Evaluating the Potential of Chimeric IL-15 on Re-activation of CD8+ T-cells in Tumor Model

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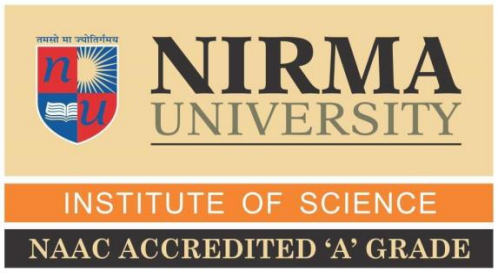

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ABBREVIATION

Ab: Antibody

Ag: Antigen

APCs: Antigen presenting cell

APS: Ammonium persulfate

BCR: B-cell Receptor

BPB: Bromo phenol Blue

BSA: Bovine Serum Albumin

CD: Clusters of differentiation

CTLs: Cytotoxic T Lymphocyte

DC: Dendritic cell

DF: Dilution factor

DMEM: Dulbecco's Modified Eagle Medium

Ecl: Enhanced chemiluminescence

ELISA: Enzyme-linked immune sorbent assay

ELU: Elution

EQU: Equilibrium

ETBr: Ethyl bromide

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

FCS: Fetal calf serum

FSC: Forward scatter

IFN-γ: Interferon gamma

IL15: Interleukin 15

LPS: Lipopolysaccharide

LYS: Lysis

MHC: Major Histocompatibility Complex

MNCs: Mononuclear cells

NCBI: National Centre for Biotechnology Information

OVA: Ovalbumin

PBS: Phosphate Buffer Saline

RBC: Red Blood Cells

RPMI: Roswell Park Memorial Institute

RT: Room temperature

SDS: Sodium dodecyl sulfate

SSC: Side scatter

TAE: Tris Acid EDTA

TBST: Tris buffered saline Tween 20

TIL: Tumor infiltrated lymphocytes

TLR: Toll-like Receptor

TMB: 3,3',5,5'- Tetra methyl benzidine

TME: Tumor microenvironment

Tnaïve: T cells generated from naïve mice

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1. ABSTRACT

Our immune system has an inherent function to locate and eliminate tumors; however tumors have evolved mechanisms to evade immune surveillance with the help of several immunomodulatory pathways rendering immune cells deactivated. One of the most promising approaches in tackling cancer is by augmenting immune responses with the help of cytokines which facilitate reactivation of immune cells and help regaining the ability to eliminate malignancies.

IL-15 is one such immunotherapeutic cytokine which is first in the list among 12 immunotherapeutic drugs released by the National Cancer institute, NIH, USA. Chimeric fusion proteins have been reported to exhibit anti-tumor activity in several tumor models. Native IL has limitations like short half-life, poor bioavailability and toxicity which was overcome by developing a fusion protein having specific mutation incorporated IL-15 fuses with Fc region of IgG/2a immunoglobulin making it an ideal molecule for cytokine therapy. An Indian patent was filed for this invention and has been published (Indian Patent **Application No. 201721010096A**). In this study we examined the activation status of T lymphocytes and anti-tumor activity of this Chimeric IL-15 in tumor bearing mice when administered via IP(intra-peritoneal) and IV(intra-venous routes).

Our results show presence of IL-15 functional domains in the fusion protein which is essentially required for the biological activity. Current study also successfully shows that Chimeric IL-15 increases CD8+ T cell infiltration and an increase in proportion of activated CD8+ T cells in tumor microenvironment making it a suitable immunotherapeutic candidate for further studies on tumor models.

2. INTRODUCTION

a. Cancer and Melanoma

Cancer is abnormal cell proliferation with the ability to invade to other organs of the body. Recent trends show that cancer is the second leading cause of mortality worldwide; in 2018 an estimated 9.6 million people died from cancer.

Tumor cells exhibit six hallmarks which include:

- Uncontrolled Cell growth and proliferation
- Avoidance of Programmed Cell death
- Angiogenesis
- Metastases
- Sustaining proliferative signaling

Melanoma can be caused by a mutation in melanocytes due to environmental factors, due to certain life style and can also be inherited. Most important cause of melanoma is UV radiation, it can damage skin cell's DNA and due to certain mutations in that DNA melanoma can rise. Much of melanoma cases are seen in the USA and very few in India.

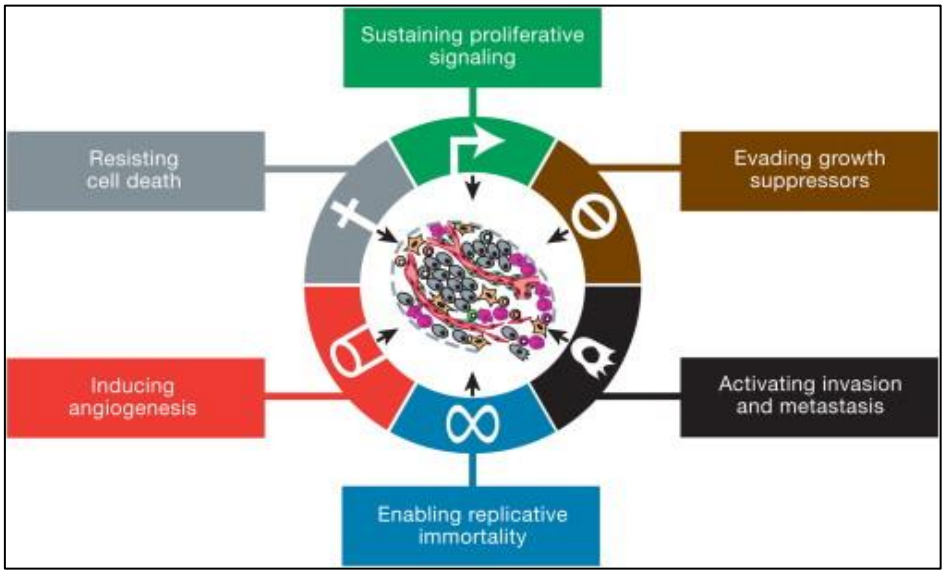


Figure 1: Hallmarks of Cancer (Hanahan and Weinberg, 2000)

b. Management of Cancer

Treatments depend on the type, location and grade of cancer as well as on patients' health. A few types of treatments are listed below:

- Chemotherapy
- Radiation therapy
- Surgery
- Cancer immunotherapy
- Laser therapy

Currently, available cancer treatments include surgery, radiotherapy, and chemotherapy. But there are associated with various adverse effects on normal cells and lack of specificity for tumor cells. From the last decades, several attempts have been made to develop different therapeutic strategies, such as immunotherapy. Immunotherapy can educate the immune system to recognize and attack specific cancer cells (a), boost immune cells to help them eliminate cancer (b), and provide the body with additional components to enhance the immune response (c). The potent modulating agents for the regulation of immune system are cytokines.

c. Tumor Microenvironment

The niche required for growth by cancer cells is very different than those found in normal cells; these cancer cells have lot of players, like growth factors and other cells which are playing a vital role in cancer development and progression from a non-metastatic to a metastatic form of cancer. This niche created by cancer cell can also be called a tumour microenvironment.

When a solid tumour grows in a body, some immune cells are attracted towards the site by their chemokine receptors such as CCR4 present in them, which will guide them from lymph node towards tumour outgrowth. In these events there are a lot of pro-tumour and anti-tumour cytokines being released in this TME, but cancer always suppress the effect of anti-tumour cytokines and also tries to induce pro-tumour cytokines. A lot of lymphocyte are successfully able to invade tumour but tumour cells have developed strategies to escape their immune surveillance by lymphocytes.**[5]**

Tumour cells have changed phenotype of macrophages that invade tumour into TAM **[5,6,7]**, it also affects TIDC via its growth factors making them impaired and paralyzed**[8]**, this causes $CD4^+$ T-cells not being getting activated. Tumour also has the ability to modify its MHC-I expression, some cases it will also express the inhibitor required to inhibit CD 8+ and NK cells, but sometimes it will also inhibit expression of TAP-1 gene which is required for MHC-I expression, making it difficult for $CD8^+$ cells to be activated.

The most important threat for TIL is the conversion of them from an active state to antolerogenic state of TIL which is mostly caused by regulatory T cells. $T_{reg}$is an important subset of T cells which in normal tissue helps in protection against autoimmune disease, which can be caused by autoreactive T and B cells. These subsets inhibit T cells activity and also inhibit its activation. Which is very helpful in normal tissues but in tumour this leads to tumour progression.

Other important problem faced by lymphocytes is the role of one Cytokine, IL-10. This cytokine inhibits the expression of MHC class II thus preventing professional APC such as DC and macrophages unable to present antigen to $CD4^+$ T cells. Various tumours have been shown to produce IL-10, including cells from melanomas, leukemia and lymphomas. **[9, 10]**

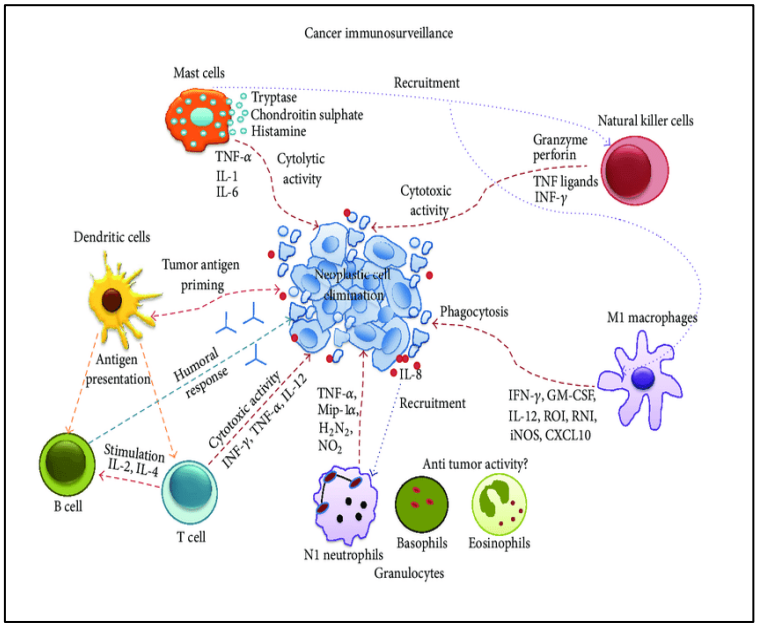


Figure 2: Tumor microenvironment and interactions with immune cells

d. Cancer Immunotherapy

Cancer immunotherapy is quickly advancing and is now called as the fifth pillar of cancer therapy. The word 'immunotherapy' includes a wide spectrum of concepts and methods which uses the immune system to tackle malignancies **[24].** There are several ways of approaching and fine tuning the immune system to eliminate malignancies, which include:

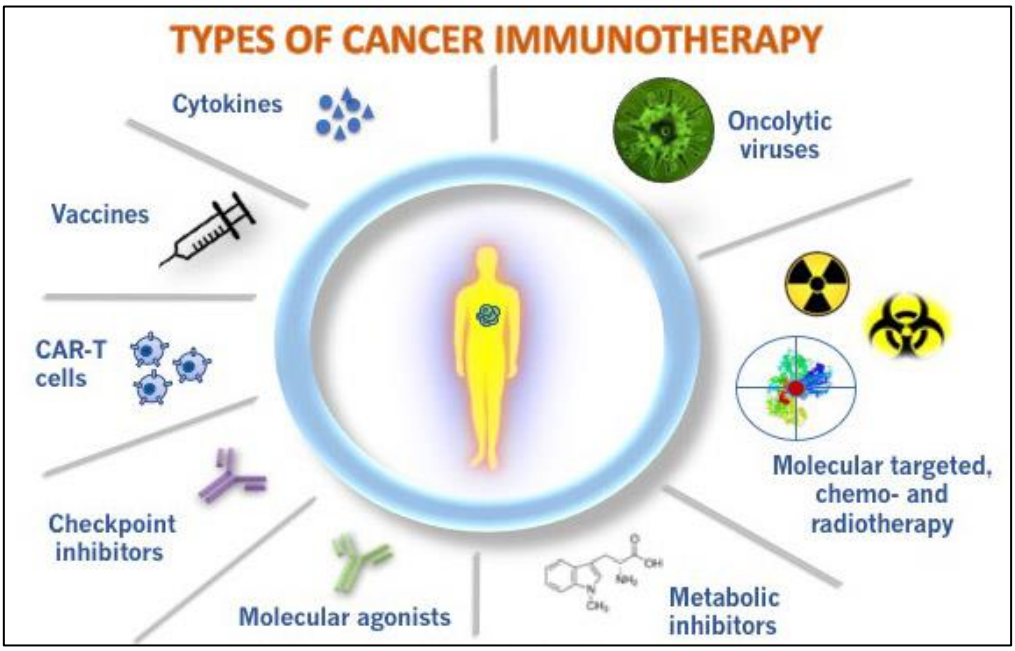


Figure 3: Types of Cancer Immunotherapy

e. Cytokines in Cancer immunotherapy

Cytokines are secreted or membrane-bound molecules that act as mediators of signaling to modulate immune responses and are produced by cells of innate and adaptive immunity in response to microbes and tumor antigens. Several animal tumor model studies have confirmed that cytokines have wide-range of anti-tumor activity. Recent years have seen a number of cytokines, including GM-CSF, IFN-γ, IL-2, IL-7, IL-12, IL-15, IL-18 and IL-21; enter clinical trials for patients with advanced cancer. **[24].**

Studies have found that IL-15 exhibits significant therapeutic activity in several pre-clinical murine models and the general mode of action includes direct activation of $CD8^+$ T effector cells in an antigen independent manner.**[25].** Initially, IL-15 was known for its role in

maintenance of memory $CD8^+$ T cells; now we have a greater understanding of its ability to generate memory T cells as well as to reactivate memory T cells during antigen challenge.

Novel strategies for improving efficacies and half-life of cytokines for better performance have been applied which includes cytokine-antibody fusion molecules**[26].** The subject molecule of this study is one such chimera which is essentially a cytokine-antibody fusion molecule imparting desirable therapeutic potential**[18].**

f. IL-15 as a cytokine and its role in immunotherapy

IL-15 is one of the major cytokines responsible of regulation of several major immune responses including activation of $CD8^+$ T effector cells. IL-15, unlike other cytokines, is widely expressed by many cell types including monocytes, macrophages, DC, fibroblasts, epithelial cells, and skeletal muscle cells, but surprisingly not by T cells. In vitro studies have shown that IL-15 is produced by the bone marrow stromal cells and activated monocytes. In humans, IL-15 mRNA was detected in many non-lymphoid tissues such as placenta, skeletal muscle, kidney, lung, liver, pancreas, cerebellum, and hippocampus suggesting its importance in other systems.

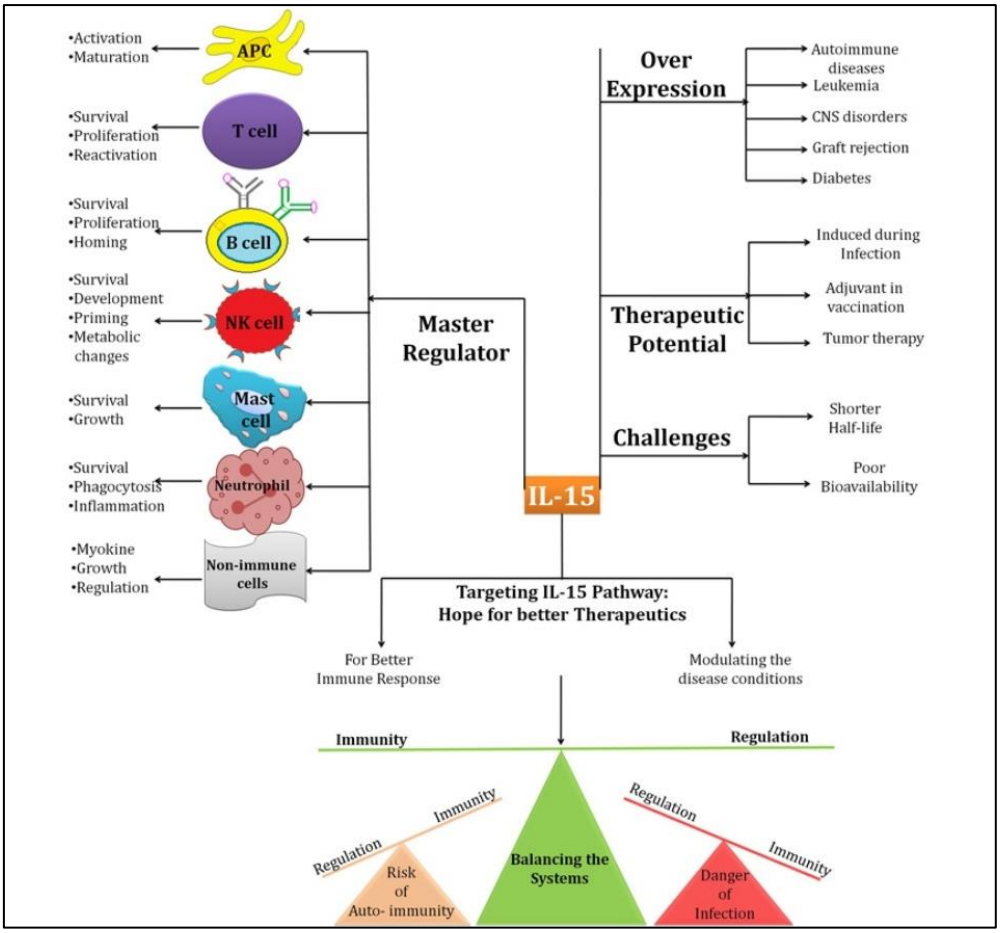


Figure 4: Tumor microenvironment and interactions with immune cells

IL-15 binds to a tri-receptor complex having the IL-15Rα, IL-15Rβ and γc chain. IL-15 stimulation induces tyrosine phosphorylation and activates JAK1 and JAK3. And activation of JAK1 and JAK3 subsequently results in STAT3 and STAT 5 phosphorylation, respectively.). IL-15 signaling pathways also involve the phosphorylation of the Src related tyrosine kinases, induction of anti-apoptotic factor Bcl-2, and stimulation of the Ras– Raf–MAP (mitogen activated protein) kinase pathway leading to Fos/Junactivation. IL-15 can also block TNFR-1 (tumor necrosis factor receptor-1)-mediated apoptosis through the competitive binding of IL-15Rα to TRAF2 **[28].**

Among the twelve anti-cancer immunotherapy drugs, IL-15 was ranked first by the National Cancer Institute, NIH, USA in 1997. IL-15 shows potent ability to maintain long-lasting T cell responses to pathogens by supporting the survival of CD8+ T cells. It is a more effective and less toxic cytokine than IL-2 for tumor therapy. It stimulates T and NK cells for increased antitumor activity. It is shown in many mouse models that IL-15 successfully

regresses the tumor burdens by activating tumor infiltrating cells and improving the in vivo antitumor activity of adoptively transferred CD8+ T cells while expanding tumor self-reactive CD8+ T cells**[27].**

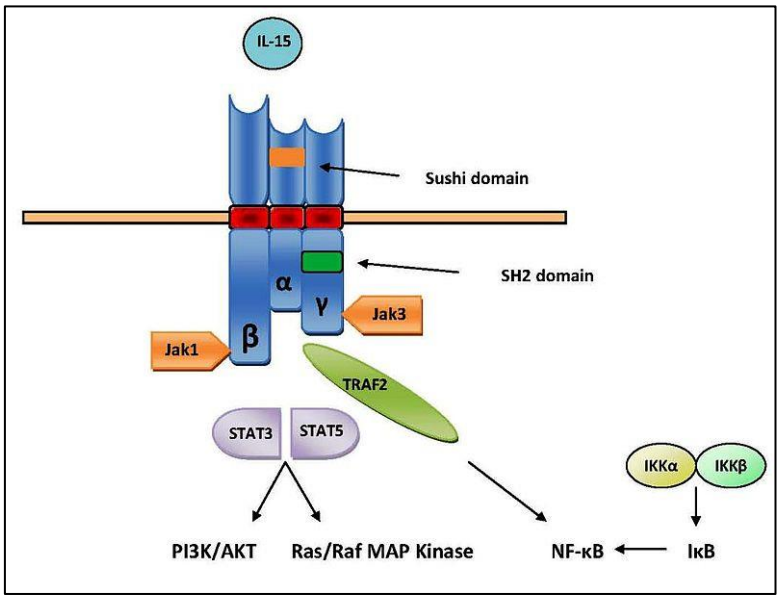


Figure 5: IL-15 Signalling pathway

g. Chimeric IL-15

The main limitation of IL-15 is its short half-life and poor bioavailability. For overcome this limitations synthesized chimeric IL-15. Chimeric IL-15 is a superagonist biologic synthesized by fusing native cytokine IL-15 with IgG2/2a base. The unique IgG2/2a base imparts a specific property of interaction with CD8+ T cells for serving in immunotherapeutic and vaccine based purposes.

The molecular architecture of the biologic is such that it favours dimer formation enhancing its stability. To address non desirable properties like shorter bioavailability and low efficacy, mutations K322A and N72D were introduced along with a linker joining native IL-15 domain and IgG2/2a base.

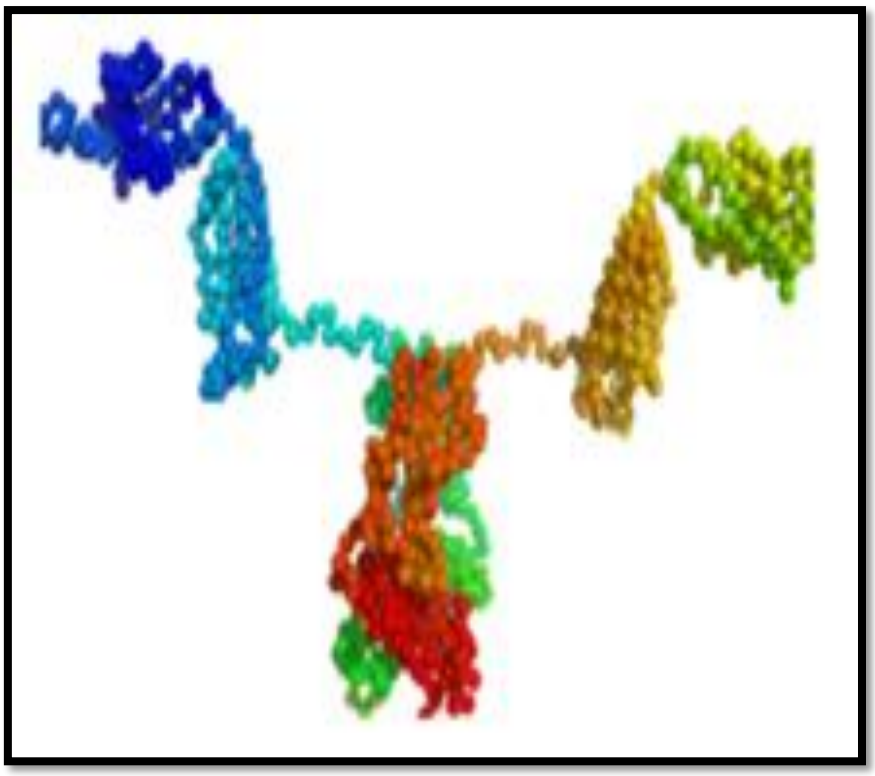


Figure 6: In-Silico image of Chimeric IL-15

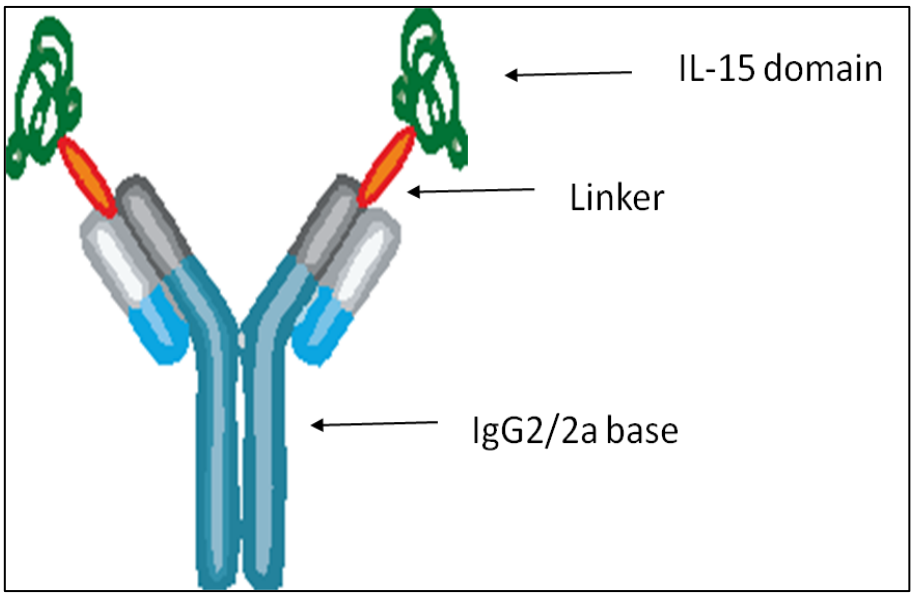


Figure 7: Chimeric IL-15

3. RATIONALE AND HYPOTHESIS

3.1 RATIONALE

Antigen presenting cell mediated presentation of tumor associated antigen and presence of Chimeric IL-15 would act as a tumor specific T-cell centric immunomodulator, reactivating cells and exhibiting anti-tumor activity.

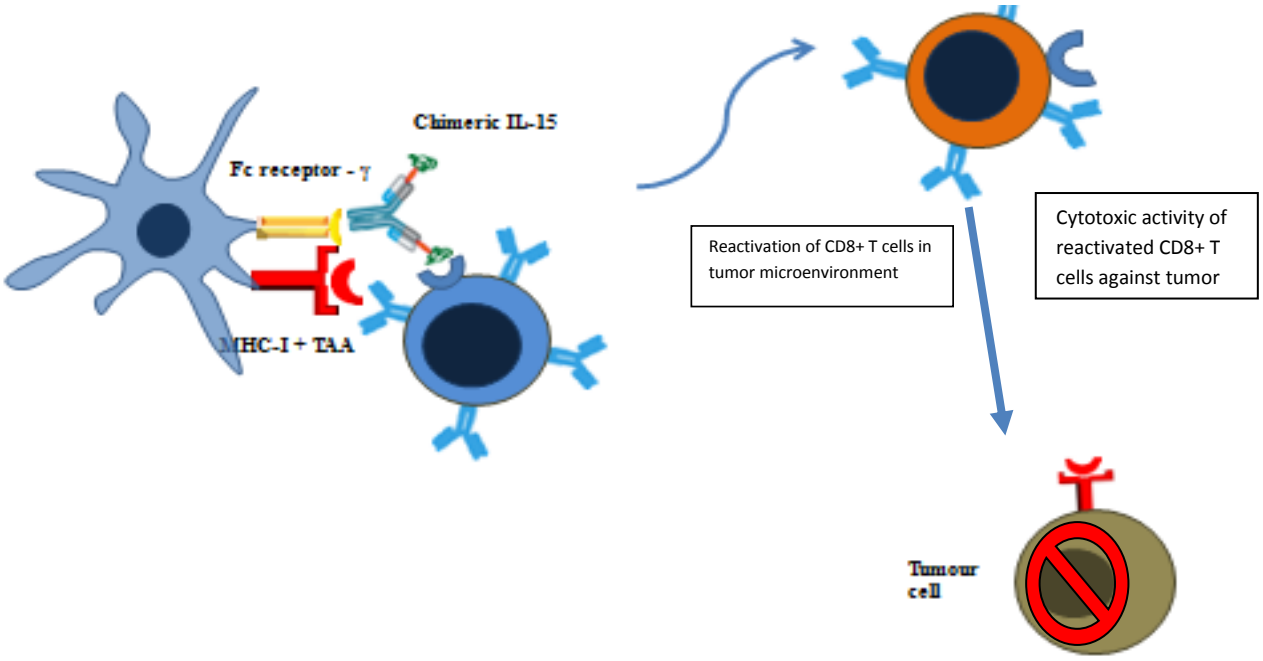


Figure 8: Mechanism of interaction of IL-15 with APC and CD8+ T cells

3.2 HYPOTHESIS

Our hypothesis is that if IL-15 is administered through IP and IV routes, there may be a significant difference in tumor regression and reactivation states of tumor-infiltrated cytotoxic T lymphocytes.

4. OBJECTIVES

Objective I : Transfection, Purification and characterization of Chimeric IL-15

a. Transfection of CHO cell lines by Chimeric IL-15 containing expression vector
b. Purification of Chimeric IL-15 by affinity purification
c. Characterization of chimeric IL-15 molecule

Objective II: Evaluation of CD8+ T cell reactivation and tumor regression upon chimeric IL-15 infusion when administered via Intra-venous and Intra-peritoneal routes.

a. To examine tumor growth upon B16f10 tumor cell line induction
b. To assess tumor regression post Chimeric IL-15 administration via IP and IV routes
c. To assess the activation status of tumor infiltrating $CD8^{+}$T cells (TILs) in tumor mouse model

5. MATERIALS AND METHODS

5.1 MATERIALS

5.1.1 PLASMID ISOLATION KIT

- Micherey-Nagel (NucleoBondXtra Midi)
- EQU buffer
- LYS buffer
- RES buffer
- Wash buffer
- ELU buffer
- 70% ethanol
- Isopropanol

5.1.2 PROTEIN PURIFICATION KIT

- Merck Millipore (Centrifugal Filter unit) 12 column
- Protein G resin
- Bind/Wash Buffer
- Elution buffer
- Neutralization buffer

5.1.3 AGAROSE GEL ELECTEOPHORESIS

- Agarose
- 1×TAE buffer
- ETBr

5.1.4 SDS PAGE

- Acrylamide/bis-acrylamide
- 10% SDS
- 1% APS
- TEMED
- pH 6.8/8.8 TrisHCL
- Distilled Water
- BPB
- Methanol
- Staining solution: 0.1% Coomassie Brilliant Blue R250 + 50% methanol + 10% glacial acetic acid

– Destaining solution: 40% methanol + 10% glacial acetic acid

5.1.5 WESTERN BLOT

- Blocking buffer:
– 3% BSA in distilled water
- Washing buffer – TBST (1x, 1000ml)
– 6.05 g Tris and 8.76 g NaCl in 1000ml distilled water
– Adjust pH to 7.5
- Antibody dilution:
– Primary antibody (PeproTech)
– Anti-murine IL15 antibody – 1:1000 dilution in BSA
– Secondary antibody (Thermo Fischer scientific)
– Anti-IgG2a antibody – 1:3000 dilution in BSA
- Ponceau S Staining Solution (100 ml, 1x):
– 0.1%(w/v) Ponceau S in 5%(v/v) acetic acid
– 0.1g Ponceau S in 5ml acetic acid and Make up to 100ml with ddH2O
- ECL kit (SuperSignal West Pico Chemiluminescent Substrate):
– SuperSignal West Pico Luminol/Enhancer Solution
– SuperSignal West Pico Stable Peroxide Solution
- X-ray film
- 20% ethanol
- Deionized water

5.1.6 CELL LINES:

a) Melanoma cell line (B16F10 cell line was a gift from Dr.Amit Awasthi's lab, Translational Health Science And Technology Institute, Delhi.)

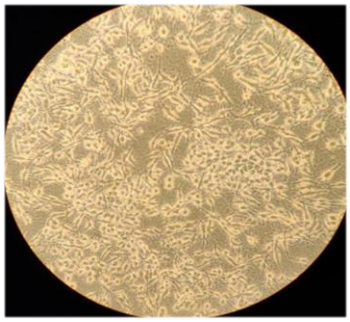


Figure 9:B16F10 cell line

Morphology	Fibroblast
Growth mode	Adherent
Growth media	DMEM, 10% FCS
Confluency of cell	70-90% in T25 flask
Stock	1*10^6 cells

Table 1: Characteristics of B16F10 cell line

b) CHO (Chinese Hamster Ovary) cell line: This cell line was revived and cultured in the cell culture facility of Institute of Science, Nirma University.

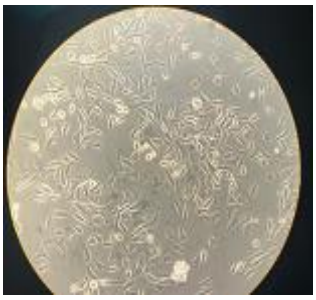


Figure 10: CHO cell line

Morphology	Fibroblast
Growth mode	Adherent
Growth media	DMEM, 10% FCS
Confluency of cell	70-90% in T25 flask
Stock	1*10^5 cells per 6 well plate and 3*10^6 cells in T25 flask

Table 2: Characteristics of CHO cell line

5.1.7 ANIMAL:

- Female C57BL/6 mice (6-8 weeks old) were obtained from Zydus Pharmaceuticals, Ahmedabad. Animals were maintained at Animal house facility of Institute of Pharmacology, Nirma University. All experiments carried out were strictly as per bioethical rules and regulations of animal care and committee of Nirma University, Ahmedabad.

5.1.8 DISSECTION OF MICE:

- Autoclaved dissection tools
- Wax tray
- 1% complete RPMI medium (HIMEDIA: cat# AL068A)

5.1.9 Immunization:

- B16F10(melanoma)cell line: 1*10^6 cells/mice(subcutaneous administration)
- Molecule – Chimeric(mouse-mouse)IL15 antigen(Intra-venous and Intra peritoneal)
- Conc. Of Chimeric IL-15 - 10µg/ml

5.1.10 CELL SURFACE STAINING AND INTRACELLULAR CYTOKINE STAINING REQUIREMENTS:

- 1% RPMI complete Media (1% FCS/FBS in RPMI media)
- 10% RPMI complete media (10% FCS/FBS in RPMI media)
- FACS Buffer/ Staining buffer (1% FCS in PBS)
- Fc Block (1:100 - normal mouse serum: FACS buffer)
- Brefeldin A
- Cytofix/ Cytoperm fixation buffer (BD biosciences®)
- 1X Perm/ Wash Buffer (10X perm/ wash buffer is diluted in double distilled water)
- 1% Formaldehyde (diluted in FACS buffer)
- Fluorescently Labeled Antibodies
- Petri Plates
- Frosted Slides
- 96 Well Microtiter Plates
- CO2 Incubator
- Centrifuge
- Flow Cytometer
- Data Analysis Software: Flow –Jo (version: FlowJo_V10_CL)

5.1.11 List of Antibodies used:

Marker	Fluorescence Antibody	Absorption Maximum(nm)	Emission Maximum(nm)
CD3	PerCP	490	694
CD4	PE	496	576
CD8	APC H7	650	785
CD107	FITC	495	518
IFN-ϒ	APC	650	660

Table 3: List of Antibodies used

5.2 METHODS

5.2.1 Plasmid isolation

1. **Cultivation of bacterial cells :**

- LB broth was prepared in 4 tubes each containing 10ml volume and inoculated with 50ml of bacterial culture in 3 tubes (Considering 1 tube as blank) and 10µl (1x) of antibiotic in all 4 tubes.
- The bacterial culture was kept in shaker at 37°C for 16-18 hours.

2. **Measure the absorbance at 600nm**
3. **Isolation of plasmid (Kit based)**

- Cells were pellet down in each tube after centrifugation.
- 250µL of Resuspension buffer was added in each tube and vortexed it for 2-3 minutes.
- 250µL of Lysis Buffer was added in each tube. Tubes were mixed gently by inverting it gently for 6–8 times. (Do not vortex to avoid shearing of genomic DNA).
- Tubes were incubated at room temperature for 5 min or until lysate appeared clearly.
- 300µL of Neutralising Buffer was added in each tube. Tubes were mixed thoroughly by inverting it for 6–8 times until blue samples turn colourless completely. (Do not vortex to avoid shearing of genomic DNA).
- Tubes were centrifuged for 5 min at 11,000 x g at 4°C.
- NucleoSpin Plasmid QuickPure Column was placed in a Collection Tube (2 mL) and the supernatant was decanted or pipette to a maximum of 700µL of the supernatant onto the column. Tubes were centrifugedfor 1 min at 11,000 x g. Flow through was discarded and placed the NucleoSpin Plasmid QuickPure Column back into the collection tube. This step was repeated to load the remaining lysate.
- 600 µLof Wash Buffer was added in each tube. Tubes were centrifuged for 1 min at 11,000 x g. Flow through was discarded and the NucleoSpin Plasmid / Plasmid (NoLid) Column was placed back into the empty collection tube.
- Tubes were centrifuged for 2 min at 11,000 x g and discard the collection tube.(Dry centrifugation)
- The NucleoSpin Plasmid / Plasmid (NoLid) Column was placed in a 1.5 mL microcentrifuge tube and 50µL Elution Buffer was added in tube. Tube was incubated for 1 min at room temperature. Again tube was centrifuged for 1 min at 11,000 x g.
- Tube was incubated at 37°C for 1 min.

5.2.2 Agarose Gel Electrophoresis

- 0.8% of Agarose gel was prepared in 60mL of TAE buffer.
- 3µL of EtBr(10mg/ml) was added and the gel was poured in gel caster along with comb and was allowed to solidify.
- TAE buffer was poured in the caster and comb was removed gently and samples were loaded in wells.
- The gel was run at 100Volt for 1 hour.
- The bands were observed in gel dock or UV lamp

5.2.3 SDS PAGE

- The glass plate and notch plate was prepared and the spacer was placed between it before casting.
- 10% separating gel was prepared and allowed it to solidify.
- Top of the gel was layered with isopropanol. This will help to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out.

Composition of Separating Gel (10%)	**Volume in mL**
Water	3.1
Acryl/Bisacryl stock solution	3.35
1.5M Tris-HCl (pH 8.8)	2.5
10% SDS	0.1
TEMED	0.01
10% APS	0.1

Table 4: Composition of separating gel

- Then remove the isopropanol.
- 6% Stacking gel was prepared.
- Stacking gel was poured on top of the separation gel and comb was placed to make wells and was allowed to solidify.
- The gel was clamped into apparatus, and fill chambers were filled with gel running buffer.

Composition of Stacking Gel (6%)	Volume in mL
Water	6.1
Acryl/Bisacryl stock solution	1.35
1.5M Tris-HCl (pH 6.8)	2.5
10% SDS	0.1
TEMED	0.025
10% APS	0.1

Table 5: Composition of stacking gel

1. **Sample preparation**

- Protein sample was treated with loading dye solution at 95°C for 5 min (Loading dye 10µl and Protein sample 10µl)
- Samples and molecular mass protein markers were loaded into wells for separation by electrophoresis.
- 150V for 20 min and 180V for 1 hour was set and allowed it to run until the loading dye reaches the end of the gel.

2. **Staining the gel**

- Gel was removed from the cassette and then stacking gel was removed.
- The gel was placed in a box and staining solution was added ~20ml till the dye covers the gel.
- The box was placed on gel rocker for 1 hour and then staining solution was removed.
- Destaining solution was added and kept on gel rocker for 1 hour till the bands became visible.

5.2.4 Western Blot

- After transferring the desired protein through PAGE on gel, gel was kept in 1x TBST buffer and meanwhile iBlot™ 2 Gel Transfer Device was prepared.
- There is generally no need for any pretreatment of the gel after electrophoresis, however, under certain circumstances, an equilibration step can improve results:

- Equilibration of the gel in 20% ethanol (prepared in deionized water) for 5–10 minutes prior to performing blotting can improve the overall efficiency of transfer for high molecular weight proteins.
- Equilibration in 10 mL of deionized water or transfer buffer for 5 minutes prior to transfer may improve transfer of mid to small molecular weight proteins.
- The transfer membrane is supplied in a ready-to-use format in the stacks without any need for pretreatment (soaking the blotting membrane in deionized water for 10-15 seconds can improve the transfer efficiency).
- Gel was stacked on the nitrocellulose membrane as given in the manual protocol by the manufacturer.
- Blotting Roller was used all over the gel to flatten any protrusions to ensure even transfer and efficient blotting.
- Stack was assembled in such a way that electrical contact is made between the device and the stack (refer the manual for actual understanding).
- The voltage was set 25V and time 10 minutes for successful transfer of bands on the membrane.
- After the completion of the transfer, Nitrocellulose membrane was removed, carrying the protein bands, carefully. Use forceps for convenience.(Gel can also be removed and stained to ensure the transfer of protein on the membrane. If bands are not seen on the gel then transfer is ensured).
- Now, to ensure the transfer of protein band on the nitrocellulose membrane, the membrane was stained with Ponceau S stain (Ponceau S stain is reversible stain, destaining is possible with the washing buffer wash for 4-5 times)
- Antibody probing was carried out.

1. **Antibody probing** – The membrane was blocked in the blocking buffer (3% BSA in TBST) for 1 hour at room temperature on a gel rocker.

- To remove the nonspecific blocking, the membrane was washed with the washing buffer for 3-5 times for 10 minutes. Then the membrane was incubated with the primary antibody at $4^{o}C$ overnight.
- Membrane was washed again with the washing buffer as per the above step. Membrane was incubated with the secondary antibody for 1 hour.

- Membrane was then again washed with the washing buffer for 3 times and X-ray of the membrane was developed by using the ECL kit (SuperSignal West Pico Chemiluminescent Substrate).

2. X-ray development by ECL kit :

- The following procedure was done in the Dark Room facility, ISNU, Nirma University.
- Membrane was placed on the x-ray film protector and in meanwhile the working solution was prepared by mixing equal amount of Peroxide Solution and the Luminol/Enhancer Solution given in the kit(Use 0.1mL Working Solution per cm^2 of membrane).
- Membrane was incubated in working solution for 5 minutes and covered it with plastic cover and protector.
- The X-ray film was exposed to the covered membrane for 5-10 minutes.
- The X-ray film was taken out and dipped in the Developer solution for 1 minute and then washed with the distilled water.
- After washing the X-ray film, it was dipped in the Fixer solution for about 1 minute until bands were observed. (Bands can be seen in the red light covered with the special cover and protector).
- Note: Whole procedure should be done in dark room; light can damage the X-ray films and ultimately the results.

5.2.5 Protein G purification

a) Bead preparation

- Protein G resin was vortexed thoroughly before adding it to the device to ensure uniform suspension.
- The collection tube cap was removed and the exchange device cap was opened.
- 200µl resin slurry was added to exchange device using wide bore tips.
- Tube was centrifuged at 1000g for 1 minute.
- 500µl of 1X Bind/Wash buffer was added and centrifuged at 1000g for 1 minute.

b) Protein Binding

- 9ml of sample was added to the exchange device.

- The device was incubated for 60 minutes at room temperature on gel rocker for gentle agitation.
- After incubation the device was centrifuged at 1000g for 1minute in swinging bucket rotor.
- 1.5ml bind/wash buffer was added and centrifuge at 1000g for 1 minute.

c) Sample Elution

- 500µl elution buffer was added and centrifuged at 1000g for 2 minutes.
- 75µl neutralization buffer was also added to adjust pH.
- Samples were stored at -20°C.

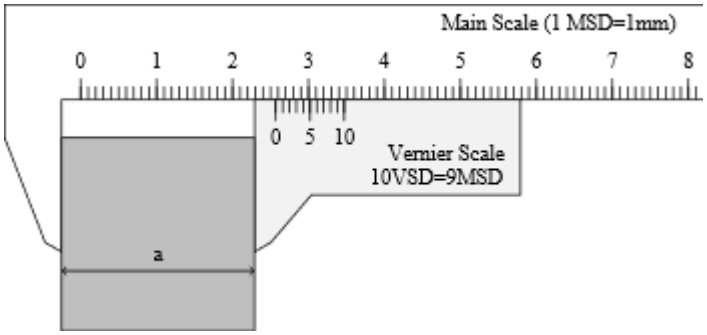
5.2.6 Transfection

- On day 0, 70-90% confluent 10^5 CHO cells were seeded in RPMI media in 6 well plate and incubated at 37°C and 5% CO_2 for 24 hours.
- After 24 hours by checking the confluency of cell, the media was discarded and ~10ml of fresh media was added to the plate.
- 14µg of plasmid DNA and 700µl of Opti-MEM media was taken in separate eppendorf and 14µl of PLUS reagent was added in eppendorf.
- The eppendorf was mixed properly by vortexing or with pipette.
- Different concentration of lipofectamine was taken (6µl/ml, 9µl/ml, 12µl/ml, 15µl/ml) and DNA mixture tube was added in Lipofectamine containing tubes.
- The mixture was incubated for 5 mins at room temperature.
- Add the mixture (DNA-lipid complex) to CHO cells.
- The cells were incubated at 37°C for 2 days.
- The transfected cells were then analyzed.
- Supernatant was collected for 3 consecutive days.
- The supernatant was stored at -20°C.

5.2.7 Vernier Caliper

- To measure the size of tumor Vernier Caliper was used.

- It is given that 10 Vernier scale division = 9 Main scale division. Thus, 1 Vernier scale division = (9/10) Main scale division = 0.9 mm i.e., distance between two successive marks on the Vernier scale is 0.9 mm.



Vernier Calliper

- Vernier caliper least count formula was calculated by dividing the smallest reading of the main scale with the total number of divisions of the vernier scale.
- Least count of vernier caliper is the difference between one smallest reading of the main scale and one smallest reading of vernier scale which is 0.1 mm or 0.01 cm.
- The size was measured by the formula:
  Main scale reading + Vernier scale reading x least count
- Tumor volume was calculated according to the formula:
  V= (length x width^2)/2

5.2.8 Isolation of Tumor Infiltrating Lymphocytes

- All the organs were collected in 10% RPMI media
- Blood was collected directly from heart and eyes.
- The upper black layer of skin was removed and snapshots were taken.
- Tumor was placed in dissociator tube and was chopped by a scissor. (Place on dissociator 3 times for 37 seconds each)
- The cells were transfered in 50 mL falcon and PBS(1x) and complete media (30-35 mL) was added in the falcon.
- The tube was centrifuged at 2000rpmfor 7 mins at 4^0C.
- The supernatant was discarded and 3mL enzyme cocktail was added and was mixed by tapping.

- The reaction was incubated at 37^0C for 1 hr.
- PBS/Media was added post incubation and was mixed thoroughly.
- The solution was passed from 0.7mm cell strainer in a new 50 mL falcon.
- The tube was centrifuged at 2000 rpm for 7 mins at 4^0C and supernatant was discarded.
- The pellet was resuspended in 1 mL 63% percoll and was transferred to 15 mL falcon.
- 2 mL of 47% percoll was slowly added for preparation of layer.
- Third layer was prepared by adding 2 mL of 33% Percoll. A sterile pipette was used for preparation of layers and do not disturb layer.
- The tube was centrifuged at 2000rpm for 30 mins at 4^0C(Acceleration – 9, Deceleration = 0)
- Buffy layer was collected post centrifugation. 5 mL syringe with a long needle (10 mL) was used for collection.
- The cells were collected by circular motion of needle and transferred into 1.5 mL microfuge tube.
- The tube was centrifuged at 2000rpmfor 3 mins at 4-15^0C.
- Supernatant was discarded and resuspended in media.
- The cells were counted and seeded into a plate.

5.2.9 Methods involved in flow cytometry

1. Cell surface staining

- Harvesting of cells: Whole spleen/ lymph node/ tumor was harvested or obtained from a normal/ immunized strain of mouse (e.g., C57Bl/6).
- Spleen/ lymph nodes were minced in sterile 1% RPMI (or 1X PBS) medium and collected in 15ml centrifuge tube. The cells were pelleted down by spinning at 500g for 5 min at 20°C.
- The supernatant was decanted and spleen cells were treated with 2 ml ACK RBS lysis solution for 3 min at 37°C. Approximately 8 ml of 1X PBS was added for washing and was mixed by inversion. The cells were centrifuged at 500g for 5 min at 20°C. (NOTE: Lymph nodes were not treated with RBC lysis buffer and PBS washing steps)
- The pellet was dissolved in chilled complete RPMI and was counted in cell counter.

- Antigen stimulation: Appropriate number of cells were seeded (0.5-0.7 million cells per well) in 200 ml complete media (10% RPMI), in 96-well plate. Desired numbers of wells were prepared according to the need.
- The cells were incubated at 37°C and 5% CO2 for required period of time (2 hours for intracellular cytokine staining and for cell proliferation (50-72 hours) according to the need.
- After incubation, the plate was centrifuged at 400-500g for 5 min at 10°C. The media was discarded directly by inverting the plate without disturbing the pellet(NOTE: The pellet was clearly seen at the base of each well).
- 200 µl of staining buffer (FACS buffer) was added and the cells were pooled in one well for duplicate samples and cells were mixed by pipetting. The plate was centrifuged at 400-500g for 5 min at 4°C. The media was discarded directly by inverting the plate without disturbing the pellet.
- The pellet was resuspended in 10 µl of Fc-block 1:100 (normal mice serum: FACS buffer). The cells were mixed by tapping the plate gently; the plate was incubated on ice for 10 minutes. NOTE: Fc-block would block the immunoglobulin Fc-receptors.
- 10 µl of antibody cocktail was added and mixed by gentle tapping the plate. The plate was kept on ice for 30-40 minutes in dark.(NOTE: It was ensured that the Fc-block and antibodies were bound well, for this reason pipetting and tapping the well were crucial steps that would give the better separation of cells in results). Dilutions were made in such a way that Fc-block was eventually diluted 1:100 times and antibodies were diluted 1:150 times in the suspension of cells.
- After incubation 200 µl staining buffer was added and centrifuged at 400-500g for 5 minutes at 4°C. Pellet obtained was washed once again with staining buffer spin same as above.
- Finally, pellet was re-suspended in 400 µl of staining buffer with 1% Formaldehyde for fixing the cells up to 48 hours at 4oC (initially 200 µl of 1% HCHO were added to each sample well and then the contents of each well were transferred to correspondingly numbered FACS tubes containing 200 µl of 1% HCHO).
- Samples were acquired using flow-cytometer. The acquired results were analysed by using the FlowJo software.

2. Intracellular staining

- Harvesting of cells: Whole spleen/ lymph node/ tumor was harvested or obtained from a normal/ immunized strain of mouse (e.g., BALB/c or C57Bl/6).
- Spleen/ lymph nodes were minced in sterile 1% RPMI (or 1X PBS) medium and collected in 15ml centrifuge tube. The cells were pelleted down by spinning at 500g for 5 min at 20°C.
- The supernatant was decanted and spleen cells were treated with 2 ml ACK RBS lysis solution for 3 min at 37°C. Approximately 8 ml of 1X PBS was added for washing and was mixed by inversion. The cells were centrifuged at 500g for 5 min at 20°C. (NOTE: Lymph nodes were not treated with RBC lysis buffer and PBS washing steps)
- The pellet was dissolved in chilled complete RPMI and was counted in cell counter.
- Antigen stimulation: Appropriate number of cells were seeded (0.5-0.7 million cells per well) in 200 ml complete media (10% RPMI), in 96-well plate. Desired numbers of wells were prepared according to the need.
- The cells were incubated at 37°C and 5% CO2 for required period of time (2 hours for intracellular cytokine staining and for cell proliferation (50-72 hours) according to the need.
- Addition of Brefeldin A 1000X solution (5.0mg/ml stock): Required concentration range 0.5g/200 µl or 1X solution. The 96-well plate was incubated at 37°C in 5% CO2 for 2 hours.
- Follow the cell surface staining according to the protocol.
- After final wash of cell surface staining, the supernatant was aspirated and the 96-well plate was agitated to disrupt cell pellets (Only gentle tapping of plate was done).
- 100 µl of Cytofix/Cytoperm Buffer (Fixation buffer) was added to each sample well and mixed by gentle pipetting. The plate was incubated for 20 minutes at room temperature. This step would fix the mouse cell morphology and permeabilize the activated cells for subsequent intracellular staining.
- 100 µl of 1× Perm/Wash buffer was added to each sample well and was mixed by gentle pipetting. The plate was centrifuged at 400 - 500g for 5 minutes at 4°C.
- Supernatant was aspirated from each sample well and the plate was agitated to disrupt cell pellets.
- Steps were repeated. (200 µl of 1× Perm/Wash buffer was added to each sample well).

- 0.5 µl/ well normal mice serum + 0.5 µl/ well each intracellular staining antibodies was added and the total volume was made up with BD Perm/Wash Buffer. 30 µl per well was added from this antibody cocktail.
- The 96-well plate was incubated for 30-35 minutes at 4°C (also can be done on ice).
- 100 µl of Perm/Wash Buffer was added to each sample well and the plate was centrifuged at 400- 500 g for 5 minutes at 4°C.
- The supernatant was aspirated and the plate was agitated to disrupt the cell pellets.
- The contents of each well were transferred to a correspondingly numbered sample tube using 200 µl of FACS Buffer (1% FCS + PBS). The final volume in each tube was made up to 400 µl using FACS Buffer with 1% formaldehyde to fix the cells.
- Samples were acquired using flow-cytometer. The acquired results were analysed by using the FlowJo software.

6. RESULTS AND DISCUSSION

6.1 Objective I

a. Transfection of CHO cell lines by Chimeric IL-15 containing expression vector.

6.1.1 Plasmid Isolation

Transformed DH5α competent cells with Mouse-mouse chimeric IL-15 clone in pcDNA3.1 plasmid vector inoculated in selective media ampicillin- LB brothand were incubated at 37°C overnight in shaking condition. Plasmid DNA was isolated after 16-18 hrs by plasmid isolation kit (NEB protocol mentioned in methodology). The size of intact plasmid was 6.8 kbp. Comparing the plasmid with the DNA ladder concluded that the plasmid was in intact form which was shown in the result.

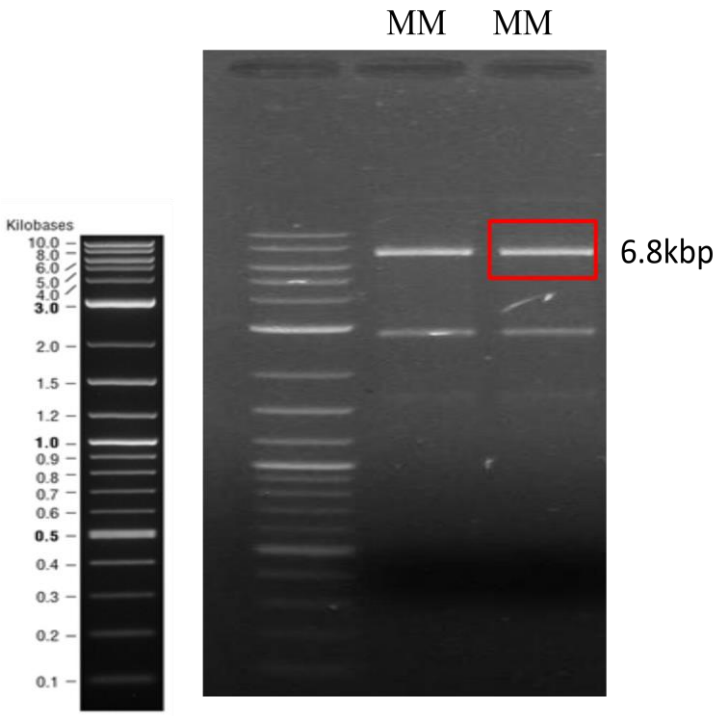


Figure11: Plasmid DNA isolated from E.coli

MM: Mouse Mouse

Absorbance of plasmid

- The absorbance of plasmid was in Nabi nano drop
- To set Blank Elution Buffer of plasmid isolation kit was used.

Concentration (ng/µl)	108.5
Absorbance at 260nm	2.170
Absorbance at 280nm	1.200
260/280 ratio	1.808

Table 6: Absorbance of plasmid

6.1.2 Transfection of plasmid into CHO cell line

To know the transfection efficiency and amount of Lipofectamine LTX required for optimum results, control transfection was performed in 24 well plate, in which pEGFP-n1 plasmid was transfected into separate wells of CHO cells with varying volumes of Lipofectamine i.e. 2µL, 3µL, 4µL and 5µL. The Transfection efficiency was measured by the GFP function of Countess cell counter® and the maximum efficiency was observed.

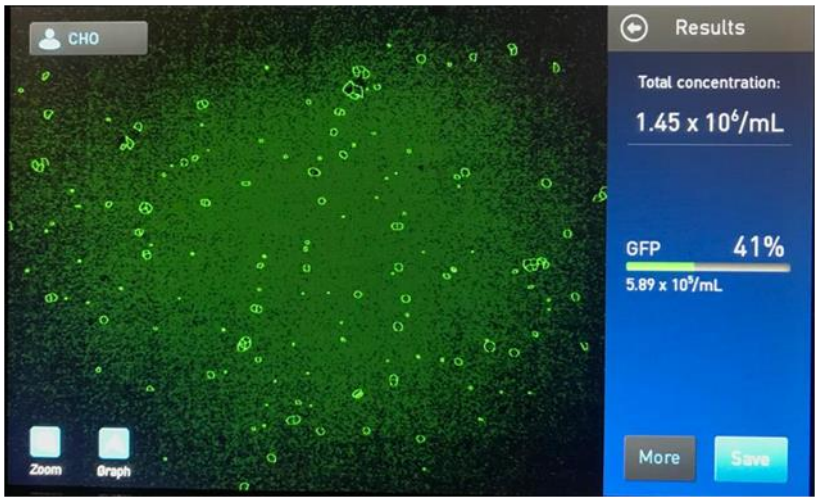


Figure 12: Transfection efficiency post pEGFP plasmid

When 4µL Lipofectamine LTX reagent® was used, thus we decided to consider 4µL as optimum volume for Transfection.

The MM (mouse IL-15 – mouse IgG/2a base) plasmid construct were transfected into CHO cells facilitated by Lipofectamine LTX reagent® to produce chimeric proteins. As the Chimeric molecule consists of IL-2 signal peptide, the desired protein is secreted in the supernatant and purification of the same would provide us chimeric IL-15 protein concentrates.

b. Purification of Chimeric IL-15 by affinity purification

Protein G based affinity chromatography was performed to concentrate the chimeric IL-15 from cell free supernatant. ~9 mL cell free supernatant was incubated with protein G beads for 1 hr at RT. The IgG2 portion of chimeric IL-15 bound to protein G beads and other CHO proteins were washed away during the washing steps. The chimeric IL-15 was eluted with elution buffer and concentration was measured by direct UV method in Nabinano-drop spectrophotometer. From 9 mL supernatant we were able to purify 760 µg/mL chimeric IL-15. (The concentration may vary with different batches of chimeric IL-15.) Purified protein product was then stored at -80^{o}C until further use.

Sample	Concentration(µg/mL)
Chimeric IL-15(9mL supernatant)	760

c. Characterization of Chimeric IL-15 molecule

Characterization of Chimeric IL-15 was carried out to confirm the composition of the molecule before proceeding for the *in vivo* experiment in tumor mouse model. Characterization was done by SDS-PAGE and western blot of the purified product. Previous characterization revealed the molecule to be a dimer of approximately 98 kDa in non-reducing SDS PAGE and 49 kDa in reducing conditions.

Molecular weight of the purified product was found to be more than 180 kDa in non-reducing gel and around 66 kDa in reducing gel. The probable reason for an increase in molecular weight would be due tetramer formation or post translational modifications like glycosylation.

To confirm the presence of desired domains(mouse IL-15 and mouse IgG/2a) in Chimeric molecule, western blot was performed in which primary antibody used was anti-mouse IL-15 antibody which was detected by HRP tagged secondary anti-IgG antibody by enhanced chemiluminescence. Pilot experiment was successful and we obtained single band on the X-ray film suggesting the presence of IL-15 in the

molecule. We could not perform another western blot with proper controls reproduce results due to inevitable conditions with regard to corona virus pandemic.

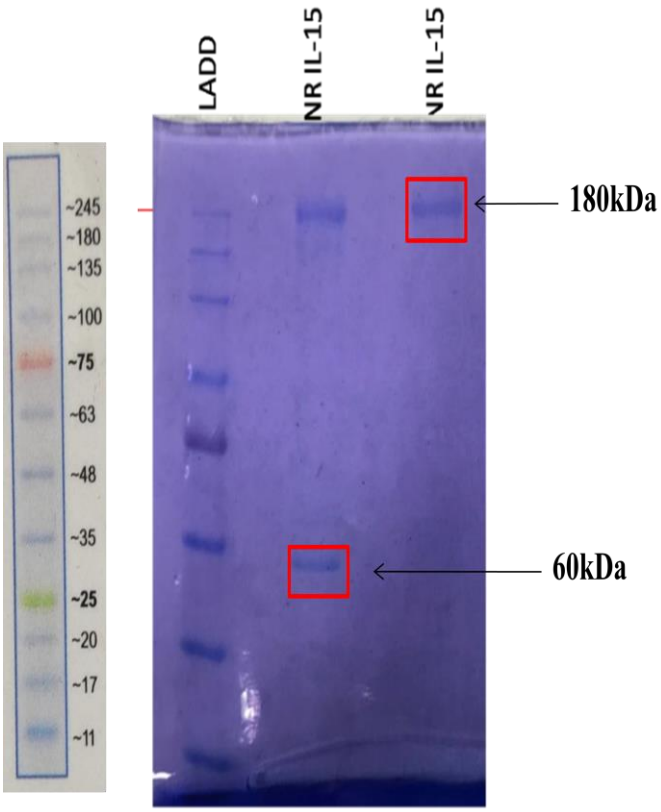


Figure13: SDS PAGE of chimeric IL-15

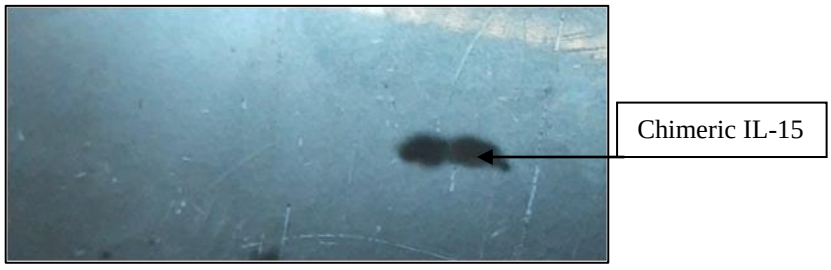


Figure14: Western Blot of chimeric IL-15

6.2 Objective II.

a. To examine tumor growth upon B16f10 tumor cell line induction

The melanoma cell line B16f10 was cultured in complete media R10 and lateral side of abdomen. The tumor induced mice were monitored daily and tumor growth was assessed using vernier callipers.

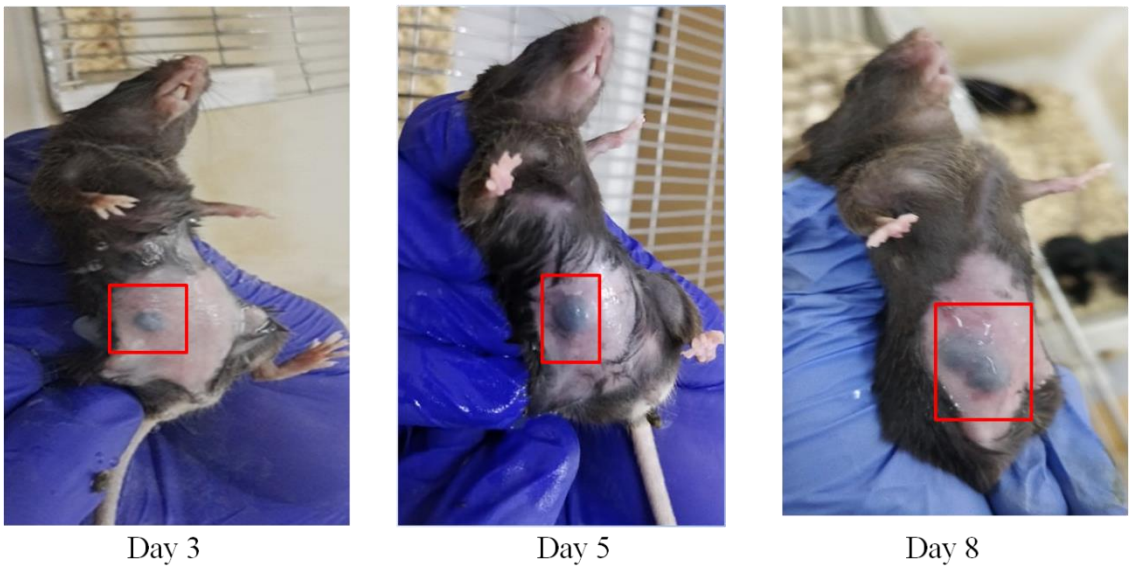


Figure15: Melanoma tumor growth in mice

Tumor size was 0.5-1.0 mm^3 by 8^{th} day post induction and Chimeric IL-15 treatment was started from 8^{th} day post tumor induction onwards. The chart given below provides a brief plan of the experiment.

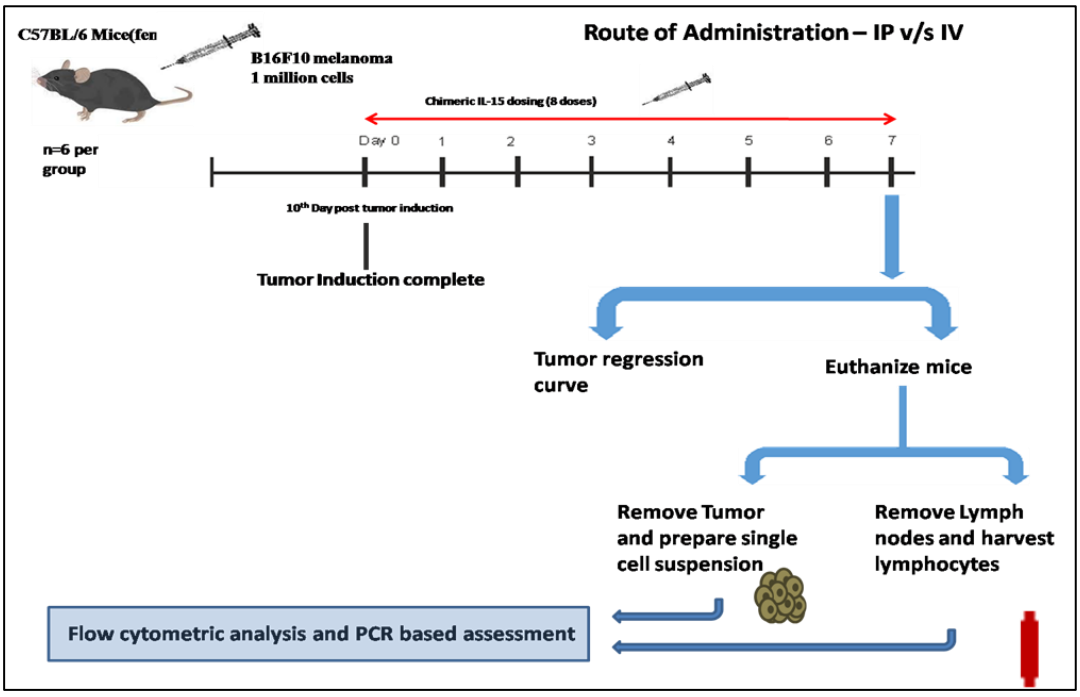


Figure 16: Work plan

The concentration of Chimeric IL-15 administered to 2 groups(per group n=6) for five doses was 5µg/mL in PBS and the rest three doses was 10µg/mL in PBS as there were

no signs of tumor regression during the first five doses. The tumor size was measured by Vernier calliper each day post tumor induction. The graph given below shows growth curve of tumor from day zero of the treatment.

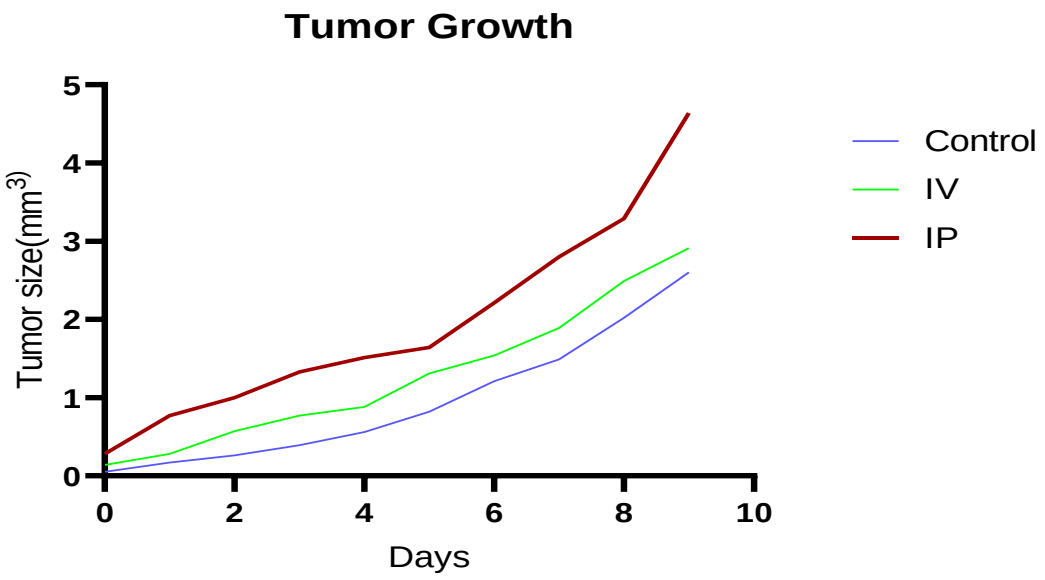


Figure 17: Tumor growth graph

There were no signs of tumor regression along the duration of treatment. The tumor volume showed negligible or no effect of treatment on the rate of growth of tumors in both the groups i.e. IV treatment group and IP treatment group.

Previously it had been shown that the tumor regression was observed in mice treated after 5 doses of 10µg/mL chimeric IL-15. There was no tumor regression observed which may be due to a lower concentration of Chimeric IL-15 administered.

b. To assess the activation status of tumor infiltrating $CD8^{+}$T cells (TILs) in tumor mouse model.

Control and experimental mice were sacrificed on 18^{th} day post tumor induction and lymphocytes were harvested from lymph node, and tumor for flow cytometric study.

The main aim of this experiment was to look for reactivation status of T- cells in tumor induced mice and the difference when Chimeric IL-15 is administered via intra-peritoneal and intra-venous routes.

There have been several reports when a different route of immunization yields different patterns of immune responses which encouraged us to consider Intra peritoneal and Intra-venous routes of administration of cIL-15 to study its effect on immune response.

To measure reactivation status of T cells, cells were surface stained and stained intracellularly with fluorochrome conjugated antibodies for cell markers CD4, CD8, CD107a and IFN-γ and were acquired by Thermofisher Scientific attune nxt® flow cytometer and data was analysed in flowjo software v10.6.2(free trial version).

Figure 18: Dissection of MICE

Common Gating strategy used to gate lymphocytes harvested from lymph node, tumor is given on the next page.

- **CD107 as an activation marker**

CD107a is a membrane marker expressed by cytotoxic T lymphocytes expressed when TCR interacts with MHC – I loaded with peptide and releases perforin and granzymes which act on target cells.

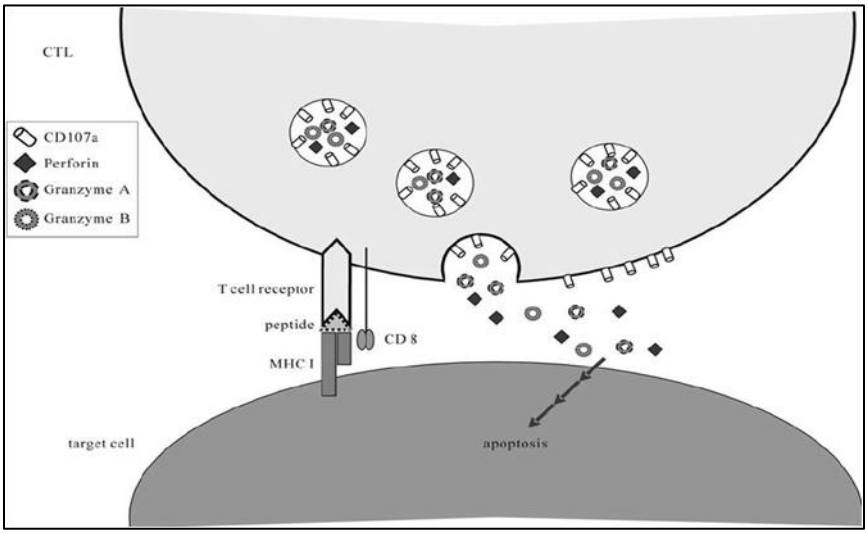


Figure19: Degranulation and CD107a

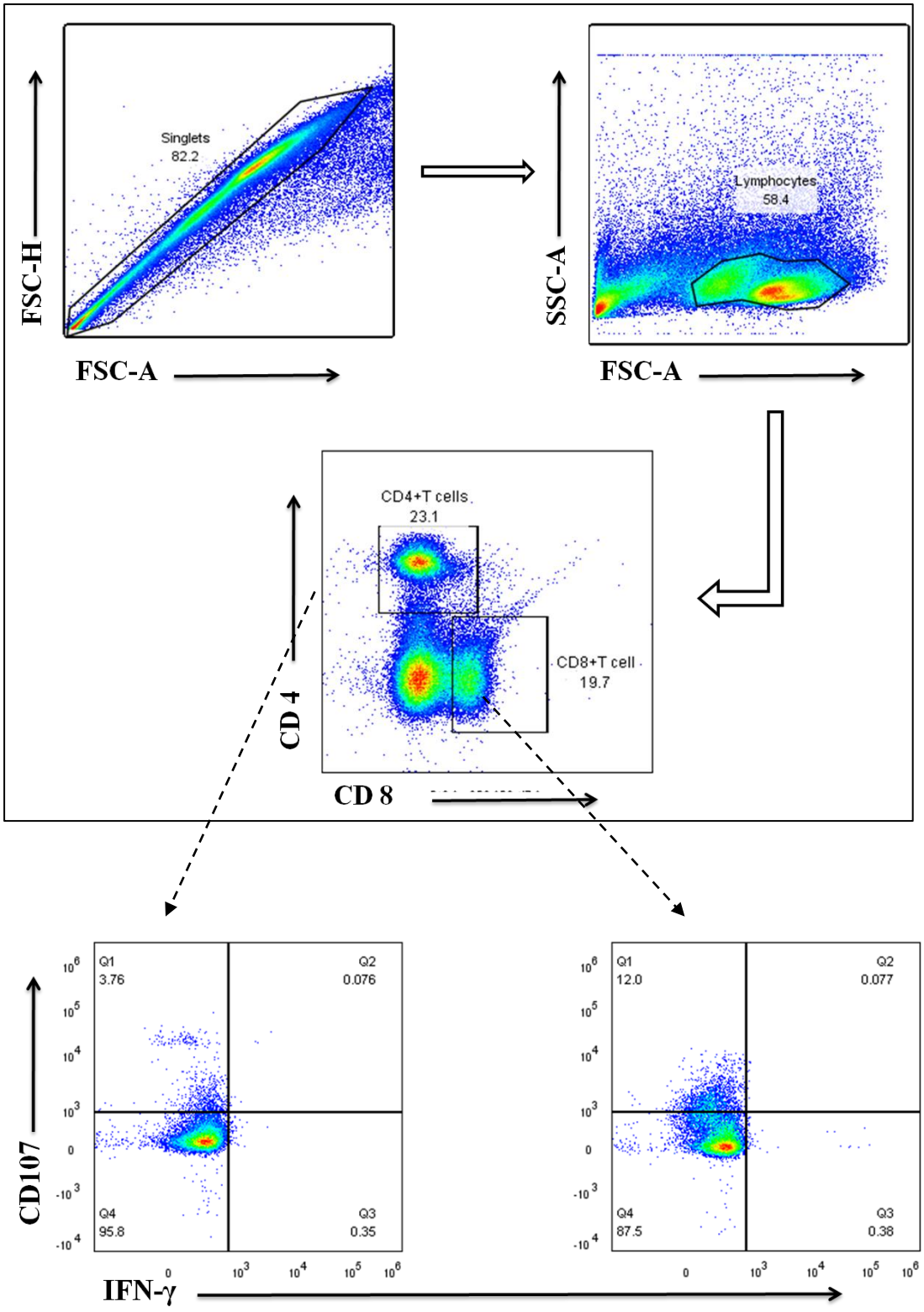


Figure 20: Schematic of gating strategy used for identifying CD8+ population expressing IFN-γ and CD107a markers from lymph node and Tumor.

6.2.1 CD8+ T cell activation status in tumor

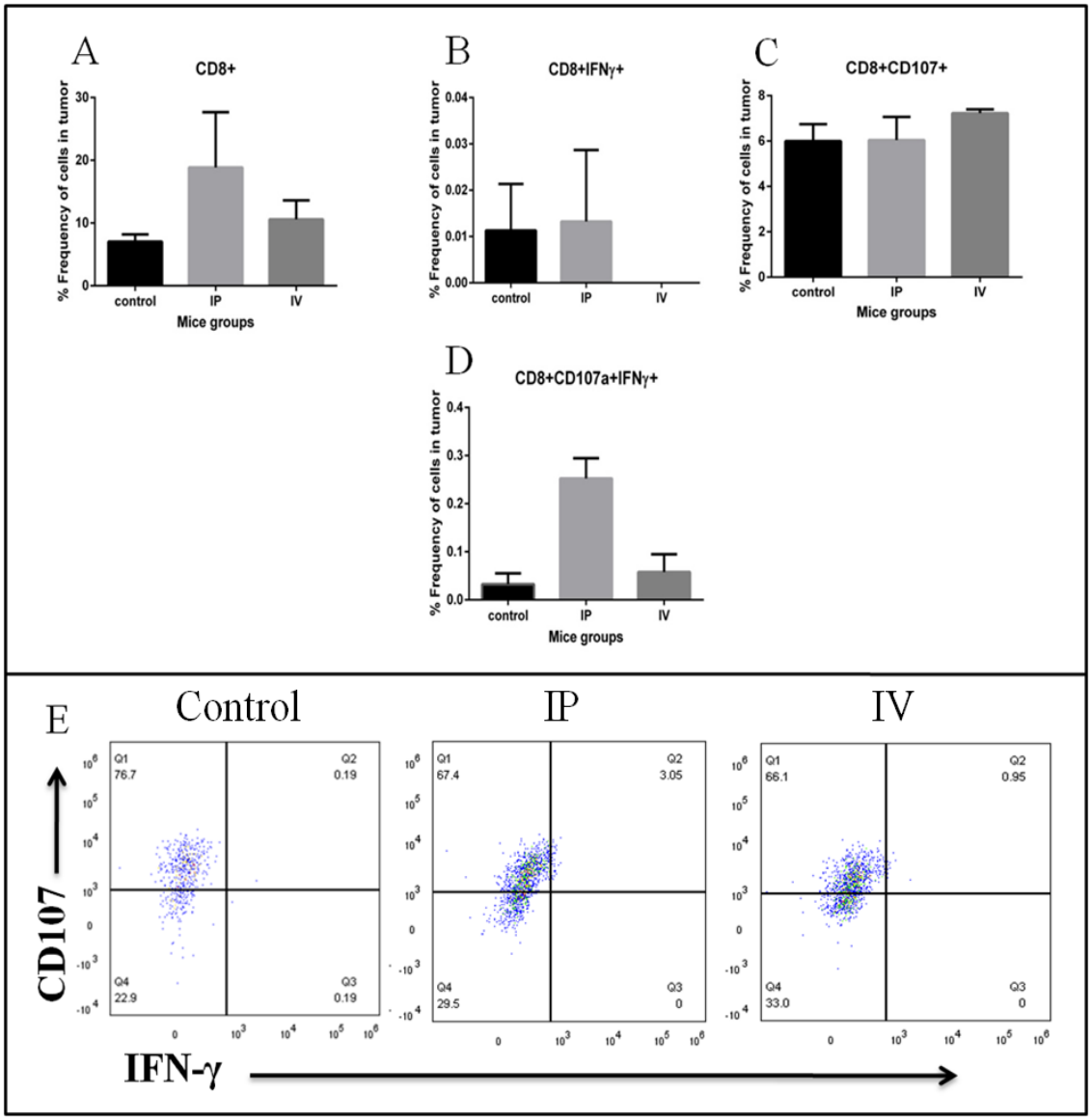


Figure 21: To assess the activation status of CD8+ T cells in tumor, tumor infiltrating lymphocytes were isolated 18th day post tumor induction and surface stained with fluorochrome conjugated antibodies against markers CD8, CD107a and IFN-γ. [A]The graph depicts the % of CD8+ cells in tumor for groups Control, IP (Intraperitoneal) and IV (intravenous)(p =).[B] IFNγ positive CD8+ cells in tumor in groups as described in (A).[C] Frequencies of CD107+ CD8 T cells[D]The graph shows percentage of double positive(CD107+IFN-γ+) cells representing activated CD8+ T cells in tumor..[E] The layouts represent dot plots of double positive (CD107a+IFNγ+) cells in control, IP and IV groups.

The results in the figure 21 indicate activation status of CD8+ T cells in tumor which suggests better CD8+ infiltration in IP treatment group as compared to that of control and IV. There are many IL 15 based chimeric molecules currently under investigation which have reported substantial increase in CD8+ T cell infiltration and activation. The studies involving these candidates have also reported significant NK cell toxicity

limiting their immunotherapeutic potential[27]. Chimeric IL-15 is designed specifically to interact with CD8+ T cells and not NK cells making it viable for immunotherapeutic purposes. In the current study it is quite evident that there is not only an increase in CD8+ T cell infiltration, but it is also evident that the percentage of double positive cells harboring activation markers CD107a and IFN-γ are significantly higher in IP treatment group indicating activated CD8+ T cells providing an insight to its potential therapeutic effect with anti-tumor activity.

Chimeric IL-15 is expected to interact with CD8+ T cells independently as well as via APC mediated presentation as shown in figure 1. APC are important part of Tumor infiltrating immune cells facilitating the migration and presentation of Tumor associated antigens in the nearest lymph node and activating T cells for elimination of malignancy. The activated T cells are supposed to take action against tumor cells but due to the immune modulatory environment, these effector cells are deactivated and hence elimination of tumor cells is inhibited. The data suggests that Chimeric IL-15 is facilitating and augmenting the CD8+ T effector cells in the tumor environment and it is also understood that Chimeric IL-15 is playing a crucial role in increasing effector CD8+ T cells infiltration in the tumor.

6.2.2 CD8+ T cell activation status in Lymph Node

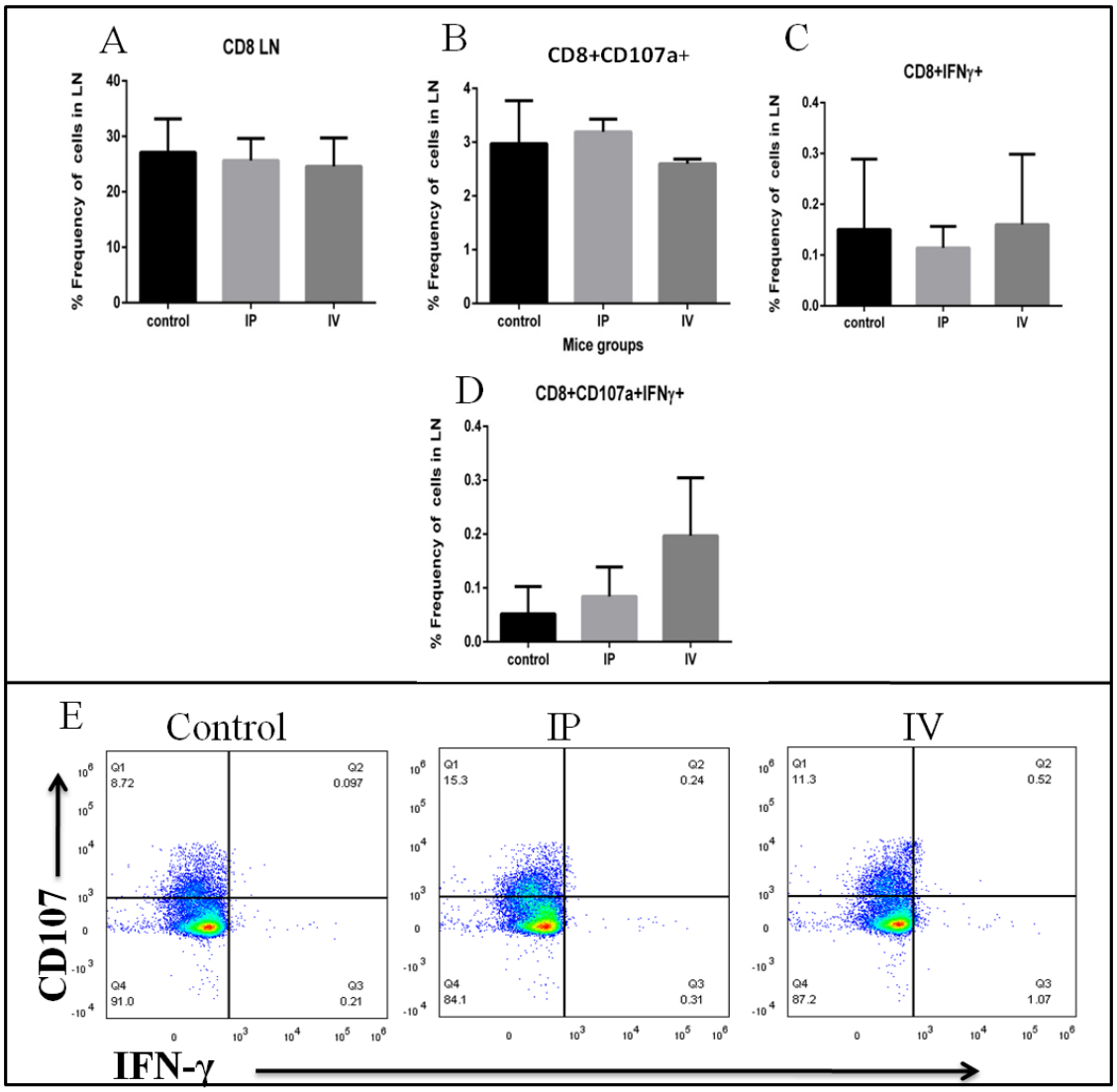


Figure 22: To assess the activation status of CD8+ T cells in lymph node, lymphocytes were isolated from lymph node on 18th day post tumor induction and surface stained with fluorochrome conjugated antibodies against markers CD8, CD107a and IFN-γ. [A] Graph shows the percentage of CD8+ T cells in the lymph node for groups control, IP(intra-peritoneal) and IV(intra-venous) [B]CD107+ cells in groups as described in [A] [C] Frequency of IFN-γ+ cells[D] The graph shows percentage frequency of double positive cells(CD107a+IFNγ+) of CD8+ cells in Lymph node[E] The layout represents double positive population in lymph node.

The immune response in space and time is very much at the core of our understanding about a cumulative action of immune system against its targets. In this particular study the target was induced melanoma tumor. The basic role play and importance of cytokine based immuno therapy is solely due to the inherent property of tumor to modulate immune responses in such a way that the defensive properties are hindered.

Obviously such modulations are especially reflected majorly in the proximity of tumor and is not expected to be systemic.

The figure 22 indicates negligible differences in the activation status of CD8+ T cells in the lymph nodes of tumor bearing mice in the control group and in the two treatment groups. It is understood that activated CD8+ T cells are predominantly present in the tumor microenvironment. T - cells when presented with antigen by antigen presenting cells get activated and migrate to the site of tumor.

6.2.3 CD4+ T cell activation status in Tumor

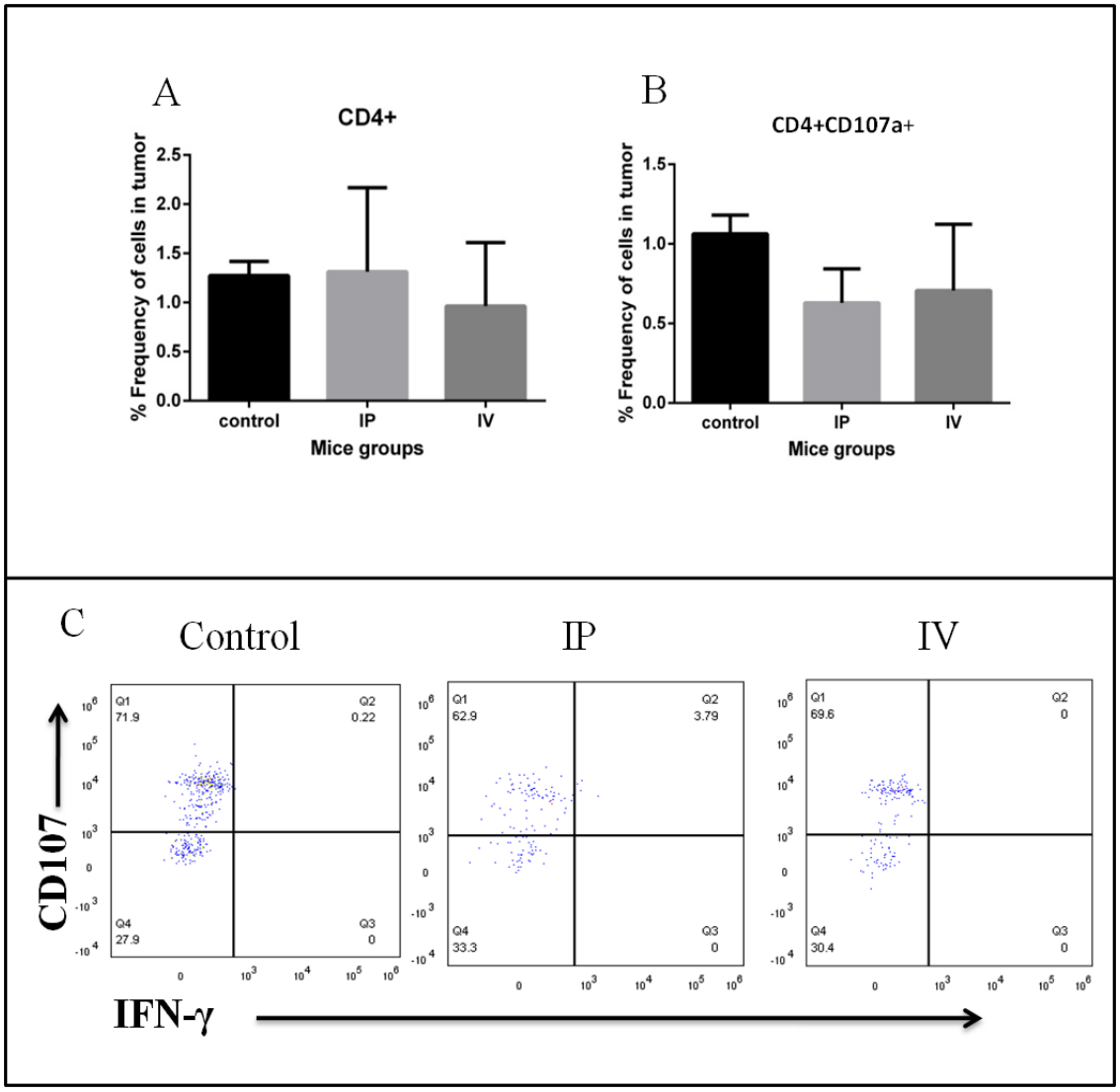


Figure 23: To assess the activation status of CD4+ T cells in tumor, tumor infiltrating lymphocytes were isolated on 18th day post tumor induction and surface stained with fluorochrome conjugated antibodies against markers CD4, CD107a and IFN-γ. [A] Graph shows the percentage of CD4+ T cells in tumor for groups control, IP(intra-peritoneal) and IV(intra-venous) [B]CD107+ cells in groups as described in [A]. [C] The layout represents double positive population (CD107a+IFNγ+) in tumor.

The schematic figure shows the plots of markers CD107a and IFN-γ which signify activation of cells in lymph node. There are several evidences suggesting the presentation of tumor associated antigens by tumor infiltrating Antigen presenting cells and production of tumor antigen specific T – cells. We assessed the activation statuses of T lymphocytes. The Chimeric IL-15 is specifically designed to interact with CD8+ T cells and hence we did not expect to observe significant changes in the activities of

CD4+ T cells in lymph node as well as tumor microenvironment. The plots clearly suggest that there are no CD107a and IFN-γ in lymph node and tumor.

Tumors can be defined as rapidly proliferating self cells. Immune system invests lot of resources in processes like negative and positive selection assuring tolerance. Because tumors are essentially self cells, the associated antigens are self in nature facilitating better interaction with MHC- I, redirecting its interaction with CD8+ T cells. This could be one of the reasons why CD4+ T cell response is less robust as compared to CD8+ T cells.

6.2.4 CD4+ activation status in Lymph node

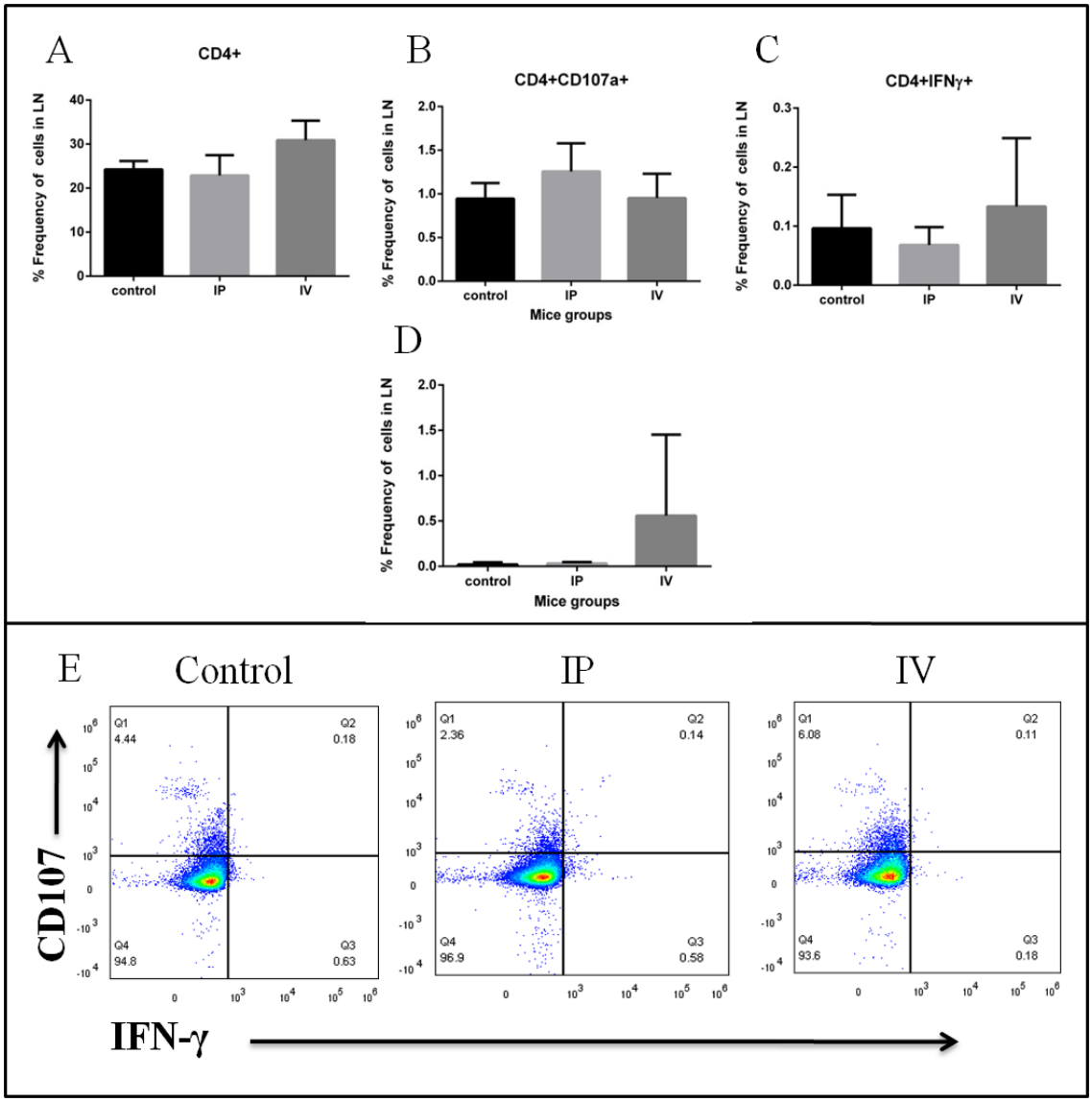


Figure 24: To assess the activation status of CD4+ T cells in lymph node, lymphocytes were isolated from lymph node on 18th day post tumor induction and surface stained with fluorochrome conjugated antibodies against markers CD4, CD107a and IFN-γ. [A] Graph shows the percentage of CD4+ T cells in the lymph node for groups control, IP(intra-peritoneal) and IV(intra-venous) [B]CD107a+ cells in groups as described in [A] [C] Frequency of IFN-γ+ cells[D] The graph shows percentage frequency of double positive cells(CD107a+IFNγ+) of CD4+ cells in Lymph node[E] The layout represents double positive population in lymph node.

Previous studies have indicated that Chimeric IL-15 is important in orchestrating CD4+ memory cells and plays a crucial role in maintenance of immunological memory[28].In this study markers of CD4+ memory T cells were not considered. There are reports suggesting cytotoxic CD4+ T lymphocytes as a part of tumor infiltrating lymphocytes[29]. CD4+ CTL have typical cytotoxic cell marker which includes

CD107a and are found to be functional when CD8+ T cells are impaired. This study shows presence of these unique cytotoxic cells in tumor and lymph node. There are no significant differences in the CD4+ and cytotoxic CD4+ T cell population in control and two treatment groups.

7. SUMMARY

The pleiotropic cytokine IL-15 presents a hope for targeting disease and there are evidences suggesting its use in infections like malaria, HIV and its role in tumor regression also is well established. The development of Chimeric IL-15 by Dr. Manoj Patidar included in silico work as well as wet lab experiments for designing and producing mutant Chimeric IL-15 with less limitations and additional desired characteristics to finely augment immune responses. The final design included IL-15 domain and IgG/2a domain adhered by a special linker which provided better flexibility, longer half-life and specificity in its action.

Our study included assessment of activation of CD8+ T cells when Chimeric IL-15 is administered via intra-venous and intra-peritoneal routes. For the study we recharacterized the molecule first by SDS PAGE and western blot and it was observed that the molecular weight of the chimeric molecule is higher than the predicted values which can probably be due to post translational modification or a tetramer formation. The reducing SDS PAGE showed that the monomer has molecular weight near to the predicted value. Further characterization of the molecule is required for confirmation of structure and properties.

B16f10 melanoma cell line was introduced in mice to study the effect of Chimeric IL-15 on reactivation of CD8+ cells in tumor model and the most effective route of administration. Tumor growth was monitored since the first appearance of tumor establishment. There was no regression observed in control as well as treatment groups during the course of treatment with Chimeric IL-15

There were no significant changes in observed as far as CD4+ T cell immune responses is considered in control and treatment groups in tumor and lymph nodes.

There was a significant increase in the frequency of CD8+ T cell infiltration and frequency of double positive (CD107a and IFN-γ) CD8+ T cells in the IP treatment group than in control and IV treatment group. These results point strongly towards the reactivation of CD8+ T cells in tumor and paves a way for adjusting number and concentration of dosing for significant regression of tumor.

Note – We had planned an objective specifically for in-vitro systems but we could not complete it due to consequences of the corona virus pandemic.

CONCLUSION

- **The molecular weight of Chimeric IL-15(dimer) is between 180 – 198 kDa.**
- **No tumor regression was observed at concentrations 5µg and 10µg in IP and IV treatment groups**
- **Flow cytometric data strongly suggested the reactivation of CD8+ T cells in tumor microenvironment**

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