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Role of GSK-3 and T-bet in anti-tumor immunity

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Ce mémoire intitulé

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Résumé

Le facteur de transcription T-bet joue un rôle central dans la régulation de la différenciation des lymphocytes T. La protéine tyrosine kinase, la glycogène synthase kinase 3 (GSK-3), inhibe l'activation des lymphocytes T et contrôle l'expression de leurs récepteurs inhibiteurs PD-1 et LAG- 3. Bien que l'inhibition de GSK-3 puisse augmenter l'expression de T-bet, l'interrelation entre T-bet et GSK-3 dans l'immunité tumorale est inconnue. Dans cette étude, nous montrons que les souris knock-out T-bet (Tbet - / -) sont compromises dans leur capacité à contrôler la croissance des cellules tumorales du mélanome B16. Cependant, l'injection d'une petite molécule inhibitrice (SMI) de GSK-3 inverse cette condition compromise entraînant le contrôle de la croissance tumorale similaire à celle observée chez les souris de type sauvage. Un examen de Tbet - / - a montré une perte de cellules dendritiques (DC) et de cellules leucocytes polymorphonucléaires (PMN) potentiellement suppressives et de lymphocytes tumoraux T (TILs) CD4 +accompagnée d'une augmentation de cellules T CD8 +. L'analyse viSNE (avancé tSNE) a en outre montré une réduction de la population effectrice expérimentée à l'antigène dans les TILs CD8 + chez Tbet -/-. Cette population est marquée par la réduction de CD44. L'inhibition de GSK-3 n'a montré aucun effet sur la perte de DC, TILs CD4 +, PMN et les TILs CD8 + ainsi que l'expression de Granzyme B (GZMB) sur les cellules T CD8 +. La seule exception était une augmentation mineure néanmoins statistiquement significative du facteur de transcription Eomesdermin (Eomes) dans les TILs CD8 +. L'étude démontre un effet compensatoire inattendu de l'inhibition de GSK-3 sur laperte de T-bet. Il reste à élucider la nature complète du parcours de cette compensation.

Mots-clés : T-bet, Glycogène synthase kinase 3 (GSK-3), CD4+, CD8+, petite molécule inhibitrice (SMI).

Abstract

The transcription factor T-bet plays a central role in regulating T-cell differentiation, while the protein tyrosine kinase, glycogen synthase kinase 3 (GSK-3) inhibits T-cell activation and controls the expression of inhibitory receptors PD-1 and LAG-3 on T-cells. Although GSK-3 inhibition can increase T-bet expression, the inter-relationship between T-bet and GSK-3 in tumor immunity is unknown. In this study, we show that T-bet knock-out (Tbet-/-) mice are compromised in their ability to control the growth of the B16 melanoma tumor cells. However, the injection of a small molecule inhibitor (SMI) of GSK-3 reverses this compromised condition resulting in the control of tumor growth similar to that seen in wild type mice. An examination of Tbet-/- showed a loss of dendritic cells (DC) and potentially suppressive polymorphonuclear leucocytes (PMN) and CD4+ cell tumor infiltrating lymphocytes (TILs) accompanied by an increase in CD8+ cells. viSNE analysis (advanced tSNE- t-Distributed Stochastic Neighbor Embedding) further showed a reduction of antigen experienced effector marker CD44 in CD8+ TILs in Tbet-/-. GSK-3 inhibition showed no effect on the loss of DCs, CD4+ TILs or the presence of PMNsor CD8+ T-cells or the loss of Granzyme B (GZMB) on CD8+ cells. The one exception was a minor but statistically significant increase in the transcription factor Eomesodermin (Eomes) in CD8+ TILs. The study demonstrates an unexpected compensatory effect of GSK-3 inhibition on the loss of T-bet.The full nature of the pathway that accounts for this compensation remains to be elucidated.

Keywords: T-bet, Glycogen synthase kinase 3 (GSK-3), CD4+, CD8+, small molecular inhibitor (SMI)

Table des matières

Liste des figures

Liste des sigles et abréviations

APC: Antigen Presenting Cell

Ag: Antigen

AP1: Activator Protein 1

APC: Adenomatous Polyposis Coli

ARG: Arginase

ADCC: Antibody Dependent Cell Mediated Cytotoxicity

AI: Aluminium

AKT: Ak strain transforming

BCR: B Cell Receptor

CTLA4: Cytotoxic T- lymphocyte- Associated protein 4

CTL: Cytotoxic T- Lymphocytes

CD: Cluster of Differentiation

CCL: C-C motif Chemokine Ligand

CAM: Cell Adhesion Molecule

DMEM: Dulbecco's Modified Eagle Medium

DC: Dendritic Cell

ECM: Extra-Cellular Matrix

EMT: Epithelial-Mesenchymal Transition

ERK: extracellular signal-regulated kinases

FCS: Fetal Calf Serum

FasL: Fas Ligand

FACS: Fluorescence-Activated Cell Sorting

FOXP3: Forkhead box P3

GZMB: Granzyme B

GM-CSF: Granulocyte- Macrophage Colony-Stimulating Factor

GSK-3: Glycogen Synthase Kinase-3

GF: Growth Factor

GITR: Glucocorticoid-Induced Tumor necrosis factor Receptor

HSC: Hematopoietic Stem Cell

HVEM: Herpesvirus entry mediator

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Ig: Immunoglobulin

IR: Inhibitory Receptor

Ig SF: Immunoglobulin Super Family

ITIM: Immunoreceptor tyrosine-based inhibitory motif

IL: Interleukin

IFN: Interferon

iNOS: Inducible Nitric Oxide Synthase

LAG-3: Lymphocyte Activation Gene-3

LAMP-1: Lysosomal-associated membrane protein 1

LPS: Lipopolysaccharide

MHC: Major Histocompatibility Complex

MALT: Mucosal Associated Lymphoid Tissue

MDSC: Myeloid Derived Suppressor Cells

MICA: MHC class I polypeptide–related sequence A

MMP9: Matrix metallopeptidase 9

MEK: MAPK/ERK kinase

NKG2D: Natural killer group 2 member D

NET: Neutrophil Extracellular Trap

NFAT: Nuclear factor of activated T cells

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NR4A2: Nuclear Receptor Subfamily 4 Group A Member 2

PMN: Polymorphonuclear leucocytes

PD1: Programmed Cell Death-1

PDL1: Programmed cell Death Ligand-1

PBS: Phosphate buffered saline

PTEN: Phosphatase and tensin homolog

PI3K: Phosphatidylinositol-3-Kinase

RBC: Red Blood Cell

RPM: Revolutions per minute

RAF: Rapidly Accelerated Fibrosarcoma.

SMI: Small molecule inhibitor

STAT: Signal transducer and activator of transcription

SCF: Stem Cell Factor

TF: Transcription Factor

TSC2: Tuberous Sclerosis Complex 2

TCR: T Cell Receptor

Th: Helper T cells

Tc: Cytotoxic T cells

Treg: Regulatory T cells

Teff: Effector T cells

TIM: T cell immunoglobulin and mucin domain-containing protein

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TNF SF: Tumor Necrosis Factor Super Family

TNFR: Tumor necrosis factor receptor

TAM: Tumor Associated Macrophages

TCF: T Cell Factor

TST: Tissue Specific T cells

TNF: Tumor Necrosis Factor

TILs: Tumor Infiltrating Lymphocytes

TME: Tumor Micro-environnement

TAN: Tumor Associated Neutrophils

TRAIL: TNF-related apoptosis-inducing ligand

VCAM: Vascular Cell Adhesion Molecule

I would like to dedicate this thesis to my daughter, my family and my friends who gave me all the love and the support I needed to complete my degree.

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Chapter 1 – Introduction

1.1 Tumor Immunology

Tumor which is also termed as neoplasm is defined as an abnormal mass of tissue which is formed when the cell grows and divides more than it should and do not die when it is supposed to. Tumors can be either benign which is not cancerous or malignant which is cancerous. Unlike benign tumors, malignant tumors acquire the property of metastasis which means to spread to the other parts of the body through blood and lymph system (1, 2, 3).

Cancer which is a complex disease is influenced by genetic and epigenetic modifications in the tumor cells and is also based on the cross talk between tumor and its microenvironment (1). Rudolf Virchow in the year 1863 predicted that there must be a hidden connection between inflammation and cancer. The cancers are classified based on origin, structure and histopathological features of tumor cell, mutational behaviour and signature genesof tumor, de-regulated molecular pathways of tumor, and also cancer invasion and metastasis (1).

A theory was proposed by Paul Ehrlich that host might be having a specialised defense system to fight the tumor cells (2); Lewis Thomas said that the humoral immune system might be able to recognize newly arising antigens from tumors (3). The widely recognised process resulting in tumor cell invasion and metastasis is termed as Epithelial-Mesenchymal Transition (EMT) (4-6). Both innate and adaptive immune systems play an important role in the modulation of metastatic cascade either positively or negatively (7).

Immune system activity in tumor micro environment is based on two concepts: immunosurveillance and immunoediting (8-10). Frank MacFarlane Burnet proposed the theory of cancer immunosurveillance (11, 12). Immunosurveillance mainly focuses on the host protection during the early stages of cellular transformation. Immunoediting is a concept where the immune system tries to protect the host and also gradually edits the tumor immunogenicity leading to 3 different phases such as cancer elimination, equilibrium and escape (13, 14).

The elimination phase mainly goes with the immunosurveillance concept where the innate and adaptive immune system components try to eliminate the transformed cells (15, 16). Equilibrium phase allows the cell variants which remain in a dormant state to escape from the elimination phase with immune ignorance (14).

Escape phase mainly results in escape of tumor cells from immune system by developing the mechanisms resulting in the loss of cognate antigen expression and gaining resistance to immune responses (12, 17). The immune cells such as natural killer cells (NK) and CD8+ T cells which are cytotoxic in nature eliminate the cancer cells which are more immunogenic in nature. This response is during early stages of tumor development (18). The less immunogenic cancer cells proliferate to clinically detectable tumors and this will impact the different subsets of inflammatory cells on tumor fate (19, 20).

A lot of study and research is being carried out to unveil the concepts of interaction between immune cells and cancer cells in tumor microenvironment (TME) (21).

1.2 Immune system

The interactive network of cells, lymphoid organs, cytokines and humoral factors is known as immune system. The main function of the immune system is to recognize the foreign antigens and eradicate them. The role of the immune system in host defence is best explained when it goes wrong; overactivity resulting in allergic and autoimmune diseases and underactivity in the severe infections and tumors (22-24).

The subtle biologic capacity of the human immune system to distinguish between self and non self and to keep the memory is achieved through the process of clonal selection. This kind of sophisticated ability is possible based on different immunoglobulins (with distinct gene rearrangement) and T cell receptors (TCRs) and the ability of T cells to recognise foreign antigens presented by MHC (Major Histo Compatibility) complexes (25-27).

1.2.1 Types of Immunity

Immunity is mainly 2 types based on specificity and speed of the reaction. They are innate immunity and adaptive/acquired immunity. The main differences between these two are:

- Antigen specific recognition system (adaptive immunity) (26),
- Lack of immunological memory (innate immunity) (22).

Innate immunity which is antigen non-specific defense system reacts immediately within several hours of encounter. This type mainly includes physical (fatty acids, epithelium, cilia and mucus, microbiological and chemical barriers, with all the non-specific immune cells like the phagocytes (monocytes, macrophages, dendritic cells), polymorphonuclear leukocytes (PMNs) (which include neutrophils, basophils, eosinophils). Complement cascade, chemokines (which induce leukocyte migration), cytokines (which modulate leukocyte function), acute phase proteins, natural killer cells (NK) are also included in this category (28-30).

Adaptive immunity mainly is antigen specific and involves B (humoral responses) and T (cell mediated responses) lymphocytes. It mainly requires antigen processing, recognition of antigenic epitopes by B and T cell resulting in their expansion and differentiation into effector/memory cells (23, 31, 32).

1.3 Origin of Immune Cells

Immune cells mainly include cells of innate and adaptive immunity.

Host defence system ontogeny starts with the hematopoietic stem cells (HSCs) present in the yolk sac during the first month of gestation. Haematopoiesis mainly occurs in bone marrow. HSCs differentiate into different cell types like granulocytes, agranulocytes as well as megakaryocytes and erythrocytes (33, 34). Lymphocytes mainly include B and T cells, given suchterminology based on their sites of maturation. T lymphocytes migrate from bone marrow to thymus for maturation unlike B lymphocytes which remain in bone marrow for their maturation where they recombine their immune receptors (22, 35, 36).

T cells which originate from the bone marrow migrate into the thymus gland for maturation and differentiation into different subtypes and also differentiate even after leaving the thymus (37-39). All the T cells are distinguished based on the receptors present on their cell surface. They are mainly involved in cell mediated immunity. They have no function until they encounter antigen (Ag) and co stimulatory molecules induced by innate immune cells. There are different types of T cells like CD8, CD4, Treg (regulatory T cells), memory T cells, natural killer T cells, mucosal associated invariant T cells (40, 41).

CD8+ cells also called as killer cells (cytotoxic T-cells) are involved in direct killing of cancer cells and clearance of viral infections (42-44). They can also take the help of various signalling proteins called cytokines to recruit other cells while mounting an immune response. CD4+ cells are called helper cells which indirectly kill the foreign cells and also utilize the cytokines for influencing regulatory B cells directly and other cells indirectly (45-46). Treg also called as suppressor cells which are mainly involved in Tolerance (mechanism adapted by T cells to prevent autoimmune diseases) (47-49). T cells have TCRs which specifically recognise the Ag present in the protein of pathogens. At resting phase, they are generally small rounded with dense nucleus and little cytoplasm (23, 26, 41).

1.4 Lymphoid organs

The B and T cells developing from the primary lymphoid tissues which are bone marrow and thymus respectively undergo gene rearrangement. At this stage, they are considered as naïve which means that they have still not encountered specific antigen within an immune response (50, 51). These cells populate the secondary lymphoid tissues which are the sites of promoting interaction of naïve B and T cells to a foreign particle which mainly include lymph nodes, spleen, tonsils, and mucosa associated lymphoid tissue (MALT) (35, 52, 53).

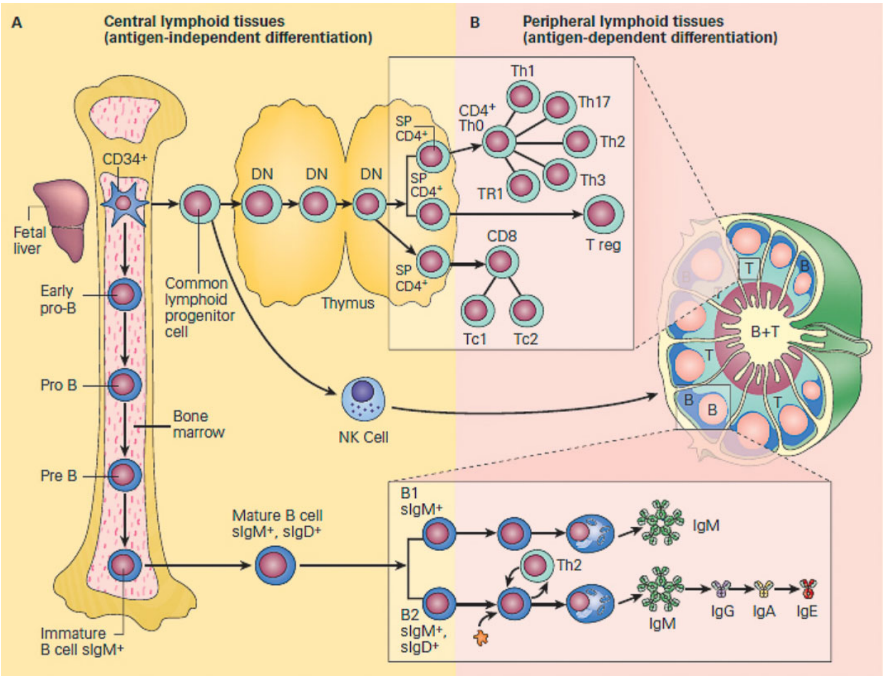


Figure 1. – T and B cell development (https://www.immunopaedia.org.za/immunology/basics/2-ontogeny-of-the-immune-system/)

Panel A shows the development of immune cells from hematopoietic stem cells in bone marrow and fetal liver and their differentiation independent of antigen in central lymphoid tissues. **Panel B** shows the migration of cells into peripheral lymphoid tissues where they differentiate after the encounter with the antigen.

1.4.1 Central Lymphoid Organs: Growth and Differentiation

Lymphopoiesis occurs in central lymphoid organs which are bone marrow for B cells and thymus for T cells. Lymphocyte precursors mainly occur in bone marrow like all hematopoietic cells. B cells complete their development in bone marrow (in fetus, B cells also develop in liver) but T cells migrate to thymus and some T cells are destined to form specialised populations within the gut epithelium which may develop directly in sites called crypto patches located under the intestinal epithelial crypts (54-56). Antigen specificity of each cell is determined early in its differentiation, when assembling of the DNA sequences encoding the variable regions of immunoglobulin like structures in their receptors (B cell receptor, BCR and T cell receptor, TCR) occurs from gene

segments (57-59). In the next developmental stage, the antigenic properties of the receptor are checked for its specificity in its immediate environment (22, 52, 53). The specificity and affinity of these immature cells decides their fate to either survive or die before maturity. Next step is positive selection of cells which bind weaklyto self-antigens and negative selection in which they receive death signal. Immunological tolerance to self-antigens is established which results in the elimination of auto reactive lymphocytes which are responsible for autoimmune diseases (47, 48). Most of the cells die either in the primary lymphoid organs or before completing maturation in secondary lymphoid organs (26, 35).

Only few survive and undergo clonal expansion (60, 61). B cells in bone marrow undergo rearrangement of their Immunoglobulin (Ig) genes with the help of bone marrow stromal cells and acquire receptors of cell surface IgM which can now interact with the antigens in the environment to undergo selection. Then they migrate to peripheral lymphoid organs for maturation. BCR and TCR are generated in central lymphoid organs independent of antigens. T cell can develop into 2 cell lineages- α: β and γ: δ based on intrinsic and extrinsic signals of the cell and also Notch and MAPK/ ERK signalling pathways. They are T cell receptor (TCR) types and defined based on their molecular programs (62-64). During the developmental stages, Ag receptor gene rearrangement occurs and results in their protein expression and also involves changes in the expression of other cell surface/intracellular proteins (65, 66). The non-lymphoid stromal cells are important in providing signals in the form of growth factors or receptor binding molecules which bind to the lymphocyte receptors (41, 67).

Bone marrow stromal cells are important for the development of B cells. So, in in vitro, bone marrow stromal cells are added as they provide soluble growth factors (GFs) which control lymphocyte proliferation and differentiation (68, 69). B cells grow in bone marrow which are stimulated by stem cell factor (SCF), which is a cytokine present on the stromal cells, Vascular cell adhesion molecule-1 (VCAM1) and Cell Adhesion Molecules (CAMs) which are the adhesion molecules interacting with the cell surface receptor tyrosine kinase kit (CD117) on B cell precursor (27, 70, 71).

1.4.2 Peripheral Lymphoid Organs: Maturation

Maturation of B cells occur in spleen, where they acquire immunoglobulin (Ig) like IgD in addition to IgM (72, 73). In these peripheral lymphoid tissues, they may encounter foreign antigen and get activated. CD45Rand CD19 are there through the development which has role in BCR signaling (72). CD43 is a cell surface protein on pro B cell and acts as adhesion molecule b (27, 36, 74).

Naïve T cells from the central lymphoid organs enter into the peripheral lymphoid organs. They acquire certain receptors named as peripheral node addressins which allow them to enter the lymph nodes by binding to the cell adhesion molecules present on the endothelial venules of the lymph nodes (75). T cells in these organs interact with cognate antigen (Ag presented by antigen presenting cells (APCs)) and are activated within 2-3 days. Ag recognition is different for Helper T cells and Killer T cells (25, 26, 40).

Innate immune cells like dendritic cells which act as antigen presenting cells (APCs) engulf the pathogens and migrate to the lymphoid tissue with the goal of initiating adaptive immune response by triggering T and B cells (28, 76).

1.5 Tolerance

Discrimination of self from non self is generally acquired during T and B cell ontogeny and maturation (77, 78). The inability to react to the self which means that the host lymphocytes are not activated when encountered with the self-tissues and cannot exert effector functions is termed as tolerance. Tolerance mainly includes central tolerance which means inability to react to self during B and T cell ontogeny and peripheral tolerance which means inability of mature T and B cell to react to self. If such tolerance is lost, it leads to autoimmune diseases and host tissue damage (26, 79, 80).

1.5.1 Mechanisms of Tolerance

Lymphocytes during their early stages of development which involves TCR re-arrangements tend to develop receptors specific to the self-antigens (65, 66). Such cells receive death signals and are eliminated via apoptosis, a process called as clonal deletion (81, 82). Some of the CD4+ T cells in

thymus receive certain signals which help them to differentiate into CD4+ T cell subset, a process known as clonal diversion. These subsets are called as natural regulatory T cells (nTreg) which express a transcription factor known as Forehead box P3 (FOXP3) (83-86). Another mechanism for tolerance is "Receptor editing" by which TCR specificity is altered to avoid the self-reactive T cells which can bind to the self-antigens and cause autoimmune diseases (87, 88). All these mechanisms are a part of central tolerance.

Lymphocytes which receive survival signals would leave thymus and enter the periphery. But unfortunately, few T cells which are reactive to the self-antigens also escape into periphery (89, 90). Such T cells are either deleted or subjected to state of anergy where they cannot respond to antigens by the peripheral tolerance. This type of tolerance is operated through one of the following 3 mechanisms (89).

- Inducing the state of anergy which means T cells are functionally inactive and cannot elicit any immune responses (77).
- Destruction of the T cells (which are reacting to the self-antigens) via apoptosis by inducing death signals (90).
- Stimulating the T cells to differentiate into induced regulatory T cells (iTreg) by transforming growth factor-β (TGF-β) in the peripheral tissues (49, 91, 92).

1.5.2 Regulatory T cells (Tregs) in tolerance

Induced Tregs and natural Tregs are similar in their immunosuppressive effector function except for the fact that they are developed at different sites (86, 91). This subset of CD4+ T cells suppress immune cells based on the site and type of immune response and by using either direct or indirect mechanism. They evade immune responses by transferring cAMP to conventional T cells (Tcons) or by generating immune suppressive adenosine (93). They also produce cytokines like IL-10, IL-35, TGF-β which suppress the immune cells. They block IL-2 for T cells or induce death by granzyme-perforin release which are serine proteases inducing apoptosis in the target cell (94, 95).They also block the co-stimulatory receptors (CD80 and CD86) on DCs via CTLA4 binding and affect the antigen presentation to T cells. Tregs over express inhibitory receptors (IRs) to interrupt T cell-APC

interactions and suppress immune responses of Teff (effector T cells) cells. Tregs also suppress the transcription factors like NFAT and NF-κB which are present downstream of TCR signaling (89, 96).

1.6 Co-receptors

The B and T lymphocytes definitely require 2 signals for their activation. They not only require primary signal provided by APCs but also require secondary signal obtained by co-receptors (97, 98). The reactivity of T cells to self-antigens results in auto immune responses (89). Such responses are prevented by the specialised immune cells by maintaining the homeostatic balance between Teff (effector T cells) and Treg (regulatory T cells) which is based on the role of co-signalling receptors (co-stimulatory and co-inhibitory receptors) which help in modulating autoimmune and anti-tumor immune responses (41, 99).

Bi-signaling mechanism is the main process in T cell activation and expansion. Primary signal involves binding of Ag specific TCRs on T cells to cognate antigen presented by APCs. Secondary signal is acquired from co receptors interacting with the co-stimulatory/co-inhibitory ligands expressed on APCs (102). B cells also require bi-signaling mechanism for their activation with primary signal from foreign Ag (soluble or membrane bound) and secondary signal by the CD40-CD40L interaction via T cell help (74).

Based on the structure, co-receptor signaling molecules which are the receptors on T cells can be divided into two categories (103, 104);

1) Immunoglobulin superfamily (Ig SF),

2) Tumor Necrosis Factor Superfamily (TNF SF).

Ig super family contains immunoglobulin domain and based on this extracellular domain structure, it includes CD28 (T cell specific surface glycoprotein), cell adhesion molecule PVR-like (CD155) and T-cell immunoglobulin and mucin (TIM). TNF super family has TNF homology domain (cysteine rich) and include, OX40 (CD134), 4-1BB (CD137), CD27, glucocorticoid-induced TNFR-related protein (GITR) etc., (100, 101).

Co-signaling receptors are categorised into co-stimulatory and co-inhibitory molecules based on

their function. Major co-stimulatory molecules include CD28, 4-1BB, OX40, inducible T cell co stimulator (ICOS), TNFR-II, GITR and etc, whose role in immune response are Ag priming of T cell followed by its expansion, survival, and differentiation resulting in effector functions (105). Not all these molecules are expressed on naïve T cells except CD28. The strong signal generated through primary signal (TCR signal) and secondary signal via TNF-RII, GITR, OX40 affected Treg selection and differentiation and also facilitated Treg impairment in thymus (104, 106).

Co-inhibitory receptors on the contrary, behave completely opposite to costimulatory signaling molecules and inhibit the T cell above mentioned processes. Most important co-inhibitory receptors are Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4), Programmed Cell Death 1 (PD1), T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), Lymphocyte Activating 3 (LAG3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT) to prevent over reactivity of the immune response. In fact, both signaling molecules co-ordinate with each other to maintain proper immune response (107-109). Many co inhibitory receptors are over expressed by Tregs which include PD1, ICOS, TIGIT, LAG3, TIM3, TNF-RII, and 4-1BB. Other receptors like GITR, CTLA4, OX40 are also expressed by Tregs (99, 110, 111).

Tumor-associated macrophages (TAMs) and Myeloid-derived suppressor cells (MDSCs) also express co-inhibitory receptors to supress the immune surveillance feature of the immune cells. Tumor infiltrating Tregs, TAMs and MDSCs contribute to immune suppressive microenvironment in tumor (45).

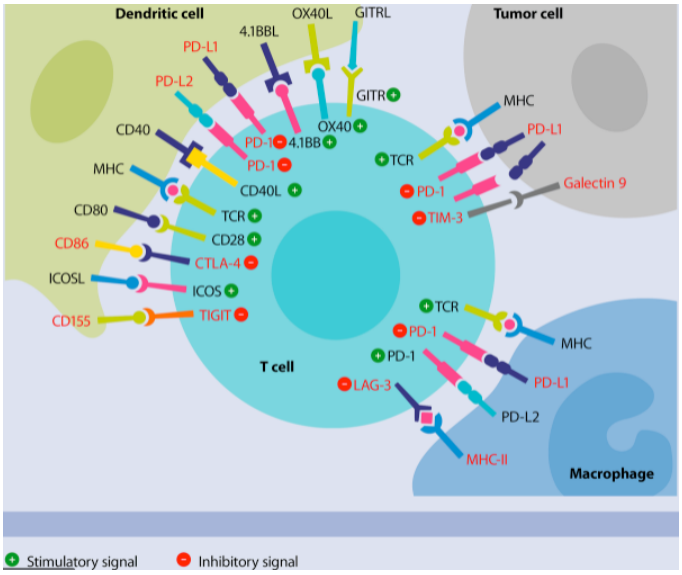


Figure 2. – Courtesy:https://www.miltenyibiotec.com/US-en/applications/flow-cytometry-applications/immunecheckpoint-analysis.html#scrollto_anchor1

Co-signaling receptors on T cells include co-stimulatory and co-inhibitory receptors which are the important regulators required to avoid auto immunity and maintain immune homeostasis. Some of these receptors are also involved in tumor progression. Co-stimulatory receptors promote T cell development and differentiation but co-inhibitory receptors behave in an opposite manner to the co-stimulatory receptors.

1.7 Transcription factors

T cell development mainly involves wide range of changes in gene regulatory network of transcription factors. Connection between TCR signalling, cytokines and transcription factors is still an important area of interest to explore (67).

1.7.1 TCF 1/7- T cell specific transcription factor

It is a transcription factor present on naïve and stem like T cells. Useful for T cell development and differentiation into memory T cell subsets. Apart from its major role in T cells, it also plays a prominent role in NK cell and innate lymphoid cell development. When complexed with β-catenin, results in transcription of Wnt target genes via Wnt/ Catenin canonical pathway. TCF1 knock out is shown to block T cell differentiation. Found on human T cell memory. TCF1 expression on human CD8+ T cells means that they have self-renewing capacity (112, 113).

1.7.2 TOX

It is highly expressed in thymus which is the home for T cell development. It is a nuclear factor which is highly expressed in tumor specific T cells (TST) and exhausted T cells. Knock out of TOX in TST abrogated T cell exhaustion. TOX expression is stimulated by chronic T cell receptor stimulation and expression of TOX, TOX2, NR4A2 and NR4A3 is induced by NFAT in the absence AP1 binding in exhausted T cells. TOX induced exhaustion program might result in prevention of activation, differentiation and effector functions of T cells in cancer cells (114, 115).

1.7.3 NFAT, AP1, NF-κB

Nuclear factor of activated cells (NFAT) are of different types like NFAT1, NFAT2, NFAT4 and contributea prominent role in adaptive immune response (116). This specific transcription factor is involved in T cell activation and also T cell exhaustion. These are activated downstream of TCR signaling via calcium signal. They use different transcriptional program in T cell activation and exhaustion (117).

Along with NFAT, the other two TFs involved in T cell activation and differentiation are AP1 (Activator Protein-1), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (118). Primary (TCR signal) and secondary signal (Co stimulatory receptors signal) result in activation/attenuation of these downstream TFs which result in either activation or suppression of T cell activation (102, 119).

1.7.4 Eomesodermin

The two main TFs which are mainly involved in adaptive anti tumoral immunity are T-bet and Eomes. They help in Tc1 differentiation and also block the alternative fates of T cell differentiation like Tc2 or Tc17 which have suppressive roles (120, 121). In absence of T-bet, TCF1 high expression upregulates the Eomes expression driving T cell differentiation to central memory phenotype (112).

1.7.5 T-bet (T cell specific T-Box transcription factor)

T-bet protein is expressed in T cells and is a master transcription factor regulator in the expression of IFNγ producing CD4+ T cells (i.e., specifically Th1 cells) and also other immune cells like cytotoxic T cells, natural killer cells, B cells and also dendritic cells (122). T-bet mainly regulates CD4+ T cell differentiation into Th1 specific cell type of T cells. It directly binds to the promoter region of IFNγ gene and activates its expression. It regulates the production of IFNγ in CD4+ and NK cells. T-bet deficient Th cells resulted in augmented expression of IL-2, which is a T cell growth factor (123). T-bet knock out mice show susceptibility to viral infections and tumor progression. One research group has shown that upon melanoma cell injection (to induce tumor formation),

there is tumor suppression leading to apoptosis of cancer cells proving that T-bet is essential for control of tumor development and metastasis by suppressing the expression of IL2 expression and inducing IFN γ production by Th1 type cells (123, 124).

It is shown in another study that T-bet and Eomesdermins are master regulators of Th1 phenotype. T-bet is found specific to Th1 cells but Eomes are more specific to CD8+ T cells and less expressed in CD4+ T cells (121). Knock out of T-bet results in increased cancer metastasis (73). Role of T-bet is known to have its regulation in innate immunity particularly Natural Killer (NK) cell function in cancer. T-bet and Eomes regulate adaptive immunity against cancer by promoting migration of CD8+ T cells enhancing IFNγ production and suppressing inflammatory IL17 production (121). Knock out of T-bet and Eomes resulted in lower expression of CXCR3 in T cells and resulted in drastic decrease of tumor infiltrating T cells T-bet/Eomes deficient tumor infiltrating CD8+ T cells resulted in increased expression of IL17 and decreased production of IFNγ (126).

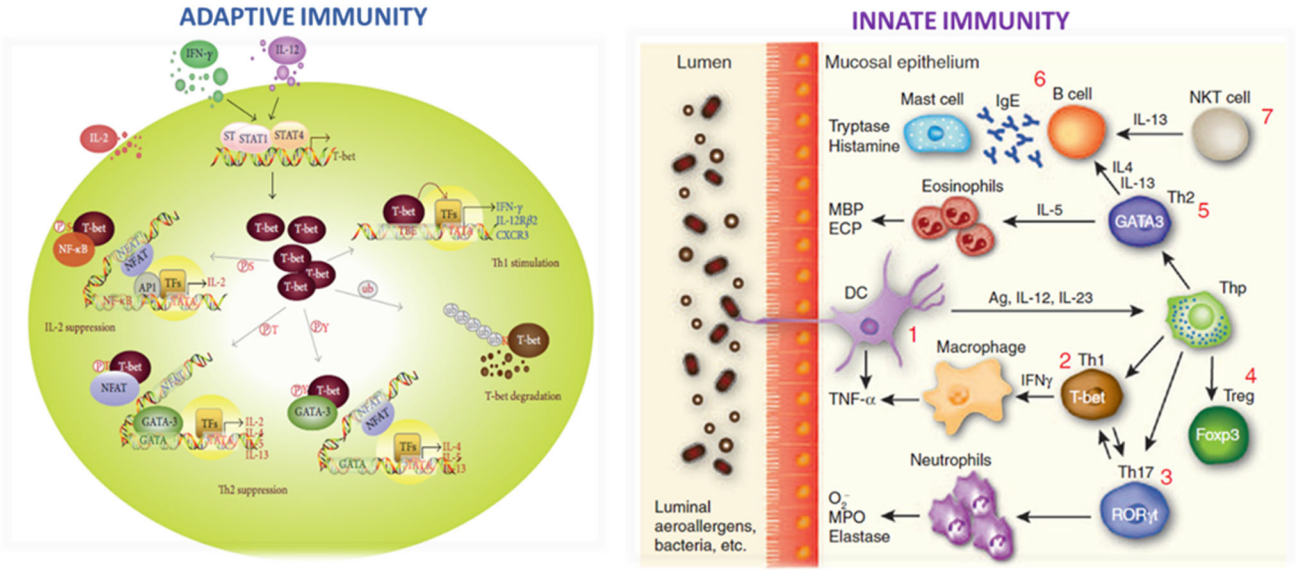


Figure 3. – T-bet role in adaptive and innate immunity (72, 74).

Multiple roles of T-bet in Th cell differentiation (adaptive immunity) and mucosal immune responses (innate immunity). T-bet is expressed by both the cell types from lymphoid and myeloid lineages. It regulates mucosal homeostasis via dendritic cells and other innate lymphoid cells

which shows its role in innate immunity and also regulates Th1 differentiation of adaptive immune system via chromatin -modifying enzymes recruitment.

1.8 GSK-3 (Glycogen Synthase Kinase-3)

GSK3 (Glycogen Synthase Kinase 3) is not only a key enzyme which regulates glycogen synthase in the glycogen metabolism pathway but also plays important role in proliferation of different immune cells, structural protein modifications, regulation of other metabolic pathways and is involved in signalling pathways. GSK-3 has emerged as a potential therapeutic target for a broad spectrum of cancer because of its role as a positive and negative regulator in cancer. GSK-3 is involved in regulating the T cell development either directly or indirectly (130).

GSK-3 comprises two isoforms of serine/threonine kinases known as GSK-3α (51 kDa) and GSK-3β (47 kDa) that are constitutively expressed and encoded by different genes (131). These isoforms share high similarity in their kinase domains (98%), but they differ in their N- and C-terminal regions (132).

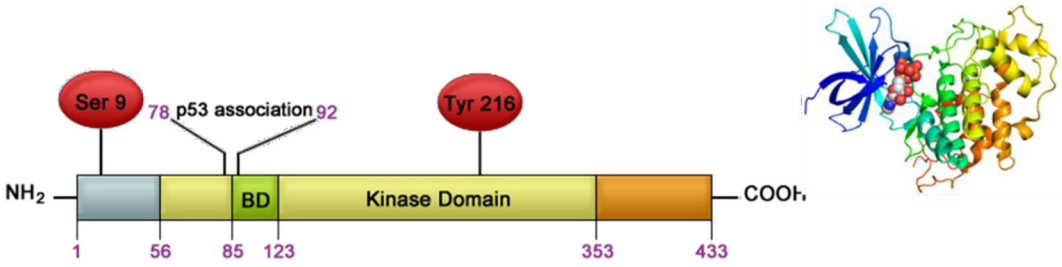


Figure 4. – Structure of GSK-3.

GSK3 has N-terminal for ATP binding and C-terminal for kinase activity and the 3D model shows that it is bilobed. C-terminal has tyrosine which is essential for GSK3 activity. It has 2 isoforms- GSK-3α and GSK-3β. Phosphorylation at tyrosine 279 in GSK-3α and phosphorylation at tyrosine 216 in GSK-3β indicates maximum activity of GSK3. It is inhibited by phosphorylation at serine 21 in GSK-3α or phosphorylation at serine 9 in GSK-3β present on N terminal.

Phosphorylation by GSK-3 affects the activity of several substrates and therefore, in a context-dependent manner, it could act as a tumor suppressor molecule and also as a tumor promoter molecule regulating proteins involved in cancerous growth (133). For instance, this kinase along with APC and Axin together, also called as destruction complex, possesses an important role in the WNT/β-catenin pathway by promoting proteasomal degradation of β-catenin, a crucial transcription factor that regulates the expression of several genes promoting cell growth (111). Downregulation of GSK-3 activity allows the translocation of β-catenin to the nucleus to interact with the T cell factor, TCF1, which in turn upregulates the proliferative capacity of T cells (113, 134).

The transcription factor NF-κB which is involved in innate immunity is also found to be regulated by GSK-3. GSK-3 can modulate NF-κB through its post-transcriptional regulation and more specifically GSK-3β was shown to influence the binding of NF-κB to different target promoters via regulation of epigenetic mechanisms (135). The GSK-3 inhibition is shown to downregulate STAT3 which is a transcription factor which bindsto MICA promoter and upregulating MICA expression which results in increasing the sensitivity to NK cell mediated cytotoxicity (136).

The effect of GSK-3 on glucose uptake and GLUT1 are mediated by mTOR/TCS2 dependent pathway. This kinase is implicated to have a suppressing effect on glucose uptake via GLUT1 expression. This mechanism is mediated by TSC2/ mTOR which is also shown to have negative regulation on glucose uptake and GLUT1 expression. Ectopic GLUT1 expression increases T cell proliferation. GSK-3 may have its negative effect on T cell proliferation via GLUT1 expression (137).

This kinase also controls many features like cancer stemness, Epithelial Mesenchymal Transition (EMT), and resistance to therapy by phosphorylating various upstream and downstream elements involved in different pathways such as PTEN/PI3K/MTOR/Akt, Ras/Raf/MEK/ERK, Notch, Hedgehog, TP53 and several microRNAs (138). GSK-3β overexpression and nuclear localization lead to TRAIL and TNFα resistance which is mediated via the anti-apoptotic factor BCL-XL (139). A study has shown that GSK-3 has the potential to modulate the ability of IFN-β for IL-10 induction (140).

In addition, GSK-3 affects several transcription factors (TF) that regulate proliferation, survival and differentiation of progenitor T cells, survival of regulatory T cells (Tregs) and memory CD8+ Tcells (141).

In T cells, the inhibition of GSK-3 reduces their motility and increases their cytotoxicity activity (142). The use of GSK-3 inhibitors reduces the expression of PDCD1 via upregulation of T-bet and increases Granzyme B and Lamp-1 production and the cytotoxicity activity, as well as compensating the lack of CD28 stimulation (128, 143, 144). The use of GSK-3 inhibitors in combinationwith anti-PD-1 therapies increase the implanted tumors mice survival as well as a better control on the tumor growth (128). This cooperativity between these 2 approaches can be explained by the reduced transcription of LAG-3 after GSK-3 inhibition (145).

All the above information suggests that targeting GSK-3 can overcome the “exhausted” state of T cells and also promote the proliferation and differentiation of T cells in the TME to improve the antitumor immune response.

1.9 Tumor micro-environment (TME)

Tumor micro environment (TME) mainly has different types of immune cells which behave differently and may have tumor promoting or tumor antagonizing function (146, 147). Even though immunecells have tumor antagonizing function to target and kill cancer cells in the early phase of tumorigenesis, the cancer cells gradually tend to escape from the cytotoxic activity of immune cells and immune surveillance by various mechanisms (148, 149).

The main 6 hallmark capabilities of cancer include: (150)

- limitless replicative potential,
- self-sufficiency in growth signals,
- sustained angiogenesis
- evading apoptosis,

- tissue metastasis/invasion
- insensitivity to anti-growth signals

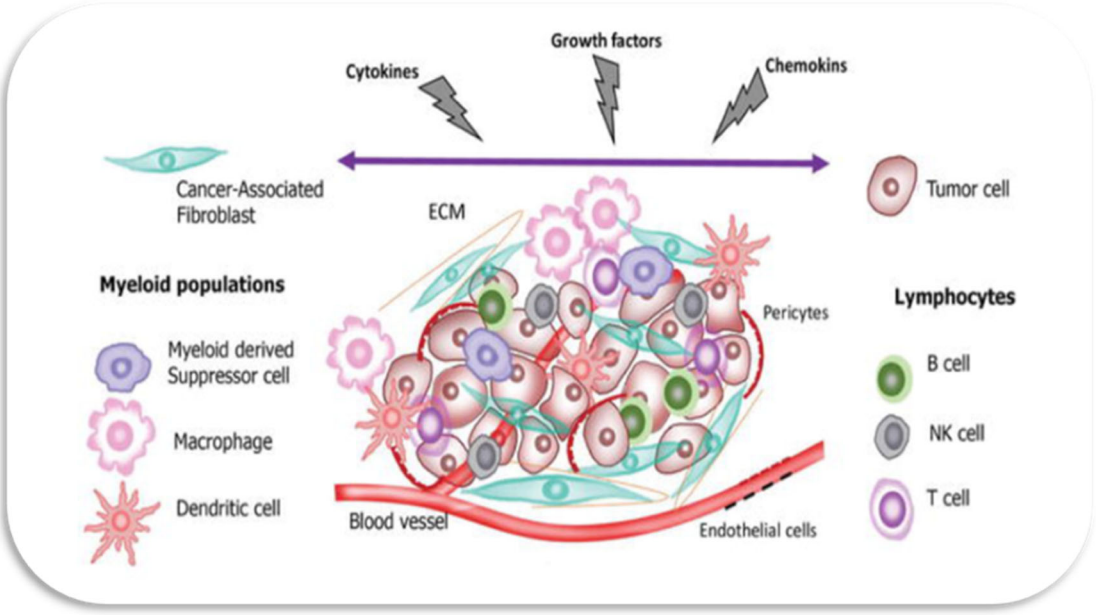


Figure 5. – Different immune cells in Tumor micro-environment (TME) (151).

TME has a complex network with mediators like growth factors, cytokines and chemokines, stromal cells such as endothelial cells, stromal fibroblasts, immune cells like lymphocytes, microglia, macrophages, tumor cells and TME components which are non-cellular like hyaluronan, laminin, fibronectin, collagen etc. The cross talk between the tumor cells and TME involves interaction with extracellular matrix or cell to cell contact or the mediators involved in this interaction.

TME not only has cancer cells but also a repertoire of cancer-associated fibroblasts immune cells, endothelial cells and stromal cells (152). A series of immunotherapy strategies were developed on the basis of immune evasion mechanisms and clinical applications were made in the past decades. Immunotherapy started gaining attention due to the immune system complexity to attack the cancer cells (153).

1.10 Tumor antagonizing immune cells

The effector T CD8+ CD4+cells (including cytotoxic T cells and effector T cells), dendritic cells (DCs), natural killer (NK) cells, N1-polarized neutrophils and M1-polarized macrophages are categorized in the tumor-antagonizing immune cells (154, 155).

1.10.1 Cytotoxic CD8+ T cells (CTLs)

T cells are studied intensively besides Tumor associated macrophages (TAMs) in various types of cancers (156, 157). Cytotoxic CD8+ T cells (CTLs) differentiate into effector CD8+ T cells whose activity is direct killing when presented with antigens from DCs with major histocompatibility complex class I molecules (MHC-I) (157-159). Activated CTLs can migrate with the influence of chemokines (such as CXCL9 and CXCL10) secreted by DCs into the inflamed milieu through the expression of CXCR3 (160, 161). CD8+ T cells priming in the secondary lymphoid organs is initiated by the interaction between the ligands (CD70 and CD80-CD86) on DCs and the receptors (CD27 and CD28) on CD8+ T cells. The CD8+ T cells also get signals from Helper T cells either through CD40-CD40L interaction which results in their proliferation or through DC activation to present tumor antigens to CD8+ T cells and activate them (162-164). CD4+ T cells also play role in differentiation of CD8+ T cells into memory CTLs (165-167).

CTLs in the “activation” state, would kill the target cells either by Fas- Fas ligand (FasL)-mediated apoptosis or by induction of granule exocytosis (granzyme-perforin) and they also induce the cytotoxicity in cancer cells by secreting interferon (IFN-γ) and tumor necrosis factor α (TNFα) (79, 158). T cell is activated by bi-signaling mechanism: the first one is from the T-cell receptor (TCR), and the second one is from other co-receptors known as immune checkpoints. The CTLs in the TME would also enter an “exhausted” state, which results in T cell dysfunction and tumor development (154).

1.10.2 Natural killer (NK) cells

Another important subset of tumor-antagonizing immune cells and innate immune system that mediate the immunosurveillance of tumor are NK cells. They account 5–15% of circulating lymphocytes and are phenotyped as CD3−CD56+ cells in humans (168, 169). The function of NK

cells within TME is cytolytic activity which is similar to the CD8+ T cells and are brought by the chemo attractants secreted by dendritic cells to the cancer tissues (170). NK cells kill the tumor mainly by releasing perforin and granzymes to induce the apoptosis of the target cells and through activation of TRAIL-FASL pathways or production of TNFa (171). Apart from above action,NK cells attack cancer cells by secreting pro-inflammatory cytokines and chemokines (including TNFa, IL-6, IFN-γ, GM-CSF and CCL5) and may promote antigen cross-presentation to CTLs (169, 172, 173). The study says that the receptor NKG2D on NK cells recognise the ligands on the tumor cells and playsan important role in Immunosurveillance (174, 175). Blocking the signals from inhibitory immunecheckpoints (e.g., PD-1, CTLA-4, NKG2A, Tim-3, TIGIT, CD96, KIR2DL-1/2/3) should restore the anti-tumor potential of NK cells (169).

1.10.3. Dendritic Cells (DCs)

DCs mainly function as professional antigen-presenting cells (APCs) and act as crosslinks between innate and adaptive immunity. They can present both endogenous and exogenous antigens and also provides second signal for T cell activation (176, 177).

They also interact with NK cells and B cells (76, 169). Active and matured DCs are infiltrated to the sites of tumor immediately and then, they activate and recruit the other immune effector cells (178). The DCs are inactivated by cancer cellsby inducing the expression of PD-1 (179, 180). Therefore, DC-based vaccines alone or in combination with the PD-1 blockades might help to treat cancer by restoring their antigen-presenting function (181).

1.10.4. Macrophages

One more important type of innate immune cells within TME are macrophages which are derived from circulating monocytes after tissue extravasation. They are generally divided into proinflammatory polarization (M1-polarized driven by LPS and IFNδ) which on activation produce pro-inflammatory cytokines and reactive oxygen/nitrogen species, which are crucial for tumor cell killing and host defense and are therefore considered as ‘good’ macrophages (182). Anti-inflammatory polarization (M2-polarized driven by IL-4 or IL-13) which in contrast, not only produce anti-inflammatory cytokines and suppress the immunosurveillance against tumor cells,

but also promote angiogenesis, lymphangiogenesis, EMT, cancer cell proliferation and ECM remodelling to promote the tumor progression and metastasis, impairment of effector T cell activity, inhibition of DCs maturation (183, 184).

1.10.5. Neutrophils

Neutrophils are another type of innate immune cells that are also found infiltrating many types of tumors which are mainly involved in defending the infections through a series of mechanisms and inducing the inflammatory response by secreting many cytokines, neutrophil extracellular traps (NETs) (185-187). Theoretically, neutrophils are considered as potent anti tumor effector cells since they secrete various cytotoxic and antimicrobial compounds (neutrophilic granules), cytokines and chemokines which can recruit other cells which have antitumor activity. They destroy tumor cells using the mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC) which is a cytolytic process. This process involves effector cells of the immune system which lyse the target cell whose surface antigens are bound by specific antibodies (188). In contrary, evidences indicate that the tumor-associated neutrophils (TANs) may help tumor progression. And similar to the antitumor (M1) and pro tumor (M2) phenotype of macrophages, TANs are divided into N1 and N2 phenotypes. The N2-polarized neutrophils are morphologically similar to granulocytic or polymorphonuclear MDSCs (PMN-MDSCs), which are tumor-promoting immune cells and N1 are anti tumor phenotype (189, 190).

1.11 Tumor promoting immune cells

Tumor-promoting immune cells mainly consisting of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (14, 154, 155).

1.11.1 Regulatory T cells

Tregs as a member of T cell family play a key role for maintenance of peripheral tolerance and immune homeostasis (191, 192). Tregs express transcription factor FOXP3 which is a reliable marker in addition to CD4+ marker on their cell surface (193, 194).The suppressive action of Tregs is based on contact dependent which involves expression of CTLA4, PD1, PDL1, LAG3, CD39/CD73

and contact independent which involves production of TGF-β, IL-10, adenosine, galectin-1, prostaglandin E2, and sequestration of IL-2 mechanisms (79).On one hand, Tregs can suppress autoimmune disease by suppressing over-reactive immune response. But on the other hand, the Tregs may prevent the effective response of CTLs, macrophages, neutrophils, CD4 T cells, NK cells in cancer cells (195, 196).

1.11.2 B cells

The tumor promoting/tumor-antagonizing role of B cells in TME is contradictory (197-199). We know that B cells also behave as efficient antigen presenting cells and produce cytokines that coordinate with cytotoxic activity of T cells (200, 201). But there are some studies showing that B cells are involved in tumor promotion by secretion of TGF β and IL-10 and also recruit MDSCs, inhibit CTL function and enhance the angiogenesis (201-203). Now the area of interest is to identify the subtypes of B cells in TME and also to find out the phenotype of B cells before and after their entry into tumor micro environment.

1.11.3 Myeloid Derived Suppressor Cells (MDSCs)

Myeloid derived suppressor cells (MDSCs) are immature cells of myeloid origin. They are granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) (204-207). The M-MDSCs are similar to M2-polarized macrophages whereas PMN-MDSCs are morphologically similar to N2-polarized neutrophils which all together come into the category of tumor promoting immune cells (208). They inhibit the effector function of T cells and also regulate innate immune responses. Apart from inhibiting immune cells, MDSCs are also involvedin non-immunological functions like promoting angiogenesis through the production of vascular endothelial growth factor (VEGF), MMP9, prokineticin 2, cancer cell invasion and metastasis (209, 210, 211). Thestudy shows that they can also regulate aerobic glycolysis of breast cancer. The MDSCs could alsoinhibit the T cell function through production of inducible nitric oxide synthase (iNOS), reactive oxygen species, arginase (ARG) and immunosuppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β) (138, 139).

All these pro-tumorigenic and anti-tumorigenic cells are present in TME interacting with the tumor cells and resulting in either sensitivity or resistance to the tumor. Many studies are still going on to understand such type of cells in TME and also various therapeutic inventions are being made to block such tumor promoting cells and help the body to fight the cancerous cells (212, 213).

1.12 Hypothesis and Objective

The Rudd lab has found that the down-regulation or inhibition of GSK-3α/β selectively inhibits PD-1 (pcdc1) transcription in CD8+ T-cell (130, 144). Small interfering RNAs (siRNAs) and small molecule inhibitors (SMIs) resulted in reduced PD-1 transcription and augmented OT-1 CD8+ cytotoxic T-cell (CTL) killing by a remarkable 5-10 fold (128). Further, GSK-3 SMIs were as effective as PD-1 blockade in the clearance of virus (144) and in limiting B16 melanoma and EL-4 lymphoma tumor growth in mice. Gsk-3-/- mice also limit B16 melanoma pulmonary metastasis to the same extent as Pcdc1-/- mice (128, 130, 144). Further, a single dose of GSK-3 SMI SB415286 down-regulates PD-1 for 10-14 days in human andmouse CD8+ cells (129). The downregulation was seen primarily in CD8+ T-cells, and not on FoxP- 3+ or conventional CD4+ T cells (128, 144). This finding underscores differences in signalling between the two subsets.

Inactivation of GSK-3 has shown to have its effect on transcription of T-bet (Tbx21) which resulted in its increased expression. T-bet is found to have binding sites on the promoter region of PD-1 (pcdc1) and inhibit its transcription (143) which has also been described by the Wherry lab (215). Negative regulation of the Pdcd-1 promoter by Tbet has also been described bythe Wherry lab (215). However, the full range of targets for GSK-3α/β blockade in T-cell functionis unclear. Several pathways include the Wnt pathway for the activation of TCF1/7 (129, 130) as well as the ability of T-bet to up-regulate several genes including those for the induction of interferon-gamma1, and granzyme B transcription (127, 144).

In this thesis, I proposed to examine the relative involvement of T-bet and GSK-3 in the control of tumor growth. Also define aspects of the immune response in Tbet-/- mice associated with increased susceptibility to B16 melanoma tumor growth. To understand the mechanism

underlying basis for the ability of GSK-3 small molecule inhibitor (SB415286) to reverse the compromised state of Tbet-/- mice.

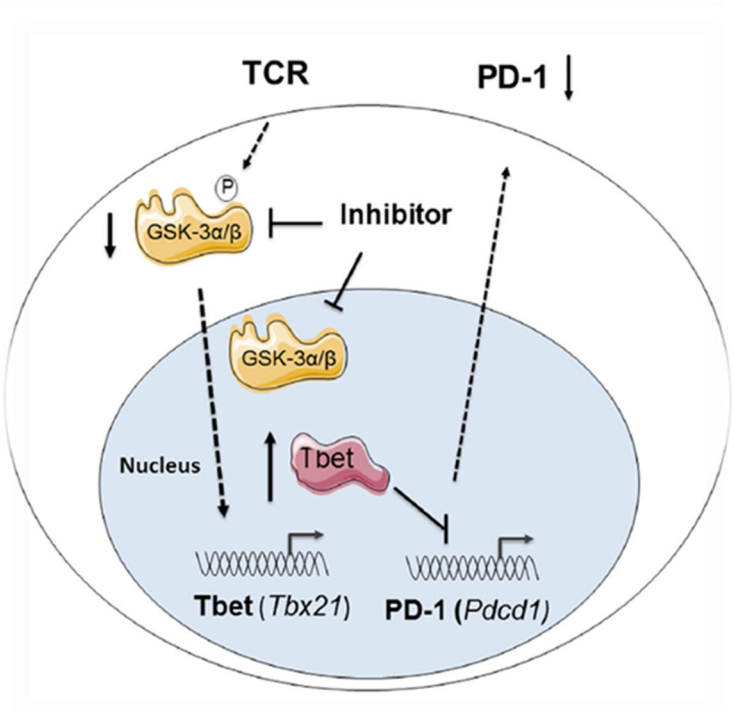


Figure 6. – Schematic representation of downregulation of PD1 by T-bet via its upregulation by GSK-3 small molecule inhibitor (143).

The figure shows the effect of GSK3 inhibition on T-bet and PD1 which are important for T cells.

It explains that blocking GSK3 results in upregulation of T-bet which is a transcription factor.

This factor binds to the promoter region of PD1 and inhibits its transcription.

Chapter 2 – Materials and Methods

2.1 *In vivo* studies

2.1.1 Mice

C57BL/6 (wild type) and Tbet knock out mice were used for the *in vivo* experiment. The mice were infected when they are 7-8 weeks old. Mice were bred in the animal facility of Maisonneuve-Rosemont Hospital Research Centre (APC approval 2017-JA-001).

2.1.2 T-bet knockout Mice

Correctly targeted ES cells were injected into blastocysts and the resulting chimeric males were bred to C57BL/6 females. These mice were backcrossed to C57BL/6 mice for at least 8 generations. Upon arrival at The Jackson Laboratory, mice were bred to C57BL/6J (Stock No. 000664) for at least one generation to establish the colony" (216) (https://www.jax.org/strain/004648)

2.1.3 Cell lines

B16 melanoma cells over expressing PDL1 were grown in DMEM supplemented with 10% FCS, L-glutamine.

2.1.4 Therapeutic experiment

Wild type mice and T-bet knock out mice (each group with 12 mice) were used for the *in vivo* studies to understand the effect of GSK3 inhibition (SB415286) and T-bet in the context of melanoma micro environment. The mice were shaved with a clipper and injected intradermally with B16 melanoma PDL1 (50,000cells/50 ul of DMEM). After 2 days of post-implantation, mice were monitored to check for the tumor growth every other day. 90% of mice in each group started

showing visible tumors on the 7th day of post implantation. Mice were randomized based on tumor size within each genotype groups into control and SB Treatment subgroups on the 7 dpi. The treatment subgroup (6 mice) received 400 ug of SB41 i.p. every other day while untreated controls received vehicle. The treatment groups were injected with same concentration of SB41 every 2 days till day 13th post implantation (4 injections in total).

2.1.5 Harvest of Tumor Infiltrating Lymphocytes (TILs) from tumor

Mice were harvested at day 14. Tumor masses were removed, minced, and digested with collagenase and hyaluronidase digestion solution containing 2.5 mg/ml collagenase I, 1 mg/ml collagenase IV, 0.25 mg/ml hyaluronidase IV-S, 300 mg/ml DNase I, and 0.1mg/ml HEPES in RPMI 1640 mediumat 37°C for 30 min in water bath with manual shaking for every 5 min. The pieces were then gently pressed between the frosted edges of two sterile glass slides, and the cell suspension was filteredthrough a 70-mm cell strainer (BD Biosciences) to remove debris and separate cell clumping.

0.3 ml of supernatant obtained is retained to check for all the immune cells and the rest were loaded on to the ficoll-Paque for mononuclear cell separation (1.070 g/l). The cloudy layer formed from ficoll density gradient centrifugation is aspirated, washed and used for flow cytometric analysis. Single cell suspension before ficoll separation was used to check for the different immune cell lineages among Tumor infiltrating leukocytes (TILs). Cells from ficoll gradient separation were used to characterize T cell infiltration, to check for the different markers like co stimulatory receptors, co inhibitory receptors, transcriptional factors, cytolytic enzymes and cytokines by flow cytometry.

2.1.6 Flow cytometric analysis (FACS)

Antibodies against CD45-AF700, TCRb-BUV395, CD4-PERCP/CY5.5, CD8a-BV650, CD44-BV605, CD62L-APCCY7, PD1-BV421, Lag3-APC, CTLA4-PE DAZZLE, TCF1-AF647, Eomes-PE DAZZLE, Tbet-PECY7, Ki67-FITC, granzymeB-PE, perforin-APC, FasL-BV421, IFNγ-FITC, TNFa-PECY7, TOX-PE, Tim3-BV786 were purchased from Biolegend, eBioscience (San Diego, CA). Flow cytometric

analysis was performed using a LSRII Fortessa flow cytometer (BD Biosciences, San Jose, CA).

2.2 In vitro CD8+ T cell differentiation

Spleens were collected from C57BL/6 WT, Tbet KO mice. Spleens were crushed in RPMI media with 10% FCS and filtered through 70 um cell strainer (BD Biosciences) to remove debris and separate cell clumping. Then the splenocytes were treated with 1x red blood cell (RBC) lysis buffer (Biolegend) and incubated for 5min at RT to remove red blood cells. Cells were washed and counted in a haemocytometer.

Splenocytes (4 million) were stimulated with 2.5 ug/ml plate-bound anti-CD3 (clone: 145-2c11) (coating for 1 hr at 37C and 2 ug/ml of soluble anti-CD28 mAbs in the presence of anti–IL-2Ra (5 IU) in 12 well plate along with 1uM SB415286 and 2 ug/ml anti PD1 (clone: J43). After 24 hrs, cells were again treated with same concentrations of SB41 and anti PD1. After 48 h, cells were re-plated to new wells without anti-CD3 and anti CD28 and with freshly added IL-2 (20 U/ml) for another 48 h along with same concentrations of SB41 and anti PD1. After 24 hrs, the cells were stained for different markers and analysed by FACS.

2.3 FACS staining protocol

The cells are transferred to falcon 5 ml tubes and washed with 1x phosphate buffered saline (PBS). Then they are stained 1x BV510 (viability stain) (1:1000) and incubated on ice for 20 min covered with aluminium foil. Thenafter incubation, the cells are washed with ice cold 2% FACs buffer (PBS with fetal calf serum (FCS)). Centrifuged and thepellet is collected. Then the cells are fixed with ice cold 2% para formaldehyde (PFA) and again washed with ice cold 2% FACs buffer (PBS with FCS). Then stained with the surface markers and incubated for 30-40 min on ice covered with Al foil on the shaker. Washed with ice cold 2% FACs buffer and then the cells arefixed with fix perm for 45 min on ice on shaker (1:4 dilution in perm buffer) for permeabilization of nucleus. Washes are carried on with 1x permeabilization buffer for 2 times. then, intracellular markers are prepared in 1x perm buffer and added to the cells. Incubated on ice covered with Al foil for 30-40min on shaker and carried on with 2X washes with 1x perm buffer and last wash with 2%FBS buffer. Finally, cells are re suspend in 100uL of 2% FBS buffer for FACs analysis.

Chapter 3 – Results

3.1 *In vivo* studies

3.1.1 B16 tumors grow more in Tbet-/- mice, a defect that is corrected by the injection of the small molecule inhibitory GSK-3

The figure mainly shows that the T box transcription factor T-bet is important for the suppression of tumor growth. This is evident when seen that the tumor growth is greater in Tbet-/- mice than in the wild type mice. The finding is consistent with a study by Gacerez et al., group using a different tumor model (217). GSK-3 SMI (SB415286) has already shown to inhibit the catalytic activity of GSK-3 (143). Here, the graph shows that GSK-3 SMI in the absence of T-bet results in the suppression of tumor growth in Tbet-/-. Surprisingly, this result shows that GSK- 3 SMI compensates for the absence of T-bet and reduces the tumor growth in Tbet-/-.

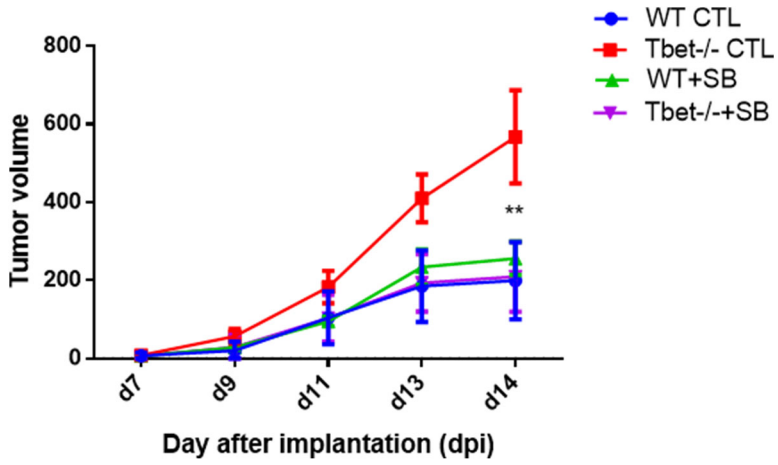


Figure 7. – The *in vivo* experiment was done with Wild type mice and T-bet knock out mice (each group with 12 mice). Flow cytometric analysis shows that that Tbet-/- are less resistant to B16 tumor growth. Tumor volume statistics where the P value < 0.05 (P value= 0.003) shows significant difference between the non-treated groups of WT and T-bet-/- and treated group in Tbet-/-. There is no much difference among treated and non-treated groups of WT. This

represents that the T-bet is involved in suppression of tumor growth and the treated groups of T-bet-/- show that GSK-3 SMI is required for regression of tumor growth in the absence of T-bet observed from day 7 to day 14.

The error bars are the graphical representations of variability in the sample size (six mice per group). It is calculated based on standard error of the mean (SEM). SEM measures how far the sample mean (average of all the six mice in each group) of the data is likely to be from the true population mean.

3.1.2 The absence of T-bet affects not only T cells but also other immune cells:

The figure (8a) shows that T-bet is useful for proliferation of dendritic cells (DCs) and polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs). Reduced number of DCs in the absence of T-bet might result in DCs losing their ability to stimulate IFNγ and suppress IL-4 production by Th2 cells (218). T-bet is expressed in DCs suggesting that the effect is a direct effect on DCs and not an indirect consequence of regulation by other cell types. Increased number of PMN-MDSCs (which are morphologically similar to N2 polarized neutrophils) in Tbet-/- may result in suppressing the T cell responses (219). These results suggest that the change in these immune cell numbers may contribute to the tumor progression in the Tbet-/- mice as shown in Fig 7. GSK-3 SMI seems to show no effect on these cells which implicates that GSK-3 SMI is choosing some other strategy in anti tumor immunity in Tbet-/- mice.

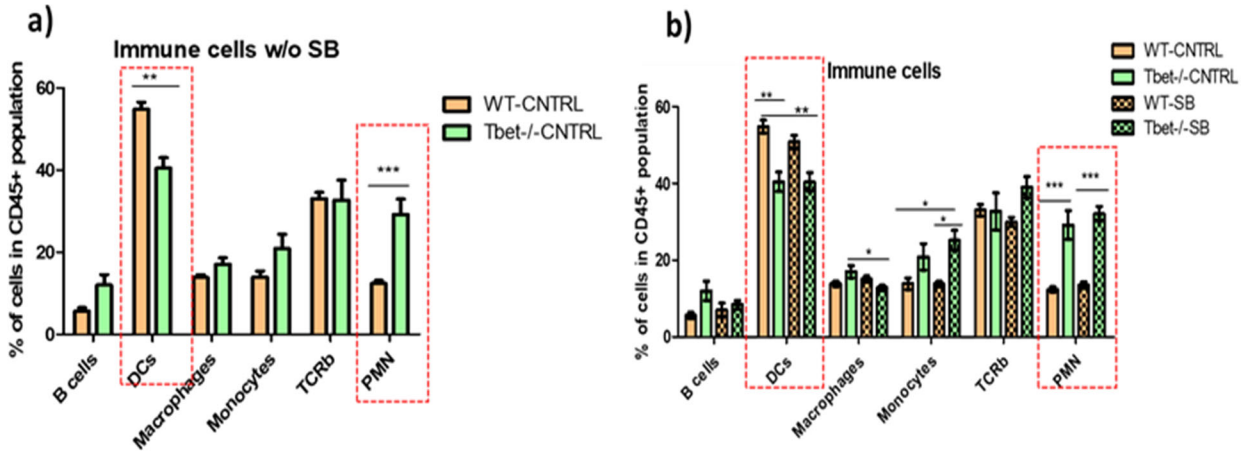


Figure 8. – a) TILs from Tbet-/- mice show normal levels of T-cells but with fewer Dendritic cells (DCs)and increased polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs). The P

value< 0.05 in DCs (P value= 0.0059) and PMN-MDSCs (P value= 0.0002) population among the WT and Tbet-/- represents significant difference which contributes to the fact that the Tbet-/- are prone to progression of tumor growth in absence of T-bet. b) Since the P value is not less than 0.05, there is no much significant difference in DCs and PMNs among treated and non-treated groups. GSK-3 small molecule inhibitor (SB415286; GSK-3i) had no effect on DCs or PMNs neither in wild type nor Tbet-/-.

3.1.3 No significant effect of T-bet or GSK-3 SMI on the total T cell numbers in TILs:

T-bet is required for T cell development and effector differentiation (more precisely CD4+ T cell population). The figure (9) w.r.t treated and non-treated groups in the presence and absence of T-bet do not show any effect of T-bet or GSK-3 SMI on total T cell numbers.

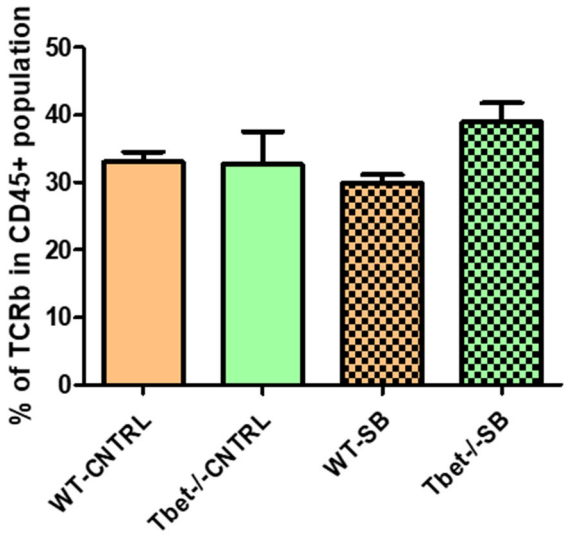


Figure 9. – The flow cytometric analysis explains that there is no effect of T-bet or GSK-3 SMI on the total T cell numbers in TILs. The P value greater than 0.05 (P= 0.2147) shows that there is no significant difference in total T cell numbers between the groups.

3.1.4 T-bet plays an important role in development of IFNγ producing CD4+ T cells:

T-bet essentially is involved in development of IFNγ producing CD4+ T cells resulting in eliciting adaptive immune responses (220, 222, 226). Figure (10a, 10b and 10c) shows that there is marked decrease in CD4+ TIL count in Tbet-/- consistent with the past observation that T-bet is important for CD4+ Tcell development and effector differentiation. This paradigm also operates in TILs from tumors. Surprisingly, GSK-3 SMI had no effect on this number, neither in presence nor in absence of T-bet. It is seen that there is an increase in CD8+ T cells in absence of T-bet and no effect of GSK-3 SMI on this number (fig:11a, 11b). These two figures give us an information on already existing study that T-bet is essential for CD4+ T cells but the increase in CD8+ T cell number in Tbet-/- shows that T-bet may have an inhibitory effect on CD8+ T cells (since there is decrease of CD8 population in the presence of T-bet in WT). It also shows that GSK-3 SMI effect on tumor growth in Tbet-/- is not being influenced by the loss of CD4+ T cells.

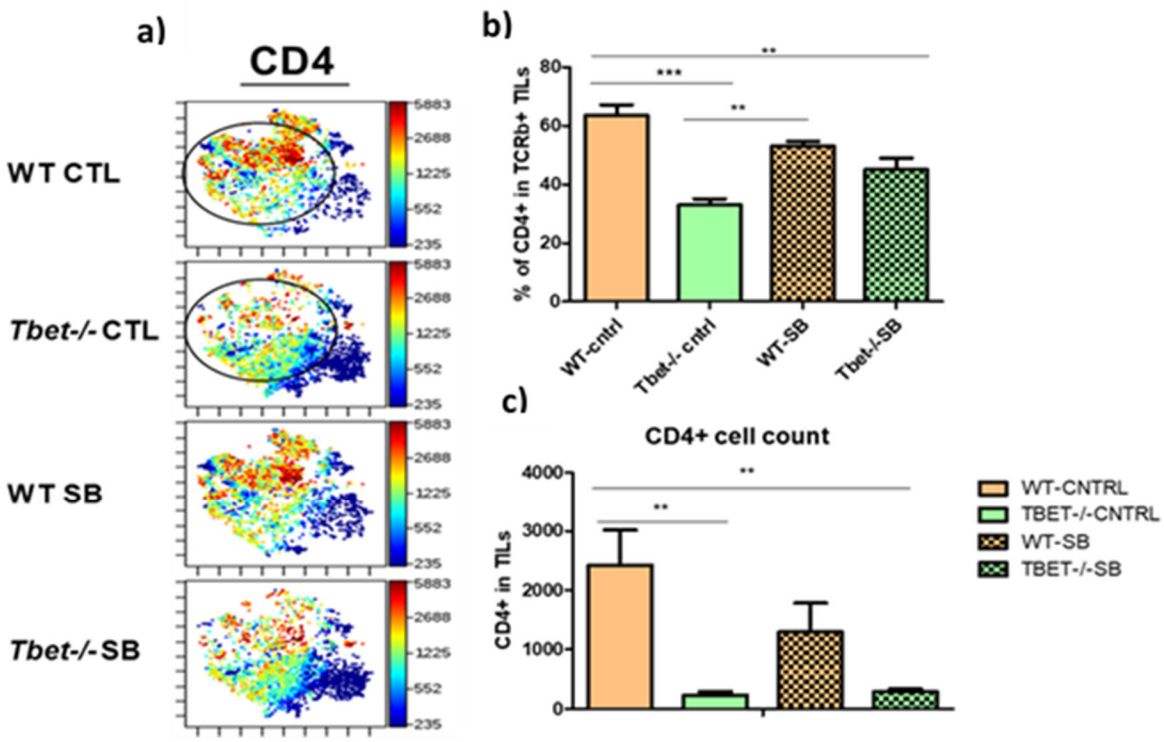


Figure 10. – a) viSNE analysis plot and flow cytometric analysis represent that Tbet-/- mice are characterised by a loss in absolute and relative numbers of CD4+ T-cells in B16 tumors. viSNE analysis is an advanced t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm and it is

a two-dimensional representation of single cell data analysis with separation between immune subtypes and capturing the heterogeneity of markers within populations, thus highlighting transitional cell populations.

The P value of 0.0029 shows that there is a significant difference in CD4 numbers among WT and Tbet-/- which proves that T-bet is essential for CD4+ T cell development. c)GSK-3 SMI had no effect on CD4+ T cell numbers as the P value represents no statistical difference.

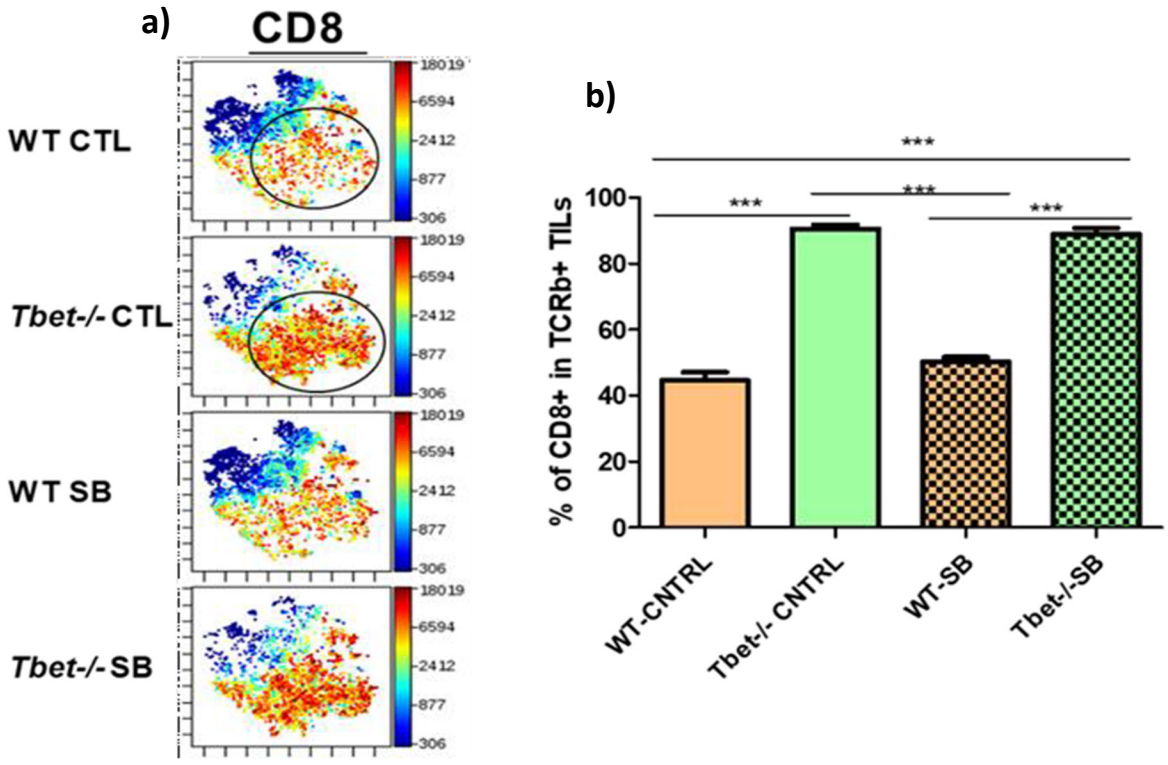


Figure 11. – a) The P value = 0.0001 shows a significant difference among WT and Tbet-/- in CD8 population. This is also evident from viSNE plot that T-bet-/- is characterized by an increase in CD8+ T-cell number in B16 tumors. b) Since P value is not less than 0.05, there is no much difference in GSK-3 SMI treated groups. It basically shows that T-bet is needed for CD4+ TILs but T-bet may have an inhibitory effect on CD8+ T cells.

3.1.5 Absence of T-bet increased the presence of progenitor CD8+ T cells with T cell Factor (TCF1) and Eomes expression

The figure (12a) shows that the loss of T-bet resulted in the increase in the presence of TCF1+CD8+ TILs. It seems that since there is no protection of Tbet-/- mice against tumor growth (fig 7) does not reflect on the fact of having high CD8+ T cell number as seen in fig 11b. Progression of tumor growth in Tbet-/- mice indirectly tells us that CTL activity is less and high CD8+ T cell number might be because of mostly progenitor phenotype with high expression of TCF1. The naïve CD8+ T cells with high TCF1 in the absence of T-bet seems to have high expression of Eomes (as shown in fig 12b) resulting in possible memory precursors (223).

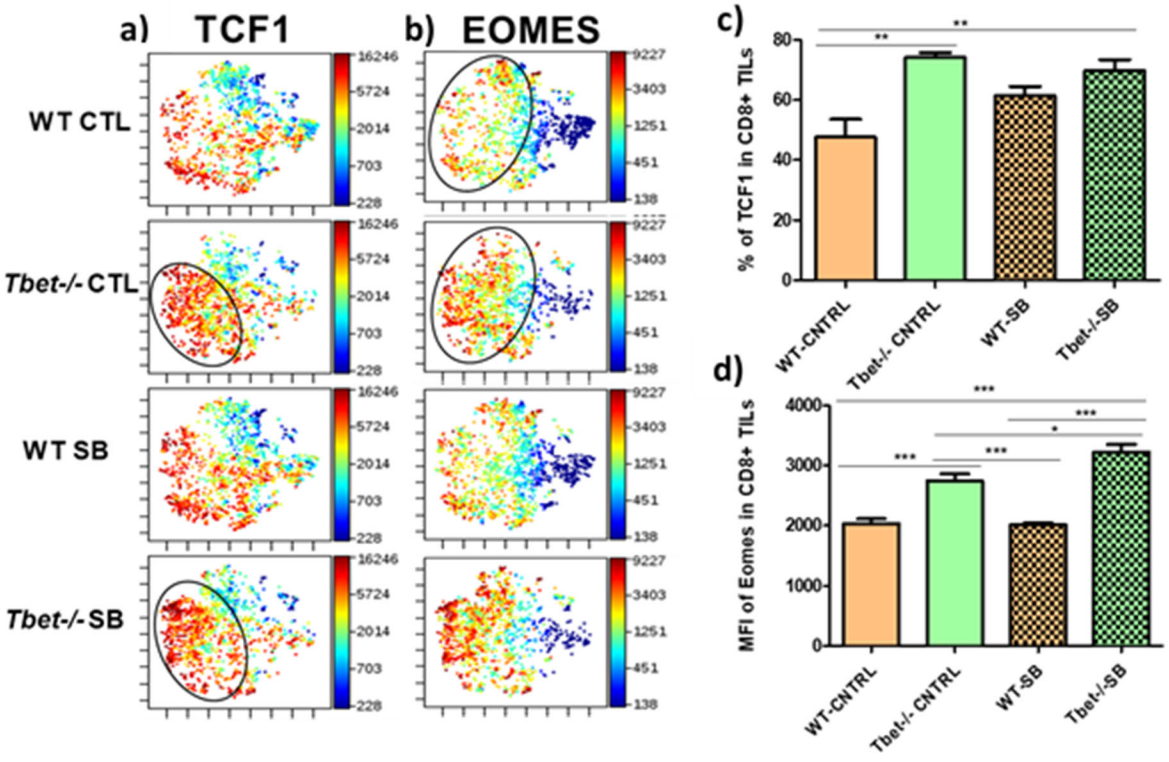


Figure 12. – a,c) viSNE plot and flow cytometric analysis show that T-bet-/- mice is characterised by an increase in the progenitor transcription factor TCF1. b,d) The viSNE plot and FACs analysis represents that Tbet-/- show increased Eomes on CD8+ TILs. GSK-3 SMI seems to have its effect on the expression of Eomes in Tbet-/-.

The results from the figures (12) show that GSK-3 SMI has no significant effect on TCF1 expression (P= 0.1537) but has on Eomes (P value<0.05) in Tbet-/- implicating the differentiation of T cells into memory precursors. T-bet-/- mice is characterised by an increase in the progenitor transcription factor TCF1. This proves that T-bet is needed to drive differentiation (i.e., Th1). So, may be most of the CD8+ population seems to be progenitors and may be blocked for the activation process in the absence of T-bet.

3.1.6 Reduced CD44 expression on CD8+ T cells in Tbet-/- is not reversed by GSK-3 SMI

GSK-3 SMI had no effect on CD44 expression. Less CD44 expression may indicate that only a small proportion of T cells seem to underwent a proliferative expansion that led to an antigen experienced phenotype especially in the absence of T-bet.

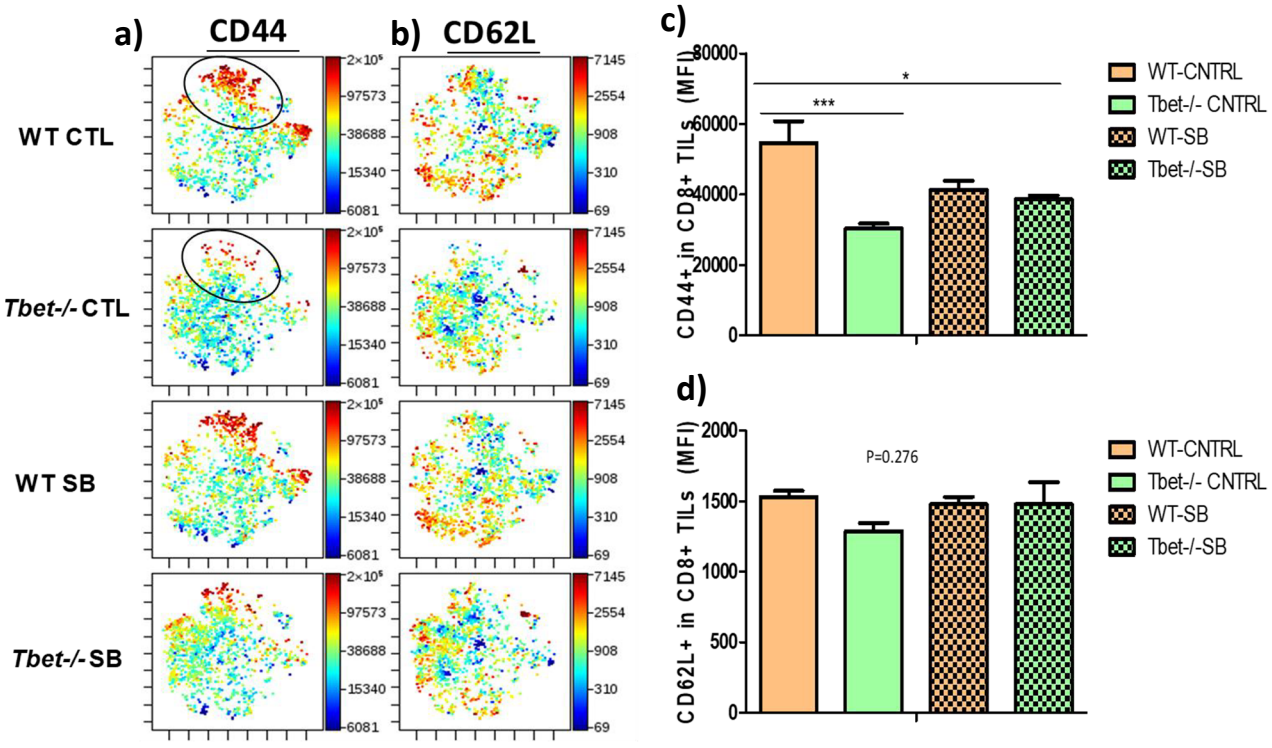


Figure 13. – viSNE analysis shows that Tbet-/- CD8+ T-cells have reduced CD44 expression which is characteristic of antigen-experienced effector T-cells but GSK-3 SMI did not reverse this.

This can stand as a reason why most of the CD8+ T cells in Tbet-/- (fig: 11a) are progenitors and why there is tumor growth in Tbet-/- (fig:7). Central effector memory (CEM) seems to have no such great variation with GSK-3 SMI as treatment does not show any increase in the expression of CD44 and CD62L (markers for CEM). The P value= 0.0001 in flow cytometric analysis among Tbet-/- and WT shows that there is a significant decrease of CD44 population in Tbet-/- but the P value in bar graphs does not seem to show significant difference statistically between treated and non-treated groups.

3.1.7 Less exhaustion seen in the absence of T-bet may not support the fact that CD8+ T cells are really on the verge of exhaustion path:

Scott et al., proved that TOX expression in T cells is an indication of exhausted state. Over-expression of T-bet results in repression of TOX in EL4 melanoma according to Page et al., (221).

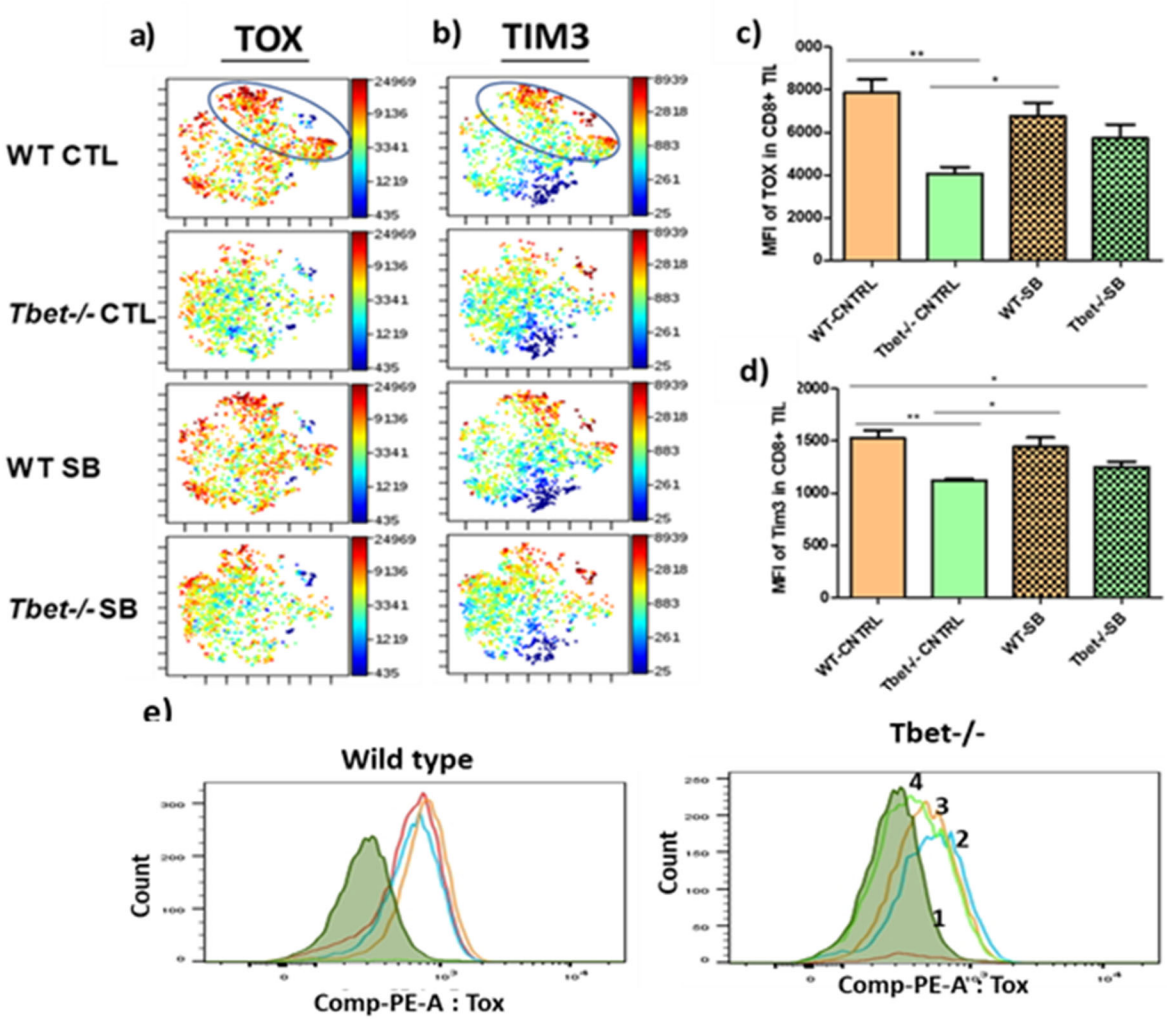


Figure 14. – a,b) Tbet-/- CD8+ T-cells showed reduced markers of T-cell exhaustion in TILs (viSNE plots), c,d) The flow cytometric analysis also shows that there is a decreased expression of exhaustion markers in Tbet-/-, e) Tox expression on day 1, 2, 3, 4, after activation of splenocytes (in vitro studies) show that this exhaustion marker decreases gradually by day 4 in Tbet-/- .

The P value= 0.0022 in flow cytometric analysis shows that there is a significant decrease of the exhaustion markers in Tbet-/-. But to the contrary to the existing studies, absence of T-bet has shown to reduce expression of TOX in my study both *in vivo* and in vitro studies in fig 14a and 14e respectively. Interaction between TOX and T-bet can be an area of interest. viSNE plot in fig (14a,b) also shows that TOX and TIM3 (which is also a marker of exhaustion) has a lower expression in absence of T-bet in CD8+ T cells. Since the P value shows no significant difference in treated groups, GSK3 SMI seems to have no effect on exhaustion markers.

3.1.8 Absence of T-bet results in loss of GZMB and FasL but GSK-3 shows a trend to increase FasL expression

The in vitro and in vivo data showed that absence of T-bet resulted in loss of GZMB and FasL. Xin et al., showed that Tbet is needed for GZMB and IFNγ expression.

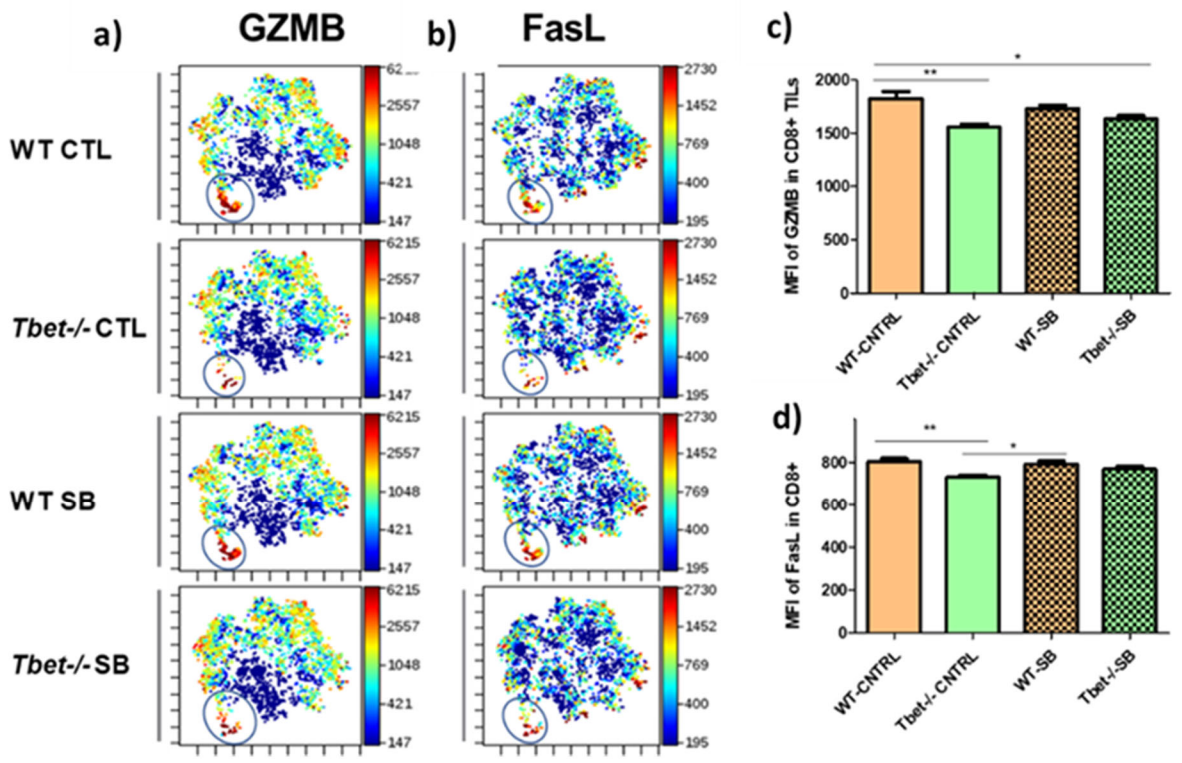


Figure 15. – a,c) Tbet-/- mice show a loss of GZMB. Study shows that T-bet is involved in upregulationof GZMB. GSK-3 SMI has no effect on GZMB. b, d) Tbet-/- mice show a loss of FasL and GSK-3SMI shows a trend to increase FasL P value= 0.015 in GZMB population among WT and Tbet-/- show that there is a significant reduction of GZMB in Tbet-/-. But the loss of GZMB is not reversed by GSK-3 SMI. Absence of T-bet in Tbet-/- resulted in loss of expression of FasL (223, 224) with P= 0.02. But GSK-3 SMI seems to have a slight effect on FasL expression as seen in viSNE plot which is not very significant as shown in flow cytometric analysis. Replicating the experiment could clarify the effect of GSK-3 SMI on FasL.

3.1.9 GSK-3 SMI resulted in decrease in the expression of co-inhibitory markers

It is already proved that GSK-3 inhibition resulted in downregulation of PD1 by Rudd lab (130, 144). Here, the flow cytometric analysis show that there is decrease in the expression of PD1 with GSK-3 SMI. Expression of CTLA4 which is a co-inhibitory receptor on T cells is also seen to slightly reduce in Tbet-/- which is not very significant.

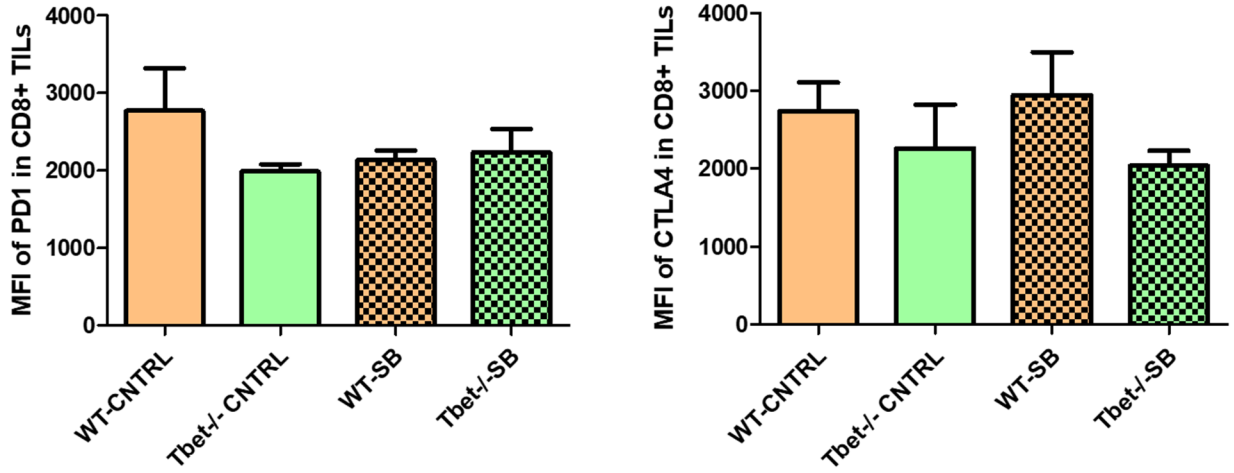
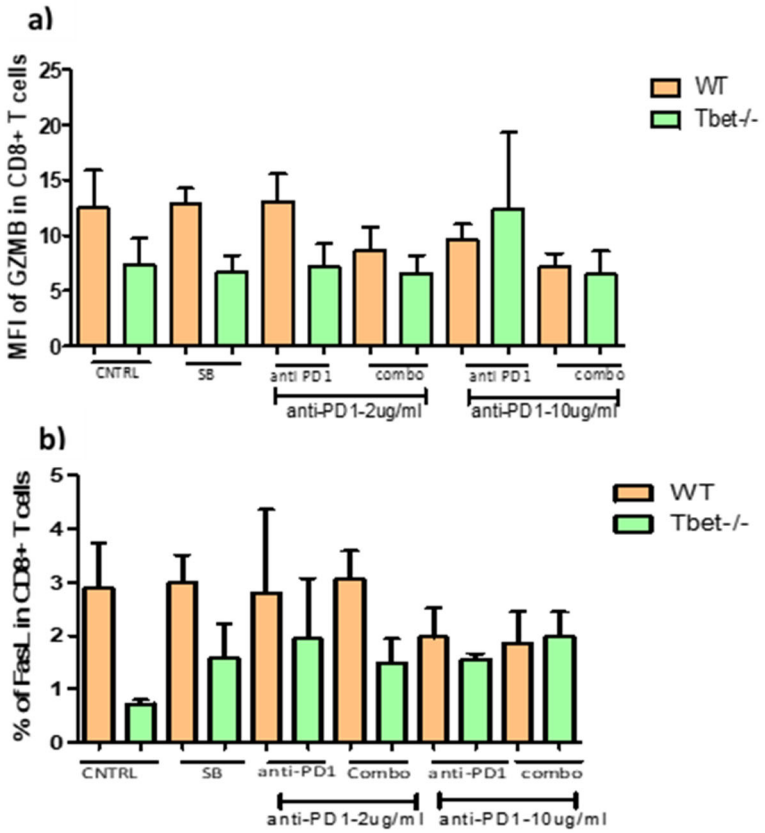


Figure 16. – Tbet-/- and GSK-3 SMI both reduce PD-1 expression. GSK-3 SMI effect is consistent withprevious paper from Rudd lab (90). The P value in the flow cytometric analysis shows significant difference ($P<0.05$) in PD1 expression among WT and Tbet-/-. This data represents that T-bet is involved in regulating PD1 expression. The WT control and WT treated group shows that the GSK3 is playing a role in regulating PD1 expression. There seems to be a slight decrease in CTLA4 expression among WT and Tbet-/- control groups. GSK3 SMI seems to have no effect.

3.2 In vitro studies

3.2.1 CD8+ T cells differentiation from Wild type and Tbet-/- mice spleen Six Spleens (3 from each group) were collected from C57BL/6 WT, Tbet KO mice for the in vitro experiment. This experiment was repeated more than 3 times to check for the consistency of the data. Loss of GZMB, FasL, IFNγ in Tbet-/- activated CD8+ T cells (invitro studies) seems to be consistent with *in vivo* data and consistent with T-bet control of their expression. With GSK-3 SMI, there was no effect on IFNγ and GZMB as seen also in invivo studies. Similar to the in vivo data, GSK-3 SMI showed slight trend to increase FasL in Tbet-/- as seen in fig: 15 b&d. These results show that T-bet is essential for GZMB, FasL and IFNγ expression. GSK-3 SMI could show some effect on FasL expression similar to invivo experiment. Therapy with anti-PD1 antibodies seems to have no effect on IFNγ and GZMB but a bit increases in FasL expression in Tbet-/-. But the combinational therapy (combo) with GSK-3 SMI and anti PD1 shows no much effect when compared to individual treatments.



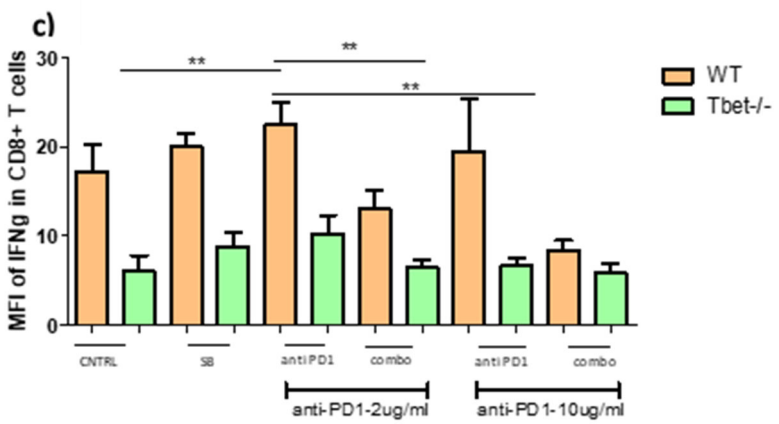


Figure 17. – a,b,c) Loss of GZMB, IFNγ and FasL expression in Tbet-/- activated CD8+ T cell population.GSK-3 SMI and anti PD1 show a slight increase in expression of FasL in Tbet-/-. But no much effect is seen with combinational therapy. The P value < 0.05 shows significant difference in the expression of GZMB, IFNγ and FasL among WT and Tbet-/- control groups. GSK3 SMI and anti PD1 treatments showed no much effect on their expressions and also their P values had no significant difference.

TCF1 expression in Tbet-/- activated CD8+ T cells is higher and is not affected by GSK-3 SMI which is similar to in vivo data. Also, expression of PD1 shows slight variation with GSK-3 SMI but gradually it is reduced with anti-PD1 antibodies and more effectively reduced with combinational therapy. The invitro flow cytometric analysis results show that there is consistency with in vivo viSNE plots and flow cytometric analysis data.

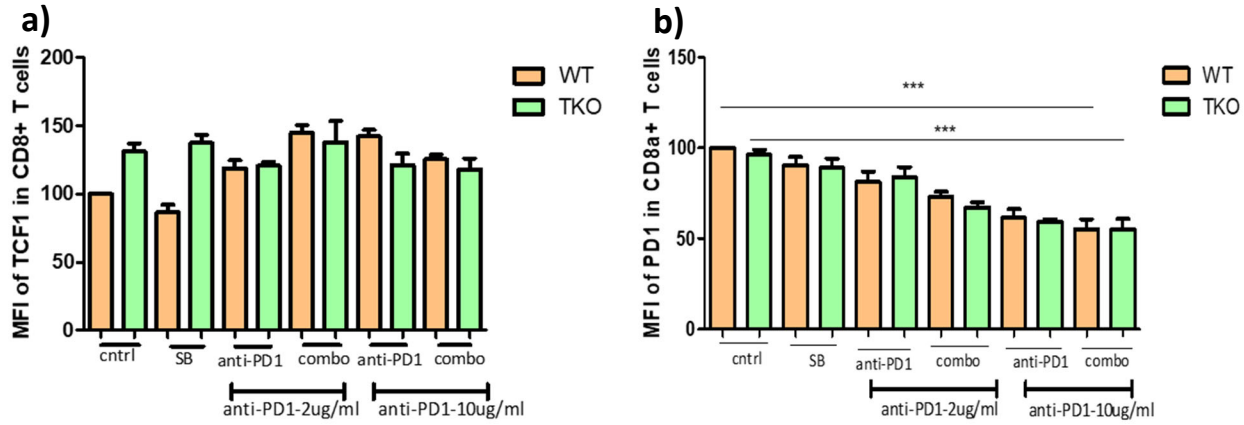


Figure 18. – a) TCF1 seems to have high expression in absence of T-bet and GSK-3 SMI seems to have no effect on TCF1. The P value < 0.05 shows significant difference in TCF1 in Tbet-/- and WT control group.

b) Expression of PD1 seems to decrease with anti PD1 therapy and more effective with combinational therapy. The P value= 0.0015 shows significant difference among WT and Tbet-/- control groups with anti PD1 treatment.

Chapter 4 -Discussion

Previous work from many labs have underscored the importance of T-bet in the differentiation of CD4+ Th1 cells and the function of CD8+ T-cells of the immune system (224-226). T-bet controls the generation of the CD8+ cytotoxic effector cell where the loss of T-bet in OT-I T cell receptor transgenic mice results in a reduced cytotoxicity and a marked shift in cytokine secretion profiles (223, 225). It was therefore not surprising that Tbet-/- mice were more susceptible to B16 tumor growth which proves that T-bet is essential for tumor suppression. It also agrees with the work of Lu et al 2013 using adoptive cell therapy.

This study is one of the first to show the need of T-bet in the rejection of solid tumors and to define the nature of the immune response. Our work showed that the defect in protecting against tumor growth in Tbet-/- mice was correlated with decreased number of dendritic cells (DCs) and increased presence of suppressive PMNs (tumor promoting cells) which are polymorphonuclear MDSCs (PMN-MDSCs) and are morphologically similar to N2-polarized neutrophils which are also pro-tumor phenotype. They also include eosinophils, basophils and mast cells. The loss of DCs is consistent with the role of T-bet in the generation of mature DCs for antigen presentation (227). T-bet is also expressed in human monocytes and myeloid DCs (224, 228). The loss of function could be due to either subset change (effector/ memory) or a direct effect on T-cell such as CD4+ T-cells which showed a marked loss of 10-fold. T-bet is also responsible for the production of IFNγ (228), a cytokine needed for DC maturation and other functions. T-bet also suppresses Th17 development (229, 230).

The surprising aspect of this work is that the inhibition of GSK-3 could reverse the defect in protection against tumor growth. The result was also surprising given that the lab had shown that the ability of GSK-3 SMIs to enhance anti-tumor effects was correlated with the upregulation of T-bet and its inhibition of PD-1 transcription (130, 143, 144). This indicated that there is also a T-bet independent pathway by which the GSK-3 kinase can promote tumor rejection. Although I was not able to define the target responsible, GSK-3i had no effect on CD4 numbers and also it

seems that this number has not affected GSK-3 SMI to reduce the tumor in *Tbet-/-*. However, it could have restored IFN gamma production which needs to be checked in thefuture. In *Tbet-/-* mice, I noted a surprising increase in CD8+ TILs. However, most of them were progenitor-like with higher TCF/Eomes (possible memory) and lacking CD44 (antigen experienced cells) and also GZMB/FasL (effector molecules). Loss of Granzyme B and FasL in *Tbet-/-* show that there is progression of tumor growth. GSK-3 SMI seems to have no effect on the loss of GZMB in*Tbet-/-* but has shown a slight trend to increase in FasL expression. There was therefore no obvious effect of GSK3i on CD8+ effectors other than increasing their representation in tumors. Over-expression of T-bet results in repression of TOX in EL4 melanoma according to Page et al., (221). But to the contrary, absence of T-bet has shown to reduce expression of TOX in my findingboth in invivo and invitro studies.

CD8+ T Cells that co express RORγt and T-bet are functionally impaired (232). The observation in my experiment about the restoration of protection in the Tbet-/- mice against tumor growth by GSK-3 inhibition is still unclear. The increase in Eomes, or changes in cellular function of PMNs or Tregs should be more explored to understand the underlying mechanisms by which GSK-3 SMI is actually having its effect in regression of tumor growth in the absence of T-bet.

Further work will be needed to identify the key pathway responsible for immune cell rejection of tumors even in the absence of T-bet. For this adoptive T-cell transfer of Tbet-/- T-cells will be needed in order to see whether the inhibition of GSK-3 in these cells is sufficient. If not, the likely target of GSK-3 might reside in other immune cells.

5.Future perspectives

Modulation of T-bet may play a crucial role in anti-tumor immunity by affecting the presence of suppressor myeloid cells. More research can be done in this area to understand the behaviour of immune cells which are controlled either directly or indirectly by T-bet.

Defects due to the loss, or reduced T-bet function, may be corrected by GSK-3 SMI therapy. This area seems to be interesting. As seen with the expression of PD1, there can be some connection between T-bet and GSK-3. This can be better studied using Chromatin Immuno Precipitation (ChIP) analysis to understand the effect of GSK-3 on different transcription factors which affect the transcription of T-bet or effect of T-bet on transcription of different proteins which interact with GSK-3. The pathway connecting T-bet and GSK-3 can be revealed.

Effect of GSK-3 on tumor promoting cells like regulatory T cells may provide more information about regression of tumor growth in the absence of T-bet. This area can be interesting as how Tregs which lack T-bet expression are controlled by GSK-3. Role of GSK-3 on Tregs suppressive behaviour in the presence and absence of T-bet might be crucial in anti tumor immunity.

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