# Role of mycobacterial protein, PPD in modulating TLR induced T-cell response

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# ABBREVIATIONS

Ag: Antigen

AP-1: Activator Protein 1

APC: Antigen Presenting Cell

Bcl-2: B cell Leukemia 2

BM: Bone marrow

CD: Cluster of Differentiation

CM: Central memory

DC: Dendritic Cell

DEPC: Diethyl pyrocarbonate

EDTA: Ethylene Diamine Tetra acetate

ELISA: Enzyme Linked Immuno Sorbant Assay

EM: Effector memory

EtBr: Ethidium Bromide

FACS: Fluorescence associated cell sorter

IFN-γ: Interferon γ

IL-15: Interleukin 15

IL-15R: Interleukin 15 Receptor

IL-7: Interleukin 7

IL-7R: Interleukin 7 Receptor

JAK: Janus kinase

LLCM: Long Lived Central Memory

LPS: Lipopolysaccharide

LTA: Lipoteichoic acids

MAPK: Mitogen Activated Protein Kinase

MHC: Major Histocompatibility ComplexmIL- 15: Mouse( Mus musculus) Interleukin 15

mIL -7: Mouse( Mus musculus) Interleukin 7

MOPS: 3-[N-morphino]- propanesulfonicacid

MyD88: Myeloid Differentiation Factor 88

NFKB: Nuclear Factor Kappa B

NK cells: Natural Killer cells

NKT cells: Natural Killer like T cells

PAMP: Pathogen Associated Molecular Pattern

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

PGN: Peptidoglycan

PI3K: Phosphatidylinositol-3 kinase

PKB: Phosphokinase B

Poly I: C: Poly inosinic: cytidinic acid

PRR: Pattern Recognition Receptor

RT: Reverse Transcription

RT-PCR: Reverse Transcription Polymerase Chain Reaction

STAT: Signal Transducer and Activator of Transcription

TAE buffer: Tris Acetate EDTA buffer

TCR: T cell Receptor

TH cells: T helper cells

TLR: Toll like receptors

TlR: Toll-like IL-I Receptor

Tm: Melting Temperature

TNF: Tumor Necrosis Factor

TRAF: TNF Receptor Associated Factor

TRAF: TNF Receptor-associated factor

TU: Tubercullin Unit

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# INTRODUCTION

## 1.1 Innate and adaptive immunity

## 1.2 Development of the cells of immune system

## 1.3 T-cell activation and differentiation:

## 1.4 1.4 TLR ligand and their receptor

## 1.5 Adjuvants (IFA and CFA)

## 1.6 Purified protein derivatives (PPD)

## 1.7 TLR ligands used in our study

a) LPS

b) Zymosan

# INTRODUCTION

## 1.1

Host defence against invading microbial pathogens is elicited by the immune system, which consists of two components: innate immunity and adaptive immunity. The innate immunity provides the first line of defines against infection. It consists of cellular and biochemical defence mechanisms that are in place even before infection and are poised to respond rapidly to infections.

In adaptive immunity, B and T lymphocytes utilize antigen receptors such as immunoglobulins and T cell receptors to recognize non-self. Adaptive immune response against an antigen is generated within five or six days after the initial exposure

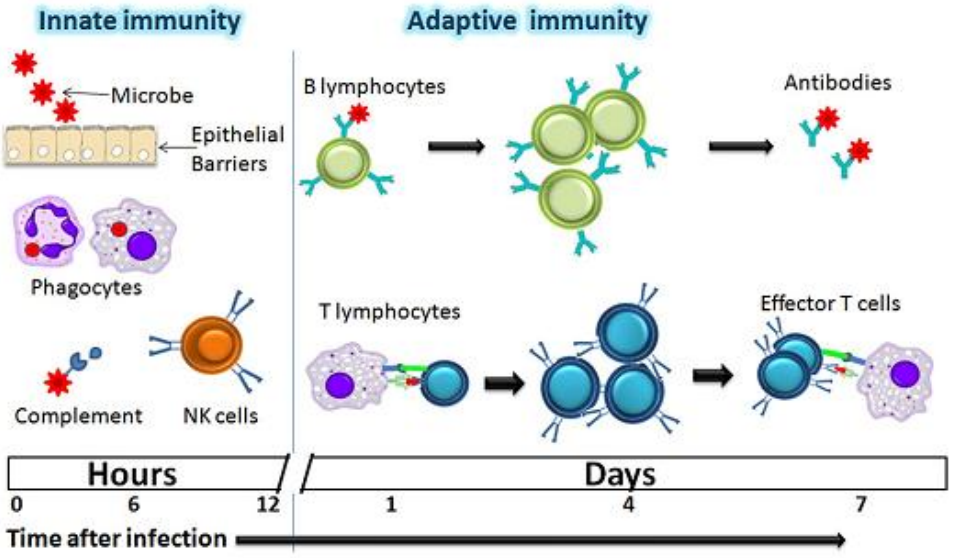



**Figure1: Kinetics of Innate and adaptive immunity**

to that antigen. It responds to the infection with a high degree of specificity as well as the remarkable property of "memory", that provide long-lasting immunity against the re-infection. Because the adaptive immune system takes longer to be activated pathogens get favourable condition for their rapid growth. During that period the innate immune system takes responsibility by acting immediately to encounter the infections. Thus innate immunity mainly performs two function. One is the initial response to microbes (antigen) and second is stimulation of adaptive immunity by processing and presenting the antigen to T-cell. This processing and presentation is done by APC (antigen presenting cells like macrophages and dendritic cells) The innate and adaptive

immune system is linked through direct cell contact and through interactions involving chemical mediators such as cytokines and chemokines.

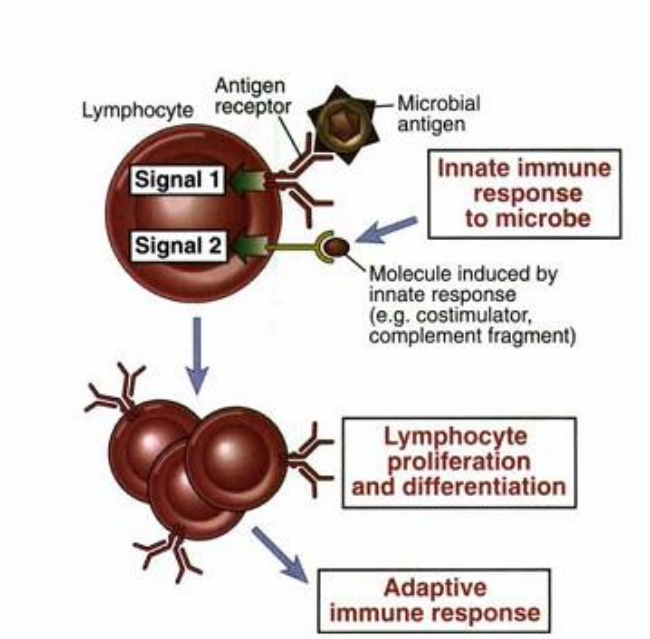



**Figure 2:- Stimulation of adaptive immunity by innate immune response**

(Cellular and molecular immunology by Abul K. Abbas 6th edition)

**1.2 Development of the cells of Immune system:**

All cells of the immune system have their origin in the bone marrow and they include myeloid (neutrophils, basophils, eosinpophils, macrophages and dendritic cells) and lymphoid (B lymphocyte, T lymphocyte and Natural Killer) cells, which differentiate along distinct pathways

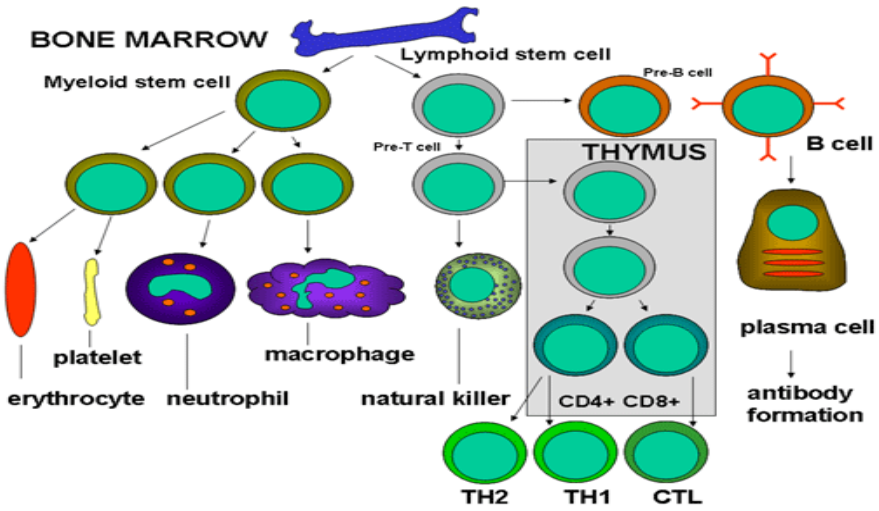



**Figure 3: Development of the cells of immune system**

Bone marrow precursor T-cells enter the thymus through blood circulation and undergo the process of maturation and become a naive T-cell. After the activation these naive T-cells differentiate into antigen specific effector T-cells

### 1.3 T-cell activation and differentiation:

Bone marrow precursor T-cells migrate to thymus and undergo the process of maturation to become naive T-cells. After maturation these naive cells moves to secondary lymphoid organ, where they encounter antigen presented by mature dendritic cells on class I and II MHC molecules and thus become activated. This results in the expansion of the antigen specific lymphocytes the differentiation of these cells into effector and memory T cells

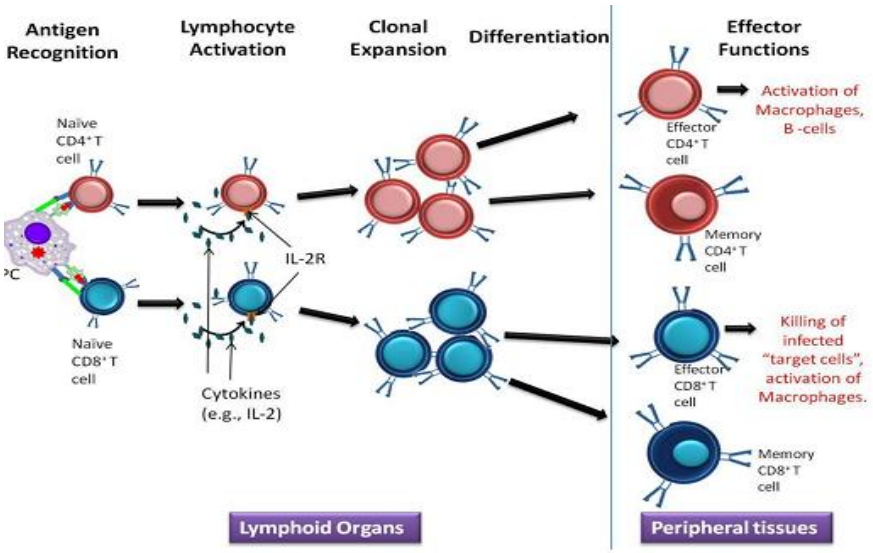



**Figure 4**: **Phases of T-cell responses**

When APC present antigen to T-cell by MHC molecule, they also provide a complex set of co-stimulatory signal to T-cell and helps them to undergo vigorous clonal expansion and acquire effector function. In adaptive immunity, B and T lymphocytes utilize antigen receptors such as immunoglobulins and T cell receptors to recognize non-self. Adaptive immunity takes five to six days to generate response against antigen. But after activation they form memory cells which provide long lasting immunity against the re-infection.

Effector $CD4^+$ T-cell thus produced respond to antigen by producing cytokines that have several actions like activation of macrophages and B lymphocytes, and CD8+

CTLs respond by killing other cells. These cytokines control events of T-cell activation at various stages of T-cell responses.

There are three cytokines- IL-2, IL-7 and IL-15 which have been shown to have integral roles in CD8+ T-cell memory generation and maintenance. Although their functions might be overlapping in some cases, their activities on T-cells can occur either simultaneously or at temporally distinct points during an immune response

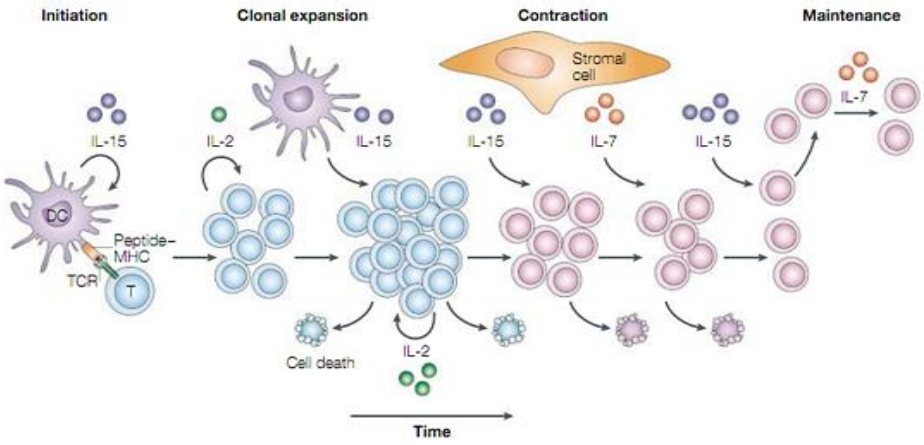



**Figure 5: Cytokines control T-cell differentiation at different phases.**

The antigen in our system is recognised by the cells of innate immunity like APC and dendritic cells. These cell recognise the specific pattern on pathogens i.e. PAMPs (pathogen associated molecular patterns) with the help of receptor molecules present on their membrane called Toll like receptors(TLRs)

### 1.4 Toll Like Receptors (TLRs)

TLRs are membrane-bound molecules that recognize microbial components on the surface or within extracellular compartments of antigen presenting cells (APCs). TLRS are pattern recognition molecuale, which recognise specific pattern on pathogen and activate innate immune system, which in turn stimulate adaptive immune system.

These TLRs binds to TLR ligand like lipopolysaccharide (LPS) from Gram-negative bacteria.LPS is a TLR ligand which binds to TLR4.Till now at least 11 mammalian TLRs have been identified among which TLR1-9 are conserved between the human and mouse.

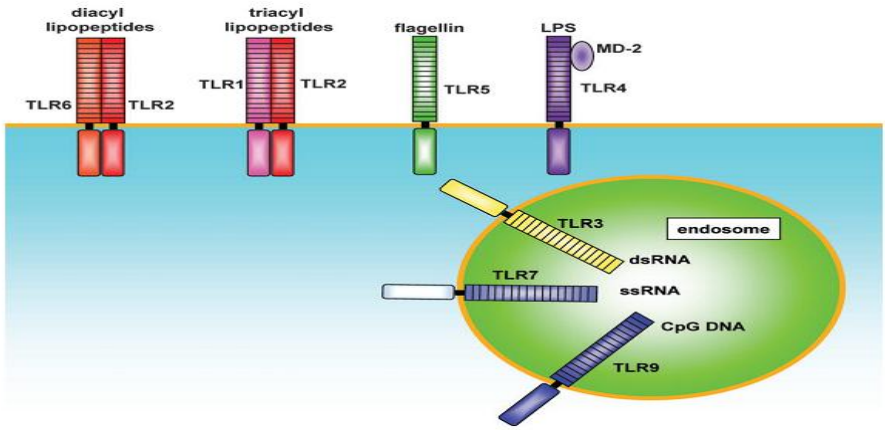



**Figure 6:TLRs and TLR ligands**

TLR Ligands are the microbial components which bind with different TLRs and activate specific TLR pathways. Stimulation of TLRs by microbial components triggers expression of several genes that are involved in immune responses, like genes for inflammatory cytokines and co-stimulatory molecules

**Table 1**: Toll like receptors (TLRs) and their ligand(TLR ligand)

| S.no | Toll like receptors (TLRs) | TLR ligand recognised by TLRs |
|---|---|---|
| 1 | TLR2+TLR6 | diacyl lipopeptide |
| 2 | TLR2+TLR1 | triacyl lipopeptide |
| 3 | TLR4 | Gram –ve LPS,fungal mannans, parasitic phospholipids |
| 4 | TLR5 | Bacterial flagellin |
| 5 | TLR9 | CpG DNA |
| 6 | TLR3 | Virsl ds RNA |

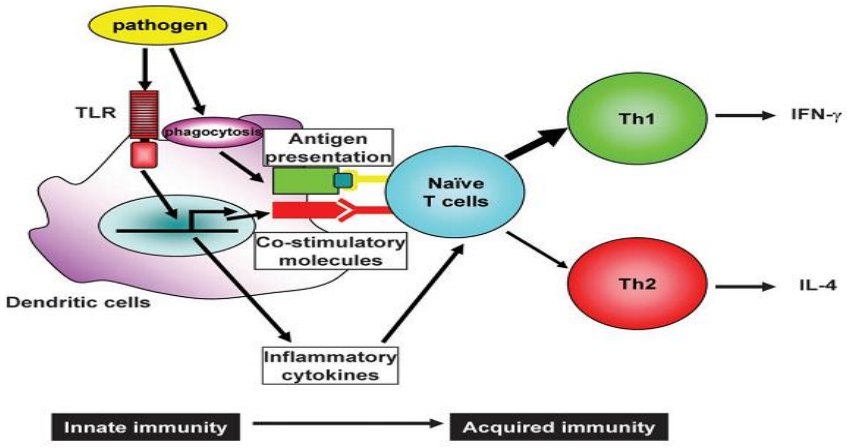



**Figure 7**:**Pathogen recognition of TLRs and processing and presentation of antigen to T-cell**

The molecular mechanism lying behind the expression of various gene is the dimerization of TLRs. TLR2 is shown to form a heterophilic dimer with TLR1 or TLR6, but in other cases TLRs are believed to form homodimers. Dimerization of TLRs triggers activation of signaling pathways, which originate from a cytoplasmic TIR domain.

TLRs leads to expression of many gene that give signals to other cells of immune system to respond the antigen in a better way. This is the basis on which adjuvants works and helps in enhancing the immune response against pathogens.

**1.5 Adjuvants:-**

The word adjuvant comes from the Latin word adjuvare, which means to help or to enhance. The role of innate immunity in stimulating adaptive immune responses is the basis of the action of adjuvants, which are compound that when administered with antigen potentiates adaptive immune response, both humoral & T-cell mediated response, against co-administered antigen. The main function of adjuvants is to induce inflammatory response that will help in triggering adaptive immune response. In absence of adjuvant even higher dose of antigen cannot induce productive immune response, which can be achieved with low amount of antigen if adjuvant is used.

Adjuvants are useful in experimental immunology and in clinical vaccines. Many adjuvants in experimental use are microbial products, such as **killed mycobacteria** and **LPS,** that engage TLRs and elicit strong innate immune responses at the site of antigen entry.

Alum is one of the few adjuvants approved for human use in most of the countries worldwide.Alum induce a good antibody (Th2) response but it has a little capacity to stimulate cellular(Th1) immune responAse which is so importent for protection against many pathogens.Alum is thought have a role in neurodegenerative diseases such as Alzheimer's disease.Pure recombinant or synthetic antigens used in modern day vaccines are generally far less immunogenic than older style live or killed whole organism vaccines.So there is a need for safer and more effective adjuvants suitable for human use.

Since last few decades, adjuvants like IFA (Incomplete Freund's adjuvant) and CFA (Complete Freund's adjuvant) are in use for experimental work and preclinical vaccine preparation to enhance immune response. Its mode of action, however, is still not very clear.

IFA is essentially a paraffin oil containing mannide mono-oleate as a surfactant.. When IFA is mixed with aqueous solution of antigen it forms water in oil emulsion where antigen is in water phase. This water in oil emulsion slowly releases antigen and help to induce immune response effectively (Alfonso Billiau et.al 2001). CFA contain an additional factor to oil i.e. heat killed *Mycobacterium tuberculosis.* It is more potent than IFA. It is used in primary immunization to potentiate adaptive immune response by triggering innate immunity

IFA stimulates innate immunity, as evident by transiently increased resistance to bacterial infection (Castro et. al., 1993).It also induces the expression of cytokines, predominantly tumor necrosis factor α (TNF- α) in regional lymph nodes (Mussener et. al., 1995). Besides inducing the above responses, CFA containing mycobacteria attracts macrophages and other cells to site of injection and enhances immune response.

Various components of heat killed mycobateria interact with various TLR which activates innate immune system and attracts macrophages and other cells to the site of infection and enhances immune response

**Active components of CFA:**

- Heat shock protein (HSPs), the intracellular protein found in both prokaryote and eukaryote, bind to TLR4, resulting in an LPS like effect on mononuclear phagocytes (Kol et. al., 2000; Ohashi et. al., 2000).
- Glycolipids typical for mycobacteria trehalose dimycolate (TDM) and lipoarabinomannan (LAM). LAM is the agonist of the TLR2 (Golenbock et. al., 2001).
- CpG oligodeoxynucleotides, present in heat killed mycobacteria have been shown to strongly stimulate innate immune mechanisms, including production of cytokines by mononuclear phagocytosis (MPCs), maturation, and activation of APCs (Weiner, 2000).
- Muramyl-di peptide (MDP) is also immuno-modulator component of CFA which is the building block of peptidoglycan component of the bacterial cell wall. MDP was found

in expansion of granulocyte-macrophage progenitors in spleen, and proliferation of multi potential stem cells in bone marrow (Galelli et. al., 1983).

**Mechanism of Immuno-potentiation by CFA:-**

- **Enhancement of antigen uptake by APCs:** Water in oil emulsion of antigen causes slow delivery of antigen to immune system, hence increased persistence of antigen in system.
- **Complex set of signal:** The mycobacteria in CFA are currently assumed to be recognized by PAMP (pathogen-associated molecular pattern) receptors on various immunocompetent cells and thus to provide the stimulus for these cells to release mediators and express membrane receptors (collectively designated as "danger" signals) that will lead to Th1-type skewing of ongoing immune responses
- **Cytokine and chemokine induction**: Cytokines observed to be induced in the early phases following exposure to CFA (or mycobacteria) are TNF-α, IL-12, IL-6, IFN-γ, and several chemokines. Mycobacterial components are known to target MPCs and DCs (involving TLRs) and to induce production of monokines, in particular IL-12 and TNF-α (Mussener et. al., 1995).

  CFA is a more potent adjuvant because it contains heat killed *mycobacterium*. The antigens present on *mycobacterium* are mainly responsible for the generation of enhanced immune response. PPD (purified protein derivatives) is one of the major mycobacterial protein antigen. These antigens boost the response by providing complex set of signals to immune cells.

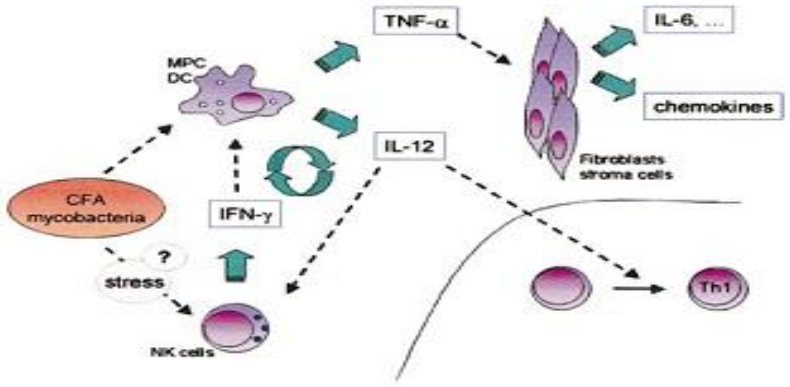



**Figure 8: cytokine and chemokines induction by CFA**

**1.6 PPD (Purified protein derivatives)**:

Tuberculin Purified protein derivatives(PPD) is an antigenic preparation derived from *Mycobacterium tuberculosis*, the causitive agent of tuberculosis.It has had a widespread clinical and epidemiologic, and investigative use. It is used as an in vivo diagnostic aid for detection of mycobacterium tuberculosis infection. It is basically used in the measurement of T cell reactivity in persons exposed to TB or vaccinated with BCG.

PPD is prepared by precipitation of proteins from heated Mycobacterium tuberculosis cultures and composed of soluble protein antigens.

PPD contains multiple antigenic component.One such component , a small protein or a large peptide designated as antigen 7, was identified as major constituent of PPD.D-Arabino and D-mannan is major polysaccharide constituent of PPD.(Yu Ma and Thomas M. Daniel JSTOR 1983) .

PPD stimulate innate immunity (Jenefer M. Blackwell et al. 2004); elicit strong Th1-cell response which can be identified by production of IFN-γ, IL-2, and TNF-α, TNF-β. PPD also act as immunogen carrier (De Silva BS 1999) and helps in inducing effective adaptive immune response. It has potential in vaccine preparation as an adjuvant.(De Silva et.al., 1999). The effect of PPD in modulating TLR induced T-cell response is not yet clear.

**In the proposed study we want to test whether enhanced antigen specific immune response in presence of PPD is due to modulation of TLR induced response of adjuvants or due to modulation of innate immunity by TLR ligands.**

**1.7 TLR Ligands used in our study:**

We have used following two TLR ligand in different combination with antigen.

- **Lipopolysaccharide** (LPS)

LPS is the major constituent of cell wall of Gram negative bacteria. It is a potent immunostimulator.It is a TLR4 ligand recognised by TLR4 receptor.

LPS stimulates APCs to release various cytokines e.g., IFN, TNF-α, IL-12 and also elicit both $CD4^+$ and $CD8^+$ T-cell response (David et. al., 1997). HSP60 elicits potent inflammatory response in innate immune cells. HSP60 signalling mediated by TLR4 (LPS) release inflammatory mediators such as IL-6, IL-15, TNF-α (Ohashi et. al., 2000). LPS induces upregulated expression of both IL-15and IL-15Rα by splenic

dendritic cells and stimulation of antigen specific T cell proliferation (Mattei et. al., 2001).

- **Zymosan**

  Zymosan is a yeast cell wall particle. It is a TLR2 ligand recognised by TLR2 receptor. It has potential to activate macrophages, monocytes, and leukocytes resulting in the stimulated secretion of inflammatory products including TNF-α, IL-8, hydrogen peroxide, and arachidonic acid. Zymosan also induce NF-κB activation(Sato et. al., 2003). It is also able to induce maturation of dendritic cells *in vitro* and to stimulate the production of IL-12, provide a link between innate and adaptive immune response.

**We wants to mimic CFA by using by using LPS (which represent TLR4), zymosan (which represent TLR2 of mycobacteria) and PPD which is a major mycobacterial protein antigen**.

When two or more antigens are injected together they can respond in either way, they enhance the immune response against each other or they dampen the response against each other. But it is well known that in the case of multivalent vaccine like BCG two or more antigen mixed together enhances the immune response against each other.

**So Further we want to test the idea whether one antigen from a pathogen can influence the immunogenicity of another antigen.**To test this idea we have taken two protein antigen chicken Ovalbumin and hen egg white lysozyme (HEL) which are the from same source

# MATERIALS AND METHODS

## 2.1. MICE

## 2.2. TLR LIGANDS

- **LPS**
- **ZYMOSAN**

## 2.3. ANTIGENS

- **OVALBUMIN**
- **PPD**
- **HEN EGG WHITE LYSOZYME (HEL)**

## 2.4. IMMUNIZATION PROTOCOL

**2.4.1 PROTOCOL OF FIRST OBJECTIVE**
**2.4.2 PROTOCOL OF SECOND OBJECTIVE**

## 2.5. T-CELL PROLIFERATION ASSAY

## 2.6. RT-PCR

**2.6.1 BUFFERS AND REAGENTS**
**2.6.2 FOR CFSE DILUTION ASSAY**
**2.6.3 FOR RNA ISOLATION**
**2.6.4 FOR CDNA SYNTHESIS**
**2.6.5 FOR PCR AMPLIFICATION**
**2.6.6 FOR AGAROSE GEL ELECTROPHORESIS**
**2.6.7 FOR *IN VITRO* CULTURE**

## 2.7. PRIMERS

**2.7.1 MUS MUSCULUS ACTIN, BETA (ACTB), MRNA**
**2.7.2 MUS MUSCULUS INTERLEUKIN 15 (IL15), TRANSCRIPT VARIANT 1, MRNA**

## 2.8. PROTOCOLS

**2.8.1 RNA ISOLATION**
**2.8.2 CDNA SYNTHESIS**
**2.8.3 PCR AMPLIFICATION**
**2.8.4 IL-15 INDUCTION**

**2.1 Mice**

Male BALB/C mice (Age: 4-5 week old) were obtained from Zydus pharmaceuticals, Ahmedabad. These animals were housed in Animal house facility, Institute of Pharmacy, NIRMA University. Food and water were available ad libitum. All experiments performed in accordance with protocol approved by Animal care and use committee of NIRMA University.

Immunizations of Mice

Incomplete Freund's adjuvant (IFA) [ Banglore Genei, Cat# FIA]

IFA was used for water in oil emulsion at 1:1 ratio.

**2.2 TLR Ligands**:

1. **Lipopolysaccharide** (LPS): (Invivogen tlrl-pelps:-LPS-EB)

   Stock solution: 5 mg of LPS was dissolved in 1 ml of sterile 1X PBS.

   (Stock concentration: 5 mg/ml)

   Dose given: 10 µg/ mouse

2. **Zymosan** (Invivogen, cat. #tlrl-zyn):

   5 mg of Zymosan was weighed and dissolved into 5 ml of PBS.

   (Stock concentration: 1 mg/ml).

   Dose given: 25 µg/ mouse

**2.3 Antigen**

- **Ova Protein:**

  Chicken Ovalbumin (Merck, cat.# 32467 1gmcn)

  Stock solution: 40 mg of Ova Protein was dissolved in 1.0 ml Sterile PBS.

  (Stock concentration:40mg/ml).

  Dose given: 200 µg/ mouse

- **PPD (Purified Protein Derivative):**

PPD was purchased from Span Diagnostics, Surat, Gujarat.

Stock Concentration: 10TU/0.1 ml

Dose given: 0.2 µg/ mouse

- **Hen Egg white Lysozyme (HEL):** Merck (4403-1GMCN)

40 mg of HEL was dissolved in 1ml sterile PBS.

Stock concentration:40mg/ml

Dose given: 100 µg/ mouse

**2.4 Immunization Protocol**

**2.4.1 Objective 1**:-**Role of PPD in modulation of TLR induced T-cell responses.**

Following combinations of TLR ligands with antigen and/or PPD were injected to

Mice intraperitonealy

**Table 2**: Immunization protocol for first objective

| S.no. | Combination of TLR ligand | Serum collection for IL-15 ELISA | | Number of mice sacrificed for T-cell assay | |
|---|---|---|---|---|---|
| | | 24 hr | 72 hr | $10^{th}$ day | $10^{th}$ day repeat |
| 1 | OVA+IFA | 3 | 3 | 3 | 3 |
| 2 | PPD+OVA+IFA | 3 | 3 | 3 | 3 |
| 3 | OVA+LPS+IFA | 3 | 3 | 3 | 3 |
| 4 | PPD+OVA+LPS+IFA | 3 | 3 | 3 | 3 |
| 5 | OVA+ZYMOSAN+IFA | 3 | 3 | 3 | 3 |
| 6 | PPD+OVA+ZYMOSAN+IFA | 3 | 3 | 3 | 3 |
| 7 | OVA+LPS+ZYMOSAN+IFA | 3 | 3 | 3 | 3 |
| 8 | PPD+OVA+LPS+ZYMOSAN+IFA | 3 | 3 | 3 | 3 |

**2.4.2 Objective 2**: **whether one antigen from a pathogen can influence the immunogenicity of another antigen of same origin**

To test this idea we have taken two antigen ovalbumin and Hen egg white lysozyme (HEL), which are having same origin and injected these antigens in different combination with TLR ligands

**Table 3: Immunization protocol for second objective**

| S.no. | Combination of TLR ligand | Serum collection for IL-15 ELISA at 72 hours | Number of mice sacrificed for T-cell assay |
|---|---|---|---|
| 1 | OVA+ IFA | 3 | 3 |
| 2 | HEL + IFA | 3 | 3 |
| 3 | HEL+OVA+IFA | 3 | 3 |
| 4 | OVA+LPS+ ZYMOSAN +IFA | 3 | 3 |
| 5 | HEL +LPS+ZYMOSAN+IFA | 3 | 3 |
| 6 | HEL +OVA+LPS+ZYMOSAN+IFA | 3 | 3 |

**2.5 T-cell Proliferation Assay:**

The measurement of T cell responses is an important parameter to determine status of immune system. Usually T cell assays are done by measuring:

1. Cell proliferation
2. Synthesis of Intracellular Cytokine or secreted cytokine

Cell dilution assay can be done by using either thymidine assay or CFSE dilution assay. The classical method of measuring T cell proliferation has been done via uptake of [3H] thymidine during the final hrs of 3-5 day culture. But this method demands handling of radioisotopes which is not very safe. Moreover the interpretation of results is very subjective. The difficulties and limitations of this method have been overcome by CFSE dilution assay

Carboxyfluorescein succinimidyl ester (CFSE) is an effective and popular means to monitor lymphocyte division. CFSE covalently labels long-lived intracellular molecules with the fluorescent dye, carboxyfluorescein. Thus, when a CFSE-labelled cell divides, its progeny gets half the number of carboxyfluorescein-tagged molecules and thus each cell division can be assessed by measuring the corresponding decrease in cell fluorescence via Flow cytometry.

CFDA-SE is carboxy-fluorescein-diacetate-succinimidyl-ester is a lipophilic non-fluorescent compound. It diffuses into the cells passively. The acetate groups are removed by esterase activity inside the cell yielding highly fluorescent compound, which binds random secondary groups of all cellular proteins and ultimately label the cells

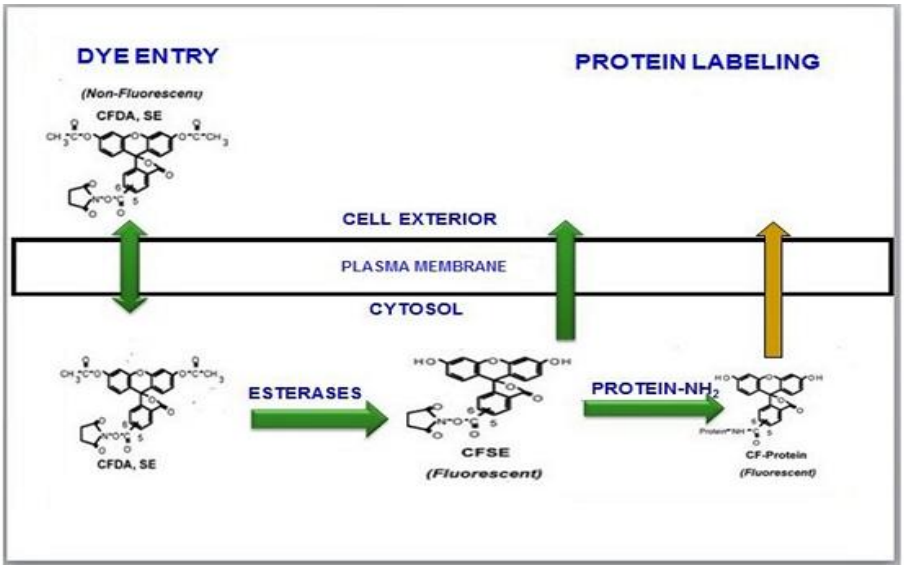



**Figure 9**:-**Mechanism of action of CFSE**

The capacity of CFSE to label lymphocyte populations with a high fluorescent intensity of exceptionally low variance, coupled with its low cell toxicity, make it an ideal dye to measure cell division. Since it is a fluorescein-based dye it is also compatible with a broad range of other fluorochromes making it applicable to multi-color flow cytometry.

This method is based on analysis of CFSE dilution upon *in vitro* recall stimulation with antigen. Analysis of proliferation was combined with the use of monoclonal antibodies directed against the lymphocyte surface markers like CD4, CD8, CD3 etc. This assay helps analyzing proliferation of cells as they divide in response to antigenic or mitogenic challenge and their fate division.

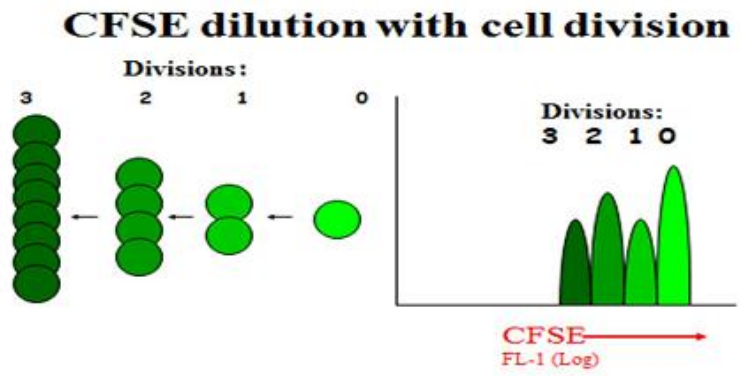



**Figure 10: CFSE dilution with cell division**

Upon antigenic or mitogenic challenge, cells start to proliferate and by this they lose fluorescence in step-wise manner with each cell division. Reduction in fluorescence intensity can be quantified by flow-cytometry. Thus, there are many advantages to use CFSE cell proliferation including determination of proliferation of specific cell population.

**Labelling cells with CFSE:**

- Lymph node obtained from mice was minced in sterile 1X PBS and 1% glucose solution. The cells obtained were pellet down by spinning at 1000 rpm for 10 mins. Cells in pellet were treated with 1 ml RBC lysis solution and kept for 3 min. (Optional step if lymph node is used). Repeat the washing step using 1X PBS.
- Cells were pellet down and then dissolved in chilled complete RPMI and taken for counting under microscope. Based on the counting definite volume of cell was taken in a way to stain $3*10^6$ cells with 5µM CFSE.
- To stain the cells uniformly dye is not added directly to cells as dye being denser will not stain cells uniformly. For this reason $3*10^6$ cells were made up to certain volume in one tube and 5mM CFSE dye made in another tube with prewarmed complete RPMI. Both the tubes were mixed together in a way that working concentration of dye is 5µM (CFSE dye is photosensitive so avoid intense light during CFSE staining).
- Cells were incubated at $37^0$C and 5% $CO_2$ for 10 min during CFSE staining in $CO_2$ incubator.
- The staining is quenched by addition of 2 volumes of chilled complete RPMI 1640 to the stained cells. The cells were pellet down at 1000 rpm for 10 min. Pellet is resuspended in the complete RPMI and spin as before, repeating washing step twice.
- Finally cells in pellet were resuspended in media in a way that 100µl of $3*10^5$ cells are dispensed to the each well of culture plate. 100µl of different concentrations of antigen (OVA or PHA) are added to 96 wells culture plate. The culture plate is incubated at $37^0$C and 5% $CO_2$ for 72 hours in $CO_2$ incubator.
- After 72 hours cells were washed from the wells with 1% FACS buffer and collected in eppendorfs and volume was made upto 1 ml.

Cells were pellet down by spinning at 1200 rpm for 7 mins and resuspended in minimum volume. Then 10µl of 1:50 diluted normal mouse serum was added and

mixed by tapping the tubes and by pipetting. Cells were kept for 10 mins on ice. After which 10µl 1:75 times diluted antibodies were added, mixed well by pipetting and tapping the tubes and kept on ice for 30 mins.

Note: Ensure that the serum and antibodies are bound well, for this reason pipetting and tapping the tubes are crucial steps that would give the better separation in the results.

- Dilutions are made in such a way that serum is eventually diluted 1:100 times and antibodies are diluted 1:150 times in the suspension of cells.

  Note: Keep eppendorf in dark while antibody staining
- Volume was made upto 1 ml with 1% FACS buffer and centrifuged at 1200 rpm for 7 mins. Pellet obtained was washed once again with 1% FACS spin same as above.
- Finally the pellet was resuspended in 500µl of 1% FACS. 50µl of 1% Formaldehyde was added for storage up to 24 hours at $4^0$C. Samples were acquired using flow Cytometer (BD Canto) at GCRI, Ahmedabad. The acquired results were analyzed using the software FlowJo (7.6.5).

**2.6 Buffers and Reagents required**:

**2.6.1 For CFSE dilution assay:**

1. Lymph node or spleen
2. Frosted slide for preparing single cell suspension
3. Sterile dissection tools and petriplate
4. Sterile complete RPMI media (with 10% FCS)
5. Sterile RPMI washing media (with 1% FCS)
6. $CO_2$ incubator
7. Swinging rotor clinical centrifuge
8. CFSE [Carboxyfluorescein succinimidyl ester (cat# no. 21888-25MG-F, Sigma)]
9. Phytohaemagglutinin (PHA)
10. Flow Cytometer (BD FACS Canto II at GCRI, Ahmedabad)
11. Flowjo software (version 7.6.5) to analyse result

12. 96 Well U-bottom culture plate.

13. Fluorescently labelled antibodies

- APC Rat Anti-Mouse CD4 (BD Bioscience, cat# 553932)
- PE Rat Anti-Mouse CD8a (BD Bioscience, cat# 554714)
- PerCP Hamster Anti-Mouse CD3 (BD Bioscience, cat# 553975

**2.6.2 *In vitro* culture**

1. Phosphate buffer saline, PH 7.4 (PBS)

2. Complete RPMI 1640 media (10% FCS) (HI GlutaXLtm RPMI-1640 AL751G-20X50NO)

3. Washing RPMI 1640 media (1%FCS)

4. U-bottom 96 well culture plate

5. Ovalbumin (0µg/ml, 0.1µg/ml, 1.0µg/ml, 10µg/ml )

6. RBC lysis buffer

7. $CO_2$ Incubator

8. Frosted slides

**2.6.3 Reverse Transcription and Polymerase Chain Reaction (RT-PCR):**

For RNA isolation

1. Trizol or Tri reagent (cat# no. T9424-200ML, Sigma)

2. Diethyl pyrocarbon, DEPC (cat# no. D5758, Sigma )

3. Isopropanol

4. RNA precipitation solution

5. 75% Ethanol

6. Formamide

7. Chloroform

8. Cuvette for measuring O.D. at 260 nm and 280 nm

9. RNA isolation hood.

**2.6.4 For Agarose gel electrophoresis:**

1. RNA or DNA sample
2. 5% (for RNA) and 1%(for DNA, PCR products) Agarose gel
3. Autoclaved Mili Q water
4. 1X TAE buffer
4. Electrophoresis unit
5. Gene ruler 100bp DNA ladder, Fermentas (cat.# SM0241)
6. 6X loading dye
7. Gel Documentation system.
8. Gel loading dye (Bromo phenol blue)
9. Ethidium bromide (EtBr)

**2.6.5 For cDNA synthesis:**

1. RNA template
2. First Strand cDNA Synthesis Kit, Fermentas (cat. # K1612)
3. PCR machine (eppendorf thermal cycler)

**2.6.6 For PCR amplification:**

1. cDNA template
2. Forward IL-15 primer (sigma)
3. Reverse IL-15 primer (sigma)
4. Forward and reverse β-actin primer (eurofins mwg operon)

5. PCR Master Mix 2X, Fermentas (cat. # K0171)
6. PCR machine (Eppendorf)
7. Primer designing tool: IDT(Integrated DNA technology)

   Web address: http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer

**2.7. Primers:**

**2.7.1. Mus musculus actin, beta (Actb), mRNA**

**NCBI Reference Sequence: NM_007393.3**

**>gi|145966868|ref|NM_007393.3| Mus musculus actin, beta (Actb), mRNA**

**Table 4**: **β-actin primer detail**

| **Primer 5'→ 3'** | **Primer Sequence 5'→ 3'** | **%GC** | **$T_m$ ($^OC$)** | **Primer Length** |
|---|---|---|---|---|
| **Forward** | TGGAATCCTGTGGCATCCATGAAAC | 48 | 64 | 25 |
| **Reverse** | TAAAACGCAGCTCAGTAACAGTCCG | 48 | 64 | 25 |
| **Product Length: 349** | | | | |

**2.8.2. Mus musculus interleukin 15 (Il15), transcript variant 1, mRNA**

NCBI Reference Sequence: NM_008357.2

**Table 5**: **IL-15 primer detail**

| **Primer 5'→ 3'** | **Sequence (5'→3')** | **Primer Start Position** | **%GC** | **$T_M$ ($^oC$)** | **Primer Length (Nucleotide)** |
|---|---|---|---|---|---|
| **Forward** | GACAGTGACTTTCATCCCAG | 732 | 50 | 61.1 | 20 |
| **Reverse** | CCAAGTGGCTCATTATCTCC | 1128 | 50 | 61 | 20 |
| Product Size: **397 bp** | | | | | |

## 2.8 PROTOCOLS

### 2.8.1. RNA Isolation Protocol:

1. Preparation of tissue sample for RNA isolation:

- **100 mg** tissue was homogenized for 20-30 sec with **1 ml Tri reagent** in homogenizer.
- Centrifuged at 10,000 rpm for 10 min at 4°C
- Tri reagent is a denaturant, it denature the cell wall protein and lyse the cell.

2. Supernatant was taken and **250µl chloroform** was added. Mix well and incubate for 10 minutes at room temperature (RT).

- This help dissociating proteins present in cell extract.
- Centrifuge at 12,000 rpm for 15 min at 4°C

3. After centrifugation three phases will obtain. Take out the uppermost layer.

- The **upper aqueous layer** contains RNA, middle layer will contain DNA and lowest layer will contain protein.
  Note: DNA and protein are extracted out in the organic phase while RNA remains in the aqueous phase.

4. Precipitation of RNA

- To the upper aqueous layer **200 µl RNA precipitating solution** + **250 µl Isopropanol** was added.
- Mix gently for 2-3 minutes for 10 min at RT
- Centrifuged at 10,000 rpm for 15 min at 4°C
- This would precipitate out **RNA in the pellet form**.

4. The pellet thus obtained was washed twice with 1 ml of **75% ethanol** (prepared in DEPC treated water) by centrifuging it at 8000 rpm for 5 min at 4°C

- This wash is given to remove excess Isopropanol used for RNA precipitation.

5. Supernatant was discarded pellet obtained was allowed to air dry.

6. Finally pellet was dissolved in **20 µl formamide** and stored at -20°C or in liquid nitrogen.

Facts:

1. The yield of total RNA depends on the tissue or cell source, but it is generally 4-7 µg/mg starting tissue or 5-10 µg/$10^6$ cells.

2. Dissolve the precipitated RNA in deionized formamide and store at -20°C.Formamide provides a chemically stable environment that also protects RNA against degradation by RNases. Purified, salt free RNA dissolves quickly in formamide

up to concentrations, samples of the RNA can be analyzed directly by gel electrophoresis, RT-PCR, or RNase protection saving time and avoiding potential degradation. (Chomczynski,1992)

3. If necessary, RNA can be recovered from formamide by precipitation with 4 volumes of ethanol or by diluting the formamide fourfold with 0.2N NaCl & then adding the 2 volumes of ethanol.

**2.8.1.1 Agarose Gel Electrophoresis:**

1.5% agarose gel is suitable for resolving RNA.

- 1.5% agarose gel was prepared in 1X TAE.
- 1X TAE was used as electrophoresis buffer
- Electrophoresis unit was installed and loaded with 1X TAE
- Sample was loaded with Bromophenol Blue dye in the propotion of 2:1
- Gel was run at 100V 20 mins.
- Gel picture were taken in Gel documentation system.

**2.8.1.2 Spectrophotometric analysis:**

The absorbance of RNA sample was measured at 260 nm and 280 nm. The nucleic acid concentration was calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.

$$A = €Cl,$$

Where, A= absorbance at a particular wavelength

C= concentration of nucleic acid

L= length of the spectrophotometric cuvette (1 cm)

E= extinction coefficient (€ for RNA is $0.025(mg/ml)^{-1}cm^{-1}$)

For RNA Absorbance of 1at 260 nm ($A_{260}$ value 1) corresponds to 40µg/ml of single stranded RNA. $A_{280}$ an idea about the amount of protein present in isolated RNA. The $A_{260}/A_{280}$ ratio is used to assess RNA purity. An A260/A280 ratio of 1.82 is indicative of highly purified RNA. RNA samples were diluted with DEPC- water and A260 was measured. And concentration was calculated using following formula:

$$\text{RNA concentration (µg/µl)} = \frac{A_{260} \times \text{Dilution Factor} \times 40}{1000}$$

random hexamer are the primers that can be used for cDNA synthesis. Oligo dT primers specifically binds to poly-A tailed mRNA, while random hexamer non-specifically binds to all those RNA which are having sequence complementary to random hexamer.

**Composition of Reaction 2.8.2 cDNA Synthesis:**

RT-PCR (reverse transcription-polymerase chain reaction) is a technique used to study the expression of gene at mRNA levels. It is the most sensitive technique for mRNA detection and quantitation.

Reverse transcriptase is a RNA-dependent DNA polymerase enzyme isolated from retrovirus. Oligo dT and **Mixture for RT**

For cDNA synthesis various components were added following order

**Table 6**: Composition of Reaction Mixture for RT

| S.no. | **Reagents** | **Volume** |
|---|---|---|
| 1 | Template DNA | * |
| 2 | Oligo dT primer | 1µl |
| 3 | nuclease-free Water | Upto 11µl |
| | Total | 11µl |

*Volume of template RNA is adjusted according to RNA concentration to be used for cDNA synthesis

| | **Reagents** | **Volume** |
|---|---|---|
| 4 | 5X reaction Buffer | 4µl |
| 5 | Ribolock RNase Inhibitor | 1µl |
| 6 | 10mM dNTP Mix | 2µl |
| 7 | M-MuLV Reverse Transcriptase | 2µl |
| total volume | | 20µl |

For cDNA synthesis tubes containing above mixture were incubated for 60min at $37^0$C (If oligo dT is used) and reaction was terminated by incubating at $70^0$C for 5min.

If random hexamer is used above mixture should be first incubated at $25^0$C for 5min followed by 60 min incubation at $37^0$C

**2.8.3 Polymerase chain reaction (PCR):**

**Composition of Reaction Mixture for PCR**

**Table 7:** Composition of Reaction Mixture for PCR

| S.no. | Reagent | volume |
|---|---|---|
| 1 | PCR master mix | 12.5 |
| 2 | Template cDNA | 2µl |
| 3 | Forward primer | 1 µl |
| 4 | Reverse Primer | 1 µl |
| 5 | Nuclease free water | Upto 25µl |

After mixing all reagents give a short spin for proper mixing of all components. Above tube was placed in thermal cycler having with below mentioned PCR profile.

**PCR profile for β-actin**

**Table 8:** PCR profile for β-actin

| S no. | Step | Temperature ($^o$C) | Time | Number of cycle |
|---|---|---|---|---|
| 1 | Initial denaturation | 95 | 3 min | 1 |
| 2 | Denaturation | 95 | 30 sec | 35 |
| 3 | Annealing | 56 | 45 sec | |
| 4 | Extension | 72 | 60 sec | |
| 5 | Final extension | 72 | 10 min | 1 |

**PCR profile for IL-15**

**Table 9:** PCR profile for IL-15

| S no. | Step | Temperature $^o$C) | Time | 1 |
|---|---|---|---|---|
| 1 | Initial denaturation | 95 | 3 min | 35 |
| 2 | Denaturation | 55.8 | 30 sec | |

| | | | | |
|---|---|---|---|---|
| 3 | Annealing | 72 | 45 sec | 1 |
| 4 | Extension | 72 | 60 sec | |
| 5 | Final extension | 95 | 10 min | 1 |

**2.8.3 IL-15 induction:**

***In vitro* induction of IL-15:**

1. Isolation of spleenocytes was done by mincing through two frosted slides with 1X PBS and 1% glucose in petriplates

- Cells were spin down at 1000rpm for 10 mins, pellet was obtained.

2. Supernatant was discard, **pellet** was dissolved in 3 ml complete **RPMI media**.

3. Cells were divided in required number of wells, and RPMI and **10% FCS** were added in each well.

- Note: FCS promotes and increases the degree of adherence of cells onto culture plate.

4. Cells were kept for incubation for **4 hours** at 37'C and 5% $CO_2$

- Note: Lymphocytes would be in the suspension while macrophage and DC adhered to culture plate.

5. **Lymphocyte suspension was** carefully removed and extra RPMI was added for culturing of Macrophage and DC.

6. Cells were incubated **overnight** at 37'C, 5% $CO_2$.

- Note: Macrophage will be adhered and DC in the suspension.

7. **DC were removed** from the suspension by repeating a wash with PBS and transferred to sterile tube.

8. To the Macrophage was added 0.5ml **Trypsin-EDTA** per well and kept for **3** minutes.

9. Reaction was stopped by adding 3 ml RPMI.

10. Macrophage were transferred into sterile tube and centrifuged at 1000 rpm for 10 mins to pellet down the cells.

12. Pellet was dissolved in minimum volume of RPMI and taken the cell for counting.

Spleen was dissected cells were harvested and total RNA was isolated13. According to the cell count, pellet was dissolved in desired volume of media. Then the cells were divided in the required number of tubes.

14. Cells were stimulated with **LPS-10ng/ml** and **Poly I:C- 50ug/ml**.

15. Cells were incubated for **4 hours** at 37°C and5% $CO_2$.

16. Cells were obtained in pellet by centrifuging at 1000rpm for 10mins.

17. Supernatant was discarded and **pellet** was dissolved in left volume of supernatant.

18. Adding 1 ml **Trizol** to the tube and cells were transferred in the eppendorf and proceeded for **RNA isolation** by Trizol method.

**2.8.4 Enzyme-Linked Immunosorbent Assay (ELISA):**

ELISA is a powerful and sensitive technique for the detection of antigen or antibody. The principle of this is same as that of radio immuno assay (RIA) i.e.an enzyme conjugated with an antibody reacts with a colourless substrate to generate a coloured reaction product and intensity of colour thus produced will give the concentration of antigen in the sample.

A number of variations of ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody, like direct ELISA, indirect ELISA, sandwich ELISA.

At a time interval of 24 hours and 72 hours after the immunization ~0.5 ml of blood was collected from three mice of each group by retro-orbital bleeding. Blood was allowed to clot for 4 hours at room temperature and then incubated at $4^0$C for better recovery of serum.

Serum was collected after centrifugation at 1500 rpm for 7 min. and IL-15 ELISA was performed using Ray biotech IL-15 ELISA kit.

# RESULTS AND DISCUSSION

In the natural condition host might be encountered by more than one organism, so in this study we want to check the influence of one antigen on the immune response generated against the other antigen.PPD (purified protein derivatives) is a major mycobacterial protein antigen of CFA. Not much study is done about the role of PPD in the adjuvant activity of CFA. So here our objective is to study how PPD influencing the immune response generated against Ova.

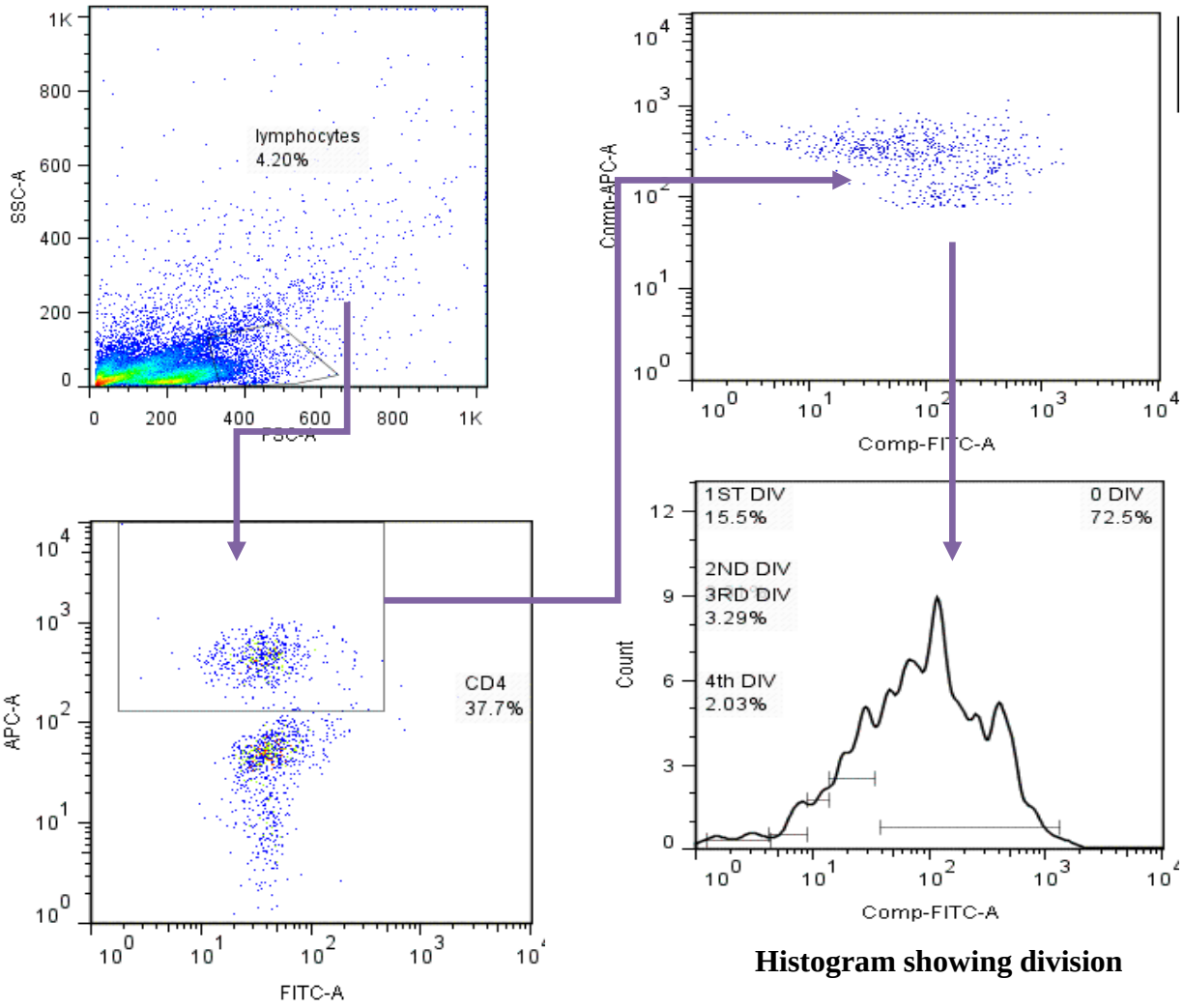
Multivalent (polyvalent vaccine) vaccine is designed to elicit an immune response either to more than one infectious agent or to several different antigenic determinants of a single antigen. In natural condition when an organism is infecting any host, host immune system will be encountered with different antigens of that organism. So our next aim was to study whether two antigenic protein from same origin influence immune response generated by one antigenic protein. For that we used Ova (chicken Ovalbumin) and HEL (hen egg lysozyme) from the same source i.e. egg.

.

- **PPD shown enhanced antigen (Ova) specific $CD4^+$ and $CD8^+$T-cell responses**.

Eleven days after the post immunization cells were harvested from draining lymph node and were labelled with CFSE dye and T-cells were challenged *in vitro* with increasing concentration of Ova protein (0, 0.1,1.0 ,10 µg/ml) . Following 72 hr stimulation, $CD4^+$ and $CD8^+$ T-cells were labelled with anti-CD4 APC and anti-CD8 PE and analysed by Flow Cytometry.

**Scheme of analysis:** **$CD4^+$ Cells gating**




**Histogram showing division**

**Figure 11: Scheme of analysis**

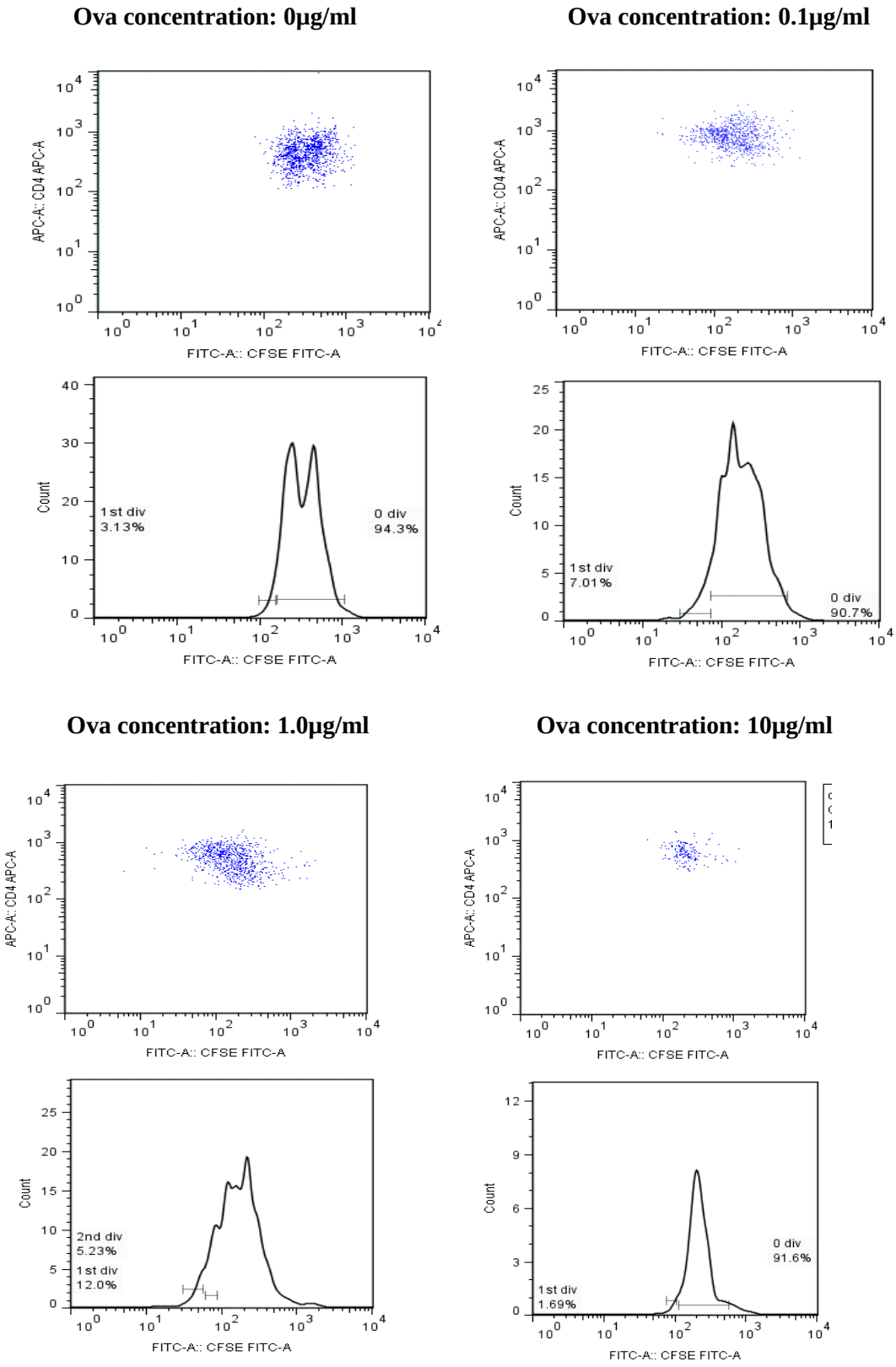
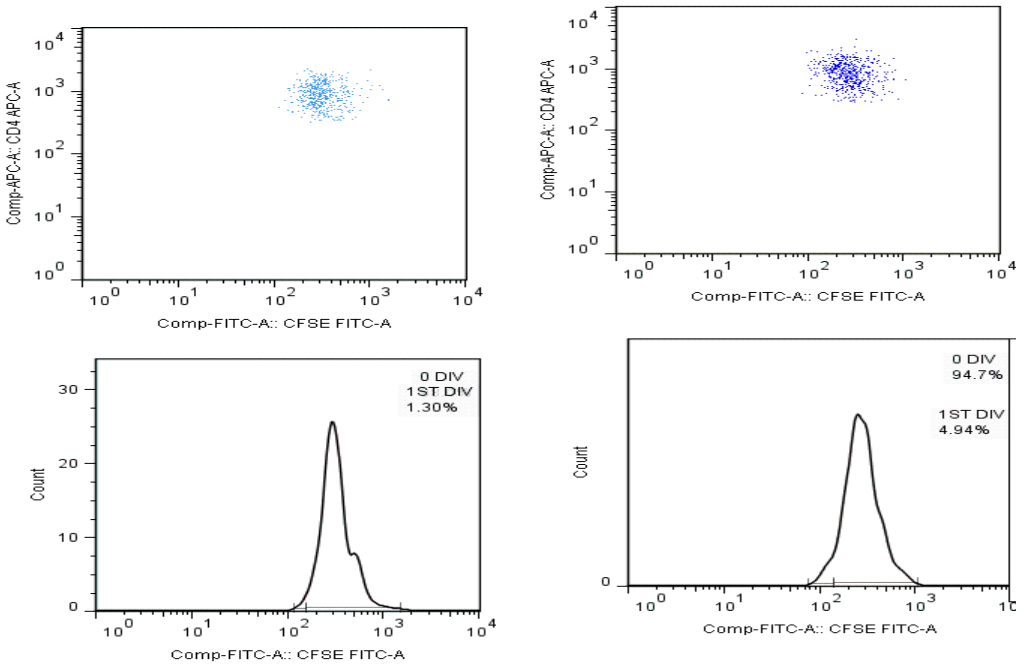



**Figure 12: Mice were immunized with Ova, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0 , 10 µg/ml)**

**Ova concentration: 0µg/ml** **Ova concentration: 0.1µg/ml**




**Ova concentration: 1.0µg/ml** **Ova concentration: 10µg/ml**

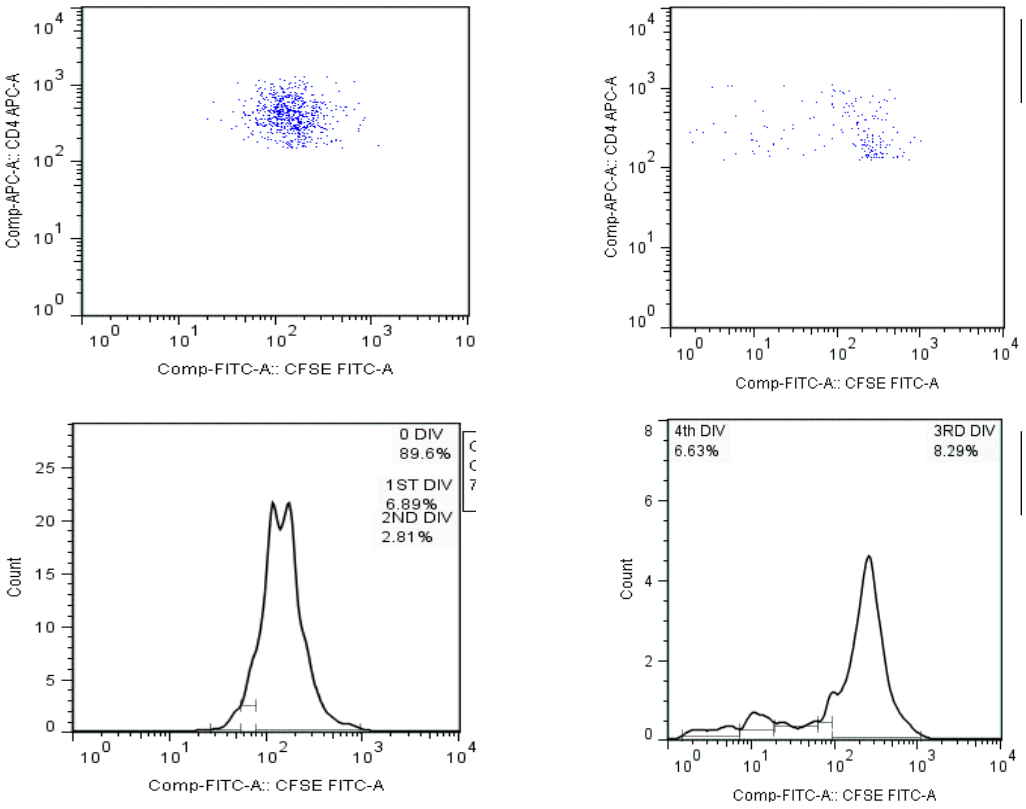



**Figure 13: Mice were immunized with Ova+PPD, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0,10 µg/ml)**

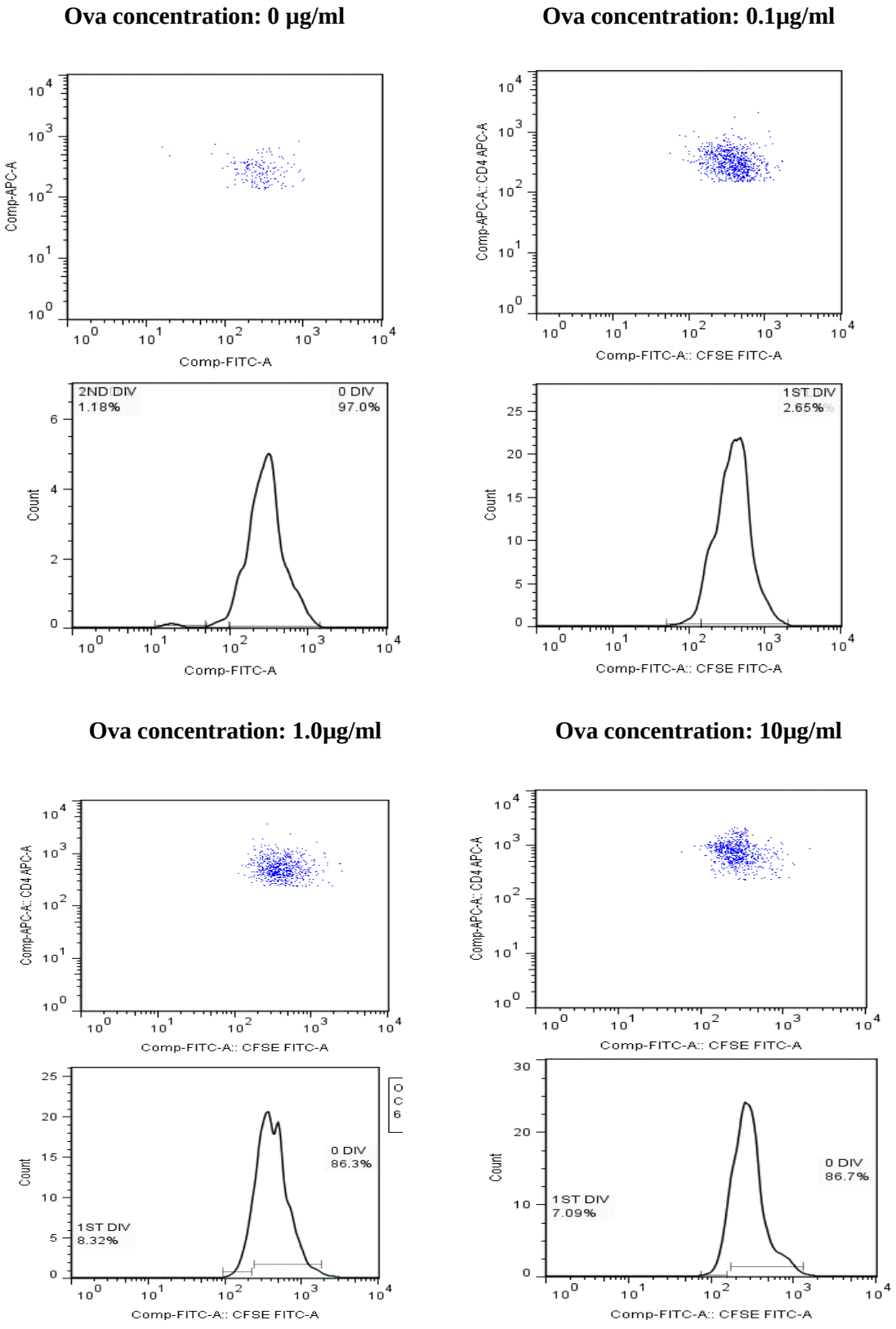
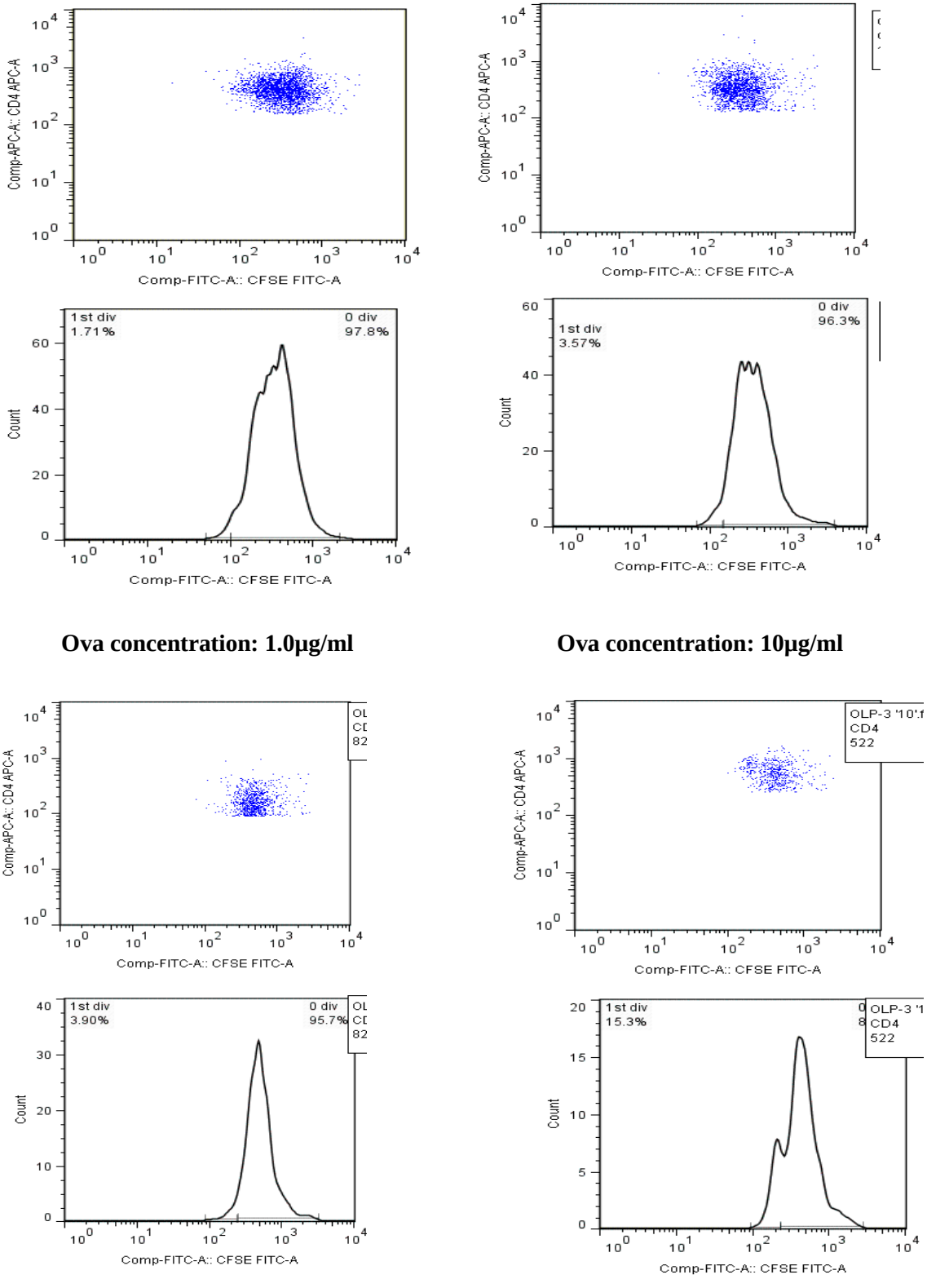



**Figure14: Mice were immunized with Ova+LPS, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0,10 µg/ml)**

**Ova concentration: 0µg/ml** **Ova concentration: 0.1µg/ml**




**Ova concentration: 1.0µg/ml** **Ova concentration: 10µg/ml**




**Figure 15: Mice were immunized with Ova+LPS+PPD, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0, 10 µg/ml)**

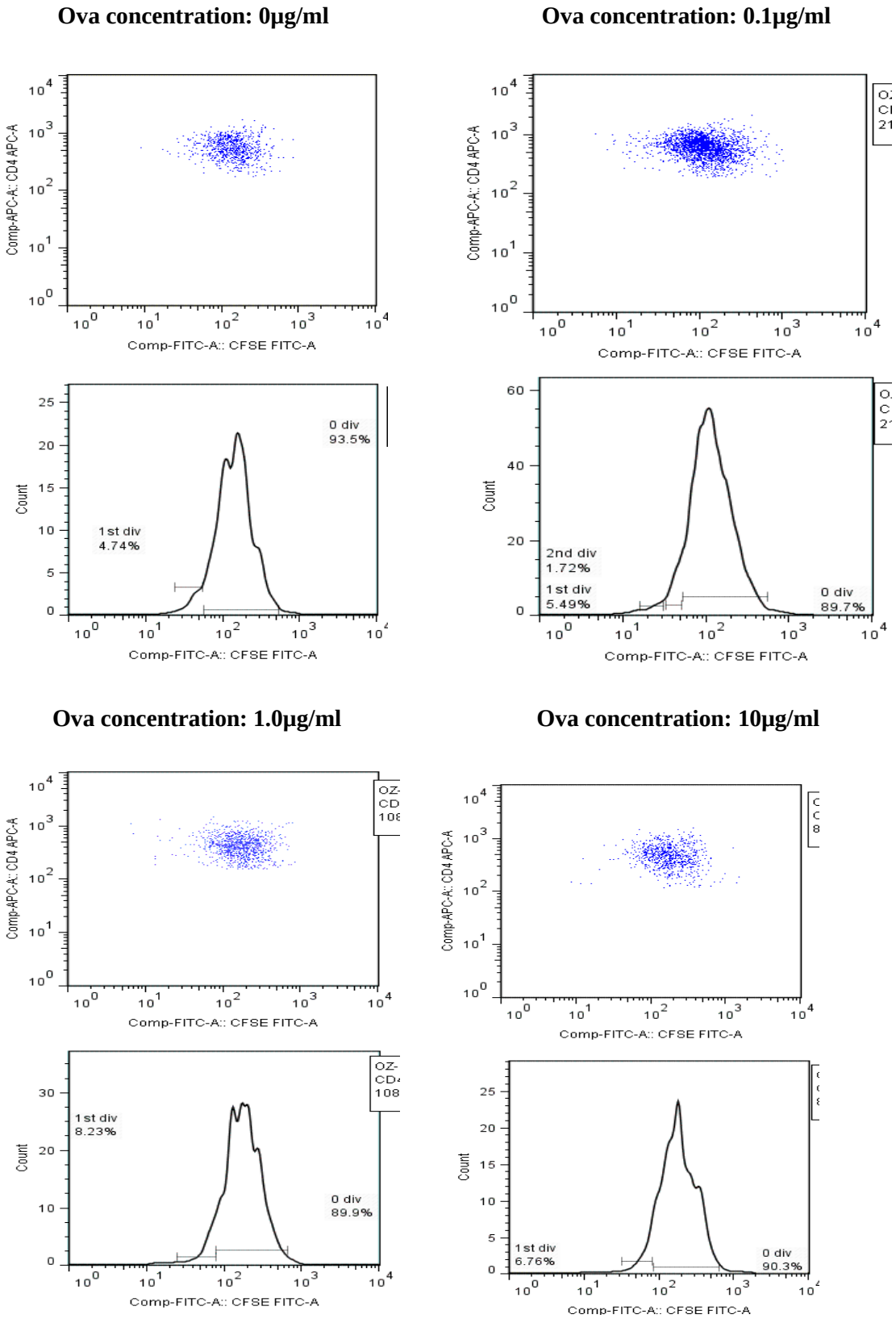
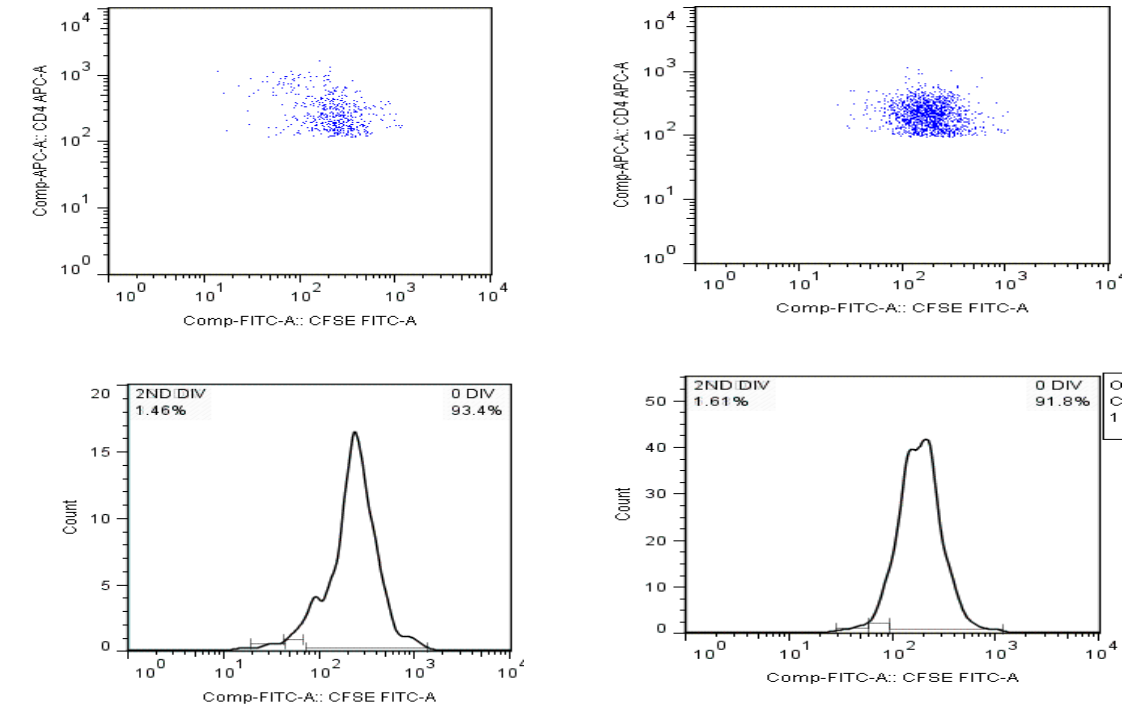



**Figure 16: Mice were immunized with Ova+ Zymosan, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0,10 µg/ml)**

**Ova concentration: 0µg/ml** **Ova concentration: 0.1µg/ml**




**Ova concentration: 1.0µg/ml** **Ova concentration: 10µg/ml**

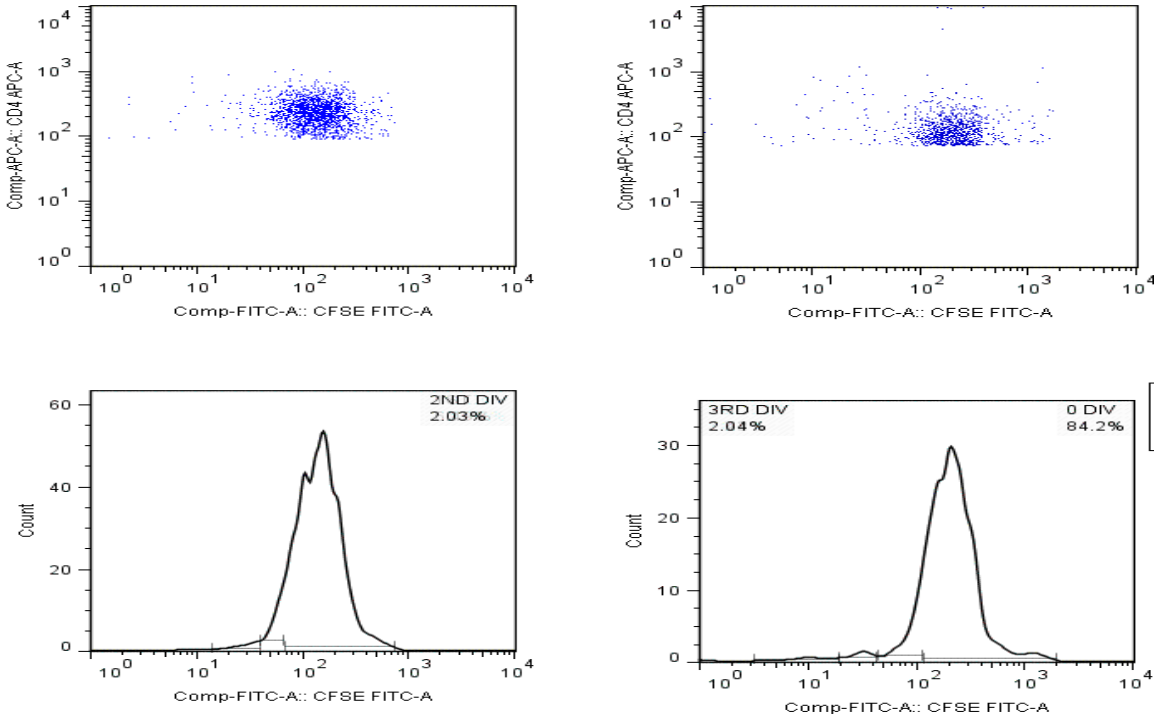
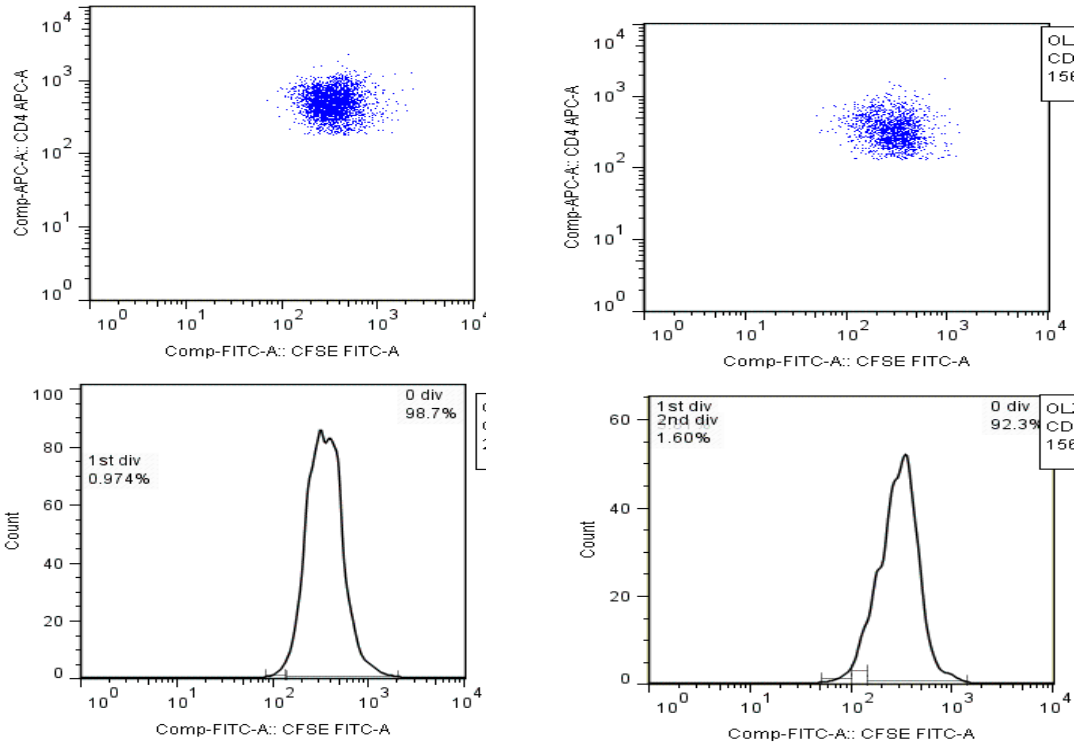



**Figure 17: Mice were immunized with Ova+Zymosan +PPD, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0, 10 µg/ml)**

**Ova concentration: 0µg/ml** **Ova concentration: 0.1µg/ml**




**Ova concentration: 1.0µg/ml** **Ova concentration: 10µg/ml**

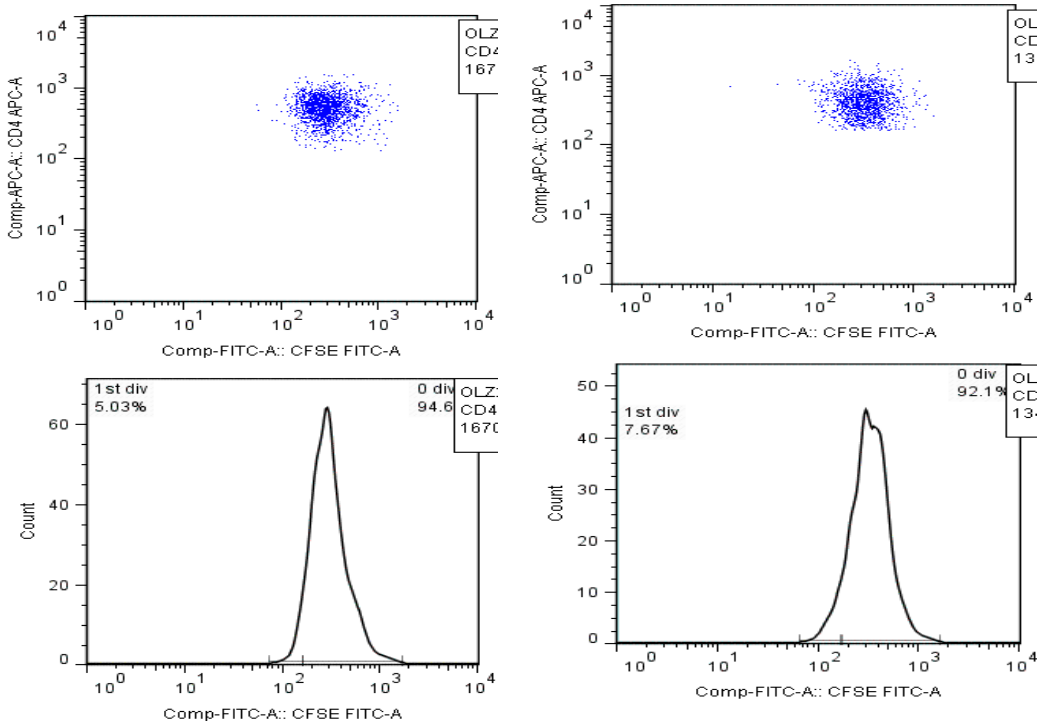
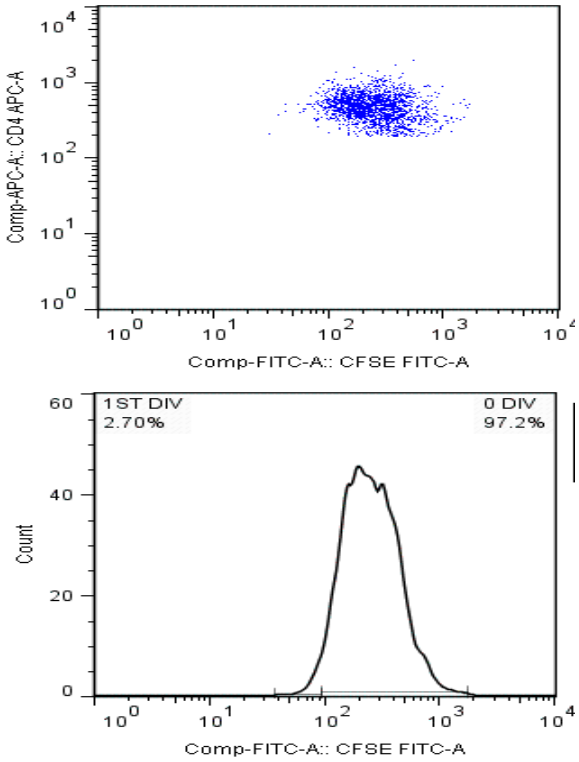
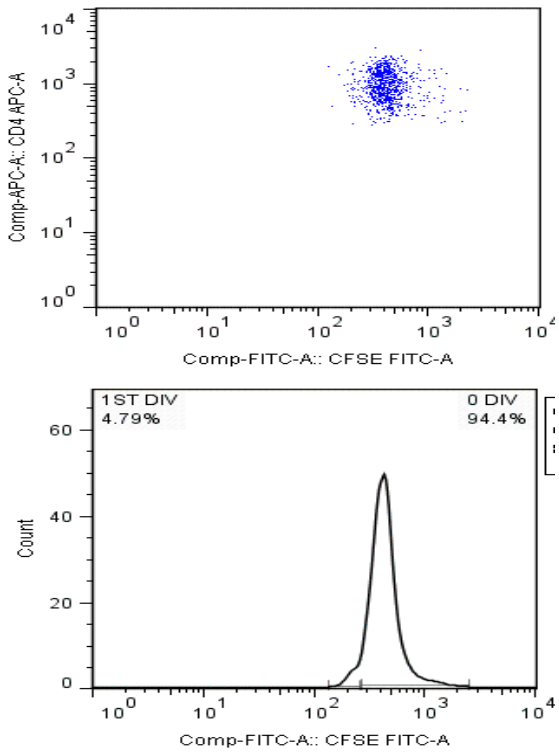



**Figure 18: Mice were immunized with Ova+LPS+Zymosan, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0, 10 µg/ml)**

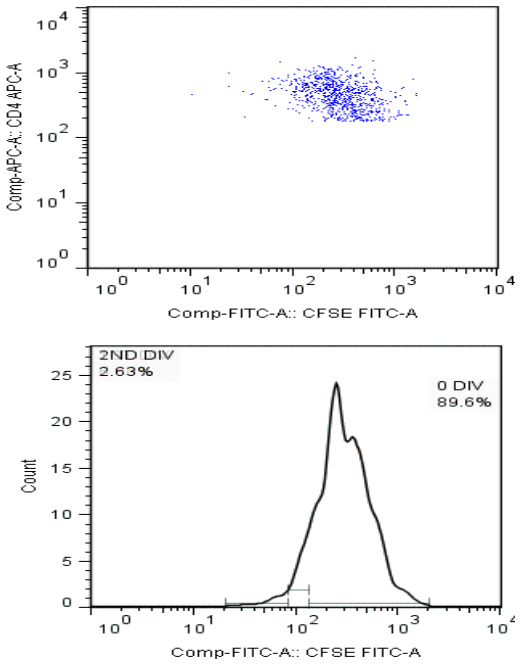
**Ova concentration: 0µg/ml**




**Ova concentration: 0.1µg/ml**




**Ova concentration: 1.0µg/ml**




**Ova concentration: 10µg/ml**

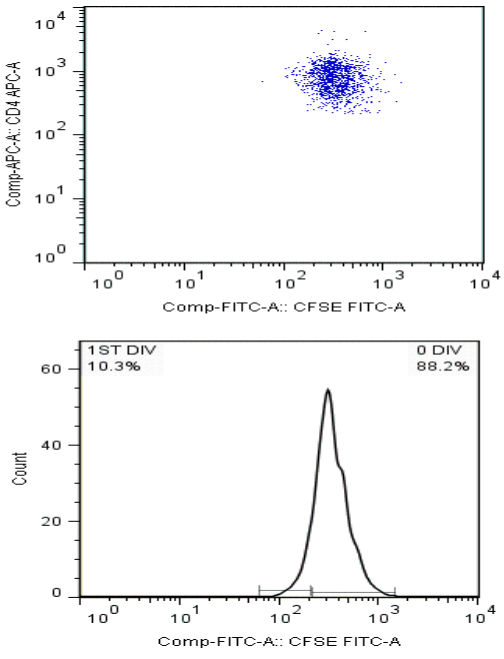



**Figure 19: Mice were immunized with Ova+ LPS+ Zymosan+ PPD, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0,10 µg/ml)**

PPD (purified protein derivatives) is a major constituent of CFA (Freund's complete antigen), CFA also contain TLR ligands like lipoarabinomannan (TLR2), heat shock protein (TLR4) etc. So we used zymosan (TLR2 ligand) and LPS (TLR4 ligand) for our study in different combination. Aim of our study was to show that how proteins of different origin can influence the immune response generated against another antigenic protein and whether PPD modulates TLR induce immune response or not. So we immunized mice with Ova as model antigen and measured CD4 T-cell proliferation by CFSE dilution assay.

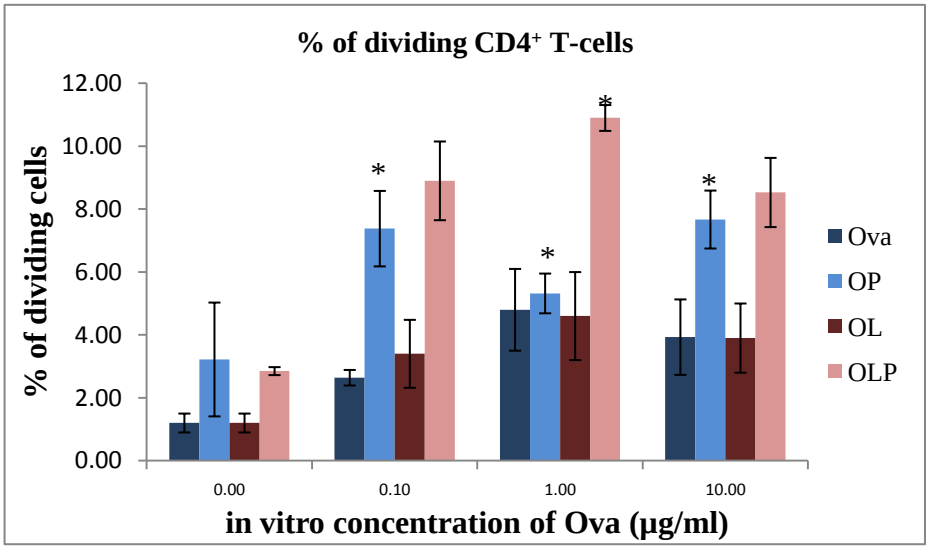
**Table: % of dividing$CD4^+$ T-cells present in each division with respect to total dividing cell (TDC) and Total cell (dividing + Non dividing cells) and number of division cells has undergone upon *In vitro* challenge with Ova protein**

**A=% of dividing cells relative to total dividing cells; B=% of dividing cells relative Total cells (dividing + Non-dividing cells)**

| | | ***In vitro* challenge with Ova (µg/ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Combinations** | **Number of division cells has undergone** | **0 µg/ml** | | **0.1 µg/ml** | | **1.0 µg/ml** | | **10 µg/ml** | |
| | | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** |
| **Ova** | 1 | **75.0** | **0.9** | **87.3** | **2.6** | **91.7** | **4.5** | **3.9** | **100.0** |
| | 2 | **25.0** | **0.3** | **12.7** | **0.4** | **8.3** | **0.4** | **0.0** | **0.0** |
| | 3 | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** |
| **Ova+PPD** | 1 | **100.0** | **0.7** | **100.0** | **2.5** | **83.1** | **3.7** | **46.0** | **3.5** |
| | 2 | **0.0** | **0.0** | **0.0** | **0.0** | **16.9** | **0.8** | **29.9** | **2.3** |
| | 3 | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **17.2** | **1.3** |
| **Ova +LPS** | 1 | **75.0** | **0.9** | **100.0** | **1.3** | **100.0** | **4.6** | **100.0** | **3.9** |
| | 2 | **25.0** | **0.3** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** |
| | 3 | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** |
| **Ova+LPS+PPD** | 1 | **100.0** | **0.9** | **100.0** | **1.8** | **100.0** | **2.0** | **100.0** | **8.3** |
| | 2 | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** |
| **Ova+Zymosan** | 1 | **100.0** | **2.5** | **86.4** | **3.0** | **100.0** | **4.4** | **100.0** | **3.6** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | **0.0** | **0.0** | **13.6** | **0.5** | **0.0** | **0.0** | **0.0** | **0.0** |
| **Ova+Zym+PPD** | 1 | **81.0** | **1.6** | **89.7** | **3.7** | **86.4** | **3.4** | **49.6** | **1.5** |
| | 2 | **19.0** | **0.4** | **10.3** | **0.4** | **13.6** | **0.5** | **28.1** | **0.9** |
| | 3 | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **22.3** | **0.7** |
| **Ova+LPS+Zym** | 1 | **100.0** | **0.5** | **87.9** | **3.0** | **100.0** | **2.6** | **100.0** | **4.0** |
| | 2 | **0.0** | **0.0** | **12.1** | **0.4** | **0.0** | **0.0** | **0.0** | **0.0** |
| **Ova+LPS+Zym +PPD** | 1 | **100.0** | **1.4** | **100.0** | **2.5** | **83.9** | **3.7** | **100.0** | **5.5** |
| | 2 | **0.0** | **0.0** | **0.0** | **0.0** | **16.1** | **0.7** | **0.0** | **0.0** |

**Figure 20: % of dividing Ova specific CD4$^+$ T-cell after *in vitro* challenge with Ova (µg/ml): Mice were immunized with below mentioned combination and T-cell response was measured by CFSE dilution assay using flow cytometer**

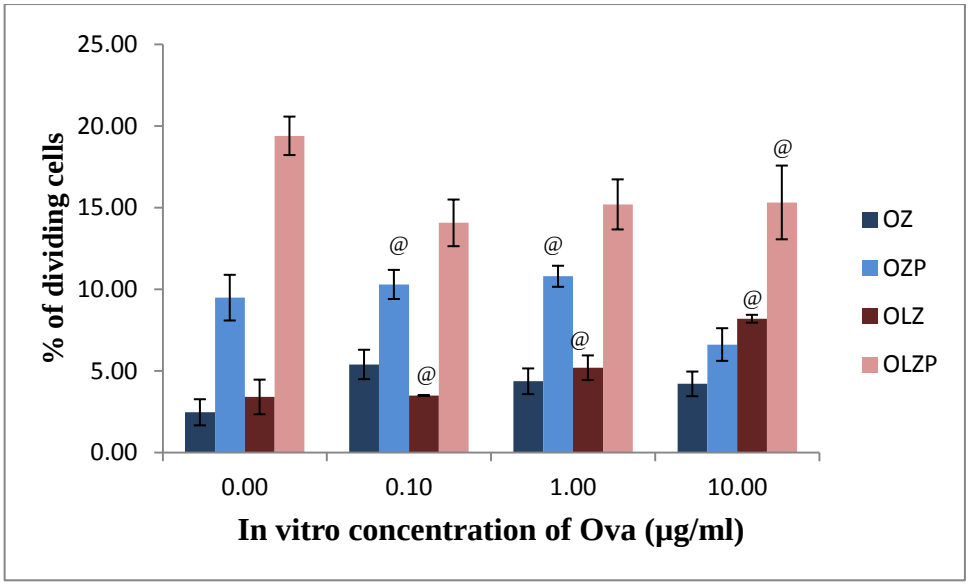



Values are mean± SD of 3 separate experiments (n=3mice per group)

*ANOVA* followed by Student's- Newman Keul's Test

*p<0.05 – As compared to Ova

$ p<0.05 – As compared to HEL




Values are mean± SD of 3 separate experiments (n=3mice per group)

*ANOVA* followed by Student's- Newman Keul's Test

@ $p<0.05$ – As compared to OZ

**Figure 21: % of dividing Ova specific $CD4^+$ T-cell after *in vitro* challenge with Ova (µg/ml) mice were immunized with below mentioned combination and T-cell response was measured by CFSE dilution assay using flow cytometer**

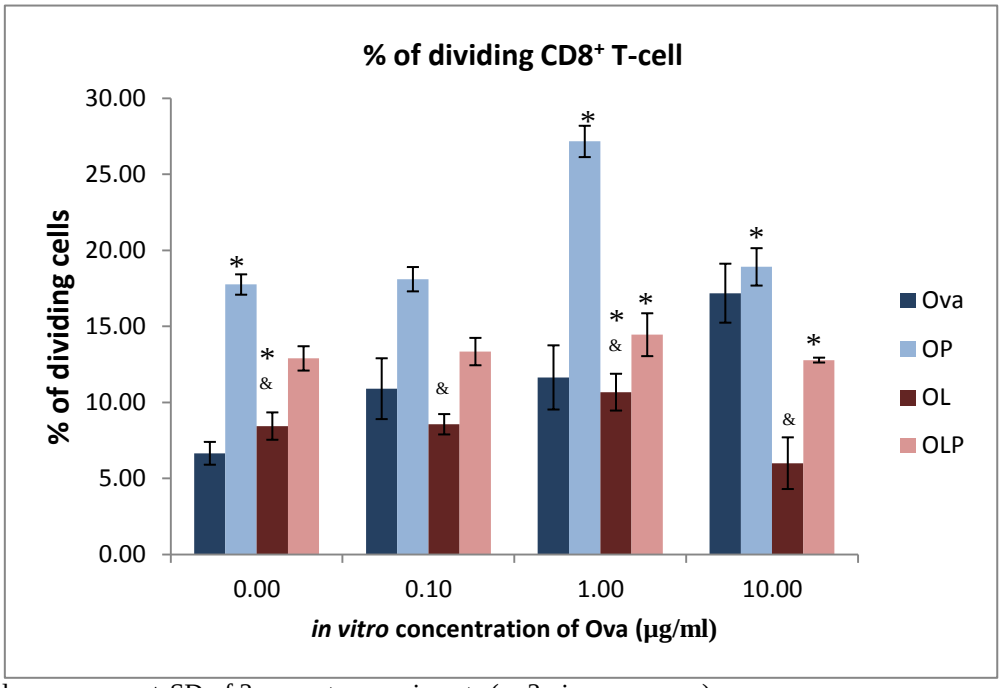
**Table: % of dividing$CD8^+$ T- cells present in each division with respect to total dividing cell (TDC) and Total cell (dividing+ non dividing cells) and number of division cells has undergone upon *In vitro* challenge with Ova protein**

| | | ***In vitro* challenge with Ova (µg/ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Combinations** | **Number of division cells has undergone** | **0 µg/ml** | | **0.1 µg/ml** | | **1.0 µg/ml** | | **10 µg/ml** | |
| | | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** |
| **Ova** | 1 | 100.00 | 6.65 | 94.49 | 10.32 | 86.29 | 10.05 | 85.86 | 17.11 |
| | 2 | 0.00 | 0.00 | 5.51 | 0.60 | 13.71 | 1.60 | 10.77 | 2.15 |
| **Ova+PPD** | 1 | 58.61 | 10.40 | 94.34 | 17.09 | 86.97 | 23.62 | 83.82 | 23.62 |
| | 2 | 35.71 | 6.34 | 4.88 | 0.88 | 11.15 | 3.03 | 11.95 | 3.03 |
| | 3 | 4.78 | 0.85 | 0.78 | 0.14 | 1.88 | 0.51 | 4.23 | 0.51 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 4 | 0.91 | 0.16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Ova +LPS** | 1 | 100.00 | 12.87 | 100.00 | 18.39 | 100.00 | 15.24 | 75.70 | 10.53 |
| | 2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 24.30 | 3.38 |
| **Ova+LPS+PPD** | 1 | 100.00 | 3.14 | 70.41 | 2.73 | 90.45 | 4.25 | 90.05 | 3.21 |
| | 2 | 0.00 | 0.00 | 29.59 | 1.15 | 9.55 | 0.45 | 9.95 | 0.35 |
| **Ova +Zymosan** | 1 | 94.24 | 13.78 | 60.69 | 8.86 | 86.30 | 17.65 | 76.11 | 9.62 |
| | 2 | 5.76 | 0.84 | 30.34 | 4.43 | 12.09 | 2.47 | 22.67 | 2.86 |
| | 3 | 0.00 | 0.00 | 8.97 | 1.31 | 1.62 | 0.33 | 1.21 | 0.15 |
| **Ova+Zym+PPD** | 1 | 84.19 | 11.30 | 52.30 | 10.67 | 71.00 | 15.73 | 75.20 | 18.50 |
| | 2 | 15.81 | 2.12 | 42.31 | 8.63 | 27.67 | 6.13 | 24.80 | 6.10 |
| | 3 | 0.00 | 0.00 | 5.39 | 1.10 | 1.32 | 0.29 | 0.00 | 0.00 |
| **Ova+LPS+Zym osan** | 1 | 82.28 | 2.54 | 45.67 | 7.38 | 74.80 | 10.95 | 91.02 | 19.89 |
| | 2 | 17.72 | 0.55 | 46.72 | 7.55 | 17.57 | 2.57 | 7.68 | 1.68 |
| | 3 | 0.00 | 0.00 | 7.61 | 1.23 | 7.63 | 1.12 | 1.30 | 0.29 |
| **Ova+LPS+Zym +PPD** | 1 | 58.80 | 6.30 | 68.86 | 12.82 | 85.08 | 18.81 | 70.56 | 12.05 |
| | 2 | 33.63 | 3.60 | 26.80 | 4.99 | 12.60 | 2.79 | 26.94 | 4.60 |
| | 3 | 7.57 | 0.81 | 4.35 | 0.81 | 1.52 | 0.34 | 2.50 | 0.43 |
| | 4 | 0.00 | 0.00 | 0.00 | 0.00 | 0.80 | 0.18 | 0.00 | 0.00 |

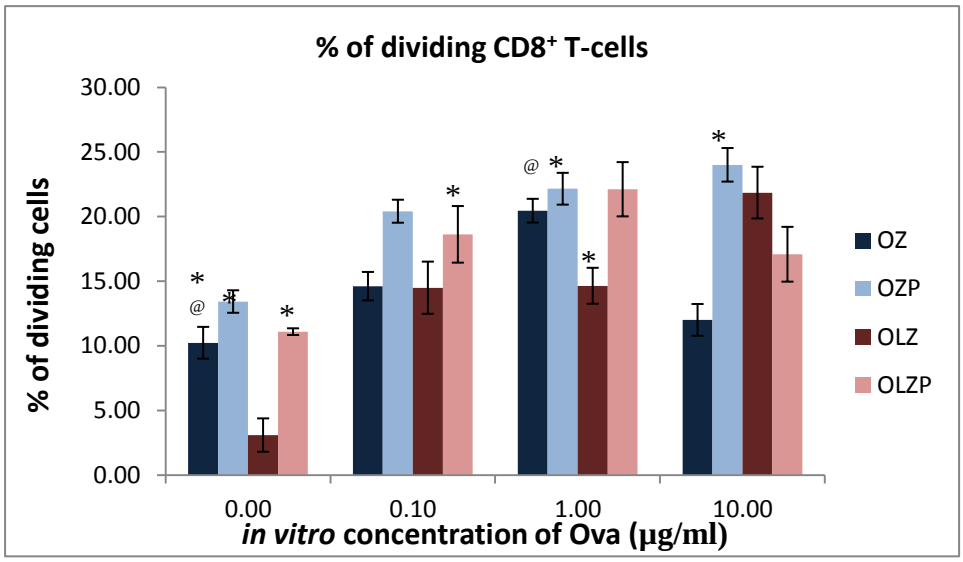
**A=% of dividing cells relative to total dividing cells; B==% of dividing cells relative to total cells (dividing+ Non-dividing cells)**

Ova=Ovalbumin; LPS= Lipopolysaccharide; Zym=Zymosan; PPD= purified protein derivatives




Values are mean± SD of 3 separate experiments (n=3mice per group)
*ANOVA* followed by Student's- Newman Keul's Test
*p<0.05 – As compared to Ova
& p<0.05 – As compared to OL

**Figure 22: % of dividing Ova specific CD8$^+$ T-cell after *in vitro* challenge with Ova (µg/ml) mice were immunized with below mentioned combination and T-cell response was measured by CFSE dilution assay using flow cytometer**




Values are mean± SD of 3 separate experiments (n=3mice per group)
*ANOVA* followed by Student's- Newman Keul's Test
p<0.05 – As compared to Ova
@ p<0.05 – As compared to OZ

**Figure 23: % of dividing Ova specific CD8$^+$ T-cell after *in vitro* challenge with Ova (µg/ml), Mice were immunized with below mentioned combination and T-cell response was measured by CFSE dilution assay using flow cytometer**

- **PPD is enhancing T-cell proliferation in the presence and absence of TLR ligands**

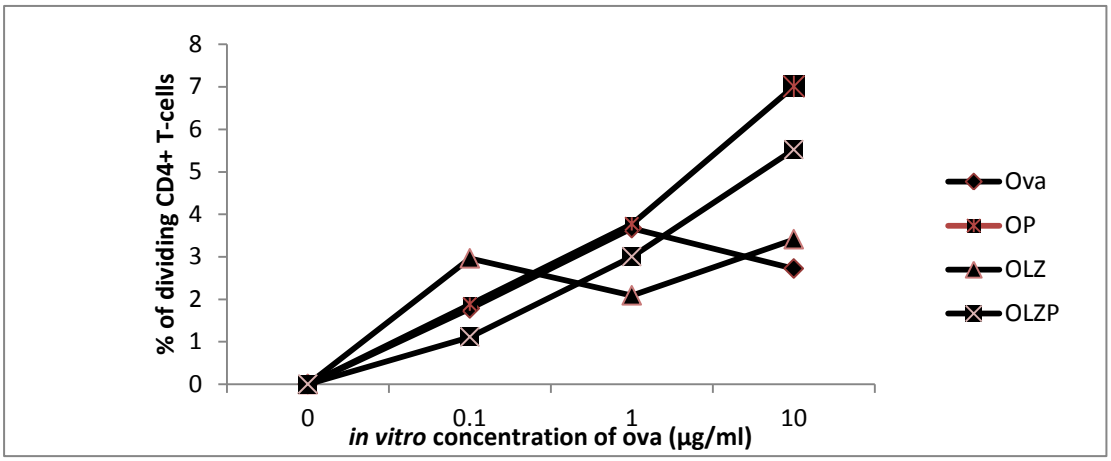



**Figure 24: % of dividing ova specific CD4$^+$ T-cells at different *in vitro* Ova challenge.**

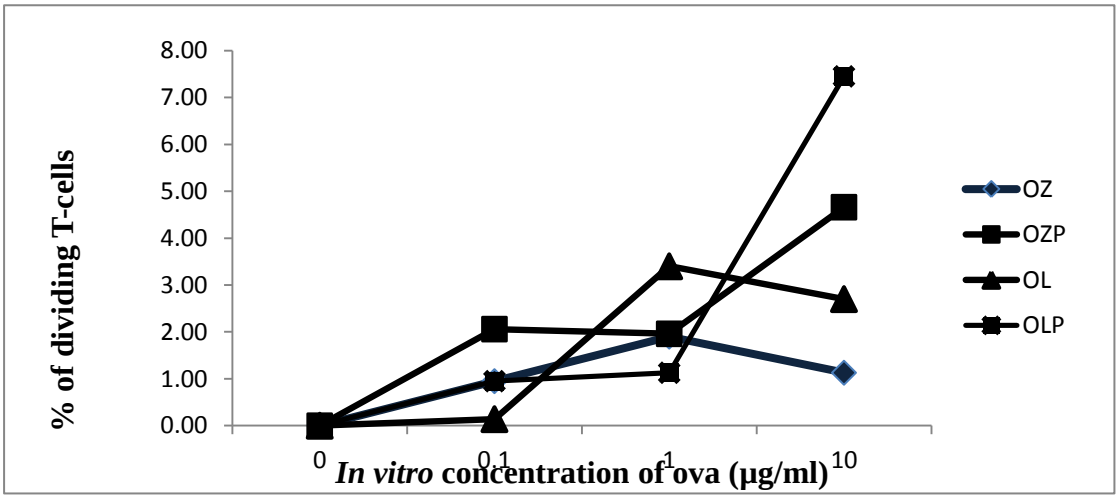



**Figure 25: % of dividing ova specific CD4$^+$ T-cells at different *in vitro* Ova challenge.**

From our study we found that PPD is positively modulating response generated against Ova. When Ova alone was injected cells has undergone only two division cycle and the % of responding cells in second cycle was only 25%, but when injected with PPD, presence of PPD has pushed cells 17% of responding cells to third division.

Similar is the case when Ova and LPS were injected with or without PPD. In the absence of PPD cells has undergone only two division cycle and only 13% of the responding cells were in second division, while in the presence of PPD has pushed the cells to 3 division cycle and the % of responding cells in third division was 23.3%

So our results suggest that PPD is enhancing TLR induced immune response and our study also shows that not only TLR ligands of CFA is responsible for adjuvant activity of CFA but PPD (purified protein derivatives) of *Mycobacterium tuberculosis* contributes majorly to make it as a strong adjuvant.

We further suggest to study molecular details that play a crucial role in modulating TLR induced response via PPD. It is known that all TLR ligands except TLR3 mediates their action via TRAF6 and NF-kB protein in MyD88 dependent and MyD88 independent pathway. So we assume that PPD modulates TLR induce response via this pathway and for that we suggest to measure TRAF6 protein level by immunoprecipitation.

- **PPD upregulates interleukin-15 (IL-15) expression, in the presence or absence of TLR ligand**

| **S. no.** | **Combinations** | **Concentration of IL-15 (pg/ml)** | |
|---|---|---|---|
| | | **After 24h** | **After 72h** |
| 1 | Ova | 60.98 | - |
| 2 | Ova+PPD | 90.79 | - |
| 3 | Ova+LPS+Zymosan | 115.18 | 102.53 |
| 4 | Ova+LPS+Zym+PPD | 256.94 | 102.08 |

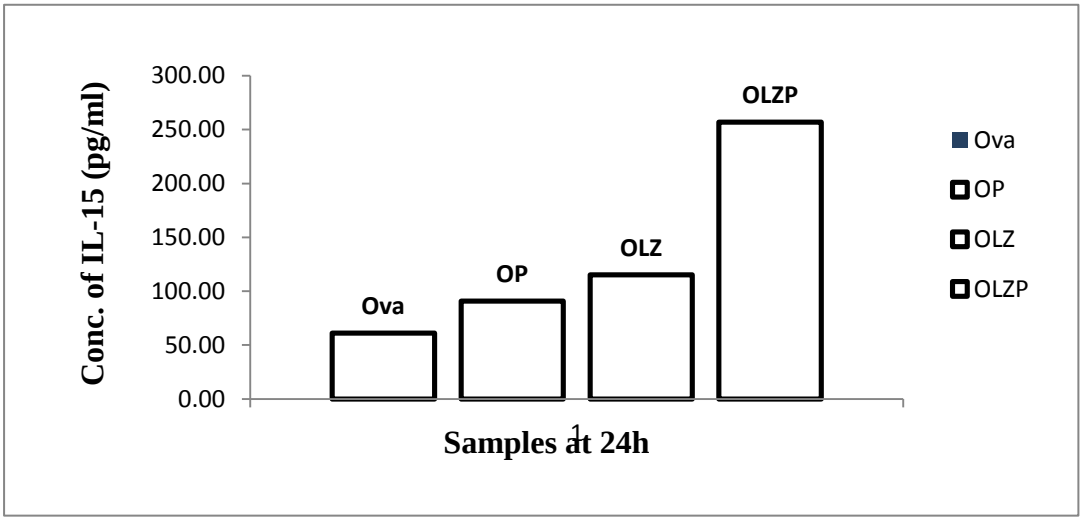


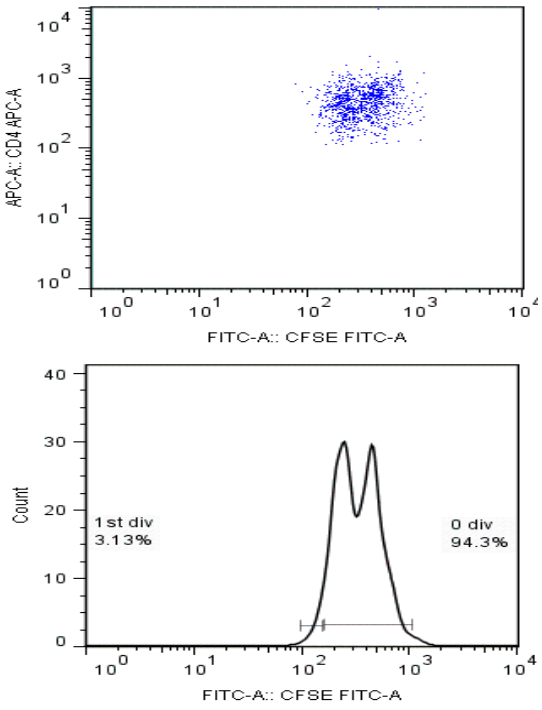

**Figure 26: Amount of IL-15 produced after 24 of immunization, blood was collected 24 hours post immunization and serum was separated and IL-15 level was measured by using ELISA kit (Ray Biotech)**

When only Ova was injected the amount of IL-15 produced was quite less as compared when Ova was injected with PPD. Similar is the results when Ova, LPS and Zymosan were injected with or without PPD, with PPD the amount of IL-15 produced was significantly higher than without PPD. This data suggest that PPD is upregulating IL-15 expression.

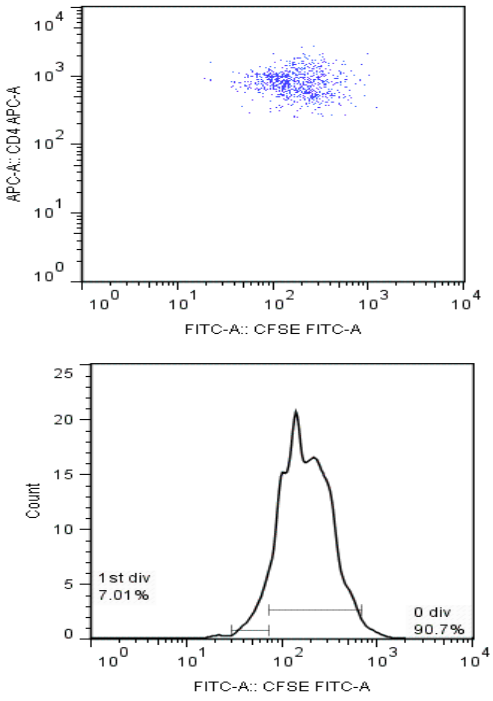
- **Hen egg lysozyme (HEL) injection modulates the $CD4^+$ and $CD8^+$ T-cell response generated against another antigen (Ova).**

Eleven days after the post immunization cells were harvested from draining lymph node and were labelled with CFSE dye and T-cells were challenged *in vitro* with increasing concentration of Ova protein (0, 0.1,1.0 ,10 µg/ml) . Following 72 hr stimulation, $CD4^+$ T-cells were labelled with anti-CD4 APC and analysed by Flow Cytometry

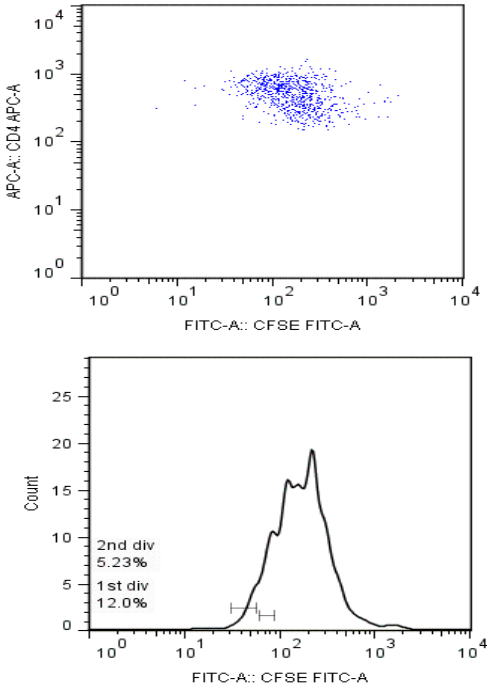
*In vitro* Ova concentration: 0µg/ml




*In vitro* Ova concentration: 0.1µg/ml




*In vitro* Ova concentration: 1.0µg/ml




*In vitro* Ova concentration: 10µg/ml

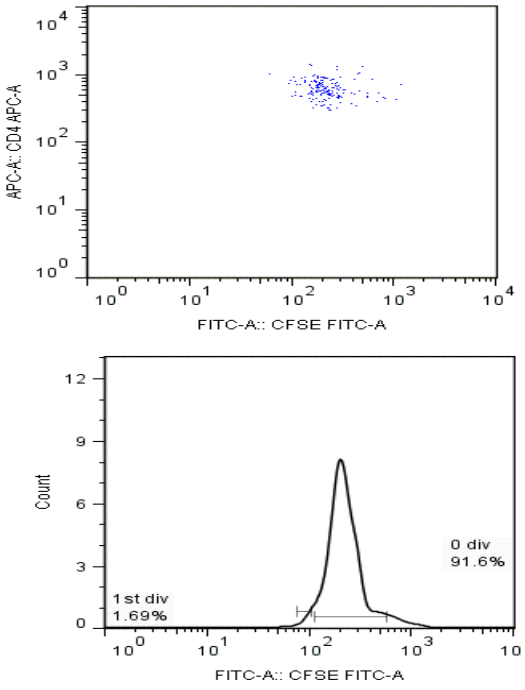
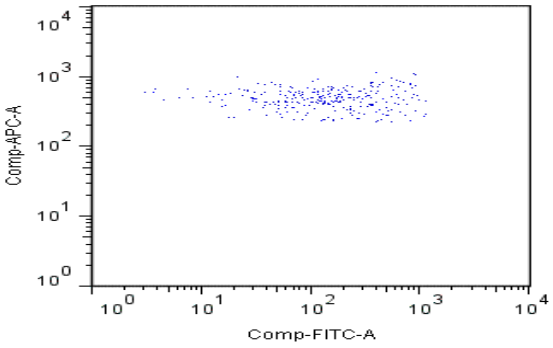
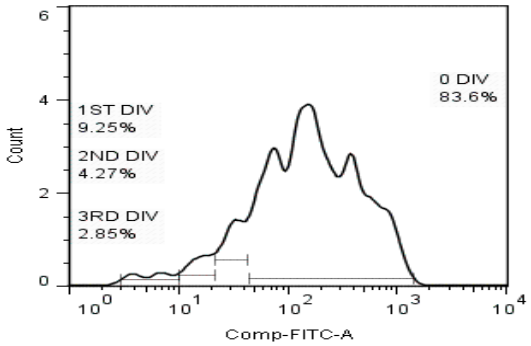


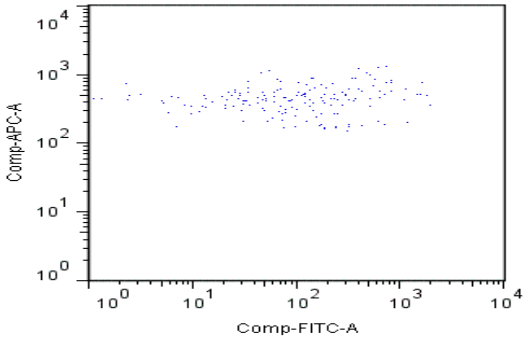

**Figure 27: Mice were immunized with Ova, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0, 10µg/ml)**

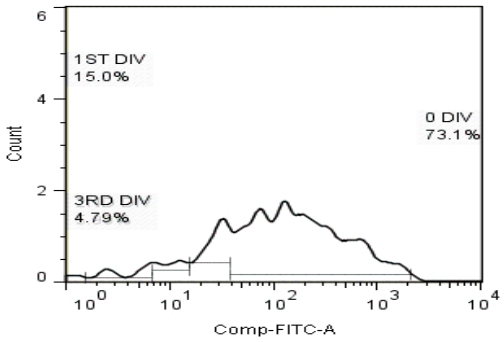
*In vitro* Ova concentration: 0µg/ml



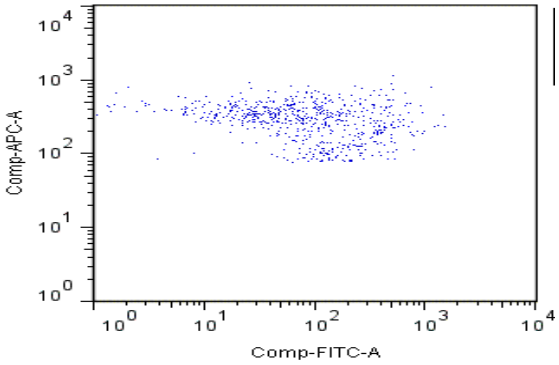



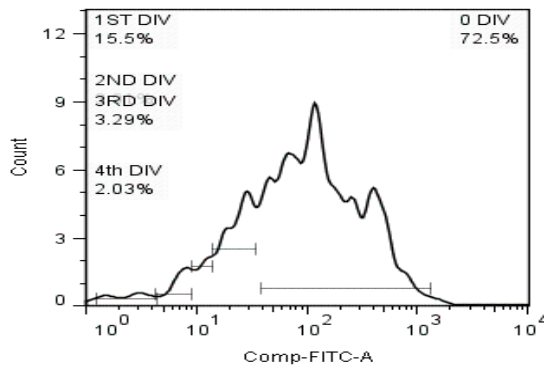

*In vitro* Ova concentration: 0.1µg/ml



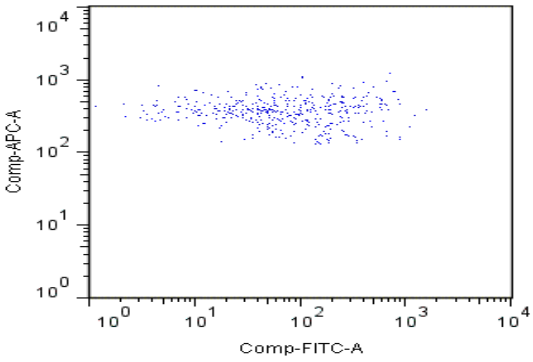



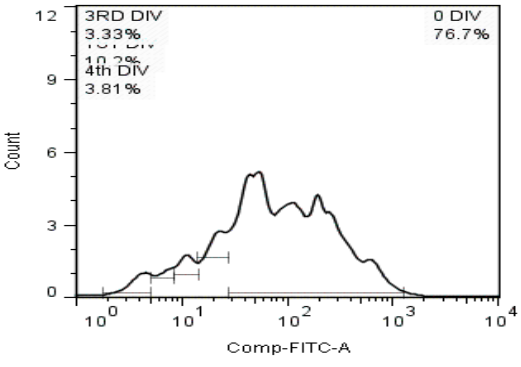

*In vitro* Ova concentration: 1.0µg/ml




*In vitro* Ova concentration: 10µg/ml




**Figurer 28: Mice were immunized with HEL, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0,10 µg/ml)**

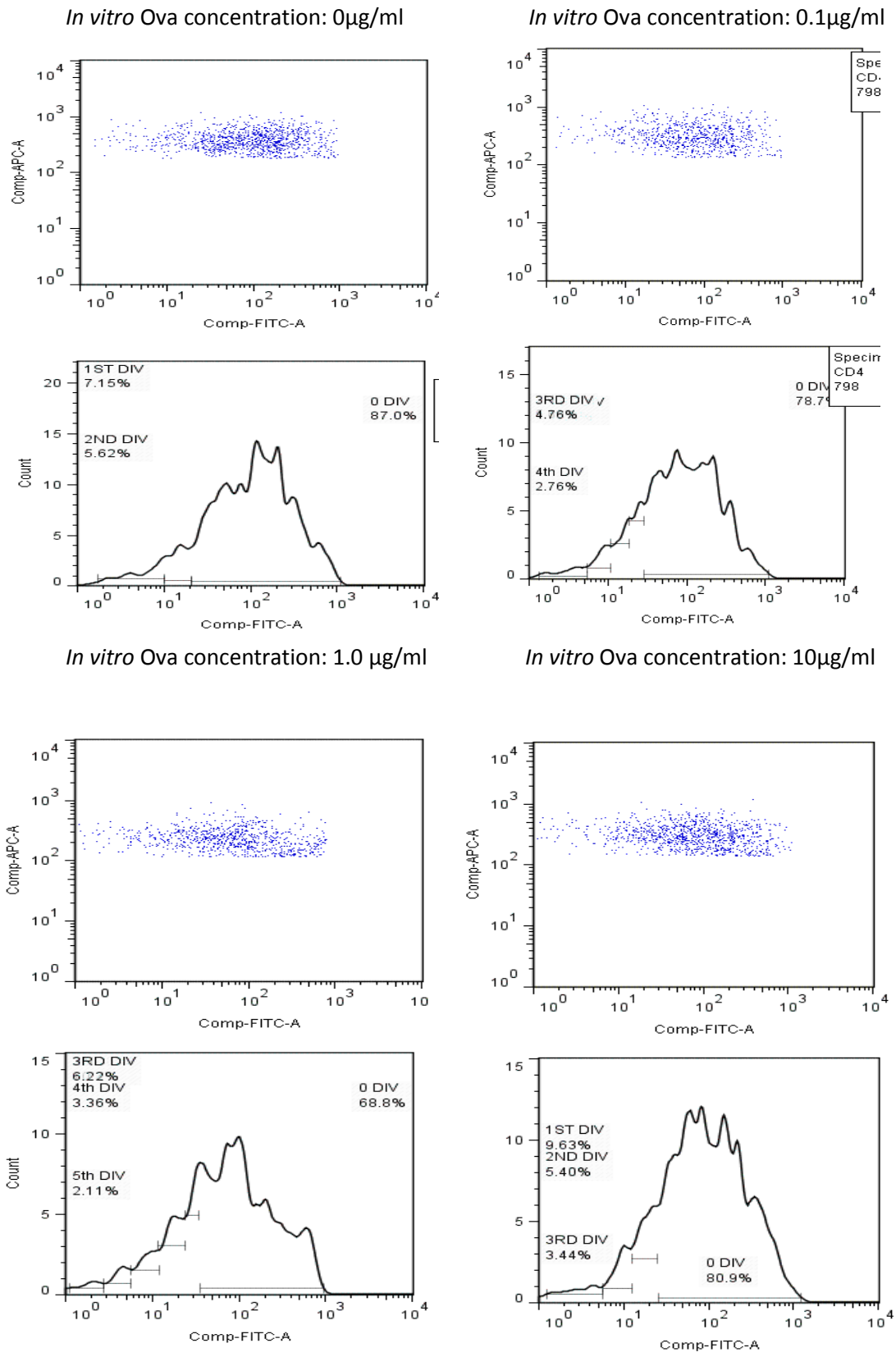



**Figure 29: Mice were immunized with Ova+ HEL, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0,10 µg/ml)**

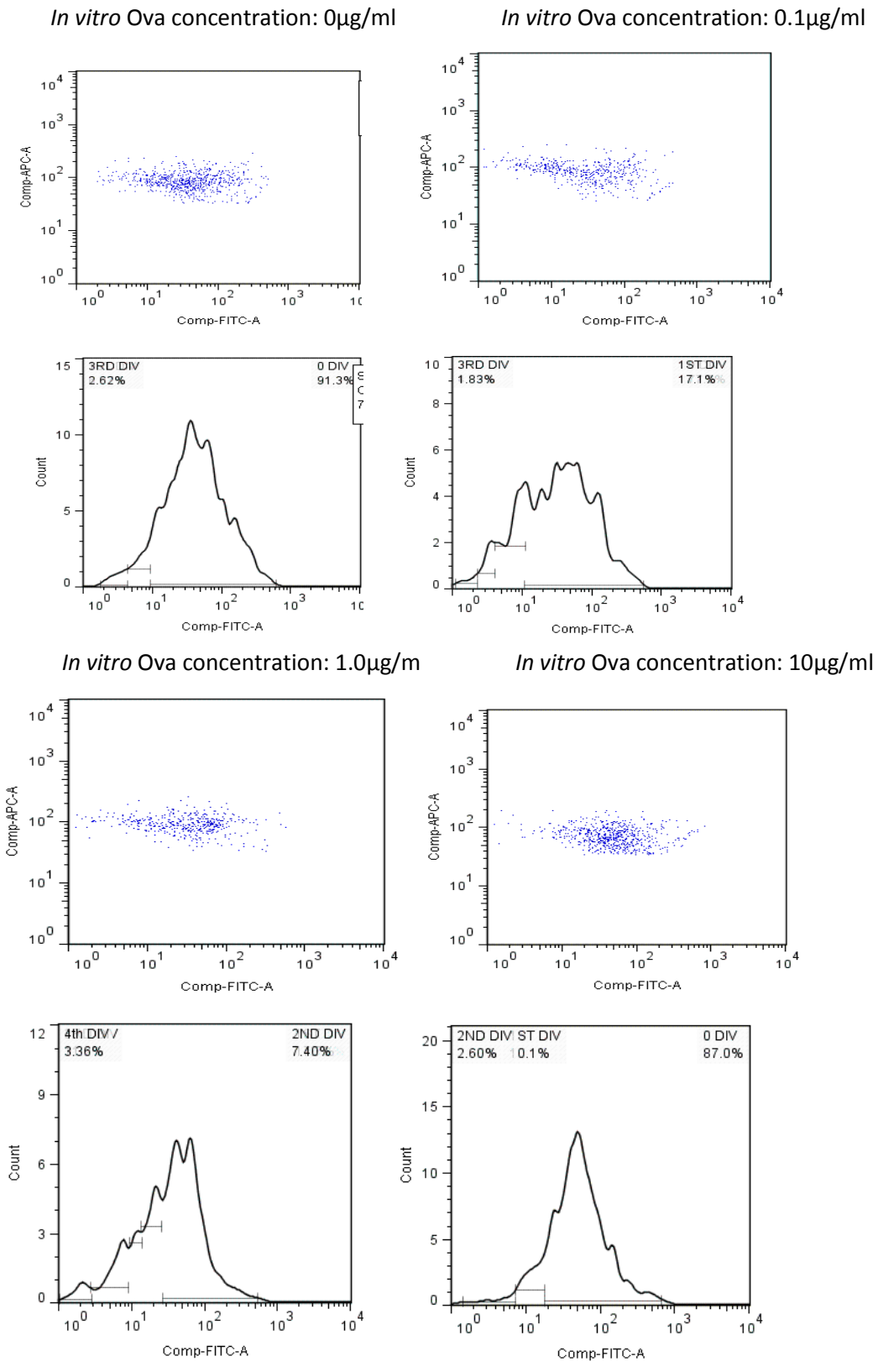



**Figure 30: Mice were immunized with Ova+LPS+Zymosan, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0, 10µg/ml)**

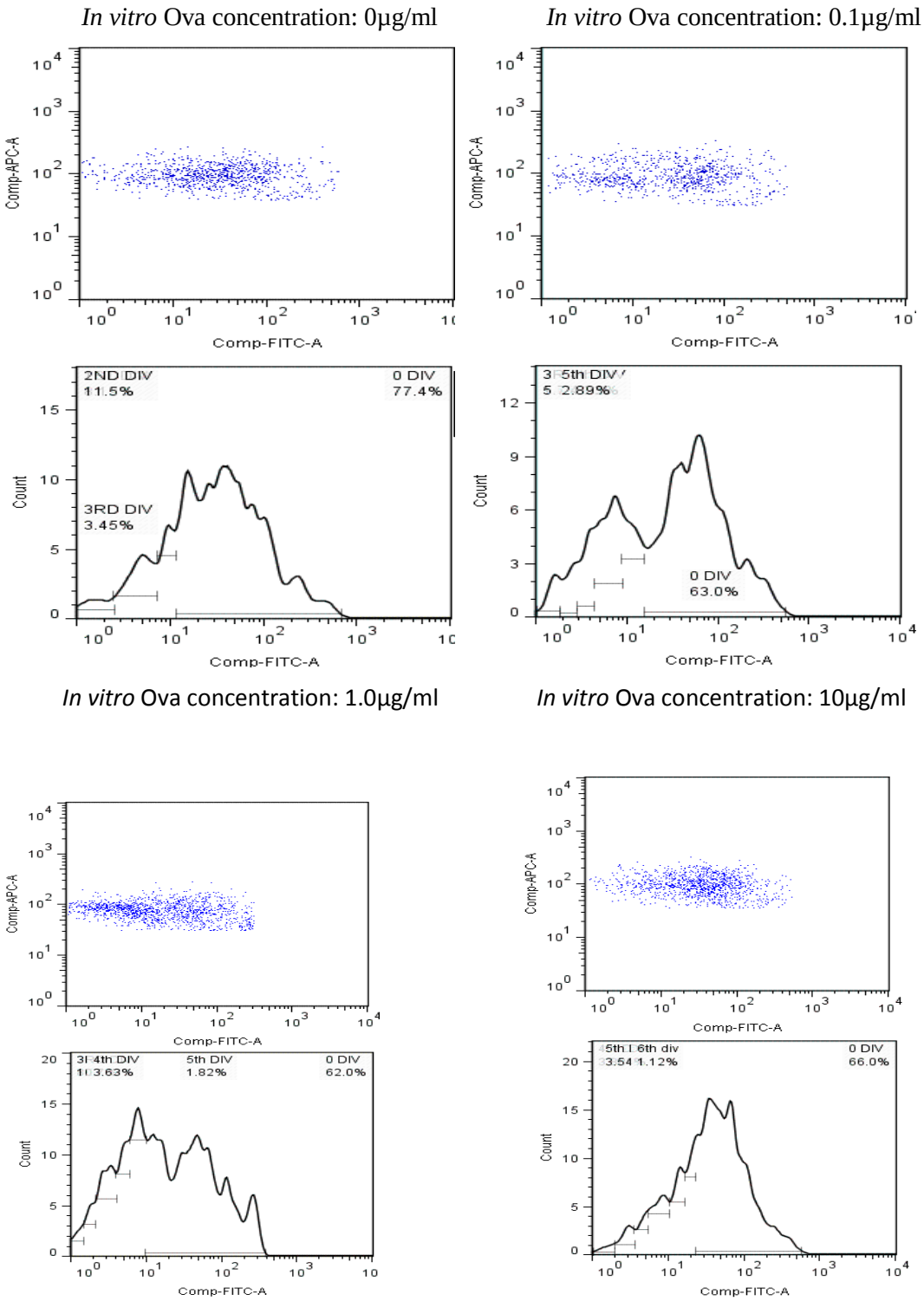



**Figure 31: Mice were immunized with Ova+HEL+LPS+ Zymosan, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0,10 µg/ml)**

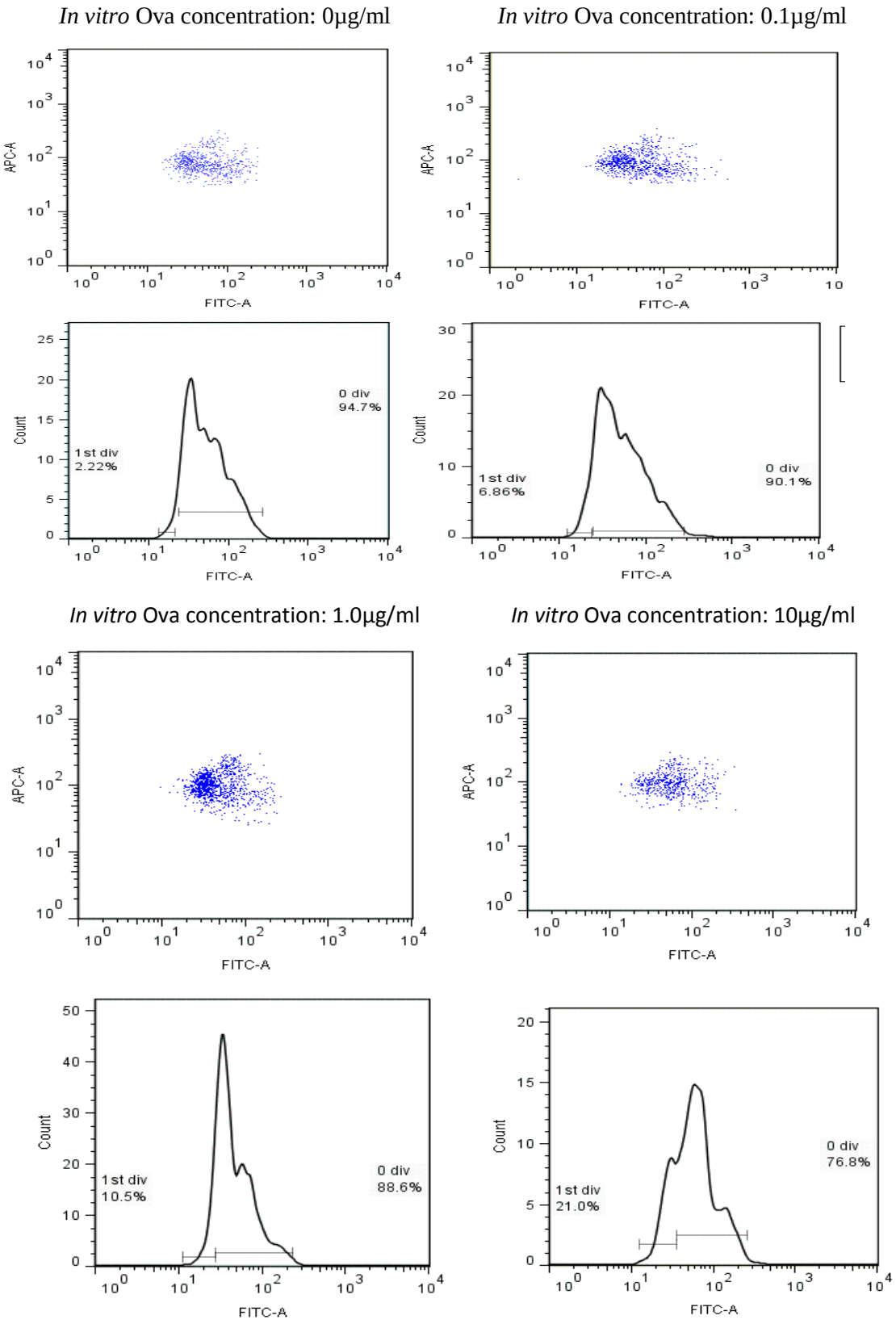



**Figure 32: Mice were immunized with HEL+LPS+Zymosan, T-cell isolated from draining lymph node and challenged i*n vitro* with Ova (0, 0.1, 1.0, 10µg/ml)**

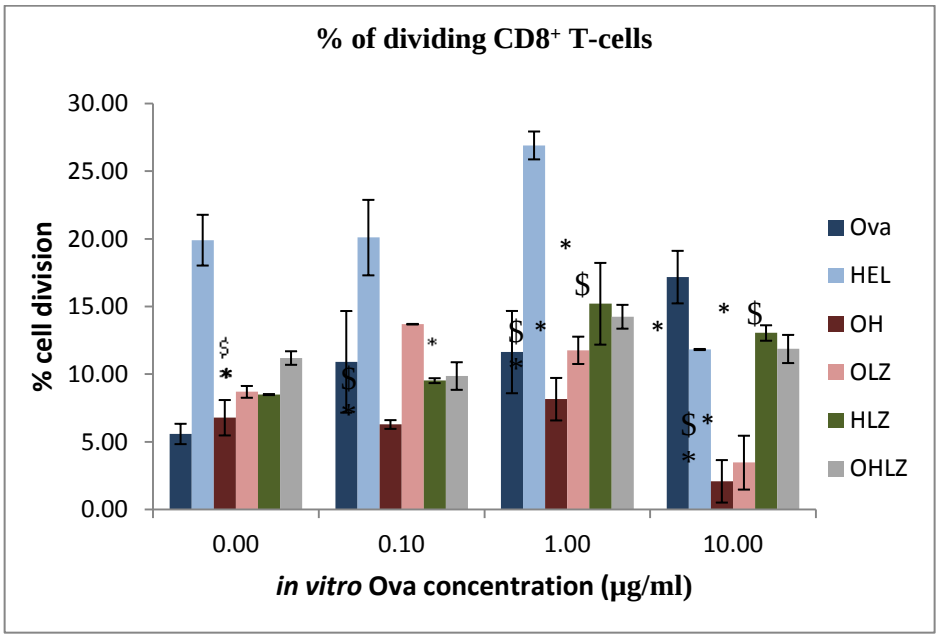
Multivalent (polyvalent vaccine) vaccine is designed to elicit an immune response either to more than one infectious agent or to several different antigenic determinants of a single antigen. So our next aim was to study whether two antigenic protein from same origin influence immune response generated by one antigenic protein. For that we used Ova (chicken Ovalbumin) and HEL (hen egg lysozyme) and immunize mice intraperitonealy with two TLR ligands (LPS and zymosan) and two antigenic proteins i.e. Ova and HEL and measured CD4 T-cell proliferation by CFSE dilution assay using flow cytometer.

**Table: % of dividing $CD8^+$ T-Cells present in each division with respect to total dividing cell (TDC) and Total cell (Dividing+ Non dividing cells) and number of division cells has undergone upon *In vitro* challenge with Ova protein.**

**A=% of dividing cells relative to total dividing cells; B==% of dividing cells relative to total cells (dividing+ Non-dividing cells)**

| Combination injected | | *In vitro* challenge with Ova (µg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Number of division | 0 µg/ml | | 0.1 µg/ml | | 1.0 µg/ml | | 10 µg/ml | |
| | | A | B | A | B | A | B | A | B |
| HEL | 1 | 77.42 | 1.82 | 95.75 | 15.47 | 75.68 | 2.67 | 78.37 | 5.82 |
| | 2 | 12.90 | 0.30 | 3.09 | 0.50 | 16.22 | 0.57 | 19.59 | 1.45 |
| | 3 | 9.68 | 0.23 | 0.77 | 0.12 | 5.41 | 0.19 | 1.63 | 0.12 |
| | 4 | 0.00 | 0.00 | 0.39 | 0.06 | 2.70 | 0.10 | 0.41 | 0.03 |
| | 5 | 0.00 | 0.00 | 0.00 | 0.00 | 0.23 | 0.01 | 0.00 | 0.00 |
| | 6 | 0.00 | 0.00 | 0.00 | 0.00 | 0.11 | 0.00 | 0.00 | 0.00 |
| Ova+HEL | 1 | 85.71 | 4.24 | 58.92 | 3.71 | 81.93 | 2.50 | 82.61 | 3.56 |
| | 2 | 10.71 | 0.53 | 27.62 | 1.74 | 16.87 | 0.52 | 17.39 | 0.75 |
| | 3 | 3.57 | 0.18 | 11.51 | 0.72 | 1.20 | 0.04 | 0.00 | 0.00 |
| | 4 | 0.00 | 0.00 | 1.96 | 0.12 | 0.00 | 0.00 | 0.00 | 0.00 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **HLZ** | 1 | **96.82** | **8.23** | **88.89** | **4.30** | **95.49** | **14.52** | **43.73** | **5.71** |
| | 2 | **3.18** | **0.27** | **11.11** | **0.54** | **4.14** | **0.63** | **56.27** | **7.34** |
| | 3 | **0.00** | **0.00** | **0.00** | **0.00** | **0.37** | **0.06** | **0.00** | **0.00** |
| **Ova+HEL+LPS+ Zymosan** | 1 | **75.60** | **8.47** | **74.63** | **7.36** | **75.80** | **5.20** | **78.20** | **9.28** |
| | 2 | **22.42** | **2.51** | **23.13** | **2.28** | **23.35** | **3.10** | **18.05** | **2.14** |
| | 3 | **1.54** | **0.17** | **2.24** | **0.22** | **0.85** | **0.20** | **2.79** | **0.33** |
| | 4 | **0.44** | **0.05** | **0.00** | **0.00** | **0.00** | **0.00** | **0.97** | **0.11** |
| **Ova+LPS+ Zymosan** | 1 | **98.98** | **9.22** | **100.00** | **13.73** | **98.67** | **11.62** | **100.00** | **3.47** |
| | 2 | **1.02** | **0.10** | **0.00** | **0.00** | **1.33** | **0.16** | **0.00** | **0.00** |




Values are mean± SD of 3 separate experiments (n=3mice per group)

*ANOVA* followed by Student's- Newman Keul's Test

*p<0.05 – As compared to Ova

$ p<0.05 – As compared to HEL

**Figure 33:% of dividing Ova specific CD8$^+$ T-cell after *in vitro* challenge with Ova (µg/ml), mice were immunized with below mentioned combination and T-cell response was measured by CFSE dilution assay using flow cytometer**

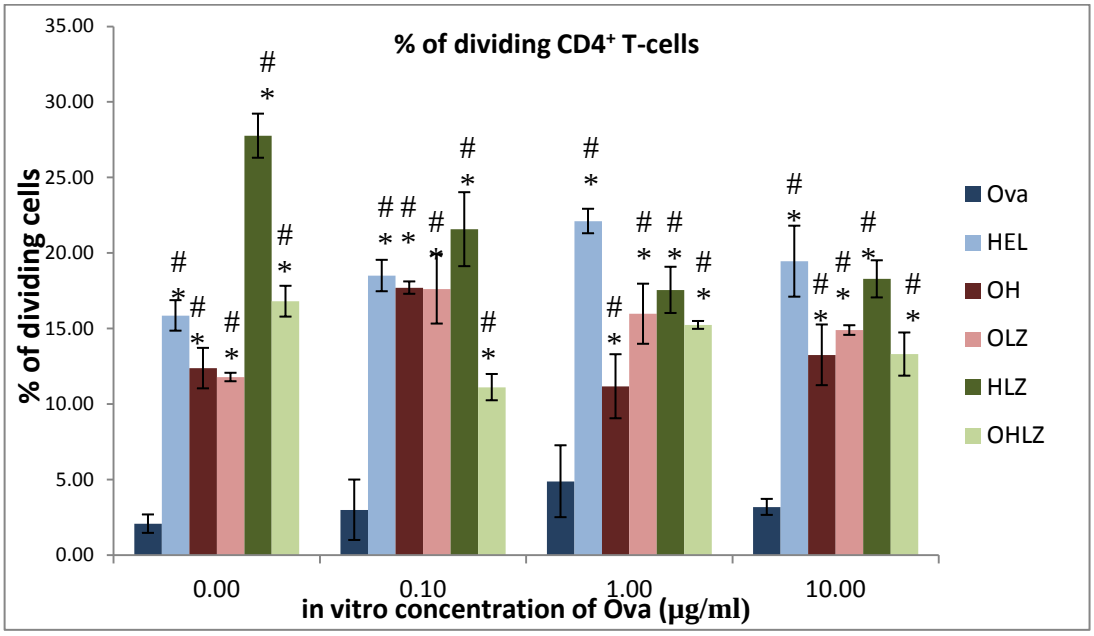
**Table: % of dividing $CD4^+$ T-Cells present in each division with respect to total dividing cell (TDC) and Total cell (dividing+ non dividing cells) and number of division cells has undergone upon *In vitro* challenge with Ova protein.**

**A=% of dividing cells relative to total dividing cells; B==% of dividing cells relative to total cells (dividing+ Non-dividing cells)**

| Combination | | *In vitro* challenge with Ova (µg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ova | Number of division | 0 µg/ml | | 0.1 µg/ml | | 1.0 µg/ml | | 10 µg/ml | |
| | | A | B | A | B | A | B | A | B |
| | 1 | 100 | 2.07 | 100 | 12.79 | 87.17 | 7.9 | 100 | 3.18 |
| | 2 | 0.00 | 0.00 | 0.00 | 0.00 | 12.82 | 4.23 | 0.00 | 0.00 |
| | 3 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| HEL | 1 | 79.75 | 13.79 | 56.44 | 14.31 | 52.66 | 11.64 | 29.22 | 7.6 |
| | 2 | 14.56 | 2.52 | 22.09 | 5.60 | 25.08 | 5.54 | 58.90 | 15.67 |
| | 3 | 4.75 | 0.82 | 17.18 | 4.35 | 16.93 | 3.74 | 9.36 | 2.4 |
| | 4 | 0.95 | 0.16 | 4.29 | 1.09 | 5.33 | 1.18 | 2.51 | 0.66 |
| | 5 | 0.00 | 0.00 | 0.46 | 0.12 | 2.12 | 0.47 | 0.86 | 0.22 |
| | 6 | 0.00 | 0.00 | 0.31 | 0.08 | 0.18 | 0.04 | 0.23 | 0.06 |
| Ova+HEL | 1 | 56.54 | 8.07 | 41.92 | 7.42 | 54.61 | 6.10 | 100.00 | 13.25 |
| | 2 | 43.46 | 6.20 | 42.24 | 7.48 | 40.59 | 4.53 | 0.00 | 0.00 |
| | 3 | 0.00 | 0.00 | 15.84 | 2.80 | 4.80 | 0.54 | 0.00 | 0.00 |
| | 4 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| HLZ | 1 | 61.73 | 18.38 | 100.00 | 21.59 | 100.00 | 13.89 | 100.00 | 20.03 |
| | 2 | 38.27 | 11.39 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 3 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Ova+HEL+LPS+ | 1 | 64.22 | 10.79 | 39.77 | 4.42 | 40.3 | 3.1 | 65.16 | 8.86 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Zymosan** | 2 | 19.85 | 3.34 | 39.02 | 4.34 | 42.5 | 3.6 | 18.36 | 2.50 |
| | 3 | 13.73 | 2.31 | 18.01 | 2.00 | 13.2 | 1.2 | 12.93 | 1.76 |
| | 4 | 2.21 | 0.37 | 3.19 | 0.35 | 3.6 | 0.3 | 3.56 | 0.48 |
| | 5 | 0.00 | 0.00 | 1.69 | 0.19 | 0.4 | 0.2 | 2.68 | 0.36 |
| | 6 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.86 | 0.12 |
| **Ova+LPS+ Zymosan** | 1 | 65.54 | 7.72 | 62.81 | 11.07 | 55.85 | 8.92 | 64.03 | 9.53 |
| | 2 | 20.95 | 2.47 | 24.76 | 4.36 | 23.04 | 3.68 | 29.48 | 4.39 |
| | 3 | 9.46 | 1.11 | 8.71 | 1.53 | 15.56 | 2.48 | 5.07 | 0.75 |
| | 4 | 4.05 | 0.48 | 3.72 | 0.66 | 5.55 | 0.89 | 1.43 | 0.21 |
| | 5 | 2.45 | 0.29 | 0.52 | 0.09 | 0.88 | 0.14 | 0.00 | 0.00 |
| | 6 | 0.44 | 0.05 | 0.36 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 |

**Figure 34: % of dividing Ova specific CD4$^+$ T-cell after *in vitro* challenge with Ova (µg/ml), mice were immunized with below mentioned combination and T-cell response was measured by CFSE dilution assay using flow cytometer**




Values are mean± SD of 3 separate experiments (n=3mice per group)

*ANOVA* followed by Student's- Newman Keul's Test

*p<0.05 – As compared to Ova

# p<0.05 – As compared to OLZ

- ***In vitro* Ova challenge is helping more number of cells to undergo subsequent cell division cycle**

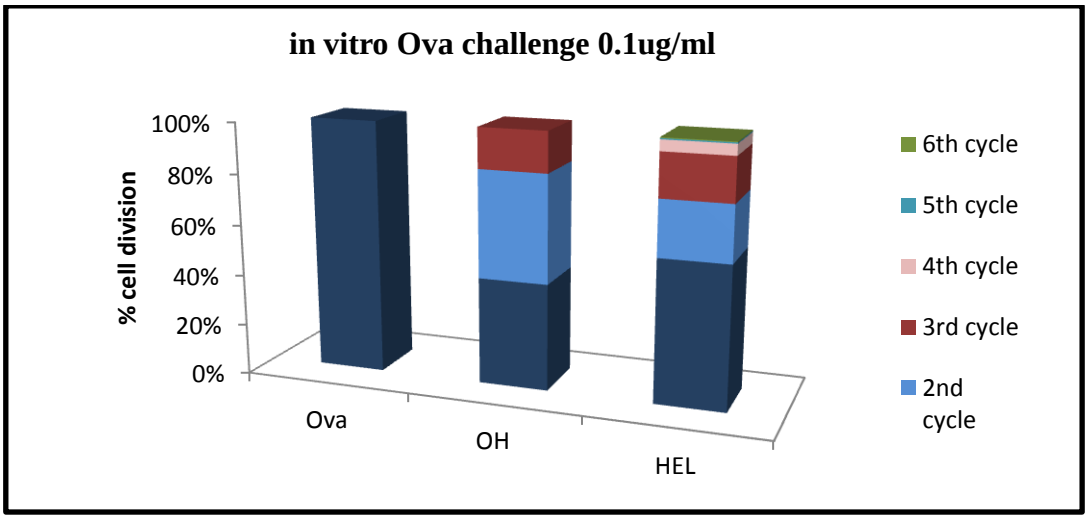



**Figure 35: % of division Ova specific $CD4^+$ T-cells has undergone at 0 µg/ml *in vitro* Ova concentration (each colour indicate one division cycle)**

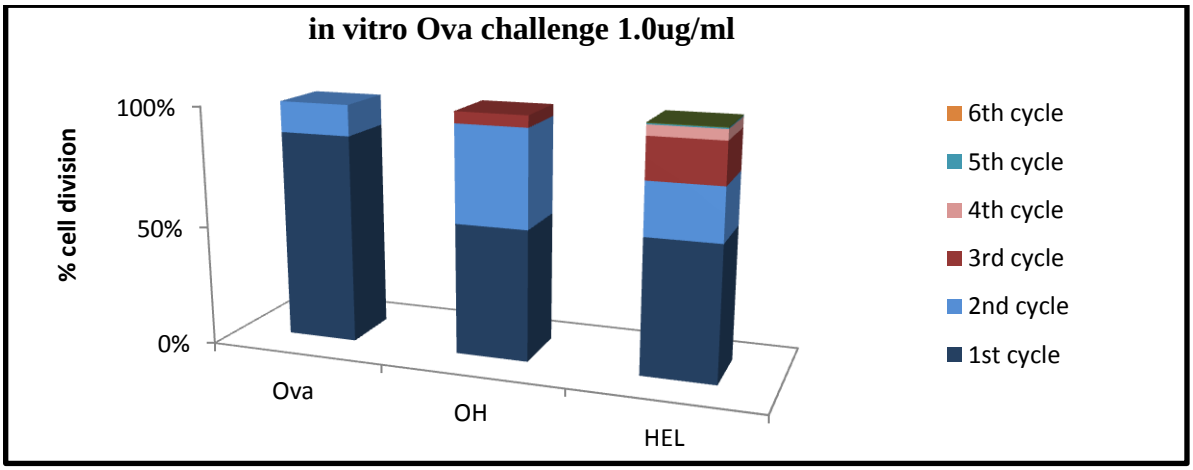



**Figure 36: % of division Ova specific $CD4^+$ T-cells has undergone at 1.0 µg/ml *in vitro* Ova concentration (each colour indicate one division cycle)**

- **Increasing *in vitro* Ova challenge in presence of TLR ligands, decreases T-cell proliferation**

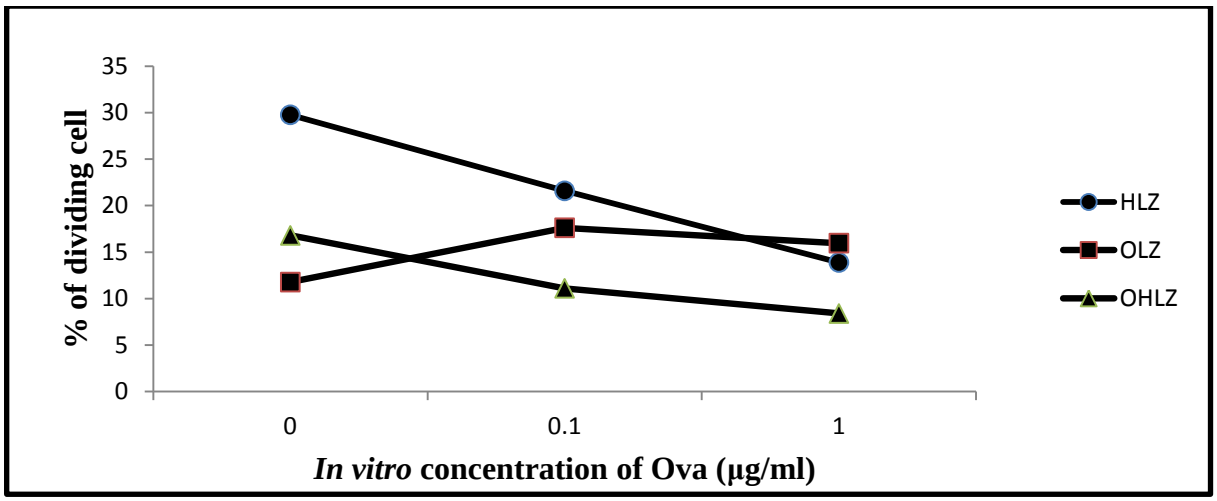



**Figure 37: % of dividing Ova specific CD4$^+$ T-cells with increase in *in vitro* Ova challenge**

Most of the Ova specific T-cells taken from mice immunized with Ova alone get stuck in first division even upon the *in vitro* challenge with Ova protein, but in the HEL alone as *in vitro* challenge by the Ova protein increases in the concentration from 0 to 10 µg/ml, it is helping cells to undergo the further division cycles upto 6 cycle, which suggest that *in vitro* challenge by Ova might be upregulating the MHC molecule which present more HEL specific epitope to specific T-cell, or it is increasing the contact time between MHC-epitope complex and TCR (T-cell receptor) and leads to higher proliferation or it may be due to cross-reactivity of Ova protein. One another possibility may be that after *in vitro* challenge by Ova, it is engulfed and processed by APCs (antigen presenting cells) and providing such specific cytokines rich niche i.e. signal 3 which in turn leads to T-cell proliferation. But when both Ova and HEL together are injected in vivo we observed that now cells are not pushed much for proliferation like in case of HEL alone but it is limited to 3 division cycles only, which might be due to dilution of cytokines signals i.e. signal 3 which is generated after *in vitro* challenge by Ova protein, but at the same time it is sufficient enough for more proliferation compared to the Ova alone.

**IL-15 ELISA:**

| S. no. | Combination injected | Concentration of IL-15 (pg/ml) At 72 h post immunization |
|---|---|---|
| 1 | HEL | 95.53 |
| 2 | Ova+HEL | 87.40 |
| 3 | HEL+LPS+Zym | 171.64 |
| 4 | Ova+HEL+LPS+Zym | 71.14 |

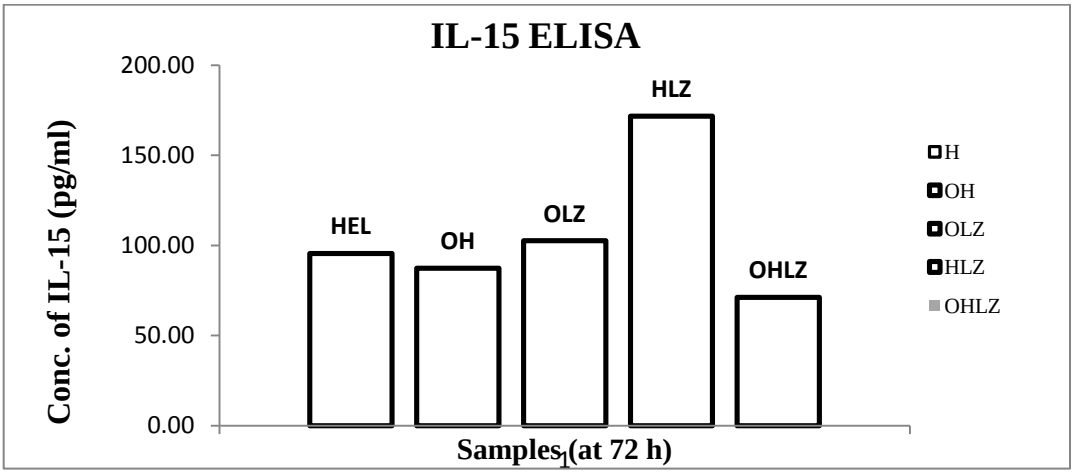



**Figure 38: Amount of IL-15 produced after 72 post immunization**

The difference in the amount of IL-15 produced in HEL, OH and OLZ was not quite high, but the difference is very significant between HLZ and OHLZ. When Ova is injected with HLZ the IL-15 level has reduced significantly, suggesting that there are some unknown interaction between HEL and Ova.

Further our aim was to co-relate the proliferation of T-cell to innate immunity. The APCs (Antigen presenting cells) process antigen and present epitopes to naïve T-cells. During this phase many immune as well as non-immune cells produce cytokines such as IL-2, IL-7, IL-15 etc, which play an important role in T-cell proliferation and survival at many stages of immune response. To investigate the expression of IL-15 we isolated mRNA from splenic dendritic cell stimulated with TLR ligands and performed RT-PCR.

### 3.2 RT-PCR

#### 3.2.1. Standardization of RNA isolation by Trizol method:

RNA was isolated from spleen sample by Trizol method. To check the integrity of isolated RNA electrophoresis was performed using 1.5% Agarose gel electrophoresis.

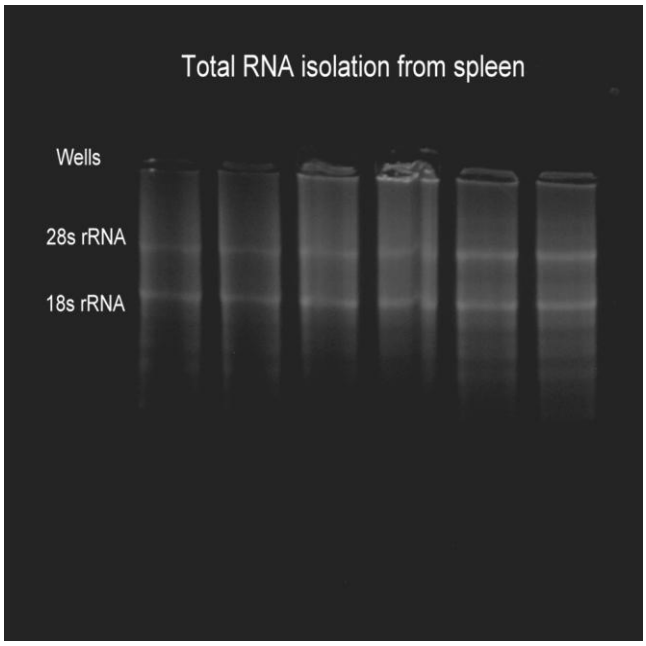



**Figure 39: Electrophoresis of isolated RNA sample**

Ideally 28S, 18S and 5S rRNA should be observed in the Agarose gel. And according to the standard observations, the intensity of 28S rRNA should be highest and that of 18S and 5S rRNA should in decreasing manner.

The expectation was matched in the above gel-electrophoresis, suggesting that RNA isolated by our protocol were of good quality. The presence of intact 28S and 18S rRNA band indicate that the mRNA in the total sample is intact and not degraded by RNase.

#### 3.2.2 Spectrophotometric analysis of RNA sample:

The concentration and purity of RNA sample was determined spectrophotometrically taking A260/A280nm ratio. Nucleotides give maximum absorbance a 260 nm($A_{260}$) it reflects the concentration of RNA in our sample, While protein gives maximum absorbance at 280nm ($A_{280}$nm). Ratio of absorbance at 260 and 280nm ($A_{260}/A_{280}$)

indicates the purity of RNA sample. $A_{260}/A_{280}$ ratio in the range of 1.8-2.0 indicates a highly purified RNA preparation.

Absorbance and concentration of above mentioned RNA sample is given below:

| **Sr. No.** | **Sample** | **Absorbance at 260nm $A_{260}$nm** | **Absorbance at 280nm $A_{280}$nm** | **$A_{260}/A_{280}$nm** | **Concentration (µg/ml)** |
|---|---|---|---|---|---|
| **1.** | **Sample 1** | 0.126 | 0.078 | 1.62 | 2.52 |
| **2.** | **Sample 2** | 0.259 | 0.125 | 2.07 | 5.18 |
| **3.** | **Sample 3** | 0.217 | 0.129 | 1.68 | 4.34 |
| **4.** | **Sample 4** | 0.298 | 0.142 | 2.09 | 5.96 |
| **5.** | **Sample 5** | 0.281 | 0.137 | 2.05 | 5.62 |
| **6.** | **Sample 6** | 0.286 | 0.172 | 1.66 | 5.72 |

Dilution factor- 500

Table: Absorbance at 260nm, 280nm and $A_{260}/A_{280.}$

## 3.3 Standardization of cDNA synthesis:

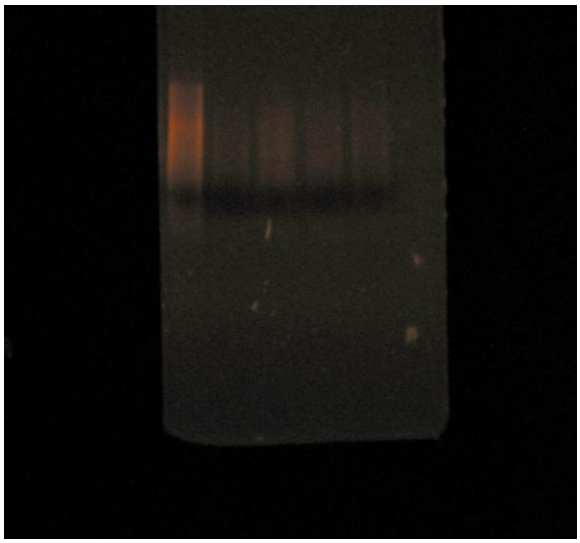


**Figure 40: electrophoresis of cDNA transcribed from isolated RNA**

cDNA synthesis was carried out for the RNA sample at 37°C for 1 hour where we could successfully Reverse Transcribe the RNA sample of 1.33 µg/ml into cDNA and was observed by gel electrophoresis using 1.2% Agarose gel.

### 3.4 Standardization of PCR amplification:

PCR was carried out using **control gene**, **β actin**. Primers for β-actin were designed whose product size was **349bp**.

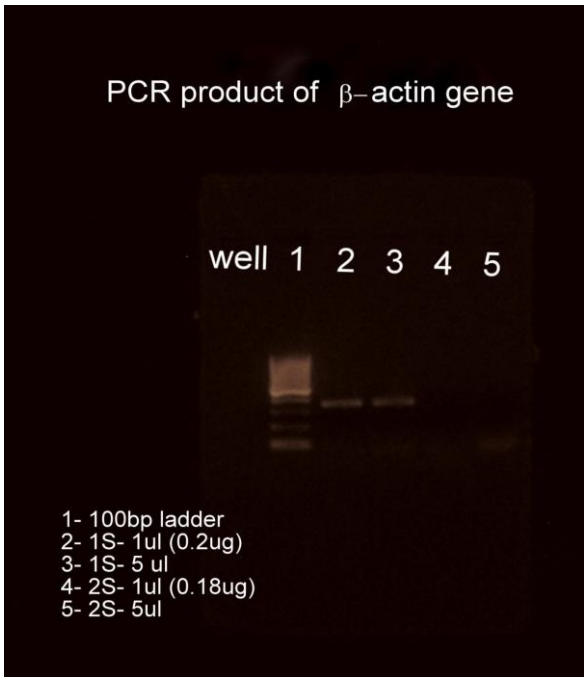



**Figure 41: Electrophoresis of PCR for β-actin at 56°C**

PCR for control gene (**β-actin**) was performed at **56°C** where we could successfully obtain amplified bands for RNA sample with concentration **0.20 ug** (lane 2 and 3) with template volume 1 ul and 5 ul. Electrophoresis was carried out using 1% agarose gel.

### 3.5 *In vitro* induction of IL-15:

**Dendritic cells** were isolated from spleen using protocol as given above. Dendritic cells were stimulated with **LPS 10 ng/ml** and **Poly I:C 50 ug/ml**, **LPS(5ng/ml)** + **Poly I:C (25ug/ml)**. After 4 hours RNA was isolated by the protocol as given above. The electrophoresis of the isolated RNA samples was carried out using 1.5% Agarose gel electrophoresis.

**a. RNA isolation from splenic dendritic cells stimulated with TLR ligands**

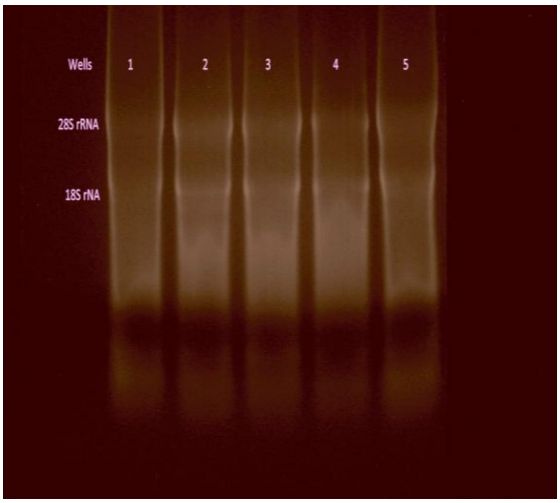



**Figure 42**: **RNA from splenic dendritic cells**

Ideally 28S rRNA and 18S rRNA band should be observed in the agarose gel. According to the standard observation, the intensity of 28S rRNA is higher than the 18S rRNA.The expectation is matched in the above agarose gel, where we could successfully isolate RNA from **control** (lane 1) and **LPS** (lane 2), **Poly I:C** (lane 3), **LPS+Poly I:C** (lane 4), **LPS II**( lane 5) induced sample.

**.5.1 Polymerase Chain Reaction of DC cells stimulated with TLR ligands (*in vitro*)**

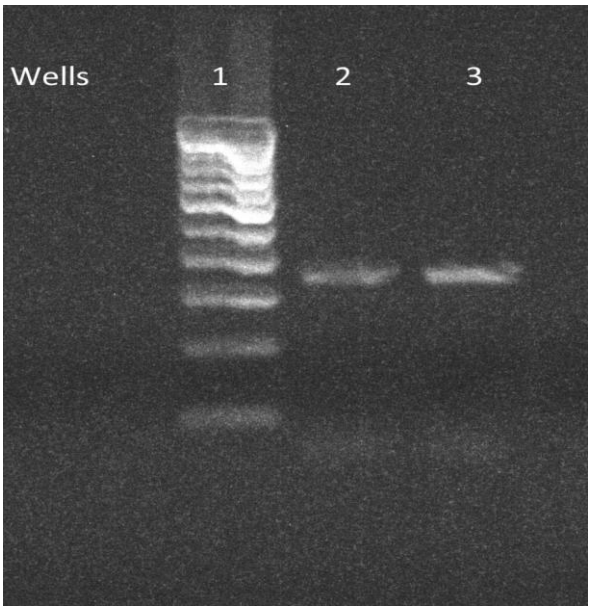



**Figure 43: PCR run for β-actin**

PCR was carried out for control gene **(β-actin)** at 56'C. Electrophoresis was carried out using 1% agarose gel where we could successfully obtain band in both, **control** (lane 2) and **LPS** (lane 3) induced sample run against the **ladder** of **100bp.** Our control gene has the product size 349 bp, which we could match against the ladder.

## SUMMARY

In the natural condition host might be encountered by more than one organism, so in this study we want to check the influence of one antigen on the immune response generated against the other antigen.PPD (purified protein derivatives) is a major mycobacterial protein antigen of CFA. Not much study is done about the role of PPD in the adjuvant activity of CFA. So here our objective is to study how PPD influencing the immune response generated against Ova. And to test this idea whether one antigen from pathogen can influence the immunogenicity of another antigen, we developed a model that mimic the infection and measured the T-cell response against the targeted antigen. We found that PPD enhances antigen (Ova) specific $CD4^+$ and $CD8^+$T-cell responses. And our study also shows that not only TLR ligands of CFA is responsible for adjuvant activity of CFA but PPD (purified protein derivatives) of *Mycobacterium tuberculosis* contributes majorly to make it as a strong adjuvant. We further suggest to work on the molecular details of the immuno-modulatory activity of PPD to induce strong cellular responses

Multivalent (polyvalent vaccine) vaccine is designed to elicit an immune response either to more than one infectious agent or to several different antigenic determinants of a single antigen. In natural condition when an organism is infecting any host, host immune system will be encountered with different antigens of that organism. So our next aim was to study whether two antigenic protein from same origin influence immune response generated by one antigenic protein. For that we used Ova (chicken Ovalbumin) and HEL (hen egg lysozyme) from the same source i.e. egg. We found that Hen egg lysozyme (HEL) injection modulates the $CD4^+$ and $CD8^+$ T-cell response generated against another antigen (Ova).We also found that HEL alone is helping more number of the cells for further division compared to Ova alone, which might be due the *in vitro* challenge by Ova protein provides cytokines rich niche for T-cell proliferation or due to the cross-reactivity of Ova protein. But when both Ova and HEL injected *in vivo* cell proliferation is less in comparison to HEL alone.

Further our aim was to co-relate the proliferation of T-cell to innate immunity. We have standardized the protocol for RNA isolation and got high quality RNA. We have standardized the conditions of reverse transcriptase (RT)-PCR for control gene, β-actin. To standardize the condition for IL-15, we performed cDNA synthesis with different RNA concentration and with various template concentrations. We also measured systemic IL-15 level using ELISA-kit, collecting blood 24h and 72h post immunization. And we found that PPD upregulates interleukin-15 (IL-15) expression, in the presence or absence of TLR ligand.

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## APPENDIX

- **DEPC- treated water:**

  Prepare 0.1% v/v DEPC added in to sterile Nuclease Free Water

  (Milipore). Keep for 1 hour at 37°C until the dissolution of DEPC in water.

- **RNA Precipitation Solution:**

  1.2 M Nacl

  0.8M Trisodium Citrate

  Dissolve in DEPC-treated water.

- **50 X TAE**:

  **Tris acetate-EDTA: (PH: 8.4)**

  Tris base: 24.20 g

  Glacial acetic acid: 5.7 ml

  EDTA: 3.72 g

  Prepared in sterile Nuclease Free Water (Milipore).

- **Gel loading dye:**

  Preparation for 10 ml dye

  50% glycerol -5 ml

  10mM EDTA(ph-8.0)- 37mg

  0.25%w/v BPB- 25 mg

- **Ethidium Bromide:**

  EtBr: 2mg/ml

  Prepare in DEPC-treated water, protect from light and store at 4'C

- **Phosphate Buffered Saline(PBS): (1X)**

  PH: 7.4

  Preparation for 300 ml

  NaCl- 2.4 g

  KCl- 60 mg

  $Na_2HPO_4$: 432 mg

  $KH_2PO_4$: 72 mg

- **RBC lysis buffer:**

  Preparation for 200ml

$KHCO_3$: 0.2 g

$NH_4Cl$: 1.658 g

EDTA: 7.4 mg

- **CFSE:**

  Stock: 5mM

  Stock was prepared by dissolving 2.78 mg CFSE in 1 ml Dimethyl Sulphoxide (DMSO).

- **PHA:**

  Stock: 1mg/ml

  Stock was prepared by dissolving 1 mg in 1 ml sterile Nuclease Free water (milipore)