Understanding the Mechanism of Cancer Progression in Tumor Hosts with Type I Diabetes with Reference to the Alteration in Cancer Immune-Surveillance: Correction by NLGP

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Thesis submitted for the degree of
Doctor of Philosophy (Science)
Index No.: 112/18/Life Sc./26
Department of Life Science and Biotechnology
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This is to certify that the thesis entitled "Understanding the mechanism of cancer progression in tumor hosts with type I diabetes with reference to the alteration in cancer immune-surveillance: Correction by NLGP" submitted by Mr. Anirban Sarkar who got his name registered on 05.03.2018 for the award of Ph. D. (Science) degree of Jadavpur University (Index No. 112/18/Life Sc./26), is absolutely based upon his own work under the joint supervision of Dr. Rathindranath Baral and Dr. Anamika Bose and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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Anirban Sarkar

ABBREVIATIONS

A

AKT	Protein Kinase B (PKB or AKT)
ALL	Acute Lymphoblastic Leukemia
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
APC	Allophycocyanin (fluorochrome)
APS	Ammonium Persulfate
ARG	Arginase
ASC	Adipocyte Stem Cell
ATP	Adenosine Triphosphate

B

BCL	B-cell Lymphoma
BCL-xL	B-cell Lymphoma Extra Large
BSA	Bovine Serum Albumin

C

CAA	Cancer Associated Adipocyte
CAF	Cancer Associated Fibroblast
CAR	Chimeric Antigen Receptor
CCL	CC Chemokine Ligand
CD	Cluster of Differentiation
cDNA	Complementary DNA
CEA	Carcinoembryonic Antigen
ConA	Concanavalin A
COX	Cyclooxygenase
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte-Associated Antigen 4
CXCL	CXC Chemokine Ligand
CXCR	CXC Chemokine Receptor

D

DC	Dendritic Cell
DEPC	Diethyl Pyrocarbonate
DLBCL	Diffused Large B-cell Lymphoma
dMMR	Mismatch Repair Deficit
DMSO	Dimethyl Sulfoxide
DN	Double Negative
DNA	Deoxyribonucleic Acid
DPX	Distyrene Plasticizer Xylene

E

ECAR	Extracellular Acidification Rate
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial to Mesenchymal Transition

F

FASL	Fas Ligand
FADD	Fas-associated Death Domain
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FH	Fumarate Hydratase
FITC	Fluorescein Isothiocyanate
FSP	Ferroptosis Suppressor Protein

G

G-6-PD	Glucose 6-phosphate Dehydrogenase
GH	Growth Hormone
GLUT	Glucose Transporter
GOD	Glucose Oxidase
GZMB	Granzyme B

H

HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HLA	Human Leucocyte Antigen
HRP	Horseradish Peroxidase

I

ICBT	Immune Checkpoint Blockade Therapy
IDDM	Insulin Dependent Diabetes Mellitus
IDH	Isocitrate Dehydrogenase
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin Like Growth Factor
IGFBP	IGF Binding Protein
IGFR	IGF Receptor
IL	Interleukin
IR	Insulin Receptor
irAE	Immune Related Adverse Effects
iTreg	Induced (peripheral) T Regulatory Cell

L

LAG	Lymphocyte Activation Gene
LDH	Lactate Dehydrogenase
LN	Lymph Node
LOX	Lysyl Oxidase
LSM	Lymphocyte Separating Media

M

M-CSF	Macrophage Colony Stimulating Factor
MACS	Magnet Assisted Cell Sorting
MAPK	Mitogen-activated Protein Kinase

MCP	Monocyte Chemoattractant Protein
MDSC	Myeloid Derived Suppressor Cells
MET	Mesenchymal to Epithelial Transition
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloprotease
MSC	Mesenchymal Stromal Cell
mTOR	Mammalian Target of Rapamycin

N

NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydragine
NG	Neural Glial Antigen
NGF	Nerve Growth Factor
NK Cell	Natural Killer Cell
NKT Cell	Natural Killer T Cell
NLGP	Neem Leaf Glycoprotein
NLP	Neem Leaf Preparation
NOD	Non-obese Diabetic
NOS	Nitric Oxide Synthase

O

OCR	Oxygen Consumption Rate
OXPHOS	Oxidative Phosphorylation

P

PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCX	Pyruvate Carboxylase
PD-L1	Programmed Death Ligand 1
PD1	Programmed Death 1
PDGF	Platelet Derived Growth Factor
PDK	Pyruvate Dehydrogenase Kinase
PGF	Placenta growth Factor
PI3K	Phosphoinositide 3-kinase
PKM	Pyruvate Kinase M
POD	Peroxidase
PPT	Picropodophyllotoxin

R

RAS	Rat Sarcoma
rIGF1	Recombinant IGF1
RIPA	Radio-Immunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase

S

SDS	Sodium Dodecyl Sulphate

siRNA	Silencing RNA
STAT	Signal Transducer and Activator of Transcription
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
STZ	Streptozotocin

T

T1D	Type 1 diabetes
T2D	Type 2 Diabetes
TAM	Tumor Associated Macrophage
TAN	Tumor Associated Neutrophil
Tc Cell	Cytotoxic T cell
TCA	Tricarboxylic Acid Cycle
TCF	T Cell Factor
TCR	T Cell Receptor
TDLN	Tumor Draining Lymph Node
T_{FH} Cell	Follicular Helper T Cell
TGF	Transforming Growth Factor
T_H Cell	Helper T Cell
TIGIT	T Cell Immunoreceptor With Ig and ITIM Domains
TIL	Tumor Infiltrating Lymphocyte
TIM	Translocase of the Inner Membrane
TKI	Tyrosine Kinase Inhibitor
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
Treg	Regulatory T Cell
tTreg	Thymic Regulatory T Cell

V

VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

CONTENTS

ABSTRACT

Abstract:

Epidemiological studies suggest that patients with pre-existing type 1 diabetes (T1D) have a decreased risk of developing melanoma, prostate cancer, and breast cancer, although the underlying mechanism remains to be elucidated. In translational modelling, we observed that streptozotocin (STZ) induced T1D mice exhibited restricted melanoma and carcinoma (mammary, lung and colon) growth in association with extended overall survival. Tumor-infiltrating $CD8^+$ T cells were found to be responsible for tumor growth restriction. Tumor infiltrating $CD8^+$ T cells but not tumor cells themselves exhibited higher glycolytic and cytotoxic activities in T1D hosts. Such improved anti-tumor T cell function was linked to selective upregulated expression of insulin-like growth factor 1, insulin-like growth factor 1 receptor, and phospho-mTOR in $CD8^+$ T cells in the TME. T1D patient derived $CD8^+$ T cells displayed superior activation *in vitro* after tumor antigen stimulation vs. non-diabetic $CD8^+$ T cells. Activation of T1D patient derived $CD8^+$ T cells was sensitive to targeted antagonism of IGF1R and mTOR, supporting the operational involvement of the IGF1R-mTOR signaling axis. Our results suggest that selective activation of the intrinsic IGF1R-mTOR signaling axis in $CD8^+$ T cells represents a preferred endpoint to achieving more effective immunotherapy outcomes and improved cancer patient management. Neem leaf glycoprotein (NLGP) restricts immune dependent murine melanoma, carcinoma and sarcoma tumor growth control. However, therapeutic efficacy of NLGP in tumor hosts with pre-existing type 1 diabetes has not been studied yet. We found NLGP modulates the tumor microenvironment of type 1 diabetic hosts in favor of antitumor immunity. Further study showed NLGP reduces T1D associated hepatic inflammation irrespective of tumor burden. NLGP accelerates intra-tumor $CD8^+$ T cell oxidative phosphorylation in diabetic hosts, thereby improves glucose metabolism. Further, NLGP dampens glucose uptake by tumor cells in diabetic tumor microenvironment by downregulating glucose transporter 1 (*glut 1*) expression. Overall, NLGP positively influences immune microenvironment and metabolism in tumor bearing hosts with pre-existing type 1 diabetes.

GENERAL INTRODUCTION

1. Hallmarks of cancer
2. Tumor microenvironment (TME)
3. Stromal cells of TME
4. Immune cells of TME
5. Hormones and growth factors that influence tumor growth
6. $CD8^+$ T cell metabolism
7. $CD8^+$ T cell activation
8. Type 1 diabetes (T1D)
9. Immune microenvironment in T1D, similarities with TME
10. Pre-existing T1D and risk of cancer
11. Neem leaf glycoprotein (NLGP), a natural neem leaf derived immunomodulator

1. Hallmarks of cancer

Hallmarks of cancer was first proposed in 2000 to provide a logical framework that includes all the organizing principle and diversity of neoplastic disease (Hanahan and Weinberg, 2000). Initially six hallmarks were proposed including sustained proliferative signal, evading growth suppressors, inducing angiogenesis, activating invasion and metastasis, resisting cell death and enabling replicative immortality (Hanahan and Weinberg, 2011). Hallmarks of cancer have been updated several times since then (Hanahan, 2022). We were interested in two of the emerging hallmarks of cancer, i.e. reprogramming energy metabolism and evading immune destruction.

1.1 Reprogramming energy metabolism

Cancer cells can switch their energy metabolism from oxidative phosphorylation to glycolysis, even in the presence of oxygen, the Warburg effect (Wu and Zhao, 2013; Sun *et al*., 2011). This enables them to generate ATP and biosynthetic precursors more efficiently, and also provides a survival advantage under hypoxic conditions. Interestingly, cytotoxic T cells, which augments effective antitumor functions, need to switch their metabolism towards aerobic glycolysis in order to proliferate following encounter of tumor antigen, similar to cancer cells (Menk *et al*., 2018).

1.2 Evading immune destruction

Cancer cells can evade detection and elimination by the immune system, which normally recognizes and eliminates abnormal cells. This can be achieved by downregulating MHC class I molecules (MHC-I), upregulating immune checkpoint molecules such as PD-L1, CTLA-4, and secreting immunosuppressive cytokines such astransforming growth factor β (TGF-β) (Dyck and Mills, 2017).

2. Tumor microenvironment

The tumor microenvironment (TME) is the complex and dynamic environment surrounding a tumor. It is composed of tumor cells, immune cells, fibroblasts, endothelial cells, and extracellular matrix (ECM). The TME plays a crucialfunction in tumor growth, invasion, and metastasis (Eikawa and Udono, 2017; Busk *et al*., 2011; Wu, 2017).

Tumor cells can release a variety of factors that promote the growth of new blood vessels (angiogenesis) and the recruitment of immune cells to the tumor site(De Palma *et al*., 2017; Facciabene *et al*., 2011; Yang *et al*., 2004; Jablonska *et al*., 2010). These factors include, but not limited to, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and tumor necrosis factor (TNF). Angiogenesis is essential for tumor growth, as tumors cannot grow beyond a certain size without a sufficient blood supply. The recruitment of immune cells to the tumor site can be beneficial or harmful, depending on the type of immune cells that are present. Some immune cells, such as natural killer cells (NK cells) and cytotoxic T lymphocytes (CTLs), can kill tumor cells directly. However, other immune cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), can suppress the immune response to tumor cells.

Tumor cells and immune cells compete for nutrients in the TME. Tumor cells have a higher metabolic rate than normal cells and

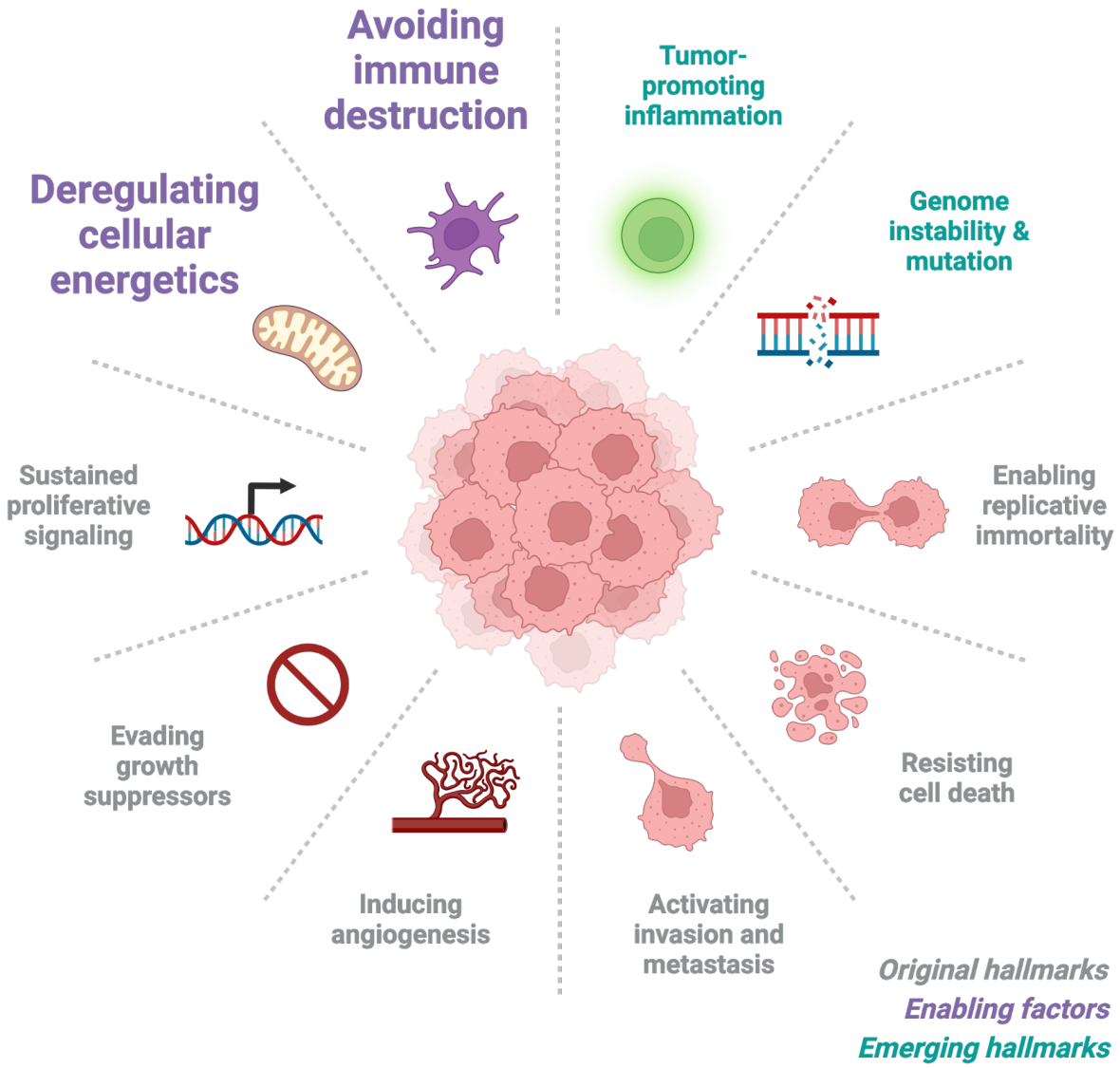

Figure 1. Graphical representation showing the original hallmarks coloured as grey, enabling factors coloured as violet and emerging hallmarks coloured as light green. Two of the enabling factors namely, avoiding immune destruction and deregulating cellular energetics are among the two hallmarks of our interest, thus presented in bigger font. Created with BioRender.com

require more nutrients to grow and divide. This can lead to nutrient competition between tumor cells and immune cells, which can impair the immune response to cancer (Palm and Thompson, 2017). Tumor cells can also release factors that suppress the immune response. These factors can make it difficult for immune cells to kill tumor cells. For example, tumor cells can release indoleamine 2,3-dioxygenase (IDO), an enzyme that breaks down tryptophan, an essential amino acid for immune cells. In addition, the TME can be hypoxic, meaning that there is not enough oxygen available. This can impair the normal functionality of immune cells and make it difficult for them to kill tumor cells (Criscuoli *et al.*, 2020; Facciabene *et al.*, 2011). Nutrient competition between tumor cells and immune cells is a complex process that is influenced by a variety of factors, including the tumor type, the patient's immune status, and the treatment.

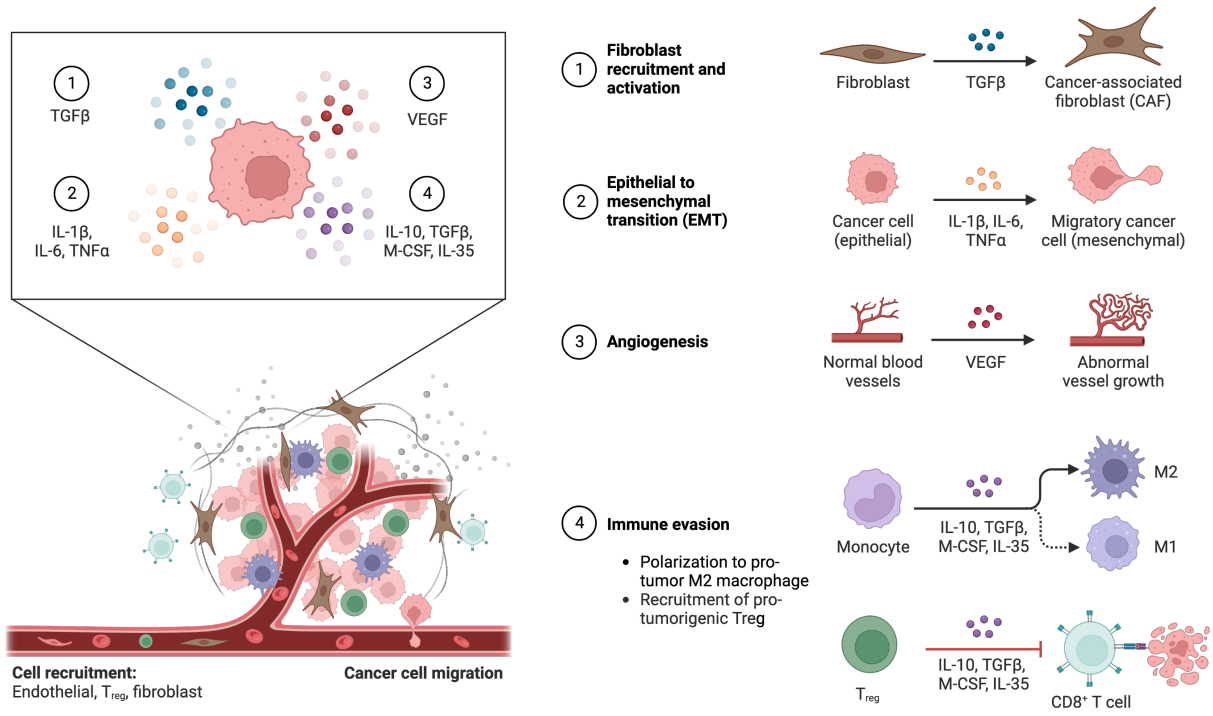

Figure 2. Tumor microenvironment (TME) comprises of not only cancer cells but also various immune and non-immune cells, which may represent up to 40% of the tumor mass. Cancer cells secrete various cytokines in TME to facilitate these immune and non-immune cells become pro-tumorigenic, which supports cancer cell survival and growth. **A. Fibroblast recruitment and activation.** In response to TGFβ, secreted by tumor cells, fibroblast cells get recruited to TME and become cancer associated fibroblasts (CAF). **B. Epithelial to mesenchymal transition (EMT).** IL-1β, IL-6 and TNFα contributes to epithelial to mesenchymal transition (EMT) of malignant cells, which aid in metastasis. **C. Angiogenesis.** VEGF secreted by malignant cells induce endothelial cells for angiogenesis, a necessary prerequisite to supply nutrients and oxygen to growing tumor mass. **D. Immune evasion.** IL-10, TGFβ, M-CSF and IL-35, secreted by tumor cells contribute to the polarization of monocytes into pro-tumorigenic M2 macrophage, instead of anti-tumorigenic M1 macrophages. These cytokines also induce the recruitment of Treg cells within TME which suppress antitumor function of $CD8^+$ T cells. these strategy adapted by cancer cells lead to immune escape and supports uncontrolled proliferation. Created with BioRender.com

The ECM is a network of proteins that provides structural support for cells and tissues. The ECM can also influence tumor growth and metastasis. The ECM can be degraded by tumor cells, which can release growth factors and other factors that promote tumor growth, a process termed epithelial to mesenchymal transition (EMT). The ECM can also provide a physical barrier that prevents tumor cells from invading surrounding tissues.The TME is a complex and dynamic environment that is constantly changing. The TME can be influenced by a variety of factors, including the tumor type, the patient's age, and the patient's immune status (Goswami *et al.*, 2013; Riaz *et al.*, 2017; Busk *et al.*, 2011; Eikawa and Udono, 2017; Wu, 2017; Yu and Ho, 2019; Banerjee *et al.*, 2014c; Chang *et al.*, 2015). The TME is an important target for cancer therapy. By targeting the TME, it is possible to disrupt tumor growth, invasion, and metastasis (Riaz *et*

al., 2017; Yu and Ho, 2019; Banerjee *et al*., 2014a; Barik *et al*., 2013b; a, 2015; Budhu *et al*., 2017). There are a number of different approaches to targeting the TME, including immunotherapy, targeted therapy, and gene therapy.

3. Stromal cells of TME

Stromal cells of TME includes:

- Cancer associated fibroblasts (CAFs)
- Adipocytes
- Neural and neuroendocrine cells
- Mesenchymal stem cells (MSCs)
- Pericytes
- Lymphatic and vascular endothelial cells

3.1 Cancer associated fibroblasts (CAFs)

CAFs are heterogeneous cells of multiple origins. CAFs are generally identified by their biomarkers. They promote angiogenesis along with tumor development and progression by releasing numerous chemokines like CXC-chemokine ligand (CXCL) and CC-chemokine ligand (CCL) and interleukins like IL-1, IL-6, IL-8 and IL-22. Chemotactic GFs (HGF, VEGF, EGF, FGF and PDGF), ECM proteins (tenascin C, fibronectins, SPARC and collagens), matrix metalloproteinases (MMPs), cyclooxygenase 2 (COX2) and lysyl oxidases (LOX) family are secreted by CAFs.

3.2 Cancer associated adipocytes (CAAs)

CAAs are characterized by loss of adipocyte differentiation, a smaller size and FSP-1 expression. They produce angiogenic factors like vascular endothelial growth factor (VEGF), interleukins like, IL-1β, IL-6, IL-8, chemokines like, MCP1, CCL2, CCL5 and TNF-α. CAAs also secrete collagen I/VI, fibronectin and MMP-11 at extremely high concentration. Paracrine and endocrine signals could be initiated by reprogrammed CAAs in close proximity to cancer cells. Adipose stem cells (ASCs) are special type of CAAs, which influence the TME by deteriorating tumorigenesis, that creates an inflammatory TME.

3.3 Neural and neuroendocrine cells

Malignant cells may induce neo-neurogenesis by secreting neurotrophins, fibroblast growth factor (FGF) and insulin-like growth factor-II (IGF-II). NE cells modulate the function of the immune system and are associated with poor prognosis.

3.4 Mesenchymal stem cells (MSCs)

MSCs are multipotent stem cells, which can differentiate into fibroblasts,pericytes, osteocytes, adipocytes and chondrocytes. MSCs are $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$ and $STRO\text{-}1^+$. They are $CD14^-$, $CD34^-$, $CD45^-$ and human leukocyte antigen $HLA\text{-}DR^-$. MSCs have immunomsuppressive properties and secrete VEGF/VEGFR to regulate the TME.

3.5 Pericytes

Pericytes known as perivascular stromal cells provides structural support to blood vessels. They function to supply vessel contractility and permeability to ensure oxygen supply and nutrient to healthy tissues. Classical pericyte marker is NG2. Clinical data correlation shows that low pericyte is associated with higher metastasis and poor prognosis leading to

increased hypoxia, tumor aggressiveness, EMT and MET receptor activation (Dasgupta *et al*., 2021).

3.6 Lymphatic endothelial cells

Lymphatic hyperplasia are driven by VEGF. Lymphatic endothelial cells may induce tumor growth by modulating the TME andby suppressing anti-tumor immune response.

3.7 Vascular endothelial cells

Vascular endothelial cells represent cells of the VEGF family. The family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D andplacenta growth factor (PGF). These ligands have different affinities towards their receptors. Theirs receptors are tyrosine kinases (RTKs), such as VEGFR1, VEGFR2 and VEGFR3, as well as co-receptors such as, neuropilins and heparan sulphate proteoglycans. VEGFA simply known as VEGF is a critical regulator of angiogenesis. Besides, VEGF may modulate cancer cells and immune cells directly (Saha *et al*., 2020; Huang *et al*., 2007). Other soluble factors present in the TME, FGFs, pericytes and PDGFs are also essential for tumor growth.

4. Immune cells of TME

The immune system is a complex network of cells, tissues, and organs that work together to protect the body from infection and disease. The immune system can also help to prevent cancer from developing in the first place. However, the immune system can sometimes fail to recognize and attack cancer cells. This can happen for a number of reasons, including:

- Cancer cells can hide from the immune system by expressing proteins (PD-L1, CTLA-4) that suppress T cell activation (Dyck and Mills, 2017).
- Cancer cells can mutate their genes in ways that make them invisible to the immune system.
- Cancer cells can release proteins that interfere with the immune system's ability to kill cancer cells.

When the immune system fails to recognize and attack cancer cells, cancer can develop and grow.

4.1 T lymphocytes

T lymphocytes are the most crucial cells of adaptive anti-tumor immuneresponse. There are mainly 2 types of T cell populations that may infiltrate TME.

4.1.1 $CD4^+$ T cells

$CD4^+$ T cells can recognize cancer cells through various mechanisms, including the expression of tumor-specific antigens, the loss of tumor-suppressor genes, and the activation of oncogenes.There are several different types of $CD4^+$ T cells, each with a distinct function (Tay *et al*., 2021).

- **T regulatory cells (Tregs),** $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) cells suppress antitumor immunity and promotes immune tolerance.
- **T_H1 cells,** produce TNFα and IFNγ, express T-bet and STAT1 transcription factors, promote antitumor immune response, drives autoimmunity.
- **T_H2 cells,** produce IL-4, IL-5 and IL-13, express GATA3 and STAT6 transcription factors.
- **T_H9 cells,** produce IL-9 and IL-21, distinguished by expression of PU.1 and IRF4 transcription factors.

- **T_H17 cells,** predominantly produce IL-17 and express STAT3 and RORγT transcription factors.

T_{FH} cells, produce IL-6 and IL-24, express BCL6 and STAT3 transcription factor.

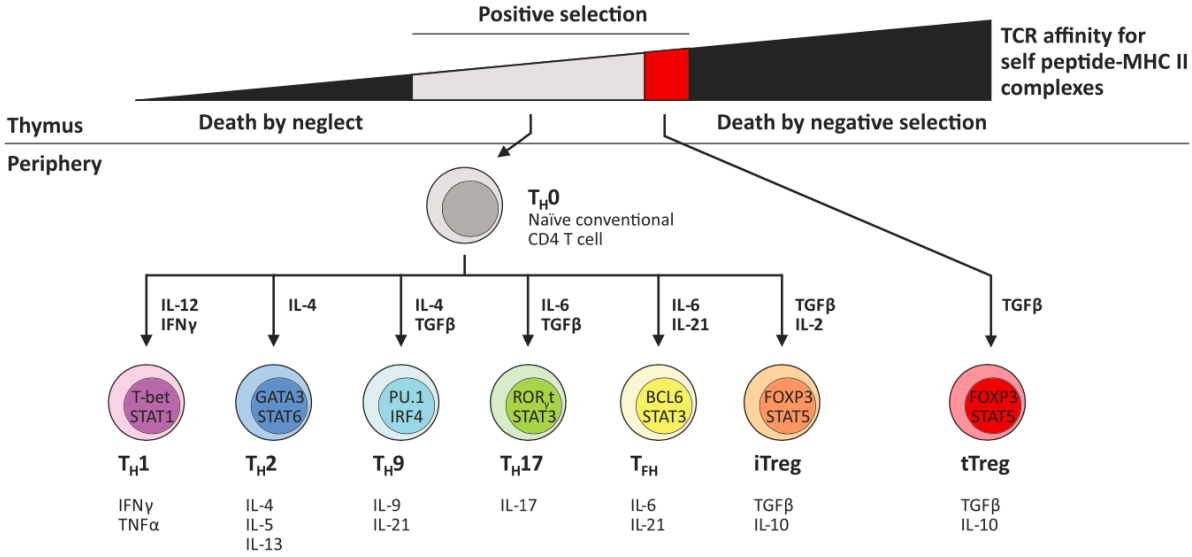

Figure 3. $CD4^+$ T cells are T lymphocytes that express T cell receptors (TCRs) recognising peptide antigens presented in the context of Class II major histocompatibility complex (MHC II) molecules. $CD4^+$ T cells express the TCR co-receptor CD4, which binds to the β2 domain of MHC II and facilitates TCR engagement with peptide-MHC II complexes on antigen-presenting cells. During thymic development, the cell fate of developing thymocytes is decided by their TCR affinity for self-peptide-MHC complexes presented by thymic epithelial cells. Thymocytes that have little to no affinity for self-peptide do not initiate activating signals from their TCR complexes and thus die by neglect. Conversely, thymocytes with high self-reactivity are negatively selected and deleted by apoptosis. Thymocytes with intermediate TCR affinities below the negative selection threshold receive positive selection via activating TCR signals and complete thymic maturation as naïve conventional T cells (T_H0). Some thymocytes with moderately high affinities to self-antigen are redirected into the regulatory T cell (Treg) developmental pathway, where they acquire immunosuppressive function to regulate tissue homoeostasis and resolution of immune responses. Upon receiving cues from the cytokine milieu together with TCR activation, naive $CD4^+$ T cells upregulate expression of key transcription factors regulating subset differentiation, which in turn drive the expression of major effector cytokines associated with each particular subtype. Key transcription factors and cytokines involved are indicated for individual subtypes. $CD4^+$ T cells augment the development of the CTL response and are required for the development of $CD8^+$ T cell immunity in their role as central co-ordinators of adaptive immunity. Unlike $CD8^+$ T cells, whose primary function is to mediate cell contact-dependent cytotoxicity of infected or malignant cells, $CD4^+$ T cells exhibit a diverse repertoire of effector functions and exhibit considerable phenotypic plasticity and heterogeneity depending on local context and microenvironment. $CD4^+$ T cells activated in the periphery can also differentiate into induced Tregs (iTregs), which are able to mediate immunosuppression similar to thymic Tregs (tTregs) (Tay *et al.*, 2021).

4.1.2 CD8+ T cells

Cytotoxic CD8+ T (CTLs) cells are major killers of pathogen and cancer cells. CTLs navigate through entire body for foreign pathogens or uncontrolled malignant cells. CD8+ T cells interacts with major MHC-I on the surface of APCs and target cells which display antigenic peptides on MHC-I molecules (Weigelin *et al*., 2021; Raskov *et al*., 2020). Upon encounter of MHC-I-peptide complex by its T cell receptor (TCR) complex, CD8+ T cells get activated. CD8 acts as a co-receptor in this activation cascade. TCR and CD8 engages with antigen peptide and MHC-I subunit respectively (Basu *et al*., 2016; Bose and Baral, 2007).

4.1.2.1 Co stimulatory signal

Following TCR mediated activation, a co-stimulatory signal from CD28 is a must in order to activate the killing machinery. Without co-stimulation, CD8+ T cells become anergic and undergo apoptosis. CD28 co-receptor of CD8+ T cells interact with CD80/B7.1 or CD86/B7.2, mostly found on DCs, macrophages and activated B lymphocytes.These molecules play a major role to determine CD8+ T cell activation. Co-stimulation lowers the stimulation threshold of naive CD8+ T cells. It alsostimulate cytokine production and cell proliferation, especially interleukin 2 (IL-2). Recent study suggests that a subpopulation of CD8+ T cells, express B7 on their surface, could self-activate the CD28 co-stimulatory signal by invaginating their membrane which accelerate antitumor immunity (Zhao *et al*., 2023). CD8+ T-cell–target-cell interactions stabilizes the motility of the CD8+ T cells on APCs or target cells. This continuous physical interaction generates mechanical force on target cell membrane which facilitates pore formation on target cell membrane by CD8 derived perforin molecules. Through the pores CD8+ T cells infuse death inducing granules, granzyme, granulysin and cathepsin C, into the target cell cytosol (Basu *et al*., 2016).

Additionally, Fas ligand (FASL), expressed on CD8+ T cells, recognize Fas receptors on malignant cells.This interaction activates Fas-associated protein with death domains (FADD). Activation of FADD leads to caspase and endonuclease activation, which leads to the fragmentation of target-cell DNA.

4.1.2.2 Immune checkpoints

PD-1 checkpoint

PD-1 is a receptor, expressed on CD8+ T cells, which mediates immune-inhibitory signals. PD-1 binds to its ligand PD-L1 and PD-L2, which are overexpressed on tumor cell surface. The PD-1–PD-L1/L2 interaction hinders the signaling of CD80-CD28 co-stimulation and suppress TCR signaling. This results into abrogated cytokine production, cell cycle arrest and diminished expression of Bcl-X_L, the pro-survival factor, in cytotoxic CD8+ T cells. PD-1 signaling leads to a rapid shift in CD8+ Tcell metabolism from glycolysis to fatty acid β-oxidation. This shift in metabolism results intointracellular reactive oxygen species (ROS) accumulation, mitochondrial damage and cell death (Raskov *et al*., 2020).

CTLA-4 checkpoint

CTLA-4 receptor binds to the same ligands as CD28. But CTLA-4 has much higher affinitythan CD28. Higher affinity of CTLA-4 resulted into outcompete of

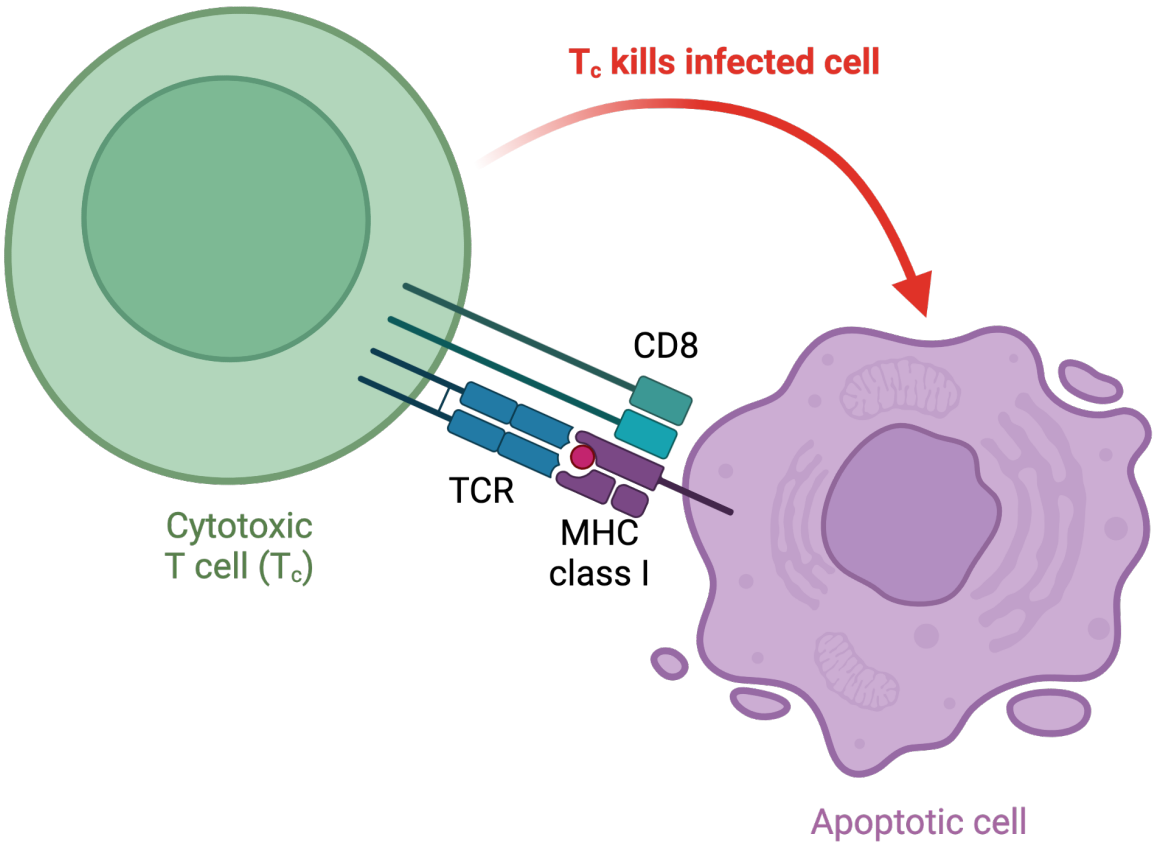

Figure 4. $CD8^+$ cytotoxic T cells recognize processed antigen peptide fragments that binds and displayed on MHC-I through TCR. CD8 co-receptor binds to MHC-I and stabilizes TCR-antigen peptide-MHC-I interaction. Successful TCR signalis followed by CD28-B7 co-receptor activation signal which activates cytotoxic T cells (T_c) to express their killing machinery. Activated T_c cells directly kill infected (or malignant) cells. Created with BioRender.com

CD28 for ligands. After $CD8^+$ T-cell activation, CTLA-4 receptor expression is upregulated. CTLA-4 signaling ensures controlled activation of $CD8^+$ T-cells. CTLA-4 activation reduces the contact time between $CD8^+$ T cell-APC by hindering $CD8^+$ Tcell movements on APCs (Raskov *et al*., 2020).

PD-1 checkpoint inhibition is effective during the effector phase in the peripheral tissues, whereas, CTLA-4 signals are effective during the priming phase of naive T-cell activation and primarily occur in lymphatic tissue. CTLA-4 knockout mice doesn't have the immune checkpoints and develop lymphocyte malignancy.

4.2 B lymphocytes

B cells are localised at the invasive margin of tumors. $CD20^+$ B cell infiltration into the TME is associated with good prognosis in human cancers but is opposite for mouse models, in which B cells inhibit tumor-specific cytotoxic T cell responses.

4.3 NK and NKT cells

Natural killer (NK) cells and natural killer T (NKT) cells, infiltrating the TME are not found to have direct contact with tumor cells. Many cancers, such as gastric, renal, colorectal, liver and lung, they have good prognosis. Although present in the TME, NK cells do not have direct tumor- killing properties.

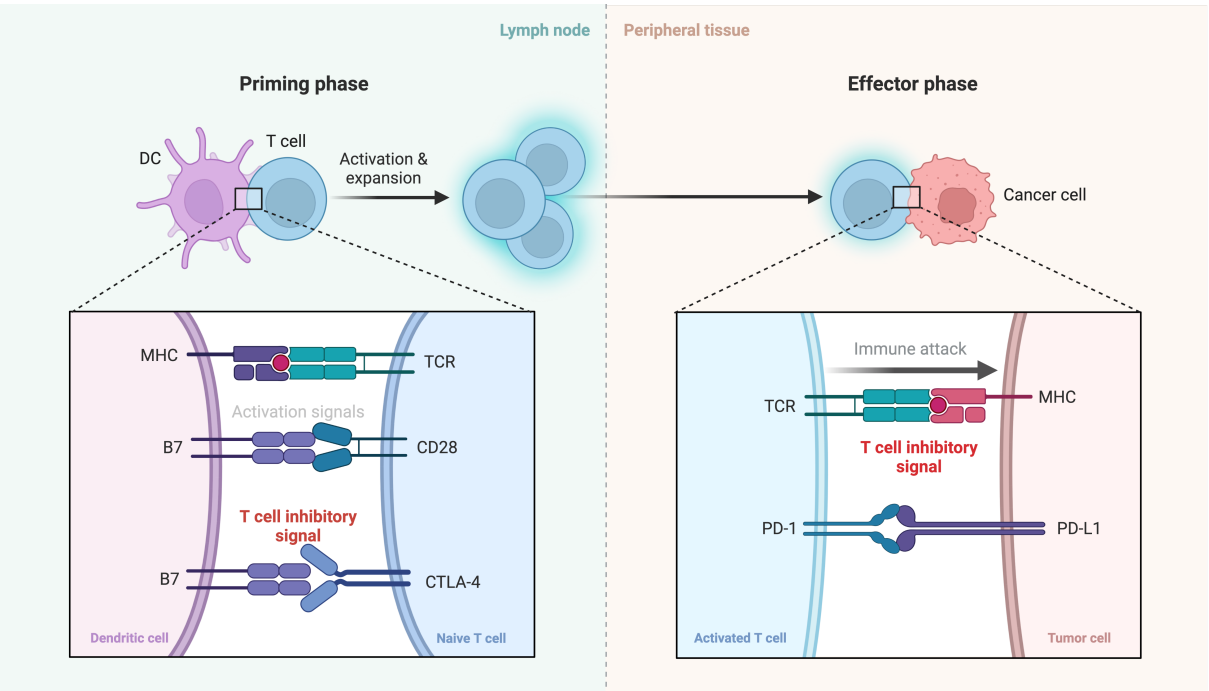

Figure 5. T cell activation could be divided into priming phase and effector phase. Priming phase, which usually takes place in the tumor draining lymph nodes involves dendritic cell mediated neoantigen peptide presentation to $CD8^+$ T Cells through MHC class I presented on DC surface. TCR on T cell surface recognizes MHC-neoantigen peptide complex. Co-stimulatory signal was generated by binding of CD28, present on Naïve T cells, with B7.1 or B7.2, present on DC. CTLA-4 mediated exhaustion takes place in the priming phase, in lymph nodes, whereas, PD1 mediated suppression of T cell function takes place in the effector phase, in tumor microenvironment. Created with BioRender.com

4.4 Tumor-associated macrophages (TAMs)

TAMs are abundant in all cancers. TAMs are associated with malignant cell migration, invasion and metastasis. TAMs are categorized by have an IL-10^{high}IL-12^{low} expression. TAMs are correlated with poor prognosis. M2 macrophages are major contributors to tumor angiogenesis, whereas M1 macrophages is characterised by pro-inflammatory cytokine production and tumour suppression (Zhang *et al.*, 2012; Goswami *et al.*, 2013; Wang *et al.*, 2018). TAMs accumulate in hypoxic and necrotic areas of tumors. It is thought that these areas attract TAMs by releasing hypoxia- induced HIF1α and VEGF.

4.5 Dendritic cells

Dendritic cells (DCs) are professional APCs. TheDCs inside TME are functionally impaired. They have migration capabilities to tumor sites with the help of GFs (VEGF and HGF), chemokines (CXCL12 and CXCL8) and antimicrobial peptide (β- defensin).

4.6 Tumor-associated neutrophils

Tumor associated neutrophils (TANs) have both pro- and anti-tumorigenic properties. Hypoxia in the TME promotes neutrophil recruitment by increasing glycolysis.

4.7 Myeloid-derived suppressor cells

MDSCs are derived from bone marrow

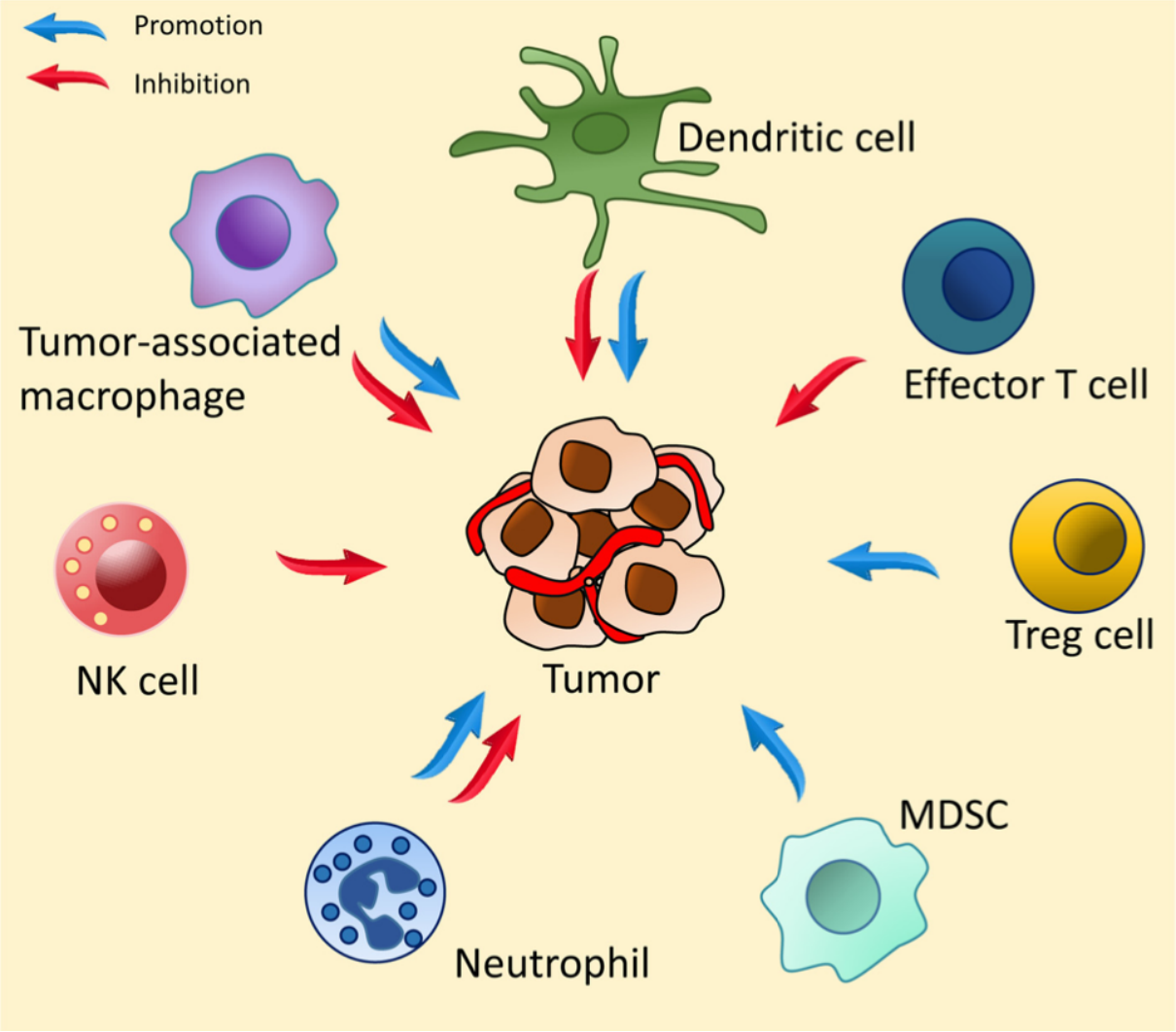

Figure 6. Tumor microenvironment comprised of not only malignant cells but also a variety of immune and non-immune cells. immune cells include dendritic cells (DCs) tumor associated macrophages (TAMs), natural killer (NK) cells, neutrophils, effector T cells, Treg cells and myeloid derived suppressor cells MDSCs). DCs, TAMs, neutrophils could play pro or anti tumorigenic functions according to their state and cytokine profile. MDSCs, Treg cells could effectively exert immunosuppressive activity through which cancer clls could evade immune system and thrive. NK cells and effector T cells on the other hand, could kill recognize and kill tumor cells directly, thus become the most important members of anti-tumor immunity. (Le *et al*., 2019)

and suppress anti-tumor immune response. Hypoxia exposed MDSCs induce HIF related signals and upregulates its functions. MDSCs activates nitric oxide synthase 2 (NOS2) and arginase (ARG1) and inhibits $CD8^+$ T cytotoxicity. They also induce the development of Tregs and the polarization of M1 to M2 macrophages (Ghosh *et al*., 2016; Porta *et al*., 2018).

5. Hormones and growth factors that influence tumor growth

Hormones are known for their ability to influence cellular growth, differentiation, function as well as metabolism. Certain cancers, like breast, endometrial, ovarian and prostate are completely dependent on certain hormones for tumorigenesis and tumor progression (Russo and

Russo, 2006). Estrogen, progesterone and androgens are the key drivers of hormone dependent carcinogenesis. Hormone receptor targeted therapies have got significant success in the past and are considered as standard therapy regime in such cancers.

Growth factors like epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), insulin like growth factor (IGF-1), platelet derived growth factor (PDGF), and transforming growth factor (TGF) have been associated with various forms of cancer and are being implicated for poor prognosis (Sachdev *et al*., 2010; Gunter *et al*., 2008b; a; Salisbury and Tomblin, 2015).

5.1 Insulin like growth factor (IGF) family and their role on tumor growth

IGF family comprised of insulin and two insulin like protein, IGF-1 and IGF-2. Insulin, secreted from pancreatic beta cells have been found to be mitogenic but not carcinogenic on cancer cells. Insulin signaling on cell is mediated through insulin receptor (IR), whereas IGF-1 and IGF-2 signaling is mediated through IGF-1R and IGF-2R respectively (Cheng *et al*., 2005). However, as insulin and IGFs share upto 60% sequence homology, their signaling pathways often overlap. Insulin could act through IR as well as IGF-1R, likewise IGF-1 could exert its signaling through IGF-1R as well as IR. All three receptors belong to tyrosine kinase receptor family. IGFs actions are regulated by six soluble IGF binding proteins (IGFBPs) that bind IGFs with high affinity and specificity. Plasma concentration of IGF-1 in human is relatively high and is approximately 150-400 ng/mL. However, only less than 1% of IGF-1 exists in ligand binding from, rest of them being bound with IGFBPs. IGF-1 is produced by almost all cell types, but the major contributing organ of plasma IGF-1 is liver. Upon stimulation of insulin signaling, hepatocytes secrete IGF-1 which then go to the circulation bound with IGFBP3, also produce by liver. Deregulation of IGF-1R signaling is associated with various kind of disease including diabetic retinopathy, diabetic neuropathy, cardiovascular disease and various forms of cancer (Salisbury and Tomblin, 2015; Sachdev *et al*., 2010). IGF-1 signaling has got significant attention due to its involvement in cancer prognosis. Therapeutic targeting of IGF-1R signal through monoclonal antibodies in cancer has got limited success as the therapy molecules interfere with insulin signaling due to their signal overlapping that results into adverse side effects and toxicities.

IGF-1R activates intracellular PI3K/AKT and RAS/MAPK pathways which are essential for malignant cell survival and proliferation. In normal physiological condition, IGF-1 signaling is tightly regulated. Genetic alterations in IGF-1R leading to varying levels of expression are found to be linked with poor prognosis. Although IGF-1R does not solely drive tumor proliferation, IGF-1R sinaling is necessary for cancer cells to achieve cellular survival and metastasis. High levels of IGF-1 have been reported in prostate and breast cancers, that may promote IGF-1R signaling and consequently activate downstream signaling. Higher IGF-1R

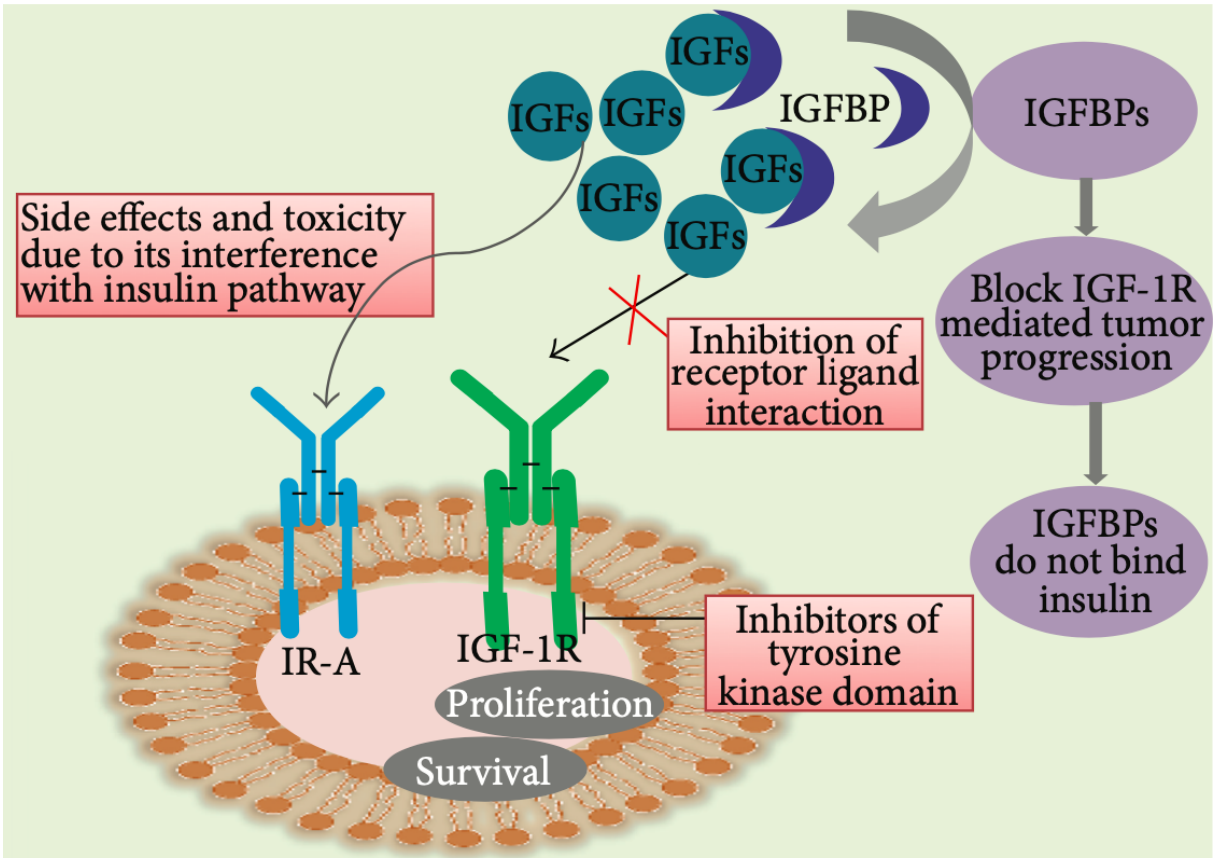

Figure 7. Insulin like growth factor 1 (IGF1) binds to its cell surface receptor IGF1R to exert its proliferative and survival signal to the cell. However, due to sequence homology, IGF1 also may binds to insulin receptors (IRs) which may lead to cellular hyperactivation and toxicity sue to interference with insulin pathway. Concentration of IGF1 in circulation is tightly regulated by insulin signaling. Upon stimulation with insulin, hepatocytes produce insulin like growth factor binding proteins (IGFBPs) which bind and transport IGFs to their respective site of function. IGFBP bound IGF1 couldn't bind to its receptor, thus it blocks IGF1R mediated signals. However, IGFBPs doesn't bind with insulin, thus no interference on insulin signaling cascade. Clinical trials have been carried out to target IGF1 signaling axis in cancer cells with limited success, largely due to toxicity and therapy resistance.

have been reported in different types of cancers, melanoma and carcinoma. Different approaches have taken into consideration to target IGF-1R signaling, like small molecule receptor tyrosine kinase inhibitors (TKIs), anti IGF-1R antibodies, small interfering RNAs (siRNAs) and adenovirus mediated blocking of IGF-1R expression on cancer cells.

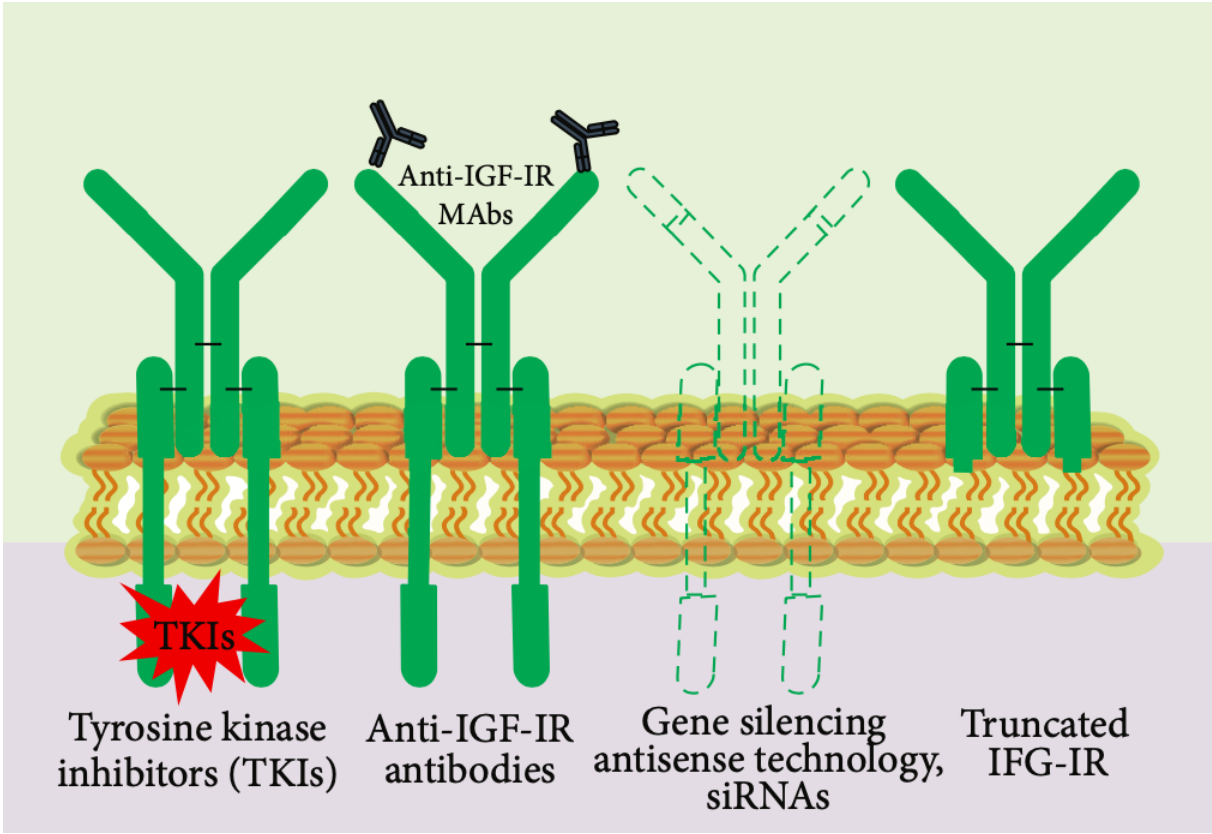

Figure 8. Different approaches were taken to target IGF1 signaling in malignant cells can be broadly categorized into four categories. First, pharmacological inhibition of tyrosine kinases (TKIs) to suppress intracellular RTK signaling. Second, anti-IGF-1R antibodies that bind to IGF-1R, causing a conformational change in the IGF1 binding region, to inhibit IGF1 signaling. Third, using gene silencing technology targeting malignant cells to silence IGF-1R expression and fourth, by introducing truncated IGF-1R to malignant cells. truncated IGF-1R lacks intracellular tyrosine kinase domain, thus inhibiting intracellular signal transduction.

5.2 IGF-1R signaling and their role on $CD8^+$ T cell activation, proliferation and metabolism

IGF-1R is expressed by T cells along with other growth factor receptors. IGF-1, IGF-2 and insulin binds to the surface of T cells. Lee *et al*. 1986 demonstrated IGF-1R on some but not T cell lines derived from patients with lymphoid malignancies. IGF-1R expression is thought to be a marker for T cell activation. Increased binding of ^{125}I-IGF-1 was demonstrated on activated human T cells compared to resting human T cells, which indicated IGF-1 can induce T cell proliferation and chemotaxis. IGF-1R levels were found to be elevated on rat lymphocytes following activation with concanavalin A in $CD8^+$ T cell subsets. Both IGF-1 and IGF-2 play crucial role in development and function of T lymphocytes. IGF-1 activates AKT and thereby enhance T cell survival. IGF-1R displays differential expression profile on T cells at different maturation stages. Quantification of receptor density revealed 66% $CD8^+CD45RA^+$effector T cells and 38% $CD8^+CD45RO^+$ memory T cells exhibited IGF-$1R^+$ phenotype. CD28

crosslinking or activation of CD80/CD86 pathway upregulates IGF-1R expression on T cells (Medyouf *et al*., 2011; Thariat *et al*., 2012; Lee *et al*., 2016; Browne *et al*., 2011). Blocking IGF-IR on TCR- and CD28-engaged T cells decreases lymphocyte survival in the presence of IL-2 (Walsh and O'Connor, 2000). Jurkat cells display enhanced IGF-1R following activation of CD28 and IGF-1 treatment facilitates resistance to Fas-mediated apoptosis. T cells isolated from cord blood showed resistance to spontaneous apoptosis and programmed cell death following activation with IGF-1.

Activation of signaling pathways downstream to IGF-1R increases lymphocyte metabolism. Enhanced glucose uptake takes place following transcriptional upregulation of Glut-1 transporters accompanied by translocation of Glut-4 on cell surface. Moreover, a paradigm shift to aerobic glycolysis is noted, similar to that observed in tumor cells. oxygen demand after activation increases rapidly but is overshadowed by the shift to glycolysis (Douglas *et al*., 2007; DiToro *et al*., 2020; Sjögren *et al*., 1999).

6. $CD8^+$ T cell metabolism

Mature naïve T cells lie in a metabolically quiescentstate in lymphoid organs and circulation until activation through T cell receptor (TCR) followed by costimulatory signals which augments metabolic switching to growth and biomass accumulation. This metabolic switching is facilitated by many fold increase in aerobic glycolysis, a process in which glucose is converted to lactate, even in the presence of sufficient oxygen to support tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS), similar to the metabolism of cancer cells (Warburg effect) in tumor microenvironment (TME) (Brown *et al*., 2019; Sharabi and Tsokos, 2020; Buck *et al*., 2015; Palmer *et al*., 2015; Chang and Pearce, 2016). Although in terms of net ATP yield, glycolysis is less efficient than OXPHOS, glycolysis facilitates generation of a number of metabolic intermediated important for cell growth and proliferation. It also provides a way to maintain redox balance (NAD^+/NADH) in proliferating cell (Brand *et al*., 2016; Cascone *et al*., 2018; Chang and Pearce, 2016; Bantug *et al*., 2018; Palmer *et al*., 2015).

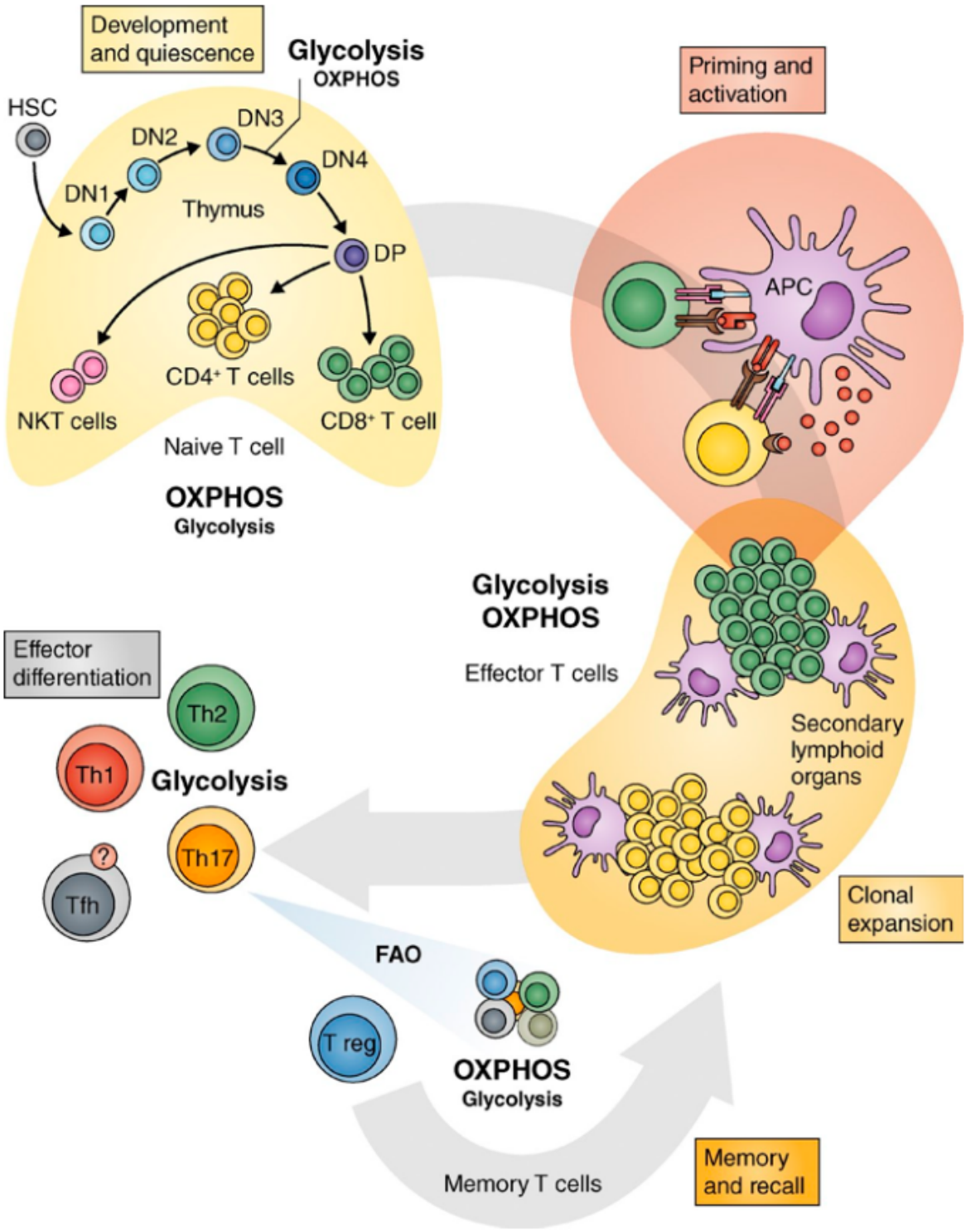

Figure 9. Metabolic switching between glycolysis, OXPHOS and fatty acid oxidation has been observed in different stages of T cell development. Developmental stages of $CD4^+$ T cells and $CD8^+$ T cells, from hematopoietic stem cells (HSCs) to CD4 and CD8 double negative cell stages (DN1, DN2, DN3, DN4) and then CD4 CD8 double positive cells, up to the generation of $CD4^+$ T cells, $CD8^+$ T cells and NKT cells, rely mostly on glycolysis. However naïve T cells mostly rely on OXPHOS and have reduced glycolysis. Upon antigen priming and activation, these cells again enter into a metabolic state where they upregulate their glycolytic activity several times along with high OXPHOS. After clonal expansion, effector T cells completely rely on glycolysis over OXPHOS, however, Treg cells have higher fatty acid oxidation and diminished glycolysis and OXPHOS (Buck *et al*., 2015).

Glucose-6-phosphate and 3-phosphogycerate produced during the course of glycolysis can be metabolised in pentose phosphate pathway and serine biosynthesis pathway respectively, donating important molecular precursor in nucleotide and amino acid biosynthesis respectively. Glucose can also enter mitochondria as pyruvate, where it is converted to acetyl-CoA and joins the TCA cycle, where it is condensed with oxaloacetate to form citrate. Glucose derived citrate can be exported to the cytosol where it is converted to generate acetyl-CoA by ATP citrate lyase for use in lipid synthesis. Metabolic shift in $CD8^+$ T cells following activation is facilitated by the activation of key metabolic regulator mammalian target of rapamycin (mTOR). mTOR exists as two complexes mTORC1 and mTORC2. Both Akt and mTOR promotes aerobic glycolysis and supports T cell growth, differentiation and function, similar to that of cancer cells (Buck *et al*., 2015).

7. $CD8^+$ T cell activation

The essential aspect of natural immune response in terms of anticancer immunity are the surveillance, detection and destruction of malignant cells. despite such immune-surveillance, cancer cells often escape the immune system by modulating the immune components. Tumor infiltrated immune cells become pro-tumorigenic or anti-tumorigenic, and the balance between these two kind of immune cells broadly defines clinical outcomes. Efficacy of immunotherapy largely depends on the degree of immune infiltration within the TME (Sharabi and Tsokos, 2020; Faubert *et al*., 2017; Wu, 2017; Chang and Pearce, 2016; De Rosa *et al*., 2017). Being the most important cells of the adaptive immune system, cytotoxic $CD8^+$ T cells are the most powerful cellular component in the anticancer immune response, thus become the backbone of cancer immunotherapy.

Antigen presenting cells (APCs) process and present antigenic peptide fragments on major histocompatibility complex class-I (MHC-I) on their surface, which is recognized by $CD8^+$ T cells. $CD8^+$ T ells recognize the MHC-peptide complex on APCs or target cells while scanning by crawling over the cells. this direct contact converts mechanical energy into biochemical energy, which plays an important role in TCR activation.

Following TCR signal, a costimulatory signal from CD28 coreceptor must be received before the cells become activated. Without the costimulatory signal the T cells become anergic and are directed to undergo apoptosis. CD28 receptors on $CD8^+$ T cells interact with CD80/B7.1 or CD86/B7.2, both of which are highly expressed on APCs, macrophages and B cells. These ligands of CD28 receptor, by binding to CD28 receptor, play a major role in lowering the activation threshold of naïve $CD8^+$ T cells and enhance cell proliferation and cytokine (particularly IL-2) production.

Hyperactivated $CD8^+$ T cell response may lead to tissue damage and autoimmunity (DiToro *et al*., 2020; Sutton *et al*., 2017; Sharabi and Tsokos, 2020; Roep, 2003; Gearty *et al*., 2022). To ensure optimal T cell activation, to enable host tolerance, and to avoid autoimmunity, $CD8^+$ T cells express a number of immune-inhibitory receptors on their surfaces, known as immune checkpoint molecules. These

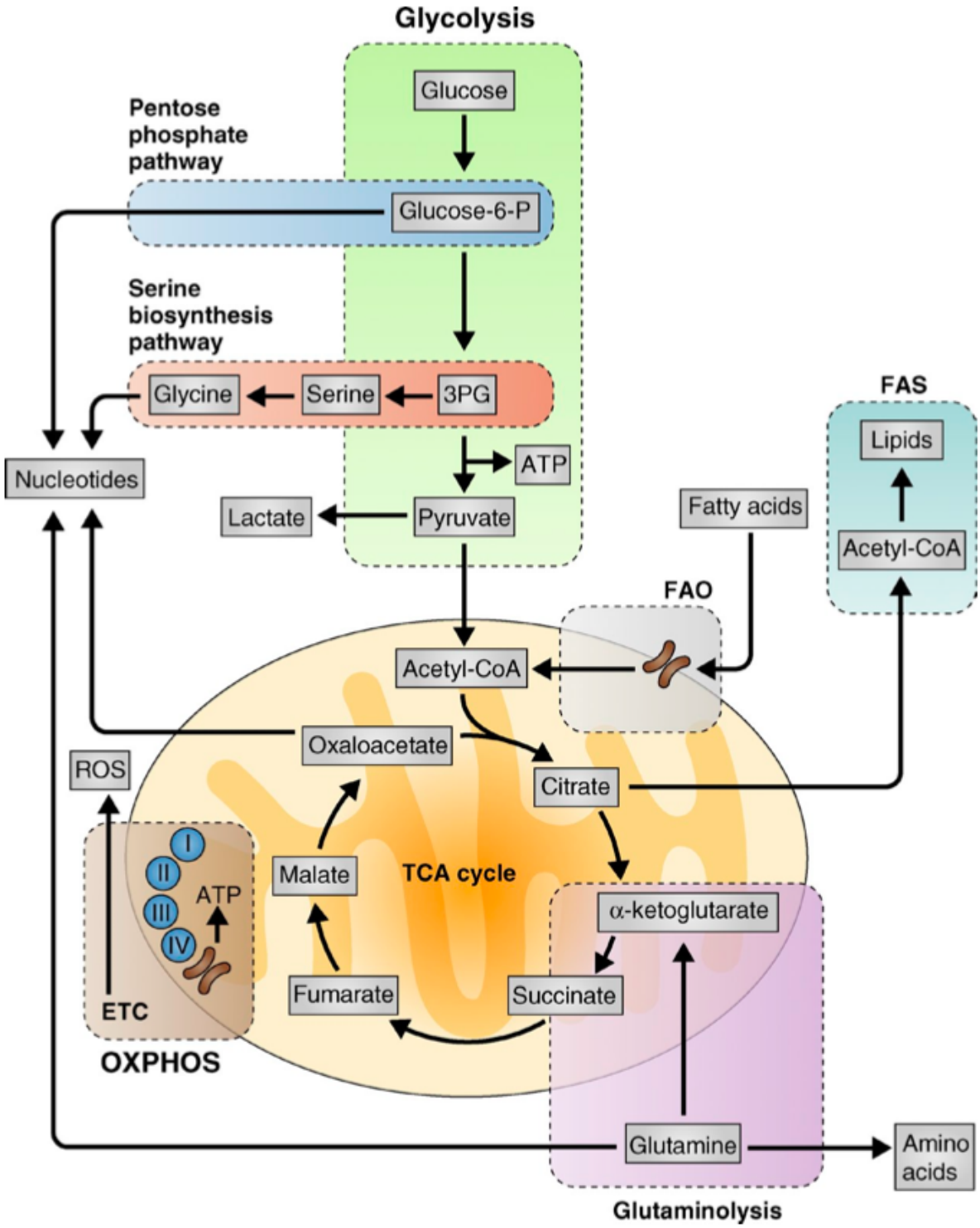

Figure 10. Schematic representation of different biochemical pathways involved in metabolism and their intersections. Glycolysis includes generation of pyruvate from glucose by stepwise chemical reactions. Glucose-6-phosphate, an intermediate of glycolysis may be utilised in pentose phosphate pathway for nucleotide biosynthesis. 3-phosphoglycerate (3-PG), another intermediate of glycolysis may be utilised to make serine (serine biosynthesis pathway) and then glycine to get into pentose phosphate pathway for nucleic acid biosynthesis. End product of glycolysis is pyruvate, which could be converted to lactate in absence of oxygen or could go into mitochondria to produce acetyl-CoA to enter TCA cycle. Fatty acid oxidation also contributes to generation of acetyl-CoA. Glutaminolysis may contribute to generation of α-ketoglutarate, which then enters to TCA cycle. Glutamine also contributes to pentose phosphate pathway (Buck *et al*., 2015).

checkpoint molecules regulate T cell hyperactivation in during the course of a vast amount of activation signal. However, a malignant tumor may exploit these checkpoint signals to promote immunosuppressive state which facilitates their survival (Hussaini *et al*., 2021). Sustained exposure to cancer neoantigens may induce the expression of immune checkpoint molecules on the surface of $CD8^+$ T cells. chronic neoantigen exposures drive T cells to a dysfunctional state called T cell exhaustion and merely removal of neoantigens doesn't necessarily restore T cell function (Wei *et al*., 2018; Dyck and Mills, 2017).

7.1 Immune checkpoints

Proper regulation of immune activation is necessary to avoid hyperactivation or autoimmune reactions. In order to achieve that, immune cells express a number of regulatory molecules, called immune-checkpoints on their surfaces. Programmed cell death receptor 1 (PD-1) and cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) are two prominent immune checkpoint molecules expressed by T cells. T-cell immunoglobulin and mucin domain-3 (TIM-3), lymphocyte- activation gene 3 (LAG-3), , T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and inducible T-cell co-stimulatory receptor (ICOS) are among the other immune-checkpoint molecules expressed by T cells (Dyck and Mills, 2017; Tian *et al*., 2022). Expressions of these molecules are now being used to monitor T cell exhaustion. PD-1 binds to programmed death ligand 1 (PD-L1) or PD-L2, and CTLA-4 binds to B7.1 or B7.2, with higher affinity than CD28, expressed on cancer cell surfaces. These receptor ligand interactions induce immune checkpoints in T cells and resulted into T cell anergy and apoptosis.

7.1.1 The PD-1 checkpoint

PD-1-PD-L1 and/or PD-1-PD-L2 interaction antagonizes CD80-CD28 co stimulation, even at a very low PD-1 expression, resulted into diminished cytokine production, cell cycle arrest and decreased transcription of anti-apoptotic $Bcl\text{-}X_L$ in cytotoxic $CD8^+$ T cells. PD-1 signaling also causes a rapid shift in $CD8^+$ T cell metabolism from glycolysis, which provides faster energy, redox balance and necessary precursors for nucleic acid biosynthesis, to fatty acid -oxidation, which leads to accumulation of reactive oxygen species (ROS), mitochondrial damage and cell death.

7.1.2 The CTLA-4 checkpoint

CTLA-4 binds to B7.1 or B7.2, same ligands as CD28, but with 20 times higher affinity. CTLA-4 expression on $CD8^+$ T cells is upregulated following T cell activation. CTLA-4 signaling regulates $CD8^+$ T cell movements and APC-T cell interaction by reducing the contact time between the cells. CTLA-4 signals primarily occur in lymphatic tissue and are effective during the priming of naïve $CD8^+$ T cell activation, while PD-1 signals occur within the peripheral tissues and are effective during the effector phase of $CD8^+$ T cell activation.

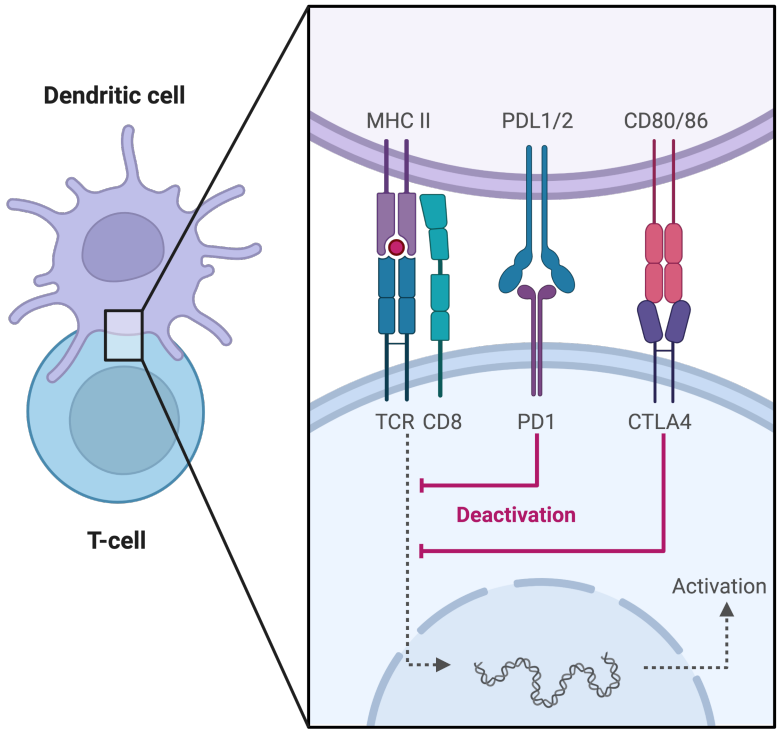

Figure 11. PD-1 and CTLA-4 are expressed on T cells and are two major immune checkpoint molecules. PD1 binds to PD-L1 or PD-L2 expressed on antigen presenting cells or malignant cells. CTLA-4 binds to CD80 or CD86 (B7.1 or B7.2) expressed on antigen presenting cells or malignant cells, with much higher affinitythan CD28. Both these binding exert inhibitory signal to T cells thereby inhibiting T cell activation. Created with BioRender.com

7.2 Immune-checkpoint inhibitor therapy

Discoveries of immune checkpoint molecules and targeting them with monoclonal antibodies have revolutionised the field of immune-oncology. Prof. James P. Allison of MD Anderson Cancer Centre, Texas, USA, and Prof. Tasuku Honzo, Kyoto University, Kyoto, Japan, shared the Nobel Prize in 2018 for their discoveries of CTLA-4 and PD-1 respectively. Following the discoveries of PD-1 and CTLA-4 and subsequent discoveries of other immune-checkpoint molecules, immune-oncology fraternity has designed a immunotherapeutic strategy in which they uses monoclonal antibodies targeting the ligands of these immune checkpoint molecules, which enhances antitumor efficacy of antitumor immune response, as well as revitalises exhausted $CD8^+$ T cells (Liu *et al.*, 2023). Below is a list of immune checkpoint blockade therapies approved or in clinical trial.

Complete response has been seen in as few as 80 days for patients who shows complete response to anti-PD-1 therapy. Higher infiltration of $CD8^+$ T cells along with increased PD-L1 expression is positively correlated with therapeutic efficacy of PD-1 blockade therapy. However, not all patients exhibit durable response to these therapies. In United States, only 13% patients respond to this immune-checkpoint blockade therapy. In addition, checkpoint blockade therapy is associated with dermatological, gastrointestinal, hepatic and endocrine

Table 1. List of immune checkpoint blockade therapies in clinical trial or FDA approve, along with their manufacturer and application in various types of cancer.

Drug	Company	Indication	Phase	Status
ipilimumab	Bristol-Myers Squibb	Metastatic melanoma	III	FDA approved
nivolumab	Bristol-Myers Squibb	Metastatic melanoma, advanced non-small cell lung cancer, Hodgkin lymphoma, head and neck cancer, kidney cancer, bladder cancer, colorectal cancer, Merkel cell carcinoma, mesothelioma	III	FDA approved
pembrolizumab	Merck & Co.	Metastatic melanoma, advanced non-small cell lung cancer, head and neck cancer, kidney cancer, bladder cancer, colorectal cancer, Merkel cell carcinoma, mesothelioma	III	FDA approved
cemiplimab	Regeneron Pharmaceuticals	Metastatic melanoma	III	FDA approved
atezolizumab	Roche	Metastatic bladder cancer, non-small cell lung cancer, head and neck cancer, Hodgkin lymphoma, Merkel cell carcinoma	III	FDA approved
durvalumab	AstraZeneca	Metastatic non-small cell lung cancer, bladder cancer, head and neck cancer, Hodgkin lymphoma, Merkel cell carcinoma	III	FDA approved
avelumab	Bristol-Myers Squibb	Metastatic Merkel cell carcinoma, advanced bladder cancer, head and neck cancer, triple-negative breast cancer, Hodgkin lymphoma, renal cell carcinoma, non-small cell lung cancer	III	FDA approved
MEDI5752	Merck & Co.	Metastatic melanoma, non-small cell lung cancer	II	In clinical trial
TSR-012	Teva Pharmaceuticals	Metastatic melanoma, non-small cell lung cancer	II	In clinical trial

mAb3111	Genmab	Metastatic melanoma, non-small cell lung cancer, head and neck cancer, kidney cancer, bladder cancer, colorectal cancer, Merkel cell carcinoma, mesothelioma	II	In clinical trial
mDC1536	Innate Immunotherapeutics	Metastatic melanoma, non-small cell lung cancer	II	In clinical trial
INO-4800	Inovio Pharmaceuticals	Metastatic melanoma, non-small cell lung cancer	II	In clinical trial
MPDL3280A	MedImmune	Metastatic melanoma, non-small cell lung cancer, head and neck cancer, kidney cancer, bladder cancer, colorectal cancer, Merkel cell carcinoma, mesothelioma	II	In clinical trial

side effects. Co-administering these drugs with one or more therapies (such as another immunotherapy or cytotoxic chemotherapy) aims to lessen their toxicity and boost their effectiveness. The approval of nivolumab in combination with ipilimumab for the treatment of metastatic melanoma, advanced renal cell carcinoma, and mismatch repair-deficient (dMMR) colorectal cancer provides proof that the combination of PD-1/PD-L1 inhibitors and a CTLA-4 inhibitor has shown potential (Schmid *et al*., 2018; Horn *et al*., 2018). By indication and line of therapy, monotherapy was compared with combination therapy in 98 clinical trials (n = 24,915) of PD-1 inhibitors. Combination therapy showed higher objective response rates in 82.7% of the trials (Schmidt *et al*., 2020).

7.3 $CD8^+$ T cell (CAR T-cell) adoptive transfer therapy

Chimeric antigen receptor T cell involves construction of a synthetic CAR by fusing single chain variable fragment (scFv) of a tumor antigen specific immunoglobulin with an intracellular signaling domain. CARs are designed in such a way that it could recognize an antigen on tumor cell (protein, carbohydrate or glycolipid) irrespective of APC-T cell interaction (Depil *et al*., 2020). CAR T cell therapy has revolutionized the treatment of elapsed or refractory acute lymphoblastic leukemia (ALL) and diffused large B cell lymphoma (DLBCL) (Brentjens *et al*., 2007). CD19 has been chosen as a target molecule on malignant cells, as expression of this molecule on the other healthy cells are very limited. In 2017, FDA approved first CD19 CAR T cell therapy for ALL and DLBCL patients. However, there have been side effects due to cytokine release (cytokine storm) which results into immune hyperactivation and neurotoxicity (Norelli *et al*., 2018; Hamid *et al*., 2013; Overman *et al*., 2018). Scientists have

coined a term as immune-related adverse events (irAEs) to describe the side effects of immune checkpoint therapies and CAR T cell adoptive transfer therapies. One of the irAEs following ICBT or CAR T cell therapy is type 1 diabetes (T1D) (Zhou *et al*., 2022).

8. Type 1 diabetes (T1D)

T1D or insulin dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by insulin deficiency due to loss of insulin producing pancreatic β cell. Insulin deficiency in T1D patients leads to hyperglycemia. Chronic hyperglycemia leads to diabetic retinopathy, neuropathy, hepatopathy and a lot of other complications (Mertens *et al*., 2021; Sempere-Bigorra *et al*., 2021; Papadopoulou-Marketou *et al*., 2017). T1D pathogenesis has mostly been found in juvenile condition, thus also named as juvenile onset diabetes. However, occurrence of T1D in adults are also not rare (Zhang *et al*., 2022; Buzzetti *et al*., 2007). A few cancer patients develop T1D as a side effect, after receiving checkpoint blockade therapy or CAR T cell therapy (Zhou *et al*., 2022). T1D is caused by the generation of autoantibodies and auto reactive $CD8^+$ T cells specific for pancreatic β cells (Buzzetti *et al*., 2007). Recent studies have shown that T1D is not a single autoimmune disease but resulted from a complex interaction of genetic, environmental, metabolic and immune related factors. Children with T1D commonly presented with symptoms of polyuria, polydipsia, weight loss and diabetic ketoacidosis. Although the classical definition of T1D is classified as juvenile onset, the disease may occur at any age, and as many as 50% of them are misdiagnosed as type 2 diabetes (T2D). Random plasma glucose concentration of more than 200 mg/dL, fasting blood glucose concentration above 126 mg/dL and/or abnormal result of oral glucose tolerance test is diagnosed as diabetes. However these tests doesn't confirm whether it is T1D or T2D. plasma insulin concentration, plasma C peptide level, β cell specific autoantibodies detectionsare necessary to differentiate T1D from T2D (Zhang *et al*., 2022; Sempere-Bigorra *et al*., 2021). Although, no single clinical feature can distinguish T1D from T2D.

9. Immune microenvironment in T1D, similarities with TME

Majority of the autoreactive T cells are eliminated by apoptosis during thymic selection. However, a few number of low avidity autoreactive T cells may escape negative selection process and reach to the periphery. Pancreatic β cell specific naïve autoreactive T cells have been found in circulation of T1D as well as heathy individuals (Rodriguez-Calvo *et al*., 2014; Pinkse *et al*., 2005). Activation of these T cells require priming by the antigens of specific peripheral tissues. However, even after activation, these autoreactive T cells remain under control by a number of immune regulatory mechanism, which involves regulatory T (Treg) cells. genetic predisposition combined with local inflammation trigger the break in immunetolerance in pancreatic β cells, leading to insulitis and β cell death (Culina *et al*., 2018). Increasing evidences suggest β cells themselves also participate in their own death by expressing neoantigens on their surfaces (Rodriguez-Calvo *et al*., 2014). These neoantigens may prime,

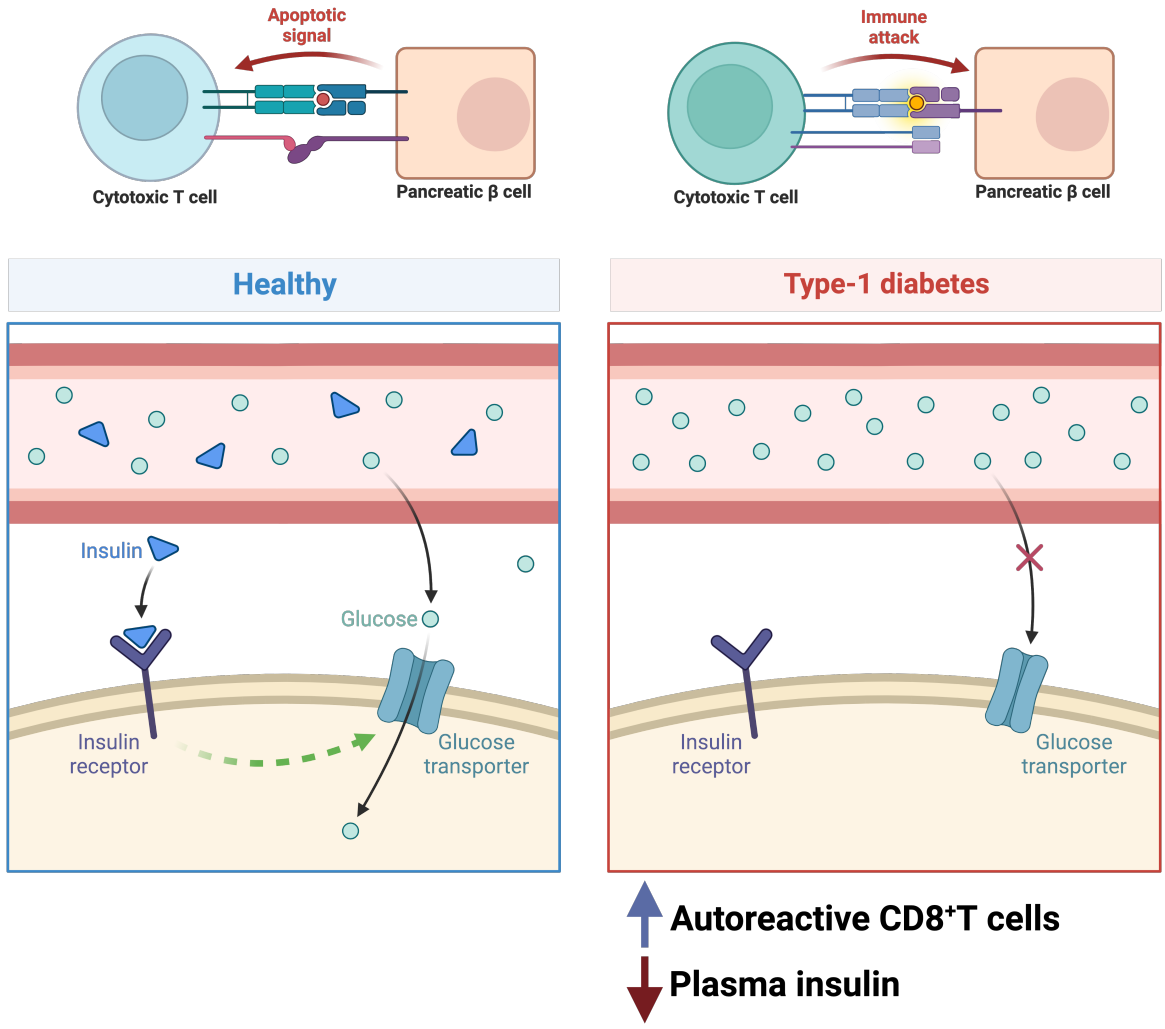

Figure 12. In healthy individuals T cells that are autoreactive to pancreatic β cells are eliminated during thymic maturation phase. Even if few β cell specific T cells escape the elimination phase during thymic maturation, they were inhibited by the expression of PD-L1 on pancreatic β cells. However, in type 1 diabetes pancreatic β cells fail to express PD-L1 and thus become subject to destruction by autoreactive cytotoxic T cells, which resulted into reduced plasma insulin. Created with BioRender.com

activate and accelerate autoreactive T cell mediated cell death. The inflammatory signals and immune responses in the islets during insulitis resembles the signals and immune responses identified at the TME during a successful antitumor immune response. Both β cells and tumor cells generate proinflammatory cytokines IL-1β, chemokines (CCL2, CXCL10) and neoantigen peptides recognized by tumor specific or autoreactive T cells respectively. Pancreatic β cells also overexpress human leukocyte antigen class 1 (HLA class 1), which subsequently precedes insulitis in T1D (Kronenberg *et al.*, 2012). Tumor cells however, may develop a way to evade immune surveillance by downregulating HLA class 1 expression.

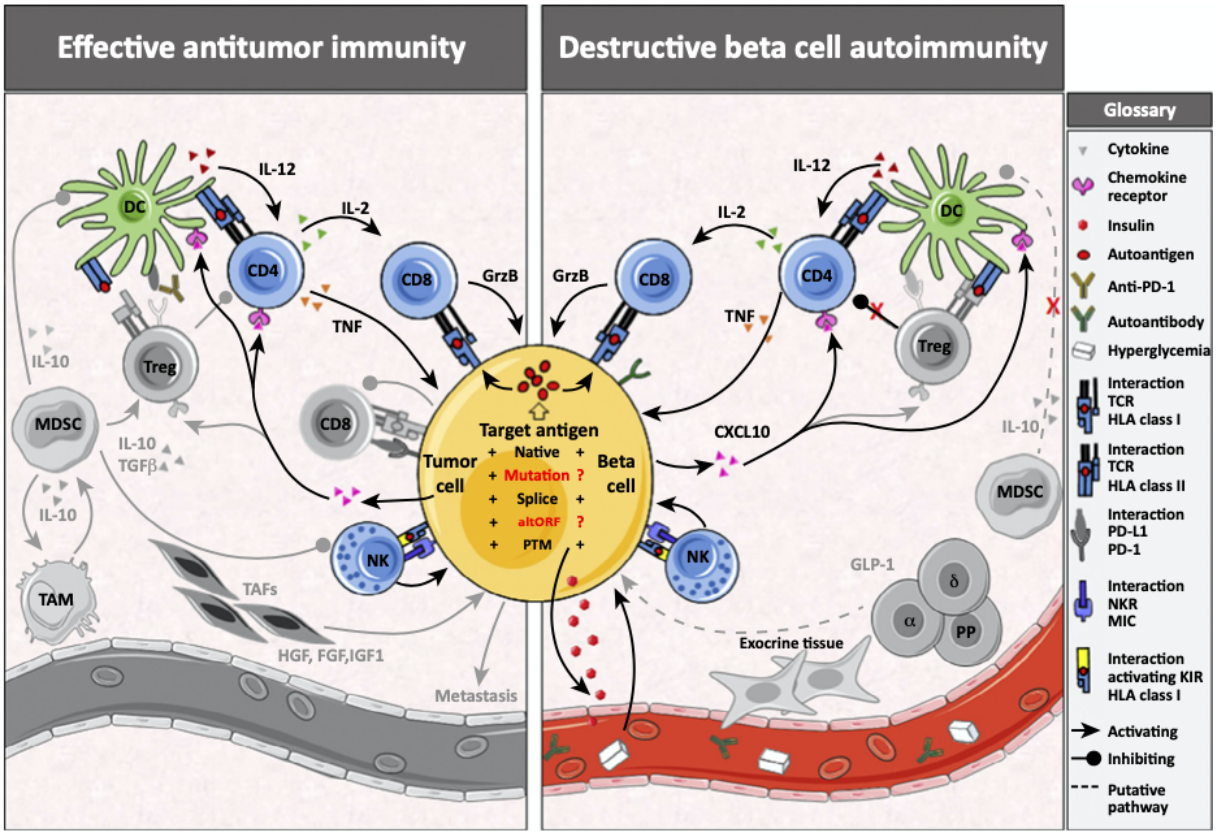

Figure 13. "The proinflammatory milieu in effective antitumor immunity highly resembles pathogenic immune responses in type 1 diabetes (T1D). Colored components are effectively engaged in target cell destruction. The tumor microenvironment otherwise suppressing antitumor immunity by downregulation of human leukocyte antigen (HLA) class I on tumor surface and recruitment of myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and tumor-associated fibroblasts (TAFs) sustaining tumor survival, and promoting tumor cell growth and metastasis (left panel, gray tones) is outweighed by a proinflammatory immune infiltrate and adaptive immunity against the target cancer cells (colored). Destruction of pancreatic beta cells in T1D follows similar proinflammatory processes (right panel, colored tones). We propose that lessons from a deleterious tumor microenvironment may guide studies investigating the roles of MDSCs, regulatory T cells (Tregs), and contribution of the beta cell microenvironment [other endocrine islet cells (alpha, delta, PP) and exocrine tissue] that may control pathogenic beta cell destruction (broken arrows). At the heart of target cell destruction, the adaptive immunity involves recognition of target cell antigens. Recent insight points to a role of modified tissue antigens, in addition to native autoantigens in both the pathogenesis of T1D and effective antitumor immunity or immunotherapy. Abbreviations: DC, dendritic cell; KIR, killer cell immunoglobulin-like receptor; NK, natural killer cell; TNF, tumor necrosis factor." (Maria *et al.,* 2016, *Trends in Endocrinol and Metabol*)

10. Pre-existing T1D and risk of cancer

The incidence of T1D is increasing annually among children; thus, there is an increasing need for further research to study its impact on inflammatory diseases, including cancer. However, the underlying mechanism by which T1D influences cancer progression is largely unknown. Meta-analysis, using PubMed and EMBASE observational

studies, suggests the association of T1D with an increased risk of cancers of the stomach, lung, pancreas, liver, ovary, and kidney, but decreased risk of melanoma, prostate, and breast cancer (Giovannucci *et al*., 2010; P *et al*., 2009). Literature is available in relation to T2D and cancer, but the association of T1D with cancer is mostly overlooked (Chen *et al*., 2017). The physiological landscapes of T1D and T2D are significantly different; thus, study outcomes based on T2DM patients might not be replicable to T1D patients. Furthermore, epidemiological retrospective studies often misinterpret T2D as T1D, which may lead to erroneous conclusions. In an earlier prospective *invivo* study of experimental rat mammary carcinoma, induction of alloxan-mediated T1D caused tumor regression in the majority of cases (Heuson and Legros, 1972). In their study, they induced experimental mammary carcinoma in rats first, then treated the with T1D causing drug alloxan. Thus, in their study, there was a possibility of direct involvement of T1D inducing drug in carcinoma regression. Lack of mechanistic insights on cancer progression in T1D hosts drive us to design this experimental setup.

11. Neem leaf glycoprotein (NLGP), a natural neem leaf derived immunomodulator

Neem (*Azadirachta indica*) is recognized as one of the most valuable plants among the traditional medicinal plants in the world, having a wide spectrum of biological functions. In 1992, US National Academy of Science designated this tree, as 'A tree solving global problem'. In ancient Sanskrit scripts, neem tree has been designated as '*Sarboroganibarani*', which means a component that can cure all kind of diseases (Council, 1992). Different components of the neem tree like leaves, bark, flowers, twigs, gum, seeds, pulp and oil were found to be beneficiary for the mankind. It possess anti-fungal, anti-helminthic, anti-bacterial, anti-viral, anti-diabetic as well as anti- cancer effects (Bose and Baral, 2018). About 20 years ago, our lab from Chittaranjan National Cancer Institute, Kolkata, India, started experimenting on the effect of an aqueous preparation of neem leaves called neem leaf preparation (NLP) on cancer cells (Haque *et al*., 2006; Baral and Chattopadhyay, 2004b; Bose *et al*., 2007; Mandal-Ghosh *et al*., 2007). Initially, we observed that NLP causes tumor growth restriction in murine Ehrlich carcinoma (EC) and B16 melanoma (B16Mel) tumor models in prophylactic settings (Mandal-Ghosh *et al*., 2007; Baral and Chattopadhyay, 2004a; Ghosh *et al*., 2009b, 2006, 2009c; a). The NLP was found to be non-toxic, hemato-stimulatory and immuno-stimulatory. NLP showed adjuvant functions in murine models in enhancement of immunogenicity of B16 melanoma antigen, breast tumor associated antigen and carcinoembryonic antigen (CEA) by induction of functional antitumor immunity (Sarkar *et al*., 2009; Baral and Chattopadhyay, 2004a; Mandal-Ghosh *et al*., 2007). Further, NLP mediated immune activation provides protection to the host from leukopenia that results from cancer chemotherapy. After few years of intense research, we identified, purified and characterized an active component in NLP responsible for these potent anti-tumor properties as a glycoprotein. We named it neem leaf glycoprotein (NLGP) (Banerjee *et al*., 2014b).

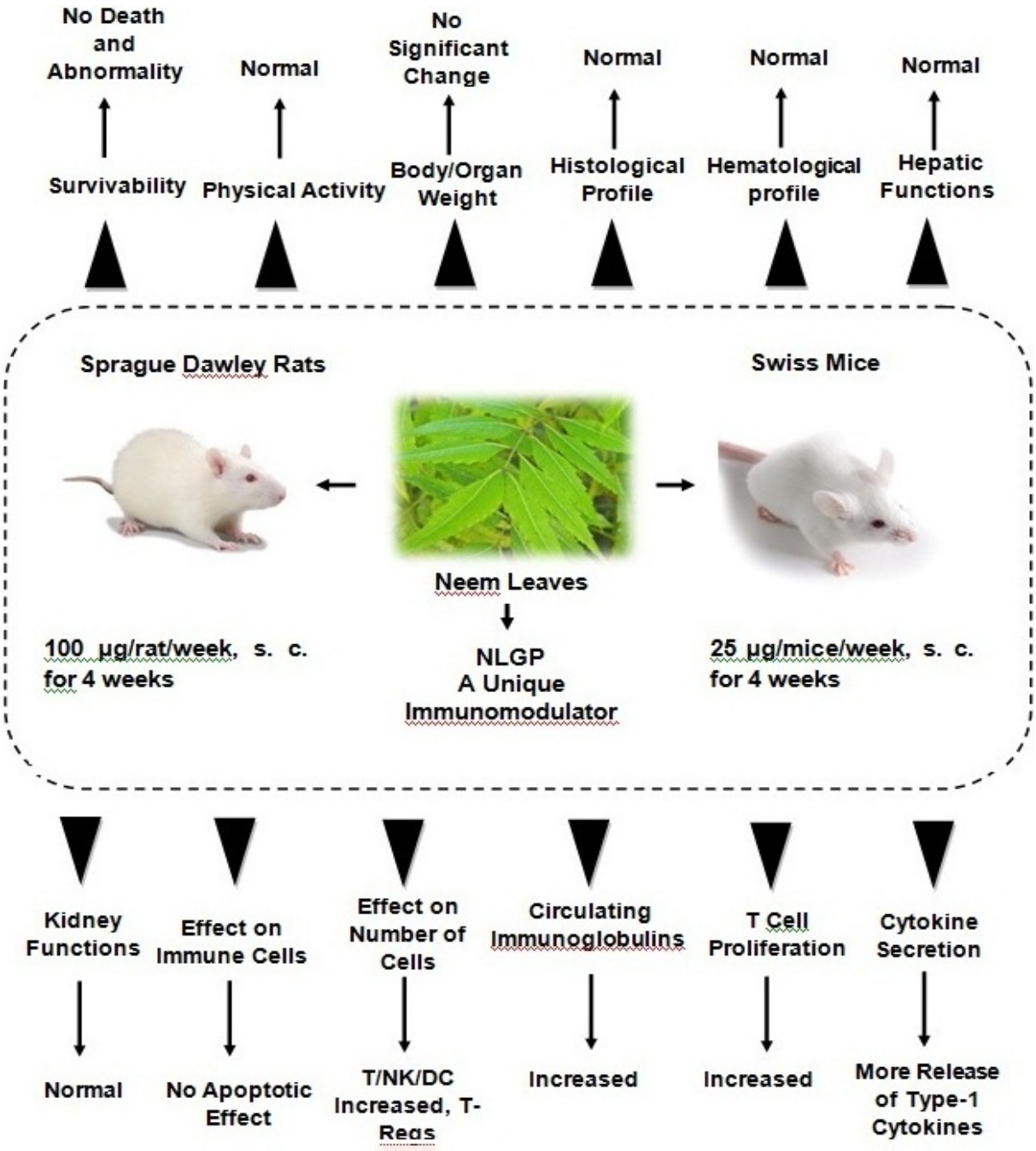

Figure 14. schematic representation describing NLGP, a unique, natural neem leaf derived immunomodulator, having anticancer potential without severe side effects. Experiments were conducted on sprague dawley rats and swiss mice and it was found NLGP is safe for murine physiology. NLGP activates cytotoxic T cells which directly kill tumor cells.

NLGP activates $CD8^+$ T cells (Bose *et al*., 2009b; Mallick *et al*., 2013), NK cells (Bose *et al*., 2009a), inhibits Tregs (Chakraborty *et al*., 2011), promotes type 1 cytokine microenvironment (Bose *et al*., 2009b) and rectify altered chemokine signaling. In addition to these immunomodulatory functions, NLGP possesses the ability to mature human myeloid (Goswami *et al*., 2010) and mouse bone marrow derived DCs (Sarkar *et al*., 2010). NLGP-matured DCs show upregulated expression of

CD83, CD80, CD86, CD40, MHCs and these DCs possess upregulated secretion of IL-12 and downregulation for IL-10, indicating maturation towards DC1, and generate an anti-tumor type 1 immune microenvironment (Goswami *et al*., 2010; Sarkar *et al*., 2010). This NLGP matured DCs effectively present carcinoembryonic antigen (CEA) to B and T cells that may be helpful in retardation of the CEA^+ tumor growth (Das *et al*., 2015a; b; Sarkar *et al*., 2009). NLGP was also observed to be effective in rectifying the dysregulated functions of DCs from cervical cancer patients(Roy *et al*., 2011).

In 2013, our group reported that NLGP have therapeutic efficacy to restrict the growth of established mouse sarcoma and melanoma by activating $CD8^+$ T cell response (Mallick *et al*, 2013b; Barik *et al*, 2013; Barik *et al*, 2015). In the same system, we also observed the increased proportion of $CD11c^+CD86^+CD80^+$ DCs after NLGP treatment (Mallick *et al*, 2014). Established sarcoma in Swiss mice was also targeted by tumor derived antigen pulsed NLGP matured DCs and such targeting is $CD8^+$ T cell dependent. TME obtained from NLGP treated sarcoma and melanoma bearing mice (NLGP-TME), is far more normalized than from untreated tumor bearing mice (PBS-TME), immunologically as well as biochemically. Therefore, NLGP has emerged as a potential beneficiary for the prophylactic as well as therapeutic treatment of different forms of cancer.

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OBJECTIVES

1. **To study how pre-existing type 1 diabetes (T1D) influence tumor growth and host survival.**
2. **To study the influence of immune cells in tumor progression in hosts with pre-existing T1D.**
3. **To study the role of pre-existing T1D in $CD8^+$ T cell and cancer cell metabolism.**
4. **Exploration of molecular signaling that influence CD8+ T cell and tumor cell metabolism in pre-existing T1D tumor bearing hosts.**
5. **To study the immunomodulatory potential of neem leaf glycoprotein (NLGP) on tumor growth and $CD8^+$ T cell metabolism in tumor bearing hosts with pre-existing T1D.**

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3.1. Materials and Methods

3.1.1. Consumables

3.1.1.1. Laboratory Reagents:

Name of Reagents	Product Description	Manufacturer
Acetic acid	CH_3COOH	SD Fine Chem. Ltd, India
Acrylamide	Acrylamide/bis-Acrylamide, 37.5:1	Sigma, USA
Agarose	For nucleic acid analysis	GIBCO BRL, USA
APS	Ammonium persulfate	Sigma, USA
Boric acid	H_3BO_3	Sigma, USA
β-Mercaptoethanol	1000X: $5.5x10^{-2}$ M in D-PBS	Life Technologies, USA
Bromophenol blue	3', 3", 5', 5" – Tetrabromophenolsulphonepthalein sodium salt	US-Biologicals, USA
BSA	Albumin bovine fraction V powder	SRL, India
Chloroform	Anaesthetic	SRL, India
Coomassie brilliant blue	Content 75% anhydrous brilliant blue dye	Sigma, USA
Collagenase	Enzyme, that breaks down collagen	Merck, USA
CytoFix/CytoPerm solution	Fixation and permeabilization solution (1X), BD Perm/wash™ Buffer (10X), Golgistop.	BD Pharmingen, USA
DAPI	Aqueous mounting media	Abcam, USA
DEPC	Diethyl pyrocarbonate, $O(COOC_2HS)_2$	Sigma, USA
Dimethyl sulfoxide (DMSO)	Methyl sulfoxide, C_2H_6OS	Sigma, USA
DMEM-low glucose	Cell culture medium	GIBCO, Invitrogen, USA; Himedia, India
EDTA	Ethylenediaminetetra acetic acid, disodium salt	SRL, India
Ethanol	C_2H_5OH (Absolute)	Merck, Germany
Ethidium bromide	2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide	Sigma, USA
FBS	Fetal bovine serum, US Origin	Himedia, India

Folin's reagent	2 (N) for estimation of proteins and phenols	Himedia, India
Glutamine	L-Glutamine	Life Technologies, USA
Glycine GR	H_2NCH_2COOH	Merck, India
Go Taq Green PCR Master Mix	DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffer	Promega, India
Sulphuric acid	H_2SO_4	Merck, India
LSM 1077	Lymphocyte separating media for human PBMCs	Himedia, India
LSM 1084	Lymphocyte separating media for mouse PBMCs	Himedia, India
Hydrochloric acid	HCl	Merck, India
HEPES	N-2-hydroxyethyl piperazine N-2 othanesulphonic acid	Sigma, USA
KCl	Potassium chloride purified	Merck, India
L-cysteine	Amino acid	Sigma, USA
L-cystine	Amino acid	Sigma, USA
Lipofectamine	Transfection reagent	Invitrogen, USA
Methanol	CH_3OH	RANKEM, India
Methylene blue	Methylthionine chloride; 3, 7-bis (Dimethyl aminophenazothionium chloride)	Sigma, USA
Orthophosphoric acid	H_3PO_4, 85%	Merck, India
OCT	Optimal cutting temperature compound	Sakura Finetek, Torrance, CA, USA
Opti-MEM	Cell culture medium	Gibco, Invitrogen, USA
Paraformaldehyde	Polyoxymethylene; $(CH_2O)_n$	Sigma, USA
PBS	Phosphate buffered saline	Life Technologies, USA
Pen-strep	Penicillin, Streptomycin, Neomycin Solution	Himedia, India
Pentothal sodium	Thiopentone sodium	Abott Laboratories Ltd, India
Protein G- sepharose	Recombinant Protein G (E. coli, Mr = 22,000) , covalently coupled to crosslinked 4% agarose beads	Sigma, USA
Rapamycin	mTOR inhibitor	Merck, USA
RPMI 1640	Cell culture medium	Gibco, Invitrogen, USA

Streptozotocin	N-nitroso-containing diabetogenic compound	Millipore, USA
Sodium chloride crystal	NaCl	Merck, India
Sheath fluid	Fluid used in flow-cytometry	BD Pharmingen, USA
Sodium bi-carbonate	$NaHCO_3$	SRL, India
Sodium dodecyl sulphate (SDS)	Detergent	SRL, India
Sulphanilamide	$C_6H_8N_2O_2S$	Merck, India
TEMED	N, N, N', N'-tetramethylethylenediamine	Life Technologies, USA
Thymidine (^3H)	Radioactive nucleoside	
TMB	Tetramethylbenzidine	BD Pharmingen, USA
TRIS buffer	Tris [hydroxymethyl] aminomethane	Sigma, USA
TritonX-100	Octylphenoxypolyethoxyethanol	Sigma, USA
Trizol reagent	Mono-phasic solution of phenol and guanidine isothiocynate	Invitrogen, USA
Trypan blue	Dye content~40% anhydrous M.W. 960.8	Sigma, USA
Tween-20	Polyoxyethylenesorbitanmonolaurate	Sigma, USA

3.1.1.2. Antibodies and recombinants

Name of target molecule	Reactivity	Fluorochrome or enzymes attached/ purified	Manufacturer
CD8	Mouse	PE	Biolegend, USA
CD8	Mouse	FITC	Biolegend, USA
CD8	Mouse	APC	Biolegend, USA
CD8	Human	Cy Chrome	BD Pharmingen, USA
CD28	Mouse	PE	Biolegend, USA
CD69	Mouse	FITC	Biolegend, USA
CXCR3	Mouse	APC	Biolegend, USA
PD1	Mouse	FITC	Biolegend, USA
LAG3	Mouse	PE	Biolegend, USA
TCF1	Mouse	PE	BD Pharmingen, USA
Insulin	Mouse	Purified	R&D Systems, USA
IGF1	Human/mouse	Purified	Invitrogen, USA

IGF1R	Human/mouse	Purified	R&D Systems, USA
β actin	Mouse	Purified	Merck, USA
mTOR	Human/Mouse/Rat	Purified	Cell Signalling, USA
p mTOR	Mouse	Purified	Merck, USA
CD4	Mouse	FITC	Biolegend, USA
FoxP3	Mouse	PE	Biolegend, USA
CD11c	Mouse	FITC	Biolegend, USA
MHC I	Mouse	PE	Biolegend, USA
CD11b	Mouse	PE	Biolegend, USA
F4/80	Mouse	FITC	Biolegend, USA
Gr1	Mouse	FITC	Biolegend, USA
IgG	Mouse	HRP	Thermo Fisher, USA
iMAG anti-mouse CD3	Mouse	Magnetic beads attached (DM)	BD Bioscience, USA
iMAG anti-mouse CD8a	Mouse	Magnetic beads attached (DM)	BD Bioscience, USA

Recombinants

Name of recombinant	Origin	Maufacturer
rIGF-1	Mouse	R & D Systems, USA

3.1.1.3. Plasticwares

Name of Plasticware	Product Description	Manufacturers
Tissue culture flasks (6/12/24/96 wells)	Tissue culture grade, either coated^{CellbindTM} or nor non-coated	BD Falcon, USA; Nunc, Denmark
Cell strainer	Used for manual cell straining.	Corning, USA
Centrifuge tubes	1.5, 2, 15 and 50 ml tubes	Tarsons Pvt Ltd, India
Centricon membrane filter	10 kDa molecular weight cut off	Millipore Corporation, USA
Dialysis sacs	Cellulose membranes	Sigma, USA
Tissue culture plates (96 wells)	round bottom, flat bottom	BD Falcon, USA
Tissue culture plates (6/12/24 wells)	Tissue culture grade, either coated^{CellbindTM} or non-coated	BD Falcon, USA

3.1.2. Buffers and kits

3.1.2.1. Buffers

Name	Composition
PBS	0.15M Phosphate buffered saline, pH 7.4
TBS	50mM Tris-HCl, 0.15M NaCl, pH 7.4
Resolving gel buffer	1.5M Tris-HCl, pH 8.8
Stacking gel buffer	0.5M Tris-HCl, pH 6.8
Sample buffer	0.06M TrisHCl, pH 6.8 containing 2% SDS, 10% glycerol, 0.025% Bromophenol blue and 5% 2-mercaptoethanol (2ME)
Electrode buffer	0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3
Transfer buffer	0.025M Tris, 0.194M glycine, 0.025% methanol, pH 8.3
Washing buffer for western blot	0.01M Tris-HCl, 0.09% NaCl, 0.1% Tween-20, pH 7.2
Washing buffer for ELISA	0.15M Phosphate buffered saline, 0.1% Tween-20, pH 7.2
Blocking buffer for ELISA	0.15M Phosphate buffered saline, 5% BSA, pH 7.4
FACS buffer	0.15M Phosphate buffered saline, 2% FBS, 0.09% Sodium azide
Buffer for RNA quantification	TE Buffer, 10mM Tris, 1mM EDTA, pH 8.0
For agarosegel electrophoresis	10X Tris Boric acid EDTA Buffer (TBE)
Lowry's solution A	4.5% sodium carbonate, 1% SDS
Lowry's solution B	2% anhydrous copper sulphate

3.1.2.2. Assay kits

Assay Kits	Product Description	Manufacturers
Glucose estimation kit	Measures glucose by glucose oxidase-peroxidase (GOD-POD) method	Autospan, India
BD Cytofix/Cytoperm™	Fixation and permeabilization solution (1X), BD Perm/wash™ buffer (10X), Golgistop	BD Pharmingen, USA
LDH cytotoxicity detection kit	Solution 1: Catalyst, Solution 2: dye solution (INT dye solution)	Roche Diagnostics, Germany

BD IMagnet™	Plastic test tube rack surrounding a strong permanent rare earth magnet	BD Biosciences, USA
Western Bright ECL HRP substrate	Luminol/enhancer solution and Peroxide Chemiluminescent peroxide solution	Advansta, USA
RevertAid™ first strand cDNA synthesis kit	Revert aid reverse transcriptase (RT), RiboLock RNAse Inhibitor, oligo (dT), random hexamer primers	Thermo Scientific, USA
Mouse insulin ELISA Kit	20X Wash Buffer , 400X HRP-Streptavidin Concentrate, 5X Assay Diluent B, Assay Diluent C, Biotinylated Anti-Mouse Insulin Antibody, Anti-Mouse Insulin coated Microplate, Mouse Insulin standard protein (Lyophilized), Stop Solution, TMB One-Step Substrate Reagent	Abcam, USA
IGF-1 ELISA Kit	20X Wash Buffer , 400X HRP-Streptavidin Concentrate, 5X Assay Diluent B, Assay Diluent C, Biotinylated Anti-Mouse IGF-1 Antibody, Anti-Mouse IGF-1 coated Microplate, Mouse IGF-1 standard protein (Lyophilized), Stop Solution, TMB One-Step Substrate Reagent	Abcam, USA
Seahorse XF Glycolytic Rate Assay Kit	Rotenone/antimycin A and 2-deoxy-D-glucose	Agilent, USA

Silencer siRNA Construction Kit	DNA Hybridization Buffer, T7 Promoter Primer, Klenow Reaction Buffer, 10X dNTP, Exo– Klenow, T7 Enzyme Mix, T7 Reaction Buffer, 2X NTP Mix, Digestion Buffer, DNase, RNase, Sense Control DNA, Antisense Control DNA, siRNA Binding Buffer, siRNA Wash Buffer, Filter Cartridges, and Tubes	Invitrogen, USA

3.1.3. Instruments

Name of the Instrument	Description	Manufacturers
Autoclave	SS with 42cm diameter	Indo Scientific, India
Balance XP56	Microbalance	Mettler-Toledo, Switzerland
Biosafety cabinet	Vertical Laminar Flow, Stage-II	Klenzoids, India
Centrifuge	Plate-3K-10; Heraeusbiofuge, Optima™ L-XP Series	Remi, India; Sigma, USA; Thermo, USA; Beckman-Coulter, USA
Cold room	4ºC constant	Blue Star, India
Cryo tank	60 L	Thermo Scientific, USA
Electrophoresis apparatus	Vertical, Horizontal	Bio-Rad, India
Flow cytometer	FACSCalibur	Becton Dickinson, USA
Freezer (-20ºC)	Low temperature freezer	Blue Star, India
Freezer (2 to 8ºC)	Refrigerator	LG, Haier, Electrolux, India
Gel-documentation system	Gel Doc XR System	Bio-Rad, USA
HPLC system	Protein pac SW300 column	Waters, USA
Hybridization oven	Shaker with rotor	Stuart Scientific, USA
Incubator (CO_2)	Heracell 240i Tri-Gas Incubators	Thermo Scientific, USA
Liquid nitrogen plant	StirLIN-1	Economy Sterling, Netherland

Micropipettes	20µl,100l, 200µl, 1000µl, 5000µl Volume withdrawal capacity	Gilson Inc. Middleton, WI, USA; Tarson, India; Thermo Scientific, FL, USA
Microscope	Light- DM1000, Inverted-Phase contrast- DM IL, Florescence- DM4000B	Leica/Leitz (Germany)
PCR system, Gene cycler	GeneAmp® PCR System 9700	Applied Bio Sytems, USA
Plate reader	Infinite 200 PRO multimode reader	Tecan (Switzerland), BioTek, USA
Seahorse XFe24 Analyzer	24 well plate extracellular flux analyser	Agilent, USA
Sonicator	UP400S	Hielscher, Germany
Spectrophotometer (UV-Vis)	Cary 300 (190-900nm)	Agilent Technologies, USA
Stirrer	Magnetic, 2MLH	Remi, India
Syringe	1ml, 2ml, 5ml, 10ml, 20ml, 50ml	Dispo van, India
Vortex	CM 101 Plus	Remi, India
Ultra low freezer (-80°C)	FW 227 F, TS586e	Heto, USA; Thermo, USA
Western blotting apparatus with power pack	Wet/Tank blotting systems	Bio-Rad, USA
β-scintillation counter	Time resolved liquid scintillation counter	Perkin Elmer, USA

3.1.4. Gene amplification primers

Name of gene	Primers 5'-3'	T_m (°C)	Primer length (base)
perforin	Forward: GATGTGAACCCTAGGCCAGA Reverse: GGTTTTTGTACCAGGCGAAA	59 55	20 20
granzyme b	Forward: TCGACCCTACATGGCCTTAC Reverse: TGGGGAATGCATTTTACCAT	59 53	20 20
ifnγ	Forward: GGTGACATGAAAATCCTGCAGAGC Reverse: TCAGCAGCGAGTGATTTTCCGCTT	58 61	24 24
il-2	Forward:GCAGGCCACAGAATTGAAAG Reverse:TCCACCACAGTTGCTGACTC	57 59	20 20

β-*actin*	Forward: CAACCGTGAAAAGATGACCC Reverse: ATGAGGTAGTCTGTCAGGTC	57 57	20 20
glut 1	Forward:CAGGTGTTTGGCTTAGACTC Reverse: GGATCAGATGCAAAGCTTTC	57 57	20 20
g-6-pd	Forward:GGCCAGTTTCTATGAGGAGTATG Reverse:GCTGATGTTGAGAGGCAGTT	65 65	23 20
pkm 2	Forward: ACTGGCATCATTTGTACCAT Reverse: GGATCAGATGCAAAGCTTTC	54 55	20 20
ldh a	Forward: CCTGAAGTCTCTTAACCCAG Reverse: CCGCCTAAGGTTCTTCATTA	54 54	20 20
pcx 1	Forward: GTAAAGACCAACATCCCCTT Reverse: ATGACATGTCCGAGGTAATG	54 54	20 20
pdk 1	Forward: CTCCTTATTGTTCGGTGGAA Reverse: TTGTATTGTCTGTCCTGGTG	55 54	20 20
pdk 3	Forward: AACTCTCGTTACTCTGGGTA Reverse: TACAGACGAGAAATTGGCAA	54 55	20 20
idh 1	Forward:GTGGAGATGCAAGGAGATGAA Reverse:ATGCAGATCCAGTTCCACATAG	64 64	21 22
idh 2	Forward:AACACCGACGAGTCCATTTC Reverse:TCAAGTAGAGCGGCCATTTC	65 65	20 20
fh 1	Forward:TGCTGAAGTAAACCAGGAGTATG Reverse:CCAAACCACCAGAGGAAAGT	64 65	23 20
igf 1	Forward: TCTGAGGAGACTGGAGATGT Reverse: TAGGTCTTGTTTCCTGCACT	54 55	20 20
igf 1R	Forward: GAGATGACCAATCTCAAGGA Reverse: CTTGTTCCCCACAATGTAGT	55 54	20 20
igf 2	Forward: GGCAAGTTCTTCCAATATGA Reverse: CTTTGGGTGGTAACACGAT	55 55	20 19
insulin R	Forward: ATACCATGAATTCCAGCAAC Reverse: ATGTTGATGATCAGGCTACC	54 54	20 20

3.1.5. Animals

Male wild-type (WT) C57BL/6J mice, male and female BALB/c mice and nude Crl:CD1-Foxn1^{nu} mice (age, 4–6 weeks, body weight, 25 g on average) were obtained from and maintained at the Institutional Animal Facility, Chittaranjan National Cancer Institute (CNCI), Kolkata, and Animal Facility, CSIR-Central Drug Research Institute (CSIR-CDRI), Lucknow, respectively. Autoclaved dry pellet diet and water were provided *ad libitum*. Animals were maintained and treated according to the guidelines established by the CPCSEA, Govt. of India, following approval from the Institutional

Animal Ethics Committee (IAEC), CNCI, Kolkata (approval no. IAEC-1774/RB-17/2017/4), and IAEC, CDRI, Lucknow (Approval No. IAEC/2019/78/Renew-0/ Dated-05/04/2019) respectively.

3.1.6. Cell lines

B16F10 (melanoma), LLC (lung carcinoma), 4T1 (breast carcinoma) and CT26 (colon carcinoma) cell lines were obtained from the National Center for Cell Sciences (Pune, India) and S180 (sarcoma) cells were maintained *in vivo* in Swiss albino mice at Chittaranjan National Cancer Institute, Kolkata. B16F10 and LLC cells were cultured in high-glucose DMEM media, 4T1 and CT26 cells were cultured in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, penicillin (1000 U/mL), and streptomycin (10 mg/mL) in a CO_2 incubator (Thermo Fisher Scientific) at 37°C with 5% CO_2 and 95% humidity.

3.1.7. Clinical samples

Random blood from type-1 diabetic (T1D) patients (n=7, median body weight 65 kg) with no other disease reported in last one month, were obtained from Doyen Diagnostics and Research Foundation (DDRF), Kolkata, India, following obtaining their written consent. Ethical approval of this study was obtained from Institutional Ethics Committee, DDRF, Kolkata (Approval No. DDRF-IEC-74517-RB-2022-1), registered under the Central Drug Standard Control Organization (CDSCO), India. Blood samples from age- and sex-matched healthy volunteers (n=5) with no reported disease for past one month were collected as control after obtaining their written consent. Blood glucose in a range of 200-550 mg/dl, HbA1c more than 7, $GAD65^+$ and $Insulin^-$ samples were included in the study. Age less than 18 years were excluded from the study. All the study were strictly adhered to the Declaration of Helsinki. Patients and healthy volunteers included in this study are of South Asian ancestry. PBMCs were separated from the blood by density gradient centrifugation following the manufacturer's protocol.

3.2. Methods

3.2.1. Neem leaf glycoprotein preparation

Extract from neem (*Azadirachta indica*) leaves was prepared by the method as described [*Chakraborty et al, 2010*]. Mature leaves of same size and color (indicative of same age), taken from a standard source, were shed-dried and pulverized. Leaf powder was soaked overnight in phosphate-buffered saline (PBS), pH 7.4. Supernatant was collected by centrifugation at 1500 rpm, extensively dialyzed against PBS, pH 7.4 and concentrated by Centricon membrane filter (Millipore Corporation, MA, USA) with 10 kDa molecular weight cut-off. Purified NLGP was checked for its quality by electrophoresis and HPLC using routine laboratory methods [*Goswami et al, 2010*]. Biological activity of purified NLGP was checked by tumor growth restriction assay before use. The protein concentration was measured by using Bradford reagent.

3.2.2. Induction of type 1 diabetes

Type 1 diabetes (T1D) was induced in 4-6 hrs fasted mice by intraperitoneal (i.p.) injection of a streptozotocin at 50 mg/ kg for five consecutive days, dissolved

in monohydrate Na-citrate buffer, pH 4.5 under anaesthesia as per the CPCSEA guidelines. Anesthesia was induced using 3.5% isoflurane for four minutes in medical air enriched with oxygen (air:oxygen 3:1). The control group received vehicle. Sucrose water (10%) was given overnight to avoid sudden hypoglycemia post injection. Mice were kept for four weeks with an autoclaved dry pellet diet and water *ad libitum* for proper diabetes induction. Induction of diabetes was confirmed by monitoring blood glucose level periodically.

3.2.3. Plasma glucose estimation

Blood sugar was measured from the plasma using the glucose oxidase-peroxidase (GOD-POD) method following the manufacturer's protocol (Autospan Glucose measuring kit, India) to evaluate hyperglycemia.

3.2.4. Solid tumor development

Solid tumors were developed in mice after four weeks of T1D induction, by inoculation of tumor (5×10^5) cells s.c. on the right flank of non-diabetic, STZ and STZ+insulin group of mice. B16F10 and LLC tumors were induced in male C57BL/6J mice. CT-26 tumors were induced in male BALB/c mice. 4T1 tumors were induced in female BALB/c mice in mammary fat pad. B16F10 tumors were also induced in male Crl:CD1-Foxn1^{nu} nude mice. After palpable tumor formation, tumor areas (mm^2) were measured regularly up to day 28 for B16F10, 4T1, LLC and CT-26. B16F10 tumors in nude mice were measured up to day 19 following the formula (length × width). Animal health, body weight (g), and blood sugar (mg/dL) of both groups of mice were monitored throughout the experiment. Tumor-bearing mice were euthanized by an overdose of ketamine HCL (160 mg/kg) + xylazine (20 mg/kg) intraperitoneally (i.p.) on days 31 and 19, respectively, as per the CPCSEA guidelines. Tumors, spleens, tumor-draining lymph nodes (TDLN) and blood were collected for downstream analyses.

3.2.5. Tumor growth monitoring

Palpable tumors were developed after 7-10 days after tumor cell inoculation to the mice. Tumor area (in mm^2) and volume (in mm^3) was measured after palpable tumor formation at three days interval up to the end of the experiment. Vernier calliper was used to measure tumor growth ~~by~~ using following formula:

$$Tumor\ area = (length \times width)$$

$$Tumor\ volume = (length \times width^2 / 2)$$

3.2.6. Survival analysis

Mice survival was monitored throughout the experiment. Data obtained from the survival experiments were analysed by Kaplan-Meyer survival analysis curve in GraphPad Prism software.

3.2.7. Magnetic-assisted cell sorting (MACS) of CD8$^+$ T cells

After surgical resection, the tumor samples were rinsed with antibiotic-containing media and minced with scissors under sterile conditions. Enzymatic digestion was performed using 1500 U/ml collagenase IV in RPMI-1640, followed by mechanical shaking. Single cells were obtained by passing the enzyme-digested tumor cell suspension through a 70 µm cell strainer. Tumor-infiltrating lymphocytes (TILs) were purified from buffy coats

by centrifugation of digested single-cell suspension over a lymphocyte separation medium made of polysaccharose and diatrizoic acid dihydrate according to the manufacturer's protocol. $CD8^+$ T cells from lymph nodes and TILs of B16F10 melanoma-bearing T1D and non-diabetic C57BL/6J mice were isolated by Magnetic-Activated Cell Sorting (MACS) using Anti-Mouse CD3 followed by BD iMag Anti-Mouse CD8a DM following the manufacturer's protocol. Purity was checked by flow-cytometry using a PE anti-mouse CD8a Antibody.

3.2.8. Adoptive transfer of MACS sorted $CD8^+$ T cells

$CD8^+$ T cells (>90% pure by flow-cytometry) and MACS sorted from T1D and non-diabetic C57BL/6J mice were adoptively transferred (2×10^5 cells) into nude tumor-bearing mice groups through tail vein (i.v.) injection under anesthesia with Ketamine HCl (80 mg/kg) + xylazine (10 mg/kg) i.p. as per the CPCSEA guidelines. Tumor progression and survival were monitored in the nude mice.

3.2.9. $CD8^+$ T cell depletion

This experiment was conducted to observe how $CD8^+$ T cell depletion alters tumor growth in STZ treated mice, also to observe whether adoptive transfer of $CD8^+$ T cells from untreated mice to STZ treated mice, and vice versa, cause tumor growth restriction. C57BL/6J mice were divided into eight groups (n=5), four groups received STZ treatment (50 mg/kg for consecutive 5 days) while other four remains untreated. Anti CD8a antibody (100µg/50µl were injected i.p on day -1 and 6 in three untreated and three STZ treated groups. B16F10 tumors were inoculated on day 1 in all groups. On day 7, one untreated and one STZ treated group received adoptive T cell from TDLN of untreated tumor bearing mice. One untreated and one STZ treated group received adoptive T cell from TDLN of STZ treated tumor bearing mice. Tumor area were measured on day 21.

3.2.10. *In vitro* antigen stimulation of clinical T1D PBMCs

This study was conducted to determine the functional capabilities of clinical T1D PBMCs. T1D PBMCs (n=7) along with non-diabetic control PBMCs (n=5) were plated in five different 60 mm petriplate (Corning) at a number of 1×10^7 cells per plate in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated FBS (Himedia). Four group of plate received tumor lysate of MCF-7 (5 µg/ml media), human breast cancer cell line and one group of plate remain as untreated. One group of plates among the four, received rapamycin (25 pM dissolved in DMSO) as mTOR inhibitor, one group of plate received picropodophyllotoxin (PPT) (0.2 µM dissolved in DMSO) as IGF1R inhibitor and another group of plated received both rapamycin and PPT. PBMCs were incubated in a CO_2 incubator (Thermo Fisher Scientific) at 37 °C with 5% CO_2 and 95% humidity for 48 hours. After that, cells were stained with fluorescent labelled antibodies for flow cytometry. Culture supernatant was preserved for ELISA.

3.2.11. RT-PCR

Total cellular RNAs from MACS sorted intra-tumoral $CD8^+$ T cells were isolated using TRIzol. Random hexamers were

used to generate the corresponding cDNA using the First Strand cDNA Synthesis Kit according to the manufacturer's protocol. Amplification of the target genes was performed using 2x green mix with gene-specific primers following the manufacturer's protocol.

3.2.12. *In vitro* silencing experiment

siRNA for mouse-IGF1R was constructed *in vitro* using the Silencer siRNA Construction Kit (Cat# AM1620, Invitrogen) according to the manufacturer's protocol. Target-specific and scrambled control siRNA (Sigma-Aldrich) were added (final concentration 50 nM) *in vitro* for 2 h in serum-starved cells in the presence of lipofectamine-2000. The primers used are listed below: The siIGF1R was used to block insulin like growth factor 1 receptor (IGF1R) expression. Rapamycin was used to block mTORC1 and mouse recombinant IGF1 was used to induce IGF1R signaling. siIGF1R, rapamycin, and m-rIGF1 were administered alone and in all possible combinations *in vitro* to $CD8^+$ T cells isolated from the TDLNs of diabetic and non-diabetic mice.

siIGF1R sense:

5'AACCCTCCTCCGGAGCCAGACCCT GTCTC3'

siIGF1R antisense: 5'AAGTCTGGCTCC GGAGGAGGGCCTGTCTC3'

3.2.13. Extracellular flux analysis

MACS sorted intra-tumoral $CD8^+$ T cells were analyzed for glycolysis and Krebs cycle by measuring ECAR and OCR, respectively, in a Seahorse XFe24 Analyzer (Agilent) using a Seahorse XF Glycolytic Rate Assay Kit following the manufacturer's protocol. Briefly, ECAR and OCR were measured at the basal level, followed by sequential injections of glucose, oligomycin, and 2-DG.

3.2.14. LDH release assay

The cytotoxicity of MACS-sorted $CD8^+$ T cells from tumors of diabetic and non-diabetic mouse cohorts against *in vitro* cultured B16F10 cells was evaluated by measuring LDH release according to the manufacturer's protocol. The S180 cell line was used as the unrelated control. The tumor cell: $CD8^+$ T cell ratios used in the experiment were 1:10, 1:25, and 1:50.

3.2.15. Protein estimation

Experimental samples as well as standard protein, BSA, containing 1-10 µg of protein, were taken in separate tubes and the volume was adjusted to 100 µl with 0.15 M NaCl. A tube without any protein was taken as control. Bradford reagent (1 ml) was added to each tube. The solutions were mixed thoroughly by vortex and allowed to stand for 2 min at 25ºC. The absorbance of each protein solution was recorded at 595 nm. A standard curve was prepared using the absorbance values of the standard protein solutions, and the concentration of the protein in the unknown samples was determined from the standard curve.

3.2.16. Enzyme-linked immunosorbent assay

Serum insulin and insulin like growth factor 1 (IGF1) levels were measured by ELISA using a microplate reader (BioTek Instruments Inc.) and respective kits following the manufacturer's protocol. For the isolation of serum, mouse blood was collected from the tail vein under anesthesia in anticoagulant-free tubes.

Blood was then allowed to clot for 30 min at room temperature, and the serum was separated from the clotted blood after centrifugation at 1000 × g for 10 min.

3.2.17. Western blot

Western blot analysis of mTOR and phospho m-TORC1 from MACS sorted intra-tumoral $CD8^+$ T cells and tumor cells were performed. Tumor cells and/or $CD8^+$ T cells were lysed in RIPA buffer, and tumor lysates (50 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel using a BioRad apparatus and transferred onto a nitrocellulose membrane for western blotting. Incubation was performed with different primary antibodies after blocking with 5% BSA. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 2h at room temperature. The ECL Western Blotting Substrate Kit was used to develop protein bands, which were visualized using Bio-Rad Chemi-Doc XRS^+.

3.2.18. Flow-cytometry

For cell surface labelling, cells were incubated with fluorescently labelled antibodies for 30 mins at 4°C in dark. Cells were washed with FACS wash buffer twice, and fixed with 1% paraformaldehyde solution. For intracellular labelling, cells were treated with BD Cytofix/Cytoperm buffer following manufacturer's protocol. Cells were then incubated with fluorescent labelled antibodies for 30 mins at 4°C in dark. Cells were washed with FACS wash buffer twice and acquired in flow- cytometer. BD LSR Fortessa Cell Analyzer (BD Bioscience) and BD FACSCalibur (BD Bioscience) were used for acquisitions and analyses. 50,000 or 100,000 events were acquired for each flow-cytometry experiments. Acquired data were analysed with FlowJo v.10 software (BD Bioscience).

3.2.19. Histology and immunohistochemistry

Tissue samples were fixed in 10% formal saline followed by gradual dehydration with 30%, 50%, 70%, 90% and absolute alcohol, each for 10 mins twice. Then tissues were placed into xylene for 10 mins and then transferred to paraffin at 60°C. Paraffin blocks containing tissues were cut into 5µm thick sections in a microtome (Leica) and stretched on a greese free glass slides. For hematoxylin-eosin staining, tissue sections were rehydrated using downgrade alcohols followed by staining with Meyer's hematoxylin for 5 mins. After that, slides were washed in running tap water, differentiated with acid water, then dehydrated with graded alcohols each for 10 mins. After incubation in 90% alcohol; eosin stain was given for 5 mins and washed in 90% alcohol. Then slides were kept in absolute alcohol for 10 min each twice followed by xylene for 5 mins. DPX was used to mount the slide with tissue sections with a rectangular cover slip.

3.2.20. Immunofluorescence microscopy and tumor tissue staining

Tumor cryo-sections adhered to glass slides were blocked in 5% BSA at RT. For intracellular staining, the sections were incubated with 0.15% Triton X-100 prior to blocking. After blocking, the sections were incubated with specific primary antibodies overnight at 4°C followed by FITC and PE-tagged secondary antibodies for 3 h at RT. The sections were then

washed and mounted with Fluoroshield and DAPI. Images were viewed under a fluorescence microscope with a 40X (NA 1.1, FN 22) objective lens at 24°C, and images were captured with an attached camera using cellSens standard software (1.18 build 16686, Olympus). The acquired images were analyzed using ImageJ software.

3.2.21. Software based data analysis and Statistical analyses

ImageJ (National Institutes of Health, Bethesda, MD, USA) was used for image analysis, brightness, contrast adjustment, and quantification of the agarose gel bands. Image Lab (Bio-Rad) was used for western blot gel scanning and analysis. The ECAR and OCR data were analyzed using the online Seahorse XF Analyzer software (Agilent). All reported results represent the mean ± SEM of data obtained from three (i*n vivo*; n = 6) or three to six (for *in vitro* assays) independent experiments. Statistical significance was established by unpaired Student's t-test (for two groups) or one-way analysis of variance followed by Tukey's post-hoc test (for more than two groups) using the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). For survival analysis, Kaplan-Meier survival analysis followed by data analysis with the log-rank (Mantel-Cox) test was used. Differences between the groups were considered significant at a p-value of 0.05. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to establish interactome maps of the expressed genes (STRING v.11.5). Graphical representations were made using BioRender Software.

CHAPTER 1

THE INFLUENCE OF PRE-EXISTING T1D ON TUMOR CONTROL AND HOST SURVIVAL

- **ABSTRACT**
- **INTRODUCTION**
- **OBJECTIVE**
- **RESULTS**
    1. Pre-existing T1D is associated with reduced growth of transplantable tumors resulting in extended overall survival
    2. STZ treatment causes high blood glucose and reduced serum insulin concentration due to permanent pancreatic β cell loss, phenotype of T1D
    3. STZ induced T1D modulates systemic cytokine landscape and promotes hepatic inflammation
    4. T1D associated reduced tumor growth is dependent on adaptive immune system
- **DISCUSSION**
- **REFERENCES**

Abstract

Epidemiological studies suggest an association between T1D and increased risk for developing cancers of the stomach, lung, pancreas, liver, ovary, and kidney, but a decreased risk for melanoma, prostate, and breast cancer. In this study, pre-existing T1D was found to be associated with reduced growth of transplantable aggressive melanoma and carcinoma tumor models resulting in extended overall survival. STZ induced T1D mice showed significantly slowed growth of B16F10, 4T1, LLC and CT26 tumors on days 21 and 28, unless they received treatment with insulin. STZ induced T1D mice bearing B16F10, 4T1, LLC or CT26 tumors also displayed a significant survival benefit when compared to untreated (control) and STZ+insulin treated groups of mice. However, T1D associated tumor control was found to be adaptive immune system dependent. T1D mice showed elevated blood glucose and reduced insulin concentration vs. control cohort. Insulin administration in T1D groups normalized blood glucose vs. insulin untreated groups. Histology and immunohistochemical study further confirmed permanent destruction of insulin producing pancreatic β cells following STZ treatment establishing T1D phenotypes. STZ treated mice also showed elevated systemic inflammatory cytokine IL-6 and hepatic inflammation, characteristics of T1D. Since adaptive immune system plays a crucial role in the progression of both cancer and T1D, modulation of immune landscape in T1D associated tumor control need to be studied.

Introduction

The incidence of diabetes is increasing globally and represents a leading cause of death annually (Carstensen *et al*., 2016). Type 1 diabetes (T1D), a major form of diabetes, is an autoimmune disease characterized by hyperglycaemia and insulin deficiency due to $CD8^+$ T cell-targeted loss of pancreatic β cells (Roep, 2003; Wiedeman *et al*., 2020a; Pinkse *et al*., 2005; Wiedeman *et al*., 2020b). Untreated T1D can lead to death (Harding *et al*., 2014). Since stem cell therapy to restore pancreatic beta cells and immune cell-based therapies designed to therapeutically treat T1D remain in their developmental stages, administration of insulin represents the sole lifesustaining therapy for patients with T1D (Van Dijk *et al*., 2016; Shapiro *et al*., 2000). Since the incidence of T1D is increasing annually among children, there is an increasing need for further research to study its impact on inflammatory diseases, including cancer. Indeed, the underlying mechanism(s) by which T1D may influencecancer progression remains largely unknown.

Animal models of T1D are essential for studying the disease and developing new treatments. Two types of T1D models are used in the research worldwide, chemically induced T1D models or spontaneously induced non-obese diabetes (NOD) model (Aldrich *et al*., 2020). Establishment of NOD mice model is a complex process. NOD mice need close monitoring, proper care, specific environment to breed and may be treated with experimental therapies to prevent or delay the onset of diabetes. Chemically induced T1D models on the

other hand is easier to develop and easier to maintain. Alloxan and streptozotocin are the two widely used chemical compound used to induce T1D in rodent models.

Low dose streptozotocin induce T1D mouse model was used to decipher the mechanism by which pre-existing T1D exerts it effect on cancer progression. Low dose was used so as to minimize STZ induced toxicity related death, due to sudden hypoglycemia, during T1D model generation. C57BL/6J and BALB/c mice both are sensitive to STZ induced T1D. To counter intra- and inter-strain STZ sensitivity, we chose only those mice having blood glucose of 250-350 mg/dl for T1D group before tumor inoculation.

Four transplantable tumor models were used viz. B16F10 (melanoma), 4T1 (breast carcinoma), LLC (lung carcinoma) and CT26 (colon carcinoma) to study tumor growth in murine T1D model. These four cell lines were chosen for their established history to form aggressive tumors which mimic human carcinogenesis when injected to their syngeneic mouse models. Tumor growth and host survival was observed in control, STZ treated and STZ + insulin treated groups.

Objective

To study the influence of pre-existing T1D on tumor control and host survival.

Results

1.1. Pre-existing T1D is associated with reduced growth of transplantable tumors resulting in extended overall survival: Following T1D induction, diabetic mice were screened for blood glucose concentration prior to tumor inoculation. T1D mice having blood glucose concentration of 250-350 mg/dl were taken for STZ and STZ + insulin groups, to counter inter-strain variation in STZ sensitivity. B16F10, LLC and CT26 tumor cell were injected s.c. orthotopically and 4T1 cells were injected in mammary fat pad, in untreated (control), STZ treated and STZ + insulin treated groups (Figure 1.1A).

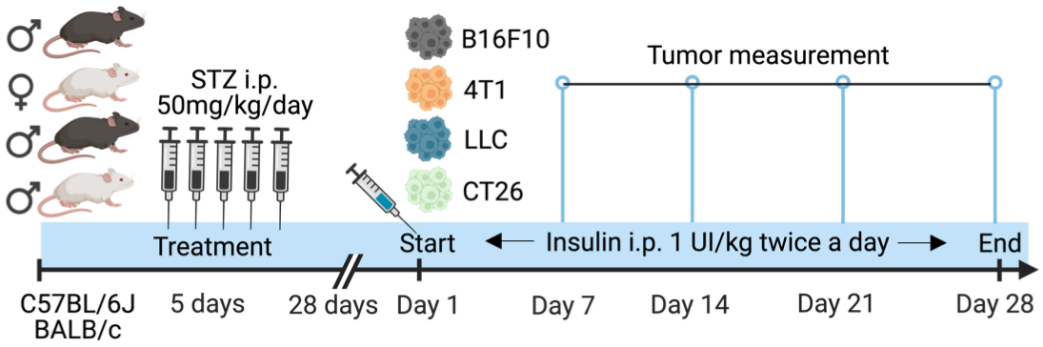

Figure 1.1: A. Schematic representation of experimental design. Mice were treated with STZ (50 mg/kg) for five consecutive days, along with vehicle treated mice as control. One group of STZ treated mice received daily twice insulin treatment (1 UI/kg) throughout the experiment. Tumor cells were injected after 4 weeks of STZ treatment. B16F10 and LLC tumors were grown on male C57BL/6J mice. 4T1 and CT26 tumors were grown in female and male BALB/c mice respectively. Each transplantable tumor models had three groups viz. control (untreated), STZ and STZ + insulin (n=6 per group).

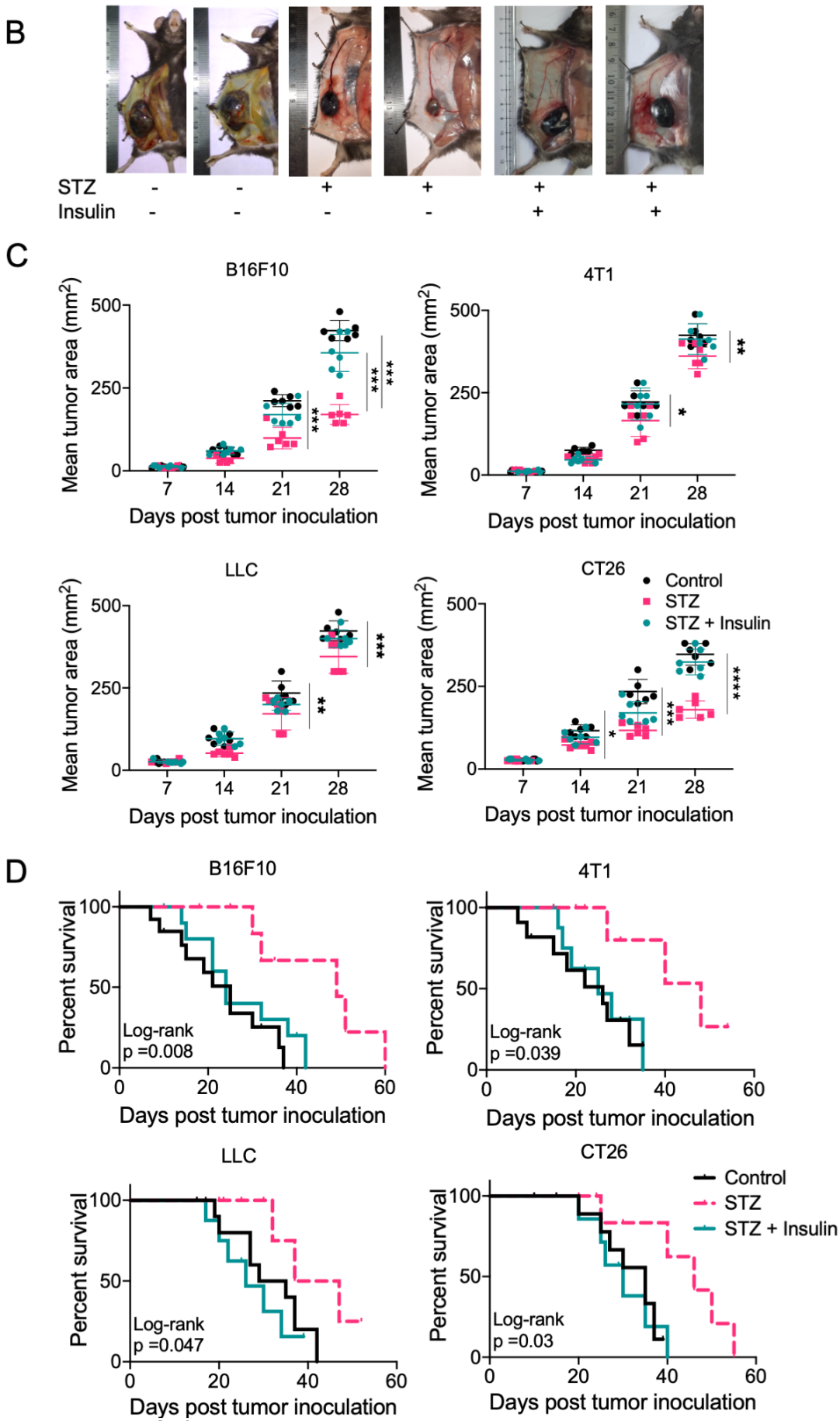
B
STZ - - + + + +
Insulin - - - - + +
C
B16F10
4T1
LLC
CT26
Mean tumor area (mm2)
Days post tumor inoculation
Control
STZ
STZ + Insulin
D
Percent survival
Log-rank p =0.008
Log-rank p =0.039
Log-rank p =0.047
Log-rank p =0.03

Figure 1.1: B. Representative photographs of B16F10 tumor bearing mice with scale (ruler) from three groups before harvest. **C.** Graphs of tumor area of all four transplantable tumor models measured on day 7, 14, 21 and 28. **D.** Kaplan-Meier survival curve of B16F10, 4T1, LLC and CT26 tumor bearing mice in control, STZ induced T1D and STZ induced T1D + insulin treated hosts. Survival curves were plotted and intergroup differences were analysed using a log-rank test. Two-way ANOVA, followed by Tukey's multiple comparisons test was performed to determine significance. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

Tumor volumes were measured once in a week for four weeks, after which tumors were harvested for further analyses (Figure 1.1B). STZ treated groups of all four transplantable tumor models showed reduced tumor growth on day 21 and day 28 vs. respective control tumor groups (Figure 1.1C). STZ treated CT26 tumor bearing mice showed tumor growth control on day 14 vs. control cohort. Insulin treatment in STZ + insulin group abolished the ability to reduce tumor growth in all four transplantable tumor models vs. respective STZ groups. Ability to reduce tumor growth in STZ groups resulted into overall survival benefit in all four tumor models vs. control (Figure 1.1D). Insulin treatment however, resulted into no survival benefit in all four tumor models vs. control cohort.

1.2. STZ treatment causes high blood glucose and reduced serum insulin concentration due to permanent pancreatic β cell loss, phenotype of T1D: Following STZ administration and before tumor cell inoculation, mice were kept for 4 weeks for proper induction of T1D. One group of STZ treated cohort were given insulin injection twice daily (Figure 1.2A). STZ treated mice showed significant reduction of blood glucose concentration as well as serum insulin vs. untreated groups (Figure 1.2B and 1.2C). Insulin administration in STZ treated group of C57BL/6J and BALB/c mice restored blood glucose, however, serum insulin level was unchanged, indicated permanent destruction of pancreatic β cells. Histologic and immunohistochemical analyses of the pancreas revealed reduced pancreatic islet content following STZ treatment in C57BL/6J mice (Figure 1.2D and 1.2E). Overall results suggest proper induction of T1D model in C57BL/6J and BALB/c mice.

1.3. STZ induced T1D modulates systemic cytokine landscape and promotes hepatic inflammation: As an autoimmune disease model, STZ induced T1D showed increase IL-6 concentration vs. untreated, in serum of both C57BL/6J and BALB/c mice, which was normalized by insulin treatment (Figure 1.3A). In contrast, serum levels of IL-17, IFNγ, TNFα and IL-10 remained comparable across the untreated (control), STZ treated and STZ+insulin treated groups. Interestingly, STZ administration significantly reduced serum IGF1 in both strains vs. untreated cohort and was normalized by insulin treatment (Figure 1.3B). Livers of STZ treated mice exhibited enlarged hepatocytes vs. untreated cohort due to inflammation (Figure 1.3C), as was further validated by increased infiltration of $CD8^+CD69^+$ T cells in hepatic microenvironment of STZ treated mice of both strains (Figure 1.3D).

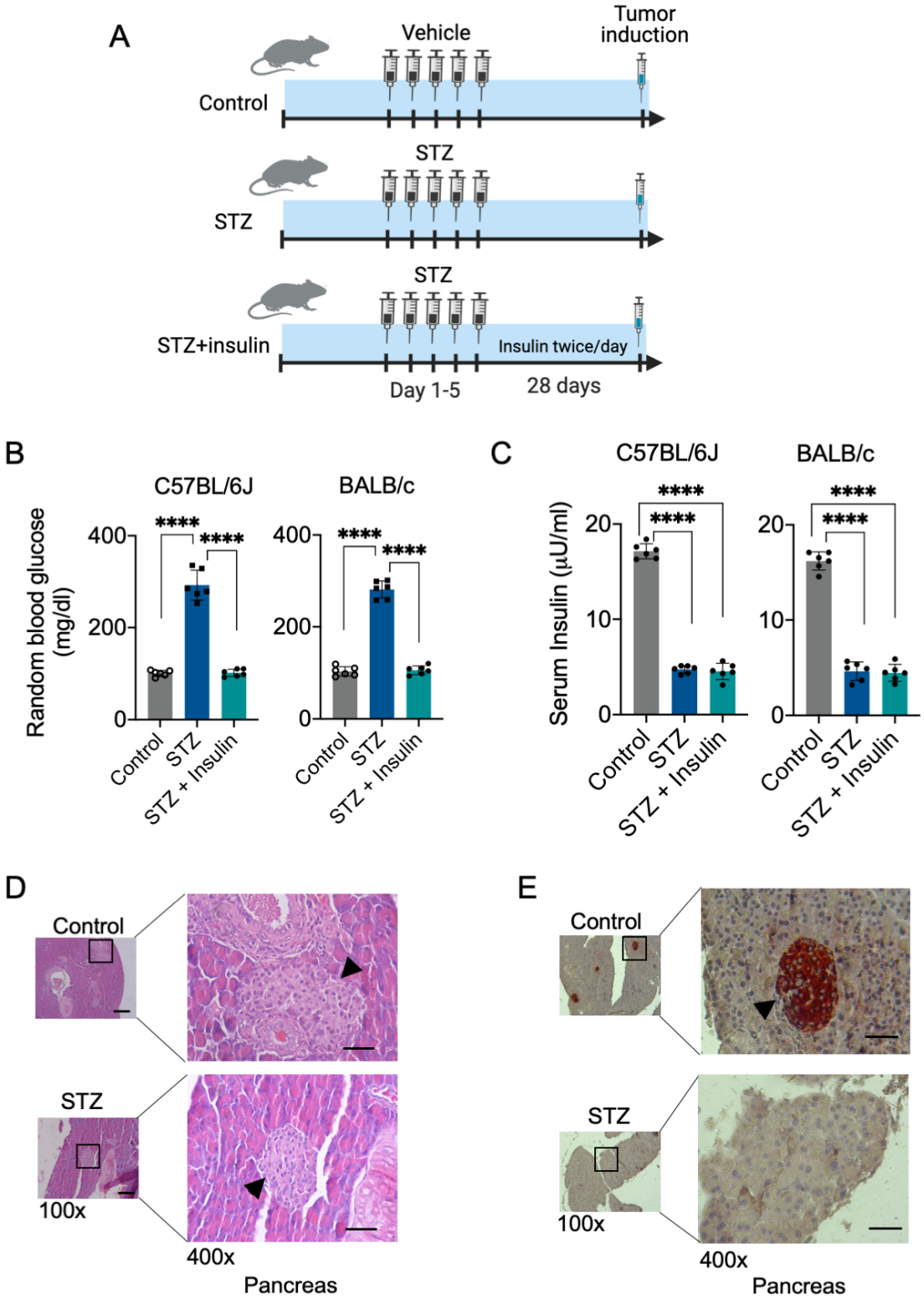

Figure 1.2: A. Schematic representation of experimental setup. Mice were divided into three groups. One group received only vehicle while other two groups received STZ i.p. for five consecutive days. One of the STZ treated groups received twice daily dose of insulin (1 UI/kg). Following STZ treatment, mice were kept for 28 days to develop T1D before tumor inoculation. **B.** Random blood glucose of C57BL6 and BALB/c mice of three different groups (control, STZ and STZ+insulin). **C.** Serum insulin of concentration of control, STZ and STZ+insulin group of mice. **D.** Histology

(hematoxylin and eosin) and **E.** immunohistochemical slides of pancreas from untreated and STZ treated C57BL/6J mice in 10x and 40x. For IHC, anti-insulin primary antibody followed by HRP tagged secondary antibody was used to stain insulin producing pancreatic islet cells (brown color). Two-way ANOVA, followed by Tukey's multiple comparisons test was performed for statistical significance. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

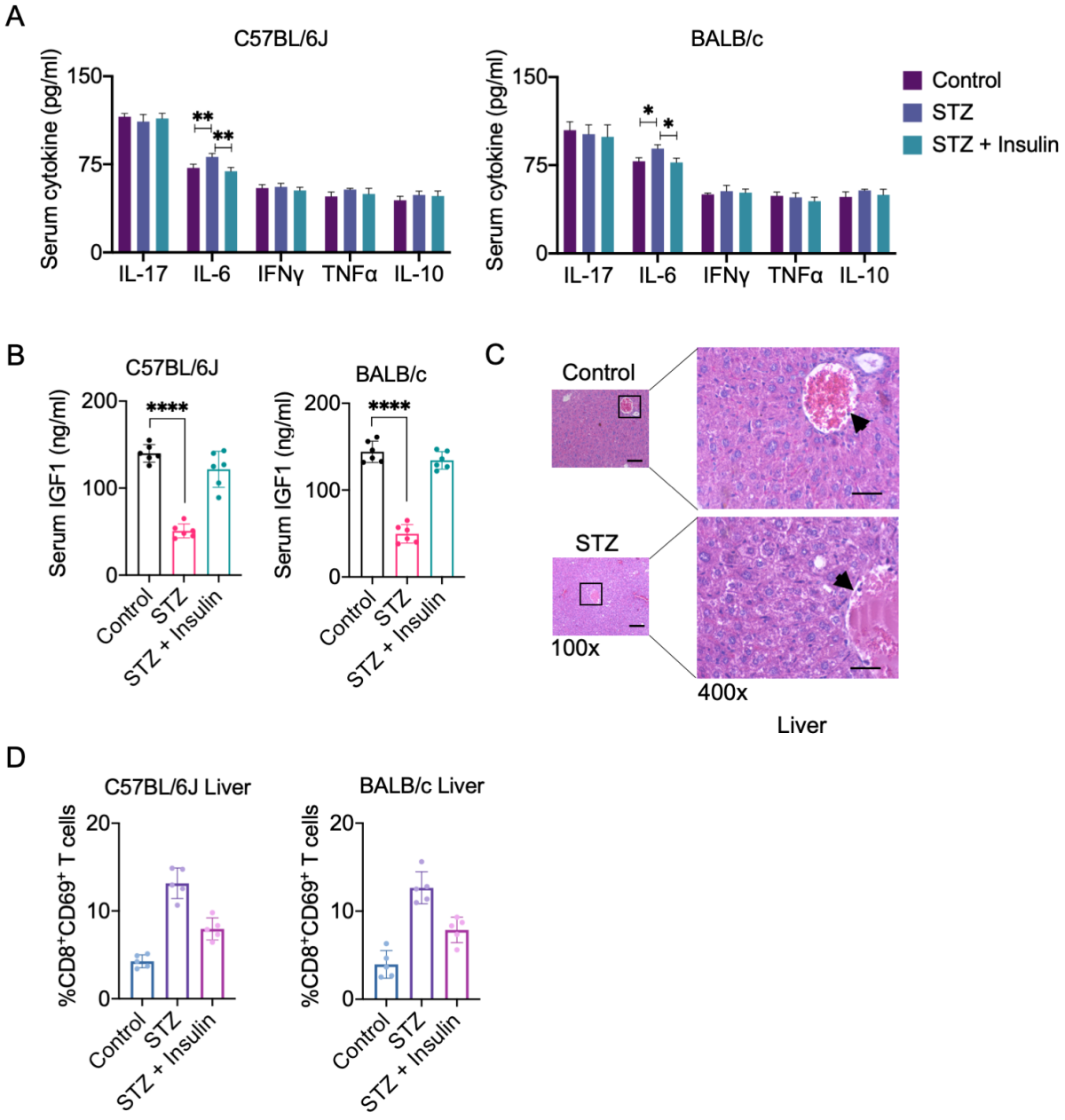

Figure 1.3: A. Serum cytokine concentrations of C57BL/6J and BALB/c mice of untreated (control), STZ treated and STZ+insulin treated groups. IL-17, IL-6, IFNγ, TNFα and IL-10 were measured in serum. **B.** Serum IGF1 concentration of control, STZ and STZ+insulin groups from two mice strains. **C.** Hematoxylin and eosin stained liver slides from untreated and STZ treated C57BL/6J mice in 100x and 400x magnification. **D.** % $CD8^{+}CD69^{+}$ T cells in liver of untreated and STZ treated groups of C57BL/6J and BALB/c mice.Two-way ANOVA, followed by Tukey's multiple comparisons test was performed for statistical significance. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

1.4. T1D associated reduced tumor growth is dependent on adaptive immune system: Considering the importance of immune regulation in T1D associated pathophysiology, next we determined whether slowed tumor growth in T1D mice was mediated by the adaptive immune system. For this, we performed analogous experiments in nude (Crl:CD1-Foxn^{nu}) recipient mice with no adaptive immune system. STZ injection in nude mice similarly showed elevated blood glucose and reduced serum insulin in serum vs. untreated control cohort (Figure 1.4A and 1.4B). Nude mice treated with STZ failed to restrict B16F10 tumor growth vs. control mice (Figure 1.4C and 1.4D) with no significant survival benefit observed vs. tumor-bearing non-diabetic nude mice (Figure 1.4E). These results support (adaptive) immune system-mediated control of tumor growth under T1D physiologic conditions that is mitigated upon insulin administration.

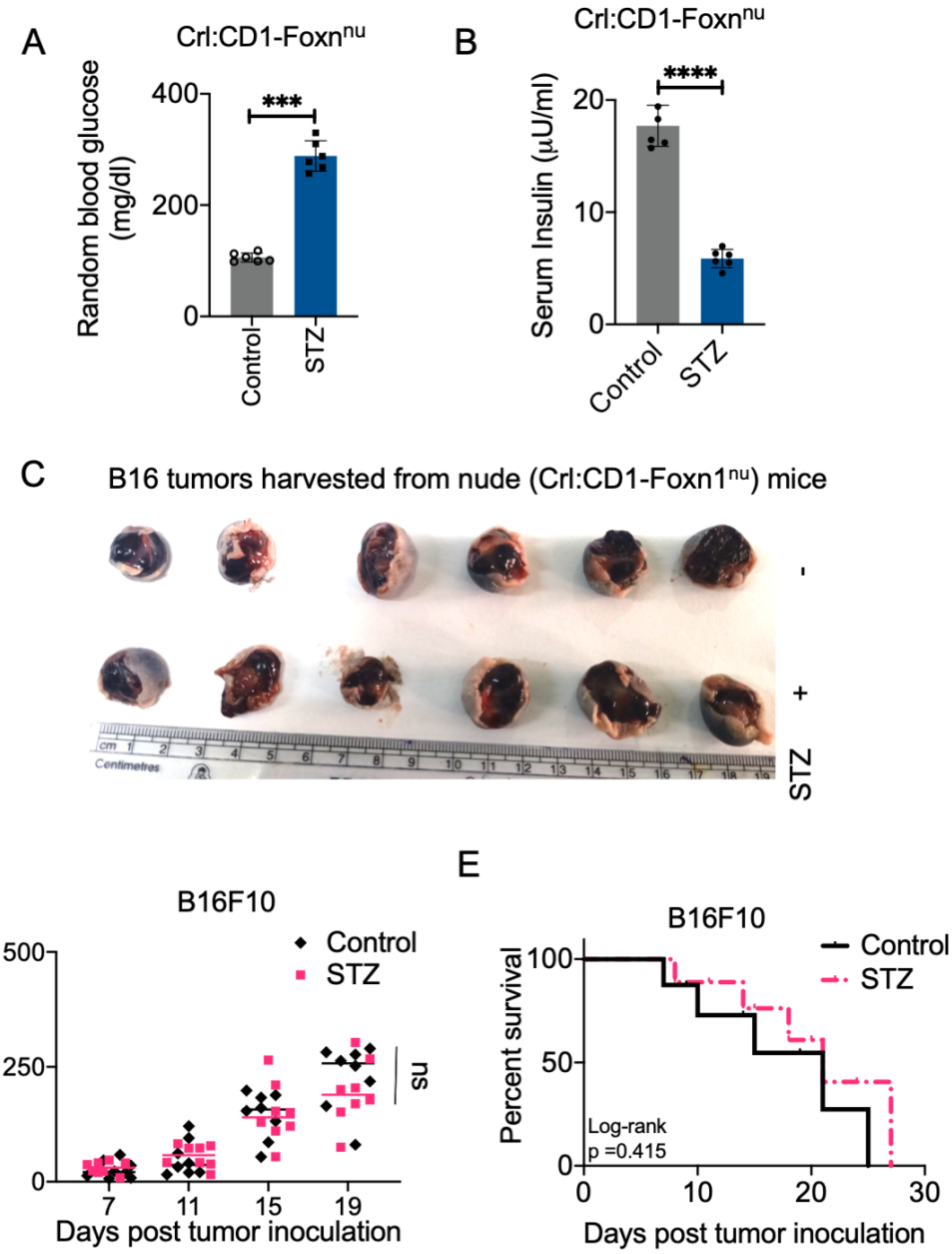

Figure 1.4: A. Random blood glucose and E. serum insulin concentration of nude mice with or without STZ treatment. **B.** B16F10 tumors harvested from nude mice of untreated and STZ treated groups (n=6 per group). **C.** Mean tumor area (mm^2) of untreated (control) and STZ treated nude mice on day 7, 11, 15 and 19. **D.** Kaplan-Meier survival curve of B16F10 tumor bearing nude mice in control and STZ induced T1D treated hosts. Survival curves were plotted and intergroup differences were analysed using a log-rank test. Multiple t tests were performed to determine significance. (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ****P $\leq$ 0.0001).

Discussion

A retrospective study by Carstensen *et al*., 2016 of 9000 cancer patients with T1D spanning 5 countries suggested a reduced risk of melanoma, breast carcinoma or prostate cancer, but an increased risk for patients developing colon, oesophageal, liver or stomach cancer. This observation indicates contextual association of T1D with varied forms of cancers. A translational study by Heuson and Legros, 1972 investigating rat mammary carcinomas in an alloxan-induced T1D model reported tumor regression in 90% of diabetic rats; however, the underlying mechanism for host protection was largely unexplored. Based on these unresolved findings, in the present study we examined the influence of streptozotocin-induced pre-existing T1D on cancer progression and associated immune alterations in a range of murine melanoma and carcinoma (i.e., breast, colon and lung) models. The streptozotocin induce T1D model was employed as this system is widely used in the field of endocrinology research (Szkudelski, 2012; Furman, 2021, 2015; Damasceno *et al*., 2014; Lenzen, 2008; Graham *et al*., 2011a; b; Wu and Yan, 2015; Ebaid, 2014; Srinivas, 2015; Cardinal *et al*., 1998; Araujo *et al*., 2022; Gorray *et al*., 1986; Luo *et al*., 2019). To nullify any direct possible effect of T1D inducing drugs on tumor cells, unlike previous studies, we induced T1D first, then injected tumor cell lines to grow tumors. Pre-existing T1D was found to be associated with better tumor control in melanoma and carcinoma models vs. control cohorts. Better tumor control also leads to better survival of T1D tumor bearing hosts. Insulin treatment in T1D tumor bearing mice, however, leads to abolishment of this effect. Further, it was found that T1D associated tumor control is adaptive immune system dependent. To find out which immune component is responsible for T1D associated tumor control, detailed immunophenotyping is necessary.

STZ selectively destroys pancreatic β cells, the only insulin producing cells in mammals (Lenzen, 2008). Low dose STZ minimizes sudden hypoglycemia induced death (Han *et al*., 2022). Mice were also given 10% sucrose in drinking water during those 5 days of STZ administration to mitigate the possibility of sudden hypoglycemia. Pancreatic β cell destruction was evident from elevated glucose and reduced insulin concentration in serum. Further confirmation of pancreatic β cell loss was evident from histology and immunohistochemistry sections of pancreas. Pancreas sections of STZ treated cohort showed no insulin secreting β cells vs. untreated pancreas. STZ induced T1D however, significantly elevates systemic IL-6 concentration which might contribute to systemic inflammation in T1D hosts (Kreiner *et al*., 2022; Rose-John, 2020;

Siewko *et al*., 2019; Pham *et al*., 2011). Hepatic microenvironment of STZ treated mice showed increase inflammation, a characteristic of autoimmune T1D. Modulation of systemic cytokine landscape and systemic inflammation are two major hallmarks of T1D in patients and low dose STZ induced diabetic model in mice mimics the T1D pathophysiology of human (Thorvaldson *et al*., 2003; Nirenjen *et al*., 2023; Sun *et al*., 2005). Furthermore, systemic IGF1 was found to be decreased in STZ treated cohort vs. untreated cohort. Insulin injection normalizes serum. IGF1 concentration. Liver is the major source of systemic IGF1, accounting for almost 80% of total systemic IGF1 concentration (Sjögren *et al*., 1999). Hepatocytes secret IGF1 upon stimulation by insulin signaling. Without insulin, IGF1 secretion from hepatocytes got diminished, resulted into reduced systemic IGF1. Systemic IGF1 concentration may play a crucial role in T1D associated tumor progression, which will be discussed in later chapters.

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CHAPTER 2

THE INFLUENCE OF IMMUNE SYSTEM IN TUMOR PROGRESSION IN HOSTS WITH PRE-EXISTING T1D

- **ABSTRACT**
- **INTRODUCTION**
- **OBJECTIVE**
- **RESULTS**
    1. T1D enhances the recruitment of activated tumor specific $CD8^+$ T cells in TME and TDLN
    2. Tumor infiltrated $CD8^+$ TILs exhibit superior cytotoxicity in T1D hosts
    3. T1D associated tumor growth control is $CD8^+$ T cell dependent
    4. T1D alleviates immunosuppressive cell population in TME
- **DISCUSSION**
- **REFERENCES**

Abstract

Pre-existing T1D facilitates recruitment of activated cytotoxic $CD8^+$ T cells into the TME of transplantable melanoma and carcinoma tumors. $CD8^+$ T cells from TME of T1D hosts exhibit superior tumor specific cytotoxicity vs. $CD8^+$ T cells from TME of non-diabetic hosts. T1D associated tumor growth restriction is $CD8^+$ T cell dependent. Systemic $CD8^+$ T cell depletion mitigates the effect of pre-existing T1D on tumor growth control. Adoptive transfer of $CD8^+$ T cells from T1D tumor bearing hosts show tumor growth restricting potential in immunocompromised tumor bearing hosts. Reduction of immune-suppressor cells from TME of T1D hosts facilitates sustained antitumor immune response.

Introduction

$CD8^+$ T cells play a major role in preventing and controlling malignant tumordevelopment and progression, withcytotoxic T lymphocytes (CTLs) capable of directly killingtumor cells (Olivo Pimentel *et al*., 2020; Wiedemann *et al*., 2006; Basu *et al*., 2016; Qi *et al*., 2019; Weigelin *et al*., 2021). Since, both in cancer and T1D, there is an immense role of the adaptive immune system, it was necessary to study how adaptive immune system reacts in co-existence of both the diseases. Limited exploration has been done so far to determine the possible influence of pre-existing T1D or T1D associated activation of CTL in the cancer setting. Meta-analysis, using PubMed and EMBASE observational studies, suggest an association between T1D and increased risk fordeveloping cancers of the stomach, lung, pancreas, liver, ovary, and kidney, but a decreased risk for melanoma, prostate, and breast cancer (Giovannucci *et al*., 2010). We hypothesized that in T1D with at least certain forms of cancer, that $CD8^+$ T cells may infiltrate tumors and mediate superior anti-tumor activity that is beneficial for patients.

Objective

To study the influence of immune system in tumor progression in hosts with pre-existing T1D.

Results

2.1 T1D enhances the recruitment of activated tumor specific $CD8^+$ T cells in TME and TDLN: Given the involvement of adaptive immunity in T1D-associated tumor growth control, T cell population in the TME was analyzed. $CD8^+$ T cells were found to be significantly upregulated amongstthe tumor infiltrating lymphocyte (TIL) population in STZ induced T1D mice bearing B16F10, 4T1, LLC or CT26 tumorswhen compared to non-diabetic tumor control cohorts, which was mitigated by treatment with insulin (Figure 2.1A). $CD8^+CD28^+$TILs isolated from B16F10, 4T1, LLC or CT26 tumor bearing STZ induced T1D hosts displayed significantly upregulated expression of CD69 when compared to their non-diabetic tumor control counterparts. Insulin treatment (STZ+insulin) reduced TIL expression of CD69 in the B16F10 tumor model but not in the 4T1, LLC or CT26 tumor models (Figure 2.1B). Remarkably, $CD8^+$ TILs

isolated from STZ treated B16F10, 4T1, LLC and CT26 tumor bearing T1D mice expressed enhances levels of CXCR3 when compared to untreated control TILs (Figure 2.1C). Insulin treatment of these STZ induced T1D tumor models normalized the population of $CD8^+CXCR3^+$ TIL in the 4T1 and LLC bearing mice, but not in B16F10or CT26 bearing mice.Tumors in T1D mice exhibited significantly higher $CD8^+CD103^+CD39^+$ TIL content compared to control groups, which was reversible upon treatment with insulin (Figure 2.1D). Higher frequencies of $CD8^+CD69^+GZMB^+$ T cells were detected in STZ treated tumor draining lymph nodes (TDLNs), but not in the spleens of B16F10, 4T1, LLC or CT26 tumor bearing T1D mice, which were counteracted by insulin treatment (Figure 2.1E and 2.1F).

2.2 $CD8^+$ T cells from tumor bearing T1D hosts exhibit superior cytotoxicity towards tumor cells: To study the cytotoxic potential of T1D $CD8^+$ T cellscytotoxicity markers on $CD8^+$ TILs were studied. B16F10 tumor bearing T1D mice expressed enhances levels of *perforin*, *gzmB*, *ifng* and *il-2* on $CD8^+$ TILs compared to untreated control TILs (Figure 2.2A). $CD8^+$ TILs isolated from STZ treated B16F10, 4T1, LLC and CT26 tumor bearing T1D mice expressed enhances levels of granzyme B (GZMB) and interferon γ (IFNγ) when compared to untreated control TILs (Figure 2.2B). Insulin treatment in T1D group reduced GZMB and IFNγ expression on $CD8^+$ TIL population.

To study the cytotoxicity towards antigen specific tumor cells, we employed CD8-MACS to isolate $CD8^+$ T cells from TDLNs harvested from B16F10, 4T1, LLC and CT26 tumor bearing mice (i.e., control, STZ induced, and STZ induced +insulin treated). Sorted $CD8^+$ T cells were cultured *in vitro* with isologous tumor cells at different effector : target (E : T) ratios, with lactate dehydrogenase (LDH) release measured as an effector cell readout. $CD8^+$ T cells from B16F10, 4T1, LLC and CT26-bearing T1D mice mediated significantly enhanced cytotoxicity against antigen specific (but not irrelevant) tumor cell targets, which was substantially reduced in $CD8^+$ T cells isolated from T1D + insulin treated tumor-bearing mice (Figure 2.2C).

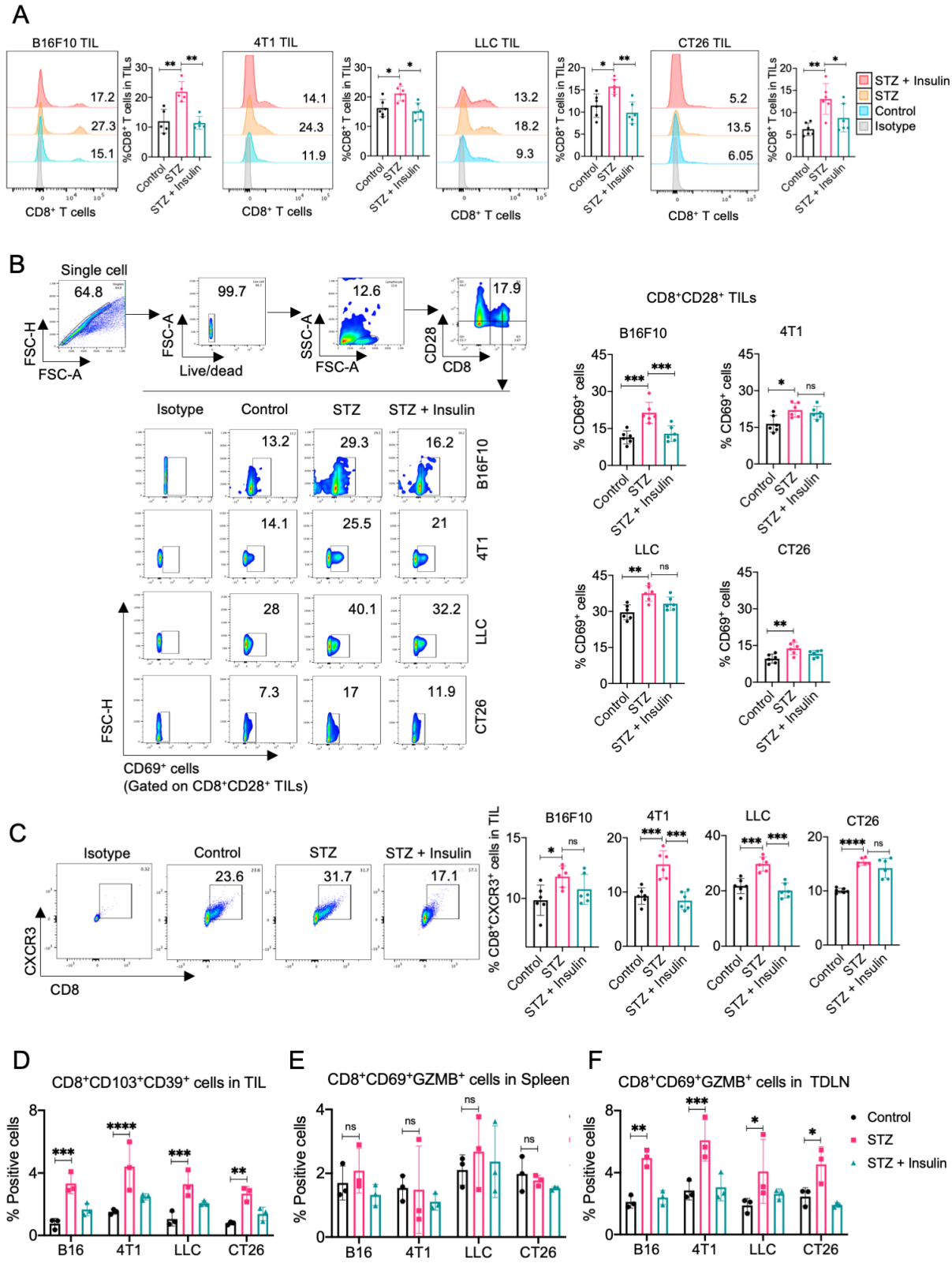

Figure 2.1: A. Histogram and graphical quantitation of CD8⁺ TIL contents in B16F10, 4T1, LLC and CT26 tumors in each recipient host evaluated (n=6 mice/group). **B.** Gating strategy for flow analysis of CD8⁺, CD28⁺ and CD69⁺ cell events along with a representative flow cytometry data set representative of TIL isolated from control, STZ treated and STZ+insulin treated hosts (n=6 mice/group) bearing B16F10, 4T1, LLC or CT26 tumors. Graphs depict the percentage of

CD69$^+$ cells amongst CD8$^+$CD28$^+$ TILs. **C.** Representative gating and graphical data reporting the percentage of CD8$^+$CXCR3$^+$ TIL in B16F10, 4T1, LLC and CT26 tumors in control, STZ treated and STZ+insulin treated hosts (n=6 mice/group). **D.** Percent CD8$^+$CD103$^+$CD39$^+$ cells in tumor infiltrating lymphocytes (TILs) from B16, 4T1, LLC and CT26 tumors of three different groups (n=3). **E.** Percent CD8$^+$CD69$^+$GZMB$^+$ cells in spleen from B16, 4T1, LLC and CT26 tumors of three different groups (n=3). **F.** Percent CD8$^+$CD69$^+$GZMB$^+$ cells in tumor draining lymph nodes (TDLN) from B16, 4T1, LLC and CT26 tumors of three different groups (n=3). One way ANOVA followed by Tukey's multiple comparisons test was performed to determine the significance of inter-group differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

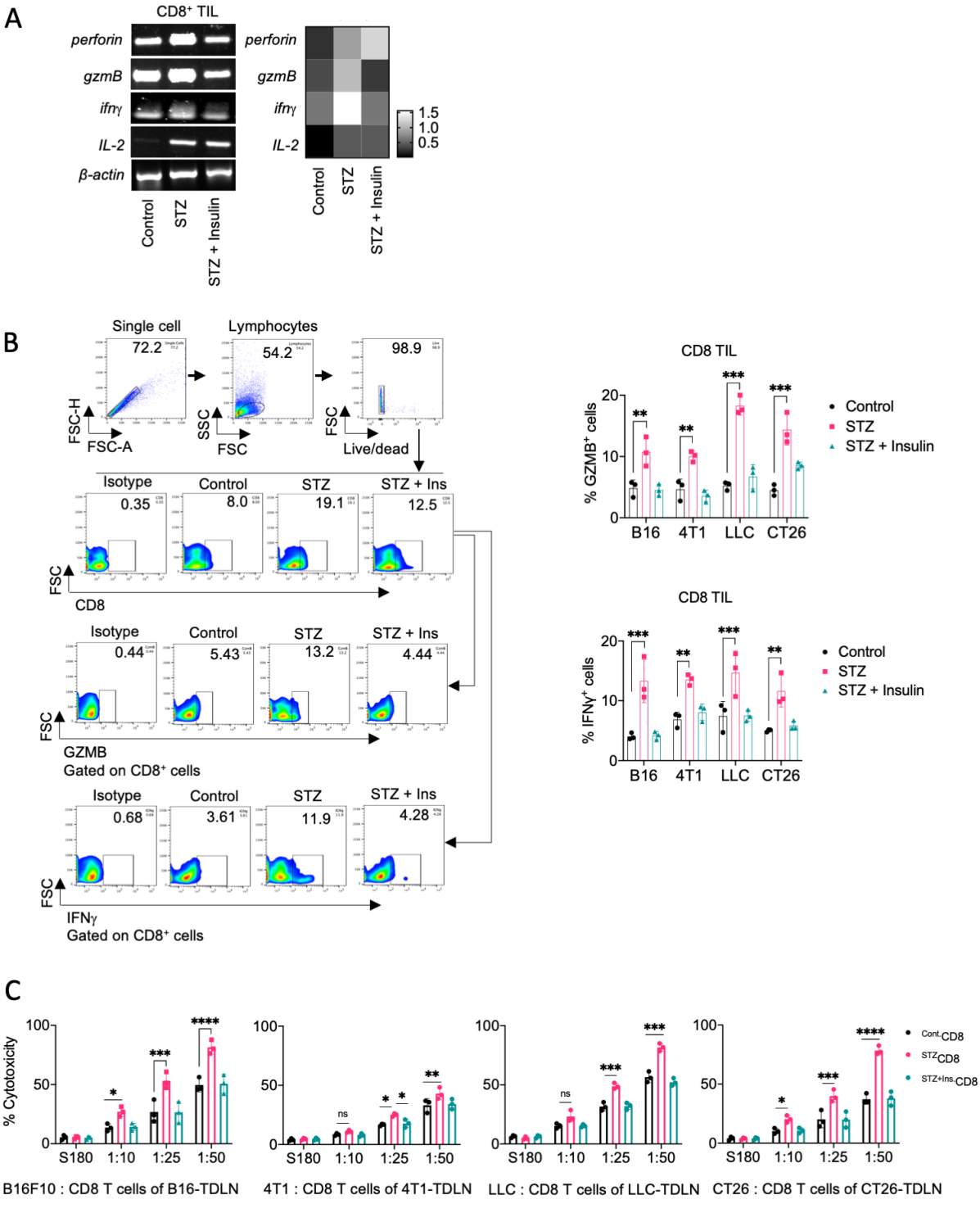

Figure 2.2: A. Heatmap depicts relative expression of *perforin, granzyme B, ifnγ and il-2* genes in $^{Cont.}CD8_{TIL}$, $^{STZ}CD8_{TIL}$ and $^{STZ+ins.}CD8_{TIL}$ cells sorted from B16F10 tumor-bearing mice. **B.** Gating strategy and representative pseudo color plots and graphs of $CD8^+$, granzyme B ($GZMB^+$) and $IFN\gamma^+$ cells in tumor infiltrating lymphocytes (TIL) of B16F10, 4T1, LLC and CT26 tumors from untreated (Control), STZ treated and STZ+Insulin treated groups. **C.** Graphs depicting the percentage cytotoxicityof model-matched tumor cells mediated by $CD8^+$ TILs isolated from B16F10, 4T1, LLC and CT26 tumors (LDH release assay). LDH release of control, STZ treated and STZ+insulin treated (n=3, repeated twice) groups were compared. Cancer cell : $CD8^+$ T cell ratios of 1:10, 1:25 and 1:50 were used. S180 sarcoma cells were used as unrelated control target. One-way ANOVA followed by Tukey's multiple comparisons test was performed to determine the significance of inter-group differences. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$)

2.3 T1D associated tumor growth control is $CD8^+$ T cell dependent: Given the direct involvement of the immune system, particularly $CD8^+$ T cells, in diabetes (Roep, 2003; Pinkse *et al*., 2005; Wiedeman *et al*., 2020a) as well as in cancer(van der Leun *et al*., 2020; Raskov *et al*., 2020), the influence of T1D on $CD8^+$ T effector cells in our tumor models was next assessed. As described above, STZ administration and maintained for 4 weeks to develop T1D in both C57BL/6J (n=6) and nude mice (n=6), with a group of nondiabetic C57BL/6J (n=6) and nude (n=6) mice serving as controls. B16F10 tumors were injected s.c.in mice. After palpable tumor formation, $CD8^+$ T cells were harvested from the TDLN of control as well as STZ-induced B16F10 bearing mice by MACS. Isolated $CD8^+$ T cells ($1x10^6$ cells/mice)from control or STZ-induced T1D mice were adoptively transferred into control or STZ induced nude mice bearing B16F10 tumors by tail veininjection (Figure 2.3A).We observed that tumor growth was reduced upon adoptive transfer of $CD8^+$ T cells from either source (vs. no transfer) in both the non-diabetic and T1D nude melanoma models. However, adoptive T cell therapy implementing $CD8^+$ T cells isolated from T1D tumor bearing mice (i.e., $^{STZ}CD8^+$ T cells) mediated significantly greatertumor growth control ($p<0.05$) in T1D hosts when compared to ACT using $CD8^+$ T cells isolated from non-diabetic tumorbearing mice (Figure 2.3B and 2.3C).

For further understanding on the role of $CD8^+$ T cells in controlling tumorgrowth in STZ induced T1D mice, systemic $CD8^+$ T cell depletion was performed (Figure 2.3D). C57BL/6J mice were divided into eight groups (n=5) with half receiving STZ treatment (50 mg/kg for consecutive 5 days) and the other four groups left untreated. Depleting anti-CD8a antibody (100µg/50µl) was injected i.p on days -1 and 6, into 3 of the untreated and 3 of the STZ induced groups. B16F10 tumors were injected s.c. on day 1 into all animals. Following palpable tumor formation on day 7, $CD8^+$ T cells were isolated from TDLN of untreated ($^{Cont.}CD8_{TDLN}$) or STZ treated ($^{STZ}CD8_{TDLN}$) tumor bearing mice. One untreated and one STZ induced recipient group received adoptive T cell therapy implementing $^{Cont.}CD8_{TDLN}$ or $^{STZ}CD8_{TDLN}$. $CD8^+$ T cell depletion in T1D but not recipient tumor bearing mice ablated tumor growth control vs. non-diabetic $CD8^+$ T cell depleted tumor bearing hosts (Figure 2.3E). Adoptive $CD8^+$ T cell therapy resulted in tumor growth restriction in both non-

diabetic and STZ treated groups receiving either $^{Cont}CD8_{TDLN}$ or $^{STZ}CD8_{TDLN}$ T cells, compared to their respective control groups received no adoptive T cell therapy. However, non-diabetic and STZ treated groups that received $^{STZ}CD8_{TDLN}$ therapy showed more prominent tumor growth restriction than non-diabetic and STZ treated groups that received $^{Cont}CD8_{TDLN}$ therapy ($p<0.01$). Collectively, these results confirmed the involvement of effector $CD8^+$ T cells in restricting tumor growth in T1D tumor-bearing hosts.

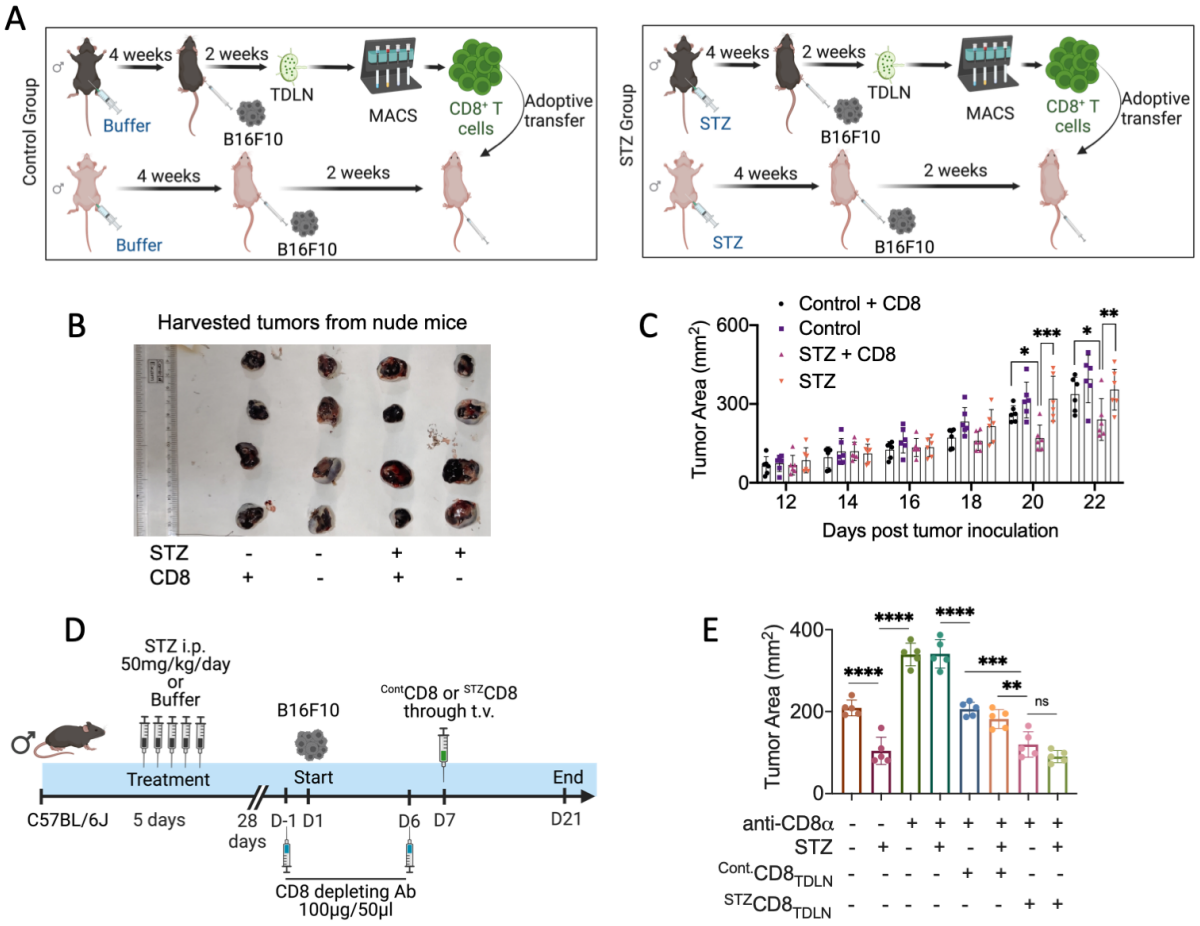

Figure 2.3: A. Schematic overview of experimental design. C57BL/6J (black) and nude (pink) mice were divided into two groups. One group received streptozotocin (STZ) i.p. (50mg/kg) for five consecutive days, while the other group received only buffer (Sodium citrate, pH 4.5), with animals subsequently maintained for four weeks to allow for diabetes induction. After four weeks, STZ treated mice with random blood sugar values in the range of 250-350 mg/dl were selected for experimental use. B16F10 cells (5×10^5 cells) were injected s.c. into C57BL/6 mice and allowed to establish palpable tumors. Two weeks after tumor injection, animals were euthanized and $CD8^+$ T cells ($^{STZ}CD8_{TDLN}$ or $^{Cont.}CD8_{TDLN}$) were isolated from tumor draining lymph nodes (TDLNs) by magnetic assisted cell sorting (MACS). MACS sorted $^{STZ}CD8_{TDLN}$ or $^{Cont.}CD8_{TDLN}$ ($2x10^5$ cells/mice) or PBS (as a control) were adoptively transferred via tail vein injection into nude mice bearing established B16F10 melanomas. Tumor growth was monitored through day 22. **B.** Representative photographs of B16F10 tumors in nude mice harvested 22 days after tumor inoculation. **C.** Mean tumor area in each of the different experimental groups (n=6) is plotted graphically over time. **D.** Control or STZ induced T1D C57BL/6J mice (n=5 mice/group) were injected s.c. with B6F10

melanoma cells on study day 1. Anti CD8a antibody (100µg/50µl PBS was injected i.p. on days -1 and 6 in the indicated groups. On day 7, cohorts of control or STZ induced T1D mice bearing B16F10 melanomas received adoptive transfer of $^{STZ}CD8_{TDLN}$ or $^{Cont.}CD8_{TDLN}$ T cells (2 x10^5 cells/mouse) via tail vein injection as indicated. Tumor area were measured on day 28 post-tumor inoculation. **E.** Mean B16F10 tumor area is reported for each experimental cohort from (D) Two way ANOVA followed by Tukey's multiple comparisons test was performed to calculate the significance or inter-cohort differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

2.4 T1D alleviates immunosuppressive cell population in TME : In extended analysesof functional status of $CD8^+$ T cells, next we studied on the checkpoint markers, we observed that $CD8^+$ TIL isolated from melanoma-bearing STZ induced T1D (vs. control) mice expressed reduced levels of the PD1 and LAG3 checkpoint molecules, but elevated expression of TCF1 (Figure 2.4A). Analyses of immune-suppressor cells in the TME indicated decreased frequencies of $CD4^+FoxP3^+$ Tregs (Figure 2.4B), $CD11b^+F4/80^+$ TAMs (Figure 2.4C) and $CD11b^+Gr1^+$ MDSCs (Figure 2.4D) in STZ induced mice bearing B16F10 tumors, with unaltered content of $CD11c^+MHC\text{-}I^+$ DCs (Figure 2.4E) when compared to control tumors. These cumulative results suggest pre-existing T1D in the tumor bearing host facilitates an immunologically "hot" TME based on preferential recruitment/maintenance of pro-inflammatory immune cell populations and exclusion/removal of regulatory cell populations which is leveraged by the absence or presence of insulin.

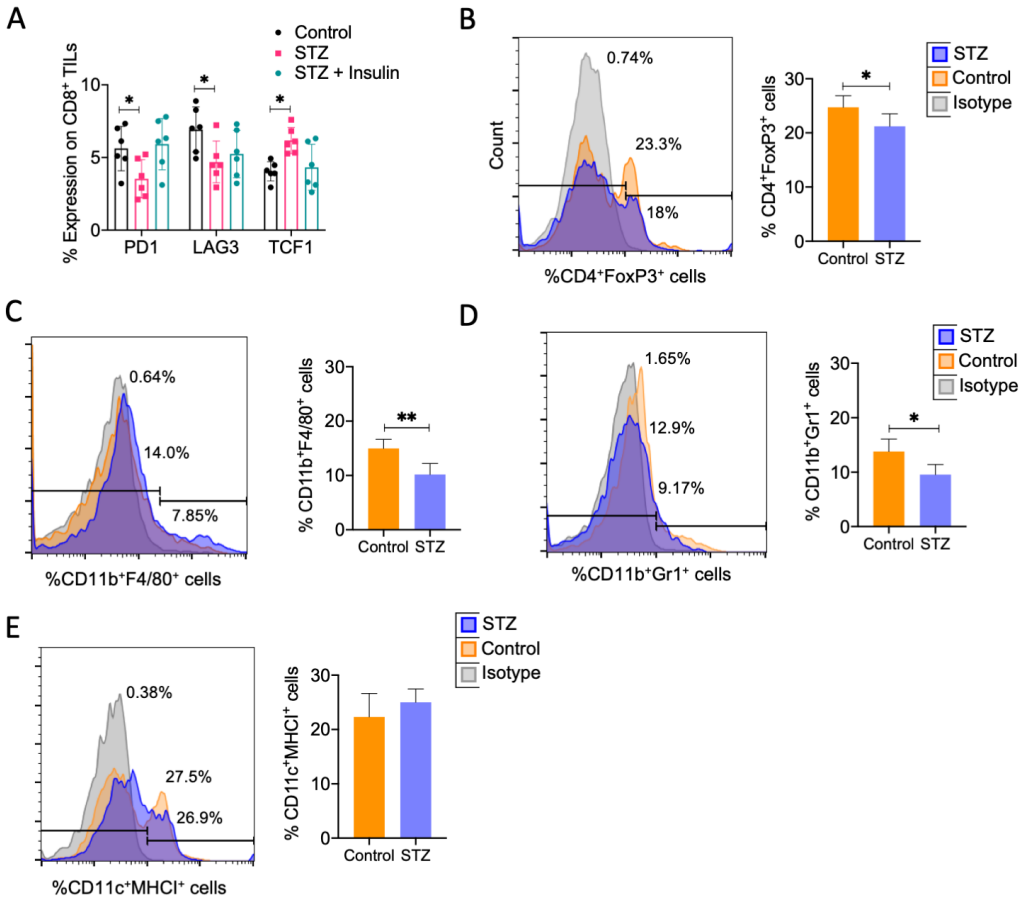

Figure 2.4: A. Percentage of $CD8^+$ TILs isolated from B16F10 tumors that co-express PD1, LAG3 or TCF1 (n=6 mice/group). One way ANOVA followed by Tukey's multiple comparisons test was performed to determine the significance of inter-group differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). Histograms and graphs of **B.** $CD4^+FoxP3^+$Treg cells, **C.** $CD11b^+F4/80^+$ tumor associated macrophages (TAMs), **D.** $CD11b^+Gr1^+$ myeloid derived suppressor cells (MDSCs) and **E.** $CD11c^+MHCI^+$ dendritic cells (DCs), from B16F10 tumors of untreated (control) and STZ treated C57BL/6J mice (n=5). Unpaired t tests were performed to calculate significance. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

Discussion

Effective adaptive immune system is a necessary prerequisite for optimum tumor control (Gajewski *et al*., 2013; Wu, 2017). T1D, which is the result of an activated immune system might play a pivotal role in restricting tumor growth in its hosts (Roep, 2003; Bluestone *et al*., 2015; Wiedeman *et al*., 2020a; Pinkse *et al*., 2005; Wiedeman *et al*., 2020b; Gearty *et al*., 2022; Fazeli *et al*., 2022; Turner and La Gruta, 2022). Pre-existing T1D showed $CD8^+$ T cell dependent tumor growth control in melanoma and carcinoma transplantable tumor models. T1D hosts of melanoma (B16F10) and carcinoma (4T1, LLC and CT26) tumors showed increased infiltration of activated and cytotoxic $CD8^+$ T cells in TME and TDLNs as evidenced by the increased expression of CXCR3 (infiltration) and CD69 (activation) on $CD8^+$ T cell surface. Further *in vitro* study on $CD8^+$ T cells from TDLNs of T1D hosts co-cultured with tumor cell lines revealed enhanced tumor cell specific cytotoxicity, but not with unrelated tumor cells, of $CD8^+$ T cells. To observe the dependency on adaptive immune system in T1D associated tumor control, similar experiments on athymic nude mice were conducted. Immunodeficient T1D nude mice failed to show tumor growth control vs. non-diabetic nude mice. Adoptive transfer of $CD8^+$ T cells from wild type T1D tumor bearing hosts to T1D nude mice restored tumor growth control vs. non-diabetic nude mice which received adoptive $CD8^+$ T cell therapy from non-diabetic wild type tumor bearing hosts. Dependency of T1D hosts on $CD8^+$ T cells in restricting tumor growth was further confirmed when systemic $CD8^+$ T cell depletion in T1D hosts failed to exert significant tumor growth restriction vs. non-diabetic $CD8^+$ T cell depleted control cohort. $CD8^+$ T cells from T1D tumor bearing hosts were found to be superior to demonstrate antitumor immunity vs. $CD8^+$ T cells from non-diabetic tumor bearing cohort. Further study on the immune-suppressor cell landscape in TME revealed decreased population of Tregs, TAMs and MDSCs in T1D hosts vs. control. Increase in DC population in TDLN further suggests better priming of $CD8^+$ T cells in TID tumor bearing host. Overall study clearly indicated the involvement of tumor specific activated cytotoxic $CD8^+$ T cells in restricting transplantable tumor growth in T1D hosts.

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CHAPTER 3

ROLE OF PRE-EXISTING T1D IN $CD8^+$ T CELL AND CANCER CELL METABOLISM

- **ABSTRACT**
- **INTRODUCTION**
- **OBJECTIVE**
- **RESULTS**
    1. T1D metabolically reprograms $CD8^+$ TILs in support of sustained immune-mediated control of tumor growth
    2. Tumor cells in T1D hosts show impaired glucose metabolism
- **DISCUSSION**
- **REFERENCES**

Abstract

$CD8^+$ TILs of T1D hosts exhibit superior glycolysis and OXPHOS in all four transplantable tumor models vs. respective control $CD8^+$ TILs. Enhanced glucose metabolism in $CD8^+$ TILs of T1D hosts is facilitated by upregulation of glucose intake and effective entry of glycolytic end product to TCA cycle. All four tumor cells of T1D hosts, on the other hand, fails to upregulate glucose metabolism due to poor glucose intake. Thus, $CD8^+$ T cells of T1D TME win the metabolic tug of war with tumor cells and exhibit sustained antitumor immune response.

Introduction

In order to achieve effective antitumor immune response, following tumor antigen encounter, $CD8^+$ T cells need to undergo robust metabolic alterations (Buck *et al*., 2015; Palmer *et al*., 2015; Bantug *et al*., 2018; De Rosa *et al*., 2017; Chang and Pearce, 2016; Yiming Yin *et al*., 2016). Activated $CD8^+$ T cells switch their metabolism towards glucose utilization (Cascone *et al*., 2018; Brand *et al*., 2016; Palmer *et al*., 2015). However, within immunosuppressive tumor microenvironment, effector $CD8^+$ T cells couldn't sustain their effector functions for a longer period of time and become exhausted and anergic (Zhang *et al*., 2020; Nagasaki and Togashi, 2022; Jiang *et al*., 2015). Exhausted and anergic $CD8^+$ T cells exhibit a metabolically quiescent stage primarily due to unavailability of nutrients and various suppressive molecules in TME (Zhang *et al*., 2022; Guan *et al*., 2023; Watowich *et al*., 2023). Tumor cells undergo rapid proliferation by upregulating glycolysis up to 10 times, which is known as Warburg effect, due to which huge amount of lactate is generated as a byproduct. Lactate makes TME more acidic which is one of the major reasons for effector $CD8^+$ T cell disfunction (Wang *et al*., 2021; Feng *et al*., 2022; Mortaezaee and Majidpoor, 2023). In the metabolic tug of war within TME, $CD8^+$ T cells lose and tumor cells win. In order to achieve superior effector function, sustained metabolic activation and sustained nutrient availability to $CD8^+$ T cells is a must. Since our study showed prolonged effector function of effector and cytotoxic $CD8^+$ T cells in TME of T1D hosts, it would be interesting to study whether these cells get some metabolic advantage or not in the TME for their prolonged antitumor activity.

Objective

To study the role of pre-existing T1D in $CD8^+$ T cell and cancer cell metabolism.

Results

3.1. T1D metabolically reprograms $CD8^+$ TILsin support of sustained immune-mediated control of tumor growth: Metabolic reprogrammingappears to represent a prerequisite for optimal $CD8^+$ T cellantitumor activity within the TME (Menk *et al*., 2018; Cammann *et al*., 2016). $CD8^+$ T cells from B16F10 tumors were isolated from STZ induced T1D ($^{STZ}CD8_{TIL}$) and control ($^{Cont}CD8_{TIL}$) mice using MACS (Figure 3.1A) and their purity confirmed by flow cytometry (Figure 3.1B). Subsequent analysis of T cell metabolomics revealed that

$^{STZ}CD8_{TIL}$ cells showed a significantly upregulated extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) when compared to $^{Cont}CD8_{TIL}$ cells (Figure 3.1C and 3.1D). As glucose metabolism is a preferred characteristic for anti-tumor CD8$^+$ T cells with regards to their therapeutic efficacy (Palmer *et al*., 2015; Cham *et al*., 2008; Ma *et al*., 2019), we next studied intrinsic T cell expression of transporters and enzymes involved in glucose metabolism (Figure 3.1E). ^{STZ}CD8$^+$ TILs showed elevated glucose transporter 1 (*glut 1*), monocarboxylate transporter 1 (*mct 1*), and pyruvate kinase M2 (*pkm 2*) expression compared to their respective untreated control CD8$^+$ TILs (Figure 3.1F). However, the expression of glucose 6-phosphate dehydrogenase (*g-6-pd*) and lactate dehydrogenase (*ldh*) differed only modestly in ^{STZ}CD8$^+$ TILs vs. control CD8$^+$ TILs. Insulin treatment of STZ induced tumor bearing mice resulted in CD8$^+$ TILs with reduced expression of *glut 1*, *mct 1* and *pkm 2* expression vs. ^{STZ}CD8$^+$ TILs in all four tumor models evaluated in this study. Expression of pyruvate carboxylase 1 (*pcx 1*), which is involved in the conversion of pyruvate (an end-product of glycolysis) into oxaloacetate, was significantly elevated in^{STZ}CD8$^+$ TILs isolated from B16F10 and LLC, but not 4T1 and CT26 tumors. Insulin treatment of STZ induced tumor-bearing mice resulted in reduced expression of *pcx 1* in CD8$^+$ TILs (vs. ^{STZ}CD8$^+$ TILs) isolated from B16F10 and LLC, but not 4T1 and CT26 tumors. Isocitrate dehydrogenase 2 (*idh 2*) expression was found differentially enhanced in ^{STZ}CD8$^+$ TILs from B16F10, 4T1 and LLC (but not CT26) vs. comparable TIL isolated from control or insulin + STZ-treated tumor bearing hosts. These results support divergent expression patterns for *pcx 1* and *idh 2* gene expression in ^{STZ}CD8$^+$ TIL across the various tumor models. In contrast, transcript levels for enzymes involved in the TCA cycle, such as pyruvate dehydrogenase kinase 1 (*pdk 1*), pyruvate dehydrogenase kinase 3 (*pdk 3*), isocitrate dehydrogenase 1 (*idh 1*) and fumarate hydratase 1 (*fh 1*) were found comparable in CD8$^+$ TILs isolated from tumors harvested from untreated (control), STZ treated or STZ+insulin treated mice.

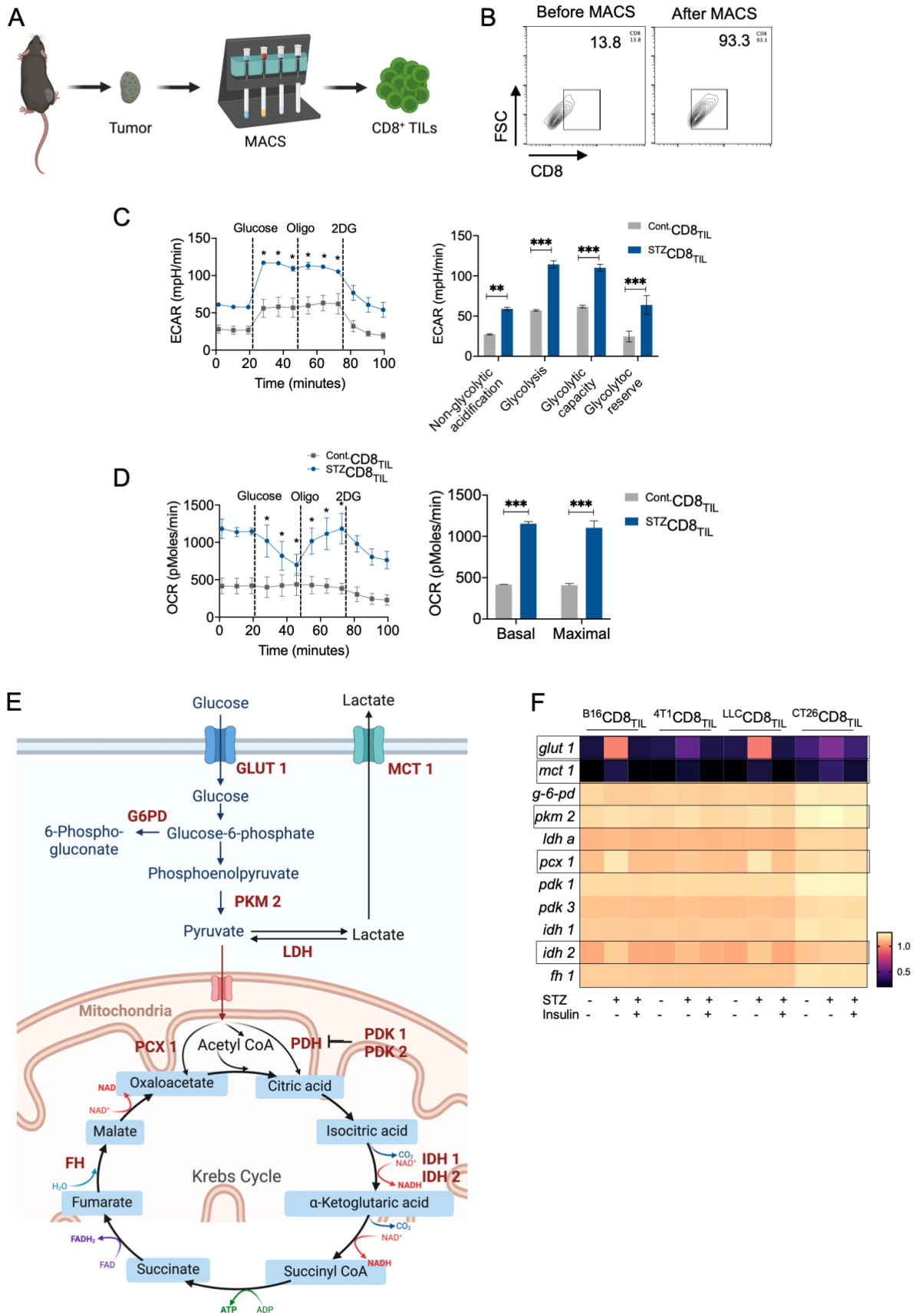

Figure 3.1: A. Schematic overview for magnetic-activated cell sorting (MACS) of CD8⁺ TIL. **B.** Flow cytometry contour plots of CD8⁺ T cells before and after MACS sorting. **C.** Extracellular

acidification rate (ECAR) and **D.** Oxygen consumption rate (OCR) of $CD8^+$ TILs isolated from B16F10 tumors (n=6 mice/group) from untreated and STZ induced T1D mice. **E.** Schematic overview of glycolysis and Krebs cycle along with the pathway associated enzymes analyzed (in red). **F.** Gene expression profiles of *glut 1* and *mct 1* transporters, *g-6-pd* and *pkm 2*, enzymes involved in glycolysis, *ldh a*, involved in pyruvate to lactate conversion, *pcx 1*, *idh 1*, *idh 2*, *fh 1*, involved in Krebs cycle, *pdk 1* and *pdk 3,* inhibitor of pyruvate dehydrogenase (PDH), with β *actin* as a control gene product, were measured by RT-PCR in $CD8^+$ TILs (n=6). Two-way ANOVA was performed to analyze the significance or intergroup differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

3.2. Tumor cells in T1D hosts show impaired glucose metabolism: B16F10, 4T1, LLC and CT26 tumor cells isolated from STZ induced T1D mice displayed decreased expression of *glut 1* and *pcx 1*, when compared to their respective counterparts in the control and STZ+insulin treated groups (Figure 3.2A). No significant alterations in expression of *mct 1, g-6-pd, pkm 2, ldh a, pdk 1, pdk 3, idh 1, idh 2 and fh1* were noted in tumor cells based on the host treatment regimen. These findings suggest that pre-existent T1D leads to selective glycolytic benefits in $CD8^+$ TIL vs. tumor cells, with this metabolic advantage in $^{STZ}CD8_{TIL}$ cells yielding sustained antitumor activity in association with superior tumor growth control *in vivo*.

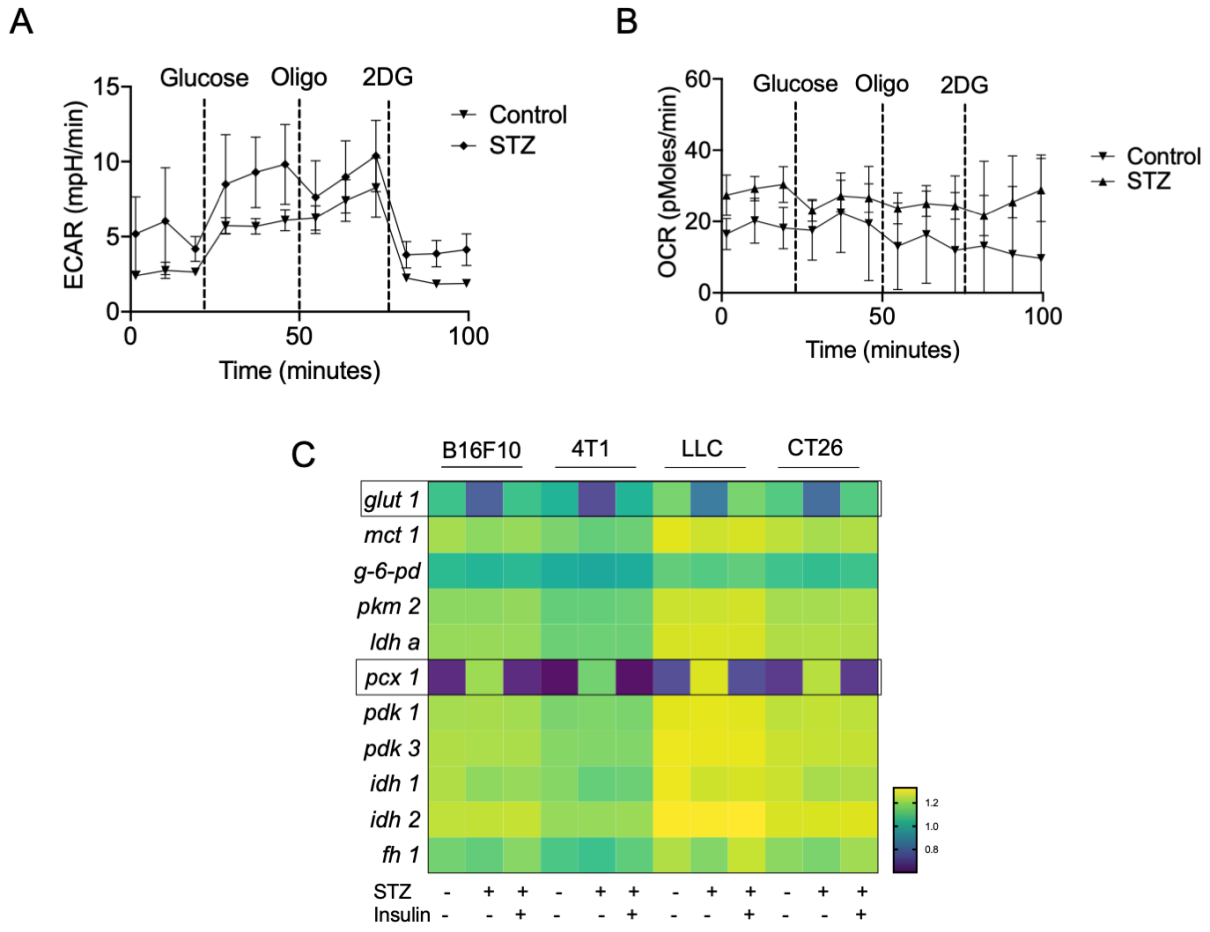

Figure 3.2: A. Extracellular acidification rate (ECAR) and **B.** Oxygen consumption rate (OCR) of B16F10 tumor cells (n=6 mice/group) from untreated and STZ induced T1D mice. **C.** Gene expression profiles of *glut 1* and *mct 1* transporters, *g-6-pd* and *pkm 2*, enzymes involved in glycolysis, *ldh a*, involved in pyruvate to lactate conversion, *pcx 1*, *idh 1*, *idh 2*, *fh 1*, involved in Krebs cycle, *pdk 1* and *pdk 3,* inhibitor of pyruvate dehydrogenase (PDH), with β *actin* as a control gene product, were measured by RT-PCR in tumor cells (n=6). Two-way ANOVA was performed to analyze the significance or intergroup differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

Discussion

Eukaryotic cells metabolize glucose via glycolysis and Kreb's cycle. Naïve $CD8^+$ T cells, following antigen encounter and activation, may increase glycolysis up to 10 times in order to achieve superior effector function (Cascone *et al*., 2018). $CD8^+$ T cells, that fail to switch their glycolytic activity fails to demonstrate effector function. Memory $CD8^+$ T cells, in contrast, rely on mitochondrial fatty acid oxidation (FAO) for their metabolic requirements (Enamorado *et al*., 2017; Youngblood *et al*., 2017). Rapid glycolysis in T cells generate large amount of nicotinamide-adenine dinucleotide phosphate (NADPH) which makes T cell cytosol and its surrounding more acidic. By measuring extracellular acidification rate (ECAR) in *in vitro* setting by putting a pH sensitive electrode close to the cells, one can indirectly measure glycolytic rate. Likewise, by measuring the oxygen consumption rate (OCR), which is solely required for the Kreb's cycle, one can measure the OXPHOS of a cell population. $CD8^+$ TILs isolated from T1D hosts showed enhanced glycolytic and OXPHOS activity vs. control $CD8^+$ TILs indicative of their metabolic switch towards rapid glucose metabolism for sustained antitumor immune response. Further, elevated gene expression of *glut 1* in $CD8^+$ TILs isolated from T1D ($^{T1D}CD8_{TILs}$) hosts validated the notion of enhanced glucose metabolism. Superior expression of *pkm 2* and *mct 1* in $^{T1D}CD8_{TILs}$ of all four tumor types vs. $^{Cont.}CD8_{TILs}$ indicated more pyruvate and lactate production, indicative of effector $CD8^+$ T cell metabolic profile. Insulin treatment mitigated T1D associated $CD8^+$ TILs metabolic switching in all four tumor models. Higher expression of *pcx 1* in $^{T1D}CD8_{TILs}$ vs. $^{Cont.}CD8_{TILs}$is indicative of superior utilization of pyruvate, the end product of glycolysis, in OXPHOS which might help $CD8^+$ TILs for prolonged antitumor immunity.

B16F10 tumor cells of T1D hosts, on the other hand, failed to upregulate their glycolytic activity even in the presence of high systemic blood glucose as evidenced by their ECAR and OCR analyses vs. control tumor cells of non-diabetic hosts. Gene expression profiling of glucose transporters and metabolic genes further revealed decreased expression of glut 1 in all four types of tumor cells in T1D hosts vs. control. Lesser glucose uptake leads to lesser glycolysis as well as lesser OXPHOS in T1D tumor cells. This was further validated by the lower expression of *pcx 1* which is responsible for the conversion of pyruvate to oxaloacetate, a key regulatory enzyme of glucose entry into OXPHOS. Cumulative findings suggested tumor cells in T1D hosts get no metabolic advantage which could sustain their rapid proliferation. Concomitantly,

lesser glucose uptake and lesser conversion of pyruvate to oxaloacetate leads to diminished glycolysis and OXPHOS respectively. T1D associated metabolic advantage of $CD8^+$ TILs in TME compared to the tumor cells might positively contribute to their enhanced and sustained antitumor immunity.

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CHAPTER 4

EXPLORATION OF MOLECULAR SIGNALING THAT INFLUENCE $CD8^+$ T CELL AND TUMOR CELL METABOLISM IN PRE-EXISTING T1D TUMOR BEARING HOSTS

- **ABSTRACT**
- **INTRODUCTION**
- **OBJECTIVE**
- **RESULTS**
    1. Pre-existent T1D in the tumor bearing host is associated with enrichment in $CD8^+IGF1R^+IGF1^+$ T cells in tumor, TDLN, spleen and blood.
    2. IGF1-IGF1R-mTOR signaling influences T1D associated metabolic activation of $CD8^+$ T cells in the tumor bearing host
- **DISCUSSION**
- **REFERENCES**

Abstract

Altered metabolic imprints associated with $CD8^+$ T cells and tumor cells are required to be explored to decipher the molecular signaling that influence T1D associated metabolic activation and enhanced cytotoxicity of $CD8^+$ T cells. Herein, we reported enhanced expression of IGF1 and IGF1R in $CD8^+$ TILs of T1D hosts. Further, $CD8^+IGF1R^+IGF1^+$ TILs were found to be in higher proportion in T1D tumors and frequency of this cell population was found to be negatively correlated to the tumor size. $CD8^+IGF1R^+IGF1^+$ T cells were also found to be in higher proportion in TDLNs of T1D but not control (only tumor and only T1D) hosts. IGF1R-mTOR signaling axis was found to be responsible for the $CD8^+$ T cell activation which was further validated in clinical T1D samples *in vitro*.

Introduction

Involvement of metabolically active and cytotoxic $CD8^+$ T cells in T1D associated tumor growth retardation was established. In order to obtain sustained metabolic activation and cytotoxicity within TME, $CD8^+$ T cells must achieve reprogrammed intracellular signaling. Intracellular signaling is a vast inter-connected network of signaling molecules starting from the cell surface or intracellular receptors to the mediator molecules and ultimately transcription factors which modulates certain gene expressions. Since T1D hosts lack a major hormone insulin, we studied those molecules, expressions of which is modulated by this hormone (Rincon *et al*., 2004; Cheng *et al*., 2005). These molecules are insulin receptor (IR), insulin like growth factor 1 (IGF1), insulin like growth factor 1 receptor (IGF1R) and insulin like growth factor 2 receptor (IGF2R). We studied the expressions of these signaling molecules in $CD8^+$ T cells within tumors, lymph nodes, tumor draining lymph nodes and spleens. In the course of the study we have found a unique T cell population i.e$CD8^+IGF1R^+IGF1^+$ TT cells, in T1D hosts involved in T cell mediated tumor growth control. We have confirmed the intracellular signaling pathway in $CD8^+$ T cells by using silencing RNA (siRNA) and specific inhibitors. Finally, we have validated our findings in T1D clinical samples *in vitro*.

Objective

To explorethe underlying molecular signaling that influence $CD8^+$ T cells and tumor cells metabolism in pre-existing T1D tumor bearing hosts.

Results

4.1. Pre-existent T1D in the tumor bearing host is associated with enrichment in $CD8^+IGF1R^+IGF1^+$ T cells in blood, TDLN and tumor: Given the importance of T1D-associated metabolic reprogramming of $CD8^+$ T cells in restricting tumor growth, we next aimed to decipher the underlying signaling mechanisms associated with improved immune-mediated control of tumor growth. Insulin-like growth factor 1 (*igf1*) and insulin-like growth factor 1 receptor (*igf1R*) expression was significantly elevated in $^{STZ}CD8^+$ TILs from B16F10, 4T1, LLC and CT26 tumors, with B16F10

TILs exhibiting the most profound degree of overexpression (Figure 4.1A). However, both C57BL/6J and BALB/c mice, after STZ treatment and under tumor-free conditions exhibited significantly reduced serum IGF1 concentrations, which could be normalized following insulin treatment (Figure 1.3B). Insulin like growth factor 2 receptor (*igf2R*) showed no significant alteration among the different groups of $CD8^+$ TILs across all tumor types. Insulin receptor (*insulinR*) was found to be downregulated in TILs from B16F10 melanomas (but not other tumors in our study) established in STZ induced T1D mice. Conversely, tumor cells recovered from STZ induced T1D mice displayed diminished *igf1*, *igf1R* and *insulinR* expression when compared to respective untreated (control) or STZ+insulin treated tumor-bearing cohorts (Figure 4.1B). In contrast, *igf2R* expression remained unchanged, and *igf1 and igf1R* expression was significantly upregulated, in $^{STZ}CD8_{TDLN}$ vs. $^{Cont.}CD8_{TDLN}$ isolated from B16F10 tumors (Figure 4.1C). These metabolic T1D-associated alterations in TDLN $CD8^+$T cells were reversed by treatment with insulin and were not observed in (non-tumor) LNs.

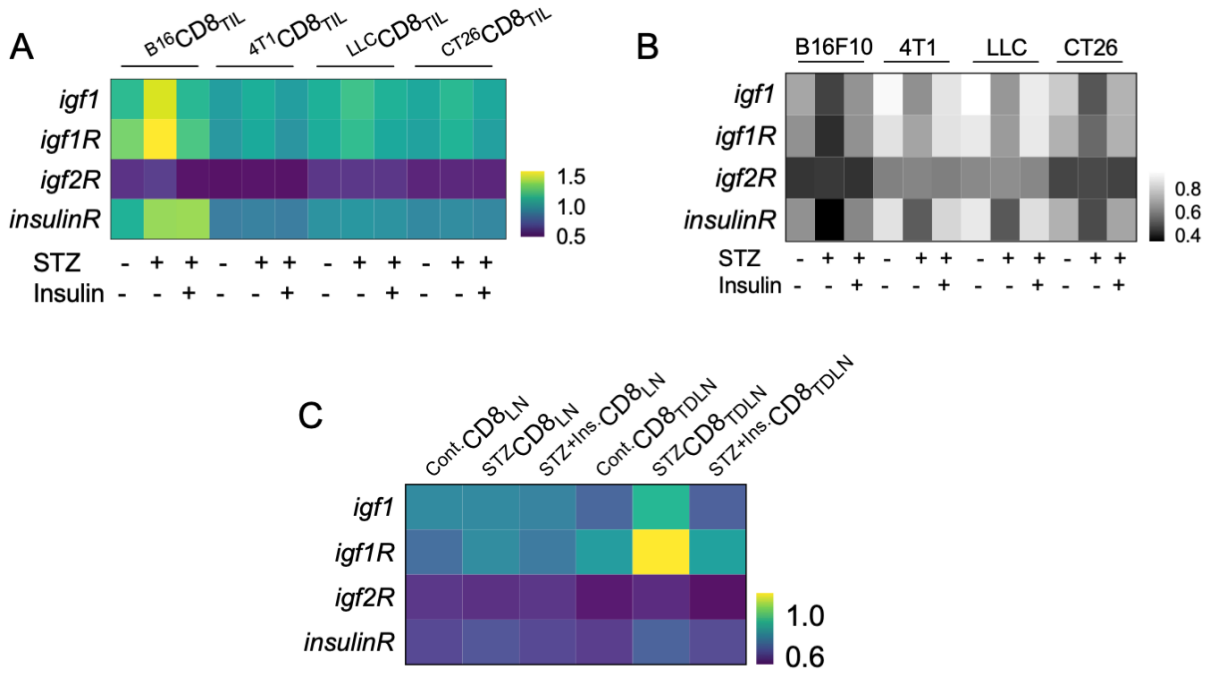

Figure 4.1: Expression of *igf1, igf1R, igf2R* and *insulinR* was analyzed by RT-PCR from **A**. MACS sorted $CD8^+$ TILs, **B**. tumor cells and **C**. MACS sorted $CD8^+$ T cells isolated from TDLNs (n=6 mice/group).

Co-expression of IGF1R and IGF1 on $CD8^+$ TILs was then analyzed by flow cytometry in the various tumor models. $^{STZ}CD8_{TIL}$ were enriched in the $CD8^+IGF1R^+IGF1^+$ T cell subpopulation when compared to $^{Cont.}CD8_{TIL}$ in all 4 tumormodels (Figure 4.1D). Insulin treatment of STZ induced T1D tumor bearing mice yielded $^{STZ+Ins.}CD8_{TIL}$ with significantly reduced frequencies of $CD8^+IGF1R^+IGF1^+$ TILs vs. $^{Cont.}CD8_{TIL}$ and $^{STZ}CD8_{TIL}$ across all models Moreover, immune-fluorescence

microscopy (IFM) analyses of B16F10 tumor sections from STZ treated T1D mice showed increased colocalization of IGF1 and IGF1R on $CD8^+$ TIL when compared to untreated B16F10 tumorsections (Figure 4.1E and 4.1F). Notably, $CD8^+IGF1R^+IGF1^+$ cells were only increased in tumor bearing mice, with the percentage of $CD8^+IGF1R^+IGF1^+$ T cells amongst total TILs determined to be inversely correlated with tumorsize (Figure 4.1G).

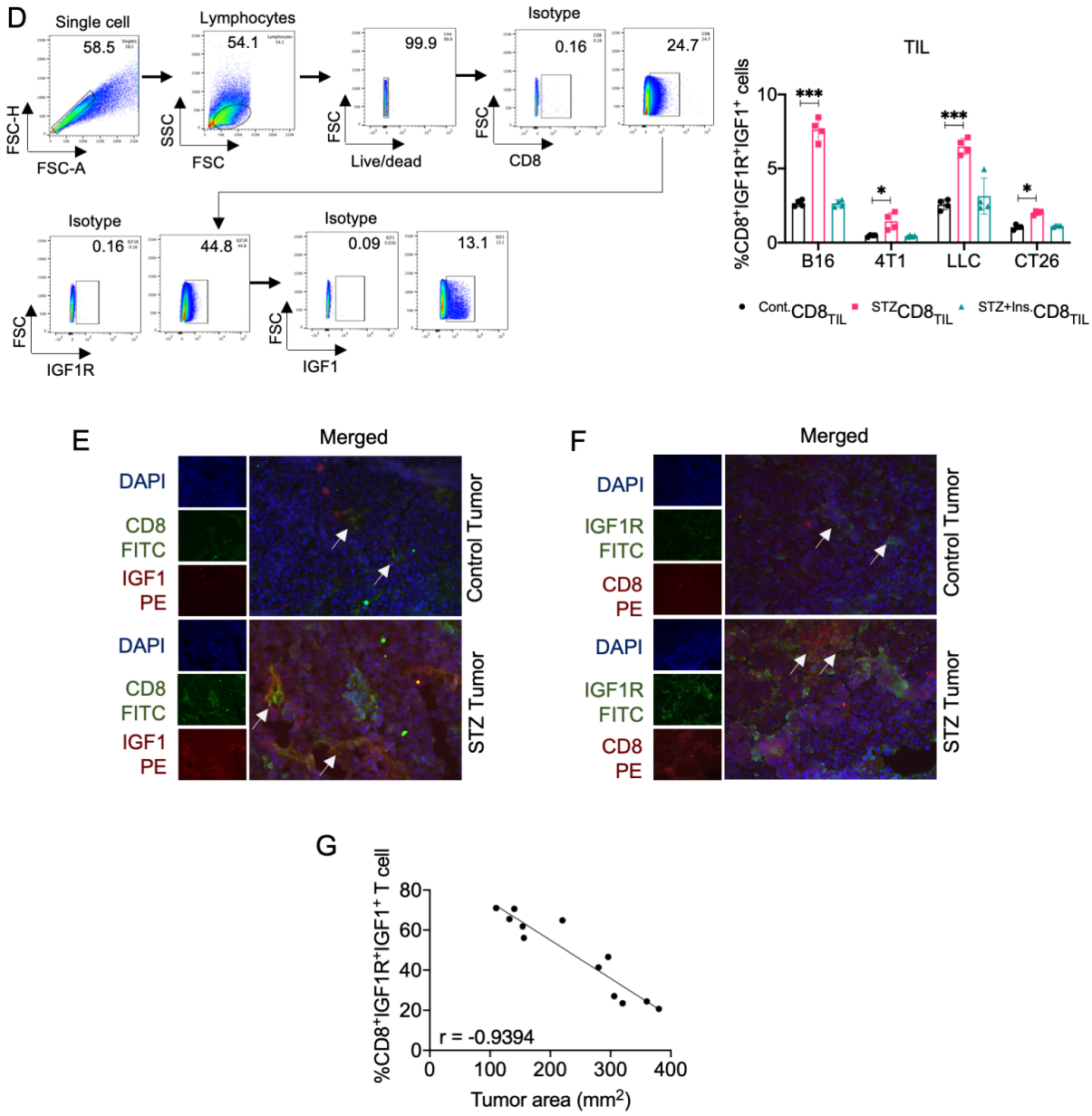

Figure 4.1: D. Flow cytometry gating and percentage of $CD8^+IGF1R^+IGF1^+$ T cells in tumors (n=4). **E.** Colocalization of CD8 and IGF1 (arrow marked) in B16F10 tumor sections harvested from control vs. STZ induced T1D mice. **F.** Colocalization of CD8 and IGF1R (arrow marked) in B16F10 tumor sections harvested from control vs. STZ induced T1D mice. **G.** Correlation analysis between tumor area and the frequency of $CD8^+IGF1R^+IGF1^+$ T cells in tumors. Two-way ANOVA was performed to test for significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

Exploration of $CD8^+IGF1R^+IGF1^+$ cell population was further extended to LNs, TDLNs, spleens and PBMCs. LNs isolated from tumor-free untreated, STZ treated and STZ+insulin treated mice did not exhibit significant difference in $CD8^+IGF1R^+IGF1^+$ T cell populations (Figure 4.1H). However, the $CD8^+IGF1R^+IGF1^+$ T cell subpopulation was elevated in TDLNs of STZ treated tumor bearing mice, regardless of the tumor model studied. Spleens of STZ (but not STZ+insulin) treated tumor bearing mice also showed upregulated frequencies of $CD8^+IGF1R^+IGF1^+$ T cells when compared to spleens harvested from control groups (Figure 4.1I). Similar results were obtained when analyzing $CD8^+IGF1R^+IGF1^+$ T cell population in PBMC in all four tumor models (Figure 4.1J).

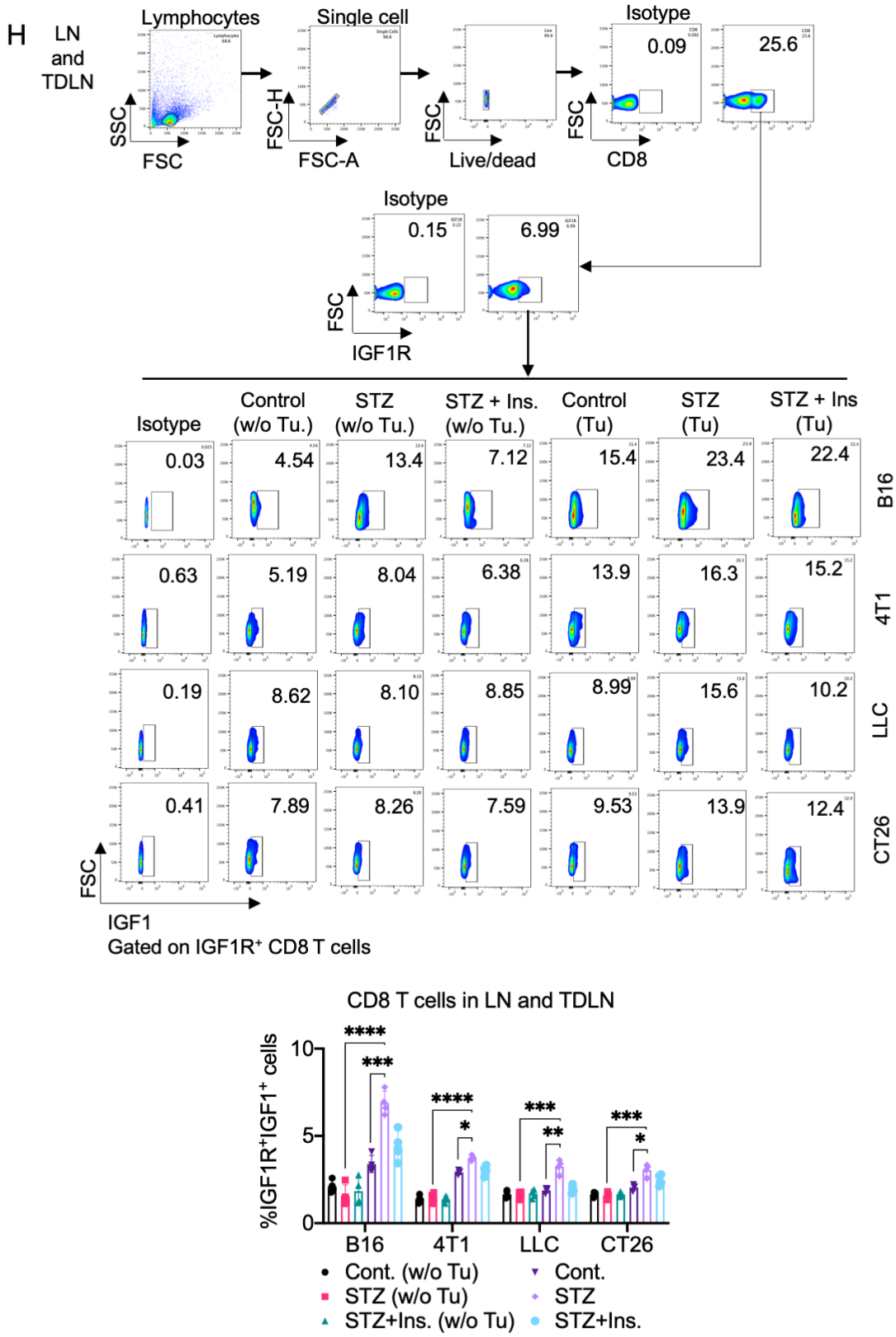

Figure 4.1: H. Flow cytometry gating strategy, representative dot plots and percentage of $CD8^+IGF1R^+IGF1^+$ T cells in lymph nodes (LNs) for hosts without tumor and tumor draining lymph nodes (TDLNs) for hosts with tumors along with isotype controls. Each experimental set with six groups were analyzed, three without tumor, and three with tumor (n=4). Two-way ANOVA was performed to test for significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

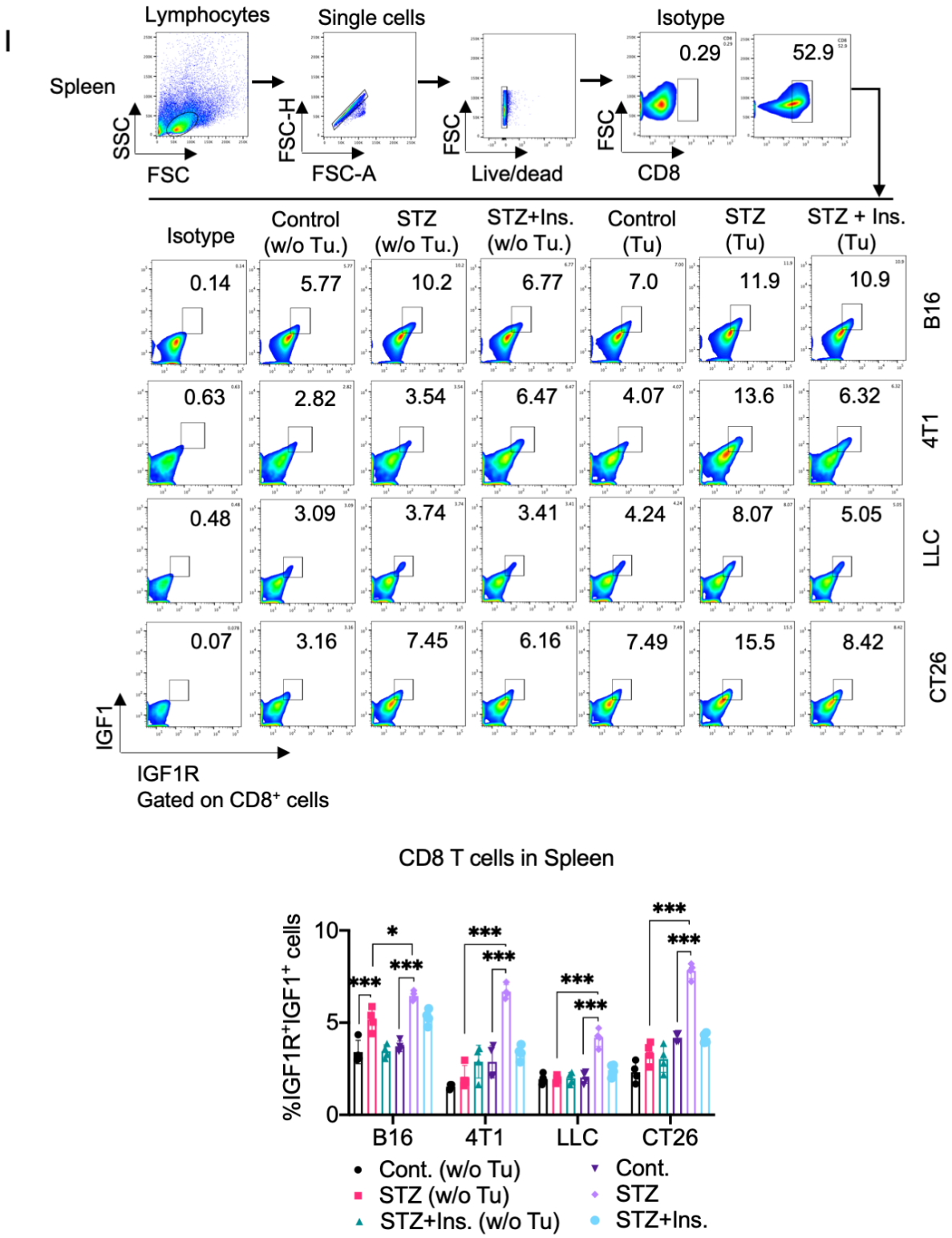

Figure 4.1: I. Flow cytometry gating strategy, representative dot plots and percentage of $CD8^{+}IGF1R^{+}IGF1^{+}$ T cells in spleen along with isotype controls. Each experimental set with six groups were analyzed, three without tumor, and three with tumor (n=4). Two-way ANOVA was performed to test for significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

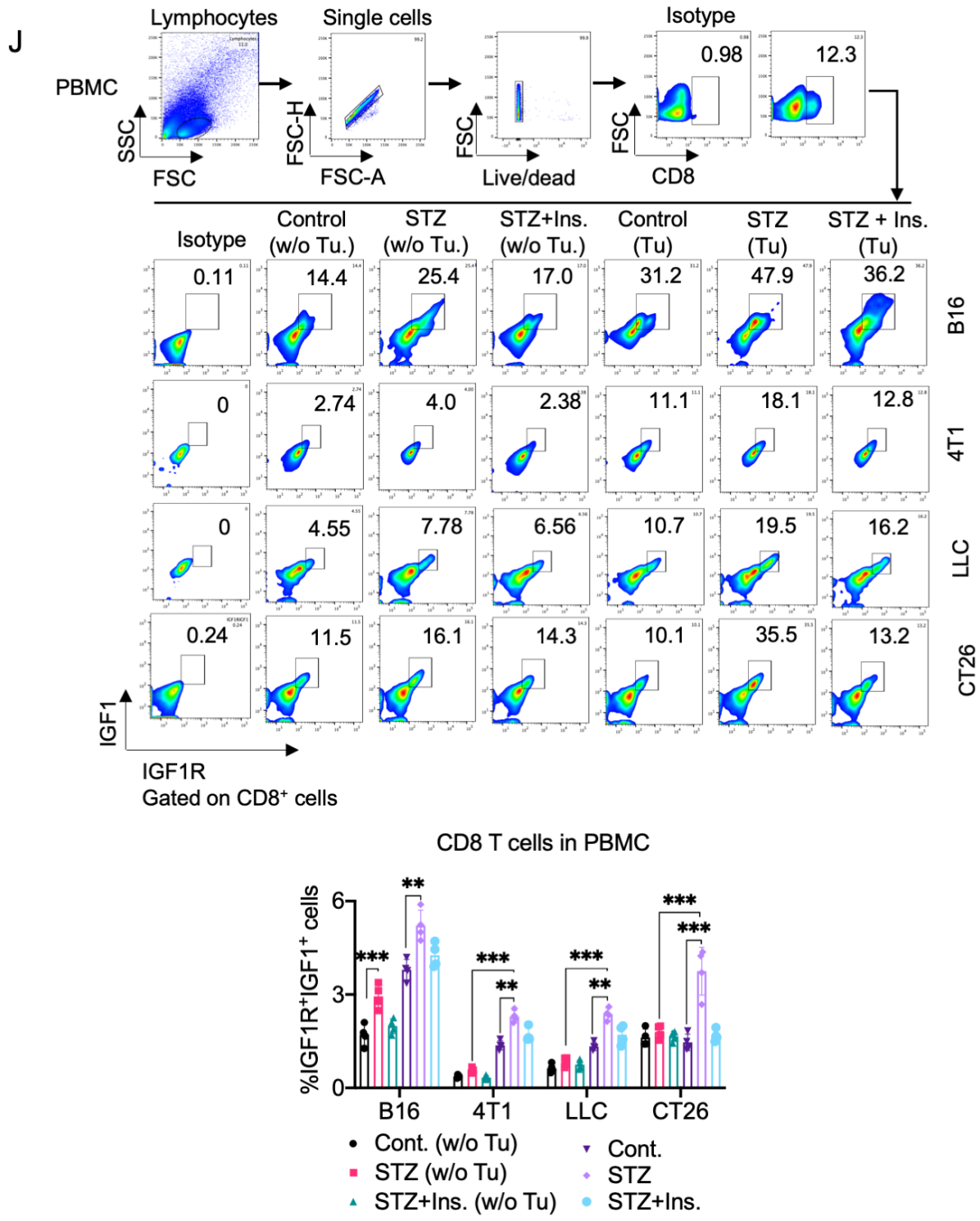

Figure 4.1: J. Flow cytometry gating strategy, representative dot plots and percentage of $CD8^{+}IGF1R^{+}IGF1^{+}$ T cells in peripheral blood mononuclear cells (PBMCs) along with isotype controls. Each experimental set with six groups were analyzed, three without tumor, and three with tumor (n=4). Two-way ANOVA was performed to test for significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

4.2. IGF1-IGF1R-mTOR signaling influences T1D associated metabolic activation of CD8⁺ T cells in the tumor bearing host: As $CD8^+$ T cells in T1D tumor bearing mice exhibit selective coordinate upregulation of intrinsic IGF1 and IGF1R expression, next we studied the associated downstream signaling pathway in these T effector cells.$^{STZ}CD8_{TIL}$ cells isolated from B16F10 melanomas expressed significantly elevated levels of phospho-AKT and phospho-mTORC1 when compared to $^{Cont}CD8_{TIL}$ cells (Figure 4.2A), with these changes selectively occurring in $CD8^+$ TIL but not tumor cells (Figure 4.2B). *In silico* protein-protein interactome analyses with STRING revealed an operational connection between IGF1, IGF1R, AKT and mTOR (Figure 4.2C).

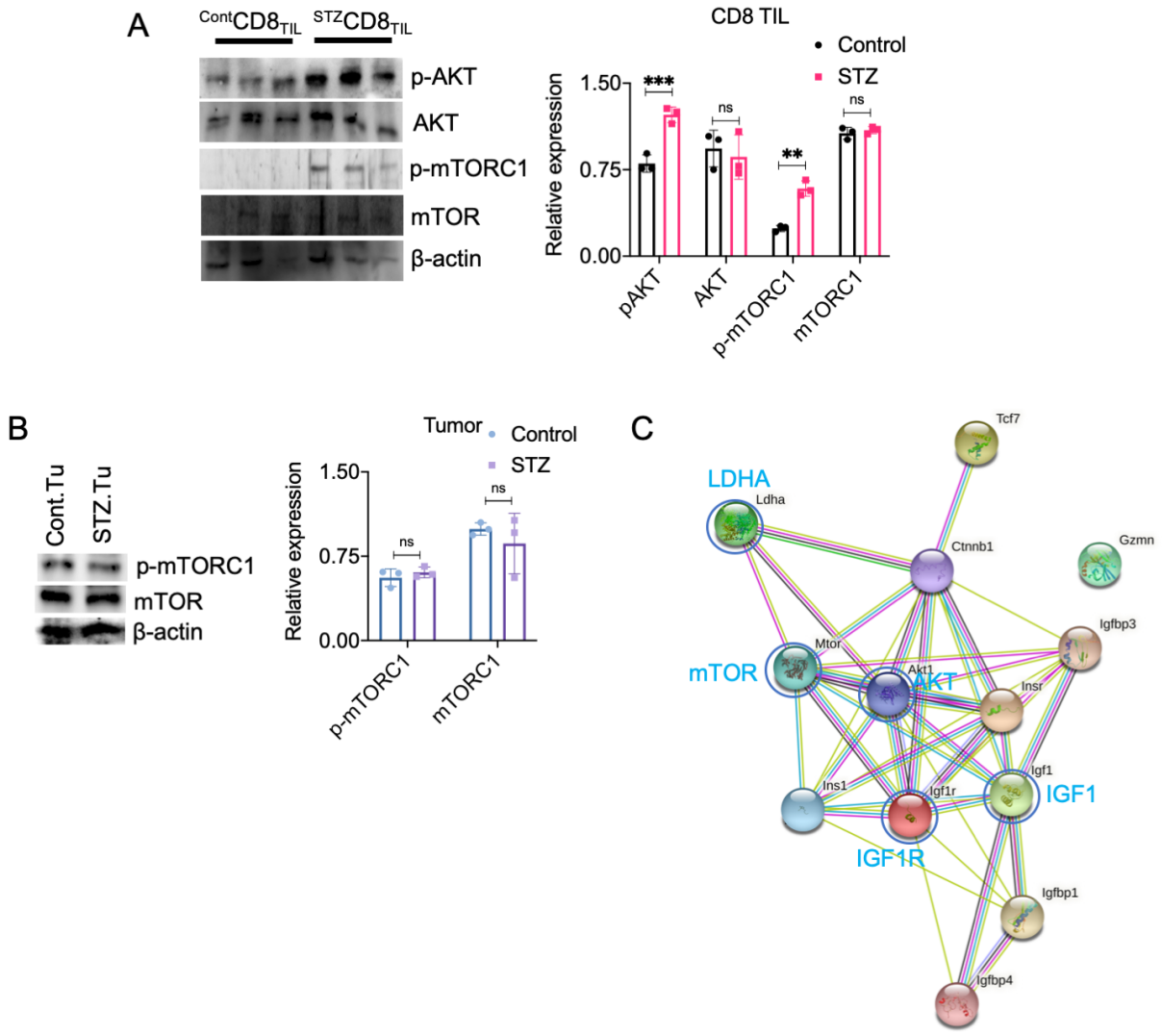

Figure 4.2: A. Western blot analysis of pAKT, AKT, mTOR and phosphor-mTORC1 (p-mTORC1) in MACS sorted $CD8^+$ TILs (n=3). **B.** Western blot analysis of phospho-mTORC1 and mTOR of control and STZ treated B16F10 tumor (n=3). **C.** Search tool for the retrieval of interacting genes/ proteins (STRING) analysis was used to study signaling interactions between IGF1, IGF1R, InsulinR and mTOR. One-way ANOVA was performed to test significance differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

To confirm the involvement of mTORC1 with upregulated IGF1/IGF1R activity in the activation of $CD8^+$ T cells, *in vitro* experiments were performed with MACS-purified $CD8^+$ T cells isolated from the TDLNs of STZ induced T1D mice bearing B16 F10 melanomas vs. control tumor bearing mice (Figure 4.2D). Recombinant IGF1 (rIGF1) was used as an IGF1R activator, with small interfering RNA against IGF1R (siIGF1R) used to block IGF1R expression. Rapamycin was used to block mTORC1-mediated signaling. $^{Cont.}CD8^+$ T cells and $^{STZ}CD8^+$ T cells from TDLNs were treated with either rIGF1 or siIGF1R or rapamycin alone or in combinations. We observed that $^{STZ}CD8^+$ T cells exhibited significant increases in expression of *igf1R, glut1, gzmB and ifn*γ after treatment with rIGF1 (Figure 4.2E). However, the expression of these genes was significantly downregulated when $^{STZ}CD8^+$ T cells were treated with either siIGF1R or rapamycin, or a combination of both agents. Flow cytometry analysis confirmed that rIGF1 treatment of $^{STZ}CD8^+$ T cells, but not $^{Cont.}CD8^+$ T cells, co-ordinately resulted in significantly upregulated expression of CD69 and IFNγ (Figure 4.2F and 4.2G). Treatment with either siIGF1R or rapamycin or both agents led to a significant diminishment in CD69 and IFNγ expression by $^{STZ}CD8^+$ T cells but not by $^{Cont.}CD8^+$ T cells. These results suggest that $^{STZ}CD8^+$ T cells from TDLN are more susceptible to targeted antagonism of IGF1R and mTOR than their non-diabetic counterparts.

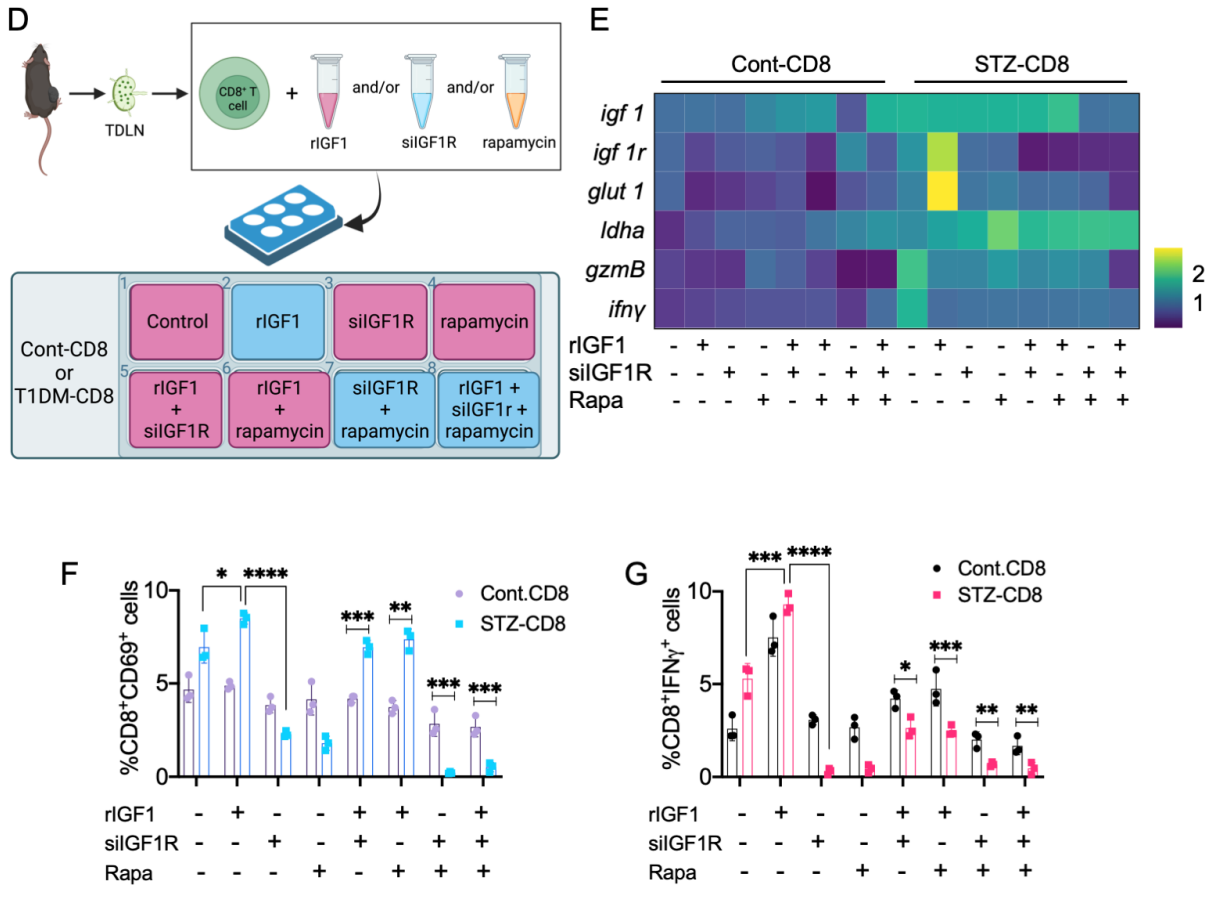

Figure 4.2: D. Schematic overview of *in vitro* experiments (n=3) to study cellular signaling in MACS sorted $CD8^+$ T cells isolated from TDLN. rIGF1 was used to stimulate the IGF1-IGF1R-mTOR signaling axis, siIGF1R and rapamycin were used to block IGF1R expression and mTORC1, respectively. **E.** Expression of *igf1, igf1R, glut1, ldha, gzmB* and *ifnγ* following *in vitro* treatment of rIGF1, siIGF1R and rapamycin, either alone or in combination. **F.** Percent $CD8^+CD69^+$ cells in $^{Cont.}CD8^+$ T cells vs. $^{STZ}CD8^+$ T cells (n=3). **G.** Percent $CD8^+IFN\gamma^+$ cells amongst $^{Cont.}CD8^+$ vs. $^{STZ}CD8$ groups (n=3).Two-way ANOVA followed by Tukey's multiple comparison test was performed to test significance of intergroup differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

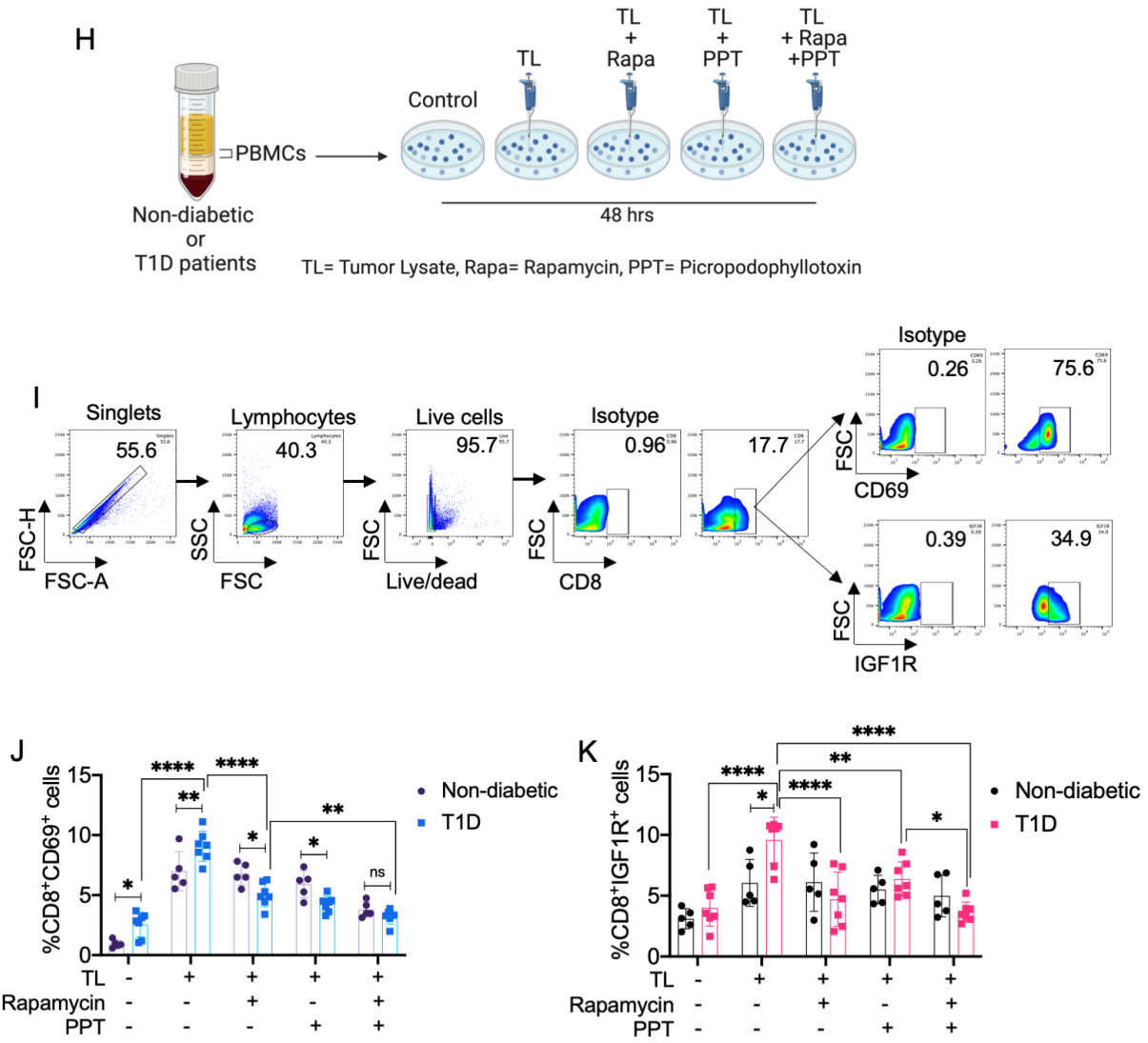

Figure 4.2: H. Schematic overview of experimental design using T1D patient blood. **I.** Flow cytometry gating on $CD8^+$, $CD69^+$ and $IGF1R^+$ cells in T1D patient PBMC. **J.** Percent $CD8^+CD69^+$ and **K.** percent $CD8^+CD69^+$ cell population in T1D patient and non-diabetic PBMC samples after in vitro treatment with tumor lysate (TL), rapamycin and picropodophyllotoxin (PPT) alone or in combination. Two-way ANOVA followed by Tukey's multiple comparison test was performed to test significance of intergroup differences. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

To confirm this finding in the clinical setting, T1D patient PBMCs, along with age and sex matched non-diabetic PBMCs, were stimulated *in vitro* with a lysate derived from the human breast cancer cell line MCF 7 in the absence or presence of rapamycin (mTOR inhibitor) or picropodophyllotoxin (PPT; an IGF1R inhibitor) or both (Figure 4.2H). Flow cytometry was performed to assess $CD8^+CD69^+$ cells and $CD8^+IGF1R^+$ status amongst "responder" T cells (Figure 4.2I). Although the $CD8^+CD69^+$ T cell population was determined to be elevated in the stimulated PBMCs from both T1D and non-T1D responders following stimulation with tumor antigens, such upregulation was most prominent in T1D PBMCs (Figure 4.2J). Interestingly, rapamycin or PPT significantly downregulated the frequency of $CD8^+CD69^+$ cells in T1D-PBMCs compared to non-T1D PBMCs, with $CD8^+IGF1R^+$ cells found to be elevated in cultures developed from both T1D and non-T1D (with T1D > non-TID) PBMCs following antigenic stimulation (Figure 4.2K). Co-treatment with rapamycin or PPT or both resulted in a significant reduction in the $CD8^+IGF1R^+$ T cell population from T1D donors vs. treatment with lysate only. These findings support the involvement of an IGF1-IGF1R-mTORC1 axis in the superior activation of (anti-tumor) $CD8^+$ T cells in T1D hosts that underlies enhanced tumor growth control *in vivo*.

Discussion

Our study revealed that $CD8^+$ TILs cells of T1D hosts but not tumor cells have higher expression of IGF1 and IGF1R. Flow cytometry analyses revealed a unique population of $CD8^+IGF1^+IGF1R^+$ T cells in T1D TILs, frequency of which is negatively correlated to tumor size. That means, $CD8^+IGF1^+IGF1R^+$ T cells might actively be involved in T1D associated tumor growth control. Further, higher proportion of $CD8^+IGF1^+IGF1R^+$ T cell population was found in TDLNs of T1D hosts, but not of control hosts. Insulin treatment in T1D tumor bearing hosts reduced the $CD8^+IGF1^+IGF1R^+$ T cell population in TILs and TDLNs. IGF1 and IGF1R shares significant structural homology with insulin and insulin receptor (IR) respectively (Cheng *et al*., 2005; Rincon *et al*., 2004). Moreover, insulin and IGF1 are involved in many overlapping signaling pathways. Liver is the major source of IGF1 in our body (Sjögren *et al*., 1999). Upon insulin stimulation hepatocytes secrete IGF1 in circulation (Dichtel *et al*., 2022). In the absence of insulin signaling, liver cells couldn't synthesize IGF1 thus, systemic IGF1 gone down (Raman *et al*., 2019). Reduced systemic IGF1 and insulin may trigger IGF1 and IGF1R expression on $CD8^+$ T cells in T1D tumor bearing hosts upon cognate antigen stimulation. Acute fasting has been reported to reduce systemic IGF1 levels, to boost systemic $CD8^+$ Tcell metabolism, and to enhance CTL-mediated cytotoxicity. During fasting, blood glucose levels are normalized there is little to no insulin in circulation. These conditions may result in decreased systemic IGF1 levels, which in turn could force $CD8^+$ T cells to activate autocrine IGF1 production and *cis/trans* activation of IGF1R-mediated signaling. *In vitro* studies have previously suggested that activated $CD8^+$ T cells produced significant quantities of IGF1 (Stentz and Kitabchi, 2004a; b). Notably, high IGF1R expression is required for

leukemia-initiating cell activity in T-cell acute lymphoblastic leukemia (T-ALL) (Medyouf *et al*., 2011).

In B16F10 melanoma models, we observed a significant upregulation of pAKT and p-mTORC1 in $^{STZ}CD8_{TIL}$ cells vs. $^{Cont.}CD8_{TIL}$ cells. Protein-protein interaction network studies further revealed an interconnection between IGF1, IGF1R, AKT and mTOR. We hypothesised that metabolic upregulation and enhanced effector function in $CD8^+$ T cells from STZ induced T1D mice might be mediated through the IGF1R-mTOR signaling axis. To test our hypothesis, we isolated $CD8^+$ T cells from TDLNs of STZ induced T1D vs. control B16F10 melanoma bearing mice. Isolated ^{Cont}. CD8 T cells and ^{STZ}CD8 T cells were treated with either recombinant IGF1 (rIGF1) or with silencing RNA against IGF1R (siIGF1R) or with rapamycin (mTOR inhibitor) alone or in combination. rIGF1 significantly enhanced activation markers on ^{STZ}CD8 T cells but not ^{Cont}CD8 T cells. ^{STZ}CD8 T cells were determined to be more sensitive to treatment with siIGF1R or rapamycin or the combination of these agents. Our results clearly indicate that the IGF1R-mTOR signaling axis underlies the enhanced effector function of $CD8^+$ T cells in STZ induced T1D tumor bearing mice. Furthermore, we validated this core paradigm in clinical samples isolated from T1D patients. In these in vitro analyses. we stimulated PBMCs from T1D vs. non-diabetic patients with a source of tumor antigen (i.e., tumor lysate). We observed significant upregulation in responder populations of $CD8^+CD69^+$ T cells and $CD8^+IGF1R^+$ T cells in cultures developed from T1D PBMCs vs. non-diabetic PBMCs. Targeted antagonism of IGF1R or mTOR or both resulted in a significant reduction in these two $CD8^+$ T cells subpopulations in T1D PBMC cultures. In vitro stimulation with tumor antigens stimulated greater $CD8^+$ T cell activation from T1D vs. non-diabetic patients. $CD8^+$ T cells from T1D patients were more sensitive to the inhibitory effects of IGF1R blockers or mTOR antagonists. This observation strengthens our hypothesis that the IGF1R-mTOR signaling axis in $CD8^+$ T cells in T1D hosts is critical to tumor growth control.

Sustained $CD8^+$ T cell fate/function are essential for the success of cancer immunotherapy, withthe success of anti-PD1 therapy link to the ability of this agent to prevent the exhaustion and premature death of ant-tumor $CD8^+$ T cells (Chakravarti *et al*., 2023). Very recently, *cis* B7:CD28 costimulatory interactions in $CD8^+$ T cells have been demonstrated to promote anti-tumor immunity, implying that $CD8^+$ T cells could boost themselves in an autocrine manner (Zhao *et al*., 2023). In a similar manner, intrinsic IGF1R-mTOR signaling in $CD8^+$ T cells may be important to the superior anti-tumor action of these effector cells in T1D patients. Interestingly, a fraction of cancer patients undergoing immune checkpoint blockade therapy (ICBT) develop immune-related adverse effects (irAEs), among which T1D is very common (Mourad *et al*., 2021). It has not yet been documented whether cancer patients who develop T1D during ICBT have a better clinical prognosis. We would hypothesize that development of T1D may enable IGF1/IGF1R/mTOR-dependent anti-tumor immunity post-ICBT therapy. The IGF1R-mTORsignaling axis could also be exploited within the context of CAR-T cell-based cancer immunotherapy,

where T cell anergy and exhaustion remain major challenges to the therapeutic efficacy of this approach in the solid cancer setting (Sterner and Sterner, 2021). Future work will define optimal targeting agents to agonize IGF1R-mTOR signaling in anti-tumor CD8$^+$ T cells for sustained therapeutic benefit in cancer patients.

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CHAPTER 5

THE IMMUNOMODULATORY POTENTIAL OF NEEM LEAF GLYCOPROTEIN (NLGP) ON TUMOR GROWTH AND CD8$^+$ T CELL METABOLISM IN TUMOR BEARING HOSTS WITH PRE-EXISTING T1D

- **ABSTRACT**
- **INTRODUCTION**
- **OBJECTIVE**
- **RESULTS**
    1. NLGP augments superior tumor control in T1D hosts with survival benefit
    2. NLGP therapy facilitates increased infiltration of activated superior cytotoxic CD8$^+$ T cells in TME
    3. NLGP modulates immunosuppressive TME in diabetic hosts
    4. NLGP reduces diabetes associated hepatic inflammation
    5. NLGP therapy accelerates glucose metabolism in CD8$^+$ T cells of non-diabetic TME
- **DISCUSSION**
- **REFERENCES**

Abstract

Neem leaf glycoprotein (NLGP) restricts immune dependent murine melanoma, carcinoma and sarcoma tumor growth control. However, therapeutic efficacy of NLGP in tumor hosts with pre-existing type 1 diabetes has not been studied yet. We found NLGP modulates the tumor microenvironment of type 1 diabetic hosts in favor of antitumor immunity. Further study showed NLGP reduces T1D associated hepatic inflammation irrespective of tumor burden. NLGP accelerates intra-tumor $CD8^+$ T cell oxidative phosphorylation in diabetic hosts, thereby improves glucose metabolism. Further, NLGP dampens glucose uptake by tumor cells in diabetic tumor microenvironment by downregulating glucose transporter 1 (*glut 1*) expression. Overall, NLGP positively influences immune microenvironment and metabolism in tumor bearing hosts with pre-existing type 1 diabetes.

Introduction

Neem leaf glycoprotein (NLGP) a neem leaf derived immunomodulator have been studied extensively for more than a decade. NLGP can restrict the murine tumor growth in prophylactic (Baral and Chattopadhyay, 2004) and therapeutic (Mallick *et al*., 2014) settings through its robust immunomodulatory properties in suppressed immune system in tumor host (Chakraborty *et al*., 2012; Bose and Baral, 2007b; Barik *et al*., 2013). NLGP interacts with an array of immune cells, including dendritic cells (DCs) (Goswami *et al*., 2010b) and macrophages (Sarkar *et al*., 2008a). NLGP matures immature DCs from healthy individuals (Goswami *et al*., 2010c) and cancer patients (Roy *et al*., 2011a). NLGP also converts M2 type tumor associated macrophages to M1 type (Goswami *et al*., 2014). It induces IL-12 from tumor associated DCs and macrophages and reduces IL-10 (Goswami *et al*., 2014; Roy *et al*., 2011b). NLGP optimizes antigen presenting cell (APC)-T cell interaction, thereby promotes antitumor T cell immunity (Bhuniya *et al*., 2020). Very recently, our lab has discovered the surface receptor of NLGP on APCs, which is Dectin 1 (Ganguly *et al*., 2024). However, therapeutic efficacy of NLGP in tumor bearing hosts with pre-existing diseases have not been studied yet. In this study we assessed the immunomodulatory potential of NLGP in tumor hosts with pre-existing type 1 diabetes (T1D).

Objective

To study the immunomodulatory potential of neem leaf glycoprotein (NLGP) on tumor growth and $CD8^+$ T cell metabolism in tumor bearing hosts with pre-existing T1D.

Results

5.1. NLGP augments superior tumor control in T1D hosts with survival benefit: NLGP has been established as a potent immunomodulator for generation of effective antitumor immunity in murine tumor models, as a result of research for more than a decade (Guha *et al*., 2020; Saha *et al*., 2020; Ghosh *et al*., 2019; Bhuniya *et al*., 2020; Ghosh *et al*., 2016; Chakraborty *et al*., 2011; Goswami *et al*., 2010a; Baral *et al*., 2005; Bose and Baral,

2007a; Sarkar *et al*., 2008b). However, effectiveness of NLGP was not studied earlier in T1D tumor bearing hosts. A total of four groups of mice were taken for the study, two non-diabetic (Control, NLGP) and two type 1 diabetic (STZ, STZ + NLGP) groups. T1D groups were treated with low dose STZ following the protocol described in Chapter 1. B16F10 tumor cells were inoculated s.c. to all four groups. One group from non-diabetic (NLGP) and one group from diabetic (STZ + NLGP) tumor bearing mice received NLGP therapy as described in Figure 5.1A.NLGP therapy effectively restricts tumor growth in non-diabetic mice as was already established from earlier studies in our lab (Figure 5.1B) (Barik *et al*., 2015). STZ treated mice group showed superior tumor control than non-diabetic mice with or without NLGP treatment. NLGP therapy significantly controlled tumor growth in T1D tumor bearing mice. Moreover, NLGP treated STZ group showed better survival compared to only STZ group (Figure 5.1C). NLGP treatment in non-diabetic group resulted into better survival further established previous observations from our lab (Bose and Baral, 2018). STZ group showed superior survival compared to non-diabetic control group as was observed in earlier chapter.

STZ treatment significantly increased blood glucose compared to non-diabetic control, and elevated blood sugar was maintained throughout the experiment (Figure 5.1D). NLGP therapy was found to have no effect on blood sugar of tumor bearing mice. Mice of both non-diabetic and diabetic tumor bearing mice showed unaltered blood sugar following NLGP therapy. STZ treated tumor bearing mice showed significant reduction in the concentration of circulating insulin (Figure 5.1E) and IGF1 (Figure 5.1F). However, NLGP administration had no effect on circulating insulin and IGF1 either in non-diabetic or T1D tumor bearing mice. Overall study elucidated the potential of NLGP in controlling tumor growth in T1D hosts independent of controlling blood sugar.

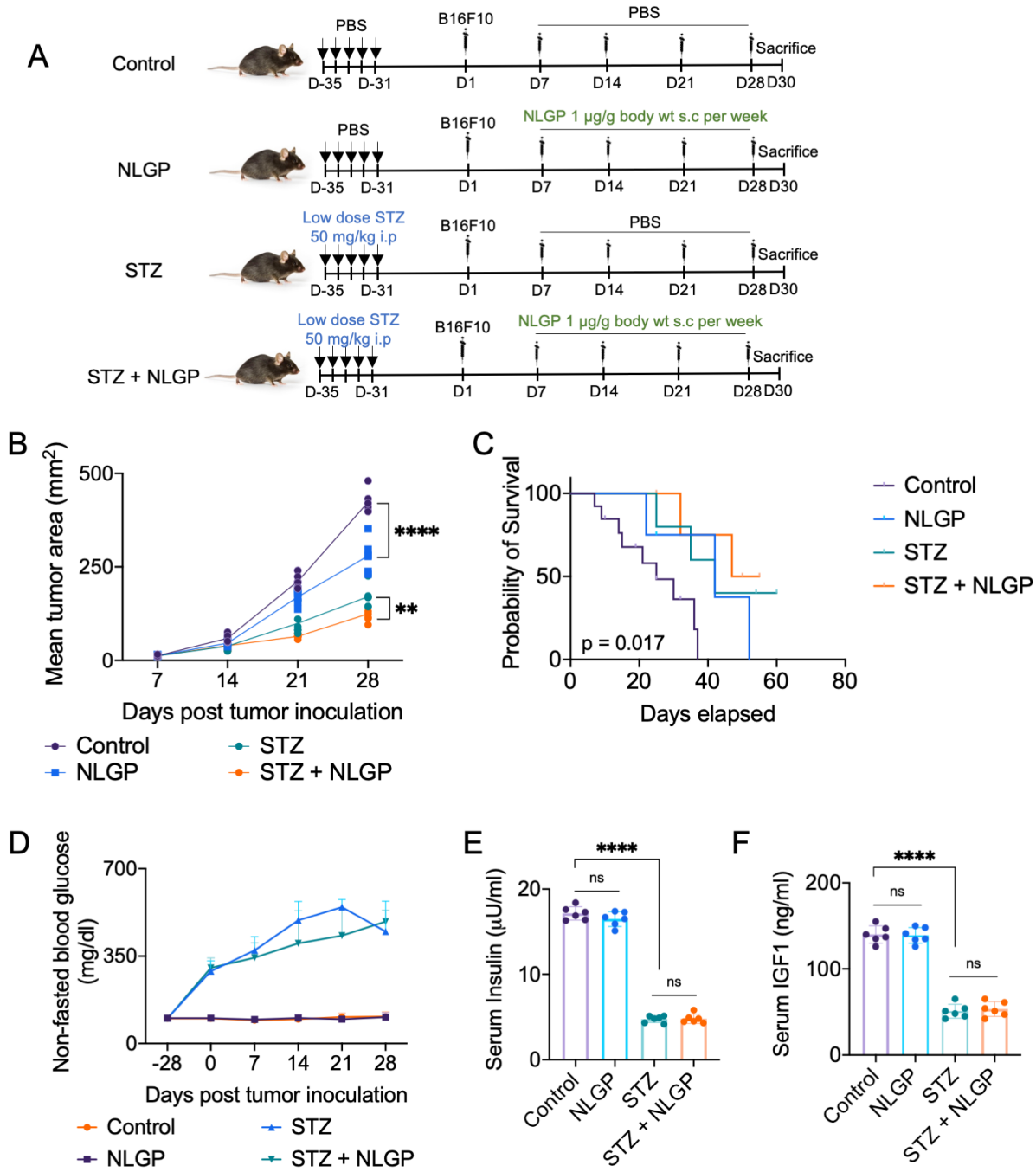

Figure 5.1: NLGP augments superior tumor control in T1D hosts with survival benefit. **A.** Graphical representation of experimental design with four groups. STZ was administered to two groups to induce type 1 diabetes. Other two groups received PBS. B16F10 tumor cells were inoculated in all four groups. One group from each diabetic and non-diabetic tumor bearing mice received four doses of NLGP therapy at 7 days interval. **B.** Mean tumor area of four groups on day 7, 14, 21 and 28 after tumor inoculation. **C.** Probability of survival curve of four tumor bearing groups followed by Kaplan-Meier survival analysis. **D.** Non-fasted blood glucose of control, NLGP, STZ and STZ + NLGP groups of mice throughout the experiment. **E.** Serum insulin (µU/ml) and **F.** serum IGF1 concentration (ng/ml) of four tumor bearing mice groups. Two-way ANOVA followed by Tukey's multiple comparison tests were performed to find out significance across groups. (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ****P $\leq$ 0.0001)

5.2. NLGP therapy facilitates increased infiltration of activated superior cytotoxic CD8$^+$ T cells in TME: NLGP treatment resulted into increased infiltration of CD8$^+$ T cells in tumor microenvironment (TME) and tumor draining lymph nodes (TDLNs) of non-diabetic but not diabetic tumor bearing groups compared to respective controls (Figure 5.2A). Chemokine marker CXCR3 was found to be elevated on tumor infiltrating CD8$^+$ T cells of NLGP treated non-diabetic but not diabetic group compared to respective controls, however, CD8$^+$CXCR3$^+$ T cell infiltration was significantly increased in both non-diabetic and diabetic TME compared to their respective control following NLGP therapy (Figure 5.2B). NLGP therapy facilitated increased infiltration of CD8$^+$CD69$^+$ T cells both in diabetic and non-diabetic TME and TDLN compared to their respective controls (Figure 5.2C). Further, infiltrated CD8$^+$ T cells from tumors of all four group of mice were sorted by magnetic assisted cell sorting (MACS) and their cytotoxicity analyzed. CD8$^+$ T cells isolated from TME of NLGP treated non-diabetic as well as diabetic groups showed enhanced expression of *granzyme* and *perforin* compared to their respective controls (Figure 5.2D). Study in earlier chapter showed CD8$^+$ T cells isolated from diabetic TME have superior cytotoxicity compared to their non-diabetic counterpart as evidenced by increased release of lactate dehydrogenase (LDH). In this study, NLGP therapy was found to be further enhanced the cytotoxicity of CD8$^+$ T cells of diabetic tumor draining lymph nodes (TDLNs), compared to their counterpart without NLGP treatment (Figure 5.2E). Cumulative results indicated NLGP therapy facilitated increased infiltration of activated cytotoxic CD8$^+$ T cells in diabetic TME.

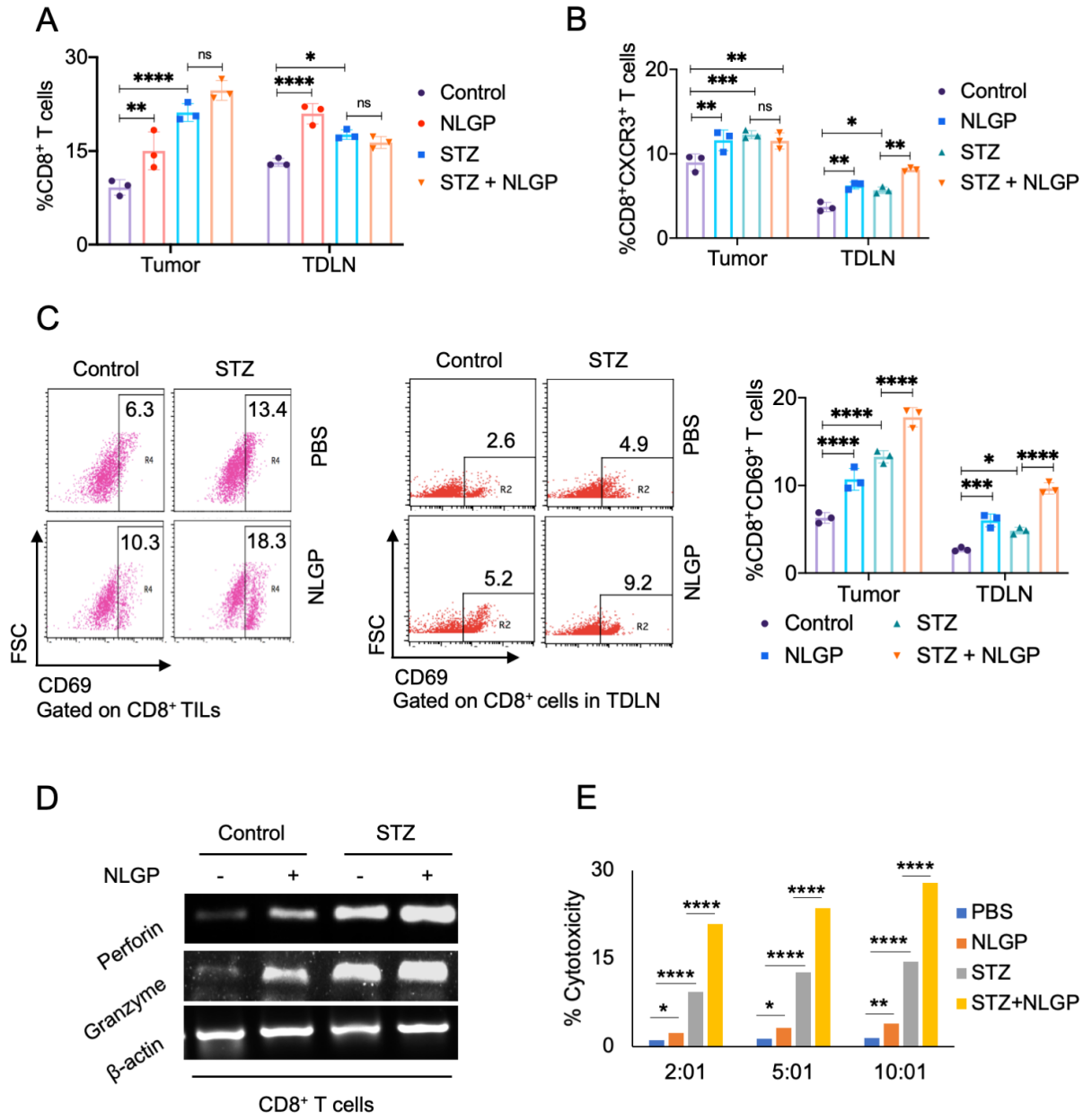

Figure 5.2: NLGP therapy facilitates increased infiltration of activated superior cytotoxic CD8⁺ T cells in TME. A. CD8⁺ T cell, **B.** CD8⁺CXCR3⁺ T cell and **C.** CD8⁺CD69⁺ T cell population in tumor and tumor draining lymph nodes (TDLNs) of control, NLGP, STZ and STZ +NLGP tumor bearing mice groups. **D.** RT-PCR bands of perforin and granzyme of sorted CD8+ T cells from TME of four mice groups. **E.** %cytotoxicity of isolated CD8⁺ T cells from TDLNs four groups. Two-way ANOVA followed by Tukey's multiple comparison testswere performed to find out significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

5.3. NLGP modulates immunosuppressive TME in diabetic hosts: Earlier studies have shown NLGP therapy to be effective in modulating immunosuppressive cell populations in TME, thereby facilitates immune dependent tumor growth restriction. In STZ treated tumor bearing hosts NLGP therapy showed immunomodulation by reducing the immunosuppressive cell populations from TME. $CD4^+FoxP3^+$Tregs (Figure 5.3A), $CD11b^+F4/80^+$ tumor associated macrophages (TAMs) (Figure 5.3B) and $CD11b^+Gr1^+$ myeloid derived suppressive cells (MDSCs) (Figure 5.3C) were found to be significantly reduced from diabetic TME with NLGP therapy compared to diabetic TME without NLGP therapy. On the other hand, $CD3^+DX5^+$ natural killer T cells (NK-T) (Figure 5.3D) and $CD11c^+MHCI^+$ dendritic cells (DCs) (Figure 5.3E) were found to be upregulated in diabetic as well as non-diabetic TME following NLGP treatment. Overall results suggested that NLGP therapy could effectively modulate diabetic TME from immunosuppressive to more immunogenic microenvironment.

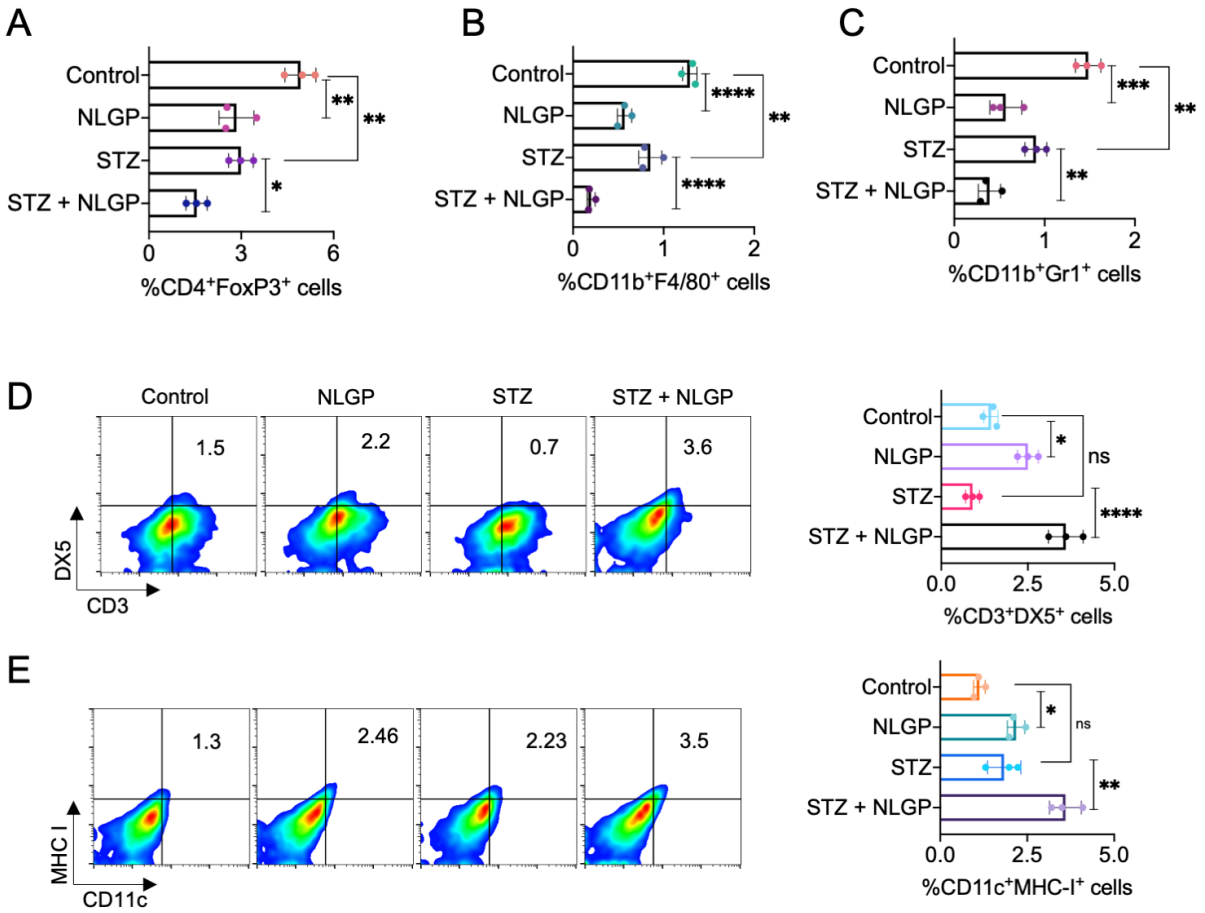

Figure 5.3: NLGP modulates immunosuppressive TME in diabetic hosts. **A.** $CD4^+FoxP3^+$Tregs, **B.** $CD11b^+F4/80^+$ tumor associated macrophages (TAMs) and **C.** $CD11b^+Gr1^+$ myeloid derived suppressive cells (MDSCs) in TME of four groups. **D.** $CD3^+DX5^+$ natural killer T cells (NK-T) and **E.** $CD11c^+MHCI^+$ dendritic cells (DCs) in TME of control, NLGP, STZ and STZ + NLGP groups of mice. Two-way ANOVA followed by Tukey's multiple comparison testswere performed to find out significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

5.4. NLGP reduces diabetes associated hepatic inflammation: T1D has been found to be associated with hepatic inflammation. Thus, we studied the immune cell populations in hepatic microenvironment in diabetic hosts with or without tumor. In our STZ treated T1D model, we observed increased infiltration of activated $CD8^+$ T cells in liver irrespective of tumor burden (Figure 5.4A). NLGP therapy was shown to reduce activated $CD8^+$ T cell population from hepatic microenvironment from both diabetic groups with or without tumor burden. Furthermore, NLGP therapy reduced NK-T cells and DCs population from hepatic microenvironment of tumor bearing diabetic and non-diabetic groups, as well as diabetic group without tumor (Figure 5.4B and 5.4C). Treg population in hepatic microenvironment was significantly downregulated in tumor bearing non-diabetic as well as diabetic groups compared to their respective controls (Figure 5.4D). However, hepatic microenvironment of diabetic mice without tumor had lower percentage of Tregs compared to control group and NLGP therapy had no effect on the Tregpopulations in hepatic microenvironment in diabetic mice group without tumor. TAM and MDSC population were significantly reduced in liver of non-diabetic mice with tumor and diabetic mice without tumor (Figure 5.4E and 5.4F). However, no such change was observed in livers of diabetic hosts with tumor. Overall observations suggest NLGP could effectively reduce inflammatory immune cell populations. In hepatic microenvironment from hosts independent to tumor burden.

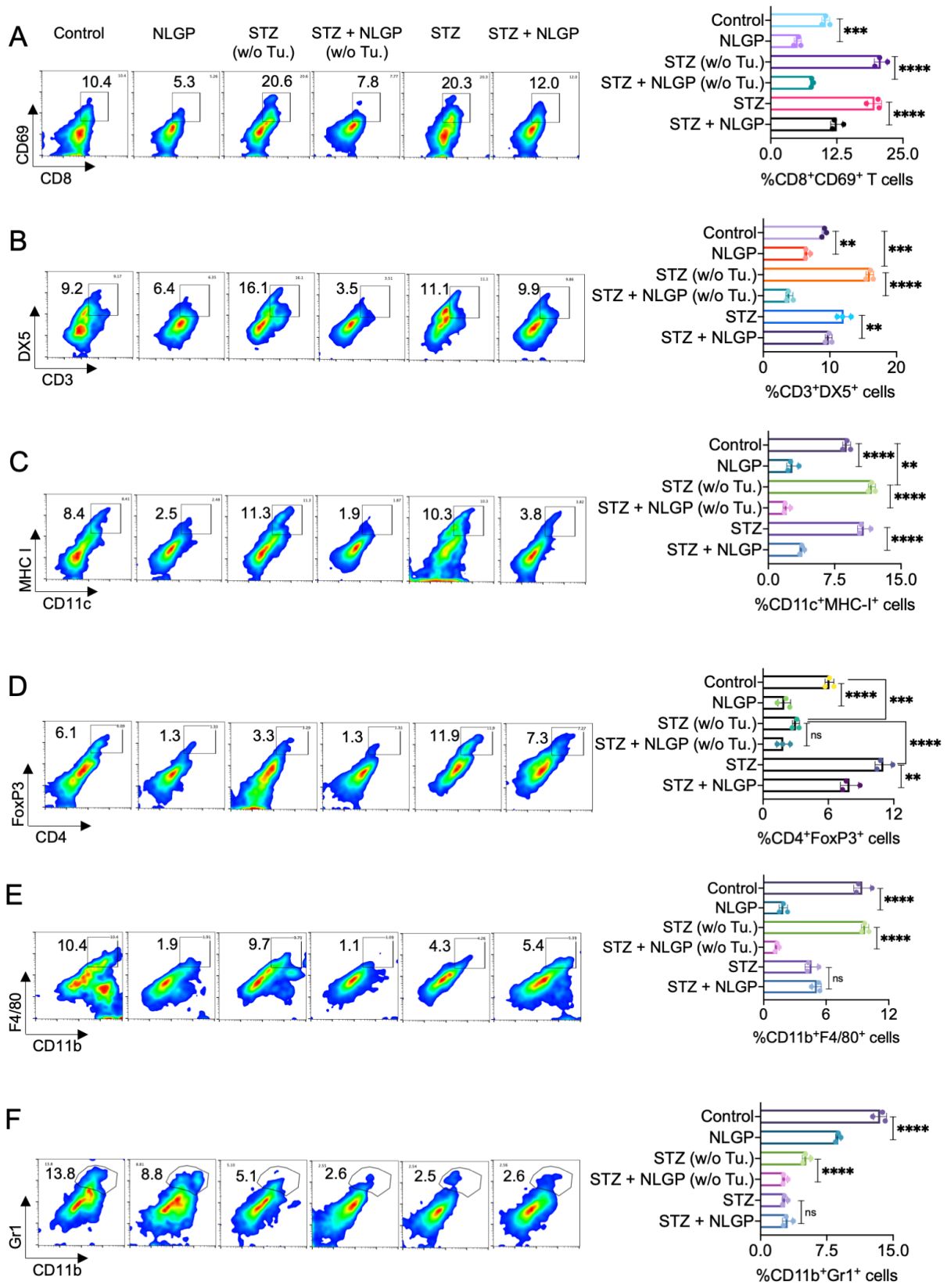

Figure 5.4: NLGP reduces diabetes associated hepatic inflammation. A. $CD8^+CD69^+$ T effector cells (T_{eff}), **B.** $CD3^+DX5^+$ natural killer T cells (NK-T), **C.** $CD11c^+MHCI^+$ dendritic cells (DCs), **D.** $CD4^+FoxP3^+$Tregs, **E.** $CD11b^+F4/80^+$ tumor associated macrophages (TAMs) and **F.** $CD11b^+Gr1^+$ myeloid derived suppressive cells (MDSCs) population in hepatic microenvironment of six mice

groups, viz., control, NLGP, STZ (without tumor), STZ + NLGP (without tumor), STZ and STZ + NLGP. Two-way ANOVA followed by Tukey's multiple comparison testswere performed to find out significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

5.5 NLGP therapy accelerates glucose metabolism in CD8$^+$ T cells of non-diabetic TME: Studied in previous chapter showed CD8$^+$ T cells from T1D TME have accelerated glucose metabolism compared to its non-diabetic counterpart. To study the effect of NLGP therapy on CD8$^+$ T cell metabolism, CD8$^+$ T cells were isolated from tumors of diabetic and non-diabetic hosts. Tumor cells were also taken out and single cells were made. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were studied to determine cellular glycolysis and oxidative phosphorylation (OXPHOS) respectively. NLGP therapy was found to increase glucose metabolism of CD8$^+$ T cells from non-diabetic TME but not CD8$^+$ T cells from T1D TME (Figure 5.5A and 5.5B). Extracellular acidification rate (ECAR) study revealed NLGP could effectively elevate glycolysis of CD8$^+$ T cells from non-diabetic TME but not CD8$^+$ T cells from T1D TME. Oxygen consumption rate (OCR) analysis revealed NLGP induced OXPHOS of CD8$^+$ T cells from non-diabetic TME both at basal and maximal level, however, CD8$^+$ T cells from diabetic TME showed significantly elevated OXPHOS only at maximal level. NLGP therapy had no effect on either glycolysis or OXPHOS on tumor cells of both diabetic and non-diabetic groups (Figure 5.5C and 5.5D). further, PCR were performed to observe any change in enzymes and transporter molecules involved in glucose metabolism (Figure 5.5E). *glut1, mct1, pkm2, pcx1 and idh2* was found upregulated in CD8$^+$ T cells, isolated from T1D TME as was observed previously (Figure 5.5F). NLGP therapy was found to have no significant effect on those genes related to glucose metabolism. However, tumor cells of NLGP treated group of both diabetic and non-diabetic tumor bearing mice showed significantly reduced *glut1* expression compared to their respective control groups. Overall results suggest NLGP could induce glucose metabolism of CD8$^+$ T cells at OXPHOS level and also may reduce glucose uptake of tumor cells by reducing glucose transports on its surface.

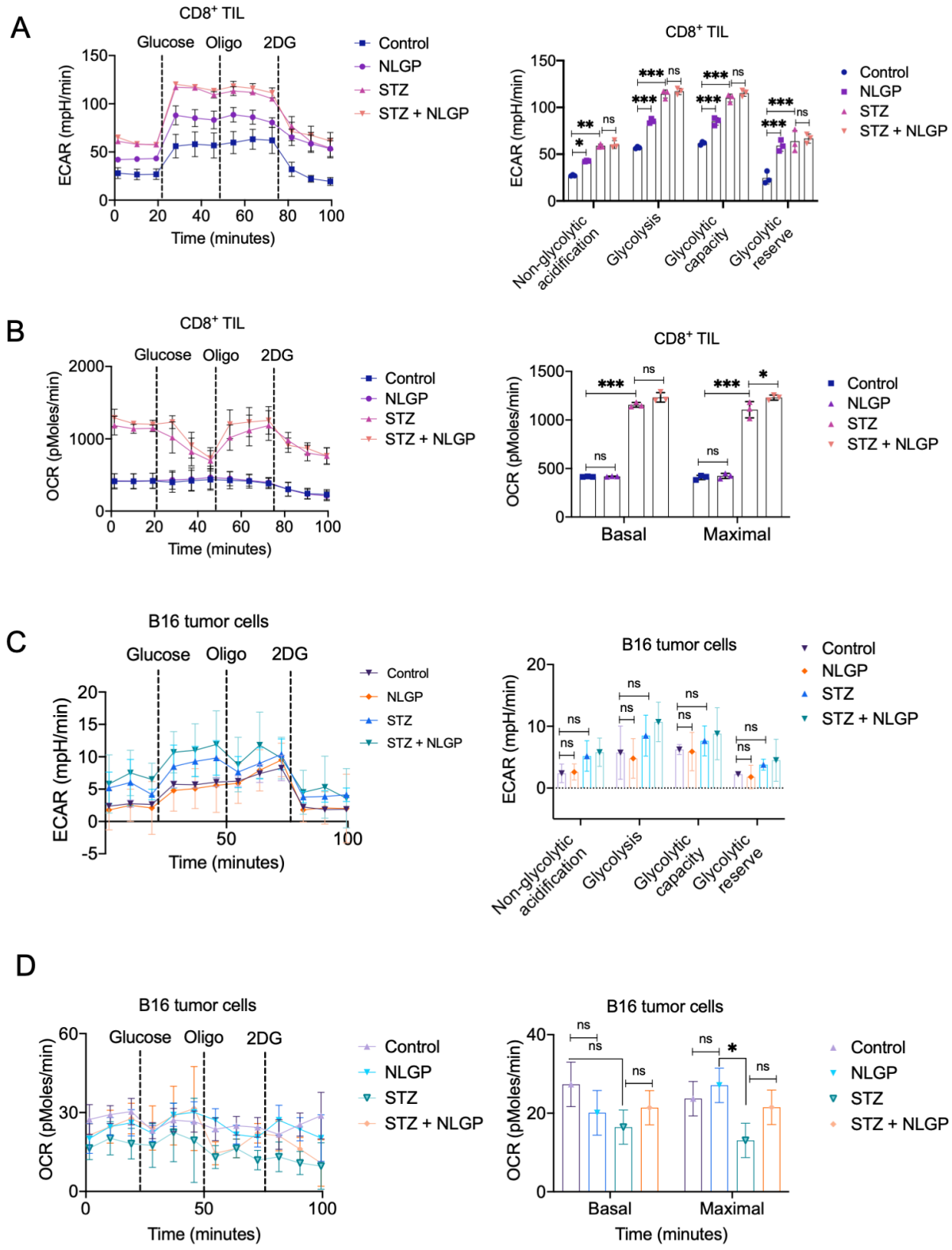

Figure 5.5: NLGP therapy accelerates glucose metabolism in CD8⁺ T cells of non-diabetic TME. **A.** Extracellular acidification rate (ECAR)and **B.** oxygen consumption rate (OCR) of CD8⁺ T cells isolated from TME of four mice groups. Glucose, Oligo and 2DG was added to the reaction plate at specific time intervals to measure non-glycolytic acidification, glycolysis, glycolytic capacity, glycolytic reserve, basal and maximal oxidative phosphorylation (OXPHOS). **C.** ECAR and **D.** OCR of tumor cells, isolated from tumors of four different groups of mice.

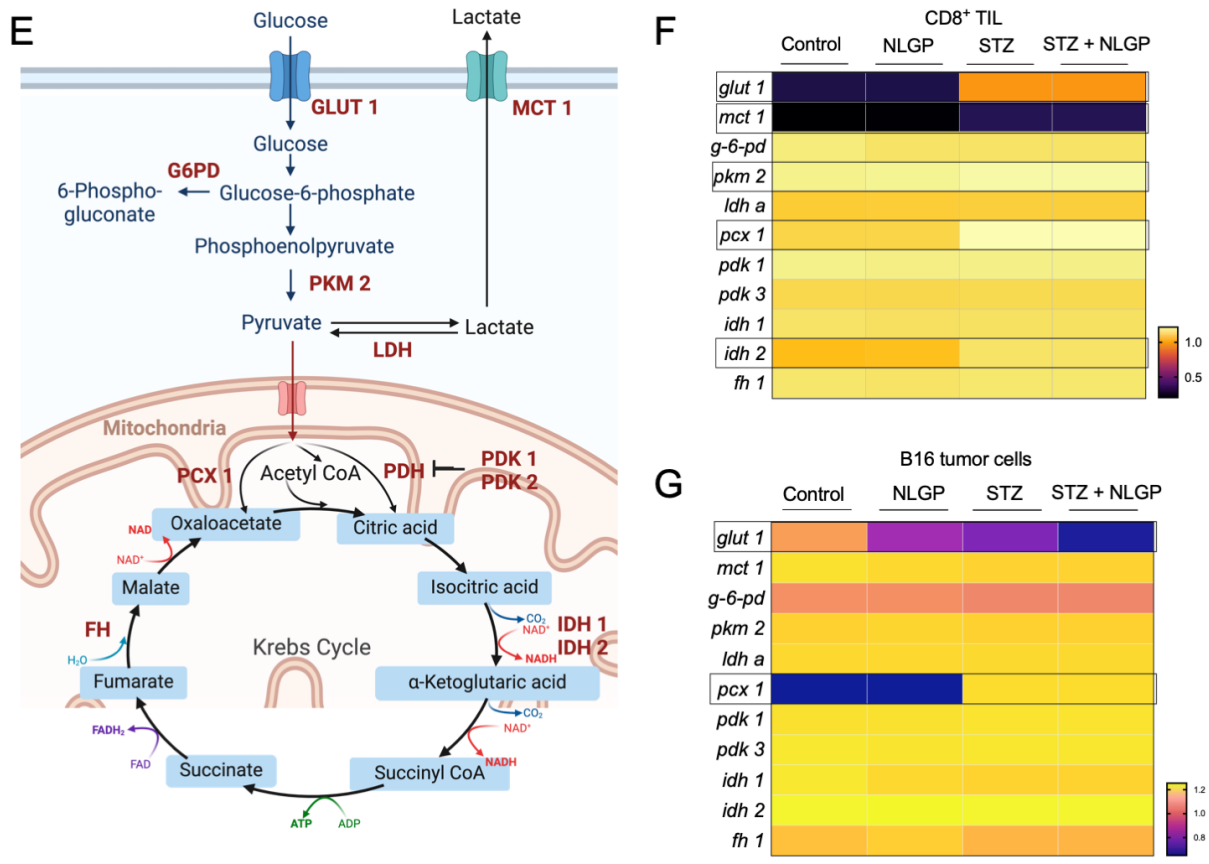

Figure 5.5: E. Therapeutic effect of NLGP on glucose metabolism were analysed by RT-PCR. Expression profiles of genes involved in glucose metabolism in **F.** $CD8^+$ T cells isolated from TME as well as **G.** tumor cells of all four tumor bearing groups. Two-way ANOVA followed by Tukey's multiple comparison testswere performed to find out significance across groups. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

Discussion

Superior antitumor immunity is necessary for efficient tumor growth control. NLGP modulates the immune components of tumor bearing hosts in such a way that favors optimum tumor growth restriction in non-diabetic as well as diabetic models. This ultimately translates into better host survival. Although NLGP has no effect on blood sugar control of diabetic hosts, it exerts its antitumor effect mostly through $CD8^+$T effector cells. NLGP therapy induces activated and cytotoxic $CD8^+$ T cell infiltration within TME of both non-diabetic and diabetic hosts, which facilitates superior tumor growth control. $CD8^+$ T cells of diabetic TME, following NLGP therapy, become metabolically more active, OXPHOS in particular, that give them a competitive edge over the tumor cells within TME. Moreover, NLGP reduces the immunosuppressive cell populations viz. Tregs, TAMs and MDSCs from diabetic TME, which facilitates efficient effector $CD8^+$ T cell function. However, T1D causes significant hepatic inflammation. NLGP therapy reduces hepatic inflammation in diabetic hosts irrespective of tumor burden. Further research with NLGP in

future might enlighten us to know the detailed mechanism by which NLGP could influence hepatic inflammation in T1D hosts. This may have larger implication in treating diabetic hepatopathy, nephropathy, neuropathy, etc.

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GENERAL DISCUSSION

- General discussion
- References

General discussion

The present study reports the novel finding that T1D predisposes the host to improved $CD8^+$ T cell-mediated control of tumor growth in a generalized, pan-cancer manner (across a range of murine melanoma and carcinoma models). Streptozotocin (STZ) selectively destroys pancreatic beta cells and is widely used to develop modelsreflective of T1D (Araujo *et al*., 2022; Furman, 2015). STZenters cells through the glucose transporter 2 (Glut 2), which is expressed at high levels on pancreatic β cells, where it operationally functions as a glucose sensor (Thorens, 2014). STZ induced T1D animals show identical pathological and molecular signatures found in T1D patients (Szkudelski, 2012; Damasceno *et al*., 2014). Intriguingly, a previous *in vivo* study suggested that alloxan-induced T1D in mammary carcinoma bearing rats could lead to the regression of tumorsin a majority of the cases (Heuson and Legros, 1972). In that study, mammary carcinomas were experimentally induced in rats, with animals then treated the with T1D inducing drug alloxan, providing conditions for direct effect of alloxan on cancer cells. We eliminated the possibility for direct anti-tumor effects in our model by discontinuing STZ treatment for 4 weeks prior to tumor inoculation (Graham *et al*., 2011). Before tumor cell inoculation, STZ treated mice displayed significantly elevated blood glucose levels and diminished serum insulin levels when compared to untreated and STZ+insulin treated mice. Upon insulin treatment, blood glucose in STZ+insulin animals was normalized, with insulin levels left unchanged (indicative of the permanent destruction of pancreatic beta cells). Importantly, we noted that reduced levels of serum IGF1 in STZ only treated mice were restored to normal levels in insulin+STZ treated mice. IGF1 in circulation primarily derives from hepatocytes upon insulin stimulation. Analyses of other serum cytokines revealed STZ treatment upregulated IL-6 in circulation and that treatment with insulin normalized levels of this chronic inflammatory biomarker. Levels of serum IL-17, IFN, TNFγ and IL-10 appeared unaltered under STZ +/- insulin treatment conditions.

Significant tumor growth control and extended host survival were observed in STZ induced T1D mice bearing any of 4 unrelated tumors that vary across histologies which was antagonized by treatment with insulin. In an attempt to evaluate the possible involvement of adaptive immune system in T1D hosts, we extended our study in athymic nude mice lacking a functional adaptive immune system. STZ induced T1D nude mice failed to control melanoma growth or extend survival when compared to untreated melanoma bearing nude mice. STZ induced T1D tumors (B16F10, 4T1, LLC, CT26) were characterized by increased infiltration of $CD8^+$ T cells enriched in a polyfunctional proinflammatory phenotype (i.e., expressing elevated levels of CD69, CXCR3, GZMB and IFNγ) and $^{STZ}CD8_{TIL}$ displayed superior anti-tumor cytotoxic activity when compared to $^{Ctrl}CD8_{TIL}$. Enrichment of $CD8^+CD103^+CD39^+$ cells amongst TIL, the $CD8^+CD69^+GZMB^+$ subpopulation amongst TDLNs in STZ induced T1D mice bearing B16F10, 4T1, LLC or CT26 tumors is indicative of improved levels of tumor antigen specific $CD8^+$ T cells in

these diabetic animals. The crucial role of $CD8^+$ T cells in tumor growth restriction in T1D mice was confirmed in adoptive $CD8^+$ T cell transfer models applied to athymic T1D tumor-bearing recipient mice and via the use of *in vivo* $CD8^+$ T cell depletion in STZ induced T1D tumor models. The superior anti-tumor efficacy of $^{STZ}CD8^+$ T cells in our Type 1 diabetic model may be causally linked to 1. the co-ordinately low levels of checkpoint molecules (PD1, LAG3) and higher expression of TCF1 associated with a less exhausted TIL effector cell populations and 2. reduced levels of immunoregulatory cell populations (including regulatory T cells and MDSCs) found in the TME of T1D host animals.

Our study findings suggest that pre-existing T1D, in the absence of insulin treatment or di*et al*terations, might have a protective role in cancer. It might be noted that insulin has an important role on cancer cell metabolism where it has been found to be mitogenic, but not carcinogenic (Gunter *et al*., 2008; Salisbury and Tomblin, 2015). Studies suggest that during and after the differentiation of naive $CD8^+$ T cells into effector T cells following antigen cross-presentation from antigen-presenting cells, $CD8^+$ T cells rely on aerobic glycolysis, similar to the Warburg effect observed in cancer cells (Menk *et al*., 2018; Palmer *et al*., 2015). We found that $^{STZ}CD8_{TIL}$ cells from B16F10 tumor bearing mice exhibit significantly elevated glycolysis and Krebs cycle activation when compared to $^{Cont.}CD8_{TIL}$ cells. Higher expression of *glut 1, mct 1, pkm 2,pcx 1 and idh 2* in $^{STZ}CD8_{TIL}$ in tumor bearing hosts further suggests intrinsic metabolic activation of $CD8^+$ TILs under diabetogenic conditions. Whereas, insulin treatment of STZ induced T1D negatively impacts metabolic genes in $CD8^+$ TILs recovered from all four tumor models examined in this study. Tumor cells in STZ induced mice on the other hand, showed diminished *glut 1* expression and increased *pcx 1* expression indicative of repressed glycolytic activity and slowed proliferative capacity. Our observations suggest that within the context of the T1D TME, $CD8^+$ TILs gain a metabolic advantage over tumor cells in association with enhanced anti-tumor $CD8^+$ T cell infiltration, activation and sustained effector function resulting in improved tumor growth control.

Further studies revealed that $CD8^+$ TILs, but not tumor cells, expressed significantly higher IGF1 and IGF1R in mRNA and protein levels. The frequency of $CD8^+IGF1^+IGF1R^+$ cells wassignificantly higher in TILs, TDLNs, spleens and PBMCs of STZ induced T1Dtumor-bearing mice but not in non-diabetic tumor hosts. Interestingly, the proportion of $CD8^+IGF1^+IGF1R^+$cells amongst TIL was found to be inversely correlated with tumor size. This observation suggestsa crucial role for this effector cell population in restricting tumor growth in T1D hosts. Untreated, STZ treated and STZ+insulin treated hosts without tumor, however, showed no significant change in the frequency of the $CD8^+IGF1^+IGF1R^+$ population in LNs, spleens and PBMCs, with the notable exception of spleen and PBMC in the B16F10 melanoma model.

IGF1 and IGF1R shares significant structural homology with insulin and insulin receptor (IR) respectively. Moreover, insulin and IGF1 are involved in many overlapping signaling pathways (Cheng *et al*., 2005; Rincon *et al*., 2004).

Although a variety of cells secrete IGF1, the major source of systemic IGF1 is the liver (Sjögren *et al*., 1999). Hepatocytes secrete IGF1 upon stimulation with insulin and growth hormone (GH) (Dichtel *et al*., 2022). T1D patients with very low or undetectable levels of insulin also exhibit reduced plasma IGF1 levels (Raman *et al*., 2019). Reduced serum insulin and IGF1 concentrations in STZ induced T1D mice may trigger enhanced expression of IGF1 and IGF1R on $CD8^+$ T cells upon cognate (tumor) antigen stimulation. This may trigger autocrine IGF1-IGF1R signaling in $CD8^+$ T cells, which may be responsible for the enhanced metabolic activation and cytotoxic potential of these cells. Acute fasting has been reported to reduce systemic IGF1 levels, to boost systemic $CD8^+$ Tcell metabolism, and to enhance CTL-mediated cytotoxicity (Yang and Wang, 2022). Glucose transporter 2 (Glut-2) which is predominantly expressed by pancreatic β cells, can sense a rise in blood glucose, which in turn stimulates insulin secretion (Low *et al*., 2021). During fasting, blood glucose levels are normalized and there isonly a little to no insulin in circulation. These conditions may result in decreased systemic IGF1 levels, which in turn could force $CD8^+$ T cells to activate autocrine IGF1 production and *cis/trans* activation of IGF1R-mediated signaling. *In vitro* studies have previously suggested that activated $CD8^+$ T cells produced significant quantities of IGF1 (Stentz and Kitabchi, 2004). These cumulative findings support the hypothesis that autocrine IGF1 signaling may be activated in $CD8^+$ T cells when systemic IGF1 is downregulated, as in the case of tumor-bearing T1D hosts. IGF1 is a potent mitogen, and high systemic IGF1 levels are associated with poor prognosis in several types of cancers (Zhang *et al*., 2021). IGF1-IGF1R signaling in tumor cells results in enhanced cell proliferation, metabolic activation, escape from programmed cell death, and metastasis. However, clinical trials using IGF1R targeting antagonists or monoclonal antibodies have shown only limited success to date (Vlahovic *et al*., 2018). IGF1 can also induce $CD8^+$ T cell activation, proliferation, and chemotaxis through the IGF1R (Douglas *et al*., 2007). Notably, high IGF1R expression is required for leukemia-initiating cell activity in T-cell acute lymphoblastic leukemia (T-ALL) (Medyouf *et al*., 2011). IGF1R is also critical for regulating Th17-Treg balance in autoimmune diseases (DiToro *et al*., 2020). Considering the differential and context-dependent effects of IGF1-IGF1R signaling across various cells and diseases, targeted modulation of such circuitry in effector $CD8^+$ T cells might be exploited for therapeutic gain in the setting of adoptive T cell-based therapy of cancer.

In B16F10 melanoma models, we observed a significant upregulation of pAKT and p-mTORC1 in $^{STZ}CD8_{TIL}$ cells vs. $^{Cont.}CD8_{TIL}$ cells. Protein-protein interaction network studies further revealed an interconnection between IGF1, IGF1R, AKT and mTOR. We hypothesised that metabolic upregulation and enhanced effector function in $CD8^+$ T cells from STZ induced T1D mice might be mediated through the IGF1R-mTOR signaling axis. To test our hypothesis, we isolated $CD8^+$ T cells from TDLNs of STZ induced T1D vs. control B16F10 melanoma bearing mice. Isolated ^{Cont}.CD8 T cells and ^{STZ}CD8 T cells were treated with

either recombinant IGF1 (rIGF1) or with silencing RNA against IGF1R (siIGF1R) or with rapamycin (mTOR inhibitor) alone or in combination. rIGF1 significantly enhanced activation markers on ^{STZ}CD8 T cells but not ^{Cont}CD8 T cells. ^{STZ}CD8 T cells were determined to be more sensitive to treatment with siIGF1R or rapamycin or the combination of these agents. Our results clearly indicate that the IGF1R-mTOR signaling axis underlies the enhanced effector function of $CD8^+$ T cells in STZ induced T1D tumor bearing mice. Furthermore, we validated this core paradigm in clinical samples isolated from T1D patients. In these *in vitro* analyses. we stimulated PBMCs from T1D vs. non-diabetic patients with a source of tumor antigen (i.e., tumor lysate). We observed significant upregulation in responder populations of $CD8^+CD69^+$ T cells and $CD8^+IGF1R^+$ T cells in cultures developed from T1D PBMCs vs. non-diabetic PBMCs. Targeted antagonism of IGF1R or mTOR or both resulted in a significant reduction in these two $CD8^+$ T cells subpopulations in T1D PBMC cultures. *In vitro* stimulation with tumor antigens generated a greater $CD8^+$ T cell activation from T1D vs. non-diabetic patients. $CD8^+$ T cells from T1D patients were more sensitive to the inhibitory effects of IGF1R blockers or mTOR antagonists. This observation strengthens our hypothesis that the IGF1R-mTOR signaling axis in $CD8^+$ T cells in T1D hosts might be critical to tumor growth control.

Neem leaf glycoprotein (NLGP) have been found to restrict murine tumor growth in prophylactic as well as therapeutic setting majorly by robust immunomodulation. NLGP interacts to DCs and macrophages and thereby reinvigorate cytotoxic T cells for optimum antitumor activity. NLGP matures immature DCs from healthy as well as cancer patients. Further study revealed NLGP convers M2 macrophages into M1 macrophages. NLGP induces expression of IL-12 and reduced IL-10 expression from tumor associated DCs and macrophages. Further exploration on the therapeutic potential of NLGP revealed tumor growth control in T1D hosts establishes our previous observations in non-diabetic tumor bearing hosts. NLGP reduces T1D associated hepatic inflammation irrespective of tumor burden in T1D hosts. This preliminary observation needs further extensive research to assess the efficacy of NLGP to treat T1D associated inflammation. Furthermore, NLGP has been found to improve glucose metabolism in intratumoral $CD8^+$ T cells and hinders glucose uptake by tumor cells in T1D hosts. Cumulative results indicate the therapeutic benefit of NLGP in T1D hosts for optimum tumor control by influencing immune microenvironment.

Sustained $CD8^+$ T cell fate/function are essential for the success of cancer immunotherapy, withthe success of anti-PD1 therapy link to the ability to prevent the exhaustion and premature death of anti-tumor $CD8^+$ T cells (Chakravarti *et al*., 2023). Very recently, *cis* B7:CD28 costimulatory interactions in $CD8^+$ T cells have been demonstrated to promote anti-tumor immunity, implying that $CD8^+$ T cells could boost themselves in an autocrine manner (Zhao *et al*., 2023). In a similar manner, intrinsic IGF1R-mTOR signaling in $CD8^+$ T cells may be important to the superior anti-tumor action of these effector cells in T1D patients. Interestingly, a fraction of cancer patients

undergoing immune checkpoint blockade therapy (ICBT) develop immune-related adverse effects (irAEs), among which T1D is very common (Mourad *et al*., 2021). It has not yet been documented whether cancer patients who develop T1D during ICBT have a better clinical prognosis. We would hypothesize that development of T1D may enable IGF1/IGF1R/mTOR-dependent anti-tumor immunity post-ICBT therapy. The IGF1R-mTORsignaling axis could also be exploited within the context of CAR-T cell-based cancer immunotherapy, where T cell anergy and exhaustion remain major challenges to the therapeutic efficacy of this approach in the solid cancer setting (Sterner and Sterner, 2021). Future work will define optimal targeting agents to agonize IGF1R-mTOR signaling in anti-tumor $CD8^+$ T cells for sustained therapeutic benefit in cancer patients.

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PUBLICATIONS

- Publications as first author
- Publications as co-author
- Abstract publications
- Oral presentations
- Poster presentations

Publications as first author

Original articles

1. **Sarkar A.**, Dhar S., Bera S., Chakravarti M., Verma A., Prasad P., Sultana J., Das J., Saha A., Bhuniya A., Guha I., Dasgupta S., Roy SS., Banerjee S., Roy S., Bhar D., Storkus WJ., Baral R., Datta D., Bose A. Experimental type 1 diabetes metabolically rejuvenates $CD8^+$ T cells for improved control of tumor growth through an IGF1-IGF1R axis. bioRxiv 2024. 04.04.588206. (Preprint, under revision in *Cancer Research*)

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2. **Sarkar A**, Dhar S, Bera S, Chakravarti M, Verma A, Prasad P, Saha A, Bhuniya A, Guha I, Roy S S, Baral R, Datta D, Bose A, Metabolic modulation of $CD8^+$ T cells regulate type-I diabetes associated melanoma growth restriction. 41stAnnual Convention of Indian Association for Cancer Research, March 2-5, 2022, Amity University, Noida.

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As first author (Abroad)

1. **Sarkar A**, Dhar S, Bera S, Chakravarti M, Verma A, Prasad P, Saha A, Bhuniya A, Guha I, Roy S.S, Banerjee S, Baral R, Datta D, Bose A. Type-1 diabetes restricts melanoma growth by reprogramming intra-tumoral T cell metabolism. ESMO Immuno Oncology Congress 2022, Dec 7-9, 2022, Geneva, Switzerland.

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AWARDS

- Awards abroad
- Awards in India
- Fellowships

Awards abroad

1. **Best poster** award in ESMO Immuno Oncology Congress 2022, December 7-9, 2022, Geneva, Switzerland.
2. **Merit Travel Grant** from European Society of Medical Oncology (ESMO) to attend ESMO Immuno Oncology Congress 2022, held on Dec 7-9, 2022 in Geneva, Switzerland.

Awards in India

1. **Best oral** presentation award in 41st Annual Convention of Indian Association for Cancer Research, March 2-5, 2022, Amity University, Noida.
2. **Third prize** in Augmenting Writing Skills for Articulating Research (AWSAR), by Department of Science and technology (DST), Govt. of India in 2019.
3. Sitaram Joglekar award for **best oral** presentation in 38th Annual Convention of Indian Association for Cancer Research, March 1-3, 2019, PGIMER, Chandigarh.
4. First prize for **best oral** in JRF category in 2nd Annual Conference on Recent Trends in Cancer Research, Early Diagnosis, Prevention and Therapy organized by CNCI, 4-5th Feb, 2019.
5. First prize for **best oral** presentation in International Conference on Immunology at SRM University, Tamil Nadu, 26-28th Sept, 2018.

Fellowships

1. Awarded **Senior Research Fellowship** by Indian Council of Medical Research (ICMR), Govt. of India, in 2022.
2. Awarded **Junior Research Fellowship** by University Grants Commission (UGC), Govt. of India, in 2017 for qualifying CSIR-UGC-JRF test June 2016 in Life sciences.

Photograph 1: With Prof. Kevin Harrington, Institute of Cancer Research, London, UK and Hon'ble Judge, ESMO Immuno-Oncology Congress'22 in Geneva, Switzerland, in front of my poster on which he had put Best Poster badge.

Photograph 2: Group photo of AWSAR '19 awardees (myself at third row second last from right) with Dr. Ramnath Kobind, the then Hon'ble President of India (front row middle), Dr. Harsh Vardhan, then Hon'ble minister of Science and Technology and Health and Family Welfare (right to the hon'ble President), Dr. Ashutosh Sharma, then Secretary, DST (second right to the hon'ble President), Dr. K. Vijayraghaban, then Principal Scientific Advisor (right to the President), Dr. Renu Swarup, then Secretary of DBT (second right to the President), Dr. Shekhar C. Mande, then Director General, CSIR (third right to the President), and other dignitaries. This photograph was taken on the AWSAR award ceremony held on 28th Feb,'20, on National Science Day.

REPRINTS

Guardian of the Malady

Mr. Anirban Sarkar*

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How many cells we have in our body? Are they more than the number of stars? asked Arko, my 12-year-old cousin brother, gazing through the starry sky.

My birthplace, Ajhapur, is a small village, about 70 km from Kolkata. After almost a year, I went to my village this weekend. Autumn has arrived. Our place has a very common problem of a frequent power cut. Today is Sunday. Like other evenings, after load shedding, Arko and I went to the roof. The moonlight had covered the entire view. We sat on a mat; branches of an old neem tree made shadow over us.

"Our body is made up of trillions of cells. One trillion means 14 zeros after 1, and the stars are approximately 1 billion trillion in the universe, which means 21 zeros after 1," I added.

"Cells are a lot more than I can ever imagine! How can they stay and work together then? Even we cannot take unanimous decisions when playing cricket!" asked Arko.

"That is one of the mysteries of nature. All of our cells work in harmony. Their division, growth, functions, etc., are highly synchronized. Any deviation from this homeostasis leads to some kind of disease," I said.

"What kind of disease is cancer then?" he asked.

"Well, that's a long story for you to understand. Cells of our body are pre-programmed when to grow, when to divide, even when to die. When a cell becomes rogue, it divides rapidly. They escape death signals and thus become immortal. These growing cells form a tumor in our bodies. They force blood vessel–forming cells called endothelial

* Mr. Anirban Sarkar, Ph.D. Scholar from Chittaranjan National Cancer Institute, Kolkata, is pursuing his research on "Immunometabolism in Cancer and its Modulation by Neem Leaf Glycoprotein (NLGP)". His popular science story entitled "Guardian of the Malady" has been selected for AWSAR Award.

Experimental type 1 diabetes metabolically rejuvenates CD8$^+$ T cells for improved control of tumor growth through an IGF1-IGF1R axis

Anirban Sarkar[1], Sukanya Dhar[1], Saurav Bera[1], Mohona Chakravarti[1], Ayushi Verma[2], Parash Prasad[3], Jasmine Sultana[1], Juhina Das[1], Akata Saha[1], Avishek Bhuniya[1], Ipsita Guha[1], Shayani Dasgupta[1], Sib Sankar Roy[3], Saptak Banerjee[1], Subir Roy[4], Debarati Bhar[5], Walter J. Storkus[6], Rathindranath Baral[1], Dipak Datta[2], Anamika Bose[1,7,*]

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Neem Leaf Glycoprotein Disrupts Exhausted CD8⁺ T-Cell–Mediated Cancer Stem Cell Aggression

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Abstract

Targeting exhausted CD8⁺ T-cell (T_{EX})–induced aggravated cancer stem cells (CSC) holds immense therapeutic potential. In this regard, immunomodulation via Neem Leaf Glycoprotein (NLGP), a plant-derived glycoprotein immunomodulator is explored. Since former reports have proven immune dependent–tumor restriction of NLGP across multiple tumor models, we hypothesized that NLGP might reprogram and rectify T_{EX} to target CSCs successfully. In this study, we report that NLGP's therapeutic administration significantly reduced T_{EX}-associated CSC virulence in *in vivo* B16-F10 melanoma tumor model. A similar trend was observed in *in vitro* generated T_{EX} and B16-F10/MCF7 coculture setups. NLGP rewired CSCs by downregulating clonogenicity, multidrug resistance phenotypes and PDL1, OCT4, and SOX2 expression. Cell cycle analysis revealed that NLGP educated–T_{EX} efficiently pushed CSCs out of quiescent phase (G_0G_1) into synthesis phase (S), supported by hyper-phosphorylation of G_0G_1–S transitory cyclins and Rb proteins. This rendered quiescent CSCs susceptible to S-phase–targeting chemotherapeutic drugs like 5-fluorouracil (5FU). Consequently, combinatorial treatment of NLGP and 5FU brought optimal CSC-targeting efficiency with an increase in apoptotic bodies and proapoptotic BID expression. Notably a strong nephron-protective effect of NLGP was also observed, which prevented 5FU-associated toxicity. Furthermore, Dectin-1–mediated NLGP uptake and subsequent alteration of Notch1 and mTOR axis were deciphered as the involved signaling network. This observation unveiled Dectin-1 as a potent immunotherapeutic drug target to counter T-cell exhaustion. Cumulatively, NLGP immunotherapy alleviated exhausted CD8⁺ T-cell-induced CSC aggravation.

Implications: Our study recommends that NLGP immunotherapy can be utilized to counter ramifications of T-cell exhaustion and to target therapy elusive aggressive CSCs without evoking toxicity.

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The immunomodulatory impact of naturally derived neem leaf glycoprotein on the initiation progression model of 4NQO induced murine oral carcinogenesis: a preclinical study

Juhina Das[1], Saurav Bera[1], Nilanjan Ganguly[1], Ipsita Guha[1†], Tithi Ghosh Halder[1†], Avishek Bhuniya[1†], Partha Nandi[1†], Mohona Chakravarti[1], Sukanya Dhar[1], Anirban Sarkar[1], Tapasi Das[1], Saptak Banerjee[1], Sandip Ghose[2], Anamika Bose[1,3*] and Rathindranath Baral[1*]

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Introduction: Murine tumor growth restriction by neem leaf glycoprotein (NLGP) was established in various transplanted models of murine sarcoma, melanoma and carcinoma. However, the role of NLGP in the sequential carcinogenic steps has not been explored. Thus, tongue carcinogenesis in Swiss mice was induced by 4-nitroquinoline-1-oxide (4NQO), which has close resemblance to human carcinogenesis process. Interventional role of NLGP in initiation-promotion protocol established during 4NQO mediated tongue carcinogenesis in relation to systemic immune alteration and epithelial-mesenchymal transition (EMT) is investigated.

Methods: 4NQO was painted on tongue of Swiss mice every third day at a dose of 25µl of 5mg/ml stock solution. After five consecutive treatment with 4NQO (starting Day7), one group of mice was treated with NLGP (s.c., 25µg/mice/week), keeping a group as PBS control. Mice were sacrificed in different time-intervals to harvest tongues and studied using histology, immunohistochemistry, flow-cytometry and RT-PCR on different immune cells and EMT markers (e-cadherin, vimentin) to elucidate their phenotypic and secretory status.

Results: Local administration of 4NQO for consecutive 300 days promotes significant alteration in tongue mucosa including erosion in papillae and migration of malignant epithelial cells to the underlying connective tissue stroma with the formation of cell nests (exophytic-hyperkeratosis with mild dysplasia). Therapeutic NLGP treatment delayed pre-neoplastic changes promoting normalization of mucosa by maintaining normal structure. Flow-cytometric evidences suggest that NLGP treatment upregulated $CD8^+$, $IFN\gamma^+$, granzyme B^+, $CD11c^+$ cells in comparison

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High monocytic MDSC signature predicts multi-drug resistance and cancer relapse in non-Hodgkin lymphoma patients treated with R-CHOP

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Introduction: Non-Hodgkin Lymphoma (NHL) is a heterogeneous lymphoproliferative malignancy with B cell origin. Combinatorial treatment of rituximab, cyclophsphamide, hydroxydaunorubicin, oncovin, prednisone (R-CHOP) is the standard treatment regimen for NHL, yielding a complete remission (CR) rate of 40-50%. Unfortunately, considerable patients undergo relapse after CR or initial treatment, resulting in poor clinical implications. Patient's response to chemotherapy varies widely from static disease to cancer recurrence and later is primarily associated with the development of multi-drug resistance (MDR). The immunosuppressive cells within the tumor microenvironment (TME) have become a crucial target for improving the therapy efficacy. However, a better understanding of their involvement is needed for distinctive response of NHL patients after receiving chemotherapy to design more effective front-line treatment algorithms based on reliable predictive biomarkers.

Methods: Peripheral blood from 61 $CD20^+$ NHL patients before and after chemotherapy was utilized for immunophenotyping by flow-cytometry at different phases of treatment. *In-vivo* and *in-vitro* doxorubicin (Dox) resistance models were developed with murine Dalton's lymphoma and Jurkat/Raji cell-lines respectively and impact of responsible immune cells on generation of drug resistance was studied by RT-PCR, flow-cytometry and colorimetric assays. Gene silencing, ChIP and western blot were performed to explore the involved signaling pathways.

Results: We observed a strong positive correlation between elevated level of $CD33^+CD11b^+CD14^+CD15^-$ monocytic MDSCs (M-MDSC) and MDR in NHL

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RESEARCH

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Neem leaf glycoprotein binding to Dectin-1 receptors on dendritic cell induces type-1 immunity through CARD9 mediated intracellular signal to NFκB

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Abstract

Background A water-soluble ingredient of mature leaves of the tropical mahogany 'Neem' (*Azadirachta indica*), was identified as glycoprotein, thus being named as 'Neem Leaf Glycoprotein' (NLGP). This non-toxic leaf-component regressed cancerous murine tumors (melanoma, carcinoma, sarcoma) recurrently in different experimental circumstances by boosting prime antitumor immune attributes. Such antitumor immunomodulation, aid cytotoxic T cell (T_c)-based annihilation of tumor cells. This study focused on identifying and characterizing the signaling gateway that initiate this systemic immunomodulation. In search of this gateway, antigen-presenting cells (APCs) were explored, which activate and induce the cytotoxic thrust in T_c cells.

Methods Six glycoprotein-binding C-type lectins found on APCs, namely, MBR, Dectin-1, Dectin-2, DC-SIGN, DEC205 and DNGR-1 were screened on bone marrow-derived dendritic cells from C57BL/6 J mice. Fluorescence microscopy, RT-PCR, flow cytometry and ELISA revealed Dectin-1 as the NLGP-binding receptor, followed by verifications through RNAi. Following detection of β-Glucans in NLGP, their interactions with Dectin-1 were explored in silico. Roles of second messengers and transcription factors in the downstream signal were studied by co-immunoprecipitation, western blotting, and chromatin-immunoprecipitation. Intracellularization of FITC-coupled NLGP was observed by processing confocal micrographs of DCs.

Results Considering extents of hindrance in NLGP-driven transcription rates of the cytokines IL-10 and IL-12p35 by receptor-neutralization, Dectin-1 receptors on dendritic cells were found to bind NLGP through the ligand's peripheral β-Glucan chains. The resulting signal phosphorylates PKCδ, forming a trimolecular complex of CARD9, Bcl10 and MALT1, which in turn activates the canonical NFκB-pathway of transcription-regulation. Consequently, the NFκB-heterodimer p65:p50 enhances *Il12a* transcription and the p50:p50 homodimer represses *Il10* transcription, bringing about a cytokine-based systemic-bias towards type-1 immune environment. Further, NLGP gets engulfed within dendritic cells, possibly through endocytic activities of Dectin-1.

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Cancer stem cell–immune cell crosstalk in breast tumor microenvironment: a determinant of therapeutic facet

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Breast cancer (BC) is globally one of the leading killers among women. Within a breast tumor, a minor population of transformed cells accountable for drug resistance, survival, and metastasis is known as breast cancer stem cells (BCSCs). Several experimental lines of evidence have indicated that BCSCs influence the functionality of immune cells. They evade immune surveillance by altering the characteristics of immune cells and modulate the tumor landscape to an immune-suppressive type. They are proficient in switching from a quiescent phase (slowly cycling) to an actively proliferating phenotype with a high degree of plasticity. This review confers the relevance and impact of crosstalk between immune cells and BCSCs as a fate determinant for BC prognosis. It also focuses on current strategies for targeting these aberrant BCSCs that could open avenues for the treatment of breast carcinoma.

KEYWORDS

breast cancer (BC), breast cancer stem cells (BCSCs), tumor microenvironment (TME), innate immune cells, adaptive immune cells

Abbreviations: ALDH1, alcohol dehydrogenase 1; APCs, antigen-presenting cells; BC, breast cancer; BRCA, breast cancer gene; BCSCs, breast cancer stem cells; CD, cluster of differentiation; CTCs, circulating tumor cells; CSF, colony-stimulating factor; DCs, dendritic cells; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; Id1, inhibitor of DNA binding 1; IL, interleukin; iDCs, immature DCs; MDSCs, myeloid-derived suppressor cells; MFG, milk fat globule; MHC, major histocompatibility complex; MIC, MHC-I chain-related protein; MMPs, matrix metalloproteinases; M-DCs, mature DCs; NK, natural killer cells; NKG2D, NK-activating receptor; PGE2, prostaglandin E2; ROS, reactive oxygen species; RNS, reactive nitrogen species; TME, tumor microenvironment; TAMs, tumor-associated macrophages; Tregs, T regulatory cells; TILs, tumor-infiltrating lymphocytes; TINs, tumor-infiltrating neutrophils.

CANCER RESEARCH | CANCER IMMUNOLOGY

Terminally Exhausted CD8$^+$ T Cells Resistant to PD-1 Blockade Promote Generation and Maintenance of Aggressive Cancer Stem Cells

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ABSTRACT

Heterogeneity within the tumor-infiltrating lymphocytes (TIL) population limits immunotherapeutic efficacy against cancer. Between two subpopulations of exhausted CD8$^+$ TILs (progenitor-exhausted; T_{PEX}, terminally exhausted; T_{TEX}), T_{TEX} cells remain unresponsive to anti–programmed cell death protein 1(PD-1) therapy. Deciphering whether and how PD-1–resistant T_{TEX} cells engage in tumor promotion could improve the response to immunotherapy. Here, we report that T_{TEX} cells actively participate in tumor progression by modulating cancer stem cells (CSC). T_{TEX} cells strongly correlated with elevated CSC frequency in poorly immune-infiltrated (CD8$^+$ TIL low) advanced human breast and ovarian carcinomas. T_{TEX} directly upregulated CSC frequency *in vitro*, which was not affected by anti–PD-1 treatment. The T_{TEX}-influenced CSCs were highly clonogenic and exhibited a multidrug-resistant phenotype, overexpressing drug efflux pumps like ABCC1 and ABCB1. These CSCs were highly invasive, displaying increased invadopodia development and elevated cofilin, CXCR4, and matrix metalloproteinase 7 (MMP7) expression. The invasive properties along with epithelial–mesenchymal plasticity of T_{TEX}-educated CSCs increased metastasis *in vivo*. T_{TEX} increased cell surface levels and activation of VEGFR2 in CSCs, and silencing or inhibition of VEGFR2 reversed the CSC-stimulatory effects of T_{TEX}. LAMP3 and NRP1 on the surface of T_{TEX} stimulated VEGFR2 in CSCs to promote aggressiveness. Cumulatively, these findings suggest that screening patients with carcinoma for both CD8$^+$ TILs and T_{TEX} frequency prior to anti–PD-1 therapy could improve patient outcomes. In addition, targeting the LAMP3/NRP1–VEGFR2 axis could be a therapeutic strategy in advanced patients with carcinoma with limited CD8$^+$ T-cell infiltration and high T_{TEX} frequency.

Significance: Cross-talk with T_{TEX} CD8$^+$ T cells mediated by the VEGFR2 axis induces aggressive properties in cancer stem cells to promote tumor progression.

Introduction

Intratumoral complexity poses a significant challenge in successful cancer management. A self-renewing rare subset of stem cells, designated as cancer stem cells (CSC), fuels such heterogeneity (1). CSCs remain arrested in a quiescent state; only transiently proliferate to generate heterogeneous malignant tumor bulk, which is generally non-CSC and can seldom foster tumors in xenograft assays (2). However, this hierarchy is not always maintained; plasticity and dedifferentiation of terminally differentiated malignant cells to CSC-like state are also evident under specific conditions (3–4). Intratumoral CSC-supportive niches protect from diverse genotoxicities, by overexpressing ABC-drug efflux proteins, enhancing DNA repair mechanisms, resisting DNA damage and upregulating antiapoptotic proteins (5–7). Tumor-intrinsic hypoxia feeds this flame via upregulation of HIF1α, conferring enhanced therapy resistance and resulting into relapse (8). CSCs also initiate metastasis and secondary tumor formation by inducing extracellular matrix (ECM) reorganization, epithelial–mesenchymal transition (EMT), neovascularization, vascular mimicry, and seeding at the secondary site (9–13).

Substantial evidence suggests the importance of immune cells in regulating CSC fate, like tumor-associated macrophage (TAM), myeloid-derived suppressor cell (MDSC) and Tregs support CSCs, while natural killer (NK) and γδT cells function against it (14–19). Immune-suppressive network increases CSC frequency, stemness, and aggressive phenotypes (20). CSC survival is further ensured due to the truncation of the effector response from infiltrated CD8$^+$ T cells. Within the tumor microenvironment (TME), CD8$^+$ T cells undergo a hierarchical loss of proliferation and effector functions, including the secretion of IL2, TNFα, IFNγ, and β chemokines, as a result of prolonged antigenic exposure and an immune-suppressive milieu. In this "exhausted" state, they show a sustained overexpression of coinhibitory receptors, such as programmed cell death protein 1 (PD-1), TIM3, LAG3, CTLA4, and TIGIT. These hyporesponsive exhausted CD8$^+$ T cells fail to regulate tumor growth (21–24).

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Tumor activated platelets induce vascular mimicry in mesenchymal stem cells and aid metastasis

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ABSTRACT

Extent of metastasis influences activation of platelets in tumor-microenvironment. Activated platelets potentiate mesenchymal-stem-cells (MSCs) to migrate in secondary metastatic sites without participation in process of invasion. Presence of higher percentage of MSCs along with activated-platelets induces formation of vascular-mimicry (VM). The pathophysiology, VM, has already been reported in multiple types of cancer including lung, ovary, melanoma etc. and related to poor-prognosis. Interaction of MSCs with platelets in cell-to-cell contact dependent manner is essential for their migration, thereby, VM. Evidences are obtained suggesting that under influence of tumor-associated-activated-platelets, expressions of vimentin, ve-cadherin are increased, along with decrease in e-cadherin on $CD105^{+}$ MSCs in both mRNA and protein levels that may help in formation of vessel like structure in VM. Adoptive transfer of MSCs along with tumor-activated-platelets causes greater B16 melanoma metastasis at lungs in comparison to MSCs with non-activated platelets. Presence of $CD105^{+}Vimentin^{+}$ MSCs in vessel like structure in the metastatic lung confirms the involvement of platelet-activated-MSCs in VM, thereby, in metastasis.

1. Introduction

Vascular or vasculogenic-mimicry (VM) [1–3], a unique pathophysiological process within tumor, provides nutritional support and oxygen, independent of the normal blood vessels [4,5]. Association of VM with aggressive tumors, e.g., melanoma, glioblastoma and carcinoma (breast, ovary, stomach, lung, and prostate) has been reported, where VM appears to be a determining factor for poor-prognosis [2,6–9] and poor-overall-survival of patients [10].

Plasticity and/or *trans*-differentiated properties of tumor-cells involved in VM have been reported, where expression of both epithelial as well as mesenchymal-markers were noticed [11,12]. Literatures have suggested that cancer-stem-cells might have the most prominent role in VM, without addressing the possible involvement of stromal cells, those are abundantly present within the tumor-stroma [13,14]. Existence of mesenchymal-markers within tumor-microenvironment (TME) indicates high probability of the participation of mesenchymal-stem-cells (MSCs) in VM. Understanding of the pathogenesis of VM and its regulation by tumor-microenvironmental component(s), like, MSCs, is poorly understood.

Platelets, cells involved in blood-clotting, are known for their role in tumor metastatic process. Platelets come in close contact of tumor cells and become activated, thus, termed tumor-activated-platelets (TAP) or tumor-educated-platelets. TAPs produce numerous filopedia and undergo degranulation to release various biologically active substances, like, VEGF, PDGF, EGF etc. [15,16]. TAPs positively influence tumor progression and metastasis by modulating epithelial-mesenchymal-transition (EMT) and angiogenesis [17,18]. Platelets, those contain either pro- or anti-angiogenic factors may either induce or prevent angiogenesis and stimulate tumor-growth. As platelets and MSCs play pivotal roles in numerous physiological (viz., wound healing, tissue homeostasis) [17,19,20] and pathophysiological phenomenon (including cancer and metastasis) [21,22], influence of TAP on MSCs' functionality is quite expected. But, not investigated in detail.

In this study, we have demonstrated the participation of TAP induced MSCs in VM and deciphered how TAPs regulate MSCs's migration, invasion, and VM like vessel formation, which ultimately influence the metastasis progression.

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Neem leaf glycoprotein salvages T cell functions from Myeloid-derived suppressor cells-suppression by altering IL-10/STAT3 axis in melanoma tumor microenvironment

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Myeloid-derived suppressor cells (MDSCs) suppress antitumor immune functions. We have observed that an immunomodulator, neem leaf glycoprotein (NLGP), inhibits tumor-resident MDSCs and enhances antitumor CD8+ T cell immunity. NLGP inhibits the number as well as functions of tumor-resident MDSCs (Gr1±CD11b±) and enhances antitumor CD8± T cell immunity by downregulating arginase 1 and inducible nitric oxide synthase production in MDSCs. Accordingly, decreased T cell anergy and helper to regulatory T cell conversion have been observed in the presence of NLGP, which ultimately augments T cell functions. Mechanistically, NLGP-mediated rectification of T cell suppressive functions of MDSCs was primarily associated with downregulation of the interleukin (IL)-10/signal transducer and activator of transcription 3 (STAT3) signaling axis within the tumor microenvironment, as confirmed by knockdown of STAT3 (by STAT3-siRNA) and using IL-10$^{-/-}$ mice. Thus, NLGP-mediated suppression of MDSC functions in tumor hosts is appeared to be another associated effective mechanism for the eradication of murine melanoma by NLGP.

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Introduction

Immunosuppression favors malignant tumor growth and metastasis. Myeloid-derived suppressor cells (MDSCs) are a group of bone marrow-derived cells of myeloid lineage directly involved in the suppression of immune responses in cancer and are recognized as Gr1+CD11b+ cells in mouse and CD14-CD11b+CD33+human leukocyte antigen – DR isotype cells in human [1]. An elevated level of MDSCs is observed in various types of cancer, including melanoma [2] and is directly correlated to the poor prognosis [3]. MDSCs mediate their suppression on T-lymphocytes in cancer either through direct contact or through a combination of multiple major mediators, such as inducible nitric oxide synthase (iNOS), arginase 1 (ARG1) [4–6], cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) [1], transforming growth factor beta (TGFβ) [4] and interleukin (IL)-10 [5] or both. MDSCs expressing ARG1 reduces the availability of L-arginine, which can result in the loss of CD3ξ expression and impaired T cell functions [1,7,8]. Reports are available showing that the production of NO by iNOS expressing MDSCs is sufficient for blocking T cell responses [1]. Furthermore, MDSCs promote the de-novo development of FOXP3+ T regulatory cells (Tregs) *in vivo* [5], in an antigen-dependent, but TGFβ-independent, manner [9].

Successful cancer immunotherapy will only be possible when systemic downregulation of suppressive factors, including suppressive cells, such as, Tregs, tumor-associated macrophages (TAMs), MDSCs, and so on, are achieved. Such therapy to break tolerance can be broadly classified based on the intervention point in the immune response process, including but not limited to: (i) adoptive transfer of immune effectors, (ii) vaccination and (iii) immunomodulation. The success rate differs according to the cancer type and is occasionally low. Counter regulatory immune suppression mechanisms by Tregs and MDSCs might be the causative factor; thus, they demand further research.

Neem leaf glycoprotein (NLGP) is a nontoxic, hematostimulatory, immunostimulatory natural immunomodulator

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RESEARCH ARTICLE

NLGP regulates RGS5-TGFβ axis to promote pericyte-dependent vascular normalization during restricted tumor growth

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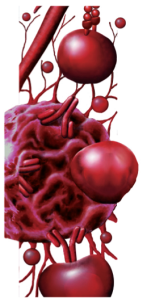
Abstract

Altered RGS5-associated intracellular pericyte signaling and its abnormal cross-talk with endothelial cells (ECs) result chaotic tumor-vasculature, prevent effective drug delivery, promote immune-evasion and many more to ensure ultimate tumor progression. Moreover, the frequency of lethal-RGS5^{high} pericytes within tumor was found to increase with disease progression, which signifies the presence of altered cell death pathway within tumor microenvironment (TME). In this study, we checked whether and how neem leaf glycoprotein (NLGP)-immunotherapy-mediated tumor growth restriction is associated with modification of pericytes' signaling, functions and its interaction with ECs. Analysis of pericytes isolated from tumors of NLGP treated mice suggested that NLGP treatment promotes apoptosis of NG2^{+}RGS5^{high}-fuctionally altered pericytes by downregulating intra-tumoral TGFβ, along with maintenance of more matured RGS5^{neg} pericytes. NLGP-mediated inhibition of TGFβ within TME rescues binding of RGS5 with Gαi and thereby termination of PI3K-AKT mediated survival signaling by downregulating Bcl2 and initiating pJNK mediated apoptosis. Limited availability of TGFβ also prevents complex-formation between RGS5 and Smad2 and rapid RGS5 nuclear translocation to mitigate alternate immunoregulatory functions of RGS5^{high} tumor-pericytes. We also observed binding of Ang1 from pericytes with Tie2 on ECs in NLGP-treated tumor, which support re-association of pericytes with endothelium and subsequent vessel stabilization. Furthermore, NLGP-therapy- associated RGS5 deficiency relieved CD4^{+} and CD8^{+} T cells from anergy by regulating 'alternate-APC-like' immunomodulatory characters of tumor-pericytes. Taken together, present study described the

Abbreviations: Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; APC, antigen presenting cell; EC, endothelial cells; NG2, neuron glial antigen 2; NLGP, neem leaf glycoprotein; PDGFRβ, platelet derived growth factor receptor beta; RGS5, regulator of signaling 5; SMA, smooth muscle cell actin; TME, tumor microenvironment.

Research Article

Immunotherapy



Neem leaf glycoprotein reverses tumor-induced and age-associated thymic involution to maintain peripheral CD8$^+$ T cell pool

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Aim: As tumor causes atrophy in the thymus to target effector-T cells, this study is aimed to decipher the efficacy of neem leaf glycoprotein (NLGP) in tumor- and age-associated thymic atrophy. **Materials & methods:** Different thymus parameters were studied using flow cytometry, reverse transcriptase PCR and immunocyto-/histochemistry in murine melanoma and sarcoma models. **Results:** Longitudinal NLGP therapy in tumor hosts show tumor-reduction along with significant normalization of thymic alterations. NLGP downregulates intrathymic IL-10, which eventually promotes Notch1 to rescue blockade in CD25$^+$CD44$^+$c-Kit$^+$DN2 to CD25$^+$CD44$^-$c-Kit$^-$DN3 transition in T cell maturation and suppress Ikaros/IRF8/Pu.1 to prevent DN2-T to DC differentiation in tumor hosts. The CD5^{int}TCR$\alpha\beta^{high}$ DP3 population was also increased to endorse CD8$^+$ T cell generation. **Conclusion:** NLGP rescues tumor-induced altered thymic events to generate more effector T cells to restrain tumor.

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Keywords: dendritic cells • IL-10 • NLGP • thymic atrophy • thymus • tumor

The cellular immune system in the tumor microenvironment not only fails to mount an effective antitumor response, but also actively enhances the intimacy with transformed cells to promote tumorigenesis [1]. On the other hand, immunotherapy enhances altered host immune response to combat against cancer [2]. The main arsenal in both scenarios is thymus differentiated T cells. Being a site for T cell differentiation, the thymus becomes targeted in cancer, though it is known to start to involute after puberty and is believed to become nonfunctional in adults. Contrary to the traditional view, recent studies with adult thymus suggest the thymic environment is maintained throughout the life [3] and inflammatory diseases such as cancer can initiate thymopoiesis, though mainly contribute Tregs expansion rather than effector T cells [4].

In context to the tumor, several *in vivo* and *in vitro* studies with tumor-induced thymic alterations observed: alterations in thymic size and cellularity; accumulation of early CD4$^-$CD8$^-$ double negative (DN)-pro T cells; enhanced apoptosis of immature CD4$^+$CD8$^+$ double positive (DP) thymocytes; loss of CD8$^+$ single positive (SP) thymocytes; alterations of thymic cytokine/chemokine gradient. All these events cumulatively diminish antitumor effector CD8$^+$ T cell pool to ultimately promote impaired cellular immunity.

Neem leaf glycoprotein (NLGP) is a neem-derived natural nontoxic immunomodulator, exhibits robust antitumor activity, chiefly by activating CD8$^+$ T cells as reported in several murine tumor models [5–7]. Corrolarily, NLGP-mediated tumor growth restriction is associated with reduction of immune-suppressor cells (regulatory T cells, tumor-associated macrophages, myeloid-derived suppressor cells and dendritic cells) [8–11] and vascular

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NLGP counterbalances the immunosuppressive effect of tumor-associated mesenchymal stem cells to restore effector T cell functions

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Abstract

Background: A dynamic interaction between tumor cells and its surrounding stroma promotes the initiation, progression, metastasis, and chemoresistance of solid tumors. Emerging evidences suggest that targeting the stromal events could improve the efficacies of current therapeutics. Within tumor microenvironment (TME), stromal progenitor cells, i.e., MSCs, interact and eventually modulate the biology and functions of cancer and immune cells. Our recent finding disclosed a novel mechanism stating that tumor-associated MSCs inhibit the T cell proliferation and effector functions by blocking cysteine transport to T cells by dendritic cells (DCs), which makes MSCs as a compelling candidate as a therapeutic target. Immunomodulation by nontoxic neem leaf glycoprotein (NLGP) on dysfunctional cancer immunity offers significant therapeutic benefits to murine tumor host; however, its modulation on MSCs and its impact on T cell functions need to be elucidated.

Methods: Bone marrow-derived primary MSCs or murine 10 T1/2 MSCs were tumor-conditioned (TC-MSCs) and co-cultured with B16 melanoma antigen-specific DCs and MACS purified $CD4^+$ and $CD8^+$ T cells. T cell proliferation of T cells was checked by Ki67-based flow-cytometric and thymidine-incorporation assays. Cytokine secretion was measured by ELISA. The expression of cystathionase in DCs was assessed by RT-PCR. The STAT3/pSTAT3 levels in DCs were assessed by western blot, and STAT3 function was confirmed using specific SiRNA. Solid B16 melanoma tumor growth was monitored following adoptive transfer of conditioned $CD8^+$ T cells.

Results: NLGP possesses an ability to restore anti-tumor T cell functions by modulating TC-MSCs. Supplementation of NLGP in DC-T cell co-culture significantly restored the inhibition in T cell proliferation and IFNγ secretion almost towards normal in the presence of TC-MSCs. Adoptive transfer of NLGP-treated TC-MSC supernatant educated $CD8^+$ T cells in solid B16 melanoma bearing mice resulted in better tumor growth restriction than TC-MSC conditioned $CD8^+$ T cells. NLGP downregulates IL-10 secretion by TC-MSCs, and concomitantly, pSTAT3 expression was downregulated in DCs in the presence of NLGP-treated TC-MSC supernatant. As pSTAT3 negatively regulates cystathionase expression in DCs, NLGP indirectly helps to maintain an almost normal level of cystathionase gene expression in DCs making them able to export sufficient amount of cysteine required for optimum T cell proliferation and effector functions within TME.

(Continued on next page)

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Results: $Fes^{-/-}$ BMDMs display stronger PRR signaling in vitro compared to WT following LPS stimulation. In vivo, we show increased tumour control and survival in $Fes^{-/-}$ mice compared to WT, which was further enhanced by stimulating ICD with doxorubicin. $Fes^{-/-}$ mice demonstrated increased CTL and NK cell activation and PD-1 positivity, which was enhanced by doxorubicin, indicating a novel role of Fes in regulating CTL and NK cell activation. Additionally, we found a shift from M2- to M1-polarized tumour associated macrophages in $Fes^{-/-}$ versus WT mice. Finally, when treated with anti-PD-1 antibody, $Fes^{-/-}$ mice demonstrated greater tumour control and survival than WT.

Conclusions: Consistent with improved overall- and disease-free survival observed in low Fes-expressing cancer patients, our results identify Fes as a potential novel therapeutic target to enhance anti-cancer immunotherapy.

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213P Type-1 diabetes restricts melanoma growth by reprogramming intra-tumoral T cell metabolism

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Background: Epidemiological studies from Bendix et al. (2016) with 5 countries and 9000 type-I diabetes mellitus (T1DM) patients with cancers showed reduced risk in melanoma, breast and prostate cancer and increased risk of oesophagus, stomach, colon and liver cancer, suggested differential consequences of T1DM with cancers of different etiopathology. However, prospective studies to decipher the possible mechanism are not well documented. In both T1DM and cancer, $CD8^+$ T cells plays crucial role and faces functional as well as metabolic alterations. Objective of this study was to evaluate the possible modulatory effect of pre-existing T1DM in melanoma growth, systemic immune landscape and T cell metabolism.

Methods: Murine T1DM model was established by using intra-peritoneal injection of streptozotocin (STZ) to C57BL/6J mice. B16F10 cells were inoculated to T1DM and non-diabetic control mice. Tumor progression and host survival was closely monitored following establishment of B16 melanoma. RT-PCR, Western-blot, Flow-cytometry and LDH release assay were used to study different immune cells, metabolic pathways etc. Athymic nude mice were used to examine the possible involvement of immune system in T1DM associated cancer progression.

Results: Pre-existence of T1DM showed restricted melanoma growth and survival benefits in murine host, however, such effect was found to be mitigated in immune-compromised mice. Significant intra-tumoral infiltration of $IFNg^+Perforin^{high}$-$GranzymeB^{high}$ $CD8^+$ T cells were observed with reduced Tregs and MDSCs in T1DM host compared to control. Moreover, pre-existence of T1DM modulates extracellular acidification rate (ECAR) and expression of enzymes associated with glucose-metabolism like PCX1, LDH, PKM2 in tumor infiltrated $CD8^+$T cells. Obtained results also pointed out the involvement of IGF1-mTOR signalling axis within $CD8^+$-effector T cells in regulation of T1DM associated tumor growth restriction.

Conclusions: Pre-existing T1DM promotes $CD8^+$ T cell dependent murine melanoma growth restriction which significantly increases tumor host survival. IGF1-mTOR signalling axis could be exploited in cancer patients with or without T1DM for therapeutic benefit.

Legal entity responsible for the study: R. Baral.

Funding: University Grants Commission, New Delhi, India. Indian Council of Medical Research, New Delhi, India.

Disclosure: All authors have declared no conflicts of interest.

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214P Location matters: Oral cancer cells at the tumor invasive border that express GARP exclude immune cells

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Otolaryngology, Head and Neck Surgery Department, Amsterdam UMC - Vrije University Medical Centre (VUmc), Amsterdam, Netherlands

Background: Failure to respond to anti-Programmed Death receptor-1 (PD-1) treatment has been linked to high levels of transforming growth factor (TGF)-β in the tumor microenvironment (TME). Since TGF-β requires activation to be functional, we evaluated the expression of the receptor glycoprotein-A repetition predominant (GARP), which facilitates TGF-β activation, on oral squamous cell carcinoma (OSCC) surgical resections.

Methods: Immunohistochemistry (IHC) for GARP and CD45 was performed on sixty-eight FFPE OSCC resection specimens. Presence (negative vs. positive) and expression pattern (diffuse or marginal) were determined. Expression patterns were linked to patient overall survival. Gene expression profiling was performed, using a Tumor-signaling (TS)360 panel, on RNA isolated from GARP negative, -diffuse and −marginal OSCC specimens (n=4 each), matched for clinicopathological features.

Results: We observed poor clinical outcome when OSCC expressed GARP on the outer rim of tumor islands located at the invasive tumor border (marginal localization) compared to tumors that lacked GARP expression or diffusely expressed GARP throughout the tumor. Gene expression analysis revealed that GARP-marginal tumors beside expressing more TGF-β1 and GARP, had enhanced expression of genes regulating "tumor-promoting inflammation", "NF-κB signaling" and "Epithelial-to-Mesenchymal transition". Based on the expression data, GARP-marginal tumors displayed increased CD45 immune cell infiltration, with myeloid cells having the most abundant cell scores. Quantifying CD45 in consecutive sections from GARP IHC revealed that while CD45 was present in the TME of GARP-marginal tumors in similar levels as GARP-negative tumors, significantly fewer CD45+ cells were able to penetrate GARP-marginal tumor islands compared to GARP-negative tumor islands within the same tumor specimen.

Conclusions: Our data suggest that infiltrating oral cancers utilize the GARP/TGF-β axis to support a pro-tumor TME and exclude infiltration of immune cells within tumor fields.

Legal entity responsible for the study: The authors.

Funding: Has not received any funding.

Disclosure: All authors have declared no conflicts of interest.

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215P Proteogenomics to guide neoantigen discovery in non-small cell lung cancer

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Background: Targeted Immunotherapy is entirely dependent on the identification of suitable TAAs or even better, of TSAs, also referred to as neoantigens. This is currently an extremely challenging task, mainly based on using experimental data from DNA and RNA sequencing technologies to generate large lists of neoantigen candidates, followed by heavy bioinformatics to predict how well each neoantigen candidate would progress through the MHC processing and presentation pathway. Up till now, most of the candidates generated in this way fail to elicit any relevant immunogenicity. The vast majority of candidates likely fail because, being based on single point mutations (as most of them are with the current state-of-the-art), and thus single amino acid variants (SAAVs), they are not that different from the respective non-mutated self-antigens tolerated by the immune system. Thus, very likely there is no T cell clone available capable of recognizing the putative neoantigen. Other problems could be due to intricacies of the MHC processing and presentation pathway, such as absence of a suitable HLA type able to present the putative neoantigen.

Methods: HiRIEF LC-MS, RNA-seq, DNA-seq (+ gene panel seq), bioinformatics pipeline (python and R).

Results: We used our proteogenomics-based pipeline to find potential neoantigens in a cohort of 141 NSCLC patients. Identified putative neoantigens were next in silico validated in 3 public NSCLC proteomic datasets and filtered based on 30 normal-tissue proteomic datasets to exclude unannotated normal peptides. After MHC-I binding prediction, our analysis revealed a list of high-confidence neoantigen candidates which will be subsequently validated in vitro and in vivo.

Conclusions: We identified a list of high-confidence non-canonical peptides in the cohort of 141 NSCLC patients which will be further validated to choose most promising candidates to advance into clinic.

Legal entity responsible for the study: Lehtio group (ONKPAT, Karolinska Institutet).

Funding: Karolinska Institutet, Scilifelab.

Disclosure: The author has declared no conflicts of interest.

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1439 TERMINALLY EXHAUSTED CD8$^+$TILS PROMOTE AGGRESSIVE CANCER STEM CELLS WHILE CONCURRENTLY EVADING ANTI PD1 THERAPY

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Background Tumor-infiltrated CD8$^+$T cell (TIL) heterogeneity is serving as one of the major hurdles in successful PD1 therapy. According to recent reports, among two subpopulations of exhausted CD8$^+$TILs, (progenitor-exhausted, CD8$^+$T$_{PEX}$; terminally-exhausted, CD8$^+$T$_{TEX}$), CD8$^+$T$_{TEX}$ do not respond to anti-PD1 therapy.[1–3] However, functional status of intra-tumoral-CD8$^+$T$_{TEX}$ remains elusive. Whether and how they participate in tumor advancement holds immense clinical importance. Given the prominence of Cancer Stem Cells (CSCs) in establishing metastatic cancer progression by evading therapies, we became interested to study CD8$^+$T$_{TEX}$ behaviour in terms of CSC regulation.

Methods CD8$^+$T$_{TEX}$ (Lin$^-$PD1$^+$TCF1$^-$) and CSC (Lin$^-$CD44$^+$CD24$^-$CSCs) frequency and their co-relation in regards to tumor advancement were analysed in human carcinomas (n=33; from 22 breast and 11 ovarian carcinoma patients). Furthermore, MACS-isolated CD8$^+$T-cells from human-PBMC or murine-spleenocytes were repeatedly exposed to tumor-lysate and tumor-supernatant in presence of antigen-loaded DCs for 120h to obtain CD8$^+$T$_{TEX}$ (PD1$^+$TIM3$^+$TCF1$^-$CXCR5$^-$IFNγ^{low}) in-vitro. These CD8$^+$T$_{TEX}$ were co-cultured with MCF7, MDAMB-231 and 4T1 cells respectively to study the influence of CD8$^+$T$_{TEX}$ on CSCs. RT-PCR, colony-formation assay, matrigel-invasion-assay, tumor-sphere assay and in-vivo tumorigenicity assay with Crl:NU-Foxn1nu athymic nude mice were utilized to characterise CD8$^+$T$_{TEX}$ influenced CSCs. ELISA, Western-blot, flow-cytometry, immune-staining, pharmacological inhibition or genetic knockdown by in-vitro and in-vivo si-RNA silencing were used to study mechanism behind CD8$^+$T$_{TEX}$- CSC cell-interaction.

Results Screening of human primary tumors disclosed that CD8$^+$T$_{TEX}$ cells remain strongly enriched across cold (low-TIL frequency) advanced-carcinomas, compared to hot (high-TIL frequency) advanced-carcinomas ($p<0.001$). Additionally, CD8$^+$T$_{TEX}$ cells positively correlated with CSC frequency ($r = 0.8809$) throughout cold-advanced carcinomas; suggesting their interdependency on tumor advancement. Furthermore, in-vitro co-culture assay as well as in-vivo adoptive transfer of CD8$^+$T$_{TEX}$ resulted in increment of intra-tumoral CSC frequency. However, this upregulation was not brought down by anti-PD1 therapy ($p<0.001$). Additionally, CD8$^+$T$_{TEX}$-influenced-CSCs exhibited increased tumorigenic and metastatic potential in athymic-nude mice. They showcased invasive and migratory phenotype with long invadopodias by overexpressing CXCR4, MMP7 and Cofilin (figure 1). These CSCs remained sustained by overexpressing OCT4, SOX2, KLF4 and NANOG. Involvement of LAMP3/NRP1-VEGFR2 axis in CD8$^+$T$_{TEX}$-CSC crosstalk was also observed (figure 2).

Conclusions Cumulative results counsel against indiscriminate use of anti-PD1 therapy. Rather a prior screening of CD8$^+$TIL and CD8$^+$T$_{TEX}$ frequency in carcinoma patients would be beneficial. Additionally, LAMP3, NRP1 and VEGFR2 could be utilized as prospective therapeutic targets against CD8$^+$T$_{TEX}$-influenced aggressive CSCs at advanced carcinoma patients with cold-tumor stroma.

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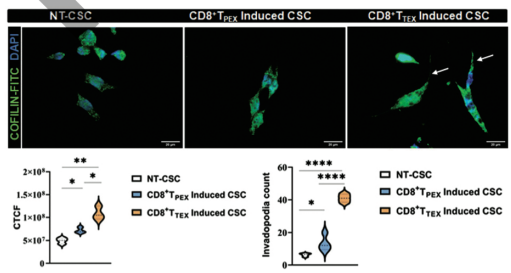
Ethics Approval All human and animal experiments were approved by Institutional Human and Animal Ethical Committee of Chittaranjan National Cancer Institute, Kolkata, India. Approval numbers are CNCI-IEC-RB-2019-6, IAEC-1774/RB-15/2017/2 and IAEC-1774/RB-19/2017/15. All patients included gave informed consent before taking part in the study.



Abstract 1439 Figure 1 CD8+TTEX induce invasive CSC generation Representative immune-fluorescence micrographs at 100x magnification for CSCs stained with Cofilin-FITC. Invadiopodias are demarked by white arrows. Corrected total cell fluorescence intensity (CTCF) and number of invadopodias were quantified and displayed as violin graphs (mean± SD). Statistical alpha value was calculated from unpaired t-test (n=4).

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P1255 TUMOR-ASSOCIATED MONOCYTIC MYELOID DERIVED SUPPRESSOR CELLS IS A POTENTIAL PROGNOSTIC BIOMARKER, PROMOTING MULTI-DRUG RESISTANCE IN NHL PATIENTS BY MODULATING IL-6/IL-10/IL-1B AXIS

Topic: 20. Lymphoma Biology & Translational Research

Sukanya Dhar[1], Mohona Chakravarti[1], Saurav Bera[1], Nilanjan Ganguly[1], Shayani Dasgupta[1], Anirban Sarkar[1], Akata Saha[1], Saptak Banerjee[1], Bhaskar Saha[2], Rathindranath Baral[1], Kalyan Kusum Mukherjee[3], Anamika Bose[1]

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Background:

Non-Hodgkin-Lymphoma (NHL), the most prevalent hematologic malignancies in the world has majorly originates from B cells. R-CHOP represents the new-standard treatment regimen for NHL. Most NHL patients initially respond to chemotherapy yielding complete response rates of 40–50%. Unfortunately, a substantial population of patients undergo relapse, resulting in poor clinical ramifications. The onset of NHL relapse evolves from several months (early relapse) to years (late relapse) after the initial remission. However, the majority of relapse occurs within two years of initial treatment. Despite considerable advancements in therapeutic concepts and techniques, disease relapse with limited response rate remains a major challenge and depicts poor prognosis in successful clinical management. Patient's response to chemotherapy varies widely from static disease to cancer recurrence and the later is primarily associated with generation of multi drug resistance (MDR) phenotypes that ultimately promotes disease progression and metastasis. However, the causes of differential responses to standard chemotherapeutic regimens and therapy failure in NHL patients are yet to be elucidated.

Aims:

To understand the influence of immune cells in differential response in NHL patients following R-CHOP-therapy.

Methods:

Peripheral blood was collected from 51 $CD20^+$ NHL patients before and after frontline chemotherapy and at the time of relapse. Clinical variables at diagnosis (age, performance status, stage of the disease, number of extra nodal lesions) were obtained to calculate prognostic indices (IPI). A panel of immune cells $CD4^+$ T cells, $CD8^+$ T cells, Cytotoxic T cells, $CD8^+CD45RO^+CD45RA^+$ Memory T cells, $CD14^+CD80^+$ M1 macrophage, $CD4^+CD25^+FoxP3$ regulatory T cells(Treg), $CD33^+CD11b^+CD14^{-/+}CD15^{-/+}$ Myeloid Derived Supressor Cells(MDSCs), $CD14^+CD163^+$ Tumor associated Macrophages (TAM), MDR phenotype P-gp(ABCB1) and MRP1(ABCC1) were studied by flow-cytometry at different phases of treatment. *In vivo* and *in vitro* doxorubicin resistance model were developed with murine Dalton's lymphoma and Raji (B cell), Jurkat cell (T cell) lines respectively and impact of responsible immune cells on generation of drug resistance were studied by RT-PCR, qPCR, flow-cytometry, colorimetric assay, gene silencing and ChIP assays.

Results:

A strong positive correlation between elevated levels of $CD33^+CD11b^+CD14^+CD15^-$monocytic MDSCs, but not $CD33^+CD11b^+CD14^-CD15^+$ granulocytic MDSC and MDR was depicted in non-responder patients compared to responder cohorts. Moreover, *in vitro* supplementation of MDSCs in murine or human lymphoma culture increases the expression of *mrp1*, *pgp* and cellular GSH level from early passage than passage without MDSCs, which is

Abstract Book Citations: Authors, Title, HemaSphere, 2022;6:(S3):pages. The individual abstract DOIs can be found at https://journals.lww.com/hemasphere/pages/default.aspx.

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ESMO Immuno-Oncology Congress 2022: Merit Travel Grant information

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Dear Mr. Anirban Sarkar,

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We wish you a successful participation and a pleasant stay in Geneva!

Kind regards,
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