiple receptors (homo and heterodimers), which in turn fine-tune the cellular responses. When one cytokine mediates distinct biological effector function on different cell types, the effect is called pleiotropic effect, whereas when two or more cytokine perform the same function are called redundant. When a cytokine induces the production of the same cytokine in specific target cells, the process is called autoinduction or cascade induction (34). When two or more cytokines augment cellular process significantly higher than the effect confers by either of the cytokine alone is called synergy. When two cytokines inhibit, each other's effect is called antagonize action (e.g., IFN-γ or IL-2 and IL-10). Cytokine refers to all the secreted proteins which help immune cells to communicate effectively among themselves. The cytokines which induce the

mobilization of the immune cells from one part to the other part of the body against concentration gradient for a specific purpose, a process called chemotaxis (20,35-38).

1.1.1.3.7. T lymphocytes:

T cell or lymphocyte is a subset of white blood cell (WBC) playing an active role in cell-mediated immunity (CMI). They develop from hematopoietic stem cells in a primary lymphoid organ, bone marrow and later migrate and mature in another primary lymphoid organ, thymus. Hence the term T cell, where T- refers to the thymus. In the thymus, T cells undergo thymic education via first positive and later negative selection and also garner different T-cell specific markers like TCR, CD3, CD4 or CD8 and CD2 (39). The thymus is a specialized multi-lobed lymphoid organ composed of an outer cortex and inner medulla, which is surrounded by a capsule. The cortex and medulla play different roles in the development of T cells. As T cells differentiate, they start moving from the outer cortex to the central medulla, thus passing through critical different inducive microenvironments for T cell development. Most of the cells entering thymus die by apoptosis, without becoming a mature naive T cell (40).

1.1.1.3.7.1. T cell lineages (αβ Vs. γδ):

T cell lineages can be divided into two classes based on the type of TCR expressed on the surface of the cell membrane, namely, αβ T cells (95%) and γδ T cells (5%). T cells express either αβ-TCR or γδ-TCR for their whole life. As the expression of pre-αβ-TCR or γδ-TCR on the surface of thymocytes results in exiting quiescent stage to proliferation stage, these processes are termed β- and γδ-selection respectively (41).

1.1.1.3.7.2. αβ T cells:

After progenitor cells commit to the T cell lineage, the next big decision lies in the commitment to progress for either αβ and γδ T cell lineage. The double negative $CD4^-$ and $CD8^-$ thymocytes are cell-cycle arrested and require TCR expression to re-enter the cell cycle. Thymocytes rearrange three out of four TCR loci: *Tcrb*, *Tcrg*, and *Tcrd*. In an in-frame *Tcrb* rearrangement, cells express TCRβ in a complex with germline encoded pre-TCRα (pT**α**) chain. This rearrangement leads to release of thymocytes from cell cycle arrest, leading to burst in cell proliferation. Co-receptors like CD4 and CD8 to get up-regulated. To arrest further rearrangement of TCR, *Tcrg* is silenced, and *Tcrd* is excised. When *Tcrα* is productively rearranged, immature double positive ($CD4^+$ or $CD8^+$) thymocytes express TCR αβ at the cell surface and can further differentiate into either Th ($CD4^+$) or Tc ($CD8^+$) lineages. Progression through the double positive stage is believed to be the hallmark of αβ lineage commitment (42).

1.1.1.3.7.3. γδ T cells:

Thymocytes that productively rearrange *Tcrg* and *Tcrd* loci express the γδ-TCR at the cell surface. Upon successful rearrangement of TCR loci, cells undergo proliferation, as in the case of αβ T cells. Most of the thymocytes circumvent progression through double positive stage and egress to the periphery with a $CD4^-$ and $CD8^-$ phenotype, or rarely through $CD4^+CD8^-$ or $CD4^-$ $CD8^+$ (43).

1.1.1.3.7.4 Positive selection of T cells:

Double-positive αβ-TCR^{low} cells are subjected to positive-affinity test with cortical epithelial cells expressing Class I or Class II MHC plus self-peptides. Only cells with a high enough affinity to bind the cortical epithelial cell get the vital survival signal. It is important to understand that both

MHC Class I/ II present self-peptide in the absence of the pathogen. Thus, it creates a self-MHC-restricted repertoire of T cells (40).

During positive selection, gene rearrangement and modification proteins, RAG-1, RAG-2, and TdT are continually expressed on T cells. These enzymes provide an opportunity to immature lymphocyte clones to rearrange different TCR α-chain genes. Since only those αβ TCR heterodimer which recognizes self-MHC will survive. This provides a unique prospect, as at least few members of a given clone can pass the positive selection, given the virtue of αβ TCR heterodimer diversity. All members of the clone which fail positive selection will eventually undergo apoptosis, within 3 to 4 days (44).

Helper or Cytotoxic T cell differentiation is also determined by positive selection. If the positive selection is made via MHC class II, it produces a CD4, Th cell. Similarly, if the positive selection is made via MHC class I, it generates a CD8, Tc cell. Binding of MHC to its respective CD4/CD8 is equally essential for positive selection.

Two different genetic mechanisms are proposed to explain T cell differentiation. The instructive model asserts that the signal received via CD4 shunts down the CD8 gene and cause the cell to segregate into a Th cell, while signals received through CD8 shunts down CD4 expression and induces Tc differentiation (45). According to the stochastic model, the cell could go equally smoothly in either Th or Tc cell differentiation. If the cell receives the first favorable correct signal during positive selection, it proceeds down the committed pathway; if it does not receive a signal through the correct co-receptor, the cell undergoes death (46).

1.1.1.3.7.5 Negative selection of T cells:

Immature T lymphocytes after undergoing positive selection in cortical regions migrate further into cortico-medullary junctional regions, where they undergo negative selection. Cortico-medullary junctions are rich in macrophages, dendritic cells and bone-marrow derived APCs with

high expression of MHC-self peptide complexes. T lymphocytes which bind self-peptide-MHC with high affinity undergo negative selection and die by apoptosis. Hence, negative selection ensures self-tolerance (47).

The paradox of MHC-dependent positive and negative selection of T cells is attempted to be explained by two opposing hypothesis. The differential-avidity hypothesis asserts that the same peptide-MHC complex delivers both signals, where the avidity of positive selection is lower, and the avidity of negative selection is higher. The differential signaling hypothesis emphasizes that two different signals are delivered during positive and negative selection (47).

1.1.1.3.7.6 T cells - Antigen Presenting Cells (APC) Interaction:

Understanding the interaction between T cells and APC assumes prime importance in the wake of consideration that it activates T cells and finally induces fully blown CMI response. Arrays of different surface proteins are expressed on the interacting T cells (TCR, CD28, CD2, LFA-1, CD40L) and APCs (MHC, CD80/86, LFA-3, ICAM 1/2, CD40). The interaction between corresponding surface receptors leads to an intercellular signaling cascade which leads to the synthesis and secretion of biochemical mediators like cytokines etc. The physical contact interface between lymphocytes and target cells/APCs is termed “Immunological synapse” (48).

APCs are a type of cells which ingest antigens, process it to polypeptides and display processed peptides through MHC, on its surface. T cells can recognize these complexes with TCR, providing primary activation signal 1 to the T cell. The interaction between CD28 of T cell and CD80/86 offers essential subsequent secondary signal, which fully activates T cell (Fig. 2). Primary activation signal (TCR-MHC) without secondary activation signal (CD28-CD80/86) leads to inactivation of T cell called T cell anergy (49).

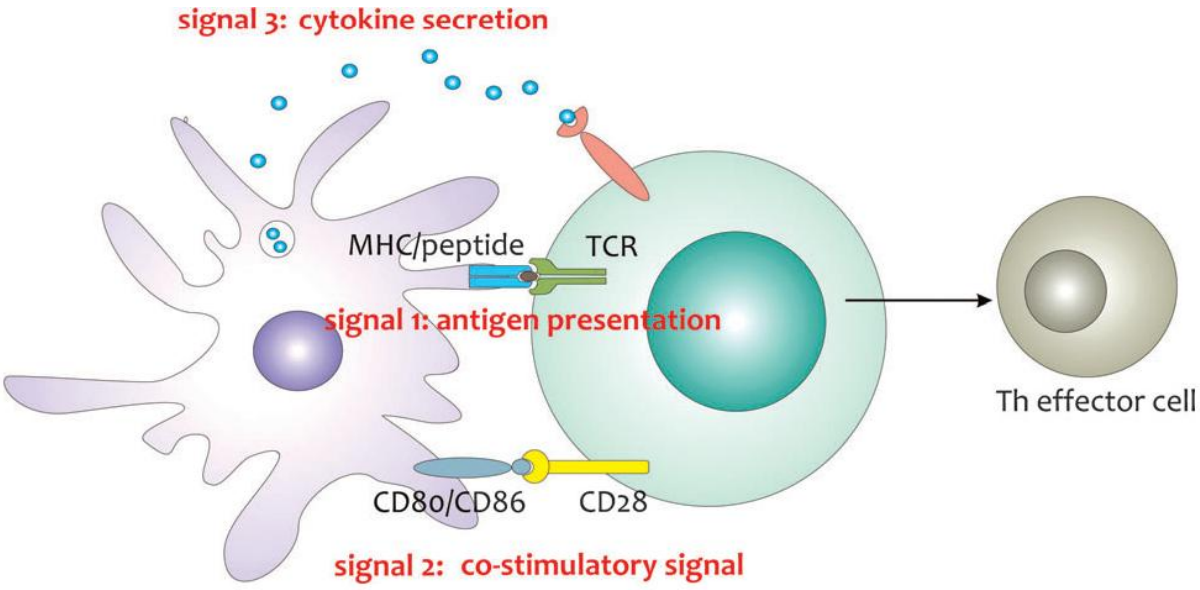


Figure 2: T cell-APC interaction resulting in effector responses: APC cells perform antigen presentation via MHC/peptide on its surface membrane which interacts with TCR of T cell, forming primary signal 1. Similarly, CD80/86 of APC interact with CD28 of T cells, establishing co-stimulatory signal 2. Both signal 1 and signal 2 are essential for Th effector cell response, effectively giving rise to signal 3, i.e., cytokine response. Figure adapted from: (50)

1.1.1.3.7.7 T Cell activation, CMI response:

T cell activation is critical for the initiation and regulation of cell-mediated immunity (CMI) response. It is an antigen-dependent process leading to proliferation and differentiation of naive T cells into effector T cells. Activation of Th cells also stimulates B cells to increase antibody responses. T cell activation requires primary and co-activating signals triggering intracellular signaling cascades and gene expression. Signal one asserts to the interaction of TCR of T cell with a peptide-MHC complex of APCs and signal two arises due to the interaction of costimulatory CD28 of T cell with B7 proteins of APCs. Both signal 1 and signal 2 are mandatory

for T cell activation. Confirmation of signal 1 without signal 2 leads to T cell inactivation called T cell anergy. This system prevents T cells from reacting to the host's own proteins (51).

1.1.1.3.7.8 T cell Differentiation:

αβ-T cells can be further divided into $CD4^+$ T cells and $CD8^+$ T cells. Resting $CD4^+$ and $CD8^+$ T cells leave the thymus and enter the circulation in G_0 phase of the cell cycle. Naive T cells are characterized by condensed chromatin, minimal cytoplasm, and almost zero transcriptional activity. Naive T cells continually recirculate between the blood and lymph systems. If the naive T cell does not encounter any antigen in lymph node, it exits through the lymph node and returns in the blood. Based on the expression of unique $CD4^+$ and $CD8^+$ markers, T cells can be classified into $CD4^+$ (Helper T cells) or $CD8^+$ (Cytotoxic T cells).

1.1.1.3.7.9. Helper T cells ($CD4^+$):

Upon encountering any antigen-MHC complex on APCs or target cells, T cells mount an effector response. T cell enlarges in size and gets transformed into blast cells, undergoing repeated rounds of cell division. Upon T cell activation, cells enter into the G_1 phase of the cell cycle. It also triggers transcription of the IL-2 gene and α chain of the high-affinity IL-2 receptor. Secretion of IL-2 cytokine and IL-2 receptor induces the activated T cell to proliferate and differentiation. Activated T cell keeps on dividing and produce a large progeny of T cells, which differentiate into memory and effector T-cell population (52).

1.1.1.3.7.10 Th1 and Th2 response:

Effector Th cells population can be further divided into Th1 and Th2 subset, depending on different panel of cytokines they secrete (Fig. 3). Th1 subset mediates classical cell-mediated functions, such as delayed-type hypersensitivity and activation of cytotoxic T lymphocytes. It

produces cytokines like IL-2, IFN-γ, TNF-α/β. Th2 subset activates B cell activation. It produces cytokines like IL-4, IL-5, IL-6, and IL-10 (53).

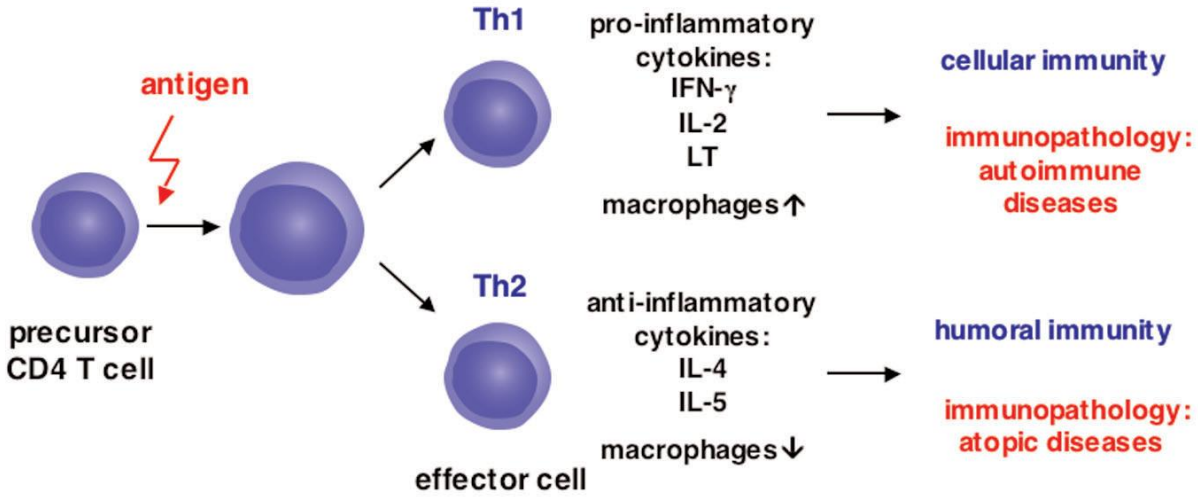


Figure 3: Th1 and Th2 responses: Upon antigenic stimulation, naïve T cells proliferate and differentiate into either Th1 or Th2 subset. Th1 effectively promotes cellular immunity and is involved in immunopathological conditions like autoimmune diseases. Th2 subset mediates humoral immunity and is involved in immunopathological conditions like allergic immune responses. Figure adapted from: (54)

1.1.1. 3.7.11 Memory T cell:

Memory T cells are antigen generated, long-lived and quiescent cells. These cells can be derived from both naive and effector T cells. On subsequent challenge with the same antigen, these cells can respond with a heightened secondary immune response. Similar to naive T cells; memory T cells also remain at G_0 phase of cell cycle. However, they appear to have less stringent conditions for activation than naive T cells. It is believed that high levels of surface adhesion molecules on Th cells enable these cells to adhere to a variety of APCs (55).

1.1.1.3.7.12 Regulatory T cells:

Regulatory T cell helps in maintaining the immunological tolerance to self-antigens which can be deleterious to the host (56). These are immune-suppressive in nature combating the potentially deleterious nature of Th cells enabling, self-checks in the immune system. Functionally, these cells help in the prevention of autoimmune diseases by maintaining self-tolerance, suppression of allergy and asthma, oral tolerance and feto-maternal tolerance. Identification of regulatory T cells remains problematic. Available Tregs markers include CD25, GITR, CTLA-4, LAG-3, CD127, and Foxp3 (57). Tregs activation is antigen-specific, which suggests that suppressive activities of Treg cells are antigen-dependent. The classification of the Treg population as a separate lineage remains controversial as the ability to suppress immune response is not exclusive to Tregs (58).

1.1.1.3.7.13. Cytotoxic T cells ($CD8^+$):

Cytotoxic T cells (CTLs) are a subset of αβ T cells, which expresses the dimeric CD8 receptor, composed of one CD8α and one CD8β chain. CTLs possess cell lytic capacity. CTLs play a critical role in the recognition and elimination of altered self-cells. CTLs are MHC class I restricted. Since virtually all nucleated cells express MHC class I, CTLs possess the capacity to recognize and eliminate any cell in the body. CTLs play a major role in immune defense against various intracellular pathogens like viruses and bacteria, and for tumor surveillance. CTLs own a number of different mechanisms to kill target cells. The first is the secretion of cytokines like TNF-α and IFN-γ, which have anti-tumor and anti-viral effects. CTLs also possess lytic cytotoxic granules: perforin and granzyme. Perforin forms a pore in the target membrane cell, and granzymes enter into target cells via perforin pores and cleave the proteins inside the cell, leading to apoptosis of the target cell. CTLs also express FasL on its surface, which then binds to the Fas receptor of target cells, initiates apoptosis (59).

1.2. Review of literature:

1.2.1. History of TLR research

In current day scenario, we are getting exposed to lots of microorganisms on a daily basis, and it is a challenge for our immune system to protect ourselves against them. To protect the host body, innate immune components (the first line of defense) play a pivotal role. Toll-like receptors (TLR), are important components of innate immunity, are responsible for recognition of several pathogen-associated molecular patterns (PAMP), i.e., lipopolysaccharide (LPS) of gram-negative bacteria, flagellin protein, peptidoglycan, endogenous ligand of host cell (viral nucleic acids) from microorganisms and danger associated molecular patterns (DAMP) from damaged tissues. TLRs are evolutionarily conserved structures which recognize their respective ligands and mediate downstream signaling cascade to generate cell-mediated immune response against invading pathogenic microorganisms (60).

Although in 1989, Charles Janeway proposed that pattern recognition receptors identify microbial products and ultimately serves as a bridge between innate and adaptive immunity (61), the discovery of TLRs initiated with the cloning of IL-1 receptor (IL-1R) in 1988 (62). IL-1 is a pro-inflammatory cytokine which was reported to induce T cell activation, acute phase response, etc. from the 1980s (63). In 1991, it was found that the cytosolic domain of IL-1 R is structurally homologous with a protein known as toll (64), which is found in *Drosophila melanogaster* and responsible for dorsoventral polarity in early embryos (26). Due to the establishment of dorsoventral polarity, it was shown that a protein named dorsal, which has REL homology domain with NF-κB family proteins, becomes functional (65). Beside this, IL-1 was also found to activate NF- κB pathway (66). Based on this ground, it was suggested that both Toll and IL-1 play vital role in NF- κB pathway-dependent manner in two different models. In support of this,

later, it was found that both Toll and IL-1 share common amino acids to promote NF-κB signaling (67).

In 1994, the next breakthrough came in the form of identification and characterization of a plant protein N, which is responsible for providing resistance against Tobacco mosaic virus (TMV). It was found that the amino-terminal domain of N protein has structural similarity with the cytosolic domain of both Toll and IL-1R. This homologous domain was named as Toll-IL-1-resistance (TIR) domain, and it became evident that structural conservation of this domain is responsible for host defense mechanism in two different kingdoms, animal, and plant (68).

From 1994, mammalian proteins that have more similarity with Toll rather than IL-1 were started to be indexed in Pubmed (62). These proteins had TIR homology domain and Leucine-rich repeats (LRR) like Toll, but in contrast to IL-1, they didn't have an immunoglobulin domain. In 1997, Medzhitov and Janeway showed that one of these toll homolog, termed as hToll when got transfected with CD4 in human monocytes led to activation of NF- κB and also activation of the gene of CD80 (69). This discovery put a significant milestone as it supported Janeway's theory about bridging the innate and adaptive immune component via PRRs. In 1998, total five mammalian toll homologs were characterized and named as Toll-like receptors, and hToll was named as TLR4 (70).

1.2.1.1 Toll-like receptor structure

Toll-like receptors are type I transmembrane proteins and have three structural segments: an extracellular leucine-rich repeat (LRR) domain, a single helical transmembrane domain, and a cytosolic Toll/IL-1 (TIR) domain (Fig. 4). Due to having significant homology in the cytosolic domain TLRs are members of a larger superfamily that includes IL-1R. However, the

extracellular domain of TLRs and IL-1R shows distinct difference in terms of structure. TLRs have LRR where IL-1R has three Immunoglobulin-like domains at the extracellular domain (71).

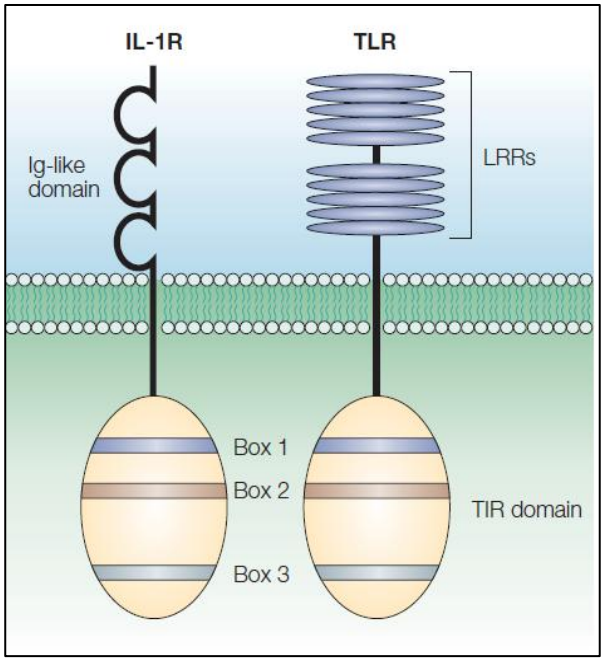


Figure 4: Structure of IL-1R and TLR: Toll/IL-1R (TIR) domain is a conserved cytoplasmic domain found in both Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs). Three highly homologous regions (known as boxes 1, 2 and 3) are present in the TIR domain. The extracellular regions of IL-1R and TLR are different. IL-1Rs have three immunoglobulin (Ig)- like domains whereas TLRs have tandem repeats of leucine-rich regions (known as leucine-rich repeats, LRR). Figure adapted from: (71)

1.2.1.1.1 Leucine-rich repeats: At LRR domain, 19-25 copies of tandem repetitive sequences are present Each repeat has 24-29 amino acids and contain two conserved sequences, the leucine-rich sequence XLXXLXLXX and $XOXXOX_4FXXLX$ (X = any amino acid, L= leucine, F =phenylalanine, O= any hydrophobic amino acid). These repetitive sequences contain a β-strand and an α-helix connected by loops. This LRR domain forms a horseshoe-like structure where various ligands interact (72).

1.2.1.1.2 Toll/IL-1R (TIR) domain: At the cytoplasmic side, both TLR and IL-1R share a conserved region of approximately 200 amino acids which is termed as Toll/IL-1R (TIR) domain (60). About 20-30% amino acids are conserved in the TIR domain. Each TIR domain comprises of three conserved box sequences. The crystal structure of human TLR1 and TLR2 show that TIR domains contain five-stranded parallel β-pleated sheets in the center, surrounded by five α-helices on each side connected by loops (73). LRR domain gets connected with TIR domain with a single time membrane spanning transmembrane helix.

1.2.1.2 TLR/IL-1R signaling mechanism

Ligand binding promotes dimerization (homo or hetero) of TLRs and conformational changes to recruite downstream signaling molecules. Downstream signaling molecules involved in TLR signaling are, myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), transforming growth factor-β (TGF-β)-activated kinases (TAK 1), TAK1-binding protein 1 (TAB1), TAB 2, and tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) (74). MyD88 consists of two domains: One is C-terminal TIR homology domain which interacts with TIR domain of TLRs to initiate downstream signaling, and another is N-terminal death domain (DD) which interacts with the death domain of IRAK4 to relay the signal. Upon ligand binding, MyD88 gets recruited as a dimer to the receptor complex (75). Total of four IRAKs, i.e. IRAK 1, IRAK 2, IRAK 3 and IRAK 4 have been characterized in the mammalian system (76), and among them, only IRAK 1 and IRAK 4 possess kinase activity. Structurally, IRAKs contain death domain in N terminus and a serine-threonine kinase domain at center. N-terminal death domain of IRAK4 interacts with the death domain of MyD88 upon ligand binding. So far six conserved members of TRAF family proteins have been identified in mammals. The structure shows they share an N-terminal-coiled-coil domain (TRAF-N) which contains a RING

(really interesting new gene)-finger/zinc-finger region, necessary for downstream signal processing and an evolutionary preserved C-terminal domain (TRAF-C), necessary for interaction with upstream signaling molecule. TRAF6 is required to process signaling information in TLR/IL1 R superfamily interacting with IRAKs as well as TNF receptor superfamily interacting with CD40 and TNF-related activation-induced cytokine receptor (TRANCER). The conserved sequence in C-terminus of TRAF6 has been deduced as P-X-E-X-X-(D/E/F/W/Y) which has been found in CD40, TRANCER and the IRAKs (77).

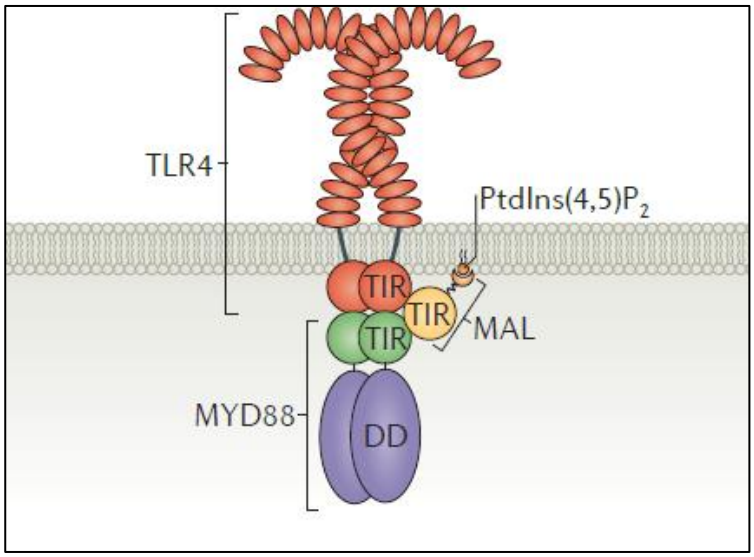


Figure 5. Interaction between TLR4 and its adaptor molecules: A schematic showing the basic structure of TLR4 where different adaptor proteins responsible for TLR4 signaling pathway interacts. MYD88 and MYD88 adaptor-like protein (MAL) interacts with Toll-like receptor 4 (TLR4) through their Toll/IL-1R (TIR) domains. Figure adapted from: (78)

1.2.1.2.1 TAK1 and TAB family proteins: TAK1, member of mitogen-activated protein kinase kinase kinase (MAPKKK) and Two TAK1 binding proteins(TAB), i.e. TAB1 and TAB2 have been shown to participate in NF-κB activation as a result of IL-1/LPS and TNF induction (79). TAB1 is required for activation of the kinase activity of TAK1 whereas, TAB2 links TAK1 and

TRAF6 resulting in TAK1 activation (80). Reports suggest that TRAF6 works as a ubiquitin ligase and triggers ubiquitylation of TAK1 interacting via RING-finger domain with ubiquitin-conjugating enzyme 13 (UBC13). A lysine63-linked polyubiquitin chain forms due to UBC13-TRAF6 complex induced TAK1 activation (81).

1.2.1.2.2 NF-κB family proteins: There are five members of NF-κB family proteins- p65 (REL-A), REL-B, cytoplasmic(c) REL, p50, and p52. These proteins present as either monomeric (active form) or dimeric form (inactive form). In the dimeric stage, NF-κB proteins stay inactive at cytoplasm due to phosphorylation by an inhibitor of NF-κB family (IκB) proteins. IκB kinases (IKK) act as a positive regulator of NF-κB signaling. It has two catalytic domains-IKK-α and IKK-β and a regulatory subunit-IKK-γ or NF-κB essential modulator (NEMO). After activation due to upstream signaling, IKK results in ubiquitin-mediated degradation of IκB, which leads to translocation of active NF-κB proteins in the nucleus (82).

TLR downstream signaling cascade follows two different adaptor molecule based pathways- one is MyD88 dependent pathway which is followed by all TLRs except TLR3, and another is TRIF dependent pathway which is followed by both TLR3 and TLR4.

1.2.1.2.3 MyD88 dependent pathway: In case of TLR4, lipopolysaccharide (LPS) of Gram-negative bacteria acts as a substrate. After binding with LPS binding protein (LBP), LPS-LBP complex is detected by CD14 molecule of the host cell. This interaction promotes the binding of LPS with MD2-TLR4 complex followed by dimerization of TLR4. Dimerization results in the recruitments of adaptor protein MyD88 followed by interaction of MyD88 with IRAK kinases resulting formation of Myddosome complex (83). In downstream signaling, IRAK1 gets auto-phosphorylated upon activation by IRAK4 (84). IRAK1 interacts with the RING domain of TRAF6 (an E3 ubiquitin ligase). TRAF6 along with UEV1A and UBC13 promotes k63-linked

ubiquitin-mediated degradation of TRAF6 itself and TAK1 protein kinase complex. As a result of this two different pathways, MAPK and IKK complex mediated NF-κB pathway get activated. Ubiquitinated TAK1 interacts with IKK complex, and IKK-β gets activated by phosphorylation. Phosphorylated IKK-β phosphorylates IκBα, a negative regulator of NF-κB and thus facilitates nuclear translocation of the active NF-κB complex (p50 and p65) to induce proinflammatory gene expression (85).

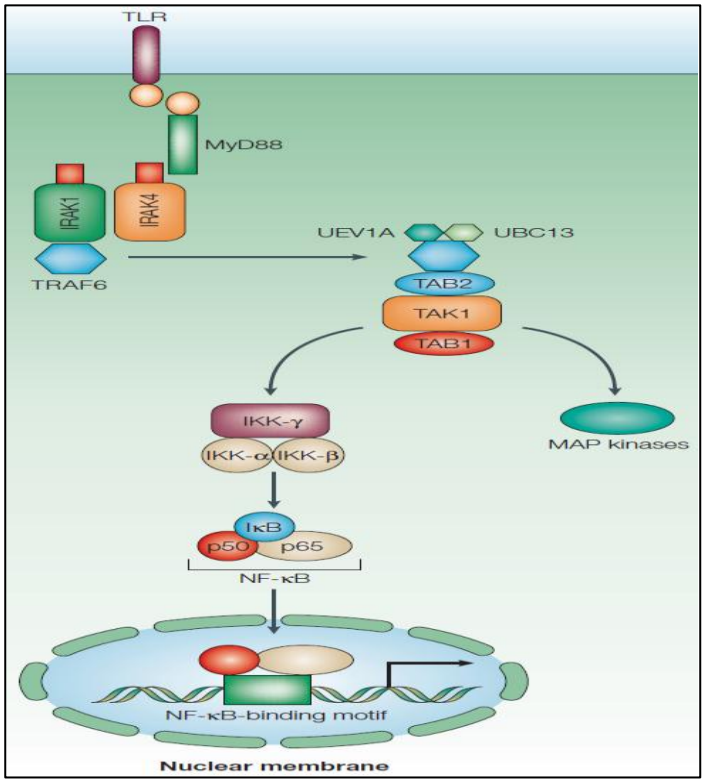


Figure 6. Basic signaling mechanism of TLR via MyD88 pathway: When TLR specific ligand interacts with TLRs it triggers the association of MyD88, which then recruits IRAK4 (IL-1R-associated kinase 4), in turn allowing its interaction of IRAK1. Phosphorylated IRAK1 then recruits TRAF6 (tumour-necrosis-factor receptor-associated factor 6). Phosphorylated IRAK1 and TRAF6 then dissociates and forms complex with different downstream signaling molecules which leads to activation of MAPK or NF-κB pathway activation. Figure adapted from: (71)

1.2.1.2.4 TRIF-dependent pathway: TLR3 and TLR4 follow TRIF mediated signaling strategy where, after ligand binding, an adaptor protein TRIF gets recruited. In the downstream pathway, TRAF6 activates RIP-1 kinase upon interaction with TRIF. Activated RIP-1 positively regulates TAK1 complex, which in turn activates NF-κB and MAPK to induce pro-inflammatory cytokine response. Beside TRAF6, TRAF3 can also be induced by TRIF and initiate downstream signaling. Activated TRAF3 results in IRF3 phosphorylation, with the help of TBK1, IKKi, and NEMO. Translocation of Phosphorylated dimeric IRF3 into nucleus results in type I interferon (IFN) response generation (85,86).

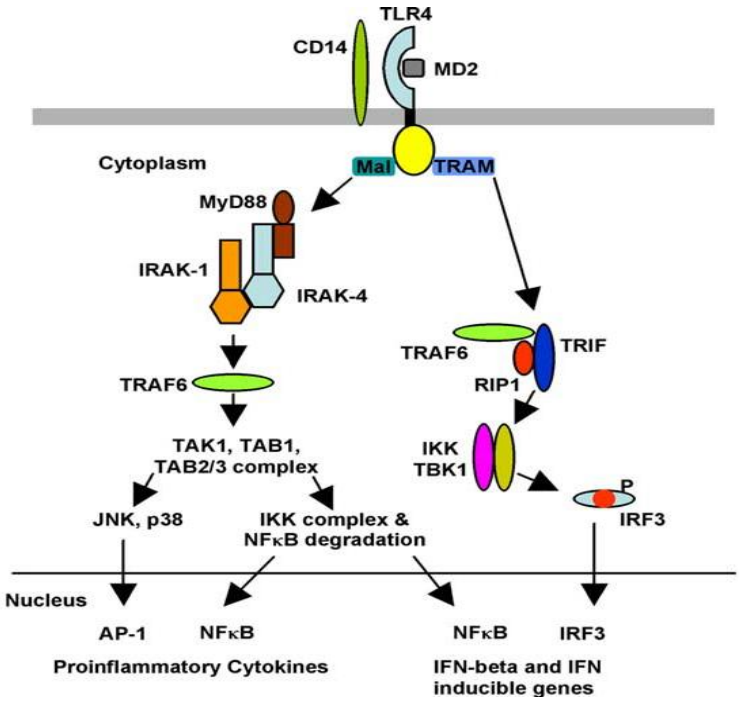


Figure 7. MyD88 dependent and independent pathway: Stimulation of Toll-like receptor 4 (TLR4) leads to the activation of two different pathways: the MyD88 dependent and MyD88-independent pathways. The MyD88-dependent pathway leads to NF-κB activation and production of inflammatory cytokines. The MyD88-independent pathway activates IFN-regulatory factor (IRF3) and late phase of NF-κB activation. Both of these lead to the production of IFN-β and the expression of IFN-inducible genes. Figure adapted from: (87)

1.2.1.3 TLR in T cells

TLR4 is a very well-studied pathogen recognition receptor in innate immune cells. It has been already known that TLR4 can direct the adaptive immune cells to elicit an immune response against invading pathogens through innate immune cells. There are reports which show TLRs can directly modulate T cell function. Several studies suggest the expression and functional significance of TLRs in T cells (88,89). Expression of different TLRs in naive and activated T cells and their role in $CD4^+$ T cells survival has been reported (90). TLR2 stimulation via Pam3CSK4 has been shown to enhance the proliferation and survival of mouse regulatory T cells (91). Transcription factor forkhead box P3 (FOXP3) was found to be regulating the expression of TLR10 in human regulatory T cells (92). However, direct TLR ligand sensitivity in T cell is a controversial topic since there are reports suggesting TLR response in T cell is costimulatory in nature (93). It has been reported that T cell adhesion and chemotaxis could be regulated by LPS (94). Moreover, evidence suggests that TLR2 and TLR4 signaling could up-regulate SOCS3 (suppressor of cytokine signaling 3) expression and downregulate T cell effector function (94). Moreover, recently there were several reports suggests that T cells also express TLR4 and there are some reports of contradiction about its role in the regulation of T cell effector function upon LPS treatment in different models (95,96). The absence of TLR4 signaling can affect $CD4^+$ T cell effector function. MyD88 signaling promotes IFN-γ production in $CD4^+$ T cells in response to bacterial infection (97). MyD88 signaling is required for effective $CD4^+$ T cell function against *Chlamydia muridarum* Genital Tract Infection in females (98). Recent evidence suggests MyD88 signaling is required for induction of Th1 and Th17 response (99). MyD88 signaling is also required for virus-specific $CD8^+$ T cell differentiation during LCMV infection (100). Classically TLRs are known to be most efficient modulators of innate immunity. However recent evidence proposes an important role of TLRs in modulating adaptive immune response. There were certain

reports which suggest that TLR4 is polarized towards TH1 response (101,102). However, the requirement of TLR4 responses towards TCR or mitogen directed acute stage T cell activation and effector function in wild-type naïve T cell population, if any, is not well reported.

Viral inhibitory peptide for TLR4 (VIPER) is an inhibitory peptide (11 aa long) specific for TLR4 derived from the A46 protein of vaccinia virus. It interacts with adaptor proteins: MyD88 adaptor-like (Mal) and TRIF-related adaptor molecule (TRAM) to inhibit TLR4-mediated MAPK and transcription factor activation. It had been shown that VIPER is able to inhibit TLR4 mediated immune response in innate immune cells such as macrophages (103). In another study, VIPER inhibited inflammatory responses elicited by *Mycoplasma pneumoniae* in mouse macrophage suggesting the role of TLR4 in the *M. pneumoniae* mediated inflammatory responses (104). Additionally, in monocyte cell line THP-1 VIPER is known to inhibit the TLR4 and CD14 mediated induction of IL-6, IL-10, and MCP-1 by electronegative LDL and ceramide-enriched LDL (CER-LDL) (105,106). Furthermore, treatment of mouse neuronal cells with VIPER was found to block TLR4 mediated CXCL1 expression and release completely. It also inhibited ICAM-1 and VCAM-1 expression on endothelial cells, and induced infiltration of neutrophils across the endothelial monolayer (107). Treatment of VIPER through intracerebroventricular route in hypertensive rat lead to reduced circulating norepinephrine levels which resulted in inhibition of delayed progression of hypertension and improvement cardiac hypertrophy and function. Further, it reduced myocardial TNF-α, IL-1β, iNOS levels, NF-κB activity, and altered renin-angiotensin system components significantly (108). This reveals the role of TLR4 in brain attenuation of angiotensin II-induced hypertensive response. Additionally, *in vivo* treatment of VIPER is also found to protect the rat from acute kidney injury mediated by LPS driven TLR4 stimulation. It also facilitates improved glomerular filtration rate, elevated renal blood flow, and a reduced renal vascular resistance, reduction in the rate of production of free radicals (reactive

oxygen species and superoxide), proinflammatory cytokine, and acute kidney injury markers (109). Collectively, these studies show the role of VIPER in understanding various TLR4 driven response in different cells and systems. In this study, we have used VIPER, as an established TLR4 signaling inhibitor to investigate the effect of TLR4 signaling disruption on T cell effector function naive T cells derived from mice and healthy human donors.

1.2.2. TRP Channels:

Transient Receptor Potential (TRP) channels are a group of ion channels which are non-selectively permeable to different cations. These channels are among the most diversified family of ion channels and are involved in a wide range of sensory and cellular functions (110). TRP channels mediated in the sensation of different type sensations like pain, pressure, cold, warm, etc. These sensations lead to regulation of several downstream cellular cascade events which are critical in function of different cell types. Functional role of TRP channels have been attributed towards different physiological events, and mutations of TRP channels have been implicated in diverse pathophysiological conditions. Stimuli such as temperature, pain and non-selectively permeable to cations warrants a study of these channels in immune cells. Moreover, recent evidence suggests the presence of TRP channels in immune cells which needs a further in-depth study (111-115).

1.2.2.1 Discovery of TRP Channels

In 1969, Cosens and Manning first reported the role of TRP channel in *Drosophila melanogaster*. They observed spontaneous formation of a group of *Drosophila melanogaster* mutant flies behaving as blind when exposed to bright illumination with the effect being reversed upon withdrawing bright light at least for a minute (116). Due to its transient response towards the sustained intense lights, Minke et al. in 1975 termed this *D. melanogaster* mutant as ‘transient

receptor potential,' otherwise called as "trp" mutant (117). Later on the term "TRP" was adapted for the whole TRP superfamily of the ion channels in 2002 by an international researchers committee (118).

The DNA carrying *trp* gene was first isolated in 1985 and reports regarding the sequencing, cloning and molecular characterization of *trp* gene were published in the year 1989 (119-121). From the amino acid sequence, *trp* gene product was suggested to be a membrane protein with up to 8 transmembrane segments. Presence of many features similar to some receptor or transport proteins distinguished this gene product from the previously reported ligand-gated and the voltage-gated ion channels (120). The role of *trp* channel as a Ca^{2+} permeable channel was established through patch clamping technique used by Hardie (122). It has been observed that the original Cosens-Manning trp mutant (trp^{CM}) appears to have rapid electroretinogram decay to baseline and moderate dark recovery kinetics while grown at 24°C as compared to 19°C suggesting that trp^{CM} is a developmental mutant with the temperature-sensitive attribute (123). This observation indicates the significance of temperature in the functioning of the *trp* channel apart from light. Discovery of the role of TRPV1 as the "Capsaicin receptor" acting in the pain pathway as a heat-activated ion channel was a major discovery in the field of TRP channel research (124).

1.2.2.2 Diversity in TRP channels

TRP ion channels are well conserved from protists, worms, and flies to humans (125-128). Based on amino acid sequence and structural homology, TRP channels are classified into 7 subfamilies (129) (**Fig. 1**). The TRP canonical (TRPC) subfamily has the closest homology to the Drosophila *trp* channels. The TRP vanilloid (TRPV) name has been designated after the founder member Vanilloid Receptor 1 (TRPV1). The TRPM subfamily is homologous to TRPM1 that was initially

named as “melastatin,” as its expression level has an inverse correlation with the melanoma cell lines (130). The name TRPMLs was derived from TRPML1, a mutation which gives rise to a neurodegenerative lysosomal storage disorder known as Mucolipidosis type IV (131). TRPPs obtained their name from ‘polycystins’ that was named after TRPP2 mutation, which cause autosomal polycystic kidney disease in case of humans (132). Presence of multiple ankyrin repeats is the major characterizing feature of TRPA subfamily. The TRPN subfamily members obtained their names from the *Caenorhabditis elegans* ‘NO-mechano-potential C’ (NOMP-C) channel. TRPN channels are found to express in invertebrates like worms and flies but not found in case of mammals. Zebrafish is the only vertebrate that harbors one of the members of TRPN family (133). TRPY (‘yeast’ TRP), the eighth subfamily is distinctly related to the other seven classical TRP channel subfamilies. TrpY1 is the only channel found in TRPY subfamily which plays the role of mechano-sensor for vacuolar osmotic pressure in yeast (134). More than 200 TRP channels across species along with 28 Mammalian TRP channels have been reported till date (135).

There are several reports predicting the presence of TRP channels in parasites (136), but there are only a few reports with experimental evidence. In *Leishmania* major, two TRPML-like genes have been reported, *viz. lmmlA* and *lmmlB* (137). Additionally, in *Schistosoma mansoni*, a locomotor activity regulator TRPA-like channel responding to TRP channel specific drugs was reported (138). These evidence indicate towards the presence of structurally and functionally diverse TRP channels in Metazoans.

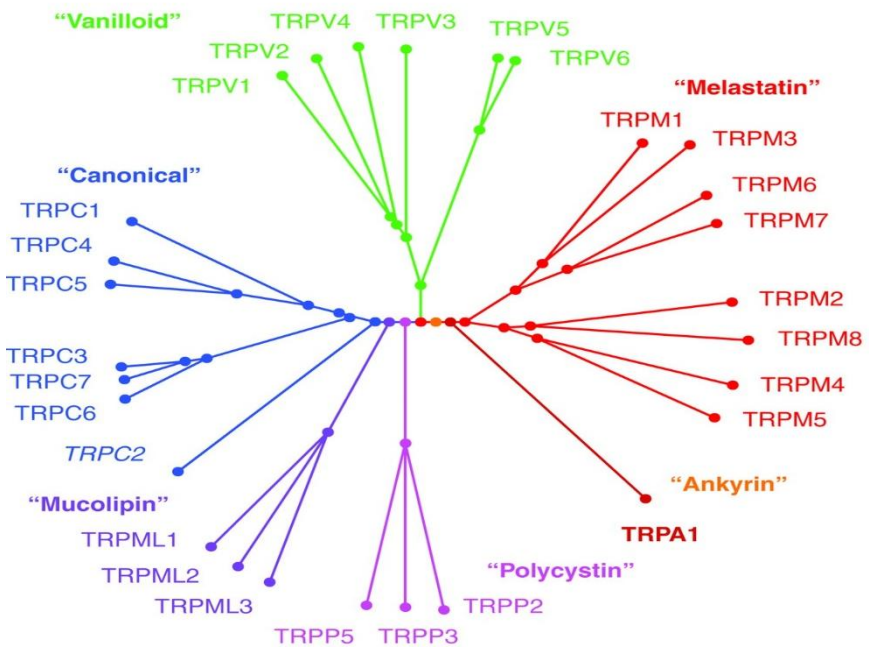


Figure 8. The TRP channel tree: The phylogenetic tree represents the Transient Receptor Potential (TRP) ion channel superfamily. TRP channels are divided into seven subfamilies which include TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin). Figure adapted from: (139)

1.2.2.3 Structure of TRP channels

The TRP channels are represented by six transmembrane helices with intracellular C-terminus and N-terminus regions. In between the 5th and 6th transmembrane regions, the pore-forming loop is present. Comparative studies between TRP channel pore loop and voltage-gated channels pore loop suggests the absence of voltage sensitivity contributing residues within the TRP superfamily (140). The intracellular N-terminus contains multiple ankyrin repeat motifs. Intracellular N- and C-terminus domains, extracellular loops contain different ligand binding sites. The "TRP-box," a conserved 25 amino acid sequence, is present immediately after the 6th transmembrane helix in many members of the TRP channels (118). The C-terminal region of TRP channels often has the "TRP-box." TRPCs possess 3 - 4, TRPAs 14 -17, TRPVs have 6 and TRPNs carry around 29

ARDs. ARDs are known to be associated with TRP channel tetramerization with being involved in molecular interactions with regulatory factors and other proteins (141,142). In several TRP channels, specific motif sequences are present with specific functions, like an EF-hand motif for sensing Ca^{2+}, sites for phosphorylation, sequences for interaction with cholesterol, Calmodulin and PIP_2 binding sites and tubulin binding sites (143-146).

It is evident that most of the TRP channels function as homotetramers. However, heteromultimeric channel formation between members of different subfamilies or the same subfamily has also been reported in some cases like in TRPCs (147) and TRPV channels (148). Heterotetrameric channels probably have absolutely different functions and properties.

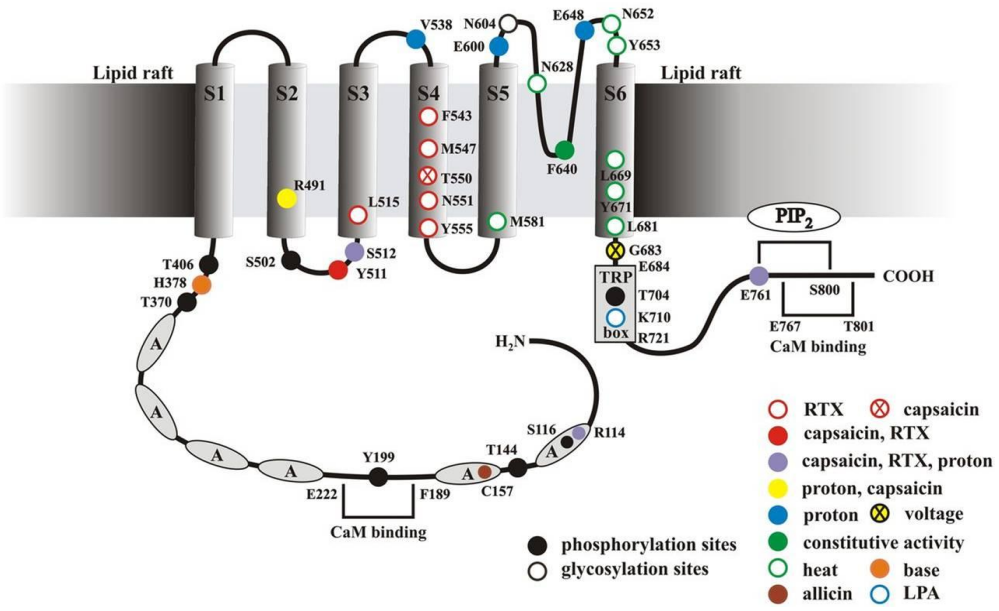


Figure 9. Schematic representation of mammalian TRPV monomer: TRPV monomer Contain six transmembrane segments where intracellular N- and C-terminus are present in the cytoplasmic side. The N-terminus contains ligand binding sites and multiple Ankyrin repeats, while C-terminus contains a conserved TRP-box motif just after the end of transmembrane 6 domain. Other TRP channels follow similar structural patterns. Figure adapted from: (149)

1.2.2.4 Functions of TRP channels

Expression of TRP channel varies largely in different individuals, tissues, and cells. Each cell type present within the tissues possibly expresses one or more kind of TRP channels. TRP channels act as non-selective cation channel and, present on the plasma membrane and also in different intracellular organelles. Most of the TRP channels are found in the plasma membrane, regulating the intracellular levels of different cations, for example, $Mg^{2+,}$ Ca^{2+}, and other trace metal ions either indirectly or directly. Many physiological processes, like homeostatic functions (thermoregulation, osmoregulation, minerals reabsorption and hormonal secretion), sensory functions (temperature sensation and nociception), motor functions are affected by the functions of the TRP channels. In many cases, the TRP channels interact with the site-specific scaffolding and signaling molecules to perform specific functions at specific locations. Extensive studies have strengthened the literature about the functional significance and localization of specific TRP channels in different cell types.

The TRPV subfamily has been studied mainly in sensory neurons and is responsible for nociception, thermosensation, and neurogenesis. TRP channels like TRPV1, TRPV2, TRPV3, and TRPV4 have been attributed to different functions associated with sensory neurons. TRPV3 expression was reported to be low in sensory neurons whereas it is predominantly expressed in skin keratinocytes (150). TRPV3 has a significant role in maintaining skin integrity, hair growth and wound healing. TRPV3 knockout mice also reveal its importance in the hair morphogenesis. This study reveals that mice lacking TRPV3 results in the wavy hair coat and curly whiskers (151). Moreover, TRPV3 has been found to be expressed in corneal epithelial cells and involved in thermosensation and wound healing (152). TRPV5 and TRPV6 are exceptionally selective for Ca^{2+} (**Fig.10**) and thus play a crucial role in reabsorption of Ca^{2+} in kidney and intestines, along

with facilitating Vitamin D3 driven keratinocyte development in the skin (153). Mutations in TRPV3 leads to a condition called Olmsted syndrome (154). TRPV4 plays the role of a mechano-sensor and osmo-sensor and has also been involved in vasculature maintenance, voiding control and bone homeostasis. TRPV4 mutation has been reported to be associated with different genetically inherited disorders of bone growth like Skeletal Dysplasia and Brachyolmia (155,156), neurodegenerative disease conditions like Scapuloperoneal Spinal Muscular Atrophy (SPSMA) and CMT2C (Charcot–Marie–Tooth disease type 2C) (157-160).

TRPC1, TRPC3, TRPC5, and TRPC6 are the members of TRPC subfamily which are ubiquitously present in neurons and brain, regulating the development of the brain and its functions in relation to neuronal plasticity (161). TRPC2 works for, determining sexual behavior and pheromone detection, whereas, TRPC4 and TRPC7 are assigned for vascular and respiratory management, respectively (161). *Trpc4*$^{-/-}$ mice study has demonstrated that TRPC4 is an essential component of Store Operated Calcium Entry (SOCE) (162). Another study in Chinese hamster ovary shows that TRPC1 is an essential component of SOCE (163). TRPC6 mutations have been reported to cause Focal and Segmental Glomerulosclerosis, a human proteinuric kidney disease (164).

Among the members of TRPM superfamily, TRPM1 serves as a light sensor in the retina and acts as a tumor repressor in melanoma cells whereas TRPM2 has a significant role for insulin release by the pancreas gland. TRPM3 acts as a sensor for steroid hormone pregnanolone. TRPM4 has a regulatory role in catecholamine secretion from chromaffin cells, histamine release by mast cells, and release of vasopressin at hypothalamus of the brain. TRPM6 and TRPM7 channels act as major Mg^{2+} reabsorption channels in intestine and kidney (165). Furthermore, these two channels have a regulatory role in maintaining intracellular levels of trace elements

including Zn^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+} (166). TRPM8 that is activated below 25°C plays the role of a cold-sensor, testosterone receptor, and menthol receptor. It is also known to be involved in prostate cancer, Parkinson's disease and Chronic Obstructive Pulmonary Disease (COPD) (167-170). Defective Mg^{2+} absorption in the intestine and renal Mg^{2+} leak is frequent when there is a mutation in TRPM6 that causes hypomagnesemia with secondary hypocalcemia (HSH/HOMG) (171).

TRPA1 is the only member of the TRPA subfamily that acts as a thermo-sensor in some species with being involved in chemosensation, itch sensation, olfactory responses, and nociception. TRPA1 is also believed to be involved in Familial Episodic Pain Syndrome (FEPS) (172).

TRPML1, TRPML2, and TRPML3 are mostly residents of intracellular vesicles where they regulate Lysosomal and Endosomal functions and Autophagy as well (173). Mutations in TRPML1causes a neurodegenerative lysosomal storage disorder called as MLIV (Mucolipidosis type IV) (174).

The members of TRPP are predominantly involved in renal functions and act as flow-sensor in endothelium. A disease called Polycystic Kidney Disease (PKD) characterized by enlarged kidneys and renal failure is caused by a mutation in TRPP2 (175). According to recent studies, during early vertebrate embryogenesis, the formulation of left-right asymmetry is affected by the TRPP2 mediated Ca^{2+} signaling (176).

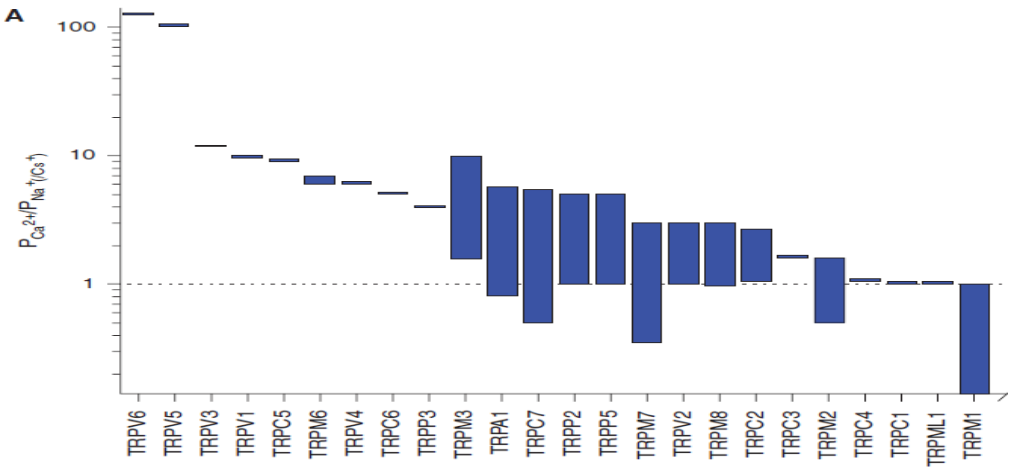


Figure 10. Ca^{2+} vs Na^{+} selectivity of TRP channels. Among the members of TRP channels, TRPV5 and TRPV6 are extremely Ca^{2+} selective with TRPV1, TRPV3, TRPV4, TRPC5, TRPC6 and TRPP3 being mildly Ca^{2+} selective. Remaining TRP channels including TRPM8 are mostly nonselective for Ca^{2+}. Figure adapted from: (139)

1.2.2.5 TRP channels in T cells

Ca^{2+} works as an important second messenger in many cells including immune cells. When immune cells were stimulated with cell-specific receptors intracellular Ca^{2+} concentration increases (177). Ca^{2+}-signaling in immune cells is important for immune regulatory functions such as gene transcription, differentiation of immune cells, and effector functions (178). Different Ca^{2+}channels play important yet different regulatory functions in immune cells like T cells. Expression of different TRP channels was reported by different research groups (179). Expression of TRPA1 mRNA and protein were detected in human T cell line Jurkat, in lymph nodes and in the spleen (180). TRPC group of TRP channels was reported to be expressed in T cell (181). TRPC1 and TRPC3 are associated with the Ca^{2+} influx in T cells during cannabinoid and TCR mediated stimulation (181,182). Up-regulation of TRPC3 mRNA expression in $CD4^{+}$ T cells after TCR stimulation was reported (183). Similarly, TRPC5 is found to be up-regulated in

activated $CD4^+$ and $CD8^+$ human T cells (184). TRPC5 is assumed to have a role during the TCR-mediated suppression of T cell effector functions in experimental autoimmune encephalomyelitis (EAE) (184) and in the NOD (non-obese diabetic) mouse model of type 1 diabetes (185). TRPC3 and TRPC6 levels are found to be upregulated in T cells of mouse having sepsis and induction of apoptosis in T cells (186).

TRPV1 expression has been shown in primary mouse and human T cells(179). TRPV1 agonist Capsaicin induces increases in Ca^{2+} levels and apoptosis in human T cells and Jurkat cells (187). The functional expression of TRPV1 has been shown in Jurkat cells by Ca^{2+} imaging and electrophysiology (188). Use of TRPV1 antagonist suggested the importance of TRPV1 for TCR-induced Ca^{2+} influx and T cell activation (189). Functional TRPV5 expression in mouse and human primary T cells and in Jurkat cells have been reported (190). TRPM2 is was found to be endogenously present in mouse $CD4^+$ T cells and can regulate TCR mediated T cell proliferation and secretion of pro-inflammatory cytokines (191). TRPM4 have been associated with regulation of T cell development towards Th1 and Th2 by differentially regulating calcium signaling and NFAT localization (192). Deletion of TRPM7 in mice showed disruption of Thymocyte differentiation in the thymus which results in decreased number of circulating T cells (193).

Ca^{2+} ions are ubiquitous universal secondary messengers in all eukaryotic cells. Immune cells like macrophages, T cells, and B cells maintain a low concentration of intracellular Ca^{2+} ions in their resting state (178). However, engagement of specific-receptors like the T cell receptor, B-cell receptor, Fc-receptors, chemokine receptors, co-stimulatory receptors, etc. present on the immune cell surface are known to induce an increase in intracellular Ca^{2+} concentration (177). The Ca^{2+}-signaling in immune cells is vital for several regulatory functions such as differentiation of immune cells, activation and effector functions (178,194). On TCR stimulation, PLC-γ1 gets

activated and in turn, stimulates IP_3 production. The IP_3 quickly diffuses into the cytosol and binds to IP_3R on endoplasmic reticulum (ER) membrane, leading to the release of Ca^{2+} from ER into the cytosol. Depletion of essential Ca^{2+} levels in ER triggers a sustained intracellular Ca^{2+} from the plasma membrane (PM) via calcium release activated calcium channels (CRAC), a process now referred as store-operated calcium entry (SOCE) (195). This increased level of intracellular Ca^{2+} is necessary for IL-2 receptor expression, and IL-2 production, which are critical for T cell activation and, such events can be effectively blocked by using Ca^{2+} chelator EGTA (196).

CRAC channel is composed of two components – stromal interaction molecule 1 (STIM1) and Orai1. Ca^{2+} sensor protein in ER is STIM1, which recognizes Ca^{2+} depletion and the pore-forming unit at PM is Orai1, composed of 4 transmembrane (TM) units. STIM1 is also a transmembrane protein with protein interaction domains at both the ER lumen and cytosol. ER lumen domain consists of a paired EF hands which recognize Ca^{2+} with low affinity. Hence, STIM1 serves as a calcium sensor. Two STIM proteins are found in mammals namely, STIM1 and STIM2. STIM1 directly interacts with Orai1 and forms final pore complex. STIM2 is important for maintaining basal Ca^{2+} levels and sustaining the late phase of SOCE. Upon ER Ca^{2+} depletion, STIM1 molecules start aggregations in clusters and preferentially localize to sites of ER-PM junctions, where they colocalize with clusters of Orai1. CRAC channels are the dominant Ca^{2+} channels present on the PM, although other Ca^{2+} channels are also present (197).

TCR induced an increase in intracellular Ca^{2+} leads to activation of various Ca^{2+} and calmodulin-dependent transcription factors. It also activates signaling proteins like phosphatase calcineurin and Ca^{2+}- calmodulin-dependent kinase (CaMK). Activated calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) family, and hence NFAT translocates to the nucleus. Within

the nucleus, NFATs are capable of cooperatively integrating with a variety of other transcription factors, thereby enabling a plethora of diverse transcriptional processes. One such instance is cytokine IL-2 production, where transcription factors NFATs/AP-1 complex integrates. NFATs are also reported to interact with regulatory T cell lineage transcription factor, forkhead box protein 3 (FOXP3). Additionally, the rise in intracellular Ca^{2+} also promotes adhesion molecules like LFA-1 interact with ICAM-1 on APCs and aid in the formation of the immunological synapse (198).

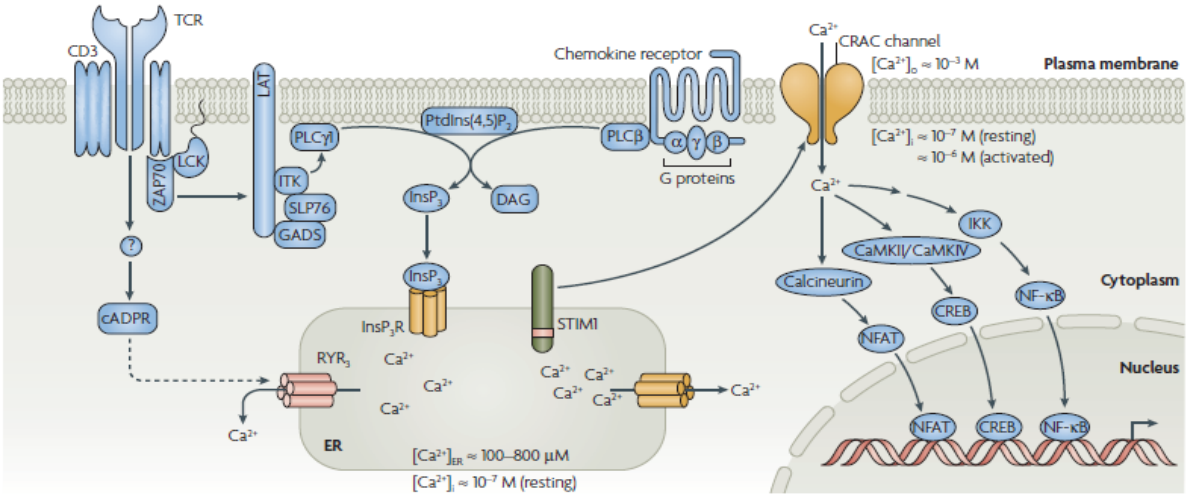


Figure 11. Calcium dynamics in T cells: The intracellular cytosolic Ca^{2+} concentration in T cells is tightly regulated and maintained at ~ 100 nM (resting cell) to ~ 1μM (TCR activated cell). In resting T cells, a sharp gradient of Ca^{2+} concentration exists between extracellular space and cytoplasm as well as in between cytoplasm and endoplasmic reticulum. Following TCR stimulation, protein tyrosine kinases (LCK, ZAP70) gets activated, triggering phosphorylation of adaptor proteins (SLP76, LAT). This leads to the recruitment and activation of PLCγ1. Similarly, GPCRs can also recruit another variant of PLCβ. Hydrolysis of membrane phospholipid (Ptdlns(4,5)P_2) to $InsP_3$ and DAG, can be carried out by both PLCγ1and PLCβ. $InsP_3$ diffuses freely into the cytosol and binds $InsP_3$Rs, on the surface of the endoplasmic reticulum, which in turn leads to the release of calcium from intracellular stores. The decrease in Ca^{2+} in the endoplasmic reticulum is sensed by (stromal interaction molecule) STIM1 proteins,

which in turn activates calcium release activated calcium (CRAC) channel. CRAC channel activation opens up the Ca^{2+} flood gates and an immediate rise in intracellular Ca^{2+}, in turn, activates enzymes like calcineurin and thereby activates NFAT, NF-kB, and CREB. These events trigger T cell proliferation and effector cytokines response. Figure adapted from: (177)

Immune cells and their activation mechanism, as well as regulation, are very diverse in nature. Ca^{2+} channels play important regulatory functions which vary according to the channel. Nevertheless, the complexity of these different Ca^{2+} channels from the perspective of different immune functions and their molecular identities are yet to be explored in details. The effect of temperature changes, on the immune activation, has been well reported (199-206). After infection, increase in body temperature is known to initiate activation of the immune system for host protection (207,208). Cytotoxic activities of T cells from adult blood and cord blood can be enhanced at slight increase of temperature (< or = 40°C) whereas it decreases further if exposed to 42°C for 1 hr (209). Effect of body temperature on different properties of T cell morphology, altered distribution in different tissues and changes in the key molecules has been demonstrated. Increased PKC activity and redistribution of PKCs within T cell via different temperature treatment have been reported (210). The benefit of mild hyperthermia and thermal stress in the enhanced immune activity is well reported (211-213). However, the exact mechanisms and molecular players involved in the process are not well understood. Moreover, latest studies have suggested the involvement of heat shock proteins (HSPs) in temperature-mediated effects on the immune cells (214). The sensitivity towards change in temperature and its function suggests thermosensitive ion channels might be associated with temperature-dependent immune modulations. Recently it has been shown that temperature-dependent activation of STIM1 (a Ca^{2+} channel) can induce Ca^{2+}-influx and modulate gene expressions associated with immune functions (215,216).

Transient receptor potential (TRP) channels are non-selectively permeable cation channels among which several members are thermosensitive in nature. So far many TRP channels have been found to be thermosensitive in nature. TRPV1 can be activated at elevated temperatures with a threshold of 43°C, (215) whereas other TRPV channels including TRPV2 (temperature threshold ≥52°C), TRPV3 (temperature threshold ≥33°C), and TRPV4 (temperature threshold ≥27°C) are known to act as thermosensors (217,218). TRPV1 has high Ca^{2+}-permeability (P_{Ca}/P_{Na}= 9.6) whereas TRPV2 (VRL-1) channel which is 50% identical to TRPV1 shows low Ca^{2+}-permeability (P_{Ca}/P_{Na}= 2.9). TRPV4 has ~40% similarity to TRPV1 and TRPV2, and it is moderately selective to Ca^{2+} (P_{Ca}/P_{Na} = 6). The "thermosensitivity" and "Ca^{2+}-permeability" property of TRP channels suggests that this group of ion channels might be associated with immune cell function which is also modulated by the change in temperature and Ca^{2+} concentration. Few reports have suggested the presence and functional role of thermosensitive TRP channels in different immune cells, particularly in macrophages and dendritic cells (112). One study suggests, Ruthenium red (10μM), a non-selective TRP channel blocker can suppress LPS-induced TNFα and IL-6 production in macrophage cells (219). Similarly, vanilloids are known to modulate expression of the genes involved in inflammatory response (such as iNOS and COX-2) in macrophages by interfering with upstream signaling events of LPS and IFN-γ (113,219). LPS-induced ERK, JNK, and IKK activation pathways are also known to be inhibited by these vanilloids (220). TRPV1 activators, capsaicin, and resiniferatoxin (RTX) are known to inhibit LPS- and IFN-γ-mediated iNOS expression and NO production (113). Capsaicin treatment was also found to inhibit transcription of LPS- and PMA-induced COX-2 expression and prostaglandin production in macrophages (220). RT-PCR and immunoblot data has confirmed the expression and functional significance of TRPV2 in macrophages (115). This study showed that

macrophages isolated from TRPV2 knock out animals (*Trpv2*$^{-/-}$) have impaired ability for particle binding and phagocytosis (115).

Form the literature study it as apparent that TLRs and TRP channels are present in different cell types including immune cells. TLRs are very well studied receptors in the context of innate immunity. Moreover, they are found to modulate the adaptive immune system indirectly through innate immune cells. Very few reports suggest the expression of different TLRs in T cells and their functional significance towards T cell function. However, the functional significance of TLRs in naïve T cells effector function is not well studied. Immune cells are known to respond differentially towards different temperature. Moreover, Ca^{2+} is an important molecule associated with key immune cell functions and their development. TRP channels are known to sensitive for change in temperature, and non-selectively permeable to cations like Ca^{2+} makes it ideal receptor for study in immune cells. Several TRP channels have been studied in immune cells like dendritic cells and macrophages. Functional role of TRP channels in innate immune cells has been reported by a few studies. However, the functional role of TRP channels in T cells is not well reported and needs further investigation. Based on the above factors we hypothesized that there might be an altered expression TLRs and TRP channels in T cells during T cell activation which might have functional significance towards T cell effector function. In this study, we have studied the expression of different TLRs and TRP channels in mouse and human T cells during resting and activated state. Further, we investigated the functional significance of these receptors towards T cell activation and its effector response, *in vitro*.

CHAPTER # 2

Hypothesis

&

Specific Objectives

2.1 Hypothesis:

There could be an altered expression of TLRs and TRPs channels in T cells that might have important implications for T cell activation and effector responses.

2.2 Objectives:

1. To study the differential expression of TLRs in T cells during resting and activation.
2. To investigate the functional role of TLRs response in T cells by using specific TLR inhibitors during T cell activation.
3. To study the differential expression of TRP channels during T cell activation.
4. To explore the functional role of TRP channels in T cell effector function by using TRP channel modulators.

CHAPTER # 3

Materials

&

Methods

3.1 Materials.

3.1.1 Animals:

Female or male BALB/c mice of 8–10 weeks old were used in the experiments. Mice were from the animal facility of National Institute of Science Education and Research (NISER). Animals were maintained in 12 h light/dark alternate cycles with food and water provided ad libitum. All experiments using animals were approved by NISER's Institutional Animal Ethics Committee according to the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

3.1.2 Antibodies:

Table 2. Details of antibodies used

Sl. No.	Antibodies (Concentration/dilution)	Company	Catalog no./Clone
1	PE Cy™5 Anti mouse CD3 (0.05μg/sample)	BD Biosciences, CA, USA	555276/17A2
2	Anti mouse CD90.2 APC(0.025μg/sample)	Tonbo biosciences	20-0903-U100/30-H12
3	Anti mouse CD25 PE(0.1μg/sample)	BD Biosciences, CA, USA	553866/PC61
4	Anti mouse CD69 PE(0.1μg/sample)	BD Biosciences, CA, USA	553237/H1.2F3
5	Anti mouse CD69 FITC(0.1μg/sample)	Tonbo biosciences	35-0691-U100/H1.2F3
6	Anti mouse CD95 PE(0.1μg/sample)	BD Biosciences, CA, USA	554258/Jo2
7	Anti mouse CD95L PE(0.1μg/sample)	BD Biosciences, CA, USA	555293/MFL3
8	Anti mouse CD284(TLR4)AF488(0.1μg/sample)	eBiosciences, CA, USA	53-9041-82/UT41

9	Anti mouse TLR2 AF488(0.25µg/sample)	IMGENX	IMG-416AF488/TL2.1
10	Anti mouse TLR5 PE(0.25µg/sample)	IMGENX	IMG-663D/85B152.5
11	*In vivo* Ready™ Anti-Mouse CD28 (1µg/ml)	Tonbo Biosciences	40-0281-U100/37.51
12	*In vivo* Ready™ Anti-Mouse CD3(2µg/ml)	Tonbo Biosciences	40-0032-U500/17A2
13	Anti human CD3 NA/LE(2µg/ml)	IMGENX	IMG-6240E/OKT3
14	Anti human CD28 NA/LE(1µg/ml)	BD Biosciences, CA, USA	555725/CD28.2
15	Anti human CD3 APC(5µl/sample)	IMGENX	IMG-6240C/OKT3
16	Isotype control APC(0.05µg/sample)	BD Biosciences, CA, USA	553932
17	Isotype control PE(0.05µg/sample)	BD Biosciences, CA, USA	559841
18	Anti-TRPV1 Polyclonal antibody (1:200)	Alomone Lab	ACC-030 0.2 ml
19	Anti-TRPV2 Polyclonal antibody (1:200)	Alomone Lab	ACC-032 0.2 ml
20	Anti-TRPV3 Polyclonal antibody (1:200)	Alomone Lab	ACC-033 0.2 ml
21	Anti-TRPV4 Polyclonal antibody (1:200)	Alomone Lab	ACC-034 0.2 ml
22	Anti-TRPA1 Polyclonal antibody (1:200)	Alomone Lab	ACC-037 0.2 ml
23	Alexa Fluor 488 F(ab')2 fragment of	Invitrogen, CA, USA	A-11070

	goat anti-rabbit IgG (1:1000)		
24	Cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb (1:500)	Cell Signaling Technology, MA, USA	9664
25	HRP-goat anti-mouse IgG (1:3000)	BD Biosciences, CA, USA	554002/NA
26	HRP-goat anti-rabbit IgG (1:3000)	BD Biosciences, CA, USA	554021/NA

3.1.3 Chemicals, reagents, and modulators:

Table 3.Details of chemicals used.

Sl. No.	Chemicals	Company	Catalog no
1	Bovine serum albumin fraction-V	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM105-100G
2	Triton X-100	Sigma Aldrich, MO, USA	MB031-500ML
3	Sodium deoxycholate	Sigma Aldrich, MO, USA	D6750-25G
4	SDS (sodium dodecyl sulfate)	Sigma Aldrich, MO, USA	L6026
5	2-mercaptoehtanol	Sigma Aldrich, MO, USA	63689
6	PhosStop™ (phosphatase inhibitors cocktail)	Sigma Aldrich, MO, USA	04906837001
7	Complete EDTA-free protease inhibitor	Sigma Aldrich, MO, USA	05892970001
8	Bromophenol blue	Sigma Aldrich, MO, USA	114391
9	Crystal violet	Sigma Aldrich, MO, USA	C6158
10	EGTA	Sigma Aldrich, MO, USA	E3889
11	Bis-Acrylamide	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB005-250G
12	Glycine	Himedia Laboratories Pvt.	MB013-1KG

		Ltd., Mumbai, India	
13	Sodium chloride	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM031-1 kg
14	Tris base	Himedia Laboratories Pvt. Ltd., Mumbai, India	TC072-1KG
15	Sodium azide	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM123-100G
16	EDTA	Himedia Laboratories Pvt. Ltd., Mumbai, India	R066-500ML
17	Glycerol	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB060-500ML
18	Sulphuric acid (H_2SO_4)	Himedia Laboratories Pvt. Ltd., Mumbai, India	AS016-500ML
19	Tween-20	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM156-500G
20	10x RBC lysis buffer	Himedia Laboratories Pvt. Ltd., Mumbai, India	R075-100ML
21	HPLC grade Methanol	Himedia Laboratories Pvt. Ltd., Mumbai, India	AS061-2.5L
22	Paraformaldehyde	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM-3660-500GM
23	Acrylamide	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB068-1KG
24	Antibiotic solution 100x liquid (10000 U Penicillin+ 10 mg Streptomycin)	Himedia Laboratories Pvt. Ltd., Mumbai, India	A001A-5x100ML
25	10x Phosphate Buffered Saline	Himedia Laboratories Pvt.	TL1032-500ML

		Ltd., Mumbai, India	
26	HiGlutaXL™ RPM-1640	Himedia Laboratories Pvt. Ltd., Mumbai, India	AL060G
27	IMDM	Himedia Laboratories Pvt. Ltd., Mumbai, India	AL070A-500ML
28	TEMED	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB026-100ML
29	Fetal Bovine Serum (FBS), Australia origin	PAN Biotech, Aidenbach, Germany	P30-1302
30	Ammonium persulfate (APS)	Bio-Rad, CA, USA	161-0700
31	20x TMB/H_2O_2	Bangalore Genei, Bangalore, India	62160118010A
32	7-AAD	BD Biosciences, CA, USA	559925
33	Trypan blue	Himedia Laboratories Pvt. Ltd., Mumbai, India	TC193
34	ConconavalinA	Sigma Aldrich, MO, USA	C0412-5MG
35	RTX	Sigma Aldrich, MO, USA	R8756-1MG
36	IRTX	Sigma Aldrich, MO, USA	I9281-1MG
37	RN1734	Sigma Aldrich, MO, USA	R0658-25MG
38	4αPDD	Sigma Aldrich, MO, USA	P8014-5MG
39	A-967079	Sigma Aldrich, MO, USA	SML0085-5MG
40	AITC	Sigma Aldrich, MO, USA	36682-1G
41	DMSO	Himedia Laboratories Pvt. Ltd., Mumbai, India	TC185-250ML
42	HiSEP	Himedia Laboratories Pvt.	LSM LS001

		Ltd., Mumbai, India	
43	Fluo4AM	INVITROGEN	F14217

3.1.4 Buffers and other reagents composition

Table 4. Details of buffers used

Sl. No.	Buffers and other reagents	Composition
1	RIPA(Radio Immunoprecipitation Assay buffer) lysis buffer	150 mM sodium chloride, 1.0% NP-40 or Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (sodium dodecyl sulphate) (w/v), 50 mM Tris, pH 8.0
2	2x Laemmli buffer	4% SDS (w/v), 10% 2-mercaptoehtanol (v/v), 20% glycerol (v/v), 0.004% bromophenol blue (w/v), 0.125 M Tris HCl, check the pH and bring it to pH 6.8
3	1x TGS or SDS-PAGE running buffer	25 mM Tris base, 190 mM glycine, 0.1% SDS (w/v)
4	1x Transfer buffer	25 mM Tris base, 190 mM glycine, 20% HPLC grade methanol (v/v)
5	4% Paraformaldehyde (PFA)	1x PBS (pH 7.4-7.6), 4% paraformaldehyde (w/v)
6	Blocking reagent Western blotting	3% BSA fraction-V in TBST
7	Tris-buffered saline (TBS)	0.0153 M Trizma HCl, 0.147 M NaCl in ultrapure water (Milli Q), pH adjusted to 7.6 by HCl
8	Tris-buffered saline Tween-20 (TBST)	0.05% (v/v) Tween-20 in 1x TBS

3.1.5 Kits:

OptEIA™ ELISA kits for IL-2, IFN-γ, TNF, were purchased from BD Biosciences (CA, USA). T cell purification kit for mouse (Catalog no.11413D), human (Catalog no.113.65D) and CellTrace^{TM} CFSE Cell Proliferation kit (Catalog no. C34554) were purchased from Invitrogen. TLR4 inhibitor peptide set (VIPER and Control Peptide) (Catalog no. NBP2-26244) were purchased from Novus Biologicals, USA.

3.2 Methods.

3.2.1 Splenocyte isolation:

Spleen was collected from 6-8 week BALB/C mice in aseptic condition followed by splenocyte isolation (221,222). The spleen was disrupted through a 70μm cell strainer in RPMI-1640 complete media and centrifuged at 300g for 5 minutes at 4^{o}C. After centrifugation supernatant was discarded and the pellet was dissolved by gentle tapping. RBCs were lysed by 1X RBC lysis buffer (HiMedia). 10ml of 1X RBC lysis buffer was used for one spleen. Cells were washed twice with 1XPBS by centrifugation at 300g for 5 minutes and suspended in RPMI-1640 complete media.

3.2.2 T cell purification:

T cell purification was done by using untouched mouse T cell isolation kit from Invitrogen according to instructions given by the manufacturer. First, 50x10^{6} splenocytes were resuspended in 500 μl of isolation buffer (1XPBS+ 2%FBS+2mM EDTA) and 100 μl of FBS was added to it. Biotinylated antibody cocktail for all the cells except T cells was added to it and incubated in ice for 20 minutes. Then cells were washed in excess isolation buffer at 300g for 5 minutes at 4^{0}C and supernatant was discarded. After washing, cells were incubated with streptavidin-conjugated magnetic beads for 15min at room temperature with gentle rolling and tilting. After 15 minutes extra isolation buffer was added, and gently pipetting was done with narrow opening tip. Then

cells were placed on a magnet for 2 minutes. The clear solution was collected in another tube followed by centrifugation at 300g for 5 minutes at 4^0C and supernatant was discarded. Purified cells were resuspended in RPMI-1640 complete media. Enrichment of the T cells was evaluated by flow cytometry and found to be around ≥96%.

3.2.3 Human PBMC isolation:

hPBMC was isolated by using HiSep (Cat no:HiSep LSM LS001) from human blood collected from healthy donors, and as per the manufacturer's instruction. In brief human blood was diluted with ice-cold 1XPBS and mixed well by Pasteur pipette. After proper mixing, the diluted blood was overlayed on 2.5ml of HiSep LSM in a 15 ml centrifuge tube which was followed by centrifugation in a swinging bucket rotor for 30 minutes at 400g with minimum acceleration and breaking. Then the lymphocyte layer was collected by sterile Pasteur pipette and washed two times with 1XPBS and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) from Himedia (Cat no: AL070A-500ML) supplemented with10% FBS.

3.2.4 TLR4 signaling pathway inhibition and Pharmacological modulation for TRP channels:

TLR4 signaling inhibitor peptide VIPER and Control Peptide were incubated with purified T cells suspended in RPMI-1640 complete media for 2h at 37^{o}C with 5% CO_2 in humidified incubators. Then cells were given ConA (5μg/ml) or TCR mediated stimulation for 36h to study T cell activation markers CD25 and CD69. Cell-free supernatant was collected and studied for T cell effector cytokines. To study the function of TRP channels in T cells different TRP channel specific inhibitors were pre-incubated with T cells for 2h followed by ConA/TCR mediated stimulation.

3.2.5 Cell viability assay.

Purified T cells were incubated with different doses of VP and CP for 36h in RPMI-1640 complete media at 37^{o}C with 5% CO_2 in humidified incubators. Cell viability was studied by the Trypan Blue exclusion method (223). Purified mouse T cells were incubated with different doses of VP and CP along with TCR/ConA stimulation for 36h. Cells were harvested and washed after 36h followed by incubation with 7AAD (BD Biosciences) for 15 mins. Cells were then acquired and analyzed in BD FACS Calibur using Cell Quest Pro software. 7AAD negative cells were considered as live cells (224,225).

3.2.6 ELISA:

Sandwich ELISA was carried out to study the secretion of cytokines by T cells. Cytokine production by the T cells was analyzed from the cell culture supernatants by the BD OptEIA™ sandwich ELISA kit (BD Biosciences, CA, USA) according to the manufacturer's instructions. Briefly, micro-wells of SPL medibinding strip immune plates were coated with ELISA capture antibodies diluted in coating buffer at 100 µl per well and incubated at 4°C for over. For optimum antibody coating, strip immune plates were sealed by plate sealer followed by incubated overnight at 4°C. Next day, individual wells were aspirated and washed 3 times with 300 µl/well ELISA wash buffer. After last wash, plates were inverted on absorbent paper to remove any residual wash buffer. Plates were then blocked with 300 µl/well ELISA assay diluent and incubated for 1h at room temperature (RT). After washing once, both samples and standards (serially diluted) were pipetted at 100 µl/well and, incubated for 2h at RT with plate sealer. After that, plates were washed for 5 times and incubated with a working detector (detection antibody+ streptavidin HRP reagent) incubated for 1h at RT with plate sealer. Then working detector were aspirated and washed 7 times (in the final wash plates were soak for 0.5-1 minute in wash buffer), followed by incubation in the substrate solution (TMB/H_2O_2) at dark without plate sealer. After

the development of blue color (usually 5-30 min) HRP-TMB/H_2O_2 reaction was stopped by stop solution (2 N H_2SO_4) and reading was taken at 450 nm within 30 minutes by Bio-Rad iMarkTM Microplate absorbance reader. The cytokine concentrations of the samples were calculated in comparison with the corresponding standard curve.

3.2.7 Flow Cytometry (FC) analysis:

Flow cytometry staining and acquisition were carried out as described earlier (221,226). In brief, harvested T cells were washed with 1XPBS by centrifugation and re-suspended in FACS buffer (1X PBS, 1% BSA, 0.01% NaN3). For surface staining, the required antibody cocktail was first made by diluting the antibodies in FACS buffer. Required antibody cocktail is added to cells resuspended in FACS buffer and incubated for 30 min on ice. After 30 min FACS buffer was added to the cells and washed by centrifuging at 300g for 5 min at 4°C. The supernatant was discarded and cells were re-suspended in FACS buffer containing 1% paraformaldehyde (w/v) and stored at 4°C until sample acquisition. Cells were acquired by the BD FACS Calibur™ flow cytometer (BD Biosciences, CA, USA) and analyzed by the CellQuest Pro software (BD Biosciences, CA, USA). A total of about ten thousand cells were acquired per sample. For analysis dot plot quadrants were set on the basis of isotype control. For intracellular staining (ICS) to study TRP channel expression T cells were first fixed with 2% paraformaldehyde (w/v). Then the cells were permeabilized with 0.1% TritonX-100 in PBS for 5 min. After that cells were blocked with 5% BSA for 1h which is followed by overnight incubation at 4°C with primary antibodies at 1:200 dilution. Primary antibodies were diluted with PBST (1X PBS with 0.1% Tween-20). Cells were washed after incubation with primary antibodies which is followed by incubation with AlexaFluor-488 secondary antibody at 1:1000 dilution. At last cells were washed

and re-suspended in FACS buffer and acquired with BD FACS Calibur™ flow cytometer (BD Biosciences, CA, USA) and analyzed by the CellQuest Pro software (BD Biosciences, CA, USA).

3.2.8 T cell Proliferation Assay:

Purified T cells are incubated with 5μM CFSE dissolved in DMSO from Invitrogen (CellTrace™ CFSE Cell Proliferation Kit, Cat no. C34554) in 1X PBS for 10 minutes at room temperature and then washed three times with RPMI media supplemented with 10% FBS. Then cells were pretreated with the desired concentration of VP and CP for 2h. After 2h cells were stimulated with ConA or TCR and incubated for 96h at 37^{o}C with 5% CO_2 in humidified incubators. Cells were harvested and washed with FACS buffer. Treated cells were acquired with BD FACS Calibur and analyzed in Cell Quest Pro software.

3.2.9 Ca^{2+} imaging:

Ca^{2+} imaging of primary murine splenic T cells was performed as reported earlier with minor modifications (227). Purified mouse T cells in their resting state were loaded with Ca^{2+} sensitive dye (Fluo-4 AM, 2 μM for 30 min) and incubated at 37^{o}C with 5% CO_2 in humidified incubators. The cell suspension was added to the live cell chamber for Ca^{2+} imaging and images were acquired every 5 sec. To study the Ca^{2+} influx cells were stimulated with specific agonists alone or in a combination with agonists and antagonists as described. To study the role of TRP channel during on TCR mediated Ca^{2+} influx, cells were pre-incubated with TRP channel inhibitors for 2 h and then stimulated with soluble αCD3 and αCD28 (10 μg/ml) for 10 min. Fluo-4 AM signal was acquired using a Zeiss LSM780 microscope, and the images were analyzed using LSM software, and intensities specific for Ca^{2+} loaded Fluo-4 are represented in artificial rainbow color with a pseudo scale (red indicating the highest level of Ca^{2+} and blue indicating the lowest levels of Ca^{2+}).

3.2.10 Immunofluorescence analysis and microscopy:

Immunofluorescence analysis and microscopy were carried out as reported earlier (228).For immune fluorescence and microscopic analysis of TRP channel expression, immediately after harvesting, T cells were diluted in PBS and fixed with paraformaldehyde (final concentration 2%). After fixation, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Then the cells were blocked with 5% BSA for 1 h. The primary antibodies were used at 1:200 dilution. To confirm the specificity of antibodies, blocking peptides were used in some experiments. The ratio (v/v) of blocking peptides used for the study with specific antibody was 1:1. Cells were incubated with primary antibodies for overnight at 4 °C in PBST buffer (PBS supplemented with 0.1% Tween-20). AlexaFluor-488 labeled anti-rabbit antibodies (Molecular Probes) were used as secondary antibodies and were used at 1:1000 dilution. All images were taken on a confocal laser scanning microscope (LSM-780, Zeiss) with a 63 9 objective and analyzed with the Zeiss LSM image examiner software and Adobe Photoshop.

3.2.11 Western Blot Analysis:

Protein expression was studied by Western blot analysis according to the protocol described earlier (229). In brief, cells were pre-incubated with VP and CP for 2h followed by TCR mediated stimulation for 4h. After 4h cells were washed once with ice-cold 1x PBS by centrifugation and the whole cell lysate (WCL) was prepared by Radio Immuno-Precipitation Assay (RIPA) lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, supplemented with protease inhibitor cocktail). After 30 min of incubation with RIPA buffer in ice with intermittent tapping at every 10 min cells were centrifuged at 13,000 rpm for 20 min at 4°C. After this supernatant was collected carefully in fresh 1.5 ml microfuge tube and stored at -80°C until further use. The protein concentration in the WCL samples was quantified by the Bradford reagent (Sigma-Aldrich, MO, USA) with different concentrations of bovine serum

albumin fraction V as standard. Equal amount of protein (10 μg/well) was loaded in the 10% SDS-PAGE after mixing with 2X sample buffer (130 mM Tris-Cl, pH 8.0, 20% (v/v) Glycerol, 4.6% (w/v) SDS, 0.02% Bromophenol blue, 2% DTT) at a ratio of 1:1 along with prestained protein marker in one well. After completion of SDS-PAGE, the gel was washed in distilled water to remove excess of salt followed by incubation in 1x transfer buffer until transfer assembly ready. Then proteins (in the gels) were blotted on to methanol activated PVDF membrane (Millipore, MA, USA) at 80 volts (constant) for 2.5hr at 4°C with the help of Bio-Rad 54 PowerPac Basic. Then the transferred membranes were blocked with 3% BSA for 2h followed by overnight incubation with the different primary antibodies at 4°C. Next day the membranes were washed with TBST for three times and incubated with the HRP conjugated secondary antibodies for 2h at RT with continuous rocking on a gel rocker. After washing with TBST for three times, the membranes were subjected to Immobilon Western chemiluminescent HRP substrate (Millipore) chemiluminescence detection, by Bio-Rad gel doc. Detection was carried out by use of Quantity one software (Bio-Rad, CA, USA) and HSP70 band was studied as loading control for all the samples.

3.2.12 Statistical Analysis:

The comparison between the groups was performed by 1 way ANOVA with Bonferroni post-hoc test. Data presented here are representative of at least three independent experiments. $p < 0.05$ is considered as statistically significant difference between the groups. (ns, non-significant; $*p < 0.05$; $**p \leq 0.01$; $***p \leq 0.001$).

CHAPTER # 4

Results

&

Discussion

Functional expression of TLR4 in T cells

Major portion of this chapter has been published as a research article:

Sahoo S. S., Pratheek B. M., Meena V. S., Nayak T. K., Kumar P. S., Bandopadhyay S., Maiti P. K., Chattopadhyay S. (2018). VIPER regulates naive T cell activation and effector responses: Implication in TLR4 associated acute stage T cell responses. *Scientific Reports,* 8:7118.

4.1.1 Results

TLRs are known to be present on innate immune cells and helps in pathogen recognition. Signaling through TLRs in innate immune cells can modulate adaptive immune cells function both *in vivo* and *in vitro*. Moreover, TLRs have been reported to be expressed on adaptive immune cells like T cells and B cells. Their functional significance on the effector response of T cells is variable in different disease conditions and model systems. TLRs expression and its function in naïve T cells during activation need to be studied further for designing effective treatment strategies during acute phage T cell activation. In this work we have studied the differential expression of TLRs during naïve T cell activation. Moreover, we have used a well established TLR4 specific signaling inhibitor peptide VIPER to understand the functional requirement of TLR4 signaling pathway during T cell activation.

4.1.1.1 Activation of naive purified T cells:

T cells were purified using mouse untouched T cell purification kit from BALB/c mouse splenocytes. After purification, purified cells were stained with αCD3 antibody and analyzed via flow cytometer for percentage of cells positive for CD3 expression. In whole splenocytes, around 37.21±0.96% cells were positive for CD3 whereas around 93.26±0.63% cells were showing CD3 expression which suggests that 93.26% of purified cells were T cells (Fig.12 a). These purified cells were used for further experiments. In order to determine the optimum time for studying T cell activation, we activated T cells and harvested the cells at different time points. Then we studied the expression of T cell activation markers CD25 and CD69 via flow cytometry. We found that at around 36 hours post activation T cell activation marker CD25 expression was at the highest level and was stabilized (Fig.12 b). However early T cell activation marker CD69 expression starts increasing after 2 hours of activation and maximum expression was observed at

around 12 hours (Fig.12 c). From the above results, we concluded that optimum T cell activation for studying T cell activation markers CD25 and CD69 was at 36 hours after activation. Throughout this work, we have studied the T cell activation markers at 36 hours post activation stimuli were given.

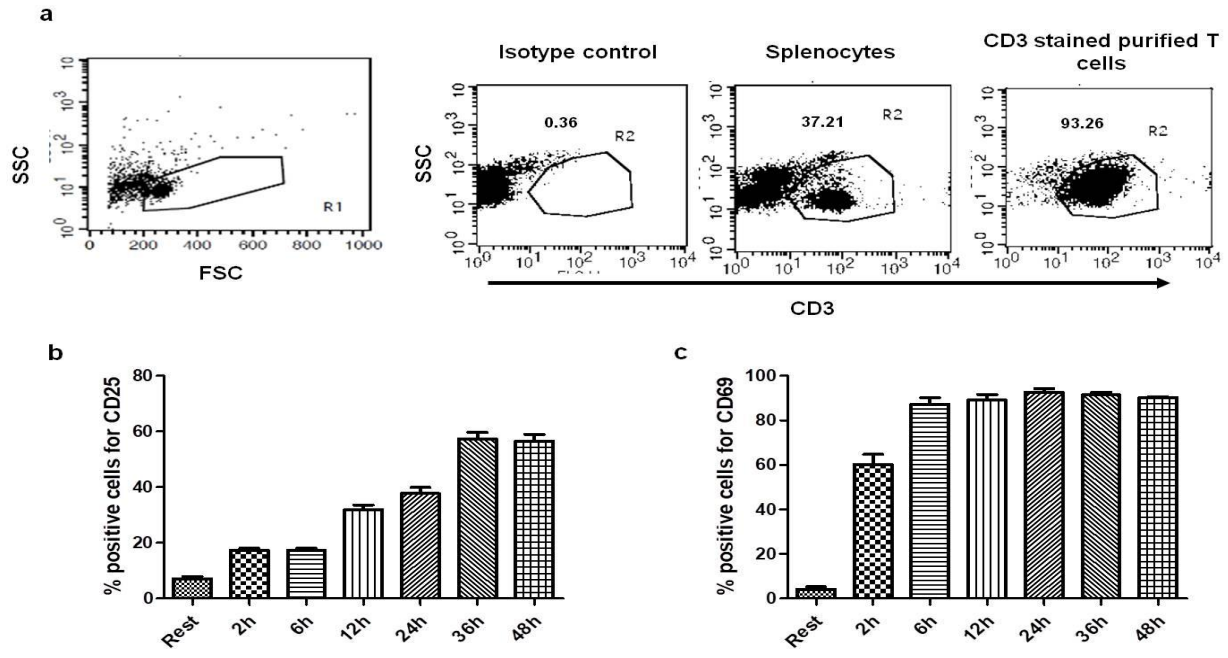


Figure 12. T cell purification and activation time kinetics: Flowcytometric dot plot showing percentage of T cells present in whole splenocytes and purified T cells from whole splenocytes (a). Bar graphs showing percent cells positive for CD25 (b) and CD69 (c) after TCR stimulation at different time points. The CD25 expression is stabilized after 36h of TCR stimulation. CD69 expression started increasing after 2h of TCR stimulation and stabilized after 6h.

4.1.1.2 TLR expression in activated and resting T cells:

In order to study the differential expression of major cell surface expressing TLRs in T cells during activation, we isolated the T cells and activated them for 36 hours. After 36 hours cells were harvested and stained with TLR4, TLR2, and TLR5 specific antibodies and analyzed for

expression of respective TLRs via flow cytometry. We observed that TLR4 expression is significantly down-regulated in activated T cells as compared to resting cells. 22.58% of resting T cells were positive for TLR4 expression whereas only 4.65% of activated cells were positive for TLR4 (Fig.13 b). However, we observed no significant change in the expression of TLR2 and TLR5 during T cell activation (Fig.13 a & c). 21.84% and 20.14% of cells were positive for TLR2 in resting and activated T cells respectively (Fig.13 a). Similarly, 2.65% and 3.51% cells were positive for TLR5 in resting and activated T cells respectively (Fig.13 c). We found that only TLR4 expression is differentially regulated during T cell activation significantly. Moreover, TLR4 has been studied in different disease conditions associated with altered T cell responses and other immune cells (95,96,230). Looking at the importance of TLR4 in different diseases and its differential expression during T cell activation, we selected it for further investigation towards its role in T cell function.

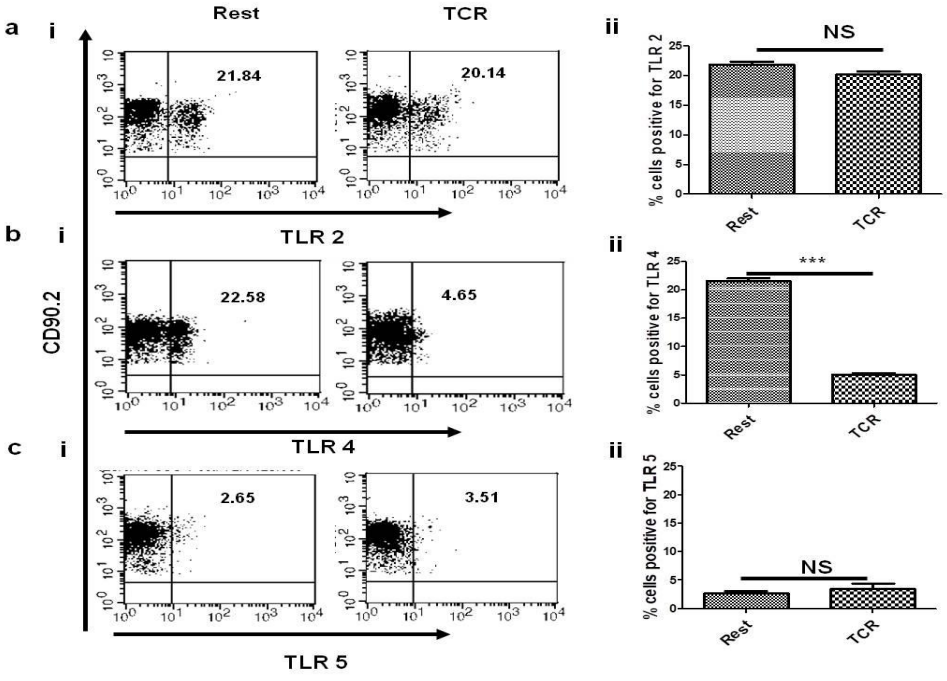


Figure 13. TLR expression in T cells: Flow cytometric dot plots showing expression of TLR2 (a, i), TLR4 (b, i) and TLR5 (c, i) in purified BALB/c mouse T cells. Bar graphs showing percent positive cells

for TLR2 (a, ii), TLR4 (b, ii), and TLR5 (c, ii) from three similar independent experiments. The TLR4 expression is significantly down-regulated during TCR mediated T cell activation whereas TLR2 and TLR5 expression didn't show any significant difference. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

4.1.1.3 Cell viability of macrophage and T cells at different dosage of VIPER.

VIPER is a peptide inhibitor, which acts by inhibiting intracellular signaling molecules related to the TLR4 signaling pathway. Seminal role of VIPER is well established about its efficacy towards TLR4 functionality as compared to other TLRs. In-depth biochemical and biophysical studies attribute towards VIPER driven TLR4 specific regulation in various cells, tissues and several disease contexts which are well reported (103,108,231). From these studies, it's apparent that VIPER is an ideal inhibitor to study TLR4 specific responses in different biological systems. We chose VIPER to study the functional significance of TLR4 towards T cell effector function.

We carried out trypan blue exclusion assay with different dosage of (Viper Peptide) VP and Control Peptide (CP) in Raw 264.7 cells to find out the suitable concentration at which cells are viable. We observed that cells were viable at concentrations from 5 to 15 µM of VP and CP (Fig.14 a). VP is an established TLR4 specific inhibitor and known to suppress LPS mediated TNF secretion in macrophages (103,104). To ensure such effect of VP in our set up on macrophage (Raw 264.7 mouse macrophage cell line) we pre-incubated Raw cells with 5µM of VP and CP as reported earlier(103) for 1h and then treated them with 250 ng/ml LPS. The cell-free supernatants were collected after 8h and ELISA was done for studying TNF secretion. VP treated Raw cells showed a significant reduction in TNF secretion as compared to cells treated with only LPS in combination with CP as reported previously (103) (Fig.14 b). Mouse splenocytes secrete TNF upon TCR mediated stimulation. In order to know the effect of VP on

splenocytes, we carried out cell viability assay by using the Trypan Blue exclusion method with different dosage of VP. It was observed that up to 5 to 15 μM concentration more than 90% cells are viable (Fig.14 c). To study the effect of VIPER on T cell survivability during activation, we incubated naïve T cells with different concentration of VIPER (VP) and Control Peptide (CP) for 36h along with ConA/TCR activation to study cell survivability vs. cytotoxicity. Then to study the effect of VIPER on T cells, we stained the cells with 7AAD. 7AAD negative cells were considered live, and dead cells will be positive for 7AAD (224,225). We found that up to 10μM of VIPER concentration nearly 100% cells are negative for 7AAD and up to 15μM VIPER concentration more than 90% of cells were negative for 7AAD (Fig.14 d). As evident from the cell viability study, most of the cells were viable at 10 μM concentration of VP and CP. We used 10 μM of VP and CP for our further experiment. To study the effect of VP on splenocytes for TNF production BALB/c mouse splenocytes were pre-treated with 10 μM VP and CP for 2h followed by TCR mediated stimulation. After 36h cell culture supernatant was collected and sandwich ELISA was performed for TNF secretion. VP treated splenocytes shows a significant reduction in TNF production as compared to cells treated with TCR or TCR along with CP (Fig.14 e). These data suggest that VP, a TLR4 signaling inhibitor can suppress TNF production by naïve mouse splenocytes during TCR activation.

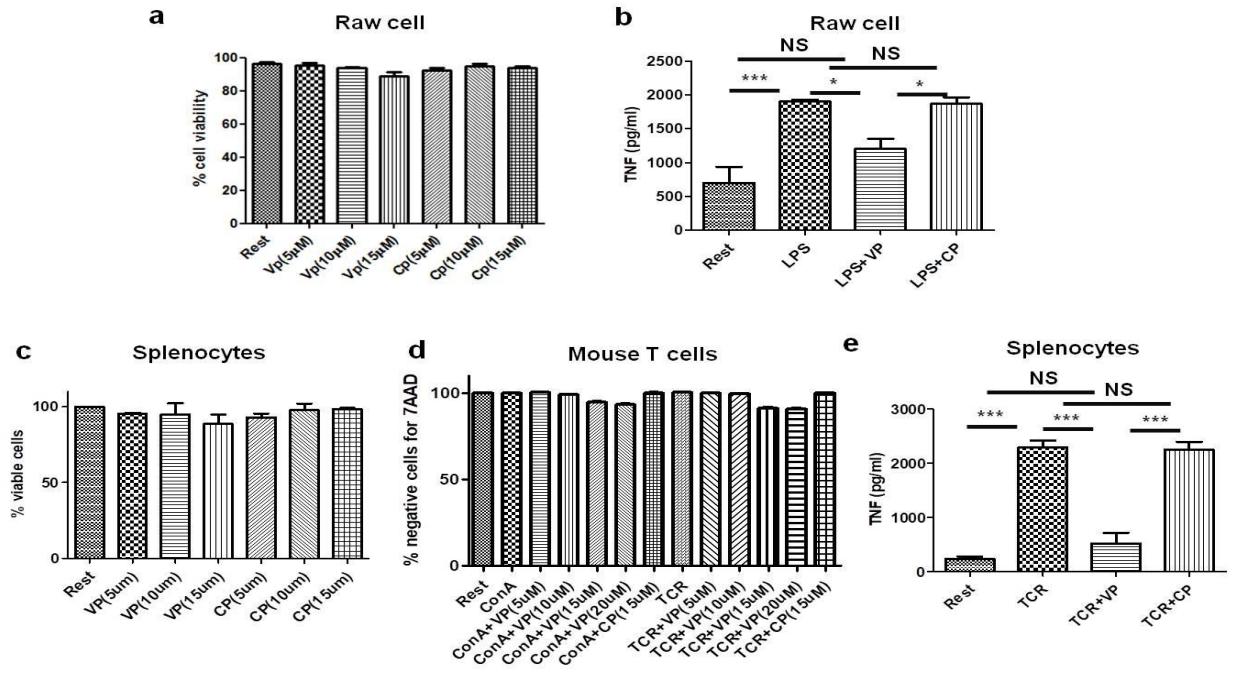


Figure 14. Cell viability and suppression of TNF secretion towards VP treatment: (a) Dose kinetic study show Raw 264.7 mouse macrophage cells are viable at high concentration of VIPER i.e.15μM. Cell viability was carried out by the Trypan Blue exclusion method. (b) VIPER suppresses TNF secretion in Raw cells during LPS mediated activation. (c) Mouse splenocytes were treated with different dose of VIPER and cell viability was studied using the Trypan Blue exclusion method. At 5 and 10μM concentration, cells are viable. (d) Bar graph represents the percent of negative T cells for 7AAD during T cell activation along with different dosage of VP and CP. (e) VIPER suppresses TNF in splenocytes activated with TCR. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. VP: VIPER, CP: Control Peptide. *P<0.05, **P<0.01, ***P<0.001.

4.1.1.4 Differential surface expression of TLR4 in ConA, TCR, and VP treated T cells.

Previous studies have suggested the expression of TLR4 on the mouse as well as human T cells (88-90,95). We have also observed the differential expression of TLR4 during T cell activation (Fig.15 a). To investigate the effect of VP on the expression of TLR4, if any, we surface stained the purified mouse splenic T cells treated with VP, CP along with TCR for 36h with TLR4

antibody. Cells were analyzed for expression of TLR4 with the help of flow cytometry. We found that 21.58 ± 0.5% of resting T cells express TLR4 on cell surface whereas during TCR mediated stimulation TLR4 expression (only 4.99% ± 0.28 cells) on the cell surface was reduced significantly (Fig.15 a). Interestingly TLR4 expression in VIPER treated cells remained similar as compared to resting cells (22.61 ± 0.18%) whereas CP treated cells showed a reduction (4.72 ± 0.27%) in TLR4 surface expression at par with TCR treated cells (Fig.15 a and b). Similar results were observed with mean fluorescence intensity (MFI) data of TLR4 expression in VIPER treated cells (Fig.15 c and d). These data suggest that VP may restore TLR4 expression during activation of naïve T cells.

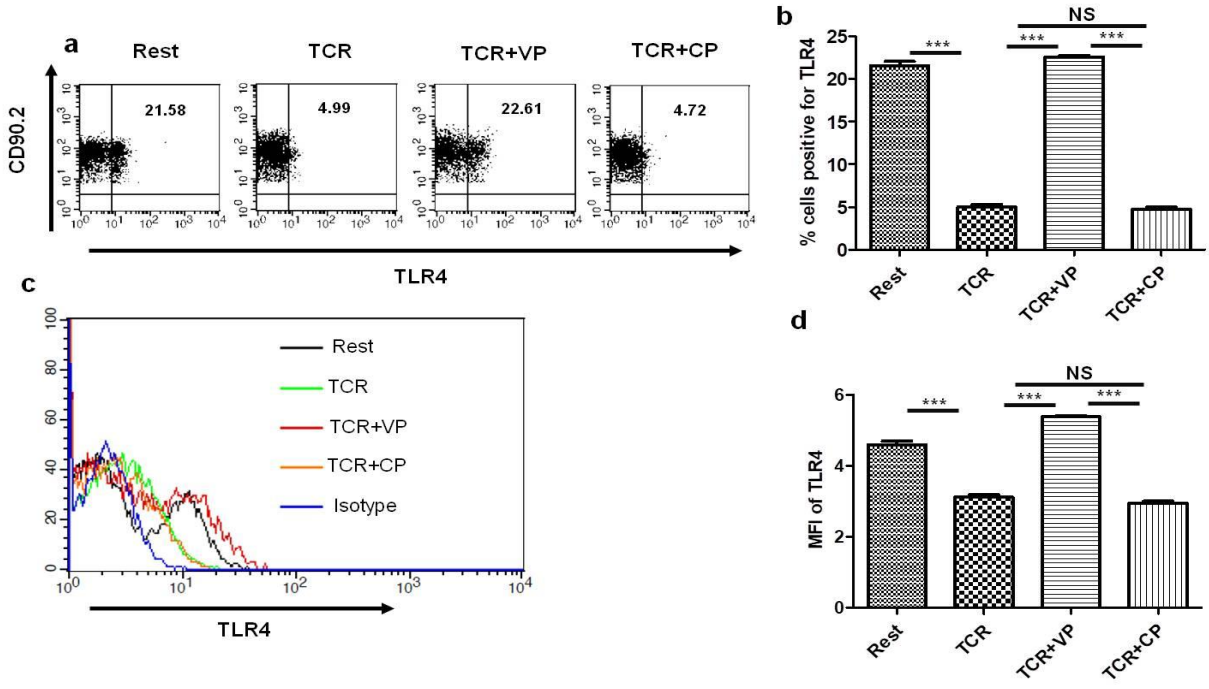


Figure 15. Differential surface expression of TLR4 during T cell activation: (a) Flow cytometry dot plot showing frequency of T cells (% positive cells) expressing TLR4 was significantly reduced during TCR mediated T cell activation, and VIPER treated cells showed restoration of TLR4 expression. **(b)** Bar graphs represent percentage positive cells expressing TLR4 from three independent experiments. **(c)** MFI plot showing TLR4 MFI is reduced in TCR activated cells whereas VIPER treated cells showed

restoration of TLR4 expression. **(d)** Bar graph representing MFI data of TLR4 expression from three independent experiments. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

4.1.1.5 VIPER downregulates T cell activation by inhibiting TLR4 signaling pathway.

As we have observed the downregulation of TNF production in splenocytes by VIPER and differential regulation of surface expression of TLR4 in purified T cells during TCR stimulation, we wanted to know further if it has any direct effect on T cells activation. For that, purified T cells were pre-treated with VP and CP for 2h and then stimulated with ConA and TCR for 36h. After 36h, cells were harvested and stained for expression of T cell activation markers CD25 and CD69. Stained cells were acquired, and CD90.2 gated cells were analyzed in Cell Quest Pro software for expression of CD25 and CD69. After ConA mediated activation 69.07 ± 8.58% cells were found to be positive for CD25 (Fig.16 a), and 70.76 ± 8.34% cells were expressing CD69 which is significantly higher than the corresponding resting cells (Fig.16 b). However, in VP treated T cells only 18.81 ± 0.83% and 20.40 ± 0.98% cells were found to be positive for CD25 (Fig.16 a) and CD69 (Fig.16 b) respectively, which shows significant down-regulation of expression of activation markers. Similarly in cells treated with only TCR 59.10 ± 6.94% and 68.12 ± 1.28% cells are positive for CD25 (Fig.16 a) and CD69 (Fig.16 b) respectively, whereas VP treated cells showed significant reduction in expression of activation marker CD25 (14.03 ± 0.05% cells, Fig.16 a) and CD69 (16.77 ± 0.08% cells, Fig.16 b). CP treated cells showed no significant change in the expression of CD25 (Fig.16 a) and CD69 (Fig.16 b) as compared to ConA and TCR treated cells. This observation suggests that VP may regulate naive T cell activation.

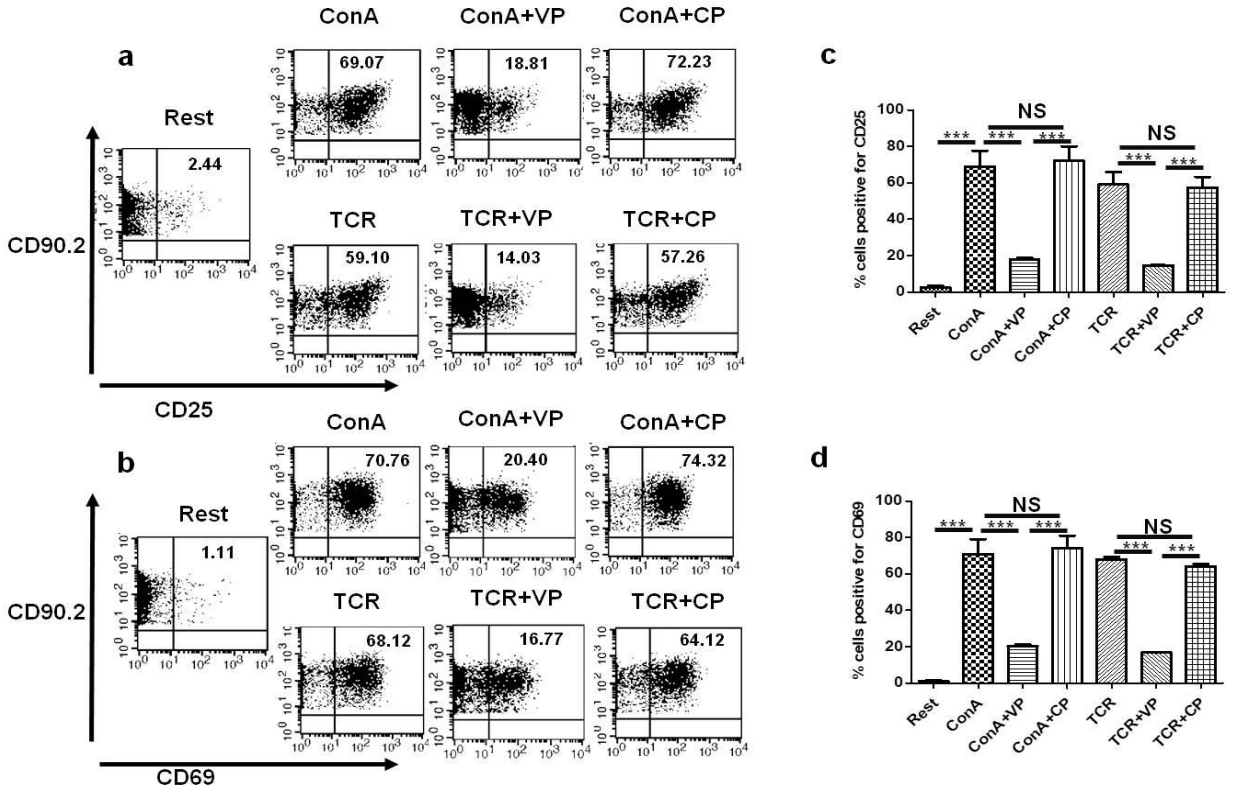


Figure 16. VIPER suppresses surface expression of T cell activation markers during T cell activation: (a) Flow cytometry dot plot showing CD25 surface expression (% positive cells) was significantly reduced in VIPER treated cells during ConA or TCR mediated T cell activation. **(b)** Similarly, the CD69 expression was also decreased in VIPER treated cells in comparison with cells activated only with ConA or TCR. **(c & d).** Bar graphs showing % positive cells expressing CD25 and CD69 respectively from three independent similar experiments Data shown are representative of three independent experiments and Bars represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

4.1.1.6 VIPER down-regulates T cell effector cytokine production during TCR and ConA stimulation.

Naive T cells are known to release effector cytokines for effective adaptive immune response during TCR and ConA stimulation (222). As we observed the downregulation of T cell activation markers with the treatment of VP, further the production of T cell effector cytokines IL-2, IFN-γ

and TNF upon VP and CP treatment during TCR and ConA mediated activation was studied. To analyze the cytokines, ELISA was performed with cell culture supernatants collected from VP, and CP pre-treated cells stimulated with ConA or TCR for 36h. We have observed that signature T cell effector cytokines IL-2 Fig.17 (a), IFN-γ Fig.17 (b), and TNF Fig.17 (c) secretions were significantly downregulated in VP treated cells as compared to CP treated cells.

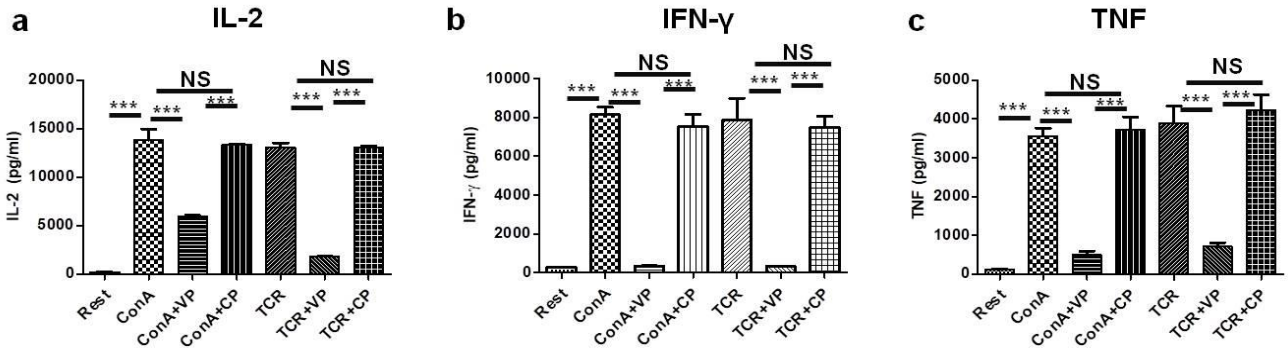


Figure 17. VIPER suppresses T cell effector cytokine responses: Cytokine secretion in pg/ml for IL-2 **(a)**, IFN-γ **(b)** and TNF **(c)** shows VIPER treated T cells produced less inflammatory cytokines as compared to cells treated with ConA, TCR or with Control Peptide. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

4.1.1.6 VIPER inhibits T cell proliferation.

TCR mediated stimulation is supposed to be followed by IL-2 secretion which helps in T cell proliferation (232). As VP down-regulated IL-2 secretion we were interested to know if it has any significant effect on T cell proliferation. We first labeled the naive T cells with CFSE as described in the material and method section and then pre-treated them with VP and CP for 2h. After 2h, cells were stimulated with ConA and TCR for 96h. We observed that T cells treated with VP showed significantly reduced proliferation as compared to cells treated only with ConA or TCR (Fig.18b). 53.71 ± 3.17% T cells showed proliferation after ConA treatment whereas only

4.93 ± 1.67 % cells showed proliferation when pretreated with VP before ConA treatment (Fig.18a). Similarly, 52.83 ± 4.58% TCR treated cells showed proliferation whereas 6.88 ± 1.28% cells pretreated with VP were proliferated upon TCR stimulation (Fig.18a). CP treated cells showed no significant difference in proliferation as compared to cells stimulated with TCR or ConA. We have also investigated the effect of TLR4 signaling inhibition on T cell proliferation of human T cells and found similar results as compared to mouse T cells (Fig.18 c & d).

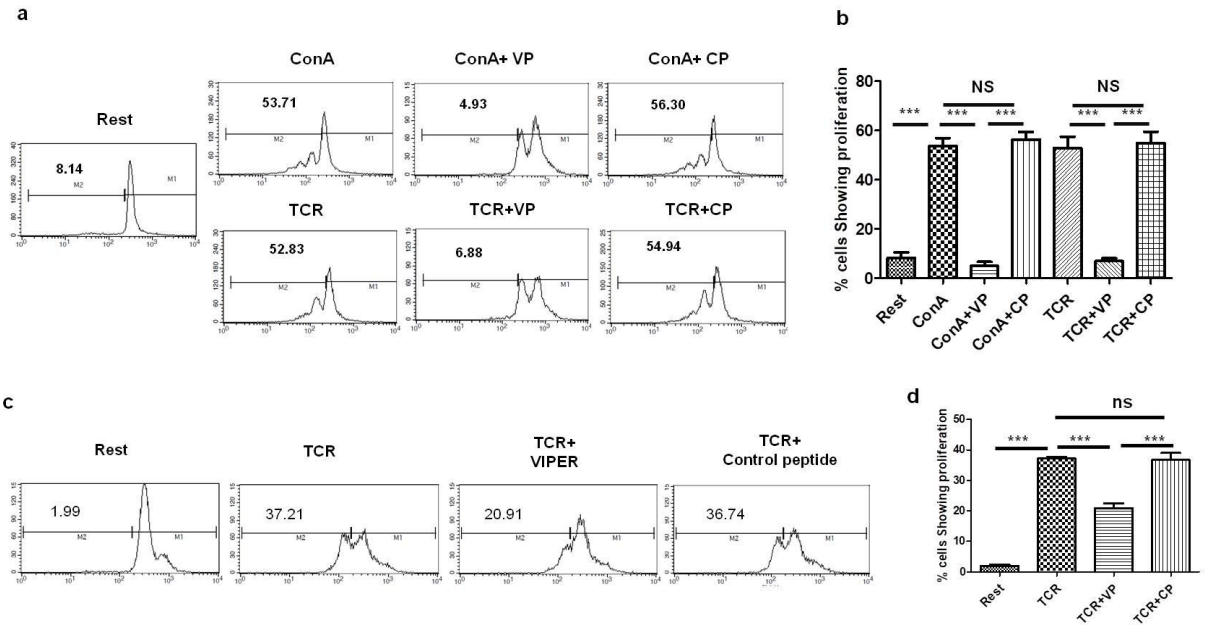


Figure 18. VIPER suppresses T cell proliferation: (a) Flow cytometry histograms represent proliferation status of purified T cells from BALB/c mice which are untreated or stimulated with ConA, ConA+VIPER, ConA+Control Peptide, TCR, TCR+VIPER and TCR+CP respectively. **(b)** Bar diagram represents % cells proliferating with different treatments from three similar independent experiments. **(c)** The proliferation of purified human T cells stimulated with TCR, TCR+VIPER, and TCR+CP respectively. **(d)** Bar diagram represents % cells proliferating from three similar independent experiments of human purified T cells. Percent cells showing proliferation is indicated above M2 gate in each histogram. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

4.1.1.7 VIPER regulates T cell activation mediated Fas and FasL expression.

T cell activation leads to the production of inflammation and effective immune response against the pathogens. However, the activated cells need to be eliminated so that the host can be protected from the adverse effect of inflammation. For this reason, optimum T cell activation is followed by Activation-Induced Cell Death (AICD) to maintain T cell homeostasis (233). As we found that VP suppresses T cell activation we were curious to study whether it has any effect on the induction of AICD or not. So we investigated the expression of Fas (CD95) and Fas ligand (CD95L) in VP treated naive T cells during TCR and ConA mediated stimulation. We observed that after 36h of ConA or TCR mediated activation CD95 and CD95L expressions were up-regulated. However, the cells pre-treated with VP showed significant down-regulation in both CD95, and CD95L expression after ConA or TCR mediated activation (Fig.19). 1.17 ± 0.52% resting cells express CD95 as compared to ConA and TCR mediated activation where it went up to 53.45 ± 2.71% and 62.38 ± 5.25% respectively (Fig.19 a). Interestingly T cells pre-treated with VP showed significantly reduced expression of CD95 (Fig.19 c). Only 2.27 ± 0.57% and 3.04 ± 0.48% VIPER treated T cells expressed CD95 with ConA and TCR respectively (Fig.19 a). Similarly, the CD95L expression was observed to be up-regulated during ConA and TCR mediated T cell activation. In resting condition 5.55 ± 0.26% cells expressed CD95L whereas during activation with ConA and TCR it significantly increased up to 33.32 ± 3.1% and 45.09 ± 3.7% (Fig1.9 b). VP treated cells showed a significant reduction in expression of CD95L (Fig1.9 d). In VP pretreated cells 18.1 ± 0.54% cells expressed CD95L when activated with ConA and 27.04 ± 1.16% with TCR (Fig.19 b). Decreased surface expression of CD95 and CD95L in VP treated T cells suggest that it may regulate the induction of AICD. Moreover, to study the induction of apoptosis due to TCR activation, we investigated the expression of executioner

caspase, cleaved-caspase 3. Cleaved caspase 3 expression was up-regulated in TCR, and TCR+CP treated cells (Fig. 19 e). Similar to the down-regulation of CD95 and CD95L expression, cleaved caspase 3 expression was found to be downregulated in VP treated T cells (Fig. 19 e).

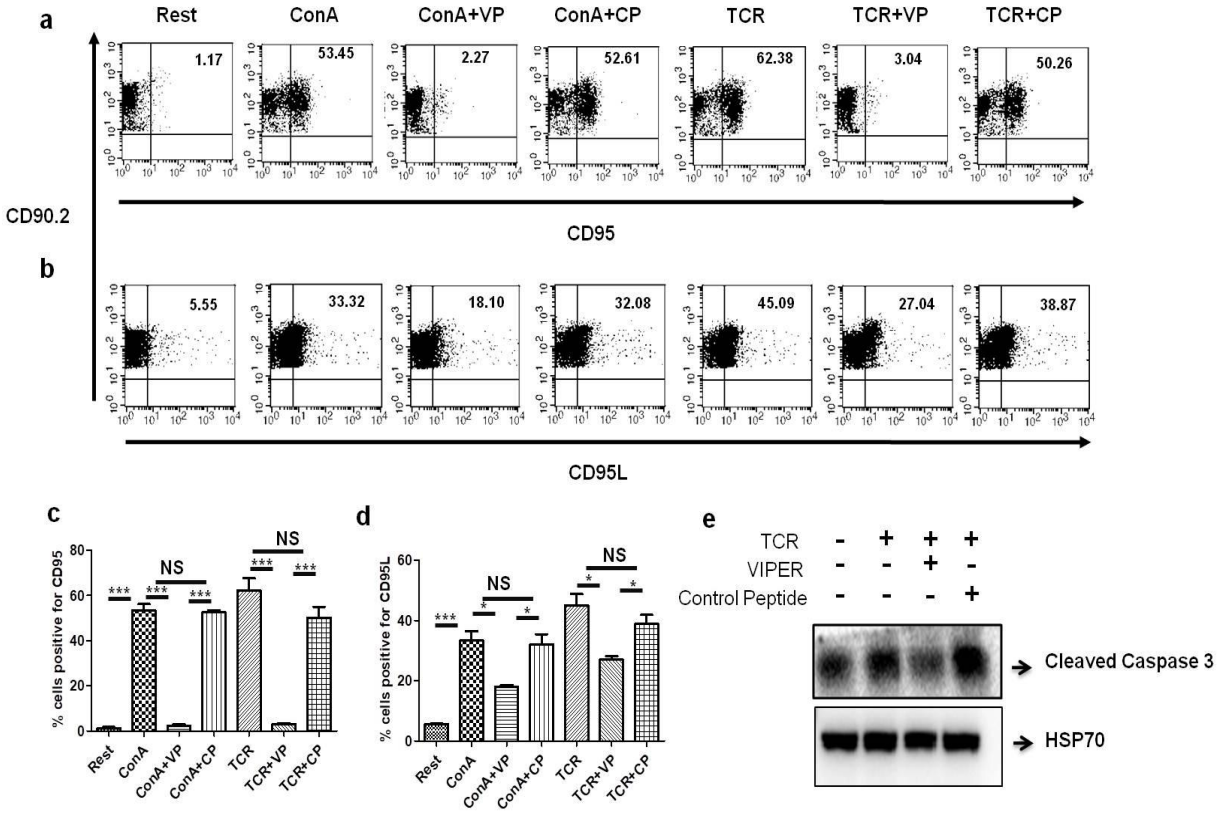


Figure 19. VIPER regulates induction of Fas (CD95) and FasL (CD95L) during T cell activation: Flow cytometric dot plot showing expression of CD95 **(a)** and CD95L **(b)** in T cells treated with VIPER and CP followed by ConA and TCR mediated activation. VIPER treated cells showed significantly reduced expression (% positive cells) of CD95 and CD95L as compared to cells treated with only ConA /TCR. Bar diagrams represent % cells expressing **(c)** CD95 and **(d)** CD95L from three similar independent experiments. **(e)** Cleaved Caspase 3 was down-regulated in VP treated cells as compared to TCR, or TCR+CP treated cells. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. * p <0.05, ** p <0.01, *** p <0.001

4.1.2 Discussion:

TLRs are one of the most widely studied receptors on the innate immune cells. Their functional significance towards the efficient functioning of innate immune cells was well reported. TLRs have been known to present in antigen presenting cells, and their modulation is important for recognition of PAMPs. TLR4 has been reported to be present on antigen presenting cells, and their modulation can alter the possible outcome of the cell-mediated immunity (101,102). TLRs have been shown to regulate innate and adaptive immune systems in diverse disease conditions like autoimmune and infectious diseases. It's known that in autoimmune diseases prolonged chronic inflammation persists as compared to acute inflammatory responses (234,235). The role of TLR4 and TLR2 has been studied and reviewed in different autoimmune conditions like RA and SLE (236-239). TLR4 had been shown to aggravate the progression of RA in mouse model whereas TLR2 has been reported to be associated with the protective role (236). Functional expression of TLR4 on various subsets of T cells in different disease condition and their role in various experimental models has been reported (95,230,240,241). However, those reports are illustrated with different experimental models and suggest a differential functional role of TLR4 on T cells. Moreover, some reports also propose that there is no such direct role of LPS, a TLR4 specific ligand, on naive T cells (242). In this study, we have observed differential regulation of TLR4 expression during T cell activation. However, we did not find any significant change in the expression of TLR2 and TLR5. As we had observed significant differential expression of TLR4 during TCR mediated T cell activation, further investigation of the possible requirement of TLR4 in naive T cell activation was required. To predict the requirement of TLR4 signaling towards T cell activation, direct modulation of TLR4 signaling in wild-type naive T cells of mouse origin or from T cells from healthy human donors is necessary.

TCR mediated activation was found to down-regulate the TLR4 expression in T cells as compared to untreated or resting cells (90,95). We have also found similar down-regulation of TLR4 surface expression on T cells during TCR driven activation. However, no change in surface expression of TLR2 and TLR5 was observed. We also observed that T cells treated with TLR4 signaling inhibitor VP along with TCR showed a similar level of surface expression of TLR4 as untreated cells suggesting that VP was actively resisting the TCR induced down-regulation of TLR4 in T cells. It's interesting to find that TLR4 inhibitor-treated cells showed a comparable level of TLR4 expression as seen in resting cells. In the presence of Control Peptide, down-regulation of TCR-driven TLR4 expression remained unaffected. Several reports suggest various important roles of MyD88; a TLR4 associated signaling adapter protein, towards T cell effector function (97-100). However, a direct role of TLR4 responsiveness towards activation of wild-type naive T cells remains scarce. Accordingly, we further investigated the effect of VP towards effector function during TCR and mitogenic activation on naive T cell. We observed that VP treated naive T cells showed considerably reduced expression of T cell activation marker CD25 and CD69 as compared to untreated cells upon TCR or mitogenic stimulation. This finding indicated towards the possible involvement of TLR4 signaling pathways during T cell activation. Optimum T cell activation is followed by secretion of effector cytokine secretion which can functionally help in different T cell functions. During the study of the secretion of effector cytokine secretion, we observed that upon TCR or mitogenic stimulation, effector cytokine secretion was significantly decreased in VP treated cells. No significant change in cytokine secretion was observed in cells treated with CP and TCR. Together these results suggest TLR4 signaling might have some regulatory function during naive T cell activation. T cell effector cytokine IL-2, secreted by activated T cells helps in T cell proliferation. During the study of TLR4 signaling inhibition on T cell proliferation, we observed that TCR or mitogen mediated

naive T cell proliferation can be regulated by VP. VP treated T cells showed reduced proliferation as compared to cells treated only with TCR or ConA. Moreover, we observed similar results with purified human T cells where VP treated cells showed a marked reduction in T cell proliferation as compared to cells treated with TCR or TCR+CP. In order to maintain the homeostasis of T cell population, optimum T cell activation is followed by clonal propagation and activation-induced cell death (AICD). AICD is mediated by expression and involvement of CD95 (Fas) and CD95L (Fas ligand) on T cells (233,243). Interestingly we observed that VP regulates upregulation of CD95 and CD95L expression during T cell activation. CD95 and CD95L were found to be increased in T cells stimulated with TCR or ConA. However, VP treated cells showed a significant reduction in activation-induced expression of CD95 and CD95L as compared to cells stimulated with TCR or ConA alone. These finding proposes that VP down-regulates T cell activation which in turn may regulate the expression of CD95 and CD95L.

It is reported that naive mouse T cells are found to express detectable level of TLR4, while it may go down during TCR activation without having the direct responsiveness of LPS on T cells (244,245). VIPER is a peptide inhibitor, which works by inhibiting intracellular signaling molecules related to the TLR4 signaling pathway. In this study, we have investigated VIPER mediated possible regulation of TLR4 responses of naïve T cell activation, if any, rather than the TLR4 receptor itself. Seminal role of VIPER is well established describing its specific efficacy towards TLR4 functionality as compared to other TLRs. In-depth biochemical and biophysical attributes towards VIPER driven TLR4 specific regulation in various cells, tissues and several disease contexts are well reported (103,108,231). Possibly the endogenous TLR4 signaling is required during T cell activation as reported earlier in the absence of LPS or other exogenous TLR4 ligand in MyD88 and TLR4 KO mice for the altered T cell responses (246,247). Recently,

$CD8^+$ T cells from a specific cohort of rheumatoid arthritis (RA) patients, unlike naive healthy donors and Systemic Lupus Erythematosus (SLE) patients, have been observed to express increased surface TLR4 expression and also found to respond upon LPS treatment (230). Interestingly, it has been previously reported that TLR4 deficiency in mice leads to down-regulation of inflammatory T cell responses (248,249).

As evident from the results, this study suggests that there might be a necessity of TLR4 signaling during naive T cell activation and effector function. In brief, we have shown VIPER driven regulation of TLR4 responses towards regulating activation, proliferation, effector cytokine production, Fas and FasL induction of naive T cells, which might have possible implication towards the pathogenic acute phase activation of T cell responses.

Functional expression of TRPV channels in T cells

Major portion of this chapter has been published as a research article:

Majhi, R. K.*, **Sahoo, S. S.***, Yadav, M., Pratheek, B. M., Chattopadhyay, S., & Goswami, C. (2015). Functional expression of TRPV channels in T cells and their implications in immune regulation. *The FEBS journal*, *282*(14), 2661-2681. *Authors contributed equally.

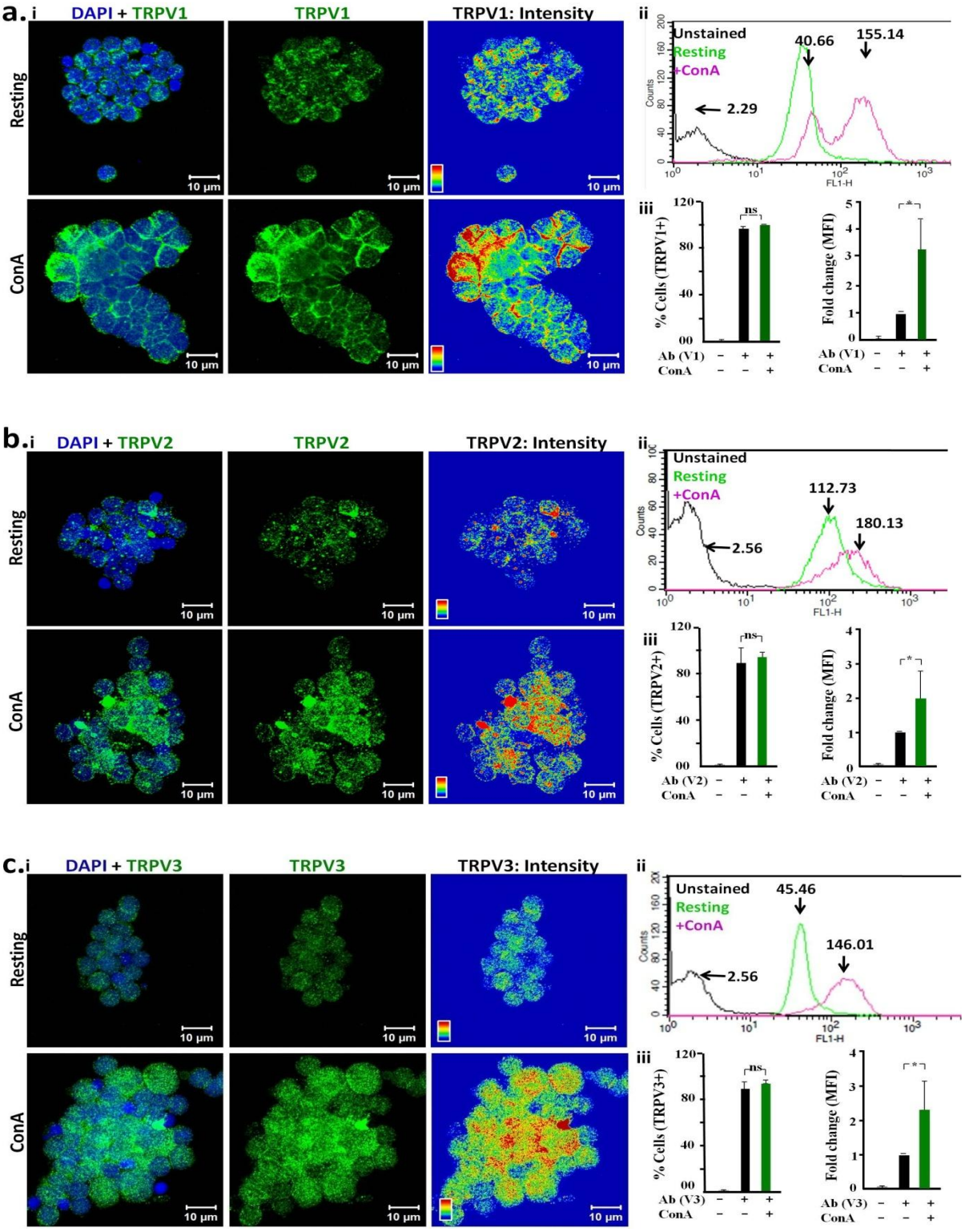
4.2.1 Results

Transient Receptor Potential (TRP) channels are a group of ion channels which are non-selectively permeable to different cations. Functional role of TRP channels have been attributed towards different physiological events, and mutations of TRP channels have been implicated in diverse pathophysiological conditions. Ca^{2+} works as an important second messenger in many cells including immune cells. TRP channels are known to be sensitive for change in temperature, and non-selectively permeable to cations like Ca^{2+} makes it ideal receptor for study in immune cells Moreover, recent evidence suggests the presence of TRP channels in immune cells which needs a further in-depth study (111-115). In this work we have studied the expression of different TRP channels in T cells during resting and activated conditions. We have used different TRP channels specific modulators to study the effect of these channels during T cell activation and its effector function.

4.2.1.1 TRPV members are expressed endogenously in primary human T cells

The expression profile of thermosensitive TRPV channels was probed in purified primary T cells from human peripheral blood mononuclear cells (PBMCs) by both confocal microscopy and flow cytometric (FC). Expression profile of TRPV1, TRPV2, TRPV3, and TRPV4 were observed in human T cells in the form of distinct punctuate (Fig. 20). TRPV channels were expressed both at the cytoplasm and at the plasma membrane. Confocal microscopy suggests that ConA-mediated activation leads to increase in the expression levels of TRPVs with a significant increase in TRPV1 and TRPV3 (Fig. 20 a and 20 c) followed by a marginal increase in TRPV2 and TRPV4 (Fig. 20 b and 20 d). Flow cytometry analysis revealed that expression (MFI levels) of TRPV channels increase several folds: TRPV1 (2.83 ± 1.28-fold); TRPV2 (2.09 ± 1.09-fold); TRPV3 (2.29 ± 0.84-fold) and TRPV4 (2.57 ± 1.54-fold) after ConA-mediated activation (Fig. 20). In control conditions, we noted that in the case of humans, >99% T cells are positive for the

expression of diverse TRPVs. Therefore, upon ConA-mediated activation, no further increase in the number of $TRPV^{+}$ T cells was observed for any of these TRPV channels.



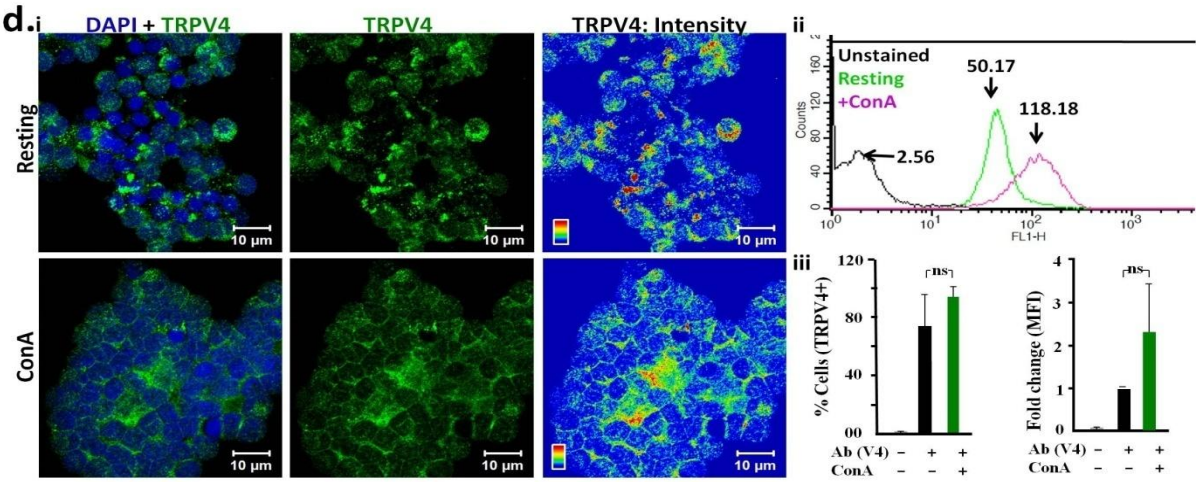
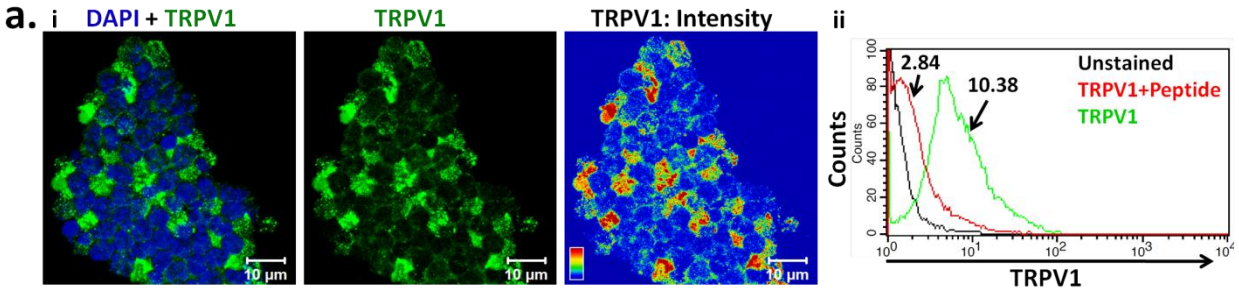


Figure 20. TRPV channels are expressed in primary human T-cells. Confocal images (**i**, left column) of cells immuno-stained with specific antibodies demonstrate the expression pattern, at resting state, for TRPV1 (**a**), TRPV2 (**b**), TRPV3 (**c**) TRPV4 (**d**) (upper panel) or after ConA-mediated activation (lower panel). TRPV channels are shown in green, and the intensity profiles of the TRPV staining are indicated in pseudo-rainbow scale. Flow cytometry profiles (**ii**) demonstrate the MFI histograms of cells positive for the TRPV channels respectively. Change in the % of positive cells and fold changes in the expression level (**iii**) of specific TRPV channels due to ConA-mediated activation are shown. The p values are: ns = non-significant; * = < 0.05 (n = 4).

4.2.1.2 TRPV members are expressed endogenously in murine splenic T cells

We next explored the endogenous expression of TRPV channels in primary murine splenic T cells. Confocal analysis of purified resting murine T cells revealed the expression of TRPV1 to TRPV4 in a pattern similar to primary human T cells (Fig. 21 a-d). $TRPV1^+$ and $TRPV4^+$ cells were higher in number than that of $TRPV2^+$ and $TRPV3^+$ cells. To explore the expression of these channels in a more quantitative manner, we performed flow cytometric analysis. Further, to confirm the antibody specificity, blocking peptides were used. These blocking peptides bind to the specific antibody. Hence, upon pre-incubation with specific antibodies, these corresponding antibodies become ineffective in binding target sequence in host cells, confirming the specificity

of respective antibodies. These blocking peptides reduce the number of cells that are positive for respective TRPV channels as well as MFI values (Fig. 21 a-d. ii) suggesting that these staining are highly specific. We have further analyzed $CD3^+$-gated cells to investigate the splenic T cell population in all our further flow cytometric experiments, if not stated specifically (Fig. 21 e). Almost 95.7 ± 3.1% of splenic T cell (in $CD3^+$ splenocyte population) is positive for TRPV1 (n=4), in resting condition. ConA-mediated activation for 36h resulted in marginal increase (97.1 ± 2.4%, n=4) in the number of $TRPV1^+$ cells. Similarly, about 92.6 ± 8% of T cells were $TRPV4^+$ (n=4) in resting conditions. The number of $TRPV4^+$ cells increases marginally (94.4 ± 6%, n=4) after ConA activation. However, the expression levels of TRPV2 and TRPV3 did not show a significant increase after ConA-mediated activation. Nearly 55.9 ± 3.8% cells are positive for TRPV2 in resting condition while 51 ± 5.9% activated T cells express TRPV2. Similarly, 86.58 ± 1.2 cells express TRPV3 in resting condition and 88.2 ± 2.5 during activated condition (n = 4). The MFI (mean fluorescence intensity) values revealed that TRPV1 expression increases to 1.47 ± 0.11 fold after ConA activation when compared to the resting conditions (from 63.99±30.64 to 95.7±49.18, n=4). The MFI values representing TRPV4 expression also increases by 1.61±0.17 fold after ConA activation (123.43 ± 65.67, n=4) when compared to the resting (79.68 ± 47.72, n=4) conditions.



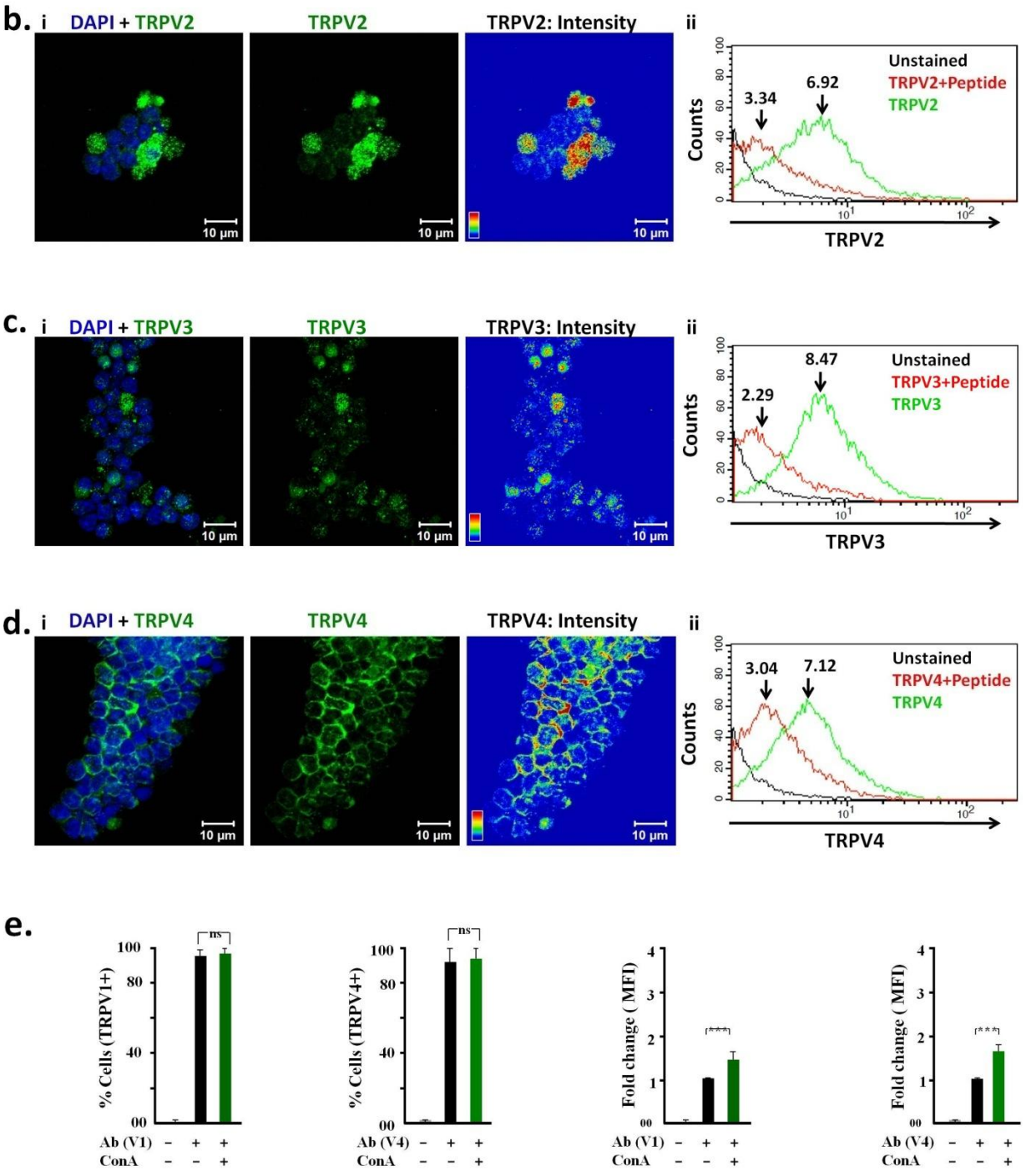


Figure 21. TRPV channels are expressed in primary murine T-cells. Confocal images (**a-d**) of cells demonstrating the expression pattern of different TRPVs (green) at resting stage. Cells were immuno-stained with specific antibodies for TRPV1 (**a**), TRPV2 (**b**), TRPV3 (**c**) and TRPV4 (**d**). Intensity profiles of the respective TRPV channels are indicated in pseudo-rainbow scale (right panel). **e.** Graphs

demonstrating the % of cells that are positive for TRPV1 and TRPV4 before and after ConA-mediated activation are shown (n = 4). Fold changes (calculated from MFI values, with respect to ConA) in the expression of TRPV1 and TRPV4 before and after ConA-mediated activation are shown in side. The p values are: ns = non-significant; * = < 0.05; ** = <0.01; *** = <0.001.

4.2.1.3 TRPV channel expressed on T cells are functional.

In order to explore if these TRPV channels are functional in T cells, we performed Ca^{2+}-imaging experiments, using purified mouse T cells loaded with Fluo4-AM (2µM). Live cell imaging did not reveal any increase in Fluo4-AM intensity with respect to time, especially in the absence of any stimuli (**Figure 22**). However, upon stimulation by TRPV1 activator, RTX, intracellular Ca^{2+} levels increased in the majority of the T cells. This RTX-mediated influx can be effectively blocked by TRPV1-specific inhibitor 5'-IRTX. Moreover, TRPV4-specific activator, namely 4αPDD also increases intracellular Ca^{2+} concentration. These results indicate that TRPV1 and TRPV4 are found to be functionally expressed in murine primary T cells and are involved in regulation of intracellular Ca^{2+}-influx.

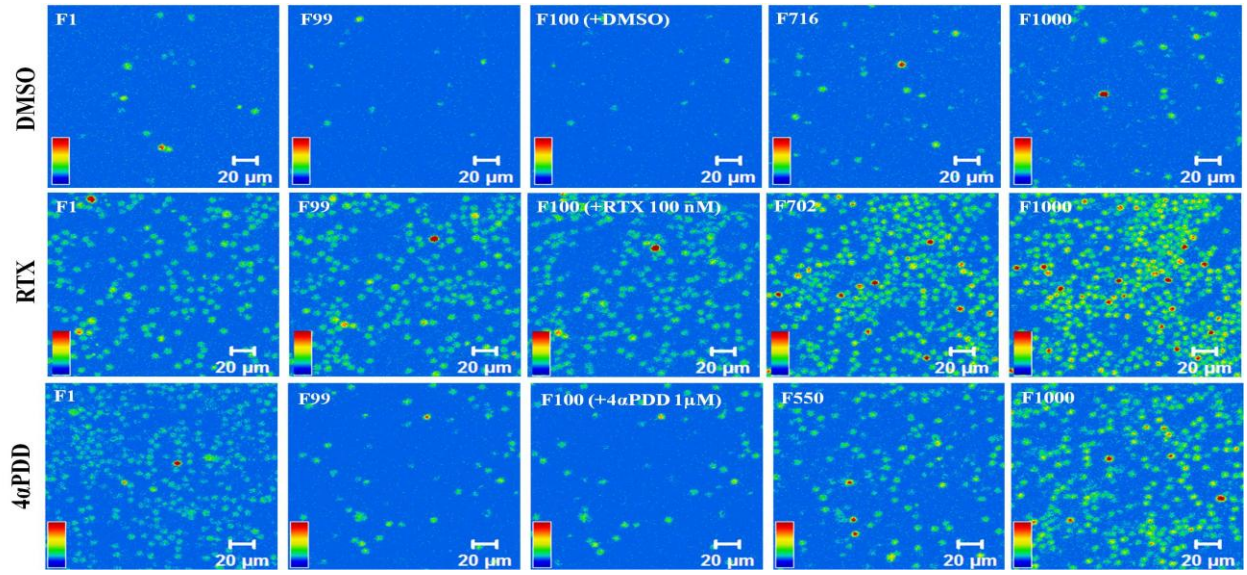


Figure 22. Pharmacological activation of TRPV1 and TRPV4 causes Ca^{2+}-influx in primary murine T cells. Time-series fluorescence images of view fields containing multiple cells loaded with Ca^{2+}-sensing

dye Fluo4-AM are depicted here. The time difference between each frame (F) is 5 seconds. The cells were treated with different pharmacological agents exactly at 100th frame (F100). Activation of TRPV1 by RTX causes an increment in the Ca^{2+}-level which can be blocked by TRPV1-specific inhibitor 5'-IRTX. Similarly, activation of TRPV4 by 4αPDD causes an increase in the concentration of intracellular Ca^{2+} which can be blocked by TRPV4-specific inhibitor RN1734. The fluorescence intensities in different time points are represented in pseudo-rainbow color. Representative results of three independent experiments are shown.

4.2.1.4 TRPV channel inhibition by pharmacological inhibitors decreases TCR mediated Ca^{2+} influx in T cells.

After TCR mediated stimulation intracellular Ca^{2+} level increases and a sustained high level of intracellular Ca^{2+} is imperative for T cell activation. So it might be possible that TCR stimulation mediated Ca^{2+}-influx is partly mediated by TRPV channels. To study the effect of TRPV channel inhibition on Ca^{2+} influx during TCR stimulation, we have carried out experiments with purified murine T cells in the presence of TRPV1and TRPV4 inhibitors, specifically with 5'-IRTX (10μM) and RN1734 (20 μM) for 36h. During TCR mediated activation intracellular Ca^{2+} level increased which is evident from the increased intensity of Fluo-4-AM as compared to resting cells (Fig. 23). T cells pre-incubated with TRPV1 and TRPV4 inhibitors are showing less Fluo-4-AM intensity upon TCR stimulation. These inhibitors were found to inhibit TCR-mediated Ca^{2+} influx in the majority of T cells (Fig. 23). 5'-IRTX (10μM) and RN1734 (20 μM) when used in combination were found to block TCR stimulated Calcium influx more efficiently than when used individually (Fig. 23). These data suggest a cooperative role of both these ion channels in regulating calcium levels in T cells during TCR mediated stimulation.

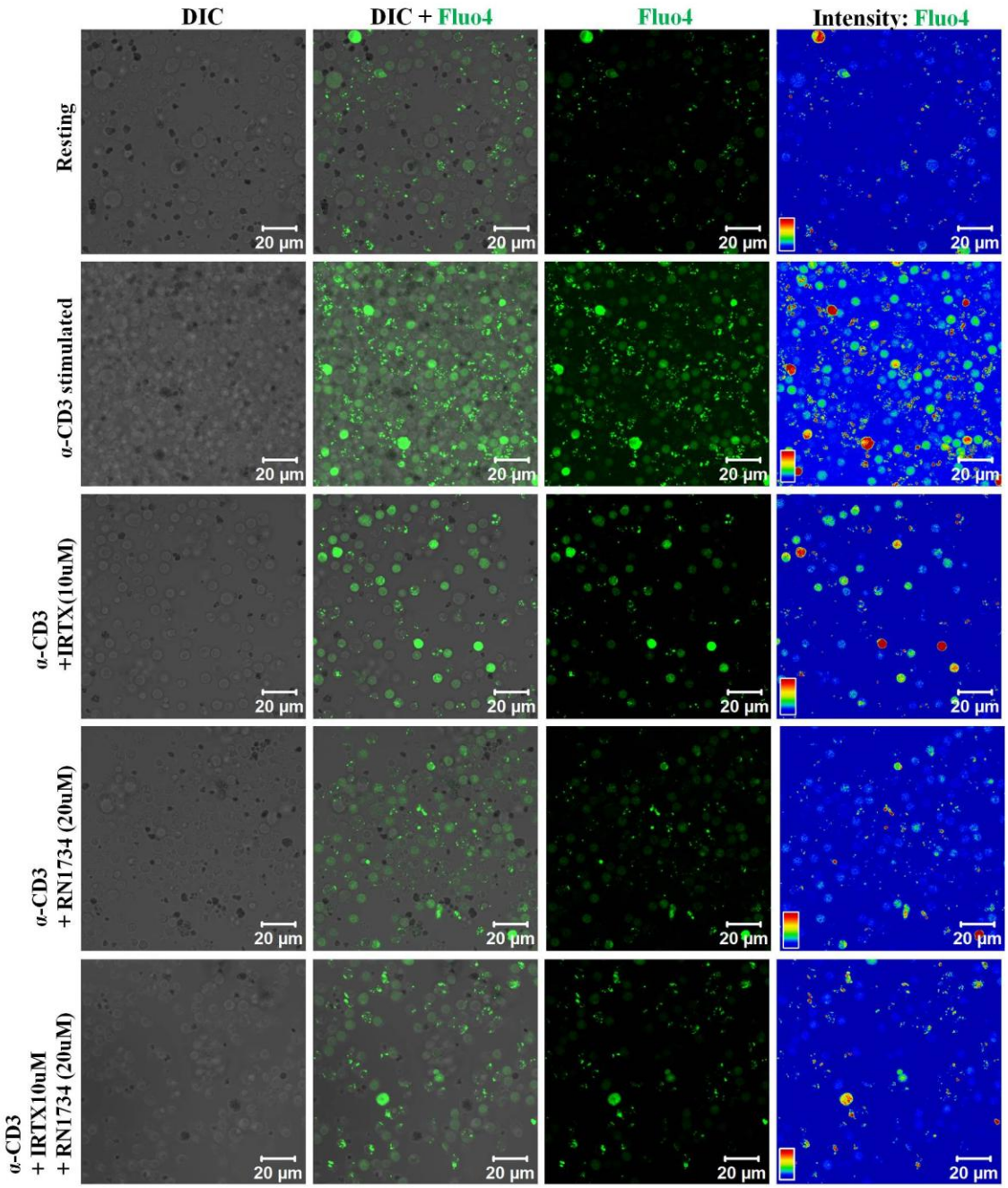


Figure 23. TRPV1 and TRPV4 are involved in the α-CD3 mediated intracellular Ca^{2+}-rise in murine splenic T cells. Fluorescence images of view fields containing resting (upper panel) or α-CD3 treated murine T cells loaded with Ca^{2+}-sensing dye Fluo4-AM. The T cells were treated with IRTX and RN1734 (specific pharmacological inhibitors of TRPV1 and TRPV4 respectively), individually or in combination. Pharmacological inhibition of TRPV1 and TRPV4 together (by 5'I-RTX and RN1734) specifically blocks α-CD3 mediated increase in the intracellular Ca^{2+} while these inhibitors used individually are less efficient

in blocking an increase in intracellular calcium levels. The fluorescence intensities are represented in pseudo rainbow color. Representative data of three independent experiments are shown.

4.2.1.5 TRPV channel inhibition downregulates TCR mediated T cell activation.

As we observed a decrease in intracellular Ca^{2+} level in T cells during TCR stimulation upon inhibition of TRPVs, we were curious to know their effect on T cell activation. To investigate the role of TRPV1 and TRPV4 in TCR-stimulation induced T cell activation we stimulated the purified mouse T cells with plate-bound αCD3 and soluble αCD28 mAbs for 36h in presence or absence of TRPV1 and TRPV4 inhibitors. After 36h we harvested the cells and checked for the expression of T cell activation markers CD25 and CD69. Percentage of $CD25^+$ cells was increased after αCD3/CD28 mAb-induced activation (6.51% in resting stage v/s 48.41% during activated stage). Co-incubation with 10 μM 5'-IRTX decreases in the number of $CD25^+$T cells (7.69%) whereas 20 μM RN1734 reduced the number of $CD25^+$T cells modestly (to 42.66%) (Fig. 24 a). However, the presence of both inhibitors (10 μM 5'-IRTX and 20 μM RN1734) results in significant reduction in cells that are $CD25^+$ (6.45%) (Fig. 24 c).

Similarly, CD69 expression in α-CD3/CD28 activated T cells was decreased upon inhibition of TRPV1 and TRPV4 channels. Percentage of CD69 positive cells was increased after αCD3/CD28 mAb-mediated activation (5.29% in resting stage v/s 56.57% during activated stage). Co-incubation with 10 μM 5'-IRTX decreases in the number of $CD69^+$ T cells (37.17%) whereas 20 μM RN1734 reduced the number of CD69 positive T cells modestly (48.48%) (Fig. 24 b). However, the presence of both inhibitors (10 μM 5'-IRTX and 20 μM RN1734) results in significant reduction in cells that are positive for CD69 (29.2%) (Fig. 24 d).

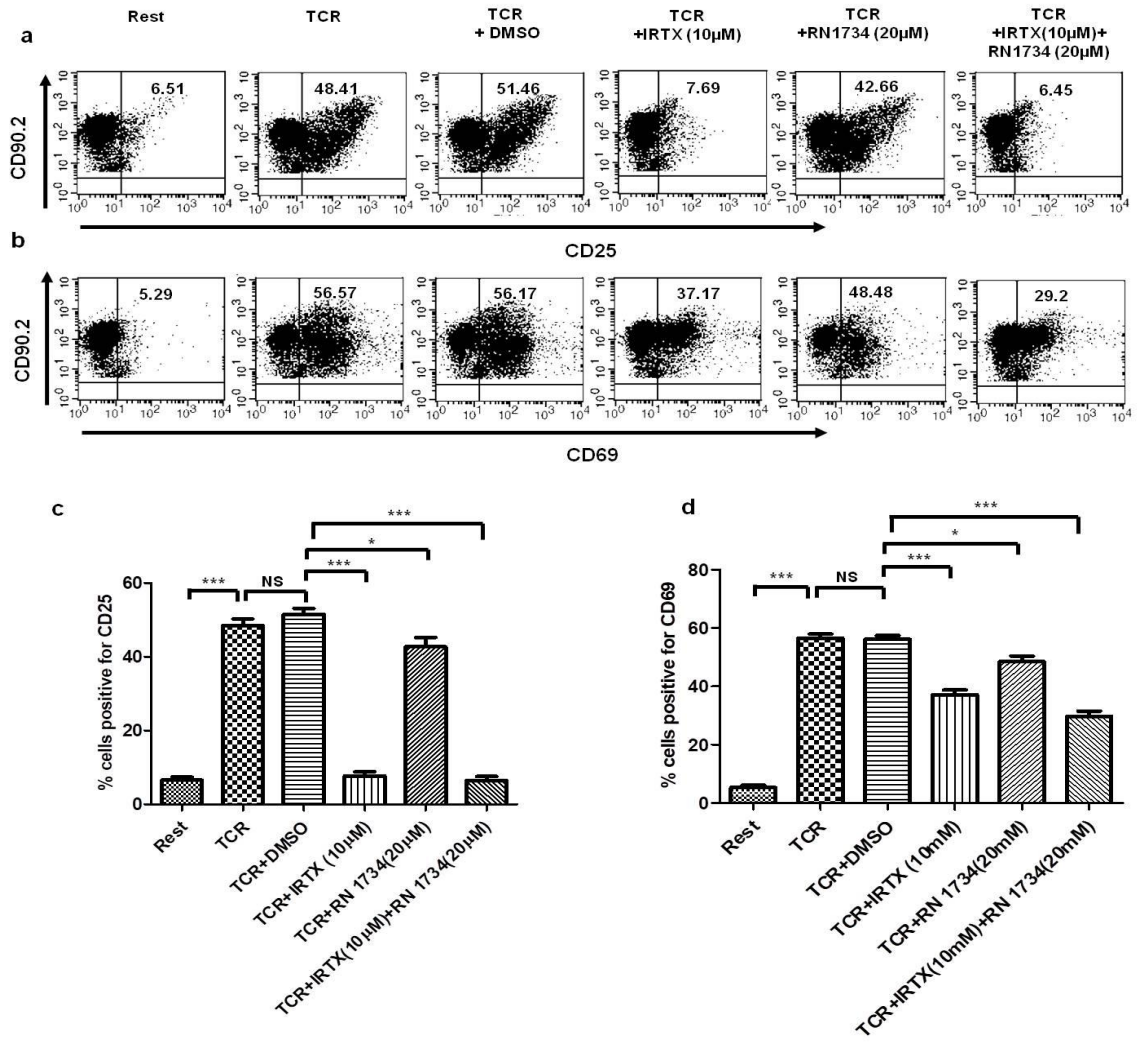


Figure 24. Pharmacological inhibition of the endogenous activity of TRPV1 and TRPV4 blocks TCR mediated T-cell activation. a. Murine T cells were pre-treated with TRPV1 and TRPV4 inhibitors at the indicated concentrations for 36h followed by α-CD3/α-CD28 stimulation and analyzed by flow cytometry for expression of T cell activation markers CD25 and CD69. The values mentioned in the upper right corner of each flow cytometric dot plot indicate the average percentage of cells that are positive for CD25 (a) and CD69 (b). One of dot plot from three similar independent experiments is shown. Bar graphs are showing the percent positive cell for CD25 (c) and CD69 (d) from three similar independent experiments (n =3). The p values are: ns = non-significant; * = < 0.05; ** = <0.01; *** = <0.001.

4.2.1.6 TRPV1 and TRPV4 inhibition downregulates T cell effector cytokine secretion and proliferation.

Since immune cell activation involves a coordinated action of several effector cytokines, we explored the role of TRP channels in cytokine secretion by analyzing the cell-free cell culture supernatants by sandwich ELISA for the presence of different cytokines released from splenic T cell culture. We observed that TRPV1 specific blocker 5'-IRTX and TRPV4 specific blocker RN1734 decreased the IFNγ production significantly as compared to cells activated with TCR only (Fig. 25 a). TRPV1 and TRPV4 inhibition alone were found to modestly decrease the production of IL-2 and TNF (Fig. 25 b & c). However, the decrease in IL-2 and TNF production was not significant as compared to TCR stimulated cells. Interestingly, when both the channels are blocked by specific inhibitors 5'-IRTX and RN1734 for TRPV1 and TRPV4 respectively the production of IL-2 and TNF was significantly decreased (Fig. 25 b & c). Moreover, we further studied the effect of decreased cytokine production on T cell proliferation during TCR stimulation (Fig. 25 d). In resting cells 2.45±0.44% cells and after TCR stimulation 45.84±1.35% cells were showing proliferation respectively. The cells treated with 5'-IRTX and RN1734 or both were showing significant downregulation of TCR mediated T cell proliferation. In TCR +5'-IRTX 13±0.89% cells were proliferating whereas TCR + RN1734 treated cells 26.74±1.66% cells showed proliferation. In cells treated with both 5'-IRTX and RN1734 along with TCR only 15.36±0.94% cells were showing proliferation. These results suggest that TRPV channel inhibition regulates T cell effector cytokine production and cell proliferation during T cell activation.

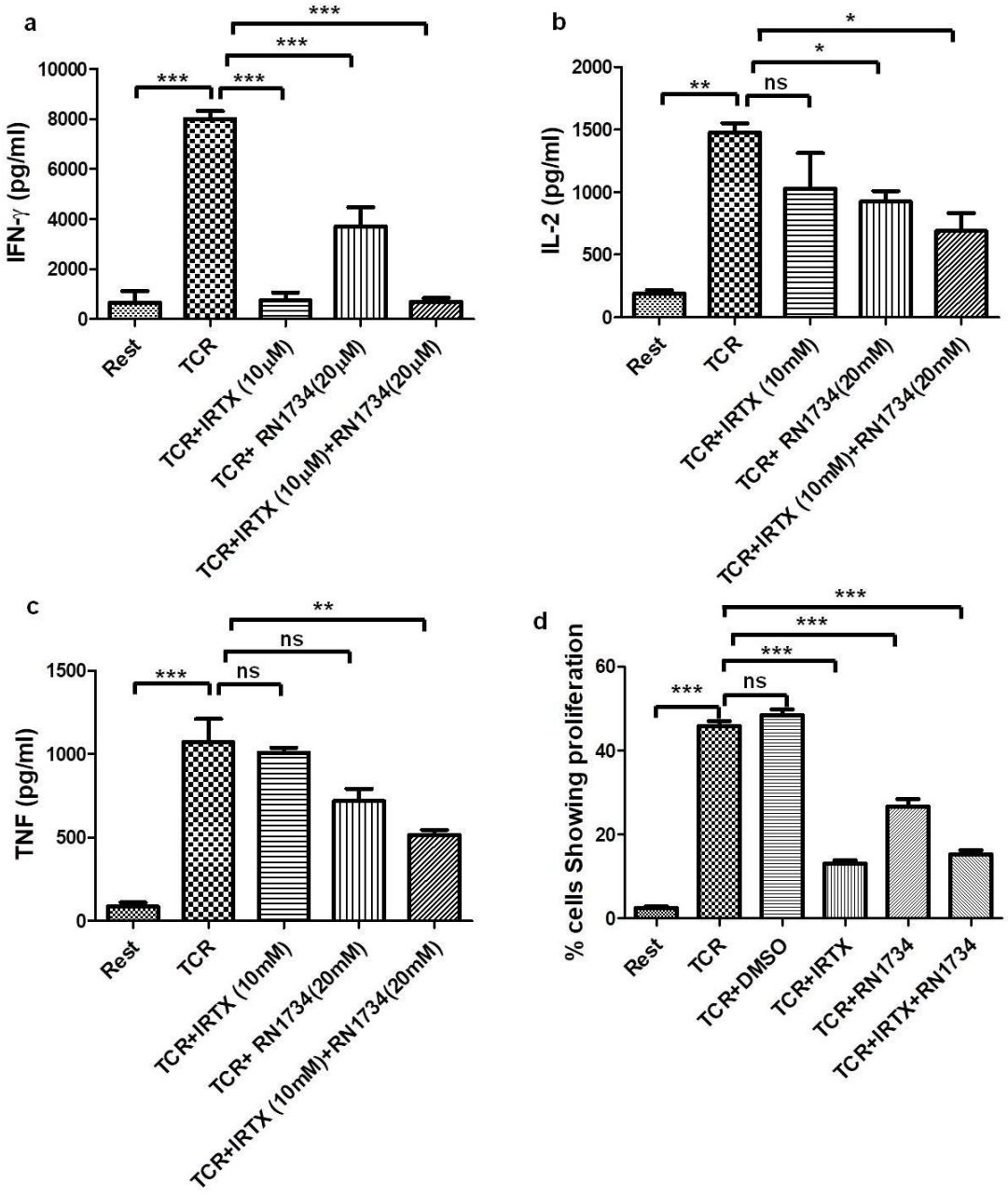


Figure 25. Pharmacological inhibition of the endogenous activity of TRPV1 and TRPV4 synergistically blocks cytokine release from T-cells and regulates T cell proliferation. Graphical bars represent the concentration (in pg/ml) of IFN-γ (**a**) IL-2 (**b**) and TNF (**c**) released from TCR stimulated murine T cells that are either pre-treated with TRPV1 inhibitor (IRTX), or TRPV4 inhibitor (RN1734), or pre-treated with both the inhibitors synergistically. **(d)** Bar graphs represent percent cells showing

proliferation after TCR stimulation with TRPV1 and TRPV4 inhibitor. The p values are: ns = non-significant; * = <0.05; ** = <0.01; *** = <0.001. (n = 3 independent experiments)

4.2.2 Discussion:

Expressions of TRP channels in different tissues including neurons are well established. However, their expression in immune cells, particularly in T cells was not well known. The effect of varying temperature on immune cells and their responses is well known. This phenomenon is studied in higher mammals to lower species like birds, amphibians, and teleost (202,250-255). TRPV1 knockout animals (*Trpv1-/-*) study has shown its role towards bacterial clearance and cytokine gene expression (256). Involvement of TRPV1 in macrophage-mediated systemic inflammatory responses including NO and ROS production and bacterial clearance was studied in TRPV1 knockout mice (257). However, the molecular mechanisms involved with TRP channel function in immune cells are not well studied. Several members of the TRPC family have been reported to be expressed in T cells (182). Different TRPC channels have been detected in T cells at the mRNA and protein level (258). Moreover, it's functional role in TCR mediated Ca^{2+} entry and proliferation has been reported earlier (181,182). Among other TRP channels, the expression and function of TRPV members in T cells remain scanty. Some reports suggest that TRPV1 mRNA was not detected in splenocytes, thymocytes, lymphocytes, purified B-cells and T cells of C57BL/6 mice. However, mRNA of TRPV2, TRPV3, and TRPV4 were found to be present in the above mentioned immune cells (259). In a different study, RT-PCR, and quantitative real-time PCR analysis showed the presence of specific mRNAs for TRPV1 and TRPV2 in human PBMCs (260). The immune-staining study showed the distribution of TRPV1 and TRPV2 in human PBMCs (261). Moreover, the channel present are found to be functional as evident from the concentration-dependent death of PBMCs by RTX (>20 μM), and that it can be blocked by inhibiting TRPV1 using 5'-IRTX (260). RT-PCR analysis has detected another TRPV member,

TRPV6 (CaT1) in Jurkat cells and it has been found to be involved in the generation of store-operated Ca^{2+}-entry, which is critical for activation of Jurkat cells (262,263). Several other studies have reported the presence of TRPV5 and TRPV6 in Jurkat and human T-lymphocytes at mRNA and protein level (264). Both these channels are reported to be involved in Ca^{2+}-conductance and cell cycle progression of T cells (190). TRPV1 mediated regulation of TCR induced Ca^{2+}-influx, T cell activation, the release of effector cytokines and colitis severity has also been shown recently (265). However, the presence and functional significance of other members of TRPV family members in the perspective of T cell activation and effector cytokine response has not been studied in details.

In this work, we observed that TRPV1, TRPV2, TRPV3, and TRPV4 channels were endogenously expressed in primary T cells from human PBMCs and purified mouse T cells. During activation with mitogenic activator ConA, we found that TRPV1 and TRPV4 expression (MFI) was modest but significantly up-regulated in purified mouse T cells. Upon activation by specific ligands of TRPV1 (RTX) and TRPV4 (4αPDD), Ca^{2+}-influx occurred in purified murine T cells, which suggests that the channels present are functional even in resting T cells. Inhibition of TRPV1 and TRPV4 by specific pharmacological inhibitors was found to decrease the TCR driven Ca^{2+}-influx of T cell to a significant level. Moreover, when the inhibitors of these channels were used in combination, they further reduced the TCR mediated Ca^{2+}-influx. TCR mediated stimulation leads to activation of T cells and effector cytokine secretion. TRPV1 and TRPV4 specific inhibitors significantly down-regulated the expression of T cell activation marker CD25 and CD69 during TCR stimulation in murine T cells. The combinations of TRPV1 and TRPV4 inhibitors show synergistic effect and almost abolish T cell activation, suggesting that both these channels may play an important role in T cell activation. Moreover, we observed a similar down-regulation of secretion of T cell effector cytokines IL-2, IFN-γ and TNF in cells treated with

TRPV1 and TRPV4 inhibitors. Both these channels are likely to be associated in the signaling pathways triggered by TCR hence inhibiting these channels had a significant inhibitory effect on CD25 and CD69 expression along with the inhibition of release of signature effector cytokines of T cells such as TNF, IL-2, and IFNγ. Even though our work establishes the significance of TRPV1 and TRPV4 in the regulation of T cell functions, the involvement of other TRP channels cannot be ruled out as a recent study indicates that TRPV5 and TRPV6 may play an important role in regulating phytohemagglutinin (PHA) mediated Ca^{2+}-influx (190).

The endogenous expression of functional TRPV channels and differential regulation in their expression in response to ConA mediated activation is important regarding the involvement of TRPV members in different forms of acute and chronic pain. This study importantly suggests a context of neuro-immune interactions. For example, production and release of different cytokines from immune cells are regulated by TRPV members and such cytokines can affect nearby peripheral neurons to induce neuronal stimulation as observed in case of inflammation and tissue injury. The diverse immune-regulatory effects and the molecular mechanism of neuropeptides and other stimulatory components can be explained by the presence of TRPV channels in T cells (266-268). Our current investigation confirms the presence of functional TRPV members in T cells and their immune-regulatory roles in T cells. Although further investigation is needed towards the *in vivo* and more in-depth studies, the current findings have immense implications in pain, immunotherapy, and regulation of neuro-immune interactions associated with TRPV channels in translational research.

Functional expression of TRPA1 channels in T cells

4.3.1 Results

4.3.1.1 TRPA1 is expressed endogenously in primary murine T cells

To find out the endogenous expression of TRPA1, we purified primary T cells and stained with specific antibody directed against the extracellular loop-1 of TRPA1. As this specific epitope is present outside, this antibody also allows us to probe for the surface expression of TRPA1 (in un-permeabilized cells) as well as total TRPA1 expression (in Triton X 100-permeabilized cells). we have probed for the surface expression as well as total expression of TRPA1 in T cells that are at resting (naïve) stage and activated with either the mitogen Concanavalin A (ConA, a lectin that acts as a mitogen and results in T cell activation) or by T cell Receptor (TCR) stimulation with α-CD3/α-CD28 antibodies (269,270).

Confocal microscopy analysis revealed that TRPA1 is endogenously expressed in resting and in activated T cells as distinct clusters (**Fig. 26 a**). In both conditions, these clusters are primarily located at the cell surface (**Fig 26 a i-ii**). The intracellular localization of TRPA1 is almost minimal as there is no significant difference in its expression level, i.e., TRPA1 present in surface versus in total cell in resting conditions. However, all the T cells do not express TRPA1 at resting state. The expression level of TRPA1 is increased in ConA activated and in TCR activated T cells (**Fig. 26 b, c**). Notably, the TRPA1-specific signal can be blocked upon pre-incubating the antibodies with their antigenic peptide indicating that the antibody used is highly specific for the epitope (**Fig. 26 a iii**).

Flow cytometric evaluation also indicates that frequency (% positive cells) of $TRPA1^{+}$ cells were increased during T cell activation (**Fig. 27 a**). The specificity of anti-TRPA1 antibody was evaluated by blocking peptide (**Fig. 27 b, c**) in resting condition, a significant number (33.22 ± 2.44%,) of T cells express TRPA1 at their surface only while almost equal number (37.16 ± 2.74%,) of cells show expression in the whole cell (both surface as well as in intracellular region)

(**Fig. 27 d**). After 36 hours of treatment with ConA, an increase in the TRPA1 positive T cells as detected by surface labeling (72.02 ± 3.76%,) and by total cell labeling (74.08 ± 0.98%,) is observed (**Fig. 27 d**). Similarly, in case of TCR mediated activation, number of TRPA1 positive T cells as detected by surface labeling (75.44 ± 1.89%,) and at whole-cell labeling (76.41 ± 3.02%,) is higher than the resting conditions (**Fig. 28 d**). However, the mean fluorescence intensity (MFI) values of TRPA1 in resting T cells as detected by surface labeling (17.25 ± 6.65,) as well as by total cell labeling (21.43 ± 9.12,) is similar (**Fig. 27 b**). In case of ConA activated T cells, the MFI representing the expression of TRPA1 at the surface (54.85 ± 17.27,) and in whole cell (60.75 ± 25.6,) is higher than that of the resting T cells (**Fig. 27 e**). The MFI representing the expression of TRPA1 in TCR activated T cells is (52.68 ± 16.4), detected by surface labeling; and 57.02 ± 21.2, detected for whole cell TRPA1) significantly higher than the expression in resting T cells (**Fig. 27 e**). These qualitative and quantitative data strongly suggests that increased surface expression of TRPA1 correlates with T cell activation process.

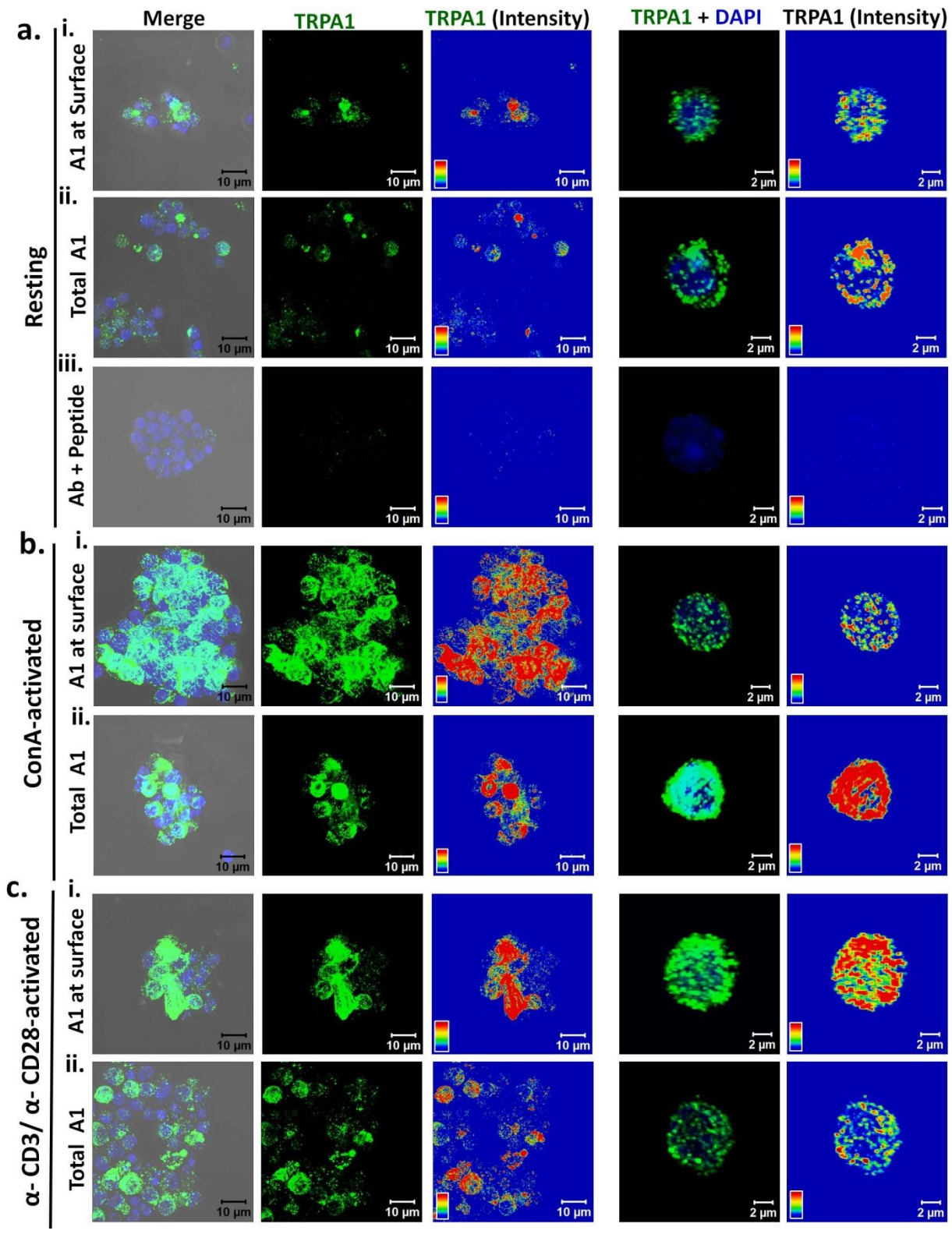


Figure 26. Endogenous TRPA1 is expressed in primary murine T cells. Confocal images of purified $CD3^+$ murine T cells are depicted. Cells have been stained with an antibody detecting the extracellular

loop of TRPA1 allowing to detect the surface expression (**a-i**, **b-i**, **c-i**) in non-permeabilized cells and total TRPA1 expression (**a-ii**, **b-ii**, **C-ii**) in permeabilized cells. Both surface level and total expression of TRPA1 in ConA-activated T cells (**b**) and α-CD3/ α-CD28-activated T cells (**c**) are higher than that in resting T cells (**a**). Staining of TRPA1 with antibody pre-incubated with its antigenic peptide (peptide control, **a-iii**) in Triton-X100 permeabilized cells results in loss of TRPA1 signal, indicating the specificity of the antibody. Fluorescence intensity of the TRPA1 signal is indicated in rainbow scale (right panels).

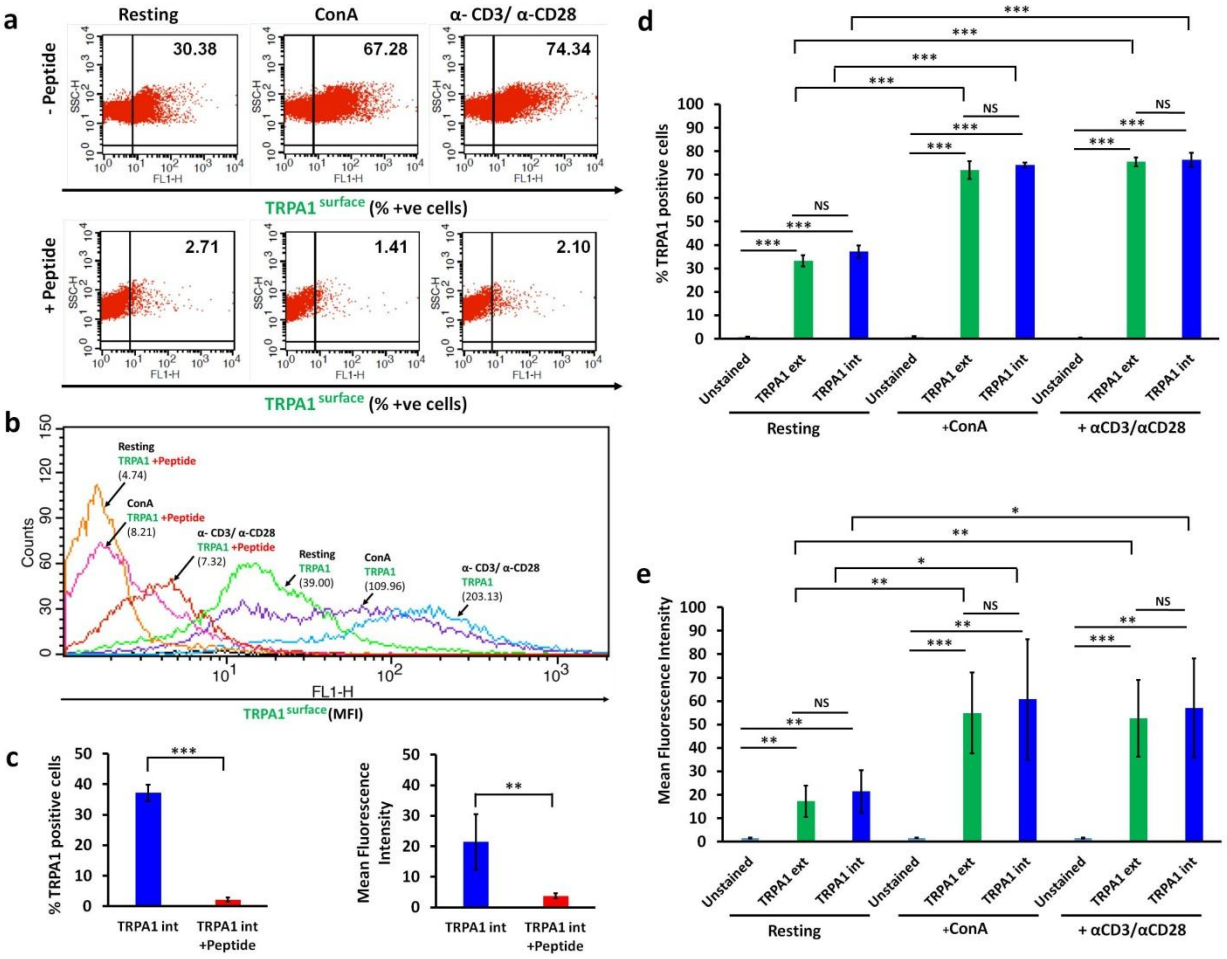


Figure 27. **TRPA1 expression is increased in activated T cells**. **a.** ConA-activated and α-CD3/α-CD28-activated T cells have a higher number of TRPA1 positive T cells than the resting T cells. **b-c**. The Mean Fluorescence Intensity plot indicates increased TRPA1 expression per cell in activated conditions (n = 4). Both dot plot (**a**) and MFI histogram (**b-c**) shows that TRPA1 signal is blocked when the same antibody is

pre-treated with specific blocking peptide. This is observed even during the activated condition, both for surface-stained (**a**, **b**) and intracellular-stained (**c**) conditions (n = 4). **d.** Flow cytometry profiles demonstrating the % of cells positive for TRPA1 channel are shown. Immunostaining of permeabilized cells reveals that ConA-activated and α-CD3/α-CD28-activated T cells have a significantly higher number of TRPA1 positive T cells than the resting T cells. The difference between ConA-activated and α-CD3/α-CD28-activated T cells remain non-significant. **e.** Similarly, the MFI values show higher TRPA1 abundance per cell (intensity) in activated T cells. The p values are: ns = non-significant; * = < 0.05; ** = <0.01; *** = <0.001.

4.3.1.2 TRPA1 activation induces Ca^{2+}-influx into T cells

In order to explore if the TRPA1 present in T cells is functional, we performed Ca^{2+}-imaging experiments (**Fig. 28**). For that purpose, we have used purified mouse T cells loaded with Fluo-4 AM. Live cell imaging revealed that in the absence of any stimuli, there is no increase in Fluo-4 intensity in the majority of the cells with respect to time. However, upon stimulation by TRPA1 activator AITC, intracellular Ca^{2+} level was increased in most of the T cells. This AITC-mediated influx can be effectively blocked by TRPA1-specific inhibitor A-967079. This confirms that functional TRPA1 is expressed in T cells.

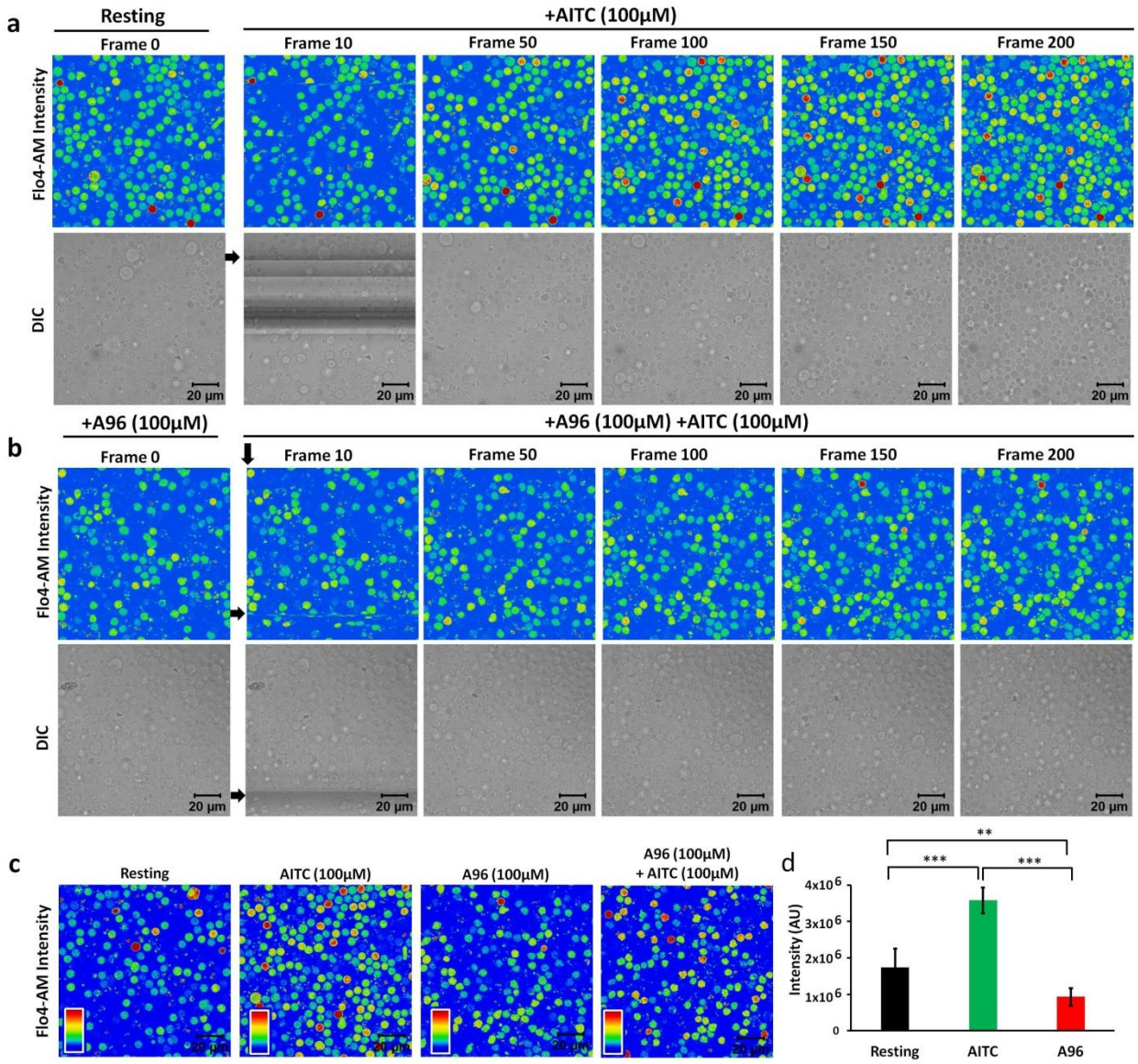


Figure 28. **TRPA1 activation induces Ca^{2+}-influx into murine T cells**. **a-b.** Fluorescence intensity images derived from time-series imaging of view fields containing multiple cells loaded with Ca^{2+}-sensing dye Fluo-4 AM are depicted here. The time difference between each frame is 5 seconds. The cells were treated with different pharmacological agents at the 10^{th} frame (F10). Activation of TRPA1 by its specific activator Allyl isothiocyanate (AITC 100uM) causes an increment in the Ca^{2+}-level (A) which can be blocked by pre-incubating the cells with TRPA1-specific inhibitor A967079 (A96; 100 µM) for 30 min (B). **c.** Fluo-4 intensity of T cells at resting stage or incubated with AITC (100µM), A96 (100µM) or combination of AITC (100µM) and A96 (100µM) for 12 hours is shown. **d.** Quantification of Fluo-4

intensity from 6 random fields after the acquisition of 200 frames (1000 second) of two independent experiments were depicted. AITC (100μM) treatment significantly increases Ca^{2+}-influx, while A96 (100 μM) treatment reduces intracellular Ca^{2+}-levels below that of resting T cells. The p values are: ns = non-significant; * = < 0.05; ** = <0.01; *** = <0.001.

4.3.1.3 TRPA1 is involved in T cell activation by mitogen or TCR stimulation

In order to explore the role of TRPA1 in T cell activation, we had activated the cells either by ConA or via TCR stimulation and probed for the expression of activation markers, namely CD25 and CD69 in the $CD3^+$ murine T cell population. The expression of these markers was probed after ConA treatment (for 36 hours) with or without TRPA1 channel modulators (**Fig. 29**). Dot-plot values from flow cytometric evaluation revealed a shift in the T cell population expressing CD25 upon T cell activation (in resting condition, 3.495 %; in ConA-activated condition, 73.79%; in TCR -activated condition, 59.07%) respectively (**Fig. 29 a, and c**). Notably, in the presence of TRPA1 inhibitor (A-967079, 100μM), T cell activation by ConA or TCR was almost significantly inhibited (**Fig. 29a, and c**).

In a similar manner, TRPA1 inhibitor (A-967079) also reduces CD69 expression. The effect of TRPA1 inhibitor on CD69 expression was also reflected by values of the percentage of CD69 positive cells (resting condition, 1.36%; ConA-activated condition, 46.33%; TCR activated 31.48%) which was reduced after treating with the inhibitor (ConA + A-967079, 16.19%; TCR + A-967079, 5.62%) (**Fig. 29 b,** and **d**).

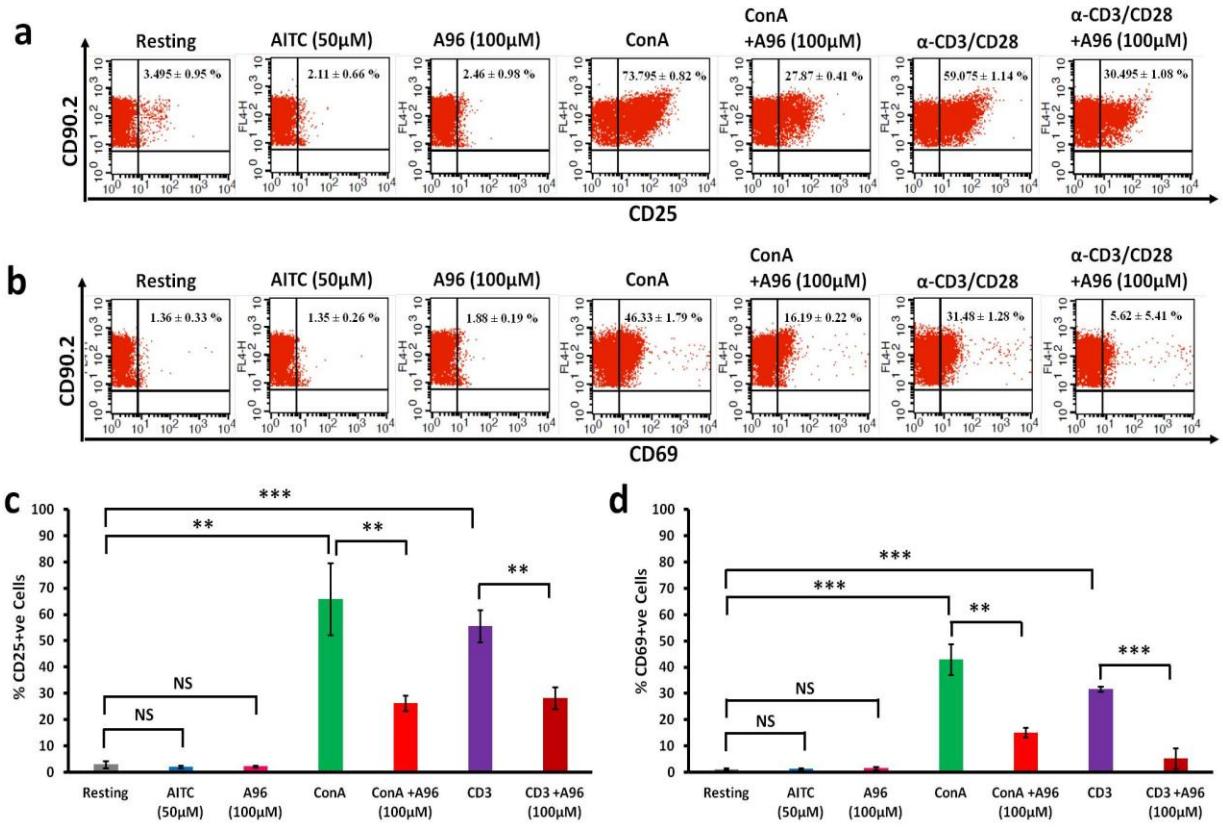


Figure 29. Pharmacological inhibition of endogenous TRPA1 blocks T cell activation. a-b. T cell activation markers CD25 and CD69 were analyzed by flow cytometry after incubating the cells with TRPA1 modulators for 36 hours. Treatment of murine T cells with AITC (50µM) or A96 (100µM) alone did not increase the percentage of $CD25^+$ cells or percentage of $CD69^+$ cells. The values mentioned in the upper right corner of each flow cytometric dot plot indicate the average number ± SD values of cells that were positive for CD25 or CD69. Representative dot plots of three independent experiments were shown. **c-d.** Treatment of T cells with A96 (100µM) along with ConA (5 µg/ml) or plate-bound α-CD3 (2µg/ml) and soluble α-CD28 (2µg/ml), decreased the percentage of cells expressing CD25 or CD69. The p values are: ns = non-significant; * = < 0.05; ** = <0.01; *** = <0.001.

4.3.1.4 TRPA1 inhibition blocks secretion of cytokines involved in T cell activation

T cell activation involves an increase in the levels of several effector cytokines like Tumor Necrosis Factor (TNF), Interferon-gamma (IFNγ) and Interleukin-2 (IL-2). We explored the role of TRPA1 in the production of these cytokines by analyzing the T cell culture supernatants by

ELISA (**Fig. 30**). TCR stimulation was found to induce high levels of TNF (**Fig. 30 a**) (Resting condition: 151.8 pg/ml; TCR-mediated activated condition: 2931.98 pg/ml) while inhibition of TRPA1 by A-967079 significantly blocked the effect of these stimulators on TNF production (TCR + A-967079 combination: 597.74 pg/ml). Similarly, TRPA1 inhibition also blocks IL-2 (**Fig. 30 b**) secretion (Resting: 62 pg/ml; TCR: 1067.4 pg/ml; TCR + A-967079 combination: 200.33 pg/ml). Moreover IFNγ (**Fig. 30 c**) production was significantly down-regulated by A-967079 treatment (Resting: 912.83 pg/ml; TCR: 10279.5 pg/ml; TCR + A-967079 combination: 1421.72 pg/ml)

Taken together the results indicate that TRPA1 might be required towards T cell activation derived effector cytokine release.

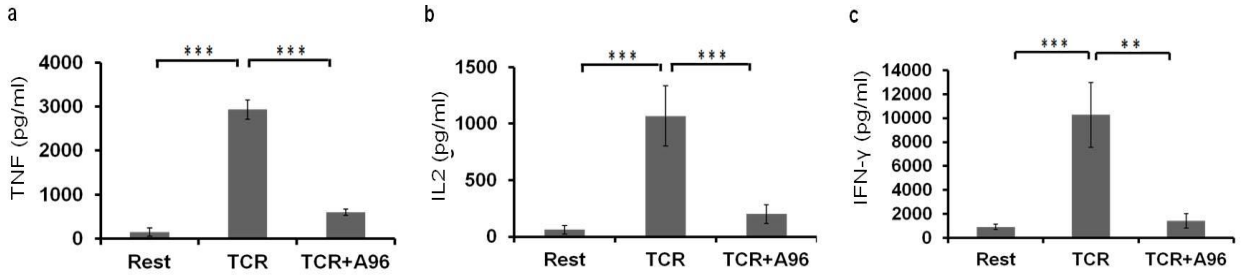


Figure 30. Pharmacological inhibition of endogenous activity of TRPA1 blocks cytokine release from T cells. a-c. Graphical bars represent the concentration (in pg/ml) of effector cytokines TNF (**a**), IL-2 (**b**), IFNγ (**c**) released from T cells around 36 hours of treatment with the indicated modulators. Incubation of TRPA1 inhibitor A96 (100μM) along with plate-bound α-CD3 (2μg/ml) and soluble α-CD28 (2μg/ml) resulted in significant reduction in the release of cytokines TNF, IL-2 and IFNγ. The p values are: ns = non-significant; * = <0.05; ** = <0.01; *** = <0.001. (n = 3 independent experiments).

4.3.2 Discussion:

T cell activation involves several distinct signaling events, and the influx of Ca^{2+}-ions is vital during this process. However, so far only a few Ca^{2+} channels have been detected in T cells,

whereas the identity of the major Ca^{2+} channels present in T cells is unknown. Also, the mode of regulation of these channels and their exact role in the context of T cell functions are largely unknown. TRPA1 can be activated by several means and also by endogenous factors. Though TRPA1 has been considered as a "cold-activated ion channel," its true thermosensitive nature is debatable. For example, it has been shown that TRPA1 is not directly gated by cold but rather gated by increased intracellular Ca^{2+}-levels as a consequence of cooling (271). It is important to mention that Ca^{2+} ions directly gate hTRPA1 in a PLC-independent fashion. This is confirmed by the fact that TRPA1 shows Ca^{2+}-induced currents even after treatment with the PLC-blocker U73122 (10 μM) (272).

In this work, we demonstrated that functional TRPA1, a non-selective cation channel is expressed in the human and murine T cells. Our results suggest that TRPA1 plays an important role relevant for T cell activation, likely by different endogenous factors. In a recent report, the functional expression of TRPA1 in murine and human $CD4^{+}$ T cells have been shown to regulate T cell activation and pro-inflammatory responses (273). However, the previous reports had suggested a pro-inflammatory role of TRPA1 towards immune activation and inflammation (274). So far, TRPA1 had been considered as one of the key regulators of neurogenic inflammation and neuropeptide release (275). It has also been reported that TRPA1 is associated with inflammation and pruritogen responses in dermatitis (276). In fact expression of TRPA1 in the peripheral sensory neurons and its involvement in the neural excitation has been demonstrated. However, limited information is available currently about the expression and role of TRPA1 in non-neuronal cells. The association of TRP channels towards inflammation and immunogenic responses has been largely found to be positively regulated during the immune-physiology of the cellular and systemic responses. TRPA1 had been reported to contribute towards inflammation induced pain and associated experimental colitis in mice models (276-279). Interestingly, recently

it has been reported that $CD4^+$ T cell-associated TRPA1 may regulate inflammation in experimental colitis model in mice (273). However, evidence for any such functional requirement of TRPA1 in T cell responses, *in vitro*, remains scanty.

In this work, endogenous expression of TRPA1 in primary murine and human T cell was observed by using TRPA1 specific antibodies. Expression of TRPA1 was found to be most predominant at the surface of these cells rather than being present in intracellular regions. We also provided evidence for the functional role of TRPA1 in immune functions. Flow cytometric analysis coupled with confocal imaging conclusively suggested enhanced expression of TRPA1 (both at surface level and in total) in activated T cells as compared to resting conditions. TRPA1 activation by specific ligands lead to increased intracellular Ca^{2+} level in purified murine T cells, confirming that this channel is present in a functional form in resting T cells. Inhibition of TRPA1 by its specific inhibitor reduces TCR and Con A-driven mitogenic activation of T cells. These results suggest that TRPA1 might be involved in the signaling pathways towards T cell activation and thus inhibiting TRPA1 inhibition has profound inhibitory effects on CD25 and CD69 expression together with the secretion of signature effector cytokines such as TNF, IFN-γ, and IL-2. TNF production is associated with several pro-inflammatory responses (280). Additionally TNF is shown to be a major mediator of different inflammatory disease conditions like colitis and rheumatoid arthritis (RA) (281,282). Moreover, it has been shown that TRPA1 facilitates TNF directed inflammatory responses in various pathophysiological conditions and blockade of TRPA1 receptors may be beneficial in reducing TNF-induced chronic pain (283,284). Interestingly, it has been shown that $TRPA1^{-/-}$ T cells may produce higher IL-2 and IFN-γ but not TNF during TCR stimulation (273). Functional role of IFN-γ as a signature Th1 cytokine is also implicated in pro-inflammatory responses and disease conditions like colitis (285). However, there are some reports which suggest an anti-inflammatory role of IFN-γ in a

mouse model of colitis (286,287). Apparently, these reports suggest that these cytokines may differentially regulate inflammatory responses in different disease conditions. Interestingly, we have found that during *in vitro* TCR activation the induction of signature Th1 and pro-inflammatory cytokines like IL-2, IFN-γ, and TNF could be down-regulated by the TRPA1 specific inhibitor, A-967079. Moreover, ConA and TCR stimulated induction of T cell activation markers like CD25 and CD69 were also found to be downregulated in presence of A-967079. Similar observations were found when TRPA1 upon activation by its specific activator (AITC) was found to increase the Ca^{2+}-levels, while A-967079 treatment was found to reduce the intracellular Ca^{2+}-levels. Moreover, it was observed that A-967079 treatment might suppress $CD4^{+}$ and $CD8^{+}$ T cell activation (data not shown). Therefore, it seems that during T cell activation, TRPA1 becomes functional and shows augmented expression and executes immune-regulatory functions, whereas, inhibition of this channel inhibits T cell activation. While our current work confirms the functional expression of TRPA1 channel and its requirement towards T cell activation, the involvement of this ion channel in T cell activation process associated with inflammatory diseases needs further investigation.

In brief, our current findings demonstrated an *in vitro* T cell activation directed functional expression and requirement of TRPA1 in T cells. This is in line with the earlier reports of inflammatory responses associated with the functional role of TRPA1 in various physiological systems. The current observation might have implication in the immunogenic and inflammatory role of T cell responses as well.

CHAPTER # 5

Summary

&

Future directions

5. Summary and future directions:

5.1 Summary:

T cell is a signature member of the adaptive immune system, and an executioner of cell-mediated immunity (CMI). The established paradigm of T cell research is abundant with seminal observations involving T cell receptor (TCR) engagement, activation/effector function, T immune-regulatory cell (Treg) responses with the altered state of adaptive immunity associated to basic and translational research. However recent evidence propose that T cell function could be associated with newly emerging fields such as TLRs and TRP channels. Immunological "sensing" through innate immune receptors like Toll-like receptors (TLRs) and "sensation" of pain, temperature and other neurological responses though transient receptor potential channels (TRP channels), which are relatively permeable to non-selective cations, have been proposed for innate immune responses. However, virtually nothing is clearly established for functional expression of TLRs and TRP channels in T cell repertoire. Accordingly, we have studied the role of TLRs and TRP channels in naïve T cells responses during T cell activation.

We have found that TLR4 expression is differentially regulated during T cell activation whereas no significant change was observed in the case of TLR2 and TLR5. Inhibition of TLR4 signaling pathway through VIPER (an established TLR4 specific inhibitor) during T cell activation reduced surface expression of T cell activation markers (CD25 and CD69) significantly. Further, it was also observed that T cell effector cytokine secretion (IL-2, IFN-γ, and TNF) and T cell proliferation followed by T cell activation was also down-regulated in the presence of VIPER as compared to control peptide. To maintain T cell homeostasis, T cell activation is followed by Activation-Induced Cell Death (AICD). We found that VIPER down-regulated the induction of Fas (CD95) and FasL (CD95L) in T cell during T cell activation. Also executioner caspase of

apoptosis, cleaved caspase 3 was found to be down-regulated in VIPER treated cells as compared to cells treated with only TCR or TCR+ Control Peptide. Thus, these results suggest an important role of TLR4 in T cell activation and effector function which might have implication towards altered immune responses during acute stage T cell activation processes.

Similarly, we investigated the expression of different TRP channels in mouse and human naïve T cells and found that TRPV1, TRPV2, TRPV3, TRPV4 and TRPA1 channels are present. During T cell activation expression of TRPV1, TRPV4 and TRPA1 channels were found to be up-regulated. Upon activation by TRPV1, TRPV4, and TRPA1 specific activators intracellular concentration of Ca^{2+} was increased in naïve T cells. During TCR mediated stimulation intracellular Ca^{2+} concentration in T cells increases which is critical for T cell activation and effector function. When cells were pretreated with channels specific inhibitors, the intracellular increase of Ca^{2+} was decreased during TCR stimulation. These results suggest that the TRP channels present in T cells are functional and contribute towards an increase in intracellular Ca^{2+} during T cell activation. TRP channel inhibitors treated T cells showed reduced expression of T cell activation markers CD25 and CD69 suggesting the functional role of TRP channels during T cell activation. Moreover, T cell effector cytokine production during T cell stimulation was also found to be reduced upon TRP inhibitor treatment. Combined all these results suggest the importance of TRP channel in T cell activation and effector function.

5.2 Future Directions:

TLRs and TRP channels are comparatively novel receptors associated with T cell biology and functions. TLRs are known to be associated with different disease conditions and can modulate T cell effector functions. In this study, we have found the direct effect of TLR4 on naïve T cell functions. However, the interaction between different TLR4 signaling molecules and molecules

involved in T cell activation can be studied for designing TLR4 specific therapeutic strategies for effective treatment of diseases associated with altered T cell effector functions. Moreover, signaling molecules like MAPK proteins associated with regulation of T cell functions can be studied during VP treatment. Direct expression of TLR4 on T cells and the effect of their modulation on T cell function in different disease models can also be studied. For TRP channels, our current work confirms the expression of functional TRPV and TRPA1 channels and their involvement in T cell functions. However, the detail regulations and involvement of these thermosensitive channels in the T cell activation process and signaling cascade need to be studied. TRP channels are known for their involvement is sensation of pain and temperature. It will be useful to study the role of different TRP channels in autoimmune diseases like Rheumatoid Arthritis where pain is a major factor. Moreover, recent studies have shown the effect of TLR4 agonist LPS on sensitization of TRPV1 in neuronal cells(288). It would be interesting to investigate the association of TLRs and TRP channels in immune cell function and regulation which might open a novel area in immunology and infectious disease biology.

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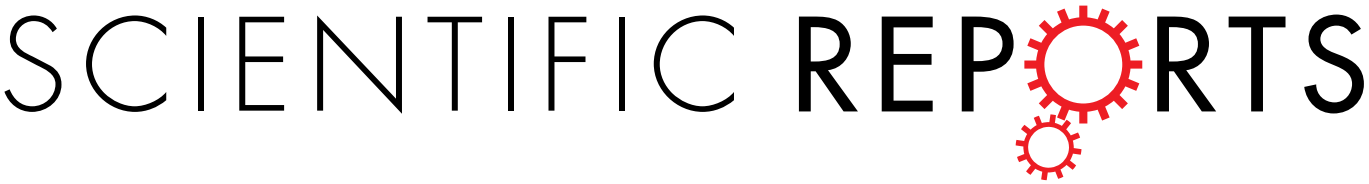
Publications



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VIPER regulates naive T cell activation and effector responses: Implication in TLR4 associated acute stage T cell responses

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Naive T cells are known to express the modest level of TLR4 while it is known to go down during TCR activation. However, information towards the requirement of TLR4 signaling during TCR or mitogenic activation of naive wild-type T cells remains scanty. Here we have investigated the endogenous functional expression of TLR4 in naive mice T cells during TCR and mitogenic stimulation in presence of VIPER peptide (VP), an established inhibitor of TLR4 signaling. As expected we found that TLR4 expression goes down during TCR and mitogenic activation. Interestingly, we observed that VP treatment restores TLR4 expression on those activated T cells. Moreover, VP was found to regulate such activation of naive T cell as evident by reduction of CD25, CD69 expression, effector cytokines (IL-2, IFN-γ, TNF) production, T cell proliferation and down-regulation of T cell activation-dependent Fas (CD95), FasL (CD95L) expression. Together, our current observation highlights a possible requirement of TLR4 responses in T cells, which might have possible implication towards the pathogenic acute phase activation of naive T cells.

An effective immune response against an invading pathogen is coordinated by both innate and adaptive immune system. Initially, it was suggested that innate immune system works for the recognition of pathogen and adaptive immune system destroy the pathogen or pathogen-infected cells and provide long-term pathogen-specific protection. Both innate and adaptive immune system may work in an interdependent manner to efficiently protect the host from disease and infection. The first step to start an immune response is to recognize the invading pathogen. For recognition of pathogens, the innate immune system has many receptors and TLRs are the most studied one. Pathogens specific conserved structures are recognized by pattern recognition receptors (PRRs)[1]. After pathogen recognition by TLRs innate immune cells starts a cascade of signaling pathways which ultimately activates the adaptive immune system[2].

TLR4 is one of the well studied TLR, which is expressed in the cell surface. It is expressed in the form of the homodimer, recognizes Lipopolysaccharides (LPS) from gram-negative bacteria, facilitated by CD14, Lipopolysaccharide Binding Protein (LBP) and Myeloid Differentiation Factor 2 (MD2) to activate downstream signalling[3]. TLR4 signal propagates through the cell membrane to activate Myeloid differentiation primary response gene (88) (MYD88) dependent pathway or TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathway in cytoplasm further cascades into nucleus resulting in activation of genes of pro-inflammatory cytokines[4].

Classically TLRs are known to be most efficient modulators of innate immunity. However recent evidence proposes an important role of TLRs in modulating adaptive immune response. There were certain suggestions that TLR4 is polarised towards TH1 response of antigen presenting cells[2,5]. Several studies suggest the expression and functional significance of TLRs in T cells[6,7]. Naive mouse T cells are found to express detectable level of TLR4 expression, while it may go down during TCR activation without having a direct responsiveness of LPS on T cells[8,9]. However, LPS has been shown to modulate the efficacy of regulatory T cells[10]. Moreover, it has been reported that T cell adhesion and chemotaxis could be regulated by LPS[11]. It has been proposed that TLR2 and

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TLR4 signaling could upregulate suppressor of cytokine signaling 3 (SOCS3) expression and downregulate T cell effector function[12]. Moreover, an apparent contrasting role of differential TLR4 signaling has been reported towards regulating inflammation associated with Tregs and $CD4^+$ T cell responses[9,13]. Recently, $CD8^+$ T cells from a specific cohort of rheumatoid arthritis (RA) patients, unlike naive healthy donors and Systemic Lupus Erythematosus (SLE) patients, have been shown to express elevated surface TLR4 expression and also found to respond upon LPS treatment[14]. However, the requirement of TLR4 responses towards TCR or mitogen directed acute stage T cell activation and effector function in wild-type naïve T cell population, if any, is not well reported.

Viral inhibitory peptide for TLR4 (VIPER) is an inhibitory peptide (11 aa long) specific for TLR4 derived from the A46 protein of vaccinia virus. It interacts with adaptor proteins: MyD88 adaptor-like (Mal) and TRIF-related adaptor molecule (TRAM) to inhibit TLR4-mediated MAPK and transcription factor activation. It has been shown that VIPER is able to inhibit TLR4 mediated immune response in innate immune cells such as macrophages[15]. In another study, VIPER inhibited inflammatory responses elicited by *Mycoplasma pneumoniae* in mouse macrophage suggesting a role of TLR4 in the *M. pneumoniae* mediated inflammatory responses[16]. Furthermore, treatment of mouse neuronal cells with VIPER was found to completely block TLR4 mediated chemokine (C-X-C motif) ligand 1 (CXCL1) expression and its release. It also inhibited intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) expression on endothelial cells, and induced infiltration of neutrophils across the endothelial monolayer[17]. Treatment of VIPER through intracerebroventricular route in hypertensive rat leads to reduced circulating norepinephrine levels which resulted in inhibition of delayed progression of hypertension and improvement of cardiac hypertrophy and function. Further, it reduced myocardial TNF-α, IL-1β, iNOS levels, NF-κB activity, and altered renin-angiotensin system components significantly[18]. This reveals the role of TLR4 in brain attenuation of angiotensin II-induced hypertensive response. Additionally, *in vivo* treatment of VIPER is also found to protect the rat from acute kidney injury mediated by LPS driven TLR4 stimulation[19]. It also facilitates improved glomerular filtration rate, elevated renal blood flow, and a reduced renal vascular resistance, reduction in the rate of production of free radicals (reactive oxygen species and superoxide), proinflammatory cytokine, and acute kidney injury markers[20]. Recently it has been shown that VIPER regulates LPS mediated $CD8^+$ T cell inflammatory cytokine responses from a cohort of RA patients unlike the T cells from healthy controls and SLE patients[14]. Collectively, these studies show the role of VIPER in understanding various TLR4 driven response in different cells and systems. However, the role of VIPER towards naive T cell activation and its regulation associated with effector function is yet to be explored.

In this study, we have used VIPER, as an established TLR4 signaling inhibitor and found that it may regulate the activation and effector function during TCR and mitogenic stimulation of naive T cells derived from mice. To our knowledge, this is the first report which shows that VIPER driven inhibition of TLR4 signaling may regulate activation and effector function of naive T cells from wild-type mice, which might have implication towards altered immune responses during acute stage T cell activation processes.

Results

VIPER suppresses TNF production in Raw 264.7 cells and mouse splenocytes. First, we did trypan blue exclusion assay with different dose of VP and Control Peptide (CP) in Raw 264.7 cells to find out the concentration at which cells are viable. We found that cells are viable at concentrations up to 15 μM of VP and CP (Fig. 1a). VP is an established TLR4 specific inhibitor reported to suppress LPS mediated TNF secretion in macrophages[15,16]. To ensure such effect of VP in our set up on macrophage (Raw 264.7 mouse macrophage cell line) we pre-incubated Raw cells with 5 μM[15] of VP and CP for 1 h and then treated them with 250 ng/ml LPS. The cell-free supernatant was collected after 8 h and then ELISA was done for TNF. VP treated Raw cells show a significant reduction in TNF secretion with respect to cells treated with only LPS in combination with CP as reported previously[15] (Fig. 1b). Splenocytes produce TNF upon TCR mediated stimulation. So we want to know the effect of VP on splenocytes. For that first we did cell viability assay by using Trypan Blue exclusion method with different dose of VP and found at 5 to 15 μM concentration cells are viable (Fig. 1c). Cell viability was also studied using 7AAD for purified mouse T cells with VP and CP in presence of TCR/ConA stimulation (Fig. 1e). We found that up to 10 μM of VIPER concentration nearly 100% cells are negative for 7AAD and up to 15 μM VIPER concentration more than 90% of cells are negative for 7AAD (Fig. 1e). As most of the cells are viable at 10 μM concentration of VP we used 10 μM of VP and CP for our further experiments. To study the effect of VP on splenocytes for TNF production BALB/c mouse splenocytes were pre-incubated with 10 μM VP and CP for 2 h. Then cells were stimulated with TCR. After 36 h cell culture supernatant was collected and ELISA was done for TNF secretion. VP treated splenocytes shows a significant reduction in TNF production when compared with cells treated with TCR or TCR along with CP (Fig. 1d). These data suggest that VP can suppress TNF production by naïve mouse splenocytes during TCR activation. 10 μM of VP and CP are used as an optimum concentration for suppression of T cell activation for all further experiments.

Differential surface expression of TLR4 in ConA, TCR, and VP treated cells. Previous studies have suggested the expression of TLR4 on the mouse as well as human T cells[6–9]. To investigate the differential expression of TLR4, if any, we surface stained the purified mouse splenic T cells treated with VP, CP along with TCR for 36 h with TLR4 antibody and analyzed with flow cytometry. We found that $21.5 \pm 0.5\%$ of resting T cells express TLR4 on cell surface whereas during TCR mediated stimulation TLR4 expression (only $4.99\% \pm 0.28$ cells) on the cell surface is going down significantly (Fig. 2a). Interestingly TLR4 expression in VIPER treated cells remained similar as compared to resting cells ($22.61 \pm 0.18\%$) whereas CP treated cells showed a reduction ($4.72 \pm 0.27\%$) in TLR4 surface expression at par with TCR treated cells (Fig. 2a,b). Similar results were observed with MFI data of TLR4 expression in VIPER treated cells (Fig. 2c,d). These data suggest that VP may restore TLR4 expression during activation of naïve T cells.

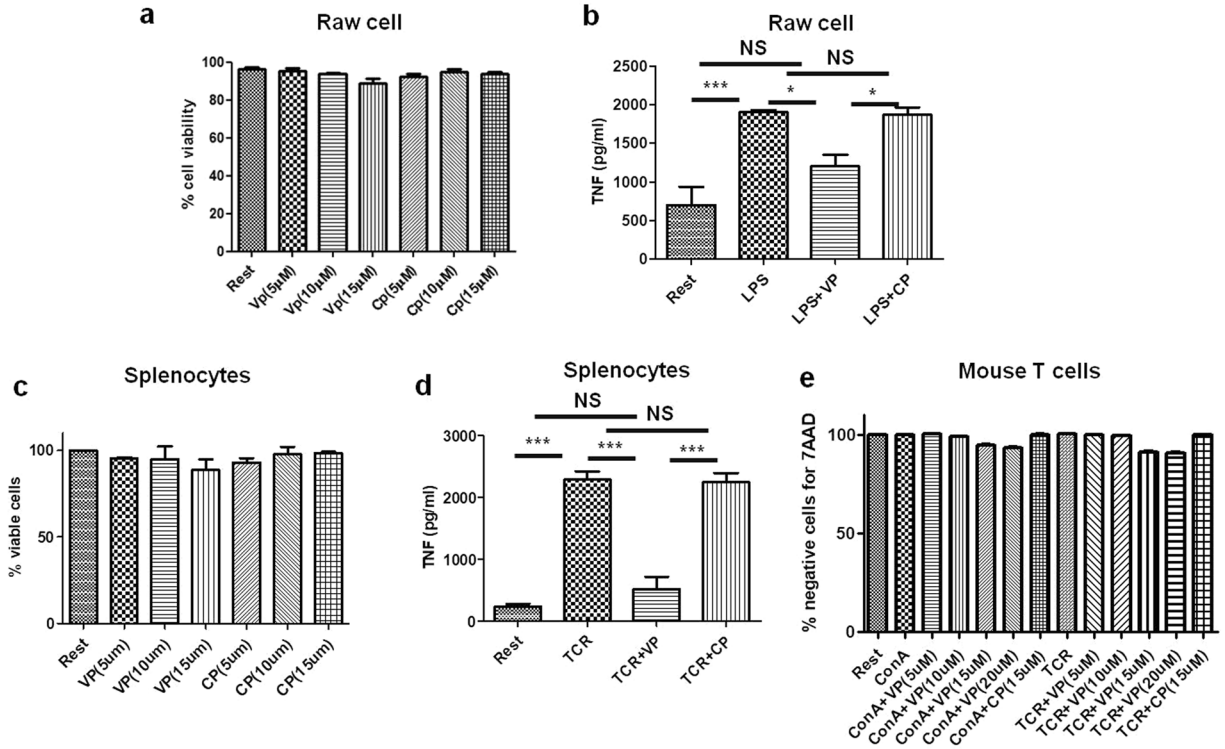

Figure 1. Cell viability and suppression of TNF secretion in Macrophages and splenocytes. (**a**) Dose kinetic study show Raw 264.7 mouse macrophage cells are viable at high concentration of VIPER i.e.15 μM. Cell viability was done by trypan blue exclusion method. (**b**) VIPER suppresses TNF secretion in Raw cells during LPS mediated activation. (**c**) Mouse splenocytes were treated with different doses of VIPER and cell viability is studied using trypan blue exclusion method. At 5 and 10 μM concentration, cells are viable. (**d**) VIPER suppresses TNF in splenocytes activated with TCR. (**e**) Percent negative cells for 7AAD shows the viability of purified mouse T cells with VIPER and Control Peptide in the presence of TCR/ConA stimulation. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. VP: VIPER, CP: Control Peptide. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

VIPER down-regulates activation of naive T cells. After observing that VIPER down regulates TNF production in splenocytes and regulates surface expression of TLR4 in purified T cells during TCR stimulation we wanted to know whether it has a direct effect on T cells activation or not. Purified T cells were pre-incubated with VP and CP for 2 h and then treated with ConA and TCR for 36 h. Cells were harvested after 36 h and stained for surface expression of T cell activation markers CD25 and CD69. Stained cells were acquired and CD90.2 gated cells were analyzed. After ConA activation 69.07 ± 8.58% cells were positive for CD25 (Fig. 3a) and 70.76 ± 8.34% cells were expressing CD69 (Fig. 3b). However, in VP treated T cells only 18.81 ± 0.83% and 20.40 ± 0.98% cells were positive for CD25 (Fig. 3a) and CD69 (Fig. 3b) respectively. Similarly in TCR treated T cells 59.10 ± 6.94% and 68.12 ± 1.28% cells are positive for CD25 (Fig. 3a) and CD69 (Fig. 3b) respectively, whereas VP treated cells showed significant reduction in expression of activation marker CD25 (14.03 ± 0.05% cells, Fig. 3a) and CD69 (16.77 ± 0.08% cells, Fig. 3b). CP treated cells showed no significant difference in CD25 (Fig. 3a) and CD69 (Fig. 3b) expression as compared to ConA and TCR treated cells. This observation suggests that VP may regulate naive T cell activation.

VIPER down-regulates T cell effector cytokine production during TCR and ConA stimulation. Naive T cells are known to produce effector cytokines during TCR and ConA stimulation[21]. Here we studied the production of T cell effector cytokines IL-2, IFN-γ and TNF upon VP and CP treatment during TCR and ConA mediated activation. Cytokine ELISA was done with cell culture supernatants collected from VP and CP pretreated cells stimulated with ConA or TCR for 36 h. We have observed that signature T cell effector cytokines IL-2 Fig. 4(a), IFN-γ Fig. 4(b), and TNF Fig. 4(c) secretion were significantly downregulated in VP treated cells as compared to CP treated cells.

TLR4 inhibitory peptide VIPER inhibits T cell proliferation. TCR mediated stimulation is supposed to be followed by IL-2 secretion which in turn helps in T cell proliferation[22]. As VP down-regulated IL-2 secretion we are curious to know if it has any effect on T cell proliferation. We first labeled the naive T cells with CFSE and then treat them with different conditions as shown in Fig. 5(a) for 96 h. We observed that T cells treated with VP were showing significantly less proliferation as compared to cells treated only with ConA or TCR (Fig. 5b). 53.71 ± 3.17% T cells showed proliferation after ConA treatment whereas only 4.93 ± 1.67% cells

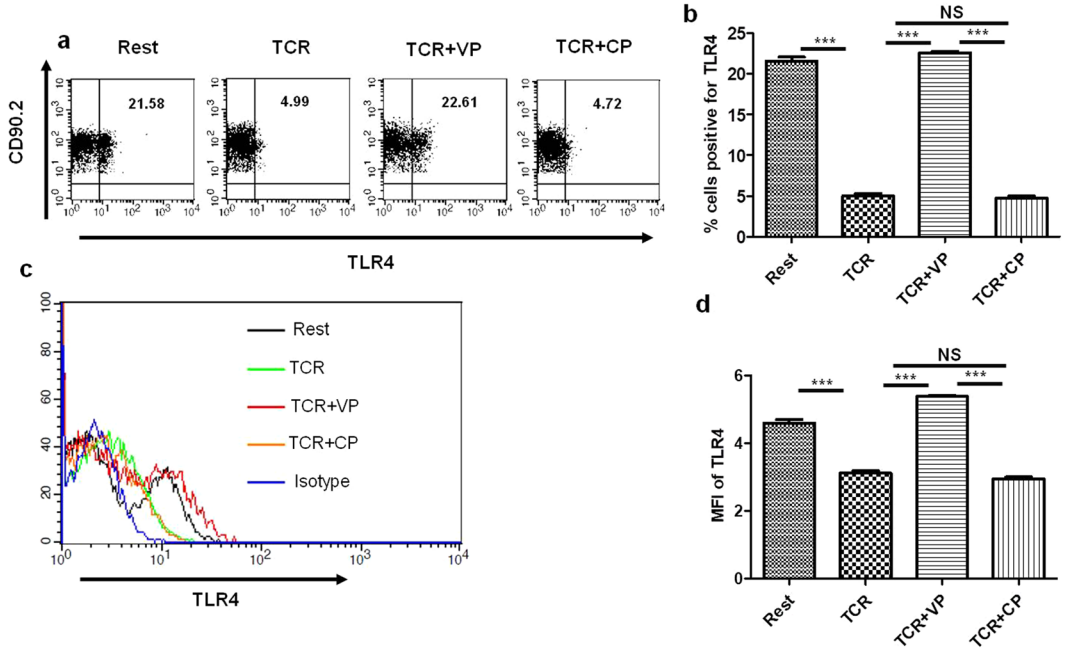

Figure 2. Differential surface expression of TLR4 during T cell activation. (**a**) Flow cytometry dot plot showing frequency of T cells (% positive cells) expressing TLR4 is significantly reduced during TCR mediated T cell activation and VIPER treated cells showed restoration of TLR4 expression. (**b**) Bar graphs showing % positive cells expressing TLR4 from three independent experiments. (**c**) MFI plot showing TLR4 MFI is reduced in TCR activated cells whereas VIPER treated cells show restoration of TLR4 expression. (**d**) Bar graph representing MFI data of TLR4 expression from three independent experiments. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

showed proliferation when pretreated with VP before ConA treatment (Fig. 5a). Similarly, 52.83 ± 4.58% TCR treated cells showed proliferation whereas 6.88 ± 1.28% cells pretreated with VP were proliferating upon TCR stimulation (Fig. 5a). CP treated cells showed no significant difference in proliferation as compared to cells stimulated with only ConA or TCR.

VIPER regulates T cell activation directed Fas and FasL expression. Optimum T cell activation is followed by Activation-Induced Cell Death (AICD) to maintain T cell homeostasis[23]. As we found that VP suppresses T cell activation we were interested to study whether it has any effect on the induction of AICD or not. So we studied the expression of CD95 and CD95L in VP treated naive T cells during TCR and ConA mediated activation. We found that after ConA or TCR mediated activation CD95 and CD95L expressions were induced. However, the cells treated with VP showed significant down-regulation in both CD95 and CD95L expression after ConA or TCR mediated activation (Fig. 6). 1.17 ± 0.52% resting cells express CD95 whereas during ConA and TCR mediated activation it goes up to 53.45 ± 2.71% and 62.38 ± 5.25% respectively (Fig. 6a). Interestingly T cells treated with VP shows significantly reduced expression of CD95 (Fig. 6c). Only 2.27 ± 0.57% and 3.04 ± 0.48% VIPER treated T cells were expressing CD95 with ConA and TCR respectively (Fig. 6a). Similarly, the CD95L expression is upregulated during T cell activation. In resting condition 5.55 ± 0.26% cells were expressing CD95L whereas during activation with ConA and TCR it increased up to 33.32 ± 3.1% and 45.09 ± 3.7% (Fig. 6b). VP treated cells showed significantly reduced expression of CD95L (Fig. 6d). In VP pretreated cells 18.1 ± 0.54% cells expressed CD95L with ConA and 27.04 ± 1.16% with TCR (Fig. 6b). Decreased surface expression of CD95 and CD95L in VP treated T cells suggest that it may regulate the induction of AICD.

Discussion

Here we have investigated the role of VIPER, a TLR4 specific signaling peptide inhibitor towards exploring the possible requirement of TLR4 in naive T cell activation *in vitro*. TLR4 has been reported mostly on antigen presenting cells and their possible modulation can alter the outcome of the cell-mediated immunity[2,5]. Functional expression of TLR4 on various subsets of T cells in different disease condition and experimental models has been reported[9,14,24,25]. TLRs have been shown to regulate innate and adaptive immune systems in different disease conditions like autoimmune diseases and infectious diseases. It's possible that in autoimmune diseases prolonged chronic inflammation persists as compared to acute inflammatory responses[26,27]. The role of TLR4 and TLR2 has been reported and reviewed in different autoimmune conditions like RA and SLE[28–31]. TLR4 had been shown to aggravate RA in mouse model whereas TLR2 has been reported to be associated with protective role[28]. However, those reports are illustrated with different experimental models and suggest a differential functional role of TLR4 on T cells. Moreover, some reports also suggest there is no such direct role of LPS, a TLR4 specific ligand, on naive

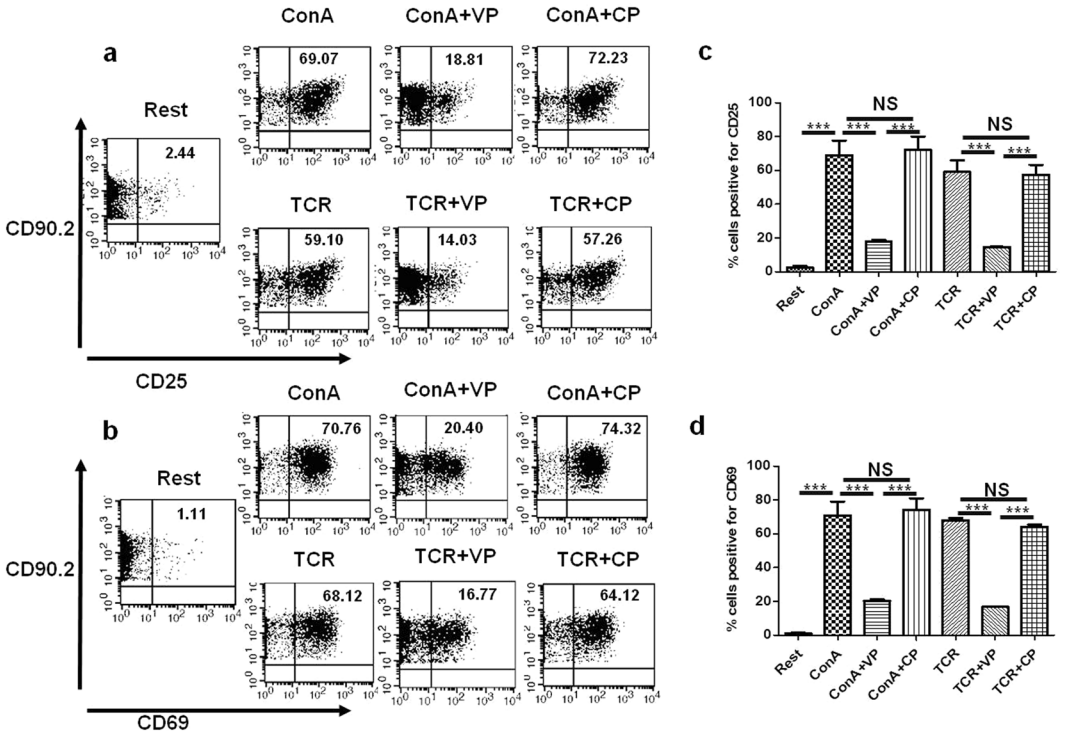

Figure 3. VIPER suppress the expression of T cell activation markers during T cell activation. (**a**) Flow cytometry dot plot showing CD25 surface expression (% positive cells) is significantly reduced in VIPER treated cells during ConA or TCR mediated T cell activation. (**b**) Similarly, the CD69 expression is also decreased in VIPER treated cells in comparison with cells activated only with ConA or TCR. (**c**,**d**) Bar graphs showing % positive cells expressing CD25 and CD69 respectively from three independent similar experiments Data shown are representative of three independent experiments and Bars represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

T cells[10]. Direct modulation of TLR4 signaling in wild-type naive T cells of mouse origin or from T cells from healthy human donors is necessary to predict the requirement of TLR4 signaling towards T cell activation if any.

TCR mediated activation can down-regulate TLR4 expression in T cells as compared to untreated cells[8,9]. We have also found similar down-regulation of TLR4 surface expression on T cells during TCR driven activation. Moreover, we observed that VP and TCR treated T cells showed a similar level of surface expression of TLR4 as untreated cells indicating that VP was actively resisting the TCR induced down-regulation of TLR4 in T cells. In presence of a scrambled peptide (CP), down-regulation of TCR-driven TLR4 expression remained unaffected. Several reports suggest some important roles of MyD88, a TLR4 associated signaling adapter protein, towards T cell effector function[32–35]. However, a direct role of TLR4 responsiveness towards activation of wild-type naive T cells remains scanty. Accordingly, we further studied the effect of VP towards effector function during TCR and mitogenic activation on naive T cell. We observed that VP treated naive T cells showed significantly reduced expression of T cell activation marker CD25 and CD69 as compared to untreated cells upon TCR or mitogenic stimulation. Similarly, we observed that upon TCR or mitogenic stimulation, effector cytokine secretion is decreased in VP treated cells. Together these results suggest TLR4 signaling might have some regulatory function during naive T cell activation. Additionally, our results showed TCR or mitogen mediated naive T cell proliferation can be regulated by VP. VP treated T cells showed reduced proliferation as compared to cells treated only with TCR or ConA. Optimum T cell activation is followed by clonal propagation and then activation-induced cell death (AICD) to maintain homeostasis of T cell population. AICD is mediated by expression and involvement of CD95 (Fas) and CD95L (Fas ligand) on T cells[23,36]. Interestingly we observed that VP regulates induction of CD95 and CD95L expression during T cell activation. This proposes that VP down-regulates T cell activation which in turn may regulate the expression of CD95 and CD95L.

In brief, our current investigation suggests that there might be a requirement of TLR4 signaling during naive T cell activation and effector function. Here we have shown VIPER driven regulation of TLR4 responses towards regulating activation, proliferation, effector cytokine production, Fas and FasL induction of naive T cells, which might have possible implication towards the pathogenic acute phase activation of T cell responses.

Materials and Method

Mice. Female or male BALB/c mice of 8–10 weeks old were used in the experiments. Mice were from animal facility of National Institute of Science Education and Research (NISER). All experiments using animals were approved by NISER's Institutional Animal Ethics Committee according to the guidelines set by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

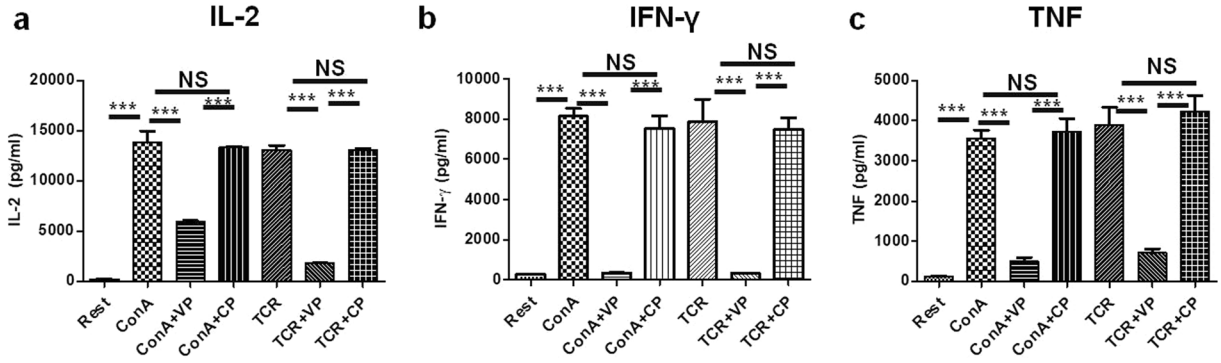

Figure 4. VIPER suppress T cell effector cytokine response. Cytokine ELISA data in pg/ml for IL-2 (**a**), IFN-γ (**b**) and TNF (**c**) shows VIPER treated T cells produce less inflammatory cytokines as compared to cells treated with ConA, TCR or with Control Peptide. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

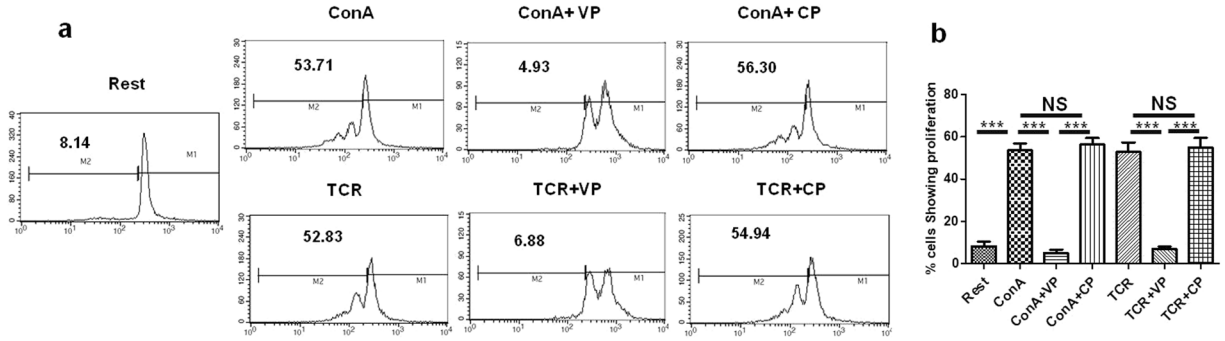

Figure 5. VIPER suppress T cell proliferation. (**a**) Flowcytometry histograms showing proliferation status of purified T cells from BALB/c mice which are untreated or stimulated with ConA, ConA + VIPER, ConA + Control Peptide, TCR, TCR + VIPER and TCR + CP respectively. (**b**) Bar diagram showing % cells proliferating from three independent similar experiments. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Chemicals, Antibodies and other Reagents. Concanavalin A (ConA) was bought from Sigma Aldrich and LPS was from HiMedia. TLR4 Peptide Inhibitor Set: VIPER and Control Peptide were obtained from Novus Bio, CO, USA, used as TLR4 signaling inhibitor. The following antibodies were used: anti-mouse CD90.2 APC from Neuprocells, CD25, CD69, CD95 and CD178 (CD95 ligand) are from BD Biosciences (San Diego, CA); TLR4-Alexafluor 488 was from eBioscience, αCD3 antibody used for the functional assay was from BD Bioscience and αCD28 was from Neuprocells. T cell enrichment kit and CFSE for T cell proliferation assay were from Invitrogen. ELISA kits from BD Biosciences were used for sandwich ELISA of TNF, IL-2, and IFN-γ.

Preparation of mouse splenic T cells. Splenocytes from BALB/c mouse spleen was isolated as reported earlier[21,37]. The spleen was disrupted through a 70μm cell strainer. RBCs were lysed by RBC lysis buffer and cells were washed with 1XPBS and suspended in complete RPMI. T cell purification was done by using untouched mouse T cell isolation kit from Invitrogen according to instructions given by the manufacturer. In brief, splenocytes were resuspended in isolation buffer and incubated with biotinylated antibody for 20 mins. Then cells were washed in excess isolation buffer and incubated with streptavidin-conjugated magnetic beads for 15 min and then placed on a magnet for 2 mins. Enrichment of the T cells was evaluated by flow cytometry and found to be ≥96%.

Cell culture. Purified T cells prepared from spleens of mice were pretreated with VP (10 μM/ml) or CP (10 μM/ml) in complete RPMI and incubated at 37 °C, 5% CO_2 humidified incubator for 2 h followed by stimulation with ConA (5 μg/ml) or TCR. 1.5×10^6cells/ml cells were cultured in 48-well tissue culture plates (500 μl/well) in RPMI media supplemented with 10% FBS at 5% CO_2, 37 °C for 36 h in humidified incubator followed by flow cytometric analysis and the cell culture supernatants were kept in −80 °C for ELISA for the respective samples.

Cell viability assay. Splenocytes were incubated with different doses of VP and CP for 36 h. Cell viability was studied by trypan blue exclusion method. Purified mouse T cells were incubated with different doses of VP and CP along with TCR/ConA stimulation for 36 h. Cells were harvested and washed after 36 h followed by incubation

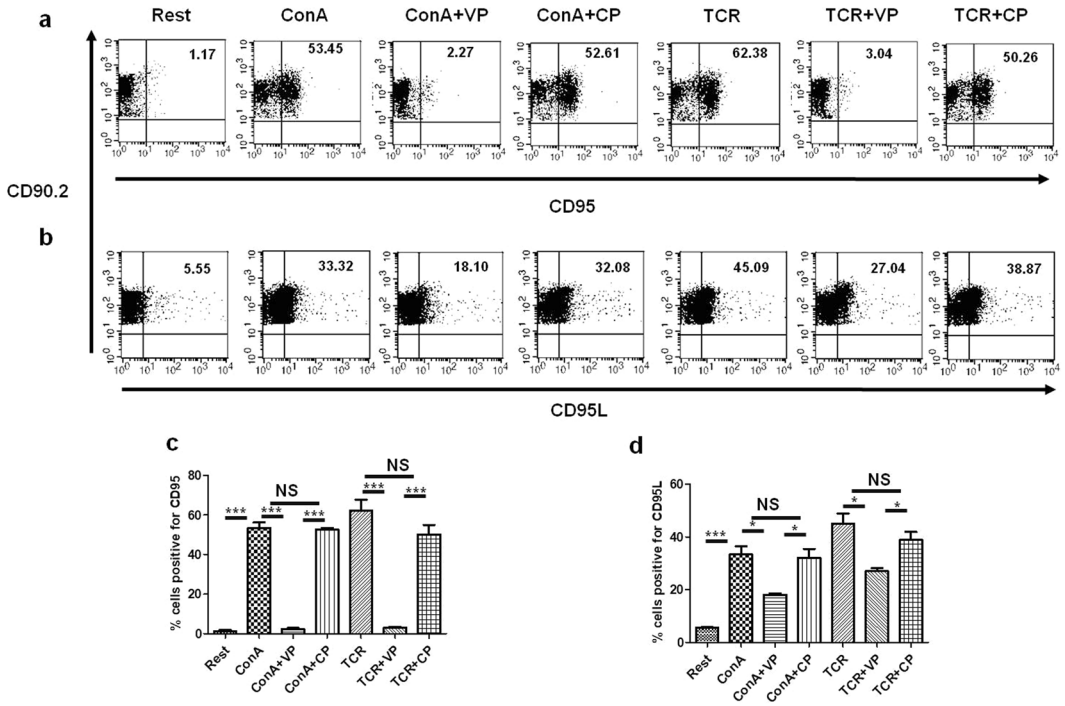

Figure 6. VIPER regulates induction of Fas (CD95) and FasL (CD95L) during T cell activation. Flow cytometric dot plot showing expression of CD95 (**a**) and CD95L (**b**) in T cells treated with VIPER and CP followed by ConA and TCR mediated activation. VIPER treated cells showed significantly reduced expression (% positive cells) of CD95 and CD95L as compared to cells treated with only ConA /TCR. Bar diagram showing % cells expressing (**c**) CD95 and (**d**) CD95L from three independent similar experiments. Data shown are representative of three independent experiments and Bars represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

with 7AAD for 15 mins. Cells were then acquired and analyzed in BD FACS Calibur using Cell Quest Pro software. 7AAD negative cells were considered as live cells.

Flow Cytometry. Flow cytometric study of T cells was carried out as reported previously[21,37]. In brief, Cells were incubated with fluorochrome-conjugated antibodies for 30 mins and then washed two times and resuspended in staining buffer. Cells were acquired using BD FACS Calibur and live gated population was analyzed by Cell Quest Pro software.

ELISA. T cells isolated from BALB/c mice were pretreated with the desired concentration of VP and CP for 2 h and then stimulated ConA or TCR (anti-CD3/CD28mAbs: 1 μg anti-CD3 mAb as plate bound and 2 μg/ml soluble form of anti-CD28 mAb)[35] and incubated for 36 h. Cell-free supernatants were collected at 36 h after culture and stored in −80 °C until further use. ELISA was done as described before[21,38] for IL-2, IFN-γ, and TNF according to manufacturer's instructions and reagents provided (BD OptEIA ELISA kit Sets). Samples were plated in duplicate.

T cell Proliferation Assay. Purified T cells are incubated with 5 μM CFSE from Invitrogen (CellTrace™ CFSE Cell Proliferation Kit, Cat no. C34554) in 1X PBS for 10 minutes at room temperature and then washed three times with RPMI media supplemented with 10% FBS. Then cells were pretreated with the desired concentration of VP and CP for 2 h. After 2 h cells were stimulated with ConA or TCR and incubated for 96 h. Cells were harvested and washed with FACS buffer. Treated cells were acquired with BD FACS Calibur and analyzed in Cell Quest Pro software.

Statistics. Data are presented as means ± standard errors of the means (SEM) of three similar independent experiments. Differences between two groups were determined by One way ANOVA using GraphPad Prism 5.03 software. A p-value of <0.05 was considered significant between groups.

Data availability. All data generated during this study are included in this published article.

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Author Contributions

S.C., S.S.S., P.K.M. designed the study. S.S.S., B.M.P., T.K.N., P.S.K. and V.S.M. performed the experiments. S.S.S., S.C. and B.M.P. wrote the manuscript. S.S.S., S.C., T.K.N., S.B. and P.K.M. reviewed and edited the manuscript.

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Functional expression of TRPV channels in T cells and their implications in immune regulation

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The importance of Ca^{2+} signalling and temperature in the context of T cell activation is well known. However, the molecular identities of key players involved in such critical regulations are still unknown. In this work we explored the endogenous expression of transient receptor potential vanilloid (TRPV) channels, a group of thermosensitive and non-selective cation channels, in T cells. Using flow cytometry and confocal microscopy, we demonstrate that members belonging to the TRPV subfamily are expressed endogenously in the human T cell line Jurkat, in primary human T cells and in primary murine splenic T cells. We also demonstrate that TRPV1- and TRPV4-specific agonists, namely resiniferatoxin and 4α-phorbol-12,13-didecanoate, can cause Ca^{2+} influx in T cells. Moreover, our results show that expression of these channels can be upregulated in T cells during concanavalin A-driven mitogenic and anti-CD3/CD28 stimulated TCR activation of T cells. By specific blocking of TRPV1 and TRPV4 channels, we found that these TRPV inhibitors may regulate mitogenic and T cell receptor mediated T cell activation and effector cytokine(s) production by suppressing tumour necrosis factor, interleukin-2 and interferon-γ release. These results may have broad implications in the context of cell-mediated immunity, especially T cell responses and their regulations, neuro-immune interactions and molecular understanding of channelopathies.

Introduction

Most cells, including immune cells, use free Ca^{2+} ions as second messenger. Resting macrophages as well as T and B cells are known to maintain a low concentration of intracellular Ca^{2+} ions [1]. However, engagement of different receptors such as the T cell receptor, B cell receptor, Fc receptors, various chemokine receptors as well as co-stimulatory receptors present on the immune cell surface are known to increase intracellular Ca^{2+} concentration [2]. In general, the Ca^{2+} signalling in immune cells is important for several regulatory functions such as differentiation of immune cells, gene transcription and effector functions [1–3]. The apparent involvement of Ca^{2+} in the contexts of immune activation, differentiation, host–pathogen interaction and other pathways of cellular response has been demonstrated by modulating the extracellular as well as intracellular free Ca^{2+} concentrations by using several Ca^{2+}-chelating agents. For example, EGTA-mediated chelation of extracellular Ca^{2+} in naive T cells inhibits rise in cytoplasmic Ca^{2+}, interleukin-2 (IL-2) produc-

Abbreviations
2APB, 2-aminoethoxydiphenylborane; 4αPDD, 4α-phorbol-12,13-didecanoate; BTP2, *N*-[4–3,5-bis(trifluoromethyl)pyrazol-1-yl]-4-methyl-1,2,3-thiadiazole-5-carboxamide; CD, cluster of differentiation; ConA, concanavalin A; DC, dendritic cell; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-6, interleukin-6; IRTX, iodoresiniferatoxin; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NADA, *N*-arachidonyl dopamine; PBMC, peripheral blood mononuclear cell; PKA, protein kinase A; PKC, protein kinase C; RTX, resiniferatoxin; TCR, T cell receptor; TNF, tumour necrosis factor; TRP, transient receptor potential; TRPV, transient receptor potential cation channel subfamily vanilloid.

tion, IL-2 receptor expression and further proliferation in response to activation stimuli [4]. In addition it is also established that concanavalin A (ConA) mediated T cell activation initiates with immediate rise in intracellular Ca^{2+} which is sustained for a longer time [5]. This higher level of intracellular Ca^{2+} is important for IL-2 receptor expression, IL-2 production, critical functions which can be effectively blocked by EGTA [5]. Similarly, depletion of intracellular Ca^{2+} regulates surface expression of T cell antigen receptor β (TCR-β) and cluster of differentiation 3-δ (CD3-δ) by enhancing degradation of these receptors in the endoplasmic reticulum [6]. As immune cells and their activation as well as regulation are very heterogeneous in nature, different Ca^{2+} channels play important yet different regulatory functions. However, the complexity of these different Ca^{2+} channels in the context of diverse immune functions and their molecular identities are yet to be explored in detail.

Like free Ca^{2+}, the effect of temperature changes, i.e. both low and high temperature, on immune activation is well reported [7–13]. Infection followed by increment in body temperature is well known for its role as activator of the immune system [14,15]. It has been shown that cytotoxic activities of T cells from adult blood as well as from cord blood can be enhanced at slightly increased temperature (≤ 40 °C) but decreased if exposed to 42 °C for 1 h [16]. The effect of body temperature on T cell morphology, altered distribution in different tissues and changes in key molecules has been demonstrated. For example, fever-like whole body hyperthermia treatment results in increased numbers of T cells in tissue with polarized spectrin cytoskeletons and uropods [17]. Such temperature treatment also induces increased protein kinase C (PKC) activity and redistribution of PKCs within T cells [17]. In fact, the benefit of mild hyperthermia and thermal stress in the enhanced immune system is now well studied; however, the exact mechanisms are not well understood [18–20]. Although recent studies have indicated that heat shock proteins are involved in temperature-mediated effects on immune cells [21], the extreme sensitivity to slight changes in temperature and precise temperature-dependent activity make thermosensitive ion channels ideal regulatory candidates for temperature-dependent immune modulation. Indeed, it has recently been shown that temperature-dependent activation of STIM1 (a Ca^{2+} channel) can induce Ca^{2+} influx and modulates gene expressions relevant for immune functions [22,23].

Transient receptor potential (TRP) channels are a group of non-selective cationic ion channels comprising various members which are thermosensitive in nature. TRP channels are known to be involved in various physiological responses, such as neuronal responses associated with sensory functions [24]. So far several TRP channels have been found to be thermosensitive in nature. For example, TRPV1 can be activated at elevated temperatures with a threshold near 43 °C [25]. Three other TRPV channels, TRPV2 (also known as VRL-1; temperature threshold ≥ 52 °C), TRPV3 (temperature threshold ≥ 33 °C) and TRPV4 (also known as VROAC or OTRPC4; temperature threshold ≥ 27 °C), also act as thermosensors [26,27]. TRPV1 reveals high Ca^{2+} permeability (P_{Ca}/P_{Na} = 9.6). The TRPV2 (VRL-1) channel is 50% identical to TRPV1 and has low Ca^{2+} permeability (P_{Ca}/P_{Na} = 2.9). TRPV3 is able to sense a warm temperature as its thermal threshold is around 33 °C and is highly selective to Ca^{2+} (P_{Ca}/P_{Na} = 12.1). TRPV4 is ~ 40% identical to TRPV1 and TRPV2 and is moderately selective to Ca^{2+} (P_{Ca}/P_{Na} = 6). The thermosensitivity and Ca^{2+} permeability in general suggests that this group of ion channels can be ideal candidates to act as molecular sensors detecting minor changes in body temperature. This property of TRPV channels seems to be relevant in the context of immune cells such as dendritic cell (DC) activation and maturation as increase in temperature as well as exposure to capsaicin are found to induce immunogenicity of the DCs [28]. Indeed, few reports have suggested the physical presence and functional role of thermosensitive TRP channels in different immune cells, especially macrophages and DCs [28]. For example, ruthenium red (10 μM), a non-selective TRP channel blocker, can suppress lipopolysaccharide (LPS) induced tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) production in macrophage cells, while blocking of TRPC channels by gadolinium (30 μM) or blocking of both TRPC and TRPM channels by flufenamic acid (100 μM) failed to do so [29]. While this particular result rules out the involvement of TRPC and TRPM family members, it does not confirm the involvement of TRPV family members in the context of LPS-induced TNFα and IL-6 production in macrophages. Similarly, vanilloids are known to modulate the expression of genes involved in inflammatory response (such as inducible nitric oxide synthase and cyclooxygenase-2) in macrophages by interfering with upstream signalling events of LPS and interferon-γ (IFN-γ) [29,30]. These vanilloids are also known to inhibit different pathways, such as LPS-induced ERK, JNK and IKK activation [31]. While the vanilloids are not strictly selective/specific and can activate several TRPVs *per se*, these results in general suggest the involvement of TRPVs in macrophage functions. The

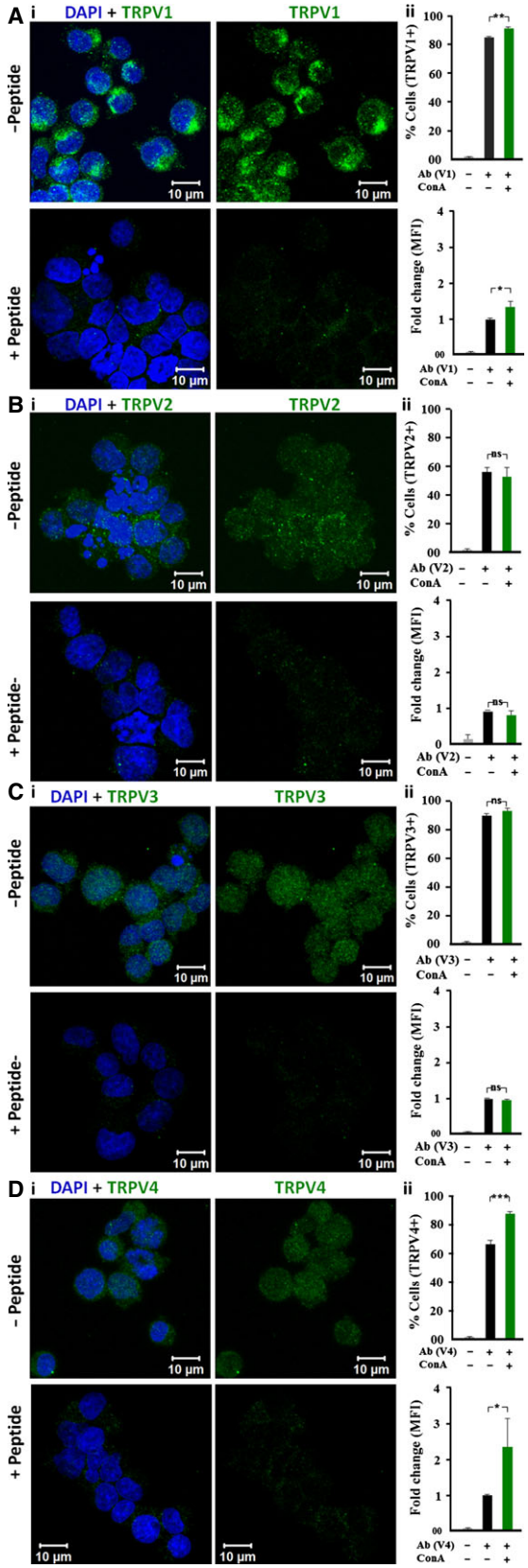


Fig. 1. TRPV channels are expressed in the human T cell line (Jurkat). Confocal images (i, left column) of cells immunostained with specific antibodies for TRPV1 (A), TRPV2 (B), TRPV3 (C), TRPV4 (D) with (lower panel) or without (upper panel) blocking peptides are shown. Flow cytometry profiles (ii, right column) demonstrating the percentage of cells positive for these TRPV channels (upper panel) are shown. Fold changes in the expression level of specific TRPV channels by ConA-driven activation are shown in the lower panel. The *P* values are: ns, non-significant; *, < 0.05; **, < 0.01; ***, < 0.001 ($n = 3$).

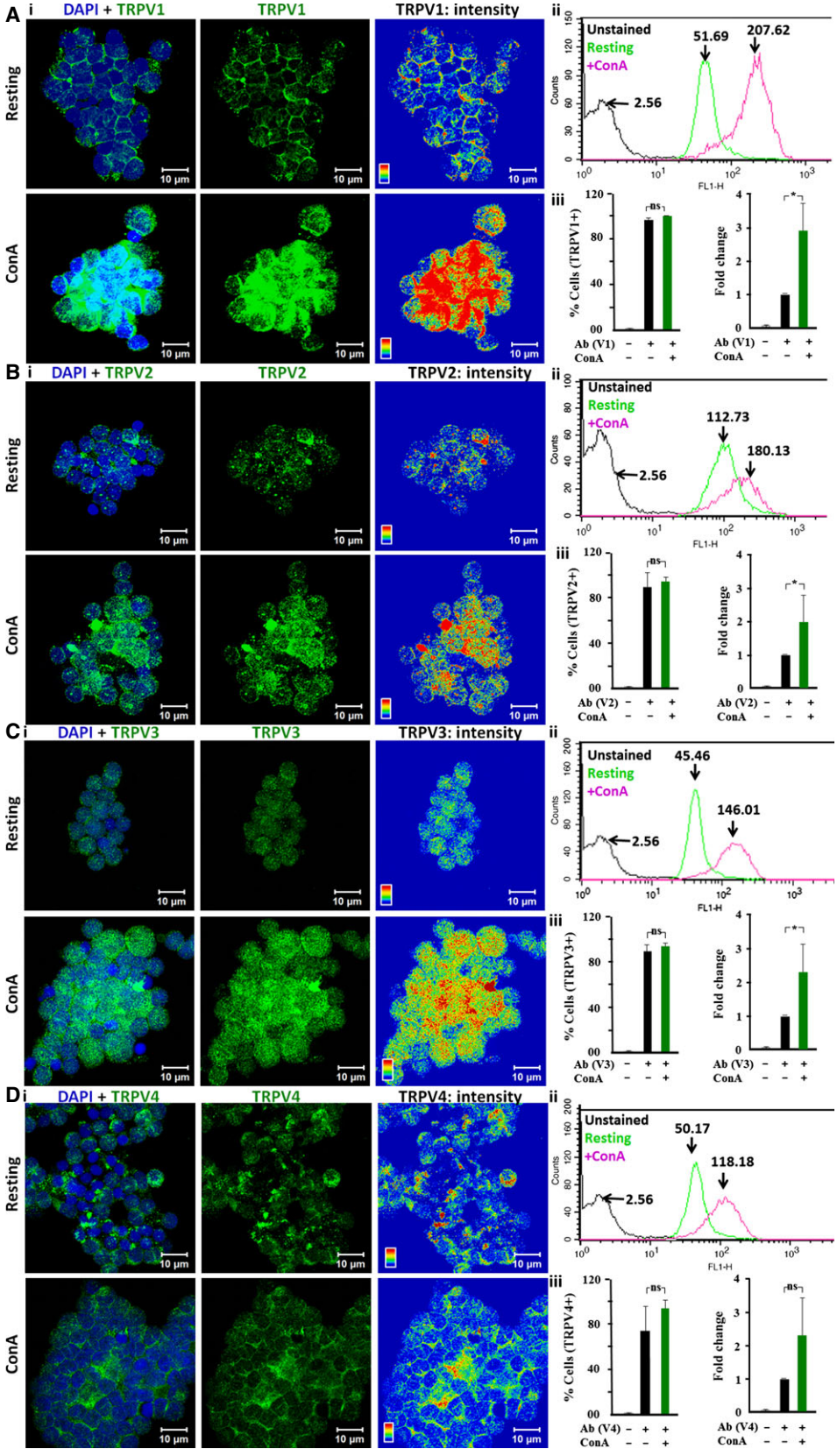
known TRPV1 activators, namely capsaicin and resiniferatoxin (RTX), are also known to inhibit LPS- and IFN-γ-mediated inducible nitric oxide synthase expression and NO production [30]. Capsaicin alone can also inhibit transcription of LPS- and phorbol 12-myristate 13-acetate -induced cyclooxygenase-2 expression and prostaglandin production in macrophages [31]. Capsaicin also exhibits anti-inflammatory properties [32]. Recently, expression of TRPV2 in macrophages has been demonstrated using RT-PCR and immunoblot techniques [33]. In agreement with this, it has been shown that murine macrophages isolated from TRPV2 knockout animals (*trpv2*$^{-/-}$) have impaired ability for particle binding and phagocytosis [33]. In a similar context, DCs are also known to express TRPV1 [28]. In agreement with this, TRPV1 activation by capsaicin leads to maturation of immature DCs as indicated by upregulation in the expression of antigen-presenting molecules and other co-stimulatory molecules [28]. Further, intradermal administration of capsaicin leads to migration of DCs to the draining lymph nodes suggesting that these channels also play a role in the chemotaxis of DCs [28].

While these reports in general suggest the expression of TRPV members in macrophages and DCs, the endogenous expression of functional TRPV channels in T cells is still not conclusive [34]. In this work we explored whether these non-selective Ca^{2+} ion channels belonging to the TRPV subfamily are endogenously present and functional in T cells. Here we report the expression and functional role of these TRPV members, especially TRPV1 and TRPV4, in the context of T cell activation and effector functions.

Results

TRPV members are expressed endogenously in human T cell line (Jurkat cells)

In order to explore the endogenous expression of TRPV channels in Jurkat cells, we performed indirect immunofluorescence staining using specific antibodies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DAPI + TRPV1
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TRPV2
TRPV2: intensity
112.73
180.13
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TRPV3
TRPV3: intensity
45.46
146.01
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D
DAPI + TRPV4
TRPV4
TRPV4: intensity
50.17
118.18
2.56
% Cells (TRPV4+)
Ab (V4)

Fig. 2. TRPV channels are expressed in primary human T cells. Confocal images (i, left column) of cells immunostained with specific antibodies demonstrate the expression pattern of TRPV1 (A), TRPV2 (B), TRPV3 (C), TRPV4 (D) at resting stage (upper panel) or after ConA-mediated activation (lower panel). TRPV channels are shown in green and the intensity profiles of the TRPV staining are indicated in rainbow scale. Flow cytometry profiles (ii) demonstrate the MFI histograms of cells positive for these TRPV channels. Changes in the percentage of positive cells and fold changes in the expression level (iii) of specific TRPV channels due to ConA-mediated activation are shown. The *P* values are: ns, non-significant; *, < 0.05 ($n = 4$).

against TRPV channels. Confocal microscopy analysis revealed the endogenous expression of TRPV1, TRPV2, TRPV3 and TRPV4 in resting Jurkat cells (Fig. 1A–D, left panels). The TRPV channels are present throughout the cytoplasm as well as on the membrane in the resting condition. The specificity of the staining was confirmed by using specific blocking peptides which either reduced or abolished the staining pattern completely (Fig. 1A–D). The specificities of these antibodies were also verified by flow cytometry, where the number of cells positive for respective TRPV channels and the mean fluorescence intensity (MFI) were lowered when specific blocking peptides were used (Fig. 1A–D, ii).

Flow cytometric analysis revealed that a significant number of cells are positive for TRPV1 (84.7 ± 0.31%, $n = 3$, $P = 5.137e\text{-}09$, Sigma), TRPV2 (55.9 ± 3.7%, $n = 3$, $P = 1.415e\text{-}05$, Alomone), TRPV3 (86.5 ± 1.2%, $P = 2.591e\text{-}08$, $n = 3$, Alomone) and TRPV4 (65.9 ± 3.31%, $n = 3$, $P = 1.052e\text{-}05$, Sigma) (Fig. 1A–D, ii, upper panel). Activation mediated by ConA, a lectin that acts as mitogen and results in T cell activation, for 36 h results in a marginal but significant ($P = 0.001315$; ANOVA test) increase in $TRPV1^+$ cell numbers (90.5 ± 0.9%). Similarly, the number of $TRPV4^+$ cells increases significantly (88.23 ± 1.53%) in the total Jurkat population after ConA activation ($P = 0.0009842$; ANOVA test). However, there was no drastic increase in the MFI values for TRPV-associated signals. Intensity of TRPV1 expression increased marginally ($P = 0.03367$, ANOVA test) by 1.31 ± 0.17 fold (52.32 ± 7.99 in the resting condition to 68.84 ± 13.95 in the activated condition) after ConA-driven mitogenic activation (Fig. 1A, ii, lower panel). Similarly, TRPV4 expression increased ($P = 0.04402$; ANOVA test) by 2.33 ± 0.79 fold after ConA activation (MFI value increased from 47.46 ± 3.25 in the resting condition to 110.26 ± 36.83 in the activated condition) (Fig. 1D, ii, lower panel). ConA-mediated activation of Jurkat cells results in no significant increase in TRPV2 expression (17.05 ± 1.04 in the resting condition versus14.84 ± 1.06 in ConA-activated cells, $n = 3$, $P = 0.06189$) and TRPV3 expression (28.02 ± 0.8 in the resting condition versus 27.7 ± 1.5, $n = 3$, $P = 0.7613$) (Fig. 1B,C, ii, lower panel). This result in general suggests that TRPV channels are expressed endogenously in Jurkat cells and the expressions of TRPV1 and TRPV4 increase modestly upon activation.

TRPV members are expressed endogenously in primary human T cells

The expression of TRPVs were probed in purified primary T cells from human peripheral blood mononuclear cells (PBMCs) by confocal microscopy and flow cytometric analysis. Distinct punctate expression of TRPV1, TRPV2, TRPV3 and TRPV4 was observed in most of the human T cells (Fig. 2). TRPVs are localized both at the cytoplasm and at the membrane. Confocal microscopy indicates that ConA-mediated activation leads to an increase in the expression levels of TRPV1 and TRPV3 (Fig. 2A,C), whereas a marginal increase in expression levels was observed for TRPV2 and TRPV4 (Fig. 2B,D). Flow cytometric analysis revealed that expressions (MFI levels) of TRPVs increase several fold [TRPV1 (2.83 ± 1.28 fold); TRPV2 (2.09 ± 1.09 fold); TRPV3 (2.29 ± 0.84 fold) and TRPV4 (2.57 ± 1.54 fold)] after ConA-mediated activation (Fig. 2). We noted that, in the case of human cells, > 99% T cells are positive for the expression of TRPVs in resting conditions. Therefore, no further increase in the number of $TRPV^+$ T cells upon ConA-mediated activation was observed for any of these TRPV channels.

Functional TRPV members are expressed endogenously in murine splenic T cells

We next explored the endogenous expression of TRPV channels in primary murine splenic T cells. Confocal analysis revealed that the expression of TRPV1 to TRPV4 in primary murine splenic T cells is similar to primary human T cells (Fig. 3A–D). The numbers of $TRPV1^+$ and $TRPV4^+$ cells were higher than those of $TRPV2^+$ and $TRPV3^+$ cells. To explore the expression of these channels in a more quantitative manner, we performed flow cytometric analysis in the presence and absence of specific blocking peptides. These blocking peptides reduce both the numbers of cells that are positive for respective TRPV channels (data not shown) and MFI values (Fig. 3A–D, ii) suggesting that these stainings are highly specific. We have analysed

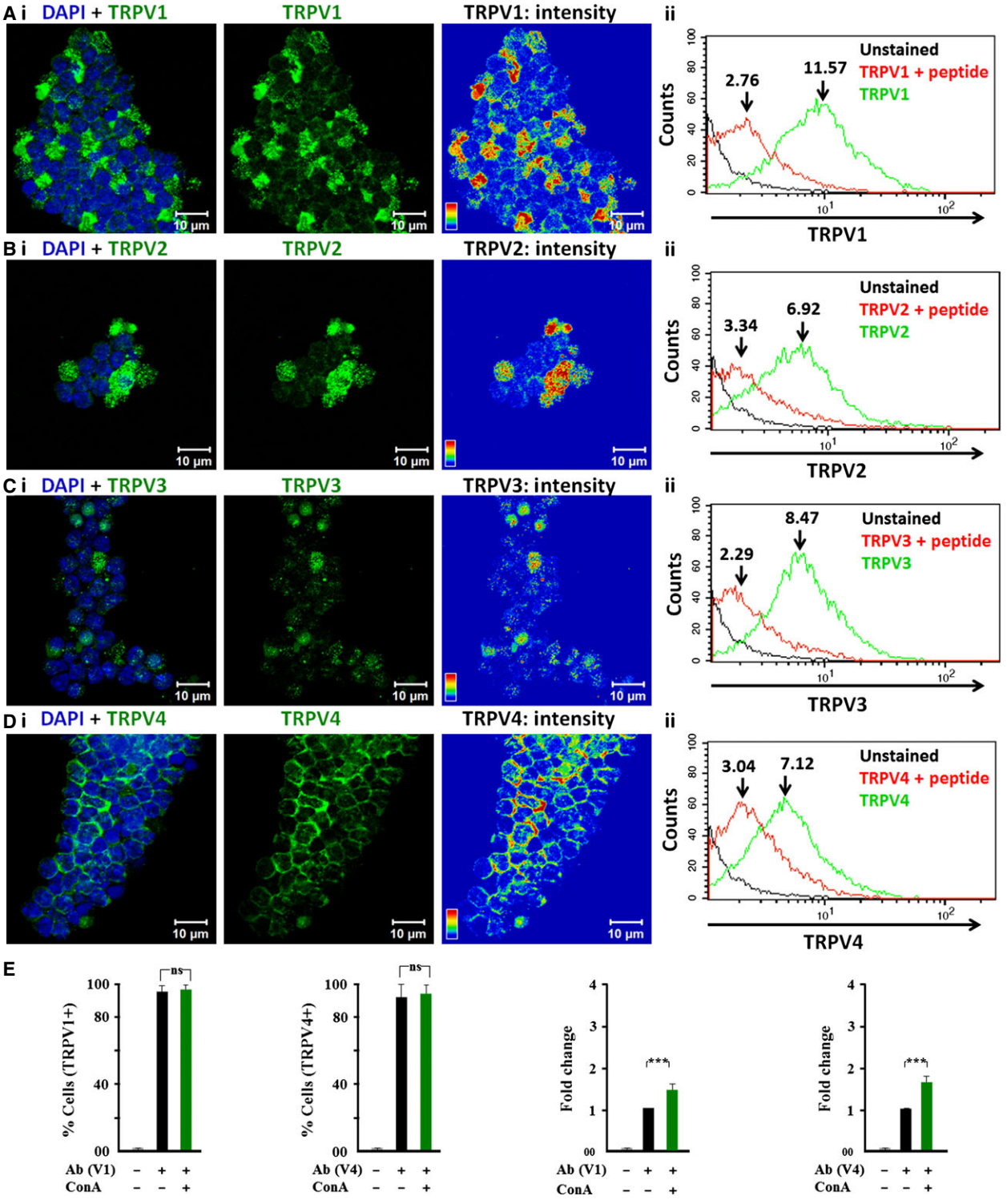


Fig. 3. TRPV channels are expressed in primary murine T cells. Confocal images (A)–(D) of cells demonstrating the expression pattern of different TRPVs (green) at resting stage. Cells were immunostained with specific antibodies for TRPV1 (A), TRPV2 (B), TRPV3 (C) and TRPV4 (D). Intensity profiles of the respective TRPV channels are indicated in rainbow scale (right panel). (E) Graphs demonstrating the percentage of cells that are positive for TRPV1 and TRPV4 before and after ConA-mediated activation ($n = 4$). Fold changes (calculated from MFI values, with respect to ConA) in the expression of TRPV1 and TRPV4 before and after ConA-mediated activation are shown. The *P* values are: ns, non-significant; *, < 0.05; **, < 0.01; ***, < 0.001.

$CD3^+$-gated cells to investigate the splenic T cell population in all our further flow cytometric experiments if not stated specifically (Fig. 3E). Nearly 95.7 ± 3.1% of splenic T cells (in the $CD3^+$ splenocyte population) are positive for TRPV1 ($n = 4$) in resting conditions. ConA-mediated activation for 36 h resulted in a marginal increase (97.1 ± 2.4%, $n = 4$) in the number of $TRPV1^+$ cells. Similarly, about 92.6 ± 8% of T cells were $TRPV4^+$ ($n = 4$) in resting conditions. The number of $TRPV4^+$ cells increased marginally (94.4 ± 6%, $n = 4$) after ConA activation. However, the expression levels of TRPV2 and TRPV3 did not increase considerably after ConA-mediated activation. Nearly 55.9 ± 3.8% cells are positive for TRPV2 in resting conditions while 51 ± 5.9% of activated T cells express TRPV2. Similarly, 86.58 ± 1.2 cells express TRPV3 in the resting condition and 88.2 ± 2.5 in the activated condition ($n = 4$). The MFI values revealed that TRPV1 expression increased to 1.47 ± 0.11 fold after ConA activation compared to the resting condition (from 63.99 ± 30.64 to 95.7 ± 49.18, $n = 4$). The MFI values representing TRPV4 expression also increase by 1.61 ± 0.17 fold after ConA activation (123.43 ± 65.67, $n = 4$) compared to the resting condition (79.68 ± 47.72, $n = 4$). Although the numbers of TRPV1 or TRPV4 positive cells do not increase significantly in activated T cells, the level of TRPV1 or TRPV4 expression per cell increases significantly (as indicated by the MFI values).

In order to explore if these TRPVs are functional in T cells, we performed Ca^{2+} imaging experiments using purified mouse T cells loaded with Fluo-4 AM (2 μM). Live cell imaging revealed no increase in Fluo-4 intensity at all in the majority of cells with respect to time, especially in the absence of any stimuli (Fig. 4). However, upon stimulation by TRPV1 activator RTX, intracellular Ca^{2+} levels increased in most of the T cells. This RTX-mediated influx can be effectively blocked by TRPV1-specific inhibitor 5′-IRTX. Application of NADA (*N*-arachidonyl dopamine, an endogenous activator of TRPV1) results in a slight increase in the intracellular Ca^{2+} concentration in the majority of cells within this time frame. This NADA-mediated influx can also be effectively blocked by 5′-IRTX. In the absence of specific modulators of TRPV2 and TRPV3, we applied 2-aminoethoxydiphenylborane (2APB) (5 μM, activator of TRPV2 and TRPV3) which could not increase the intracellular Ca^{2+} concentration in these cells [35]. Moreover, the TRPV4-specific activator 4α-phorbol-12,13-didecanoate (4αPDD) also increases the intracellular Ca^{2+} concentration. In contrast, combination of 4αPDD with TRPV4-specific inhibitor RN1734 blocks the Ca^{2+} surge in T cells. These results confirm the functional expression of TRPV1 and TRPV4 in murine primary T cells and their involvement in the regulation of intracellular Ca^{2+} influx.

T cell activation by mitogen or TCR stimulation is dependent on TRPVs and TRPV members provide additive effects on cell-mediated immunity

In order to understand the role of TRPV channels in T cell activation, we activated the T cells either by ConA or via TCR stimulation (by α-CD3/CD28). We probed for the expression of activation markers, namely CD25 and CD69, in the $CD3^+$ murine T cell population. The expression of these markers were probed after ConA treatment with or without TRP channel modulators for 36 h (Fig. 5). Flow cytometric dot-plot values of CD25 stained cells suggest a shift of the T cell population upon ConA activation (resting, 9.07 ± 0.36%; ConA-treated, 70.65 ± 1.12%). The presence of TRPV1 inhibitor, namely 5′-IRTX, inhibits T cell activation by ConA in a dose-dependent manner (data not shown). The maximum inhibition was achieved in the presence of 10 μM 5′-IRTX (7.07 ± 0.66%) (Fig. 5A). In a similar manner, TRPV4 inhibitor (RN1734) also reduced T cell activation in a dose-dependent manner (although to a lesser extent than 5′-IRTX). The maximum inhibition of ConA-mediated T cell activation achieved is with 20 μMRN1734 (60.26 ± 2.24%) (Fig. 5A). Interestingly, a combination of TRPV1 and TRPV4 inhibitors (5′-IRTX 10 μM and RN1734 20 μM) inhibited ConA-mediated T cell activation almost completely (6.44 ± 0.55%) (Fig. 5A). DMSO alone (at a concentration equivalent to 20 μM) did not have any effect on the number of CD25 positive cells (70.72 ± 0.70%). Further we found that 5′-IRTX (10 μM) and RN1734 (20 μM) may act in a synergistic manner and induce an additive effect, which was found to suppress ConA-mediated T cell activation. The effect of these TRP-channel-specific compounds was also evident from the MFI values of CD25 expression: resting T cells, 6.06 ± 0.33; ConA-stimulated, 43.80 ± 2.69; 10 μM 5′-IRTX, 5.36 ± 0.19; 20 μM RN1734, 35.94 ± 2.94; 10 μM 5′-IRTX + 20 μM RN1734, 4.86 ± 0.17 (Fig. 5B). This strongly suggests that members belonging to the TRPV family contribute towards immune activation, at least part of it being by regulating CD25 expression.

Despite stimulation by ConA (5 $\mu g \cdot mL^{-1}$, 36 h), TRPV1 and TRPV4 inhibition also suppressed the expression of CD69, which is an early activation marker for T cells (Fig. 5D). As expected, the percentage of $CD69^+$ T cells was enhanced after ConA-mediated

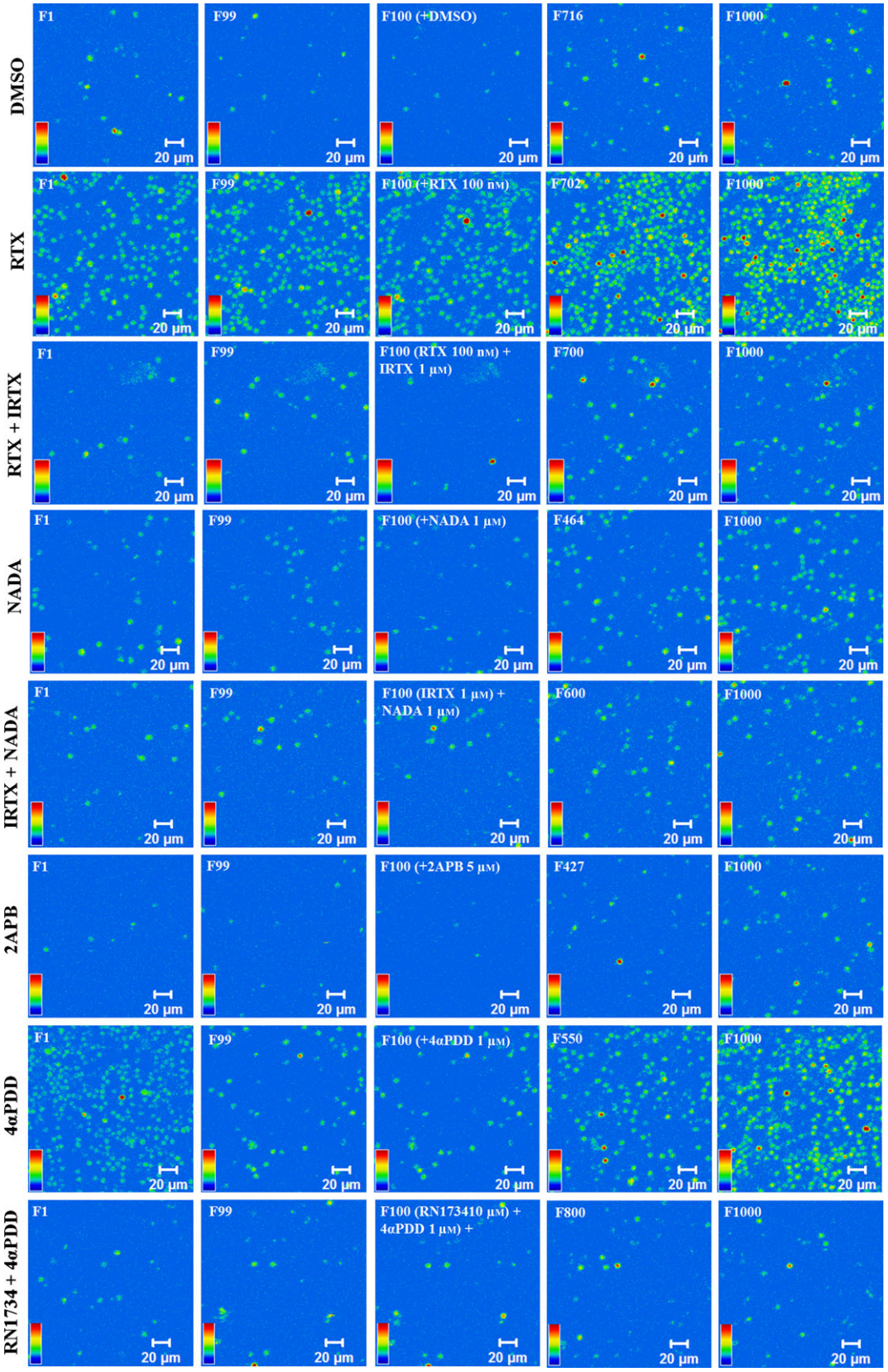
activation (4.96 ± 2.05 in resting stage versus 58.67 ± 1.65 in activated stage). Co-incubation with 10 μM 5′-IRTX decreased the number of $CD69^+$ T cells (19.32 ± 4.39) whereas 20 μM RN1734 reduced the number of $CD69^+$ T cells modestly (to 46.76 ± 1.39) (Fig. 5D). However, the presence of both inhibitors (10 μM 5′-IRTX and 20 μM RN1734) resulted in significant reduction (13.39 ± 2.68) in cells that were $CD69^+$ (Fig. 5D). TRPV channel inhibition has a profound suppressive effect on the MFI values of CD69 expression (7.60 ± 1.60 for resting T cells, 28.91 ± 1.11 for ConA-activated T cells; 10.56 ± 1.13 for 10 μM 5′-IRTX, 20.51 ± 0.73 for 20 μM RN1734, and 8.50 ± 0.66 in the presence of both10 μM 5′-IRTX and 20 μM RN1734) (Fig. 5E). This suggests that TRP channels in general and especially TRPV1 together with TRPV4 in particular may play an important role in T cell activation. However, incubation with TRPV1 activator RTX (100 nM) or with TRPV4 activator 4αPDD (1 μM) for 36 h [with or without ConA ($4\ \mu g{\cdot}mL^{-1}$)] did not show any increase in expression of the activation markers, namely CD25 or CD69 (data not shown). This probably suggests that during immune activation the endogenous activity of the TRPV1 and TRPV4 channels reaches a maximum stage where further activation by exogenous pharmacological agents may not be visible.

In order to check the role of TRPV1 and TRPV4 in TCR-mediated activation of T cells, we treated purified T cells with plate-bound anti-CD3 ($2\ \mu g{\cdot}mL^{-1}$) and soluble anti-CD28 ($2\ \mu g{\cdot}mL^{-1}$) mAbs for 48 h in the presence or absence of TRPV1 and TRPV4 inhibitors and checked for the expression of activation markers, namely CD25 and CD69. Despite stimulation by anti-CD3/CD28 mAbs, TRPV1 and TRPV4 inhibition suppressed the expression of CD25 and CD69. This correlates well with the ConA-mediated activation described above. The percentage of $CD25^+$ cells was enhanced after anti-CD3/CD28 mAb-mediated activation (4.72 ± 0.86 in the resting stage versus 42.81 ± 1.96 in the activated stage). Incubation with 10 μM 5′-IRTX decreases the percentage of $CD25^+$ cells (5.11 ± 1.28) and 20 μM RN1734 reduces the percentage of $CD25^+$ cells (37.46 ± 2.6) as compared to cells treated with only CD3/CD28 (Fig. 6A,B). However, the presence of both inhibitors (10 μM 5′-IRTX and 20 μM RN1734) results in a significant reduction (3.81 ± 0.99) in cells that are $CD25^+$ (Fig. 6A,B). Inhibition of TRPVs has a profound suppressive effect on the MFI values of CD25 expression (6.34 ± 0.30 for resting T cells; 76.20 ± 2.96 for α-CD3/CD28 activated T cells; 6.38 ± 0.49 for 10 μM 5′-IRTX; 50.31 ± 2.52 for 20 μM RN1734; 5.40 ± 0.13 in the presence of both 10 μM 5′-IRTX and 20 μM RN1734) (Fig. 6C).

Similarly, CD69 expression in α-CD3/CD28 stimulated T cells was suppressed upon inhibition of TRPV1 and TRPV4. The percentage of $CD69^+$ cells was enhanced after anti-CD3/CD28 mAb-mediated activation (2.36 ± 0.88 in the resting stage versus 49.5 ± 1.98 in the activated stage). Incubation with 10 μM 5′-IRTX decreased the number of $CD69^+$ T cells (34.13 ± 1.94) whereas 20 μM RN1734 reduced the number of $CD69^+$ T cells modestly (to 41.61 ± 2.68) (Fig. 6D,E). However, the presence of both inhibitors (10 μM 5′-IRTX and 20 μM RN1734) resulted in significant reduction (26.8 ± 2.2) in the number of cells that are $CD69^+$ (Fig. 6D,E). Inhibition of TRPVs suppressed CD69 expression in T cells (MFI values: 13.92 ± 1.06 for resting, 128.75 ± 1.85 for α-CD3/CD28 activation; 74.38 ± 6.79 for 10 μM 5′-IRTX; 75.82 ± 10.32 for 20 μM RN1734; 51.40 ± 7.29 in the presence of both 10 μM 5′-IRTX and 20 μM RN1734) (Fig. 6F).

Since immune cell activation involves a coordinated action of several effector cytokines, we explored the role of TRPVs in cytokine production by analysing the supernatants by ELISA for the level of different cytokines released from splenic T cell culture. General inhibition of TRP channels by ruthenium red significantly decreased IFN-γ production ($3373.23 \pm 1399.22\ pg{\cdot}mL^{-1}$ with respect to $7517.62 \pm 1057.42\ pg{\cdot}mL^{-1}$ IFN-γ produced due to ConA treatment, data not shown). However, inhibition of TRPV1 (by 5′-IRTX) decreased IFN-γ production to $883.40 \pm 509.69\ pg{\cdot}mL^{-1}$, whereas inhibition of TRPV4 (by RN1734) decreased the IFN-γ production to $4402.31 \pm 1760.29\ pg{\cdot}mL^{-1}$. Combination of 5′-IRTX and RN1734 resulted in a drop in IFN-γ release to $734.81 \pm 414.25\ pg{\cdot}mL^{-1}$ with respect to ConA treatment (Fig. 7A). The inhibitory role of TRPV1 and

Fig. 4. Pharmacological activation of TRPV1 and TRPV4 causes Ca^{2+} influx in primary murine T cells. Time-series fluorescence images of view fields containing multiple cells loaded with Ca^{2+}-sensing dye Fluo-4 AM are depicted here. The time difference between each frame (F) is 5 s. The cells were treated with different pharmacological agents exactly at the 100th frame (F100). Activation of TRPV1 by RTX or NADA causes an increment in the Ca^{2+} level which can be blocked by TRPV1-specific inhibitor 5′-IRTX. Similarly activation of TRPV4 by 4αPDD causes an increase in the concentration of intracellular Ca^{2+} which can be blocked by TRPV4-specific inhibitor RN1734. The effect of TRPV2 and TRPV3 activator 2APB remains insensitive. The fluorescence intensities at different time points are represented in pseudo rainbow colour. Representative results of three independent experiments are shown.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F1
F99
F100 (+DMSO)
F716
F1000
RTX
F1
F99
F100 (+RTX 100 nM)
F702
F1000
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F1
F99
F100 (RTX 100 nM) + IRTX 1 μM)
F700
F1000
NADA
F1
F99
F100 (+NADA 1 μM)
F464
F1000
IRTX + NADA
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F100 (+2APB 5 μM)
F427
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4αPDD
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F100 (+4αPDD 1 μM)
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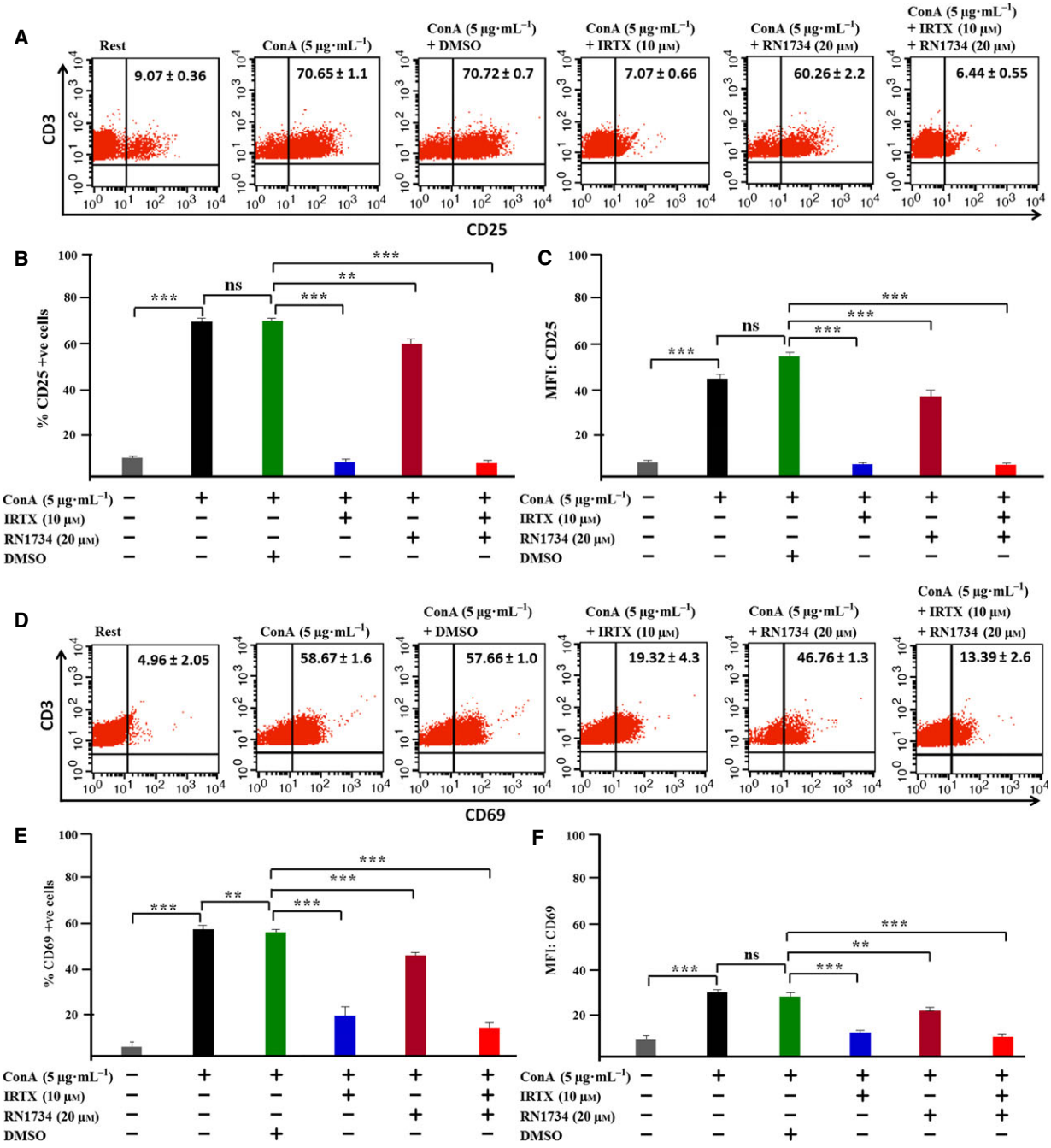

Fig. 5. Pharmacological inhibition of endogenous activity of TRPV1 and TRPV4 blocks ConA-mediated T cell activation. (A) Murine T cells were treated with ConA (4 μg·mL^{-1}) along with TRPV1 and TRPV4 inhibitors at the indicated concentrations for 36 h and analysed by flow cytometry. The values mentioned in the upper right corner of each flow cytometric dot-plot indicate the average number ± SD values of cells that are positive for CD25. Representative dot-plots of three independent experiments are shown. (B) The percentage of CD25$^+$ cells due to different treatments with respect to ConA are shown (n = 3). (C) The levels/intensity of CD25 expression (determined from MFI values) in response to inhibition of TRPV1 and TRPV4 are shown (n = 3). (D) Murine T cells were treated as mentioned above (A) and were analysed by flow cytometry. The values mentioned in the corner indicate the average number ± SD values of cells that are positive for CD69. Representative dot-plots of three independent experiments are shown. (E) The percentages of CD69$^+$ cells due to different treatments with respect to ConA are shown (n = 3). (F) The levels/intensity of CD69 expression (determined from MFI values) in response to inhibition of TRPV1 and TRPV4 are shown (n = 3). The P values are: ns, non-significant; *, < 0.05; **, < 0.01; ***, < 0.001.

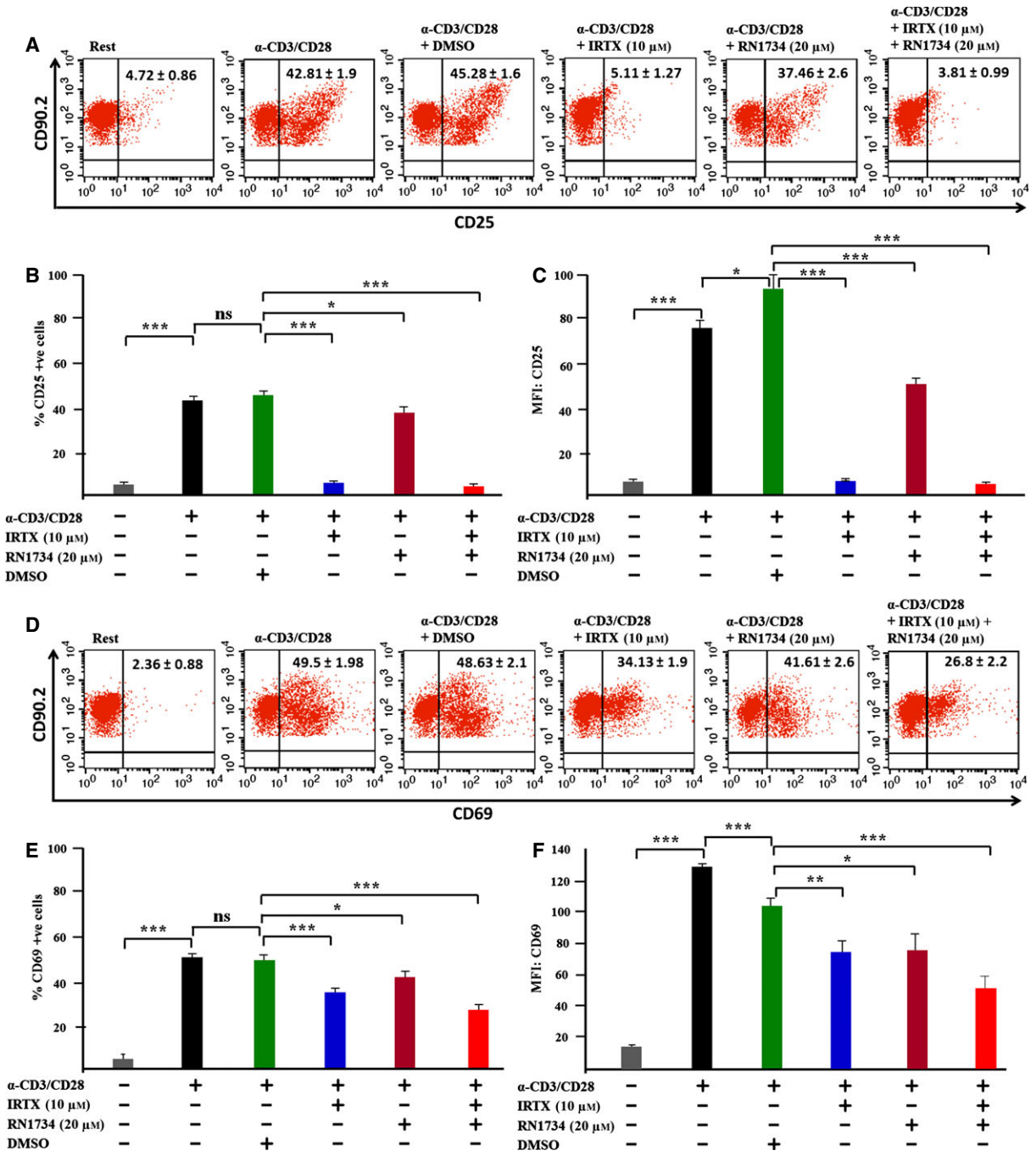

Fig. 6. Pharmacological inhibition of endogenous activity of TRPV1 and TRPV4 blocks α-CD3/α-CD28 mediated T cell activation. (A) Murine T cells were treated with plate-bound α-CD3 (2 μg·mL^{-1}) and soluble α-CD28 (2 μg·mL^{-1}) together with TRPV1 and TRPV4 inhibitors at the indicated concentrations for 48 h and analysed by flow cytometry. The values mentioned in the upper right corner of each flow cytometric dot-plot indicate the average number ± SD values of cells that are positive for CD25. Representative dot-plots of three independent experiments are shown. (B) The percentages of CD25$^+$ cells due to different treatments with respect to α-CD3/α-CD28 are shown (n = 3). (C) The levels/intensity of CD25 expression (determined from MFI values) in response to inhibition of TRPV1 and TRPV4 are shown (n = 3). (D) Murine T cells were treated as mentioned above (A) and were analysed by flow cytometry. The values mentioned in the corner indicate the average number ± SD values of cells that are positive for CD69. Representative dot-plots of three independent experiments are shown. (E) The percentages of CD69$^+$ cells due to different treatments with respect to ConA are shown (n = 3). (F) The levels/intensity of CD69 expression (determined from MFI values) in response to inhibition of TRPV1 and TRPV4 are shown (n = 3). The P values are: ns, non-significant; *, < 0.05; **, < 0.01; ***, < 0.001.

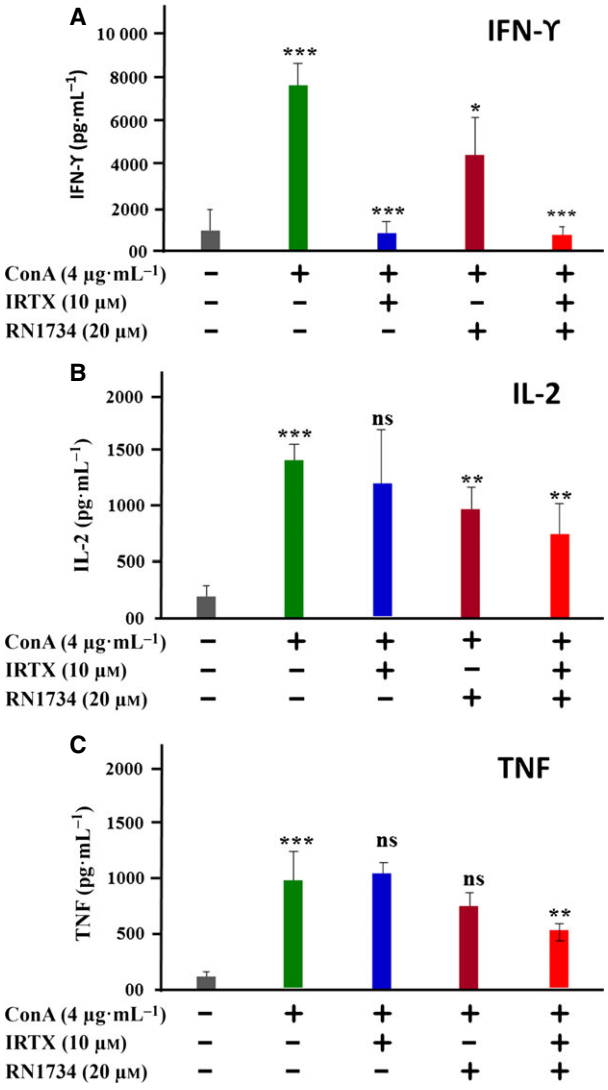

Fig. 7. Pharmacological inhibition of endogenous activity of TRPV1 and TRPV4 synergistically blocks cytokine release from T cells. Graphical bars represent the concentration (in pg·mL^{-1}) of IFN-γ (A), IL-2 (B) and TNF (C) released from ConA (4 μg·mL^{-1}) stimulated murine T cells that are pre-treated with either TRPV1 inhibitor (IRTX) or TRPV4 inhibitor (RN1734), or pre-treated with both the inhibitors synergistically. The *P* values are: ns, non-significant; *, < 0.05; **, < 0.01; ***, < 0.001 (n = 4 independent experiments).

TRPV4 inhibition also affected IL-2 release, which was 216.75 ± 61.59 pg·mL^{-1} for resting T cells and 1453.17 ± 114.02 pg·mL^{-1} in the ConA-activated condition. IL-2 concentration decreased to 1186.92 ± 513.14 pg·mL^{-1} due to 5′-IRTX alone, to 992.53 ± 183.75 pg·mL^{-1} due to RN1734 alone and to 773 ± 264.4 pg·mL^{-1} due to combination of both 5′-IRTX and RN1734 (Fig. 7B). The inhibitory roles of RN1734 alone as well as 5′-IRTX and RN1734 together on IL-2 release were statistically significant. Similarly, combination of 5′-IRTX and RN1734 showed a significant inhibitory effect on TNF production (Fig. 7C).

Since a sustained high level of intracellular Ca^{2+} is vital for T cell activation, we propose that ConA- or TCR-mediated Ca^{2+} influx is mediated at least in part by TRPV channels. In order to investigate this, we carried out experiments with purified murine T cells in the presence of TRPV1 and/or TRPV4 inhibitors [5′-IRTX (10 μM) and/or RN1734 (20 μM) for 36 h]. These inhibitors were found to inhibit ConA-mediated Ca^{2+} influx in the majority of T cells (Fig. 8). 5′-IRTX (10 μM) and RN1734 (20 μM) when used in combination blocked ConA-mediated Ca^{2+} influx more effectively than when used individually, suggesting a cooperative role of these ion channels in the regulation of Ca^{2+} levels in T cells. In agreement with the involvement of TRP channels, incubation with general TRP channel inhibitor ruthenium red (100 μM) also resulted in marked inhibition of ConA-mediated Ca^{2+} influx in T cells (Fig. 8). Similarly, inhibition of TRPV1 and TRPV4 blocks calcium influx into soluble α-CD3 stimulated (10 μg·mL^{-1}, 5 min) T cells in a similar manner to ConA stimulation (Fig. 9).

Taken together, our results suggest that TRPV members are endogenously expressed in T cells and seem to play additive as well as complex signalling cascades during immune activation by modulating events that are both Ca^{2+}-dependent and Ca^{2+}-independent.

Discussion

While the expression and functional contribution of thermosensitive TRP channels in neurons and other cells and tissues such as keratinocytes are well established, endogenous expression of these TRPVs in different immune cells, especially T cells, are clearly not known. In contrast, the effect of temperature changes on the immune response is well known. This phenomenon is observed in several species ranging from higher mammals to lower species such as birds, amphibians and even teleosts [13,36–41]. Previously, using TRPV1 knockout animals (*trpv1*$^{-/-}$) it has been demonstrated that TRPV1 is involved in bacterial clearance and cytokine gene expression [42]. Characterization of TRPV1 knockout mice by another group has also identified that TRPV1 is involved in systemic inflammatory response such as phagocytosis by macrophages, NO and reactive oxygen species production, cytokine production and bacterial clearance [43]. However, further in-depth molecular mechanisms involved in these cases were not established. In this work, by using specific antibodies and peptides we have confirmed the endogenous

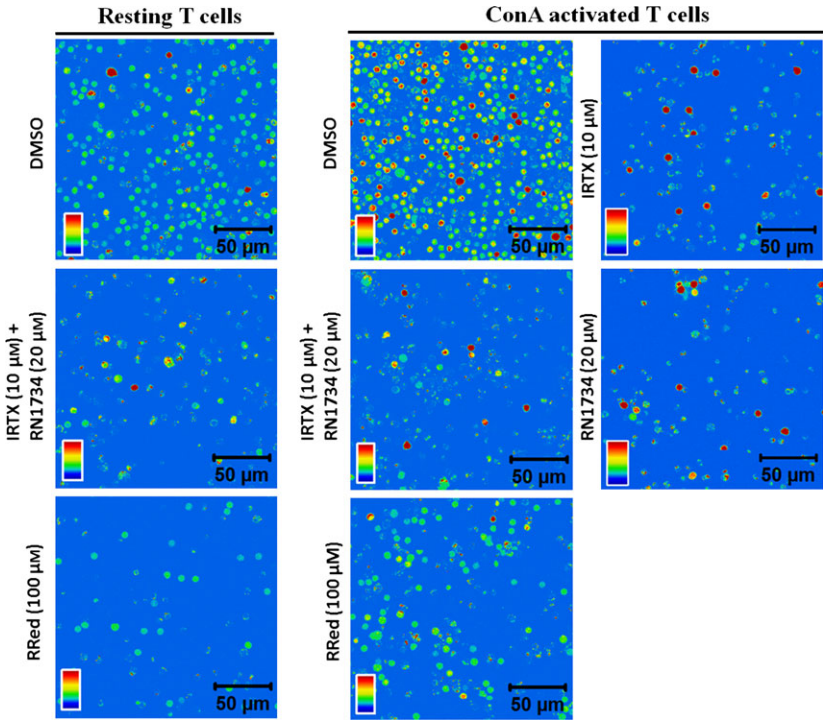

Fig. 8. TRPV1 and TRPV4 contribute in the ConA-mediated intracellular Ca^{2+} rise in murine splenic T cells. Fluorescence images of view fields containing resting (left-hand panel) or ConA-treated (right-hand panel) murine T cells loaded with Ca^{2+}-sensing dye Fluo-4 AM. The T cells were treated with IRTX and RN1734 (specific pharmacological inhibitors of TRPV1 and TRPV4 respectively), individually or in combination, separately with ruthenium red (R-Red), a common inhibitor of TRP and other ion channels. Pharmacological inhibition of all TRP channels by R-Red blocks ConA-stimulated increase in the intracellular Ca^{2+} levels. Pharmacological inhibition of TRPV1 and TRPV4 together (by 5′- IRTX and RN1734) specifically blocks ConA-mediated increase in the intracellular Ca^{2+}, while these inhibitors used individually are less efficient in blocking increase in intracellular calcium levels. The fluorescence intensities are represented in pseudo rainbow colour. Representative data of three independent experiments are shown.

expression of functional TRPV members, at the protein level in the Jurkat cell line and in primary T cells from human PBMCs and murine splenic T cells. We also provide evidence for the functional role of these channels towards T cell activation and effector functions.

Our results are in line with the few reports that have suggested the expression of different TRPs in T cells. In fact, several members of the TRPC family have been reported to be expressed in T cells [44]. In Jurkat cells and peripheral-blood-derived T lymphocytes, mRNAs encoding for TRPC1, TRPC3, TRPC4 and TRPC6 have been detected by RT-PCR [45]. Similarly, TRPC6 has been detected by western blot analysis of purified plasma-membrane fractions [45]. TRPC3 has been shown to be important for TCR-dependent Ca^{2+} entry [44] and Ca^{2+}-dependent proliferation of primary $CD4^+$ T cells [46]. Pharmacological evidence also suggests the expression of specific TRPs. For example, Δ9-tetrahydrocannabinol treatment of resting human and murine splenic T cells causes Ca^{2+} influx via TRPC1 [47]. BTP2 (a pyrazole derivative), a commonly used immunosuppressant drug, enhances TRPM4 channel activity and results in decreased Ca^{2+} influx by depolarizing lymphocytes [48].

In contrast with other TRP channels, the expression and functional pattern of TRPV members in T cells remain obscured. TRPV1 transcript could not be detected in thymocytes, splenocytes, lymphocytes, purified B cells and T cells of C57BL/6 mice [49]. However, transcripts for TRPV2, TRPV3 and TRPV4 were detected in all these immune cells [49]. In another study, by using RT-PCR and quantitative real-time PCR, specific mRNAs of TRPV1 and TRPV2 were detected in human PBMCs [50]. Further, immunostaining revealed a punctate distribution of TRPV1 and diffused cytoplasmic distribution of TRPV2 in human PBMCs [51]. In agreement with the physical expression, prolonged (24 h) activation of TRPV1 by specific activator RTX (> 20 μM) was found to induce concentration-dependent death of PBMCs, which can be effectively blocked by inhibiting TRPV1 using 5′-IRTX [50]. Previously it was also demonstrated that

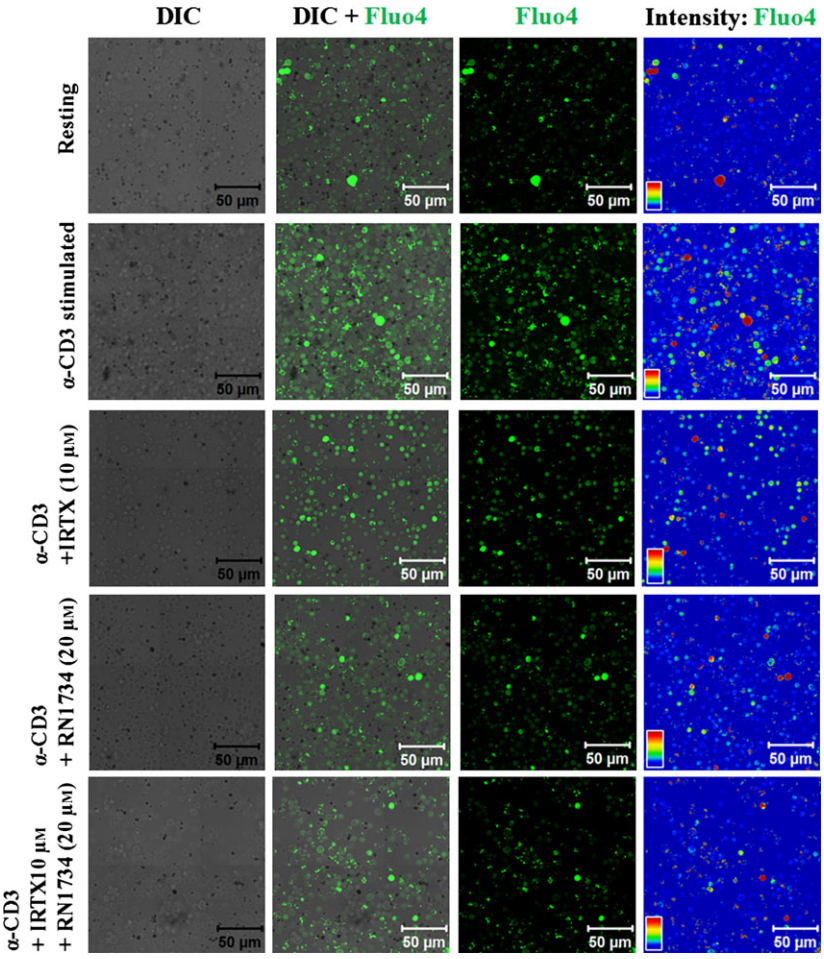

Fig. 9. TRPV1 and TRPV4 are involved in the CD3 receptor mediated intracellular Ca^{2+} rise in murine splenic T cells. Fluorescence images of view fields containing resting (uppermost panel) or α-CD3 treated (lower panels) murine T cells loaded with Ca^{2+}-sensing dye Fluo-4 AM. The T cells were treated with IRTX and RN1734 (specific pharmacological inhibitors of TRPV1 and TRPV4 respectively), individually or in combination. Pharmacological inhibition of TRPV1 and TRPV4 together (by 5′-IRTX and RN1734) specifically blocks α-CD3 mediated increase in the intracellular Ca^{2+} concentration while these inhibitors used individually are less efficient in blocking increase in intracellular calcium levels. The fluorescence intensities are represented in pseudo rainbow colour. Representative data of three independent experiments are shown.

prolonged activation of TRPV1 by capsaicin (in μM concentrations) in $CD5^+$ rat thymocytes leads to apoptotic and necrotic cell death [34]. In contrast to the thermosensitive TRPV members, TRPV6 (CaT1) has been detected by RT-PCR in Jurkat cells where it is involved in generation of store operated Ca^{2+} entry, necessary for activation of Jurkat cells [52,53]. TRPV5 and TRPV6 at mRNA as well as at protein levels have also been detected in Jurkat and human T lymphocytes [54]. Both TRPV5 and TRPV6 are involved in Ca^{2+} conductance and cell cycle progression of T cells [55]. However, the presence and functional importance of other TRPV family members in the context of T cell activation and effector cytokine response have not been explored in detail.

In this work, we demonstrate that TRPV1, TRPV2, TRPV3 and TRPV4, all thermosensitive in nature, are endogenously expressed in Jurkat cells (human T cell line), primary T cells from human PBMCs and murine splenocytes. Our results are also in line with recent reports where it has been shown that functional TRPV5 and TRPV6 are expressed in T cells [55] and that expression of these channels increases upon mitogenic activation. In this work, we show that TRPV1 and TRPV4 activation by specific ligands leads to Ca^{2+} influx in purified murine T cells, indicating that these channels are present and functional even in resting T cells. Inhibition of TRPV1 and TRPV4 by specific inhibitors was found to reduce ConA-driven mitogenic activation of T cells to significant levels. The combination of TRPV1 and TRPV4 inhibitors shows a synergistic effect and almost abolishes T cell activation, indicating that both these channels may play an important role in T cell activation. Both these channels are likely to be involved in the signalling pathways triggered by mitogen; hence inhibiting these channels had a profound inhibitory effect on CD25 and CD69 expression together with the release of signature effector cytokines such as TNF, IL-2 and IFN-γ. Moreover, we have also observed that TRPV1 and TRPV4 inhibition downregulates anti-CD3/anti-CD28 driven TCR response towards T cell activation (Fig. 6). Despite the fact that our work establishes the importance of TRPV1 and TRPV4 in regulation of certain T

cell functions, the involvement of a few other TRPV channels in these processes or other immune functions cannot be ruled out. This is especially important as a recent study indicated that TRPV5 and TRPV6 may play an important role in regulating phytohaemagglutinin-mediated Ca^{2+} influx [55].

While our current work confirms the expression of functional TRPV channels and their involvement in T cell functions, the detailed regulations and involvement of these thermosensitive channels in the T cell activation process, particularly the signalling effects, need further study. However, the involvement of different PKCs and protein kinase As (PKAs) is expected. This is due to the fact that different PKC- and PKA-mediated phosphorylations of TRPVs are typically involved with the sensitization and activation of TRPVs [56–61]. Phosphorylation of TRPVs by specific kinases at specific residues correlates well with the activation of TRPVs, Ca^{2+} influx and exocytosis-mediated cellular secretion [62–64]. Such involvement of kinases correlates well with the behavioural aspects also. For example, direct activation of PKA or PKCε mediates inflammation-induced mechanical hyperalgesia. Such hyperalgesia is decreased in TRPV4 antisense injected mice and is absent in $TRPV4^{-/-}$ mice [65].

Interestingly, while inhibiting TRPV1 and TRPV4 can efficiently block T cell activation and certain effector functions; activation of TRPV1 and TRPV4 does not increase these functions significantly. The previous studies using knockout animals also suggest that the presence of TRPV channels is required for a better immune response. Taken together, the results suggest that TRPV members present in T cells may regulate signalling events leading to T cell activation and effector functions that can be both Ca^{2+}-dependent and Ca^{2+}-independent (Fig. 10). The involvement of TRPV1 and TRPV4 in Ca^{2+}-independent signalling is intriguing as certain immune functions (such as release of cytokines) can be reduced by inhibition but cannot be increased further by activation of these channels.

It has been suggested that TRPV1 regulates various important aspects of T cell function especially in the context of thymic selection [66]. It has been reported that TRPV1 mRNA and protein are expressed in murine thymocyte subpopulations [67]. Moreover, TRPV1 activation was found to induce autophagy in thymocytes through reactive oxygen species regulated cellular pathways which have been implicated in autophagy in developing thymocytes that might regulate the survival of mature T cells and T cell developmental processes

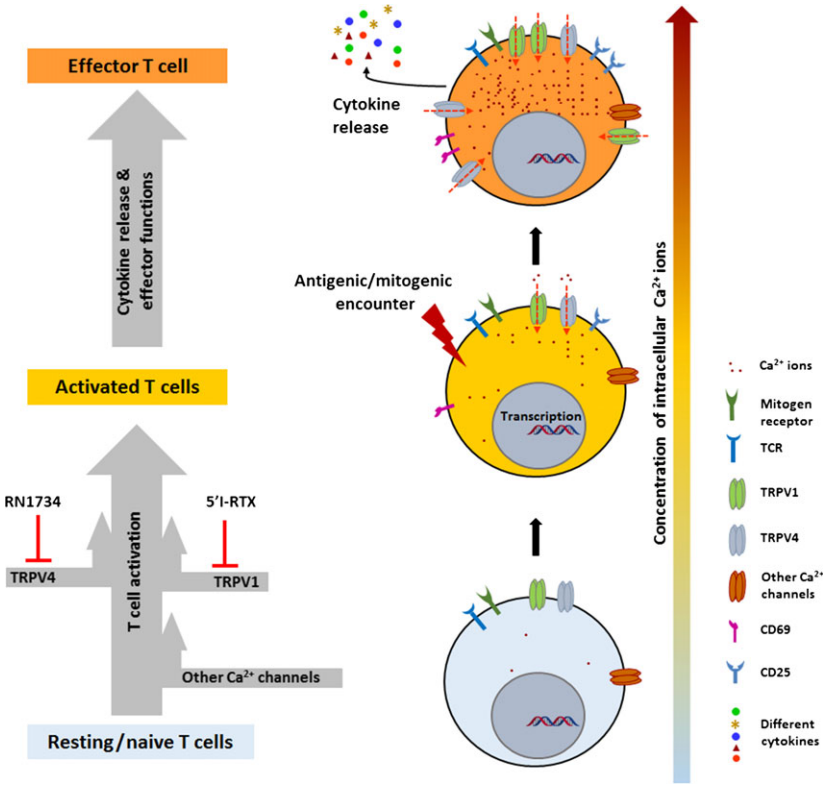

Fig. 10. Proposed model depicting involvement of TRPV channels in T cell activation and effector responses. TRPV channels present in T cells seem to be involved in diverse functions such as T cell activation, effector responses in association with cellular Ca^{2+} influx, effector cytokine production and induction of T cell activation markers (CD25, CD69). Involvement of TRPV channels in T cell activation follows sequential steps. Naive T cells with low intracellular Ca^{2+} ions express TRPV channels at lower levels. However, activation of T cells coincides with enhanced expression of TRPV channels and further increment of intracellular Ca^{2+} as well as induction of T cell activation markers along with effector cytokine production. Such activation and effector function of T cells seem to be facilitated by TRPV1 and TRPV4 as inhibition of those TRPs may restrict T cell responses. TRPV channels are likely to contribute in these T cell functions in both Ca^{2+}-dependent and Ca^{2+}-independent manners.

[67]. Very recently Bertin *et al.* have reported that TRPV1-specific T cells may positively regulate proinflammatory responses and contribute to the mouse model of colitis [68]. Moreover, it has been reported that TRPV1 may regulate Th2-biased immune response of the airways in mice through intranasal sensitization [69]. TRPVs are found to play an important role in immune regulation and various pathophysiological processes. TRPV1 has been attributed to the majority of immunoregulatory responses [43,69–75] whereas few reports are available on TRPV4 along with other TRPs and altered immune responses in experimental immunobiological investigations [49,76–78]. Together, it appears that these TRPVs are mostly involved in immunogenic responses associated with autoimmunity, inflammatory responses, and with various attributes related to disease biology. Moreover, a broadened role of the TRP members is suggested in the pathobiology of cancer, asthma, infectious diseases, neuromuscular disorders and other diseases [79–82].

The endogenous expression of functional TRPV channels and alteration of their expression in T cells in response to mitogenic activation and TCR stimulation is relevant in different forms of acute and chronic pain. This study also unravels important implications in the context of neuro-immune interactions. The direct effect of involvement of TRPV4 in responding to inflammatory mediators is established [65]. Intradermal injection of carrageenan (an inflammogen) or a soup of inflammatory mediators (containing bradykinin, substance P, prostaglandin E2, serotonin and histamine) enhanced the pain sensation in rat. However, in *TRPV4*$^{-/-}$ knockout mice or upon blocking TRPV4 expression by administration of TRPV4 antisense oligodeoxynucleotides in spinal cord, certain forms of pain sensation induced by inflammatory mediators are abolished. This indicates that inflammatory mediators engage the TRPV4-mediated mechanism of sensitization by direct action on dissociated primary afferent neurons [65]. We demonstrate that production and release of different cytokines from T cells are regulated by TRPVs and such cytokines can stimulate and affect nearby peripheral neurons as observed in the case of inflammation and tissue injury [83]. The presence of different TRPVs in T cells may also explain the immunoregulatory effects of different neurotransmitters, neuropeptides and other stimulatory components such as neuropeptide Y, substance P, calcitonin gene-related peptide, NADA, other endocannabinoids and endovanillioids as reported before [84–86].

Taken together, our work confirms the presence of functional TRPV members and elucidates the immunoregulatory roles of these channels. Although further investigation is warranted towards *in vivo* and more in-depth studies, the current findings have broad implications in pain, immunotherapy and regulation of neuro-immune interactions associated with TRPV channels in translational research.

Materials and methods

Reagents

The TRP channel modulatory drugs 4αPDD, RTX, 5′-IRTX, NADA and ruthenium red were obtained from Sigma-Aldrich (St Louis, MO, USA). 2APB and RN1734 were obtained from Tocris Biosciences (Abingdon, UK). ConA was purchased from Himedia (Mumbai, India). Rabbit polyclonal antibodies for TRPV1, TRPV2, TRPV3, TRPV4 and the specific blocking peptides (TRPV1, EDAEVFKDSMVPGEK; TRPV2, KKNPTSKPGKNSASEE; TRPV3, REEEAIPHPLALTHK; TRPV4, CDGHQQGYA PKWRAEDAPL) were purchased from Alomone Laboratories (Jerusalem, Israel). The Ca^{2+}-sensitive dye Fluo-4 AM was procured from Molecular Probes (Eugene, OR, USA). Anti-mouse CD25PE, CD69PE, CD3PE-Cy5 and anti-human CD3-PE, functional grade (azide free) CD3 and CD28 mAbs were obtained from BD Biosciences (San Jose, CA, USA). CD90.2 APC antibody was from Tonbo Biosciences (San Diego, CA, USA).

Isolation of T cells and cell culture

Murine spleen cells were obtained with the approval of the Institutional Animal Ethics Committee (IAEC protocol no. IAEC/SBS-AH/03/13/01). Mouse spleen was obtained from 6- to 8-week-old male BALB/c mice and a single cell suspension was made by passing the suspended cells through a 70 μm cell strainer. To purify the T cells, the non-adherent cells from mice splenocytes were separated after ~ 30 min of adherence to six-well plates prior to the T cell purification step. Then the T cells were purified by using BD IMag™. Mouse T Lymphocyte Enrichment Set – DM was used according to the manufacturer's instructions. The isolated cells were cultured in a 24 well (3.5×10^6 cells·well^{-1}) polystyrene cell culture plate with Iscove's modified Dulbecco's medium (IMDM) (PAN Biotech, Aidenbach, Germany) supplemented with10% FBS (Himedia). The percentage purity of the purified T cells was verified by staining cells with anti-CD3 antibody and analysing by flow cytometry. In each case the $CD3^+$ T cell population was above 95%. All the experiments were performed about 36 h after plating the cells as most of the primary T cells were found to be activated during 36–48 h of a ConA or anti-CD3/CD28 driven *in vitro* T cell activation assay. Primary murine T cells were activated with plate-bound α-CD3 (2 μg·mL^{-1}) and soluble α-CD28 (2 μg·mL^{-1}) for 48 h or with ConA (4 μg·mL^{-1}) for 36 h before harvesting for staining for T cell activation markers

(Figs 5, 6). Jurkat cells were cultured in a 24 well polystyrene cell culture plate with IMDM supplemented with10% FBS.

Human blood was collected from healthy donors and human PBMCs were isolated by using HiSep (HiSep LSM LS001, Himedia) as per the manufacturer's instruction. In brief, human blood was diluted with ice-cold PBS and overlaid on 2.5 mL of HiSep LSM (Himedia) in a 15 mL centrifuge tube. It was centrifuged in a swinging bucket rotor for 30 min at 400 ***g***. Subsequently, the lymphocyte layer was collected by sterile Pasteur pipette, washed twice with isotonic PBS and grown on IMDM supplemented with10% FBS. To purify the T cells, the non-adherent cells from human PBMCs were separated after ~ 30 min of adherence to six well plates prior to the T cell purification step. Dynabeads Flow Comp TM Human CD3 T cell purification kit from Invitrogen (Carlsbad, CA, USA) was used to purify T cells from human PBMCs as per the manufacturer's instruction. The purity of the human T cells used for the experiments was ~ 90–95% and was verified by flow cytometry.

Pharmacological modulation of cells

Jurkat (a human T cell line) cells, purified human PBMC derived T cells and purified mouse splenic T cells were activated using ConA (4 $\mu g \cdot mL^{-1}$) for 36 h. Similarly, in certain experimental conditions, cells were treated with the following TRP channel modulators: RTX (100 nM), 5′-IRTX (1–10 μM), 4αPDD (1 μM), RN1734 (1–20 μM), ruthenium red (100 μM) for 36 h. After 36 h of activation, cell culture medium was collected for ELISA and the cells were harvested by centrifugation at 500 r.p.m. for 2 min for further downstream experiments. Trypan blue exclusion assay revealed that > 95% cells were alive after incubation with TRP channel drugs (at the concentrations used in the experiments) for 36–48 h.

Flow cytometry

For probing for TRPV expression, cells were stained with the individual TRPV-specific antibodies mentioned above and subsequently flow cytometric analysis was performed as described previously [87,88]. For evaluating the profile of immune markers, mouse T cells were incubated with anti CD25 PE, CD69 PE and CD3PE Cy5 mAbs dissolved in FACS buffer (1 × PBS, 1% BSA and 0.05% sodium azide) for 30 min on ice and then washed further. Similarly the purity of human T cells was also evaluated by anti-human CD3 PE mAb. Stained cells were washed twice with the same FACS buffer before line-gated acquisition of around 10 000 cells. Stained cells were acquired with FACS Calibur (BD Biosciences). Data were analysed using CELL QUEST PRO software (BD Biosciences). The percentages of cells expressing the markers are represented in dot-plots while the MFI values represent the expression levels of the markers per cell.

Immunofluorescence analysis and microscopy

For immuno-cytochemical analysis, immediately after harvesting, T cells were diluted in PBS and fixed with paraformaldehyde (final concentration 2%). After fixing the cells with paraformaldehyde, the cells were permeabilized with 0.1% Triton X-100 in PBS (5 min). Subsequently, the cells were blocked with 5% BSA for 1 h. The primary antibodies were used at 1 : 200 dilution. In some experiments, blocking peptides were used to confirm the specificity of the immunoreactivity. The ratio (vol/vol) of blocking peptides with specific antibody was 1 : 1. All primary antibodies were incubated overnight at 4 °C in PBST buffer (PBS supplemented with 0.1% Tween-20). AlexaFluor-488 labelled anti-rabbit antibodies (Molecular Probes) were used as secondary antibodies and were used at 1 : 1000 dilution. All images were taken on a confocal laser scanning microscope (LSM-780, Zeiss) with a 63 × objective and analysed with the Zeiss LSM image examiner software and Adobe Photoshop.

Ca^{2+} imaging

Ca^{2+} imaging of primary murine splenic T cells was performed as described previously with minor modifications [89]. In brief, primary murine splenic T cells in their resting state were loaded with Ca^{2+}-sensitive dye (Fluo-4 AM, 2 μM for 30 min). The cell suspension was added to the live cell chamber for Ca^{2+} imaging and images were acquired every 5 s. For Fig. 4, the cells were stimulated with specific agonists alone or in combination of agonists and antagonists as described. For Figs 8 and 9, the cells were pre-incubated with TRP channel inhibitors for 2 h and then stimulated with ConA (4 $\mu g \cdot mL^{-1}$) or soluble α-CD3 (10 $\mu g \cdot mL^{-1}$) for 10 min. Fluo-4 AM signal was acquired using a Zeiss LSM780 microscope and with the same settings. The images were analysed using LSM software and intensities specific for Ca^{2+}-loaded Fluo-4 are represented in artificial rainbow colour with a pseudo scale (red indicating the highest level of Ca^{2+} and blue indicating the lowest levels of Ca^{2+}).

ELISA

Supernatants from the respective experiments were collected and stored at −20 °C and ELISA for cytokine markers, namely IFN-γ, IL-2 and TNF, was performed using BD Biosciences Sandwich ELISA kits as per the manufacturer's instructions. The readings were taken using a microplate reader (Bio-Rad iMARK) at 450 nm.

Statistical tests

The flow cytometric data were imported in R software for statistical analysis. The ANOVA test was done for each set of data to check the reliability and significance of the data points. $P < 0.05$ was considered statistically significant. The

significance values are as follows: ***, P between 0 and 0.001; **, P between 0.001 and 0.01; *, P between 0.01 and 0.05; ns, P above 0.05.

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Author contributions

RKM, SSS, SC and CG conceived the idea and designed all the experiments. RKM, SSS, MY and BMP performed all the experiments. RKM, SS, MY, BMP, SC and CG analysed the data. RKM, SSS, SC and CG wrote the manuscript.

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