Study of effect of Human Chimeric Il-15 on T-cell activation

A dissertation thesis submitted to

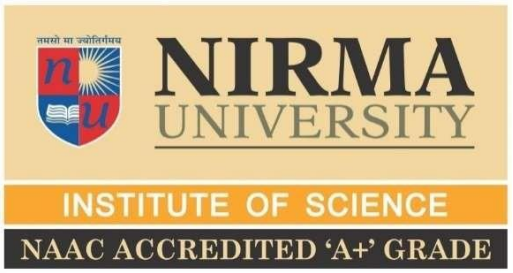
Nirma University

In partial Fulfillment for the Degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY/ BIOTECHNOLOGY



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INSTITUTE OF SCIENCE

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CERTIFICATE

There is to certify that the thesis entitled "***Study of effect of Human Chimeric Il-15 on T-cell activation***" submitted to the Institute of Science, Nirma University, in partial fulfillment of the requirement for the award of the degree of M.Sc. Microbiology/ Biotechnology, is a record of research work carried out by **Indrajit Kollai (21MBT010), Jeenal Khetani (21MBT014), Bhavya Sevak (21MBT035), Harsh Patel (21MMB017)** under the guidance of Dr. **Sonal R. Bakshi.** No part of the thesis has been submitted for any other degree or diploma.

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We, hereby declare that the dissertation work entitled **"*Study of effect of Human Chimeric Il-15 on T-cell activation*"** submitted to Institute of Science, Nirma University, is a record of the original work done by me under the guidance of **Dr. Sonal R. Bakshi** and with the support of **Mr. Devang Trivedi, Ms. Prachi Amin** and my lab members. The wokincorporated in the thesis has not been submitted for the award of any other degree, diploma, associateship, and fellowship, titles in there or any other University or other institution of higher learning.

I further declare that the material obtained from other sources has been duly acknowledged inthe thesis.

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Institutional Ethical Committee, Nirma University

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CERTIFICATE

This is to certify that the Project entitled "**Combination of Chimeric IL-15 and recombinant IL-12 as immunotherapy for lung cancer and colon cancer**" submitted by Dr. Sonal Bakshi, Institute of Science, Nirma University Ahmedabad, has been approved by the IEC, Nirma University.

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Signature with Date

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
ALL	Acute Lymphocytic Leukemia
APC	Antigen Presenting Cell
BCR	B- Cell Presenting Cell
CAR	Chimeric Antigen Receptor
CD	Cluster of Differentiation
CHO	Chinese Hamster Ovary
CTL	Cytotoxic T Lymphocytes
CTLA	Cytotoxic T-Lymphocyte Associated Antigen
DC	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ELU	Elution
EQU	Equilibrium
ETBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
HIF	Hypoxia Inducible Factor
HLA	Human Leukocyte Antigens
IFN	Interferons
IG	Immunoglobulin
IL	Interleukin
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription

LYS	Lysis
MAPK	Mitogen Activated Protein Kinase
MCL	Mantle Cell Lymphoma
MDSC	Myeloid Derived Suppressor Cells
MHC	Major histocompatibility Complex
MME	Membrane Metalloendopeptidase
NK	Natural Killer
PBMC	Peripheral Blood Mononuclear Cell
PD	Programmed Cell Death
PDGF	Platelet Derived Growth Factor
PDL	Programmed Cell Death Ligand
RPMI	Roswell Park Memorial Institute Medium
TAE	Tris Acid EDTA
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TIL	Tumor Infertility Lymphocytes
TLR	Tall Like Receptor
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

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ABSTRACT

Cancer is one of the leading causes of death in the human population after heart disease. Despite the availability of various modalities, the gravity of cancer mortality and morbidity increasing. The immune system plays an important role in the regression of cancer. Various Immunotherapeutic approach may help to overcome the burden within the tumor microenvironment. Among the various immunotherapeutic approach Cytokine-based immunotherapy has the potential to be an effective treatment option for certain types of cancer. Cytokines are proteins that help in regulation of immune responses, including the activation and proliferation of T cells and natural killer (NK) cells. Interleukin-15 (IL-15) is a cytokine that plays an important role in the immune system's response against the cancer. IL-15 activates and expands a specific type of immune cell called NK cells, which can recognize and kill cancer cells. IL-15 can also enhance the activity of other immune cells, such as T cells and dendritic cells, which can also contribute to the immune response against cancer. Previous studies data demonstrating the effectiveness and limitations of Cytokine-based immunotherapy analogs given as an exogenous immuno-oncology agent. To resolve the problem of low stability and serum half-life, earlier our lab has made modified chimeric Human IL-15 protein covalently linked with mouse IgG2 having high stability and longer half-life. *In vitro* and *In vivo* study will give an idea of the biological activity of chimeric huIL-15 signaling and $CD8^+$ T cell response in favour of activation and memory generation. To address our objective, we analyses the T-cell activation and secretion of INF-γ in the human HLA-A*02 and HLA-B*35:03 PBMCs with or without stimulation of ESAT- 6 and PRAME peptide respectively at different concentration using flow cytometry. Our data analyses demonstrate that the peptide stimulated PBMCs are secreting more IFN-γ compare to non-stimulated cells. Hence, we can conclude that the peptides designed for specific HLA were effectively activating the PBMCs. In future PBMCs will be stimulated along with or without Peptide and chimeric huIL-15 or in the presence of both and it will be looked for T-cell activation and secretion of INF-γ.

Keywords: Chimeric IL-15; Cytokines based immunotherapy; CD8+ T-cell activation;

1. INTRODUCTION

Cancer is a complex, multistep, and multifactorial disease defined by uncontrolled cell growth and proliferation, which results in the formation of abnormal mass or tumor anywhere in the body. Cancer can develop in any tissue or organ and can be caused by interplay of hereditary and environmental factors. Cancer incidence has gradually increased over the last few decades. Lung, breast, colorectal, prostate, and stomach cancer are the most prevalent types of cancer.

The term "morbidity" describes the incidence of disease or the frequency of illness within a community. The global cancer morbidity is reported to be 19.3 million new cases in 2020.

The state of being subject to death, or the number of deaths that occur in a population over a particular time period, is referred to as mortality. In 2020, there will be an expected 10 million cancer-related deaths worldwide. Lung cancer was most fatal type of cancer, accounting for the greatest number of cancer-related fatalities worldwide. Other major causes of cancer-related deaths include liver, colorectal, stomach, and breast cancer.

International Incidence of Cancer in the year of 2020:

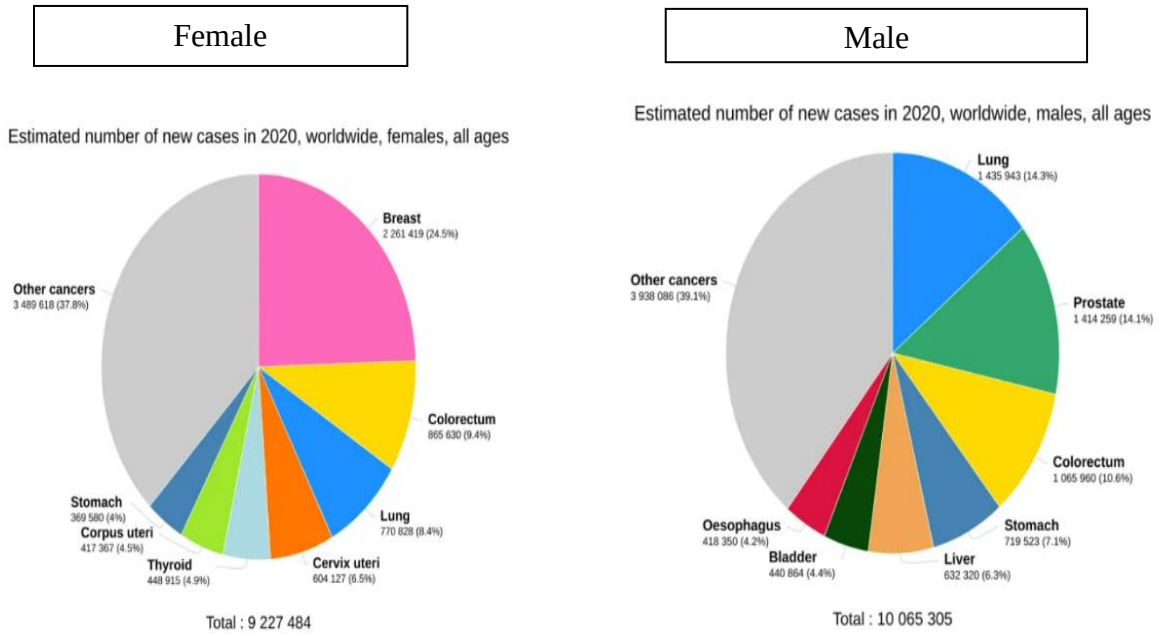


Fig 1: Incidence of cancer in male and female at global level. (Globocan 2020)

Globocan data for 2020 shows that cancer continues to be a major public health challenge. The data shows that lung cancer is the most commonly diagnosed cancer worldwide, accounting for 11.4% of all cancer cases. Other common types of cancer include breast, colorectal, prostate, and stomach cancer.

Indian Incidence of Cancer in the year of 2020:

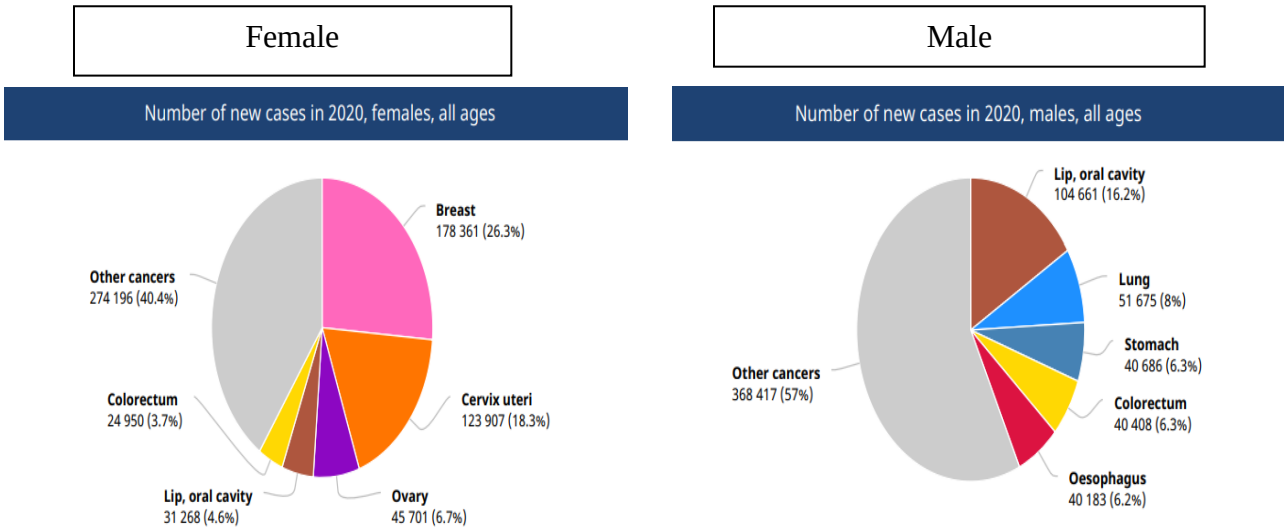


Fig 2: Incidince of cancer in india in boths the gender(Globocan 2020)

According to Globocan data from 2020, India had an estimated 1.39 million new cases of cancer, making it one of the top nations in the world with the highest cancer incidence. When compared to prior years, ther represents a significant increase in the number of cancer cases in the country. Breast cancer, lung cancer, and cervical cancer were the most prevalent types of cancer diagnosed in India, accounting for more than half of all new cancer cases.

1.1 Cancer

Cancer is an abnormal, continuous multiplying of cells. The cells divide uncontrollably and may grow into adjacent tissue or spread to distant parts of the body. The mass of cancer cells eventually become large enough to produce lumps, masses, or tumors.

Stages in progression of cancer:

- **Hyperplasia** occurs when cells within a tissue multiply faster than normal and extra cells build up. However, the cells and the way the tissue is organized still look normal under a microscope. Hyperplasia can be caused by several factors or conditions, including chronic irritation. (Cancer.net 2019)

- **Dysplasia** is a more advanced condition than hyperplasia. In dysplasia, there is also a buildup of extra cells. But the cells look abnormal and there are changes in how the tissue is organized. In general, the more abnormal the cells and tissue look, the greater the chance that cancer will form. Some types of dysplasia may need to be monitored or treated, but others do not. (Cancer.net 2019)
- **Carcinoma in situ** is an even more advanced condition. Although it is sometimes called stage 0 cancer, it is not cancer because the abnormal cells do not invade nearby tissue the way that cancer cells do. But because some carcinomas in situ may become cancer, they are usually treated. (Cancer.net 2019)

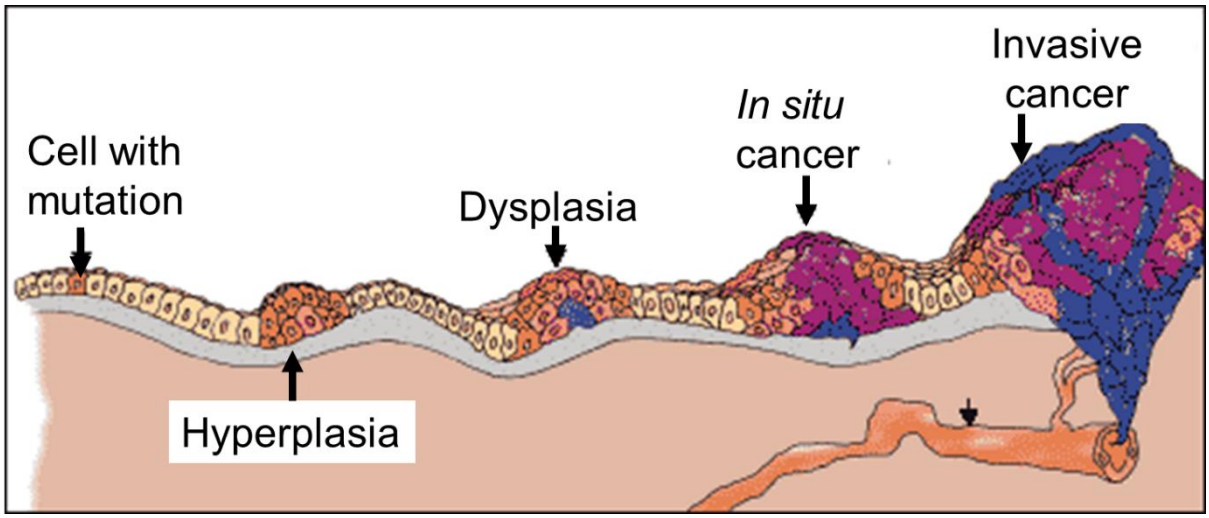


Fig 3: Metastases of cancer (W. LaMorte et el., 2016)

Cancer that has spread outside of the tissue from which it started and invaded neighbouring tissues or organs is referred to as invasive cancer, also known as infiltrating cancer. It is possible for invasive cancer cells to separate from the primary tumor and move through the bloodstream or lymphatic system to different regions of the body, where they can develop and spread by forming new tumors. Invasive cancer can be more challenging to treat and more likely to return than in situ cancer, which is contained to its initial site. Additionally, more aggressive than other cancer types, invasive cancer can grow and spread more rapidly. When describing how far along a cancer is, the stage of aggressive cancer is frequently used.

Cancer growth and progression are heavily reliant on the immune system. The basic purpose of the immune system is to detect and eradicate foreign or aberrant cells, including cancer cells. Cancer cells, on the other hand, can avoid immune surveillance by a variety of methods, including immunological checkpoint downregulation, immunosuppressive factor release, and tumor antigen mutation.

The mechanism by which the immune system detects and destroys potentially cancerous cells is known as cancer immunosurveillance. As part of its regular operation, the immune system is in charge of spotting and eliminating aberrant cells, including cancer cells. The immune system accomplishes by identifying specific proteins, referred to as antigens, on the surface of the abnormal cells as alien. T-cells and natural killer (NK) cells, two types of immune cells, are triggered when these antigens are found in order to target and eliminate the abnormal cells.

When cancer cells are present in the body but under the control of the immune system, the condition is referred to as immune-mediated tumor latency. Cancer cells are inactive and not actively proliferating in the condition, but they are still present. The immune system, which recognises and destroys cancer cells, is essential in preventing the spread of cancer.

However, some cancer cells have the ability to avoid immune detection, continue to grow, and proliferate, which causes tumors to form. Cancer cells can sometimes be kept in a dormant condition by the immune system, which can also control their growth. The can happen if the cancer cells are unable to create the blood vessels required to support their growth or if the immune system mistakenly views the cancer cells as foreign and begins to attack them.

Immune-mediated tumor dormancy can be helpful in halting the spread of cancer, but it can also be a cause of recurrence if the cancer cells start to become active once more. Understanding the mechanisms underpinning immune-mediated tumor dormancy may open up new possibilities for creating cancer treatments that use the immune system to stop the disease from coming back. Sometime cancer immunosurveillance fail which may lead to cancer progression. Cancer immunosurveillance can fail for a number of causes, including the following:

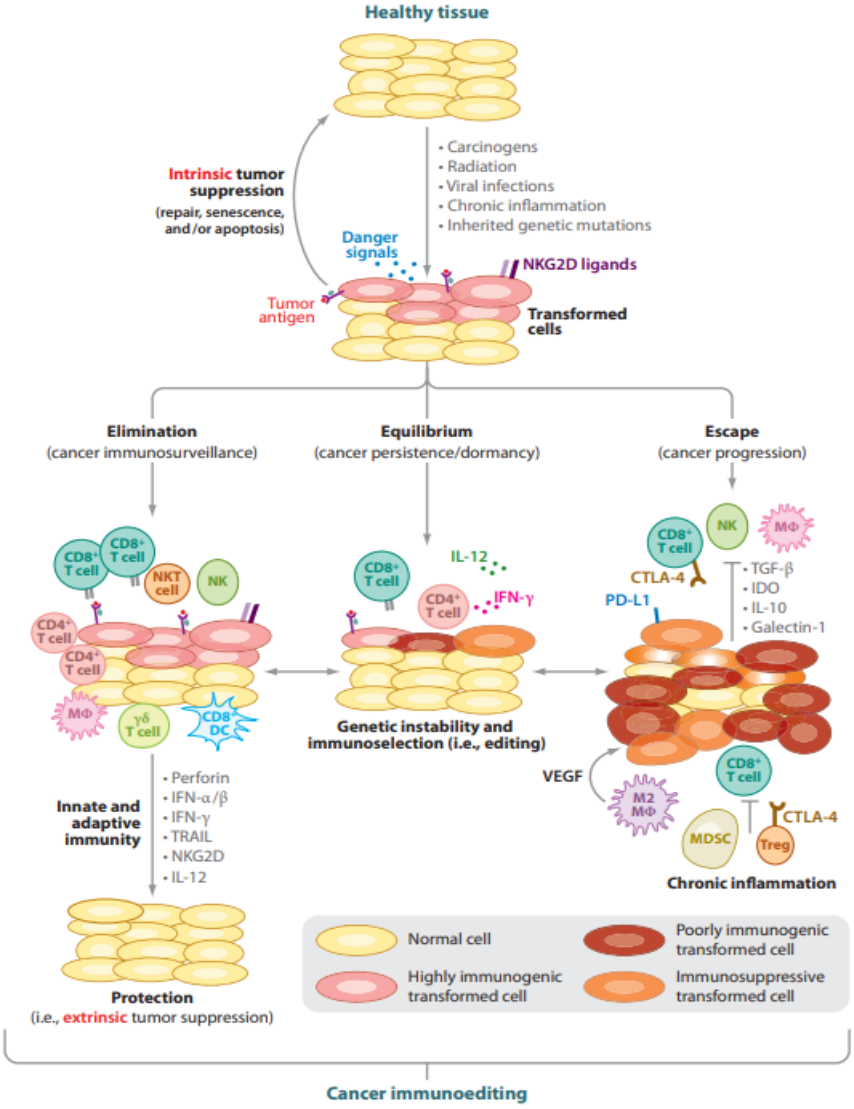


Fig 4: Natural innate and adaptive immunity to cancer (Matthew D et al., 2011)

There are multiple phases involved in the detection and destruction of cancer cells by cytotoxic T cells. First, the T cells must recognise the specific antigen on the cancer cell's surface. There is accomplished by the interaction between the T cell receptor (TCR) on the T cell's surface with the antigen-presenting molecule (MHC-I) on the cancer cell's surface. When a T cell recognises a cancer cell, it becomes activated and begins to proliferate, creating more cytotoxic T cells with the same antigen. These activated T cells travel to the tumor site and produce cytotoxic chemicals such as perforin and granezyme, which cause cancer cells to die.

Immunotherapy techniques that attempt to boost cytotoxic T cell activity against cancer cells have showed promise in the treatment of several malignancies. Immune checkpoint drugs, for example, suppress proteins that inhibit T cell activity, allowing cytotoxic T cells to kill cancer cells more efficiently. Adoptive cell treatment techniques, such as CAR-T cell therapy, entail designing T cells to produce chimeric antigen receptors (CARs) that recognise specific cancer cell antigens, resulting in increased cytotoxicity against cancer cells.

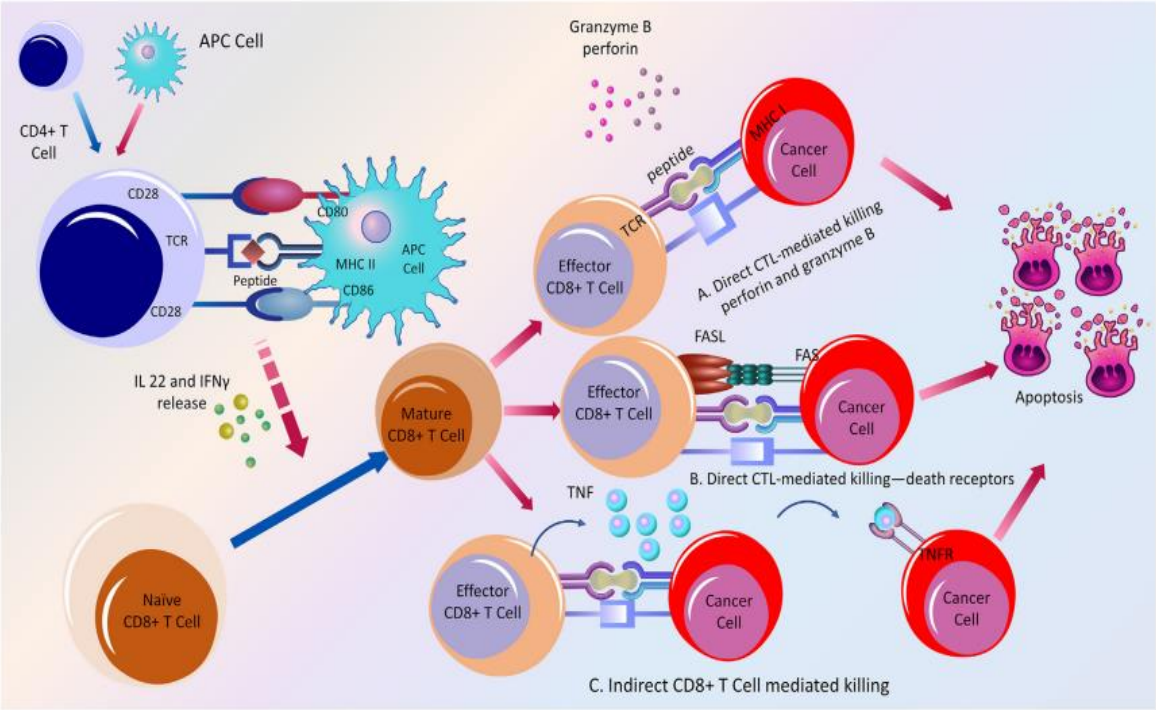


Fig 5: Cancer Pathogenesis and Therapy (Hossain, et al.,2022)

1.2 Immunotherapy

Immunotherapy is a type of cancer treatment that helps your immune system fight cancer. The immune system helps your body fight infections and other diseases. It is made up of white blood cells and organs and tissues of the lymph system.

Immunotherapy is a type of biological therapy. Biological therapy is a type of treatment that uses substances made from living organisms to treat cancer. (NCI et al., 2019)

1.3 Working process of Immunotherapy

As part of its normal function, the immune system detects and destroys abnormal cells and most likely prevents or curbs the growth of many cancers. For instance, immune cells are sometimes found in and around tumors. These cells, called tumor-infiltrating lymphocytes or TILs, are a sign that the immune system is responding to the tumor. People whose tumors contain TILs often do better than people whose tumors don't contain them.
Even though the immune system can prevent or slow cancer growth, cancer cells have ways to avoid destruction by the immune system. For example, cancer cells may:

- Have genetic changes that make them less visible to the immune system.
- Have proteins on their surface that turn off immune cells.
- Change the normal cells around the tumor so they interfere with how the immune system responds to the cancer cells.

Immunotherapy helps the immune system to better act against cancer.

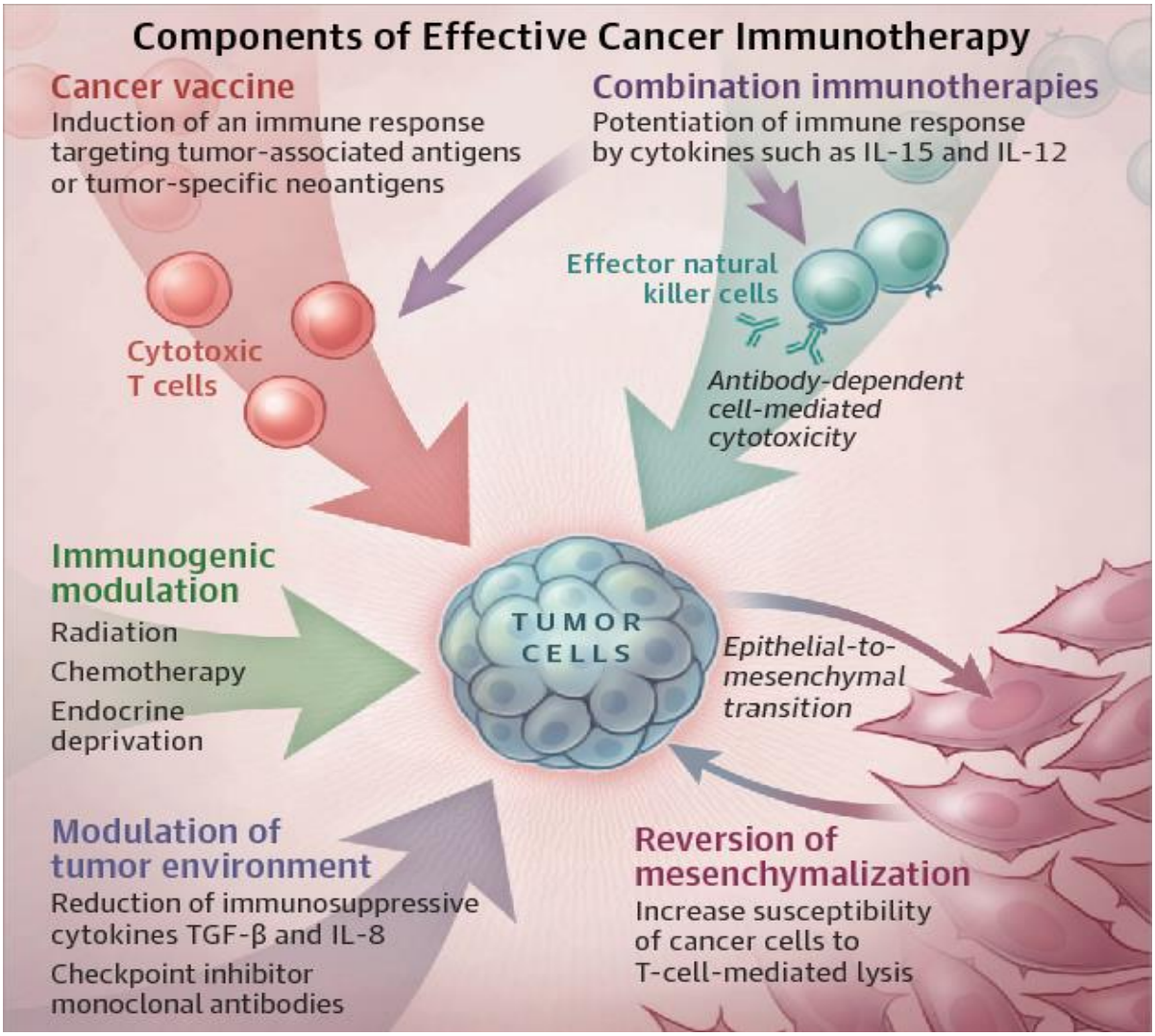


Fig 6: Components of effective Cancer Immunotherapy (J.Schlom et al., 2016)

1.4 Types of Immunotherapy

1.4.1 Immune Checkpoint Inhibitors

Immune Checkpoints Inhibitors are drugs that block immune checkpoints. These checkpoints are a normal part of the immune system and keep immune responses from being too strong. By blocking them, these drugs allow immune cells to respond more strongly to cancer.

- **Molecules working in checkpoint inhibitor Immunotherapy**

  Checkpoint inhibitor immunotherapy works by targeting molecules that regulate the immune response, particularly the checkpoint proteins that act as brakes on immune activation. Two important checkpoint proteins that are targeted in immunotherapy are CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed cell death protein 1), as well as its ligand PD-L1.

  Drugs that target CTLA-4 include ipilimumab, whereas drugs that target PD-1 include pembrolizumab, nivolumab, and cemiplimab. Drugs that target PD-L1 include atezolizumab, durvalumab, and avelumab.

  These drugs work by blocking the checkpoint proteins from interacting with their ligands, which allows for the activation of T cells and enhances the immune response against cancer cells. (NCI 2019)

- **Types of cancers that can be treated with immune checkpoint inhibitors**

  Immune checkpoint inhibitors are approved to treat some people with a variety of cancer types viz. Immune checkpoint inhibitors are approved to treat some people with a variety of cancer types viz. Breast, Bladder, Cervical, Colon, Head and Neck, Hodgkin Lymphoma, Liver, Lung, Renal Cell, Skin including Melanoma, Stomach, Rectal, any solid tumor that is not able to repair errors in its DNA that occur when the DNA is copied. (NCI 2019)

1.4.2 CAR-T Cell Therapy

CAR-T Cell therapy is similar to TIL therapy, but your T cells are changed in the lab so that they make a type of protein known as CAR before they are grown and given back to you. CAR stands for chimeric antigen receptor. CARs are designed to allow the T-Cells to attach to specific proteins on the surface of the cancer cells, improving their ability to attack the cancer cells.

- **Types of cancers can be treated with CAR-T cell therapy**

  It has been approved by the FDA for the treatment of certain types of Blood Cancers, specifically B-cell malignancies. These include:

  Acute lymphoblastic leukemia (ALL), Diffuse large B-cell lymphoma (DLBCL), Follicular lymphoma (FL), Mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL). (NCI 2019)

1.4.3 Monoclonal Antibodies

Monoclonal Antibodies are immune system proteins created in the lab that are designed to bind to specific targets on cancer cells. Some monoclonal antibodies mark cancer cells so that they will be better seen and destroyed by the immune system. Such monoclonal antibodies are a type of immunotherapy. Monoclonal antibodies may also be called therapeutic antibodies.

- **Working process of Monoclonal Antibodies**

  Monoclonal antibodies are laboratory-produced molecules that can mimic the immune system's ability to fight off harmful pathogens. When used to treat cancer, monoclonal antibodies target specific proteins or markers found on the surface of cancer cells, which can help to identify and destroy these cells.

  There are different types of monoclonal antibodies that work in different ways against cancer. Some monoclonal antibodies can block the proteins on the surface of cancer cells that help them to grow and divide, while others can attach to the cancer cells and trigger the immune system to attack them.

  In addition to their direct effects on cancer cells, monoclonal antibodies can also be used to deliver chemotherapy or radiation therapy directly to the tumor. By attaching chemotherapy or radiation therapy to the monoclonal antibodies, doctors can target the cancer cells more precisely and avoid damaging healthy cells.

  Overall, monoclonal antibodies have shown promise in treating a variety of cancers, including breast cancer, lung cancer, and lymphoma. They can be used alone or in combination with other cancer treatments, such as chemotherapy or radiation therapy. However, not all types of cancer respond to monoclonal antibody therapy, and the effectiveness of the treatment may vary depending on the individual patient's cancer and overall health. (NCI 2019)

1.4.4 Immune System Modulators

Immune system modulators are substances that stimulate or enhance the immune system's ability to fight off infections and diseases. They work by activating the body's natural defenses, including immune cells such as T cells, B cells, and natural killer (NK) cells, and increasing their production and activity.

- **Types of Immune System Modulators**

  I. **Vaccines**: These contain weakened or dead pathogens, or parts of them, which stimulate the immune system to produce antibodies and memory cells that can recognize and fight the actual infection if exposed to it later.
  BCG is a weakened form of the bacteria that causes Tuberculosis. It does not cause disease in humans. BCG is used to treat bladder cancer. When inserted directly into the bladder with a catheter, BCG causes an immune response against cancer cells. It is also being studied in other types of cancer. BCG stands for Bacillus Calmette-Guérin.

II. **Cytokines**: These are signaling molecules produced by immune cells that help regulate immune responses. Some cytokines, such as interferons and interleukins, can be used as medications to treat certain viral infections and cancers.

There are two types of cytokines that have been approved by the FDA for the treatment of certain types of cancer are:

Interferons: These are proteins that are naturally produced by the body in response to viral infections and other immune triggers. Synthetic versions of interferons, such as interferon-alpha and interferon-beta, can be used to treat certain types of cancer, such as melanoma, leukemia, and lymphoma. Interferons work by stimulating the immune system to attack cancer cells, and by inhibiting the growth of blood vessels that supply nutrients to tumors.

Interleukins: These are proteins that help regulate immune responses, including the activation and proliferation of T cells and natural killer cells. Interleukin-2 (IL-2) is a cytokine that has been approved for the treatment of advanced melanoma and renal cell carcinoma. IL-2 works by stimulating the production and activation of T cells and natural killer cells, which can attack cancer cells directly. (NCI 2019)

1.5 Clinical Data and Efficacy of Immunotherapy

Fig 7: Clinical Data of Immunotherapy (John Hopkins Medicine)

Efficacy: Immune checkpoint inhibitors significantly improved OS and PFS in both younger and older patients compared with controls, but the magnitude of benefit was clinically age-dependent. Patient's ≥ 65 y can benefit more from immunotherapy than younger patients.

1.6 Clinical Application of Cytokines in Cancer Immunotherapy

Cytokine	Secreting Cell	Cancer lmmunoregulation
IL - 2	CD4+ T cells, CD8+ T cells, NK cells, DCs Mast cells.	CD4+ T cell Differentiation CD8+ T cell cytotoxicity T cell proliferation NK cell proliferation and activation
IL - 12	DCs, phagocytes (monocytes macrophages and neutrophils)	CTL and NK cell Cytotoxicity IFN-Y secretion by T cells NKcells, ILCs antigen resentation
IL - 15	DCs, monocytes epithelial cells	CD8+ T cell, NK cell, and NKT cell cytotoxic,
IL - 18	Macrophages	Anticancer: Activation of cytotoxic T cells and NKcells perforin-mediated cytotoxicity in NKcells Pro-cancer: Tumor angiogenesis and metastasis
IL - 21	CD4+ T cells, NKT cells	CD8+ T cell, NK cell, and NKT cell cytotoxic
IFN	CD4+ T cell, CD8+ T cells, NK cells, B cells, NKT cells, professional APCs	Anticancer: Cell surface MHC class expression T cell, NK cell, and NKT cell migration into tumors Initial priming and differentiation of CTLs

Table 1: Immunostimulatory Cytokines for Cancer Immunotherapy (Suheil Albert Atallah-Yunes et al. 2022)

1.6.1 T-Cell Activation by Cytokines

When an antigen-presenting cell (APC) such as a dendritic cell presents an antigen to a T-cell receptor (TCR) on the surface of a T-cell, cytokines are released by the APC and surrounding cells.

These cytokines bind to specific receptors on the surface of the T-cell and initiate a signaling cascade that leads to T-cell activation. Cytokines can activate different types of T-cells, including helper T-cells and cytotoxic T-cells.

Helper T-cells are activated by cytokines such as interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-12 (IL-12). IL-2 is produced by activated T-cells and promotes the proliferation and differentiation of helper T-cells, leading to a stronger immune response. IL-4 promotes the differentiation of helper T-cells into Th2 cells, which are important for antibody production. IL-12 promotes the differentiation of helper T-cells into Th1 cells, which are important for activating cytotoxic T-cells. (S. Garris et al., 2018)

Cytotoxic T-cells are activated by cytokines such as interleukin-2 (IL-2), interleukin-12 (IL-12), and interferon-gamma (IFN-γ). IL-2 and IL-12 promote the proliferation and differentiation of cytotoxic T-cells, while IFN-γ enhances their ability to kill infected cells.

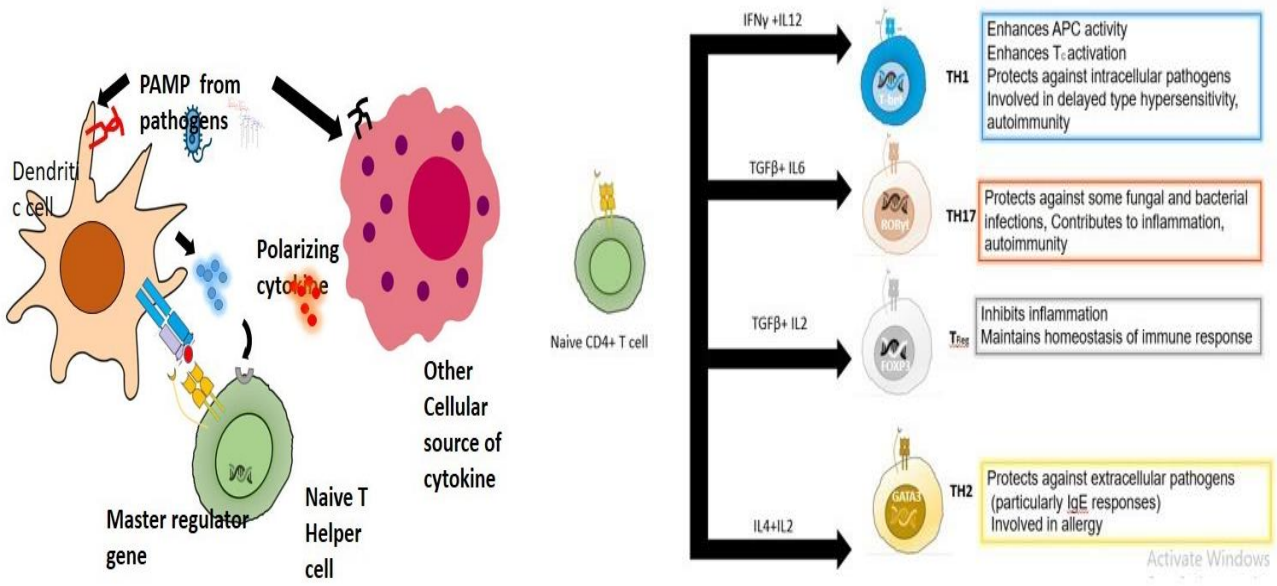


Fig 8: T-Cell Differentiation and Activation (Arpan et al., 2018)

1.7 IL-15 as a Cytokine and its role in Immunotherapy

Interleukin-15 (IL-15) is a cytokine that plays a crucial role in the immune system. It was first discovered in 1994 and is part of the family of cytokines that includes interleukin-2 (IL-2) and interleukin-7 (IL-7). Like IL-2, IL-15 is a member of the four alpha-helical bundle family of cytokines, which means that it has a similar protein structure. However, IL-15 has a unique set of properties that distinguish it from other cytokines in its family.

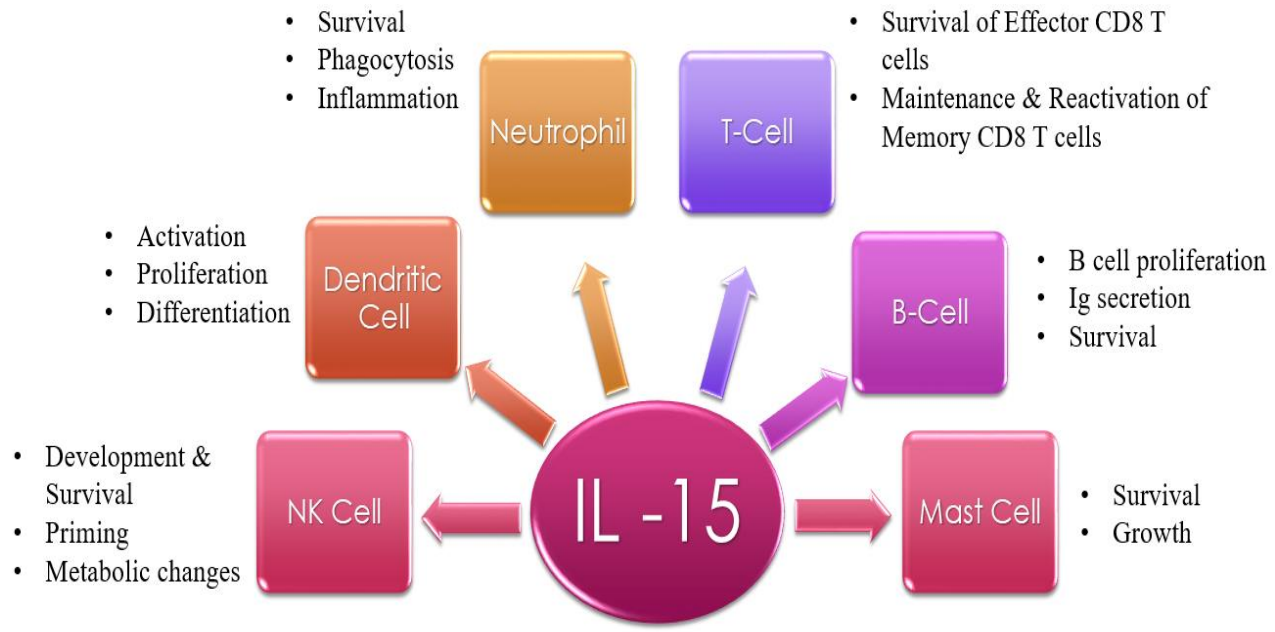


Fig 9: Role of IL – 15 (Jakobisiak, et al., 2011)

Interleukin-15 is having multiple functions and it is produced from various sources of the body and it is also having both the connection of innate immunity and adaptive immunity. It is γc-cytokine and it is also known for the T cell growth factor. It is having l4-15kDa of molecular weight with I14amino acids and it is a member of 4uhelical bundle family of cvtokine.lL-15 consists of host defense mechanism against intracellular pathogens and it also act as modulator .IL-15 is having a good memory to act against the dangerous ant1gen so it is used as immunological memory which is specific to immune system .It is also having role of differentiation of the memory T cells and it is also seen in the γc cytokine and IL-15 works as the proinflammatory cytokine which is potent in nature. It is also having role in inflammation and autoimmune disease and even it act as increasing recognition of link between development of cancer and inflammation. IL-15 act as the maintaining cytokine which help in the proliferation of NK cells, B, T lymphocytes and even helps in hematological malignancies. Its main role is to maintain the immune response which are antiviral by the regulation of IFN-aß secretion along with the proliferation of NK cells/ lt is having IL-15 SRa which is a specific receptor for expressing an antigen presenting cells and it Is having function of the dictating T cells response and in the regulation of the tissue repair and modulating inflammation homing of B cells for the activation of NK cells. (Robinson, et al., 2017)

The IL-15 receptor is a complex of three different proteins: IL-15Rα, IL-2/15Rβ, and the common gamma chain (γc). The IL-15Rα subunit binds to IL-15 with high affinity and then presents it to the IL-2/15Rβ and γc subunits, which together form the signaling complex.

The IL-15 receptor is expressed on a wide range of cells, including T cells, B cells, natural killer (NK) cells, dendritic cells, monocytes, and macrophages. IL-15 signaling plays a critical role in the development and survival of NK cells, and is also important for the proliferation and survival of memory CD8+ T cells.

The presentation of IL-15 to its receptor initiates a signaling cascade that leads to the activation of several downstream pathways, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the Phosphatidylinositol-3-kinase (PI3K) pathway. These pathways ultimately result in the transcriptional activation of genes involved in cell survival, proliferation, and differentiation.

The development of several tumor types, including melanoma, breast cancer, and leukemia, has been shown to be inhibited by IL-15. According to studies, IL-15 can improve immune cells' performance and encourage their infiltration into tumors, which increases tumor cell death and slows tumor development. (Patidar et al., 2021)

In cancer immunotherapy, cytokines based therapy serve as a powerful tools. The limitations are its short half-life & may not be that efficiently promotes the anti-tumor response because of its poor bioavailability IL-15 has dominant role in immune response IL-15 shares two common subunits C & binds heterotrimeric receptor It shows similar biological functions like IL-2 but third receptor is unique for each cytokines. Both cytokines has role to maintain differentiation & proliferation of T cells. IL-2 mainly plays role in AICD and in the maintenance of Treg especially exclude out the T cells which are auto reactive & prevent from auto immune diseases (Sakaguchi, el al., 1995). Unlike, IL-15 provides long term survival of CD8’ memory T cells and high-avidity for invading pathogens (Waldmann, et al., 2015). To make IL-15 more efficient, chimera fusion protein technology were used first "In silico" where they intent to improve activity of trans-presentation and target specificity. The chimeric IL-15 made by Fc portion of Ig which improves its stability and half-life (Maj, et al., 2013)

1.8 Chimeric Interleukin-15

The chimeric interleukin -15 is the genetically engineered protein that has been modified to give higher therapeutic outcomes and better half-life. The chimeric IL-15 is IgG2/2a molecule covalently attached to the interleukin-15 molecule, the molecule fused with the help of a linker molecule. The chimeric IL-15 has half-life of 40 hours while the naturally occurring IL-15 has half-life of 1 hour, which is 40 fold increase in half life. (Patidar et al., 2021)

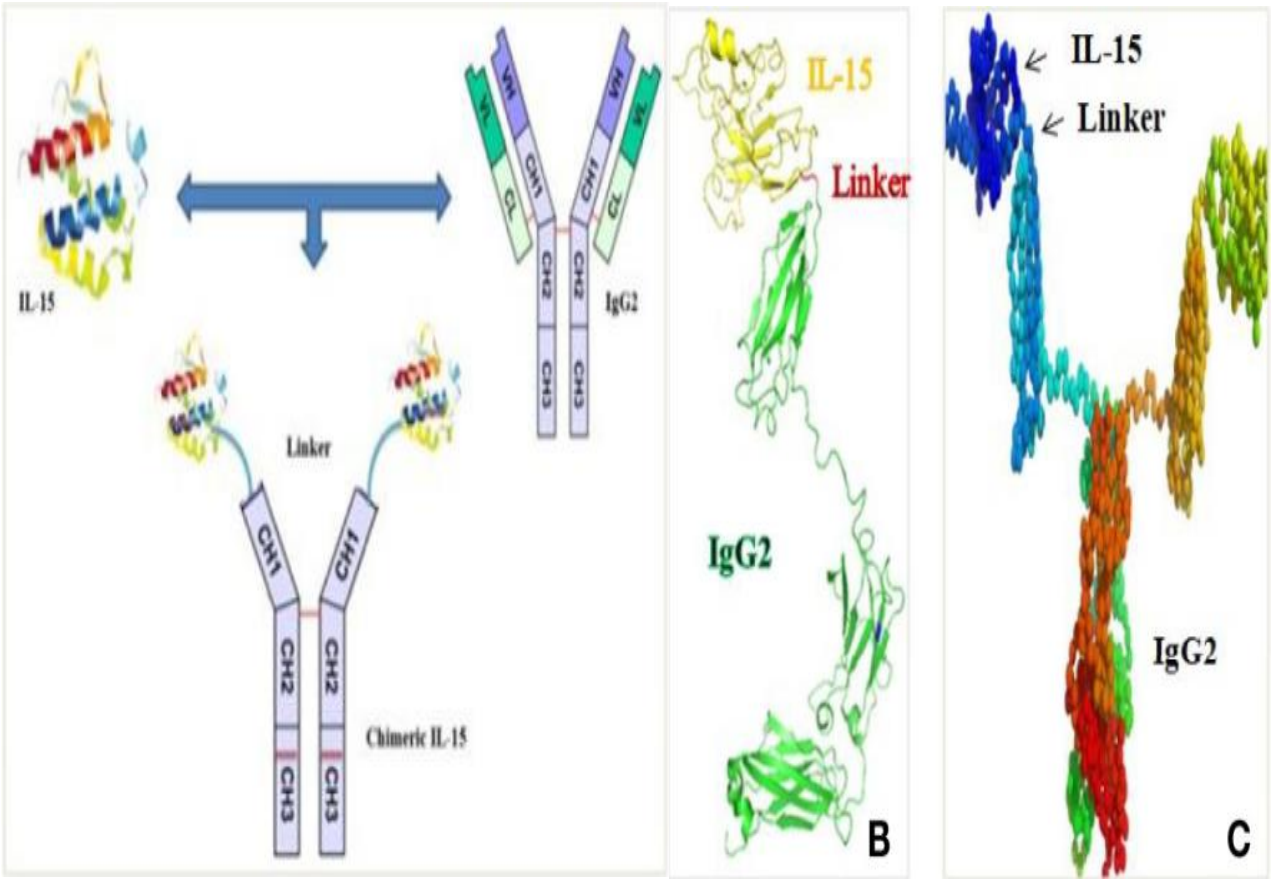


Fig 10: Dimeric Chimeric IL-15 (Patidar et al., 2021)

2. REVIEW LITERATURE

2.1 Tumor Microenvironment

2.1.1 Introduction of TME

Nowadays, Immunotherapies get attention in the treatment of many malignancies. Immunotherapy uses the host immune system; so that it can grind the cancer cells and destroy them. Immune checkpoint inhibitors (ICIs) known as first-generation antibody-based immunotherapy, role is to block the receptor/ligand interactions between different molecules involved in the regulation of T cell activation and function, such as programmed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) (Sadeghi Rad et al., 2020).

A tumor is a heterogeneous collection of infiltrating and resident host cells, secreted factors, and extracellular matrix (Nicole M. A. and M. Celeste Simon, 2020). Tumor cells make various molecular, physical, and cellular changes within the host cell so that they support tumor growth and progression (Nicole M. A. and M. Celeste Simon, 2020). The TME is an essential part of tumor initiation and progression. Several studies have been carried out to know the function of TME in cancer progression. It has been thought that the immune contexture, the cell type, the density of the cells, and the location of the cells in the TME may be associated with disease outcomes (Sadeghi Rad et al., 2020).

2.1.2 Establishment of the TME during Cancer Progression

The complexity of the TME is due to defective blood vessels and unregulated cancer cell proliferation. Several hallmarks of the TME are Acidic pH, hypoxia, endogenous H_2O_2, and the alteration of extracellular matrix (ECM), which plays a major role in tumor progression and cancer metabolism. An anaerobic glycolytic excretion of the protons (H^+) and lactate by the ATPase, monocarboxylate transporter 1 (MCT1), and MCT4 are responsible for the acidic pH. Hypoxia (partial pressure of oxygen <10 mmHg) is sometimes shown in solid tumors. Because of the uncontrolled growth of the tumor cells, the center cell mass becomes hypoxic due to the distance from the blood vessel, which ultimately leads them to nutrient and oxygen-deficient. To overcome the tumor cells need to induce hypoxia-inducible angiogenic factors, such as VEGF. In addition, the overexpression of the hypoxic response pathways mediator such as hypoxic-inducible factor-alpha (HIF-α) and HIF1β capable of binding with hypoxia response genes that are involved in tumor survival and angiogenesis (Sadeghi Rad et al., 2020).

Reactive oxide species (ROS), in specific H_2O_2, has a critical effect on various physiological processes. “Oxidative stress” is referred to as the maintenance of the low level of H_2O_2 and its associated physiological redox reaction. The low level of H_2O_2 is required for processes involved in cell proliferation, differentiation, migration, and angiogenesis. In certain pathophysiological condition also known as “Oxidative distress” the level of H_2O_2 goes above 100nM which affect the process of cell maintenance. Tumor cells take advantage of the condition and modulate signaling pathways and transcription factors, increasing cell proliferation, cell metabolism maintenance, and adapting to hypoxic stresses. (Sadeghi Rad et al., 2020)

Moreover, ECM proteins are often called the matrisome secreted by cancer cells. ECM proteins that are involved in cell-cell attachment are decreased, including laminin subunit beta-1 (LAMB1), laminin subunit gamma-1 (LAMC1), laminin subunit alpha-4 (LAMA4), and collagen alpha-1(XV) chain (COL15A1). However, the expression of matrisome is responsible for cell migration and tumor invasion, including fibronectin (FN1), cartilage oligomeric matrix protein (COMP), cathepsin B (CTSB), and collagen alpha-1(XI) chain (COL11A1), are induced during tumorigenesis (Sadeghi Rad et al., 2020).

2.1.3 Cell types in the TME

TME is made up of a heterogenous immune cell population. Figure 1 shows different cell populations in TME

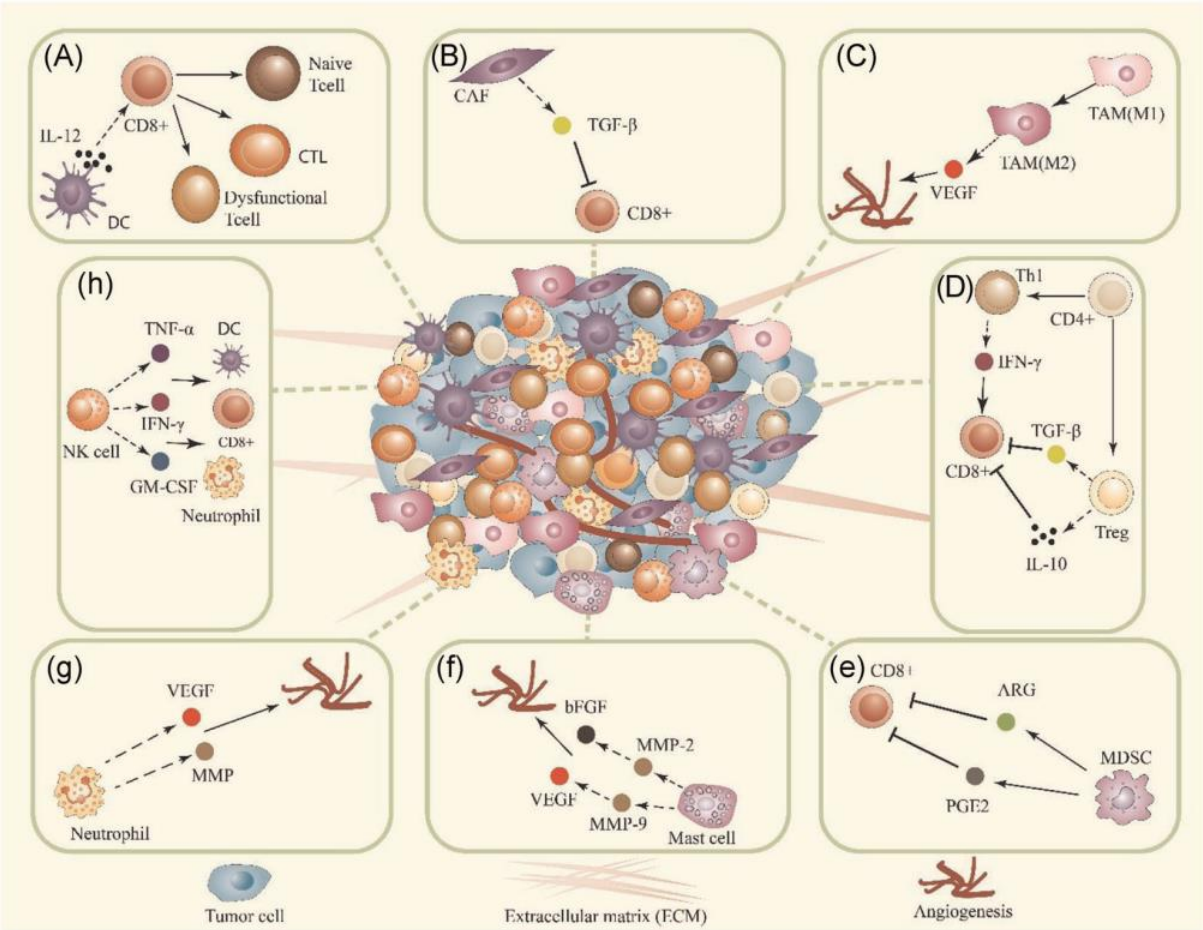


Fig 11: Different roles of TME-driven cell population in cancer progression.

(A) T-Cell (B) Cancer-associated Fibroblast (CAF) (C) Tumor-associated Macrophage (TAM) (D) $CD4^{+}$ T-Cell (E) Myeloid-Derived Suppressor Cell (MDSC) (F) Mast cell (G) Neutrophil (H) Natural killer (NK) (Sadeghi Rad et al., 2020).

I. Neutrophils

Most common type of circulatory cells is the neutrophiles in cancer patients. Two types of neutrophils are found in blood circulation:

One is circulating neutrophils, which circulate freely and are recruited into the tumor and the second is marginated neutrophils, which are bound to capillary endothelium. In TME neutrophils acts as both growth promoter and suppressor. At the very early stage of tumor growth neutrophils circulate into the tumor microenvironment and secrete cytokines such as interferon-gamma (IFN-γ), and reactive oxygen species which promotes tumor cell apoptosis. Conversely, in the late tumor stage when the tumor is developed, neutrophils promote cell growth by releasing growth factors such as VEGF and producing matrix metalloprotease (MMP)-9 to stimulate angiogenesis (Nicole M. A. and M. Celeste Simon, 2020) (Sadeghi Rad et al. 2020).

II. Mast Cells

Mast cells (MCs) are derived from pluripotent bone marrow progenitor cells and they are CD34, CD117, and CD13 positive.

MCs are present in the whole body and have various physiological and pathophysiological roles. They exploit various classes of receptors such as the high-affinity IgE receptor and G-protein coupled receptors which help MCs in the detection of inflammatory, immunologic signals. Once, MCs are stimulated they release cytoplasmic granules containing, hertamine, heparin, and granule-associated protease which result in vessel permeability, induce inflammation, and stimulate peripheral nerves. MCs are also able to activate $CD8^+$ T cells by releasing interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) at the site of inflammation. Moreover, MCs cells play a protumorigenic role in various cancer. They are capable to secrete MMP2 and MMP9 which liberate VEGF and fibroblast growth factor from ECM thus stimulating angiogenesis (Sadeghi Rad et al. 2020).

III. Myeloid-derived suppressor cells

Myeloid is formed through a process known as Myelopoiesis. Whenever any inflammation or cancer occurs the process is disturbed. Hence, stronger myelopoiesis happened which increases the migration of undifferentiated cells to the inflammatory or cancerous site. These cells show strong immunosuppressive properties and with their myeloid origin, they are known as myeloid-derived suppressor cells (MDSCs). MDSCs have two different types based on their phenotypic and morphological features; M-MDSC C reflecting monocytic progenitor cells and PMN-MDSC for polymorphonuclear. Through the secretion of immunosuppressive cytokines, the production of reactive oxygen species (ROS), and an increase in prostaglandin E2 (PGE2) and arginase (ARG) levels, MDSCs exert their immunosuppressive role. PGE2 function as a pro-inflammatory factor in tumor development and is a suppressor of host-antitumor activity. Arginase plays a key role in the reduction of T cells by downregulating T-cell receptors (Sadeghi Rad et al. 2020).

IV. Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are commonly found in TME and they promote tumor growth through nutritional and immunological mechanisms. In association with tumor development CAFs produce various growth factors such as fibroblast growth factor 2 (FGF2), and platelet-derived growth factor C (PDGFC), which enhance angiogenesis. Transforming growth factor-beta (TGF-β) is a major source of CAFs. Desmoplastic stroma is established by TGF-β during TME formation (Sadeghi Rad et al. 2020).

V. Natural killer cells

Natural killer cells (NK cells) are also referred to as patrolling cells, which continuously circulate in the bloodstream. Mainly they target virally infected host cells but also they act on tumor cells. NK cells are divided into two categories based on the function they performed. First, those that directly participate in the cell-mediated killing of tumor cells, and second, those that secrete inflammatory cytokines/chemokines such as IFN-γ and TNF-α. NK cells are highly effective in killing tumor cells within blood circulation but they are less effective in killing under TME (Nicole M. A. and M. Celeste Simon, 2020).

VI. Dendritic cells

Dendritic cells (DC Cells) are the main antigen-presenting cells. They recognize and capture the antigen and present it to T cells. DC cells have inherently anti-tumor functions (Nicole M. A. and M. Celeste Simon, 2020). Conventional DCs (cDCs) are divided into two subcategories: Conventional type 1 dendritic cell (cDC1s) play an important role within tumors by inducing tumor-specific $CD8^+$ T cells and secreting IL12. While Conventional type 2 dendritic cells (cDC2s) induce $CD4^+$ T cell response (Sadeghi Rad et al. 2020).

VII. Tumor-associated macrophages

Macrophages help in the immune response through phagocytosis and antigen presentation. Tumor-associated macrophages (TAMs) divide into two types: M1 macrophages also known as tumoricidal which are tumor suppressors, while M2 macrophages also known as tumorigenic are tumor promotors. M2 macrophages are the most dominant type of TAMs in TME (Sadeghi Rad et al. 2020). M2 secrete various growth factors including VEGF and pro-inflammatory cytokines such as TNF-α (Sadeghi Rad et al. 2020).

VIII. CD4+ T cells

$CD4^+$ T cells are involved in an adaptive immune response. They were involved in MHC class II and recognized antigens. $CD4^+$ T cells has variety of subtypes which helps in the recognition of various cells in the tumor microenvironment (Nicole M. A. and M. Celeste Simon, 2020). T helper cells (Th1) are the most studied $CD4^+$ T cells. Th1 secrete pro-inflammatory cytokines such as IFN-γ, which enhance $CD8^+$ T cells and directly stop tumor cell growth through cell cycle arrest, declining cell generation potential. Through the secretion of IL-2 and IFN-γ they promote $CD8^+$ T cells response (Nicole M. A. and M. Celeste Simon, 2020). $CD4^+$ T cells also have antitumor immunity, for ther, they differentiate into T regulatory (Treg) cells (Sadeghi Rad et al. 2020).

IX. CD8+ T cells

$CD8^+$ T cells are the main component of an adaptive immune response. They involved in MHC class I. Cytotoxic T cells are best studied $CD8^+$ T cells. Cytotoxic T cells express target cell death inducers, such as perforin 1 (PRP1), granzyme A (GZMA), and granzyme B (GZYMB) (Sadeghi Rad et al. 2020). $CD8^+$ T cells performed angiogenesis through secretion of IFN-γ which helps in suppression of tumor (Nicole M. A. and M. Celeste Simon, 2020). $CD8^+$ T cells capable to generate CD8 T memory cells which helps to give protection when tumor relapse occurs. CD8 T memory cells are two types circulatory and non-circulatory. Circulatory CD8 T memory cells divides further into stem cell memory (T_{scm}), central memory (T_{cm}) and effector memory (T_{em}). While non circulatory CD8 T memory cells are tissue resident memory CD8 T cells (T_{rm}).

Sometimes, dysfunction of ther memory cells leads to cancer progression. T_{em} cells have been associated with anti-PD-L1 activity. In the ovarian, endometrial, breast and lung cancer patient tumor infiltrated $CD8^{+}CD103^{+}$ T_{rm} cells have been reported (Fu, C., & Jiang, A., 2018).

X. T_{regs} Cells

T_{reg} cells are mainly involved in the anti-inflammatory and suppress auto-immunity responses. T_{reg} cells are composed of various different cells from which, $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs being most studied (Fu, C., & Jiang, A., 2018). However, in the context of TME T_{reg} cells are tumor promoting. For that they reduced anti-tumor immunity. For instance, they secrete IL-2 which modulate the function of NK cells. They also capable to secrete cancer cells promoting growth factors (Nicole M. A. and M. Celeste Simon, 2020). The inhibitory receptors such as CTLA-4, Tim-3, PD-1, GITR, LAG3, and BTLA are expressed by T_{reg} cells that suppresser for DCs (Fu, C., & Jiang, A., 2018).

As discussed above every immune cell has two types of action in TME either they are tumor suppressor or tumor promoter Figure 2.

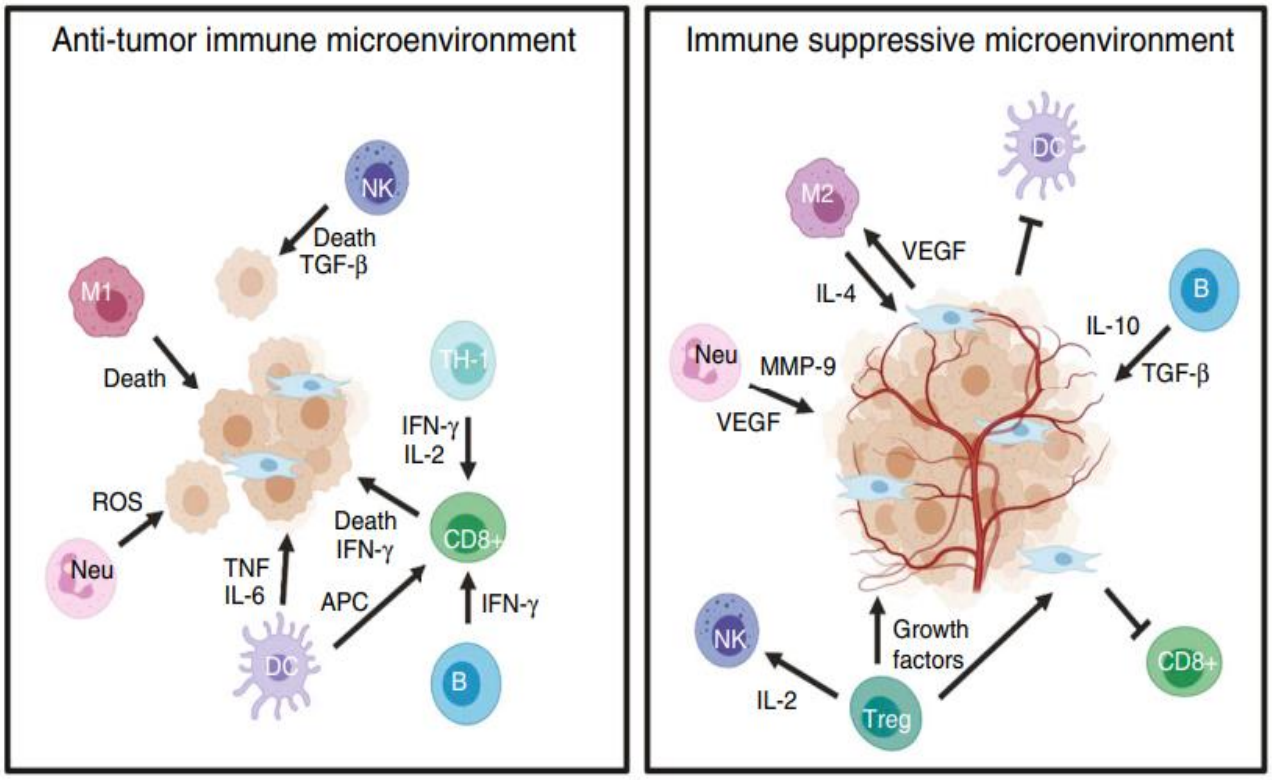


Fig 12: Impact of immune cells within the tumor microenvironment (Nicole M. A. and M. Celeste Simon, 2020).

2.2 Peptide Selection

HLA molecules are proteins located on the surface of most cells in the body that play an important function in the immune system by detecting and presenting foreign items for destruction to immune cells. HLA molecules are essential in the immune system's detection and death of cancer cells. Cancer cells can be identified as foreign by the immune system because they frequently exhibit aberrant proteins on their surface known as tumour antigens. HLA molecules can convey these tumour antigens to immune cells, triggering an immunological response against the cancer cells.

HLA classification has the potential to be utilized in the treatment of cancer to detect potential therapeutic objectives. The process of immunotherapy involves the application of drugs or other substances to amplify the immune system's capability to identify and combat cancerous cells. By detecting the particular HLA molecules generated by the cancerous cells of an individual, treatments that target these molecules and stimulate an immune reaction against the cancer can be formulated.

Amino acid chains known as peptides can bind to HLA molecules and appear on cell surfaces for identification by immune cells. Peptides with antigenic properties bind to HLA molecules and trigger an immune reaction. HLA molecules can present peptides originating from tumor antigens on the exterior of cancer cells, making them identifiable and susceptible to immune cell attack in the cancer milieu. The immune system can recognize abnormal or altered proteins present on the surface of cancer cells as foreign antigens.

Immunotherapies that are based on peptides may involve administering synthetic peptides that imitate tumour antigens. Furthermore, personalised cancer vaccines can be tailored to a patient's unique HLA type and tumour characteristics. Peptide-triggered HLA molecules provide a potential avenue for developing more potent cancer therapies by activating the immune system against cancer cells in the way. (Orenbuch, Rose et al. 2020)

In our experimental set we use a similar strategy we have selected HLA type based on a literature review for cancer HLA-B* 35:03 and as we are using BCG as an internal control the most common HLA type found in it is HLA-A*02, and corresponding to them we have selected two peptide ESAT-6 protein (AMASTEGNV) (Lalvani at el., 1998) and PRAME (FPEPEAAQP) protein. (Stanojevic at el., 2021)

3. OBJECTIVES

A. Isolation and Purification of Chimeric huIL-15

- Bacterial culture & Plasmid isolation
- Transfection in CHO cells
- Purification of IL–15 with the Protein G Affinity Chromatography

B. Activation and Proliferation of HLA-specific CD8+ T-cells

- PBMC isolation from HLA-A*02:01 and HLA-B*35:03 specific blood samples
- Treatment with Peptides & Chimeric huIL -15 to check T-Cell activation
- CFSE staining for T-Cell proliferation and Intercellular staining for Interferon Gamma secretion

4. MATERIALS

❖ Plasmid Isolation Kit

Micherey-Nagel (NucleoBond®Xtra Midi)

EQU buffer

LYS buffer

RES buffer

Wash buffer

ELU buffer

70% ethanol

Isopropanol

❖ Agarose Gel Electrophoresis

Agarose

1x TAE buffer

ETBr

❖ PBMC Cell Isolation

Blood sample

Hi-SEP™ LSM 1077 media

Trypan Blue Dye

Neubauer's chamber

❖ Cell culture Instruments

Inverted Microscope

T25 OR T75 Culture Flask

96 Well Microtiter Plates

CO_2 Incubator

Centrifuge

Complete Media

Antibiotics

Trypsin

❖ CHO Cell Line

CHO cells were grown in the laboratory condition.

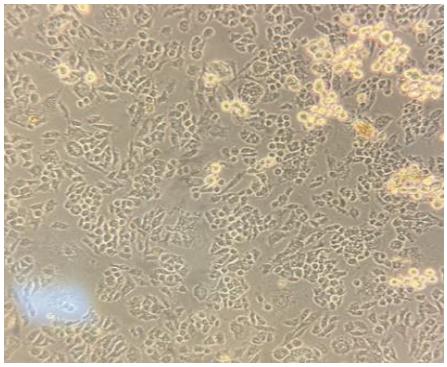


Fig 13: CHO cell line

Morphology	Fibroblast
Growth mode	Adherent
Growth media	RPMI, 10% FCS
Confluency of cell	70- 90% in T25 flask

Table 2: Characteristics of CHO cell line

❖ Cell surface Staining

10% RPMI complete media (10% Human AB serum /FBS in RPMI media)

FACS Buffer/ Staining buffer (1% Human AB serum in PBS)

Fc Block (1% Human AB serum in FACS buffer)

1 % Formaldehyde

❖ Cell surface Staining

Brefeldin A

Cytofix/ Cytoperm fixation buffer (BD biosciences®)

IX Perm wash (10X perm/ wash buffer is diluted in double distilled water)

1% Formaldehyde (diluted in FACS buffer)

Fluorescently Labeled Antibodies

Flow Cytomete

Data Analysis Software: Flow – Jo

5. METHODS

❖ Plasmid Isolation Kit

1. Cultivation of bacterial cells:

- LB broth was prepared in flask containing 100ml volume and inoculated with 100µl of bacterial culture (Considering I tube as blank) and 100µl (1x) of antibiotic in flask
- 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
- 8ml of Resuspension buffer was added in each tube and vortexed it for 2-3 minutes. 8ml 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
- Equilibrate a NucleoBond® Xtra Column together with the inserted column filter with 12ml Equilibration Buffer
- 8ml of Neutralising Buffer was added in tube. Tubes were mixed thoroughly by inverting it for 6-8 times until blue samples turn colorless completely. (Do not vortex to avoid shearing of genomic DNA
- Tubes were centrifuged for 10 min at 5000x g at 4°C and precipitate remove
- Supernatant loaded onto the NucleoBond® Xtra Column. Allow the column to empty by gravity flow
- 1st Wash: Wash the NucleoBond® Xtra Column Filter and NucleoBond® Xtra Column with 5ml Equilibration Buffer EQU
- Remove the filter
- 2nd Wash: Wash the NucleoBond® Xtra Column with 8ml Wash Buffer WASH.
- Elute the plasmid DNA with 5ml Elution Buffer ELU and collect the elute in centrifuge tube
- Add room temperature 3ml isopropanol to precipitate the elute plasmid DNA and centrifuge 4500x g for 5 min at room temperature

- Add room temperature 2ml 70% ethanol to the pellet and centrifuge 5000x g for 5 min at room temperature
- Carefully remove ethanol completely from the tube. Allow the pellet to dry at room temperature
- Dissolve the DNA pellet in a Nuclease free water and store in -20 °
- Check the purity NanoDrop spectrophotometer

❖ Agarose Gel Electrophoresis

- 1% of Agarose gel was prepared in 50mL of TAE buffer
- 5µl 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 well
- The gel was run at 100Volt for 45 minute
- The bands were observed in gel dock

❖ Kill curve

- Harvest the healthy adherent CHO cells and pellet down using centrifuge at 1000 RPM for 5 minutes
- Resuspend the cells in complete media and seed the 10^5 cells/ well in 24-well plate
  Note: Typical cell density recommended before antibiotic treatment in a 24-well plate (figure 18)
    - 10^5 cells/ ml of adherent cells
    - 10^4 cells/ ml of suspension cells
- Incubate the plate at 37° C in $C0_2$ incubator for overnight or till the cells adhered
- Replace the media with fresh media containing varying concentrations (0, 50, 100, 200, 400, 600, 800, 1000 µl/ml) of geneticin antibiotic. Maintain each concentration in duplicates
- Replace the media every 48 hours with fresh media containing antibiotic
- Culture the cells for 7 – 10 days and observe the cells under light microscope for effect of antibiotics on it

❖ PBMC Cell Isolation

- Blood Samples collected from the healthy individuals from the Institute of Kidney Disease Research Center (IKDRC) and PBMCs isolation performed within 3hr of blood sample collection
- Collected Blood diluted into 3ml of 1X Phosphate Buffer Saline (PBS).
- Diluted blood added into 2ml of Hi-SEP™ LSM 1077 media.
- Then, Centrifugation was performed at 400 rpm for 30min at 25ºC without deceleration so, PBMCs were separated based on their gradient differences
- After centrifugation carefully discards the supernatant.
- Collect the buffy coat in another tube.
- Isolated PBMCs added into 10ml 1X PBS.

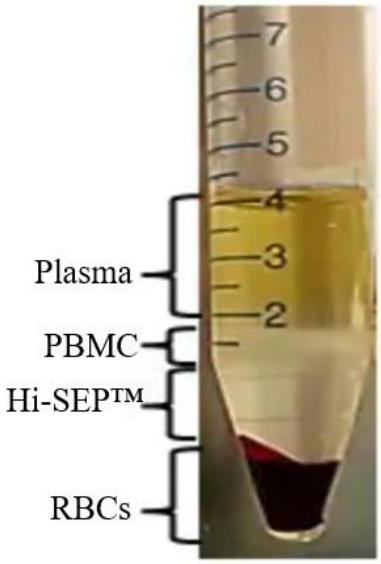


Fig 14: PBMCs isolation through density gradient centrifugation.

- 1X PBS wash is necessary to remove any other material such as Hi-SEP™ LSM 1077 that may be mixed with PBMCs due to pipette errors.
- Another Centrifugation was performed at 200rpm for 10min at 18ºC.
- Collect the pellet and discard the supernatant.
- In the pellet freezing media was added. To perform cell counting 8µl keep aside.
- For cell counting Trypan Blue which is an azo dye that selectively stains dead cells so that we easily distinguish live and dead cells.
- In 8µl PBMCs 2µl trypan blue dye was added.

- From there 1µl of cell suspension was loaded into Neubauer's chamber.
- Cell counting is done under low power (10X) light microscope in which the dead cell gets stained and the live cell remains unstained. (Bright yellow : live cells and stained blue : dead cells)

❖ Flow Cytometry

1. Cell surface staining

- Diluted blood into Hi-SEP™ LSM 1077 media. Isolated PBMCs from buffy coat
- Add 5% human serum + RPMI 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 resuspend cell in complete media
- The supernatant was decanted and pellet was dissolved in complete RPMI and was counted in cell counter
- Cell will be seeded to 96 well plate with the consideration of 10^5 cells/well
- Add 200 µl FACS buffer (1% human serum in PBS). Mix properly and centrifuge at 500g for 5 min at 4°C
- Discard the supernatant by inverting the plate gently
- The pellet was resuspended in 10 µl of Fc-block 1:100. The cells were mixed by tapping the plate gently; the plate was incubated on ice for 10 minutes
- 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 at 4°C
- Add 200 µl FACS buffer and centrifuge at 500g for 5 min at 4°C and discard the supernatant by inverting the tube

2. Intracellular staining

- After last wash (2 or 3 washes) of cell surface staining gently aspirate supernatant followed by gently tapping to the plate
- Add 100 µl of cytofix buffer and incubate at 4°C for 20 min in dark
- Add 1x perm wash (100 µl) buffer and centrifuge at 400-500g for 5 min at 4°C
- Aspirate the supernatant tap the plate gently and add 200 µl of 1x perm wash buffer followed by centrifugation at 400-500g for 5 min at 4°C
- Aspirate the supernatant tap the plate gently and add Ab cocktail (30 µl) each well followed by centrifugation at 400-500g for 5 min at 4°C

  Ab cocktail: 0.5 Serum

  : 0.5 Ab

  : Final volume make up with perm wash up to 30 µl
- Add 100 µl of 1x perm wash and centrifugation at 400-500g for 5 min at 4°C
- Aspirate the supernatant tap the plate gently and add 200 µl of FACS buffer followed by centrifugation at 400-500g for 5 min at 4°C
- Aspirate the supernatant and add 200 µl of 1% HCHO (Formaldehyde) in each tube and transfer into FACS tube

5. RESULTS

5.1 Objective 1

- **Bacterial culture & Plasmid Isolation**

  The plasmid pcDNA3.1 clone with the human-mouse chimeric IL15 in *E. coli* DH5-Alpha was used as a pure bacterial culture into the selective media LB broth added with ampicillin and incubated overnight at 37°C under shaking conditions. We measure the OD in Spectrometer at 600 nm after the 16–18 h incubation**.** Plasmid DNA was isolated by a plasmid isolation kit (NucleoBond Xtra Mini kit and NucleoBond Xtra Midi kit.)

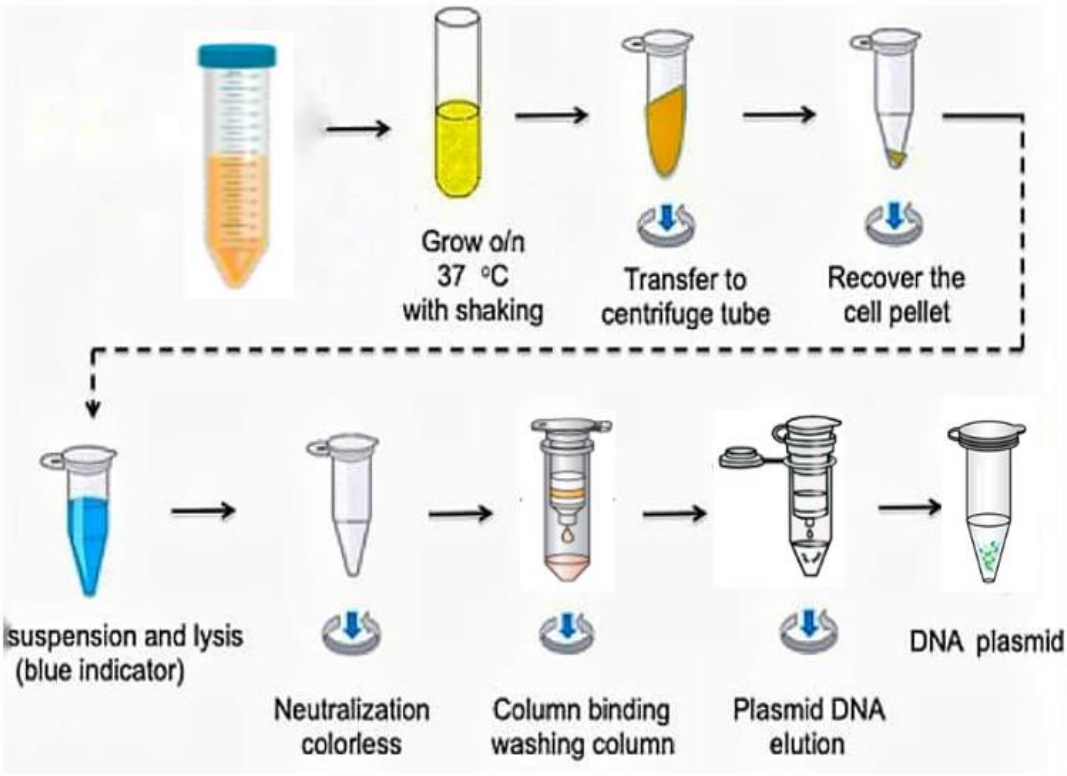


Fig 15: Plasmid Isolation by NucleoBond Xtra kit

To check the purity and concentration of plasmid we use Nano drop and electrophoresis as shown in figure 16-17 and table 3-4.

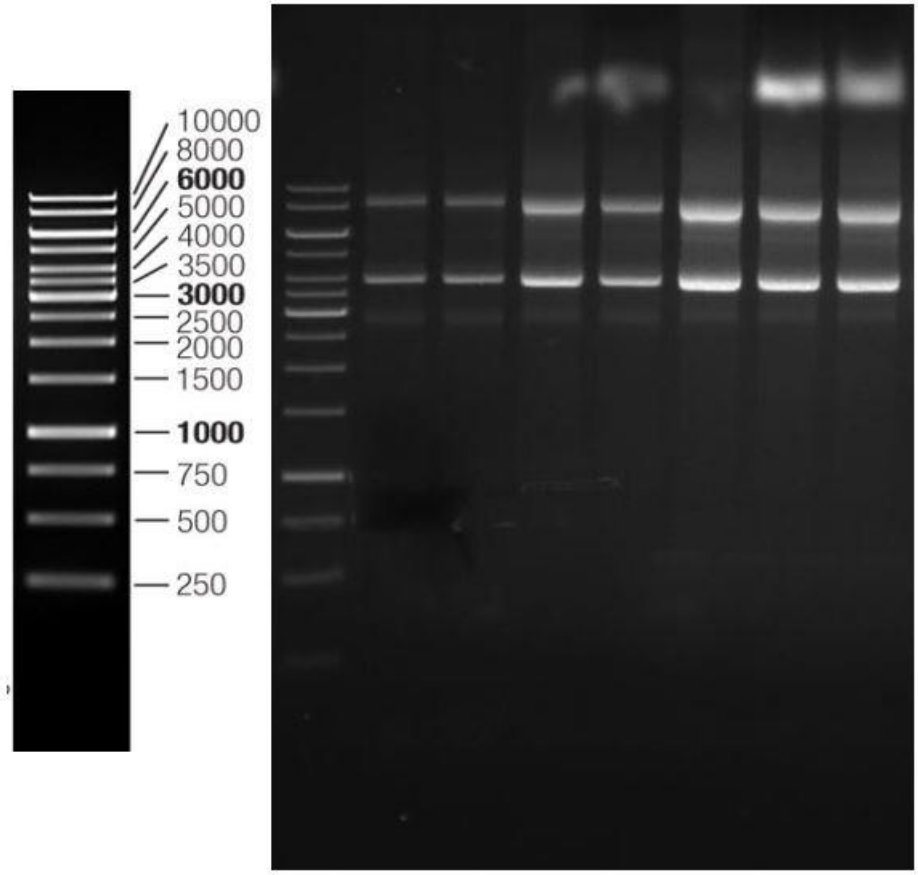


Fig 16: Plasmid isolation by Mini kit

Sample	A 260/A280	Concentration
1A	1.79	134.2 ng/µl
2B	1.80	221.5 ng/µl
3C	1.79	147.9 ng/µl
4D	1.82	176.7 ng/µl
5E	1.16	50.7 ng/µl
6F	1.79	134.9 ng/µl
7G	1.80	133.2 ng/µl

Table 3: NucleoBond Xtra Mini kit Plasmid Isolation

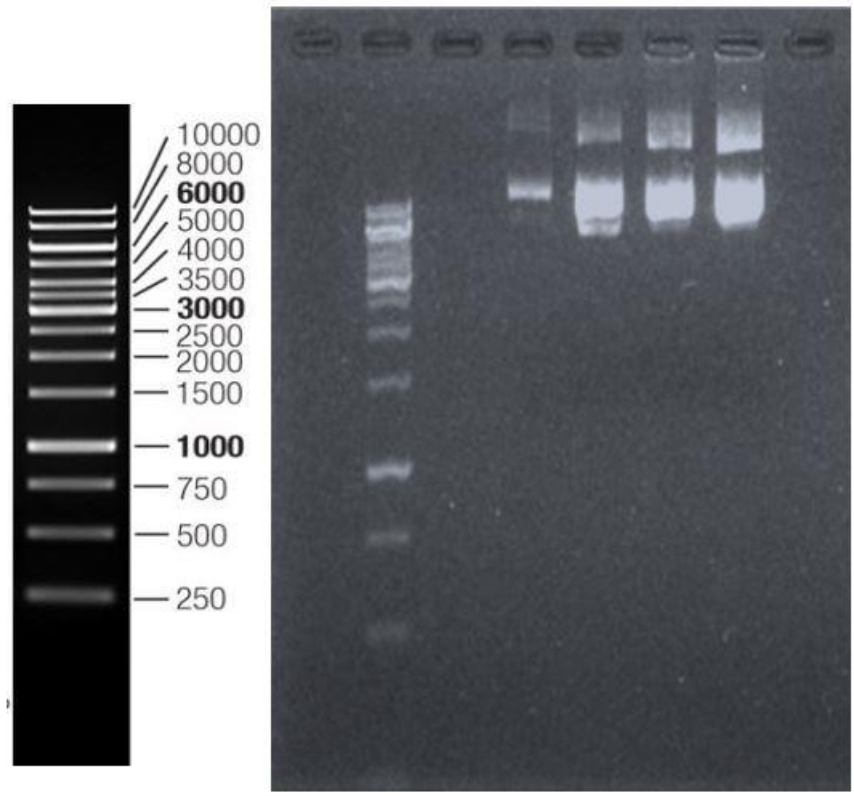


Fig 17: Plasmid isolation by Midi kit

Sample	A 260/A280	Concentration
1	1.72	22.9 ng/µl
2	1.87	1437.4 ng/µl
3	1.87	606.7 ng/µl
4	1.87	777.7 ng/µl

Table 4: NucleoBond Xtra Midi kit Plasmid Isolation

- **Transfection in CHO cells**

  For the transfection of plasmid in the CHO cell line, it is preferable to determine the optimal initial cell density for transfection and the selection of antibiotic concentration by performing a kill curve assessment. We use the RPMI media containing geneticin antibiotic at different Concentration: 0, 50, 100, 200, 400, 600, 800 and 1000 µg/ml to select the toxic antibiotic concentration for CHO cell line.

  Cells were cultured in 24-well plates along with complete RPMI media with (comprising different concentration of antibiotic) or without antibiotic for 1 week as shown in figure 18.

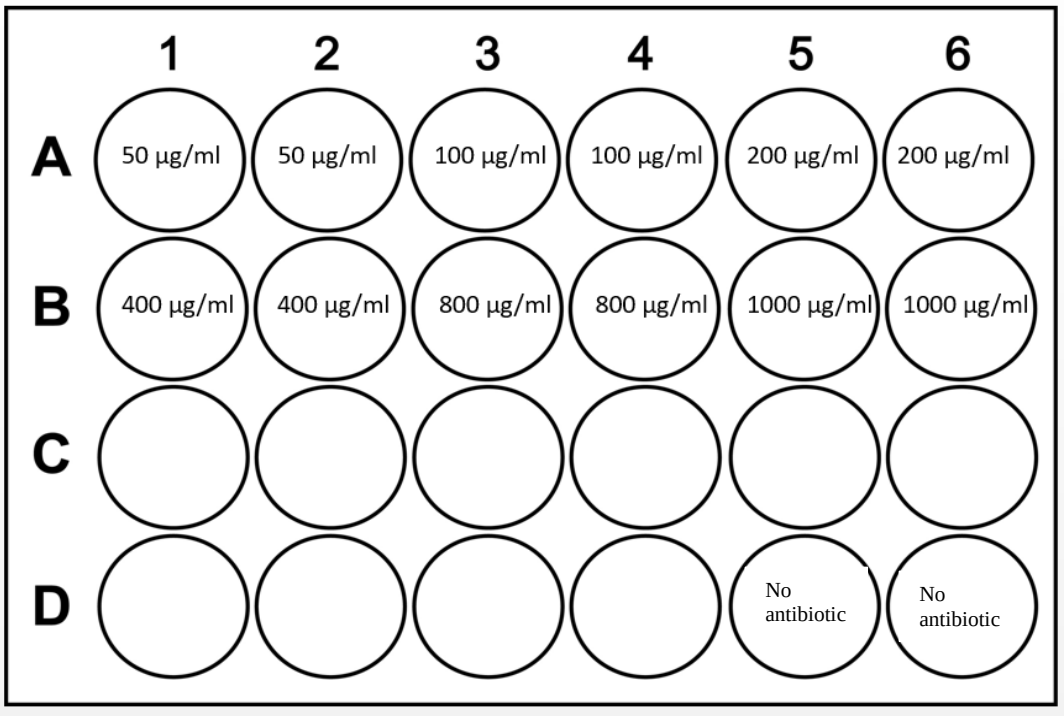
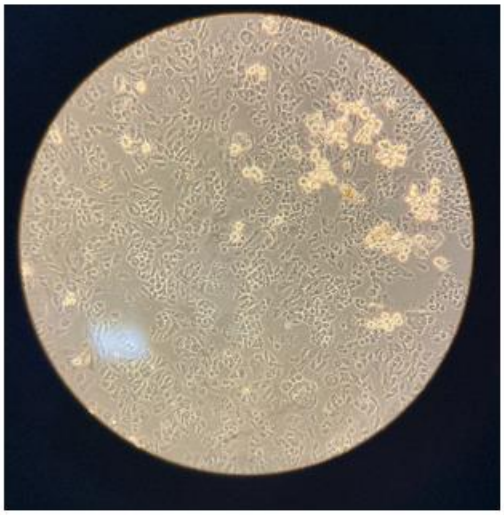


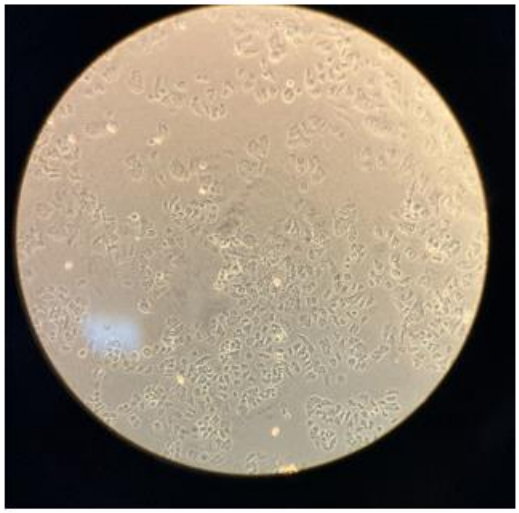
Fig 18: 24 well plate for kill curve assay

We performed kill curve assay to know the minimum concentration at which the antibiotic will be less toxic towards the cell and kill the non-transfected cell efficiently. Our results shows that 400 µg/ ml is the optimum concentration of antibiotic where cells are effectively killed as shown in figure 19. Hence, to perform transfection cells will be cultured in the media containing 400 µg/ ml geneticin antibiotic.

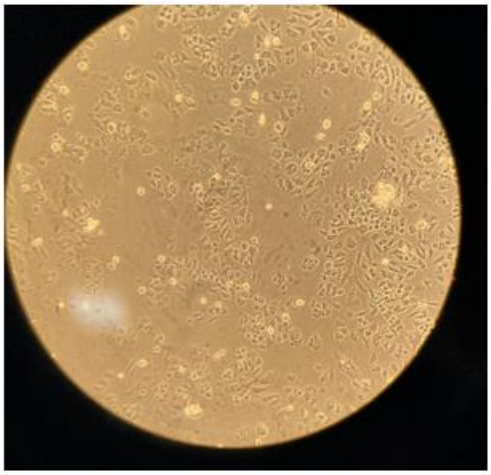
Further, to perform transfection we cultured the cells in complete RPMI media to get desired number of cells. However, due to contamination in cultured flask we could not able to proceed further.



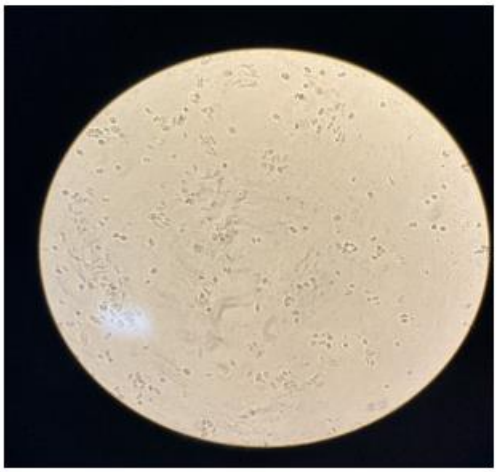
DAY-1 at 400µg/ml Conc. geneticin



DAY-3 at 400µg/ml Conc. geneticin



DAY-5 at 400µg/ml Conc. geneticin



DAY-7 at 400µg/ml Conc. geneticin

Fig 19: CHO cell line at optimum geneticin concentration.

5.2 Objective 2

- **PBMC isolation from HLA-A*02:01 and HLA-B*35:03 specific blood samples**

  To isolate the PBMC from donor blood we use density gradient method. In brief blood diluted in PBS were added in a vial containing Hi-sep and centrifuge to harvest PBMC. Further to check the viability of cells we used trypan blue and Neubauer's chamber for the cell counting as shown in table 5 and figure 20.

  According to review literature we synthesized the two HLA (HLA-A*02 and HLA-B*35:03) restricted peptides of ESAT-6 protein (BCG specific) and PRAME protein (cancer specific) to study the activation of T cells with or without peptide stimulation.

Patients No	Patient's HLA type	Cells Count
Patient 1	HLA-A*11:24 / B* 35:03	8.52 X 10^6/ 2ml
Patient 2	HLA-A*02:11 / B*40:06; 49:01	6.38 X 10^6/ 2ml

Table 5: PBMC Cell Count

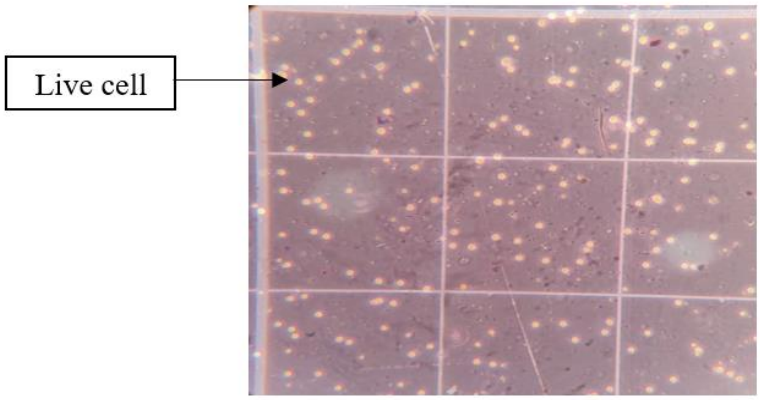


Fig 20: PBMC Cell under Microscope

- **Treatment with Peptides to check T-Cell activation**

  Our rational is to check the efficacy of the chimeric hu-IL15 in terms of activating T cells with or without peptide. Before performing the final experiment, we would like to know the optimum concentration and HLA specificity of designed peptide. To address the same, we performed the experiment in which we used three different concentrations (1 µg/ ml; 5 µg/ ml; 10 µg/ ml) of both peptides as shown in figure 21. In brief PBMCs were seeded at the density of 10^5 cells/ well and kept for overnight. Later we gave the peptide stimulation along with Brefeldin A and kept the 96 well plate at 37 ℃ for 6 hrs. After completion of incubation period, we performed the cell surface and intracellular staining according to the protocol.

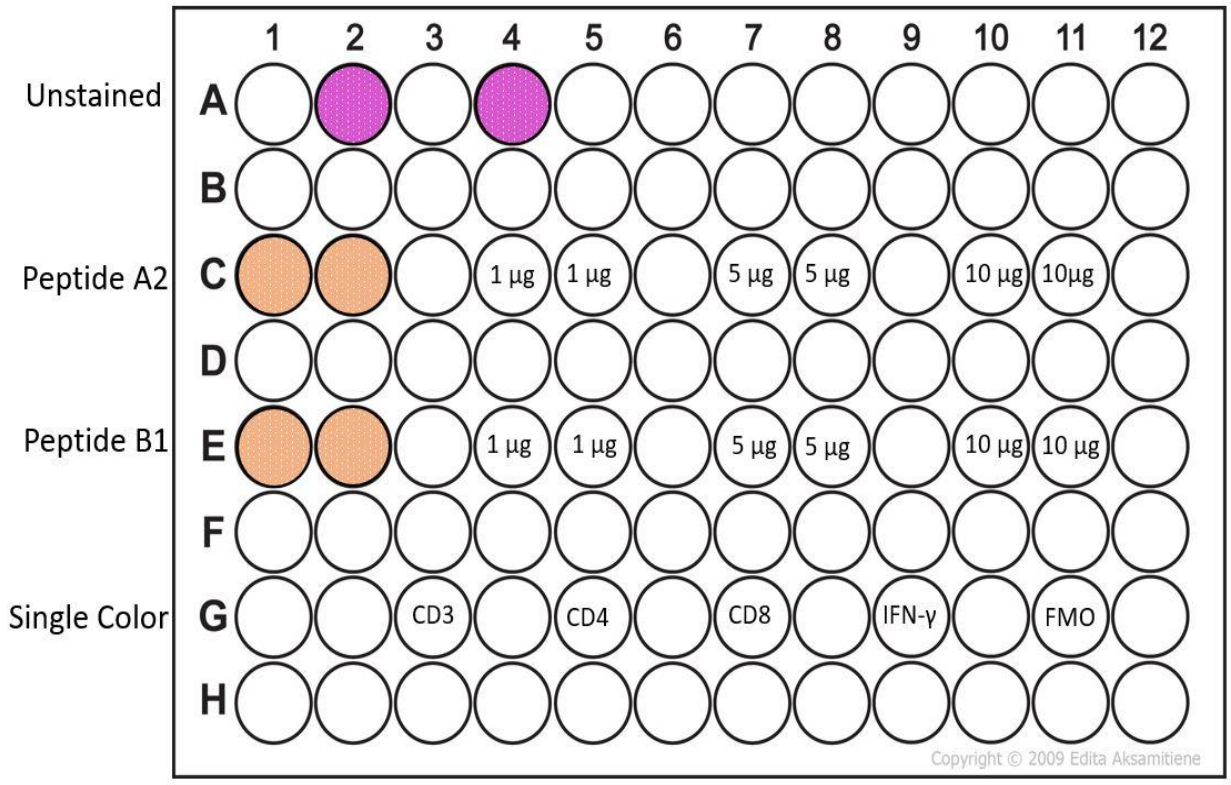


Fig 21: Strategy to check Human PBMC Activation

- **Human PBMC Activation**

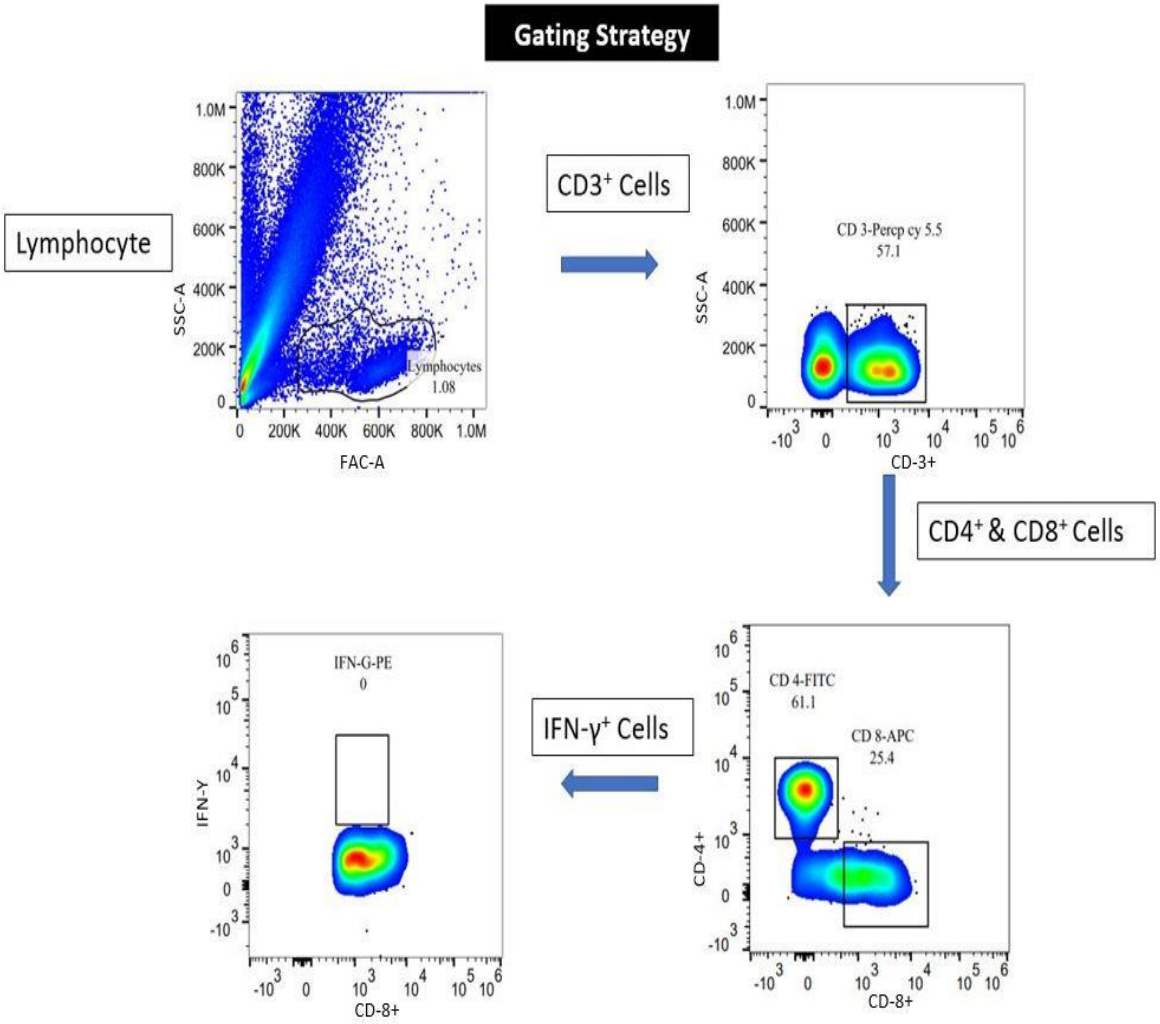


Fig 22: The common strategy to gate cell population to all the sample to look for $CD8^+$ IFN-γ^+ cells

As shown in the above figure, we first gated the lymphocyte population to look for the $CD3^+$ cells. Further, $CD3^+$ cells population were gated to obtained the $CD4^+$ and $CD8^+$ cells, and $CD8^+$ cells further gated to look for IFN-γ^+ cells.

RESULT OF ESTAT-6 PEPTIDE STIMULATION

In sample A we had HLA-A*02:01 specific PBMCs and we used the peptide ESTAT-6 to stimulate the T cells. It can be observed that there is a gradual increase in $CD8^+$ IFN-γ^+ cells as peptide concentration increasing that can be observed in the figure 23.

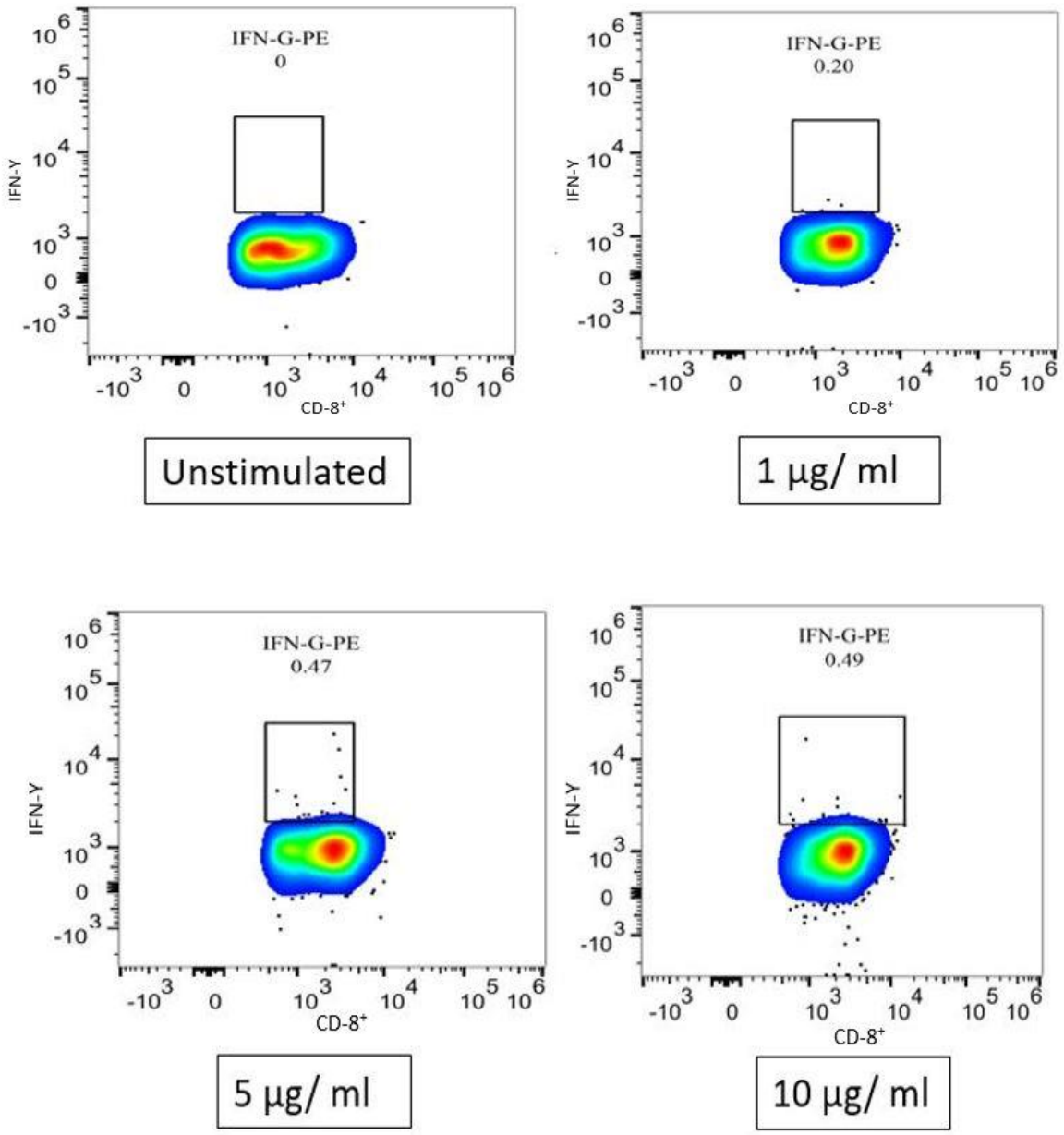


Fig 23: $CD8^+$ IFN- γ^+ Cells in Donor A PBMC stimulated with different concentration of ESAT-6 Peptide

RESULT OF PRAM PEPTIDE STIMULATION

In sample B we had HLA-B*35:03 specific PBMCs and we used the PRAME peptide to stimulate the T cells. It can be observed that we need to further optimized PRAME peptide concentration to look for increase in $CD8^+$ IFN-γ^+ cells as result were not following the trend as similar in case of ESAT-6 peptide which can be observed in the figure 24.

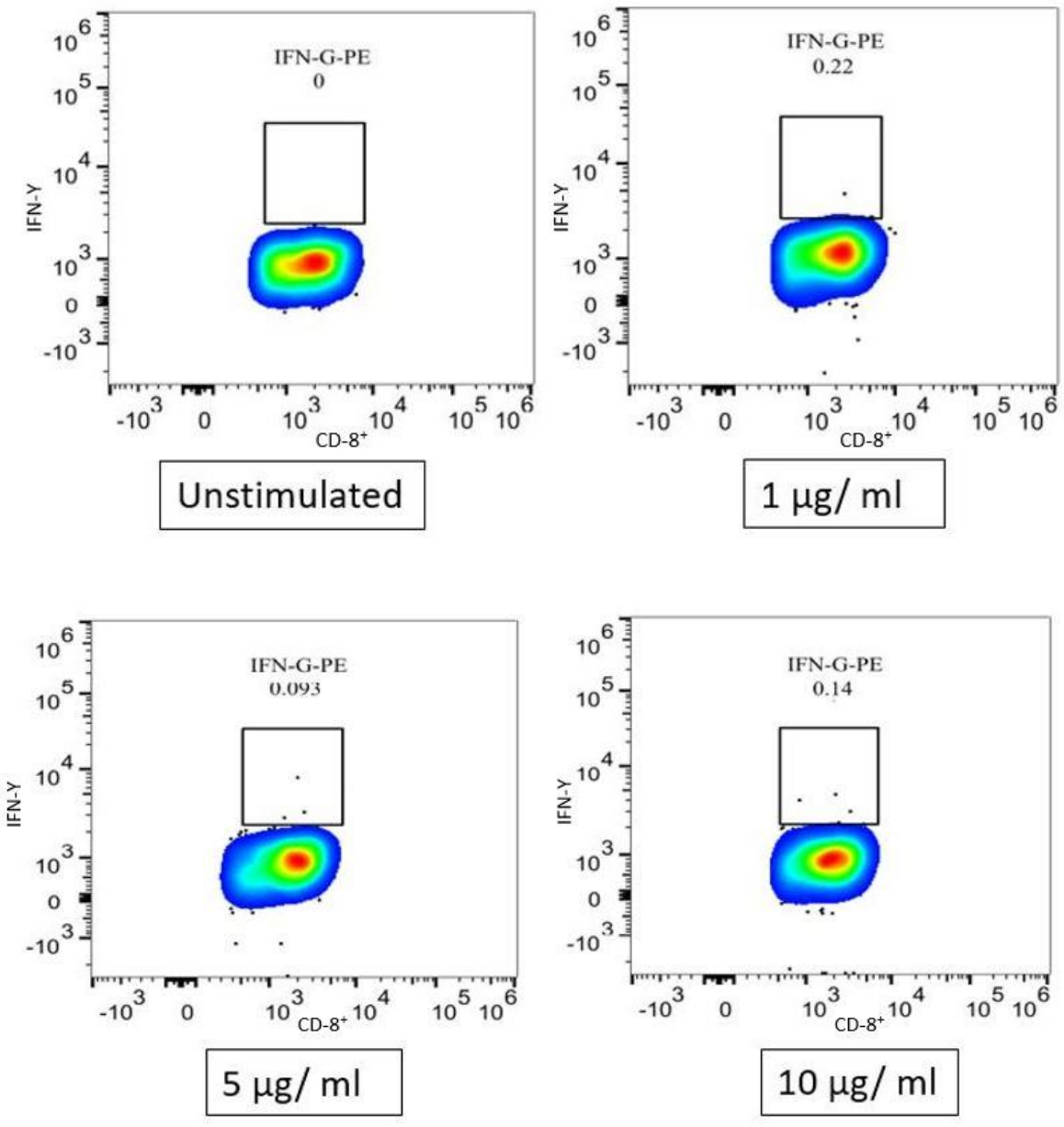


Fig 24: $CD8^+$ IFN- γ^+ Cells in Donor B PBMC stimulated with different concentration of PRAME Peptide

6. Conclusion

For T-cell activation we isolated plasmid pcDNA3.1 form *E. coli (*DH5-Alpha). And by electrophoresis we conformed that the desired plasmid was present in our bacterial culture. The concentration of isolated plasmid was checked with the help of Nano drop. There after a kill curve was performed to find out the required concentration of Geneticin selective antibiotic for the transfection of plasmid in CHO cell line. We observed that the optimum concentration for geneticin was 400 µg/ml. HLA specific human blood sample was selected for T-cell activation and isolation of PBMC cell with the help of Hi-Sep media. Cell viability was checked using Neubauer's Chamber. After stimulating the cells with peptide and observing the result of flow cytometry we observed that, in sample A, by the stimulation of peptide ESTAT-6 to HLA-A*02:01 specific PBMCs, the secretion of CD-8^+ IFN-γ^+ cells increased gradually as the concentration of peptide increases. And in sample B, the stimulation of PRAME peptide to HLA-B*35:03 specific PBMCs required further optimization.

7. Discussion

Cancer has been research hotspot for the past decade due to its high morbidity and mortality rate. As a result there are various approaches to use cure the cancer. Immunotherapy is an innovative approach for cancer therapeutics. There are several different types of cancer immunotherapy available, including checkpoint inhibitors, CAR T-cell therapy, cancer vaccines, and cytokine based therapy. Cytokine-based immunotherapy can induce long-lasting immune responses and have the potential to induce immunological memory. Cytokine-based immunotherapy remains an important area of research in cancer treatment, as it has shown promising results in improving outcomes for cancer treatment. IL-15 stimulates the growth and activation of immune cells, including T-cells and natural killer cells, which plays an important role in defense against cancer. However, IL-15 has low stability and serum half-life, and to resolve this problem earlier our lab had made a modified chimeric Human IL-15 protein covalently linked with mouse IgG2 providing high stability and longer half-life. For *In vitro* study we planned for the examining the biological activity of human chimeric IL-15 on $CD8^+$ and IFN- γ^+. For the above purpose we analysed T-cell activation on human HLA-A*02 and HLA-B*35:03 PBMCs with the stimulation of ESAT- 6 and PRAME peptide at 1µg/ml, 5µg/ml and10µg/ml concentration using flow cytometry. Our data analysis demonstrates that the peptide stimulated PBMCs are secreting more IFN-**γ** compare to non-stimulated cell. It confirms that the peptides designed for specific HLA were effectively activating the PBMCs. In future PBMCs will be stimulated with chimeric huIL-15 and it will be checked for T-cell activation and secretion of INF-**γ.**

8. Appendix

Reagent – Preparation

1) PBS buffer-1X

NACL-800mg

KCL-20mg

NA2HPO4-144mg

KH2PO4-24mg

2) 70% ethanol-15ml

100%ethannol-11ml

Distilled water – 4ml

3) FC-block -> 10µL/WELL

For, 30 well

1%Naive serum 30µl

FACS buffer-297µl

4) HCHO (1%)

HCHO (37%)-270µl

FACS buffer (1%)-9730µl

Total volume-1000µl

5) BFA ->2µl/well

For, 19 wells

BFA -42µl

Media- 168µl

Total volume- 210µl

Peptide calculation:

1) For PRAME (FPEPEAAQP)

a) 1µg / ml

Therefore, 0.2 µg/ well / 200 µl (Dilution factor 2)

So, 0.2X 2= 0.4 µg/ well / 200 µl

Therefore, 2 µg/ ml is required in a well

Stock solution: 4.95 mg/ml

Therefore, 4.95 µg/ ml, and to make 2 µg/ ml working stock 0.4 µl need to be made in 1 ml of media

b) 5 µg/ ml

Therefore 1 µg / well / 200 µl (dilution factor 2)

So, 1×2 = µg / well / 200 µl

Therefore, 10 µg / ml is required in a well

Stock solution: 4.95 mg/ml

Therefore, 4.95 µg/ ml, and to make 10µg/ ml working stock 2.02 µl need to be made in 1 ml of media

c) 10 µg/ ml

Therefore, we have to make 20 µg/ml of stock solution

So, 4.04 µl in 1 ml of media

2) For ESTAT-6 (AMASTEGNV)

a) 1µg/ml:

Therefore, we have to make a working stock solution of 2 µg/ml

Stock solution: 4.75 µg/ml

So, we have to make 0.42 µl in 1ml of media

b) 5µg/ml

Therefore, we have to make a working stock solution of 10µg/ml

Stock solution: 4.75 µg/ml

So, we have to make 2.10µl/ml in 1 ml of media

c) 10 µg/ml

Therefore, we have to make a working stock solution of 20µl/ml

Stock solution: 4.75 µg/ml

So, we have to make 4.21 µl/ml in 1 ml of media

Antibody calculation

Marker	Fluorochrome	Antibiotic	FACS buffer
CD-3	Percpcy 5.5	0.5µl	9.5µl
CD-4	FITC	0.5µl	9.5µl
CD-8	APC	0.5µl	9.5µl
INF-ɣ	PE	0.5µl	9.5µl

Cocktail of antibody

Antibody	Amount
CD-3	8.5µL
CD-4	8.5µL
CD-8	8.5µL

- Mix all the antibodies into 180 µl of FACS buffer
- Intracellular staining antibody

  Serum - 8µl

  FFNɣ-8µl

  PERUM wash-494µl

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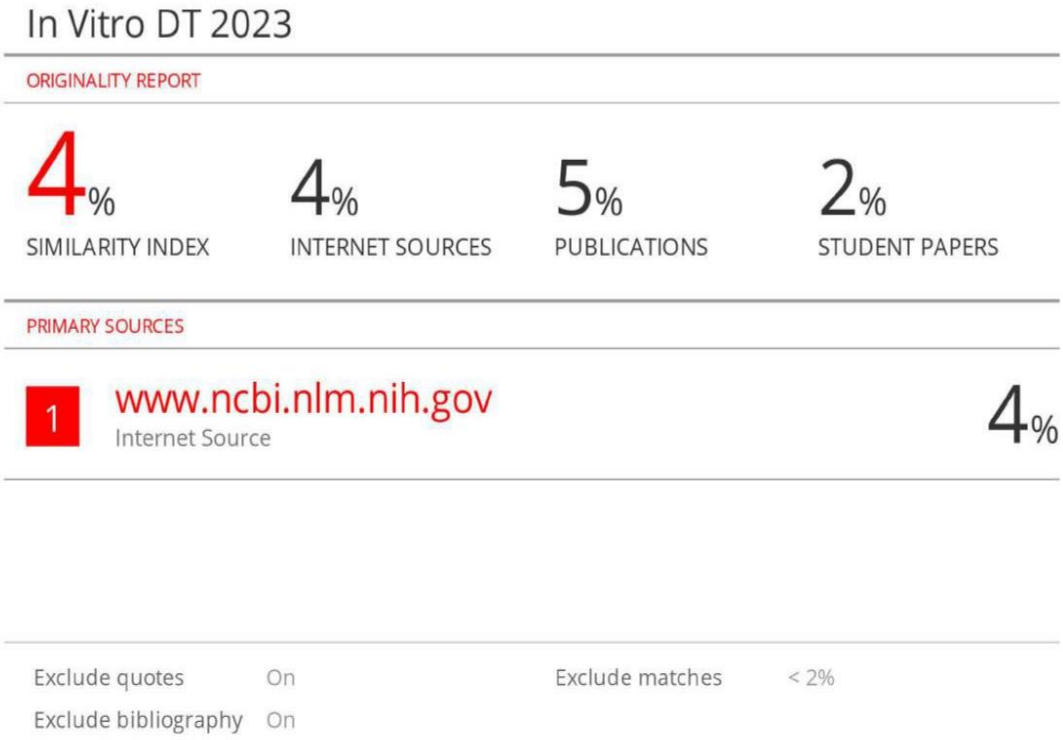
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