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T CELLS IN SOLID TUMORS: INVESTIGATING THE IMMUNOMODULATION IN THE TUMOR MICROENVIRONMENT

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T cells in solid tumors: Investigating the immunomodulation in the tumor microenvironment

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In memory of my late grandmother.

There are no two words in the English language more harmful than ‘good job’.

~Terence Fletcher, from the movie ***Whiplash***

ABSTRACT

The immune system protects human from cancer through an immunosurveillance mechanism. However, the progressive nature of tumor cells to differentiate and the complexity of the tumor microenvironment may result in the immunomodulation of immune cells. In this thesis, we aim to explore the T cell immunomodulation inside the intricate solid tumor microenvironment in patients.

First, we investigated suppressive regulatory T cells (Tregs) in urinary bladder cancer (UBC). Our group previously demonstrated a contradictory finding that a high $FOXP3^+$ tumor infiltrating lymphocyte (TIL) number correlates positively to survival. In here, we answered that $FOXP3^+$ $CD4^+$ T cells in the tumor were real Tregs which protectively regulated tumor invasiveness by suppressing MMP2 expression in tumor-associated macrophages (TAMs) and tumor cells.

Next, we explored the subset of tissue-resident memory $CD8^+$ T (T_{RM}) cells from UBC tumor. It is less known whether T_{RM} cells are effective killers of tumor cells. We revealed that tumor T_{RM} cells were epigenetically committed to express perforin. Although T_{RM} cells expressed exhaustion marker PD-1, they were not terminally exhausted. As a result, we found that an increased number of T_{RM} cells in the tumor correlated with a lower tumor stage.

Furthermore, we looked into the cytotoxic $CD8^+$ T cells in the sentinel nodes (SNs) of UBC patients. Surprisingly, we discovered that SN $CD8^+$ T cells displayed a deficiency of their cytotoxic constituent perforin, whereas granzyme B was still expressed. Thereafter, we revealed that muscle invasive UBC suppressed perforin expression using an ICAM-1/TGFβ2 – mediated pathway as an immune escape mechanism.

In the next study, we focused on the effect of standard neoadjuvant chemotherapy (NAC) and T cell responses in the SNs. We found that NAC reinforced the anti-tumor T cell activities by reducing the exhaustion in $CD8^+$ and $CD4^+$ effector T cells, which consequently increased their cytotoxicity and clonal expansion, respectively. Additionally, NAC also reduced the frequency and activation of the suppressive Tregs.

Lastly, as a result of escaping the immune destruction, tumor can grow and metastasize. In this study, we revealed that micrometastases in lymph nodes of renal tumors could be reliably detected by flow cytometry. This method is more sensitive, objective, time- and cost-effective compared to the gold standard histopathological examination.

In conclusion, T cells are modulated in the solid tumor microenvironment. By understanding the molecular and cellular aspects of T cells in this microenvironment, we may unveil new strategies for designing cancer immunotherapies in the future.

POPULAR SCIENCE SUMMARY

In the human body, the immune system acts as “the army” that fights against “the enemy” from outside. The enemies may come in the form of pathogens (viruses, bacteria, parasites, and fungi) that cause infections. Over the past decades, it has been widely acknowledged that our immune system is also responsible to protect us against the developing tumors.

The immune system consists of various cell types and each of them has different functions. In this thesis, we focus on the immune cells called T cells, which have a crucial role in the protection against tumors. In order to be able to destroy the tumors, T cells need to penetrate the tumor tissue and stay “behind the enemy lines”. However, the tumor tissue is a very complex environment, in which the characters and functions of T cells may be modulated inside it. For this reason, we aim to investigate the modulations that happen in T cells in this environment.

We characterized a T cell type called $CD8^+$ T cells, which act as the “assassins” of the tumor cells. In order to kill, $CD8^+$ T cells are equipped with weapons, which are called perforin and granzymes. We found that in the urinary bladder cancer (UBC) tumor tissue, most of the $CD8^+$ T cells were tissue-resident memory T (T_{RM}) cells. These cells were highly-trained to kill the tumor cells, even when they were displayed to be exhausted. In addition, we discovered that the tumor cells were sneaky and they could avoid the $CD8^+$ T cell killing by producing molecules that suppressed the perforin expression. Consequently, $CD8^+$ T cells lost their ability to kill, as if they only had guns without the bullets. Moreover, as the result of escaping the T cell killing, tumor can grow further and spread (metastasize) to the adjacent lymph nodes. In this thesis, we uncovered an alternative method to identify the presence of metastatic tumor cells in the lymph nodes using a method called flow cytometry. Flow cytometry could detect even a small number of tumor cells, in which standard histopathological examination routinely used in the clinic was unable to.

Furthermore, we examined another type of T cells called the regulatory T cells (Tregs). Classically, Tregs are known to suppress the function of activated effector $CD8^+$ and $CD4^+$ T cells, so that the over-reaction of T cells will not occur and damage our own normal tissues. Accordingly, the suppressive function of Tregs is bad in a T-cell mediated protection against the tumors and subsequently, the presence of Tregs in the tumor tissue may promote the tumors to grow. Surprisingly, this was not the case, since we found that in the UBC tumors, Tregs had a protective effect in regulating the tumor invasiveness. Therefore, it indicates the Janus-faced character of Tregs in the tumors.

Next, we revealed that the standard chemotherapy treatment for UBC could reinforce the anti-tumor activities of T cells from the draining lymph nodes of the tumors, which were shown to be modulated as described above. We observed that the chemotherapy reduced the exhaustion of the killer $CD8^+$ T cells and $CD4^+$ T cells. Consequently, both these cells might have better functions to control the tumors. Additionally, the suppressive Tregs were less in

number and activation after the chemotherapy treatment. This indicates the clinical importance of chemotherapy in treatment of cancer patients.

In conclusion, T cells are modulated inside the tumor tissues. By understanding the modulations that happen in T cells in this environment, we may illuminate pathways for future cancer immunotherapy development.

LIST OF SCIENTIFIC PAPERS

I. Winerdal ME*, Krantz D*, **Hartana CA**, Zirakzadeh AA, Linton L, Bergman EA, Rosenblatt R, Vasko J, Alamdari F, Hansson J, Holmström B, Johansson M, Winerdal M, Marits P, Sherif A, Winqvist O.
Urinary bladder cancer Tregs suppress MMP2 and potentially regulate invasiveness.
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II. **Hartana CA**, Bergman EA, Broomé A, Berglund S, Johansson M, Alamdari F, Jakubczyk T, Huge Y, Aljabery F, Palmqvist K, Holmström B, Glise H, Riklund K, Sherif A, Winqvist O.
Tissue-resident memory T cells are epigenetically cytotoxic with signs of exhaustion in human urinary bladder cancer.
Clinical and Experimental Immunology. 2018 Jul 15.
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III. **Hartana CA**, Bergman EA, Zirakzadeh AA, Krantz D, Winerdal ME, Winerdal M, Johansson M, Alamdari F, Jakubczyk T, Glise H, Riklund K, Sherif A, Winqvist O.
Urothelial bladder cancer may suppress perforin expression in $CD8^+$ T cells by an ICAM-1/TGFβ2 mediated pathway.
PLoS One. 2018 Jul 2;13(7):e0200079.
doi: 10.1371/journal.pone.0200079

IV. Krantz D*, **Hartana CA***, Winerdal ME, Johansson M, Alamdari F, Jakubczyk T, Huge Y, Aljabery F, Palmqvist K, Zirakzadeh AA, Holmström B, Riklund K, Sherif A, Winqvist O.
Neoadjuvant chemotherapy reinforces antitumor T cell response in urothelial urinary bladder cancer.
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V. **Hartana CA***, Kinn J*, Rosenblatt R, Anania S, Alamdari F, Glise H, Sherif A, Winqvist O.
Detection of micrometastases by flow cytometry in sentinel lymph nodes from patients with renal tumors.
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**contributed equally*

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Zirakzadeh AA, Kinn J, Krantz D, Rosenblatt R, Winerdal ME, Hu J, **Hartana CA**, Lundgren C, Bergman EA, Johansson M, Holmström B, Hansson J, Sidikii A, Vasko J, Marits P, Sherif A, Winqvist O. **Doxorubicin enhances the capacity of B cells to activate T cells in urothelial urinary bladder cancer**.
Clinical Immunology. 2017 Mar;176:63-70.
doi: 10.1016/j.clim.2016.12.003

II. Bergman EA, **Hartana CA**, Johansson M, Linton LB, Berglund S, Hyllienmark M, Lundgren C, Holmström B, Palmqvist K, Hansson J, Alamdari F, Huge Y, Aljabery F, Riklund K, Winerdal ME, Krantz D, Zirakzadeh AA, Marits P, Sjöholm LK, Sherif A, Winqvist O. **Increased $CD4^+$ T cell lineage commitment determined by CpG methylation correlates with better prognosis in urinary bladder cancer patients**.
Clinical Epigenetics. 2018 Aug 3;10(1):102.
doi: 10.1186/s13148-018-0536-6

CONTENTS

LIST OF ABBREVIATIONS

5-aza	5-Azacytidine
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCG	Baccilus Calmette Guérin
bh-SNE	Barnes-Hut Stochastic Neighbor Embedding
BID	BH3 interacting-domain death agonist
BTLA	B- and T-lymphocyte-associated protein
CA9	Carbonic anhydrase IX
Cad6	Cadherin 6
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CK18	Cytokeratin 18
CNS	Conserved non-coding sequence 2
CpG	Cytosine-phosphate-guanine
cSMAC	Central supramolecular activation complex
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T lymphocyte antigen-4
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DP	Double positive
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ETP	Early thymic progenitor
FCS	Fetal calf serum

FIP200	FAK family-interacting protein of 200 kDa
GO	Gene ontology
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine-2,3-dioxygenase
IHC	Immunohistochemistry
IFN	Interferon
IL	Interleukin
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
iTreg	Induced regulatory T cell
ITSM	Immunoreceptor tyrosine-based switch motif
LAG3	Lymphocyte activation gene 3 protein
LFA-1	Lymphocyte function-associated antigen 1
M-CSF	Macrophage colony stimulating factor
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MS	Mass spectrometry
mTEC	Medullary thymic epithelial cell
MTOC	Microtubule organizing center
mTOR	Mammalian target of rapamycin
MVAC	Methotrexate, Vinblastine, Adriamycin, and Cisplatin
NAC	Neoadjuvant chemotherapy
NF-κB	Nuclear factor- κB
NK	Natural killer
nTreg	Natural regulatory T cell
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell

PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PI3K	Phosphatidylinositol 3-kinase
PLS	Partial least squares
PRR	Pattern recognition receptor
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
S-2HG	S-2-hydroxyglutarate
S1PR1	Sphingosine-1-phosphate receptor 1
SFM	Serum and phenol red free medium
SHP-1	Src homology region 2 domain-containing phosphatase-1
SHP-2	Src homology region 2 domain-containing phosphatase- 2
SI	Stimulation index
SN	Sentinel node
SP	Single positive
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophage
TAP	Transporter associated with antigen processing
Tc	T cytotoxic cell
T_{CM}	Central memory T cell
TCR	T cell receptor
Teff	Effector T cell
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memory T cell with CD45RA upregulation
TGF-β	Transforming growth factor-β
Th	T helper cell
TIL	Tumor infiltrating lymphocyte

TNF	Tumor necrosis factor
Treg	Regulatory T cell
T_{RM}	Tissue-resident memory T cell
T_{SCM}	Stem cell memory T cell
TSP	Thymic-seeding progenitor
TSS	Transcription start site
TUR-B	Transurethral resection of the bladder
UBC	Urinary bladder cancer
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

The term “immunity” has existed for over thousand years. In the ancient Greece, Thucydides first mentioned the word immunity in the plague of Athens in 430 B.C. He wrote that the people who survived and recovered from the plague did not acquire the illness for the second time. The concept of immunity has kept developing since then. In the modern world of science, we use the term immunity to define the resistance of the body towards infectious diseases. Immunity is further described to consist of molecules, cells, tissues, and organs that together constitute the immune system with the ultimate function of eradicating pathogens. As the world of science evolves, the role of the immune system goes beyond the eradication of infections. Today, we believe that the immune system also participates in our defense against the tumors, which is the focus of this thesis.

There are two main arms of the immune system: the innate and the adaptive immune responses. The innate immune response mediates the rapid initial protection against infections, whereas the adaptive immune response is activated later during the course of infections. These two responses have a complementary action to each other. The initial induction of the innate immune response is mediated by the germline-encoded pattern recognition receptors (PRRs) upon microbial infection or tissue damage. PRRs recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). This process will in turn activate the acute inflammatory reaction for the purpose of microbial elimination and tissue repair [1]. Additionally, the innate immune response has a role in activating the adaptive immune response, which consists of T and B cells. In this thesis, we set our focus on T cells. The innate antigen presenting cells (APCs) have the capacity to present antigenic peptides to T cells via T cell receptor (TCR) and major histocompatibility complex (MHC) [2]. The process of adaptive immune response activation takes longer time compared to the innate immune activation. However, the adaptive immune response provides a specific and long-term immunity, which is essential in the immune protection against cancer.

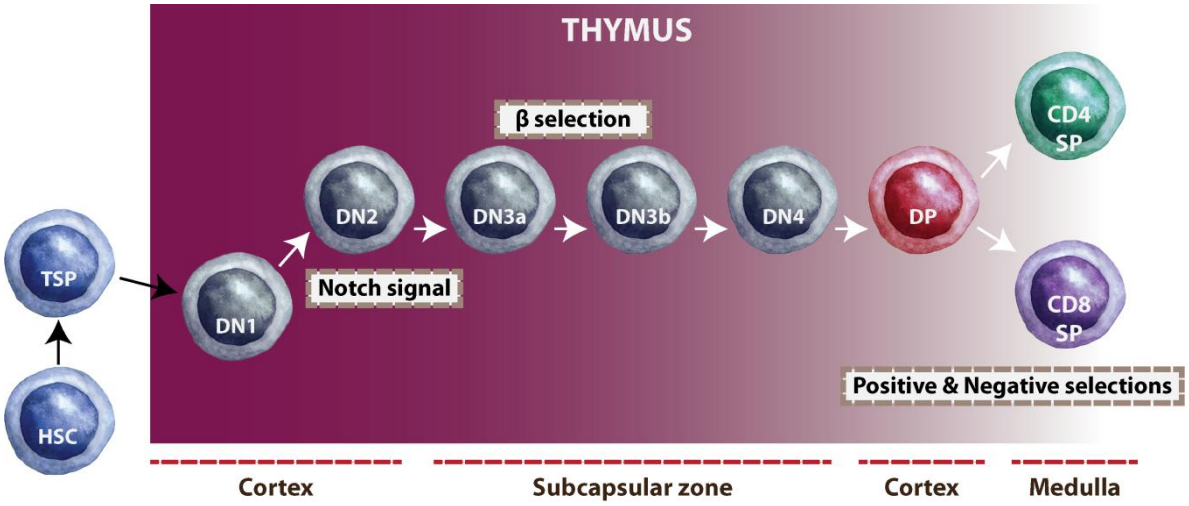
Cancer is a major global health burden. In 2013, the global cancer burden was registered to display 14.9 million incident cases with approximately 8.2 million deaths [3]. This number is projected to increase into 22.2 million incident cases by the year 2030 [4], posing for a serious threat. As the entire field of cancer research develops, it has been demonstrated that the immune system has a significant role in the cancer regression or progression [5]. This complex interplay between the tumor and immune system, especially T cells, within the intricate tumor microenvironment is something that needs further exploration.

1.1 T CELL ACTIVATION

1.1.1 T cell development

The development of T cells starts when the hematopoietic stem cells (HSCs) are constantly recruited from the bone marrow into the thymus. During their journey towards the thymus, HSCs become more and more restricted in their fate, and give rise to the earliest thymic-seeding progenitor (TSP) (**Figure 1**). As the TSP enters the thymus, it will progress into the early thymic progenitor (ETP), which is a $CD4^{-}CD8^{-}$ double negative (DN) thymocyte. The thymus provides the microenvironment for the DN thymocytes in order to develop into a T cell lineage. DN thymocytes are comprised of four differentiation stages (DN1 to DN4), based on their expression of CD25 and CD44 [6].

During the development processes, there are three important checkpoints for the thymocyte maturation into becoming T cells. The first critical checkpoint takes place in the phase of DN1 thymocyte, where the Notch signaling pathway plays an important role by inhibiting the multiple cell fate potential of a thymocyte (myeloid cell, natural killer/NK cell, and dendritic cell/DC), leading the development towards the T cell lineage [7] (**Figure 1**).



Adapted from *Koch U, et al. Annu. Rev. Cell Dev. Biol. 2011*

Figure 1. T cell development in the thymus. The development of T cells starts when the TSP deriving from HSC enters the thymus and differentiates into $CD4^{-}CD8^{-}$ DN thymocytes. In the thymus, there are three important checkpoints that shape the T cell development: Notch signal, β selection, and positive and negative selections. The locations within the thymus where each process occurs are shown. DN: double negative, DP: double positive, HSC: hematopoietic stem cell, SP: single positive, TSP: thymic-seeding progenitor.

The next checkpoint is the β selection of the TCR. This occurs during the transition between DN3a to DN3b thymocytes, in which the rearrangement of TCR genes start, which takes place in the subcapsular zone of the thymus (**Figure 1**). However, before the formation of the αβ-TCR, the developing thymocytes express the pre-TCR comprising of the rearranged TCR-β chain and the pre-TCR-α chain, due to the later rearrangement of the

TCR-α gene. The TCR-β chain, in the absence of the TCR-α chain, is capable to induce the expression of two co-receptors CD4 and CD8, yielding a double positive (DP) thymocyte [8]. After this process, TCR-α recombination is initiated and a functional TCR-αβ complex is formed and expressed by the thymocytes.

At the last checkpoint, the DP thymocytes will undergo a positive selection based on their specificity and affinity to self-peptide-MHC complexes presented by the cortical thymic epithelial cells (cTECs). The positively selected cells will then commit into either single positive (SP) CD8 cells or CD4 cells, based on their recognition to the MHC class I or MHC class II, respectively (**Figure 1**). The next process is the negative selection of the SP cells which occurs in the thymic medulla (**Figure 1**). SP cells with a high affinity towards self-antigens presented by the medullary thymic epithelial cells (mTECs) will be eliminated, in order to reduce the chance of releasing auto-reactive T cells into the periphery [9]. Following this chain of events, $CD8^+$ and $CD4^+$ T cells start leaving the thymus as naïve T cells. The naïve T cells will circulate to the secondary lymphoid organs and are ready to be presented to the peptide antigens by APCs.

1.1.2 T cell receptor activation

In the secondary lymphoid organs, naïve T cells will be activated following the presentation of peptide antigens by the APCs. This presentation is mediated by MHC molecules expressed on APCs, which present the peptides to the αβ-TCR on T cells. The MHC molecules are sub-classified into MHC class I and MHC class II, which are recognized by the $CD8^+$ and $CD4^+$ T cells, respectively [10]. Following antigen recognition by T cells, biochemical changes will take place in the cytoplasmic portion of the CD3 complex, in which the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) expressed by protein members of the CD3 complex occurs [11]. This event will lead to the downstream intracellular signal transduction.

1.1.3 Co-stimulation and co-inhibition

Nevertheless, activation through the TCR alone (signal 1) will result in an anergic state of unresponsiveness of the T cells. Therefore, an additional co-stimulatory signal (signal 2) is needed to induce a potent activation of the T cells [12]. There are several co-stimulatory molecules that have been identified. One of them is CD28. Upon TCR stimulation, CD28 will bind to its ligands CD80 or CD86, expressed on APCs, resulting in induction of the downstream PI3K signaling pathway [13]. Additionally, multiple co-stimulatory receptors have been described participating in T cell activation, such as ICOS, OX40, and 4-1BB [14, 15].

Besides the existence of co-stimulation to elicit a potent T cell activation, co-inhibitory signals are also needed to regulate the immune response. The co-inhibition acts as a peripheral tolerance mechanism in order to prevent an exaggerated T cell response that potentially could lead to harm and even autoimmunity. Two co-inhibitory molecules widely described are the cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1

(PD-1). CTLA-4 acts by setting up a competitive binding towards the co-stimulatory CD28 ligands, CD80 and CD86 on APCs [16]. As for PD-1, upon engagement to programmed death-ligand 1 (PD-L1) or programmed death-ligand 2 (PD-L2), Src homology region 2 domain-containing phosphatase-1 and 2 (SHP-1 and SHP-2) will be recruited into the immunoreceptor tyrosine-based switch motif (ITSM) in the cytoplasmic tail of PD-1 [17]. Consequently, this event will lead to a negative regulation of the T cell activation.

1.1.4 T cell lineage differentiation

Since naïve T cells are activated via the recognition of specific peptide antigens mediated by TCR-MHC complexes and co-stimulatory signals, the presence of polarizing cytokines secreted by the APCs (signal 3) will determine the lineage differentiation of $CD8^+$ and $CD4^+$ T cells [18]. The classically established T cell subsets are $CD8^+$ T cytotoxic 1 (Tc1) / $CD4^+$ T helper 1 (Th1) and $CD8^+$ T cytotoxic 2 (Tc2) / $CD4^+$ T helper 2 (Th2) [19, 20]. Priming by IL-12 cytokine will polarize naïve T cells into becoming Tc1/Th1 cells, whereas IL-4-priming will result in Tc2/Th2 cells [21] (**Figure 2**).

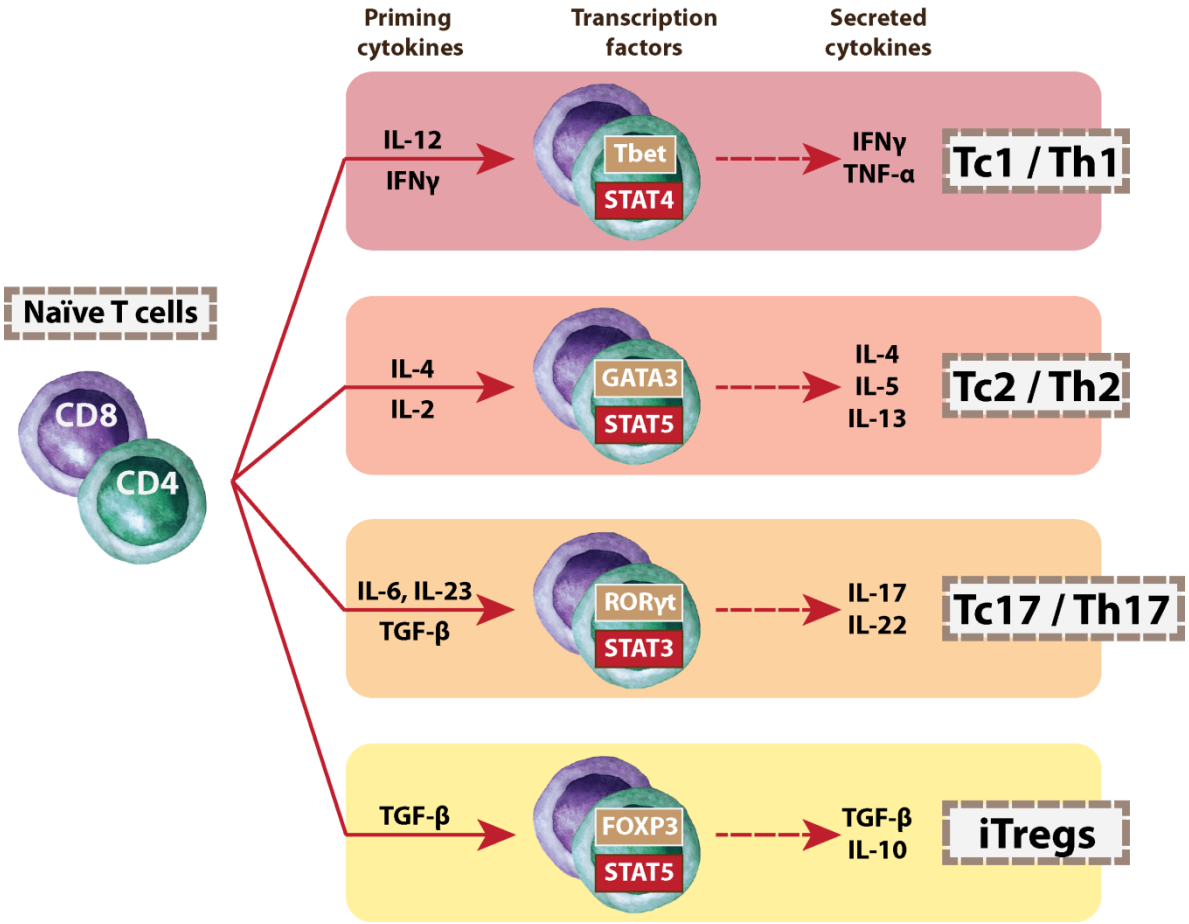


Figure 2. The lineage differentiation of T cells. Once the naïve $CD8^+$ and $CD4^+$ T cells are activated via TCR-peptide-MHC complex and co-stimulatory signals, the presence of priming cytokines will polarize the T cells into a specific lineage. Consequently, the differentiated T cells will express lineage-specific transcription factors and produce their respective cytokines. $CD8^+$ T cells will develop into Tc subsets, whereas $CD4^+$ T cells will develop into Th subsets. IFNγ: interferon-γ, IL: interleukin, iTregs: induced regulatory T cells, STAT: signal transducer and activator of transcription, Tc: T cytotoxic cells, TGF-β: transforming growth factor-β, Th: T helper cells, TNF-α: tumor necrosis factor-α.

Following the cytokine stimulation, the expression of lineage-specific transcription factors will be promoted. Expression of T-bet will promote the development of Tc1/Th1 cells and GATA3 for the development of Tc2/Th2 cells (**Figure 2**). Moreover, the downstream signaling molecules such as signal transducer and activator of transcriptions (STATs) will be differentially induced in distinct T cells subsets. In addition, the T cell subsets can be functionally distinguished by their respective cytokine production. The signature cytokines for Tc1/Th1 cells are interferon-γ (IFNγ) and tumor necrosis factor-α (TNF-α), whereas Tc2/Th2 cells produce IL-4, IL-5, and IL-13 [22, 23] (**Figure 2**).

As the study of T cell fate progresses, additional subsets of $CD8^+$ and $CD4^+$ T cells have been identified. These cells are the Tc17/Th17 cells, which express the transcription factor RORγt and the $CD8^+$ or $CD4^+$ regulatory T cells (Tregs), which are marked by their lineage-specific transcription factor FOXP3 [22, 23] (**Figure 2**).

Although having a specific distinct fate, $CD8^+$ and $CD4^+$ T cells have been demonstrated to display lineage plasticity. In $CD4^+$ T helper cells, for instance, IFNγ production can be exerted from Th2, Th17, or Tregs by stimulation using IL-12 [24, 25]. Similarly, for $CD8^+$ T cells, Tc2 and Tc17 can additionally produce IFNγ, with maintained expression of their signature cytokines [26, 27]. Hence, in the presence of proper polarizing conditions, differentiated T cells can be reprogrammed into other cell lineages, implying their situation-dependent plasticity.

1.2 EFFECTOR FUNCTIONS OF T CELLS

1.2.1 $CD8^+$ T cells

Since $CD8^+$ T cells recognize the target cells directly via MHC class I-peptide complexes, they can immediately kill the target cells using their effector cytotoxic constituents, perforin and granzymes. Upon antigenic recognition by the $CD8^+$ cytotoxic T cells (Tc1), additional molecules take charge in the formation of the immunological synapse (IS). One of the major molecules responsible in the IS formation is the lymphocyte function-associated antigen 1 (LFA-1) integrin, expressed by $CD8^+$ T cells, which will bind to its ligand intercellular adhesion molecule 1 (ICAM-1), expressed on the surface of the target cells. These two molecules firmly hold the two cells together, forming the IS, and thus optimizing the activation of the T cells. The IS formation has a lifespan of 20-30 minutes [28]. Following this event, microtubule organizing center (MTOC) will polarize towards the IS and the lytic granules containing the cytotoxic constituents will move towards the IS along the microtubules. Accordingly, the accumulation of the lytic granules below the plasma membrane occurs [29]. Next, the gap in the cortical actin opens below the central supramolecular activation complex (cSMAC), which will allow the cytotoxic constituents to be secreted into the IS [30].

Once the cytotoxic constituents are degranulated from the $CD8^+$ T cells, perforin first starts to bind to the surface of the target cells to form pores [31] (**Figure 3**). These pores will allow granzymes to enter the cytosol of the target cells and induce apoptosis. Granzyme B, the most prominent class of granzymes, promotes apoptosis in two pathways: caspases activation or BH3 interacting-domain death agonist (BID)-dependent mitochondrial permeabilization [32, 33]. Moreover, another important class of granzymes, granzyme A, promotes cell death by several mechanisms, such as mitochondrial trans-membrane potential reduction and reactive oxygen species (ROS) generation [34].

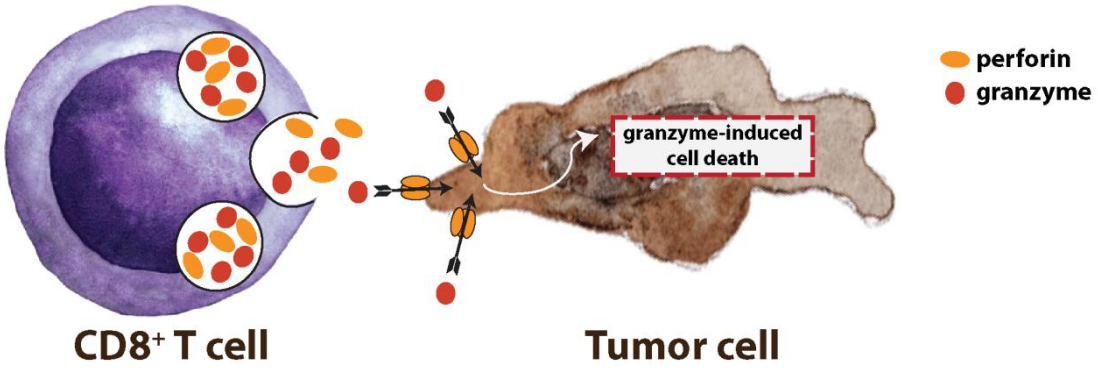


Figure 3. The cytotoxic activity of $CD8^+$ T cells. Upon the encounter of a $CD8^+$ T cell to the tumor cell, the lytic granules containing the cytotoxic constituents inside the $CD8^+$ T cell will move towards the immunological synapse and secrete perforin and granzymes. Perforin will form pores that allow granzymes to enter the cytosol of the tumor cells and induce cell death.

The importance of perforin in the cancer immunity is marked by the increased susceptibility to cancer in the people with perforin gene (*PRF1*) mutations [35]. Additionally, perforin-knockout mice have an increased frequency of spontaneously developed tumors [36]. This implies that granzyme-mediated killing of tumor cells by $CD8^+$ T cells is secondarily affected, since non-functional perforin cannot facilitate the obligated intracellular delivery of granzymes. Conversely, it was demonstrated that mice which are lack of individual granzymes remain cancer free [32], further supporting the importance of perforin for $CD8^+$ T cell-mediated tumor cell killing.

1.2.2 $CD4^+$ T helper cells

$CD4^+$ T helper cells play a crucial role in helping in the maintenance of other immune cells, such as macrophages, B cells, and $CD8^+$ T cells [37]. There have been several studies within the field in investigating the support given by the $CD4^+$ T helper cells to $CD8^+$ T cells. Classically, $CD4^+$ T helper cells provide IL-2 for $CD8^+$ T cells in order to maintain their clonal expansion [38]. In addition, $CD4^+$ T helper cells promote the maintenance of a $CD8^+$ T cell fraction as long-term memory cells, which ensures the availability of effector $CD8^+$ T cells upon recall activation [37].

Additionally, $CD4^+$ T helper cells have been described to regulate $CD8^+$ T cell responses via the CD40/CD40L pathway. CD40, expressed by the APCs, will be stimulated by CD40L that is expressed by the antigen-stimulated $CD4^+$ T helper cells. The net result is potent activation of the APCs, demonstrated by the increased expression of MHCs and co-stimulatory receptors, enhanced cytokine production, and elevated expression of chemokines. The activated APCs will in turn support the maintenance of the $CD8^+$ T cell response [39]. Moreover, it was demonstrated that the delivery of $CD4^+$ T helper cells is facilitated by a CD27/CD70 co-stimulatory pathway, expressed by $CD8^+$ T cells and APCs [40]. Hence, the interaction among $CD4^+$ T helper cells, APCs, and $CD8^+$ T cells is well-preserved.

Furthermore, in the tumor model, the presence of $CD4^+$ T helper cells results in a lower expression of co-inhibitory receptors, such as PD-1, B- and T-lymphocyte-associated protein (BTLA), and lymphocyte activation gene 3 protein (LAG3) in $CD8^+$ T cells. Consequently, by support from $CD4^+$ T helper cells, $CD8^+$ T cells will have better effector responses and anti-tumor activities, as well as an improved migratory capacity [40].

1.2.3 Regulatory T cells

T cell activation via TCR-peptide-MHC complex can lead to the generation of pathogenic auto-reactive T cells. Therefore, the mechanisms of peripheral tolerance through the co-inhibitory receptors CTLA-4 and PD-1 are needed to avoid such auto-reactivity, as described above. However, these peripheral tolerance mechanisms may not be sufficient in controlling the activated T cell response. Hence, a specific immune cell subset to regulate the T cell activity has evolved.

The groundbreaking discovery of a $CD4^+$ T cell subset with a high expression of IL-2 receptor α-chain (CD25) further supports this notion [41]. This cell subset is called regulatory T cells (Tregs). Natural Tregs (nTregs) are formed in the thymus and they possess suppressive functions [41]. Furthermore, FOXP3 has been identified as the transcription factor which is stably expressed by the Tregs and it is needed to maintain the functional suppressive phenotypes of Tregs by affecting the transcriptional programming [42]. The importance of Tregs has clinically been demonstrated since patients with mutations of *FOXP3* gene (IPEX syndrome) are lack of Tregs and suffer from severe autoimmunity and unregulated T cell proliferation [43].

Several effector mechanisms of Treg functions in suppressing the effector T cell activity have been discovered. One of them is by highly expressing CD25 on Tregs, resulting in IL-2 overconsumption. This leads to poor IL-2 stimulation of effector T cells which is needed for proliferation [44]. Moreover, Tregs express the co-inhibitory receptor CTLA-4 which inhibits the effector T cell activity [45]. CTLA-4 possesses a higher affinity than co-stimulatory receptor CD28 towards CD80 or CD86 expressed on the APCs, resulting in the inhibition of CD28 – CD80/CD86 ligation. Consequently, the co-inhibitory signal activation will deliver a negative regulatory signal to the APCs [46]. Additionally, PD-1

expression on Tregs is demonstrated to contribute in suppressing the function of $CD8^+$ T cells [47].

Moreover, other molecules such as CD39 and CD73 are expressed by the Tregs and contribute to the Treg-mediated suppression of the effector T cells. CD39 and CD73 can catalyze the generation of adenosine from the adenosine triphosphate (ATP). Correspondingly, the engagement of adenosine to its receptor on the effector T cells will induce intracellular cyclic adenosine monophosphate (cAMP) expression, resulting in limited T cell proliferation [48].

Aside from the trans-membrane molecule expression, Tregs also secrete cytokines such as IL-10, IL-35, and transforming growth factor-β (TGF-β). These cytokines also inhibit local effector T cell immune response [49]. For instance, TGF-β has been displayed to suppress $CD4^+$ Th1 cell response [50]. Other secreted proteins like granzyme B is also demonstrated to contribute in the Treg-mediated suppressive activity. Granzyme B will induce cytolysis by promoting apoptosis of the effector T cells [51].

To summarize, Tregs as part of the peripheral tolerance, have a vital role in avoiding T cell-induced autoimmunity. Several mechanisms are utilized by Tregs in order to suppress effector T cell immune response, which may be used under different conditions.

1.3 T CELL MEMORY

1.3.1 The memory formation

The classical fate of a T cell starts when a mature naïve T cell derived from the thymus met its cognate antigen in the secondary lymphoid organs. This event will result in the differentiation and clonal expansion of T cells with effector functions as explained above. However, effector T cells are short-lived and following elimination of the antigens, most of them will die by apoptosis. This process is called the contraction phase. Correspondingly, a small fraction (~5-10%) of the remaining T cells will survive and differentiate into memory T cells with the capacity to be present for a long-term within the body [52, 53].

Memory T cells have a signature surface marker expression of $KLRG1^{low}$, IL-7Rα $(CD127)^{hi}$, $CXCR3^{hi}$, and $CD62L^{hi}$ [52]. It was recently demonstrated that upon contraction phase, $KLRG1^+$ effector $CD8^+$ T cells will downregulate their KLRG1 expression in a Bach2-dependent fashion, which allows them to differentiate into different memory subsets [54]. Additionally, several sets of transcription factors are described to regulate the memory formation of T cells in a competing manner. Transcription factors such as EOMES, BCL-6, ID3, and STAT3 maintain the memory properties of T cells instead of their effector phenotypes. Conversely, the opposing transcription factors like T-bet, BLIPM1, ID2, and STAT4 counter-regulate T cells to promote effector profiles [55-57].

Furthermore, cellular metabolism is reported to regulate the memory formation of T cells

from the effector state. Following antigenic clearance, effector T cells, dependent on anabolic metabolism by glycolysis as the energy source, will switch into catabolic metabolism involving the fatty acid oxidation and the mitochondrial oxidative phosphorylation, resulting in a memory cell formation [58]. Moreover, the pathways involved in governing the effector to memory state transition are related to the effector-promoting phosphatidylinositol 3-kinase – AKT – mammalian target of rapamycin (PI3K-AKT-mTOR) and AMP-activated protein kinase (AMPK) pathways. During the memory formation, AMPK will inhibit mTOR activity and thus promote the fatty acid oxidation, needed in the memory T cell formation [58, 59]. Correspondingly, mTOR inhibition may regulate the translational programming of the ribosomal proteins, which is observed to be suppressed in the terminal effector cells, just before the contraction phase [60].

An additional mechanism involved in the T cell memory formation was further identified. S-2-hydroxyglutarate (S-2HG) is demonstrated to be produced by $CD8^+$ T cells following TCR activation. S-2HG induces transcriptional signature similarly found in the memory $CD8^+$ T cells, such as upregulation of *Cd62l*, *Cd127*, *Bcl6*, and *Eomes*, as well as downregulation of *Prdm1* (encoding Blimp1), which promotes the memory T cell formation [61].

The outstanding feature of the memory T cells is their long-term persistence within the body. Accordingly, continuous maintenance to preserve their lifespan is crucial. It was demonstrated that the maintenance of memory $CD8^+$ T cells is antigen-independent and cytokine-dependent through continuous IL-15 and IL-7 stimulation [62]. IL-15 works by sustaining the basal proliferation, while IL-7 maintains cell survival [63]. As for the memory $CD4^+$ T cells, their maintenance seems to be also supported by IL-15 [64]. However, it is still unclear whether the persistence of antigen is needed in preserving memory $CD4^+$ T cells.

1.3.2 The memory subsets

The most vital feature of memory T cells is the ability to respond more rapidly and effectively upon re-exposure to antigens. Correspondingly, the location of memory T cells is crucial for a rapid and effective immunosurveillance. Classically, two memory T cell subsets have been identified: central memory T (T_{CM}) cells and effector memory T (T_{EM}) cells. T_{CM} cells are $CD45RA^-CCR7^+CD62L^+$ and they traffic to the secondary lymphoid organs, whereas T_{EM} cells are $CD45RA^-CCR7^-CD62L^-$ and they recirculate through non-lymphoid tissues and blood [65] (**Figure 4**). Accordingly, due to the possibility of recirculation, both T_{CM} and T_{EM} cells can be found in the peripheral blood [66]. These two cell subsets have some phenotypical distinctions. T_{CM} cells own a better proliferative capacity upon activation than T_{EM} cells, but T_{EM} cells possess greater effector activities, i.e. cytokine production by $CD4^+$ T_{EM} cells and cytotoxic molecule expression by $CD8^+$ T_{EM} cells [67] (**Figure 4**).

Furthermore, some T_{EM} cells gain CD45RA expression, which are then defined as T_{EMRA} cells. T_{EMRA} cells are terminally differentiated and the upregulation of CD45RA occurs after homeostatic cytokine stimulation on memory T cells instead of antigen stimulation [68]. $CD8^+$ T_{EMRA} cells are demonstrated to express the highest amount of cytotoxic molecules like perforin [69], and they reside in the blood, spleen, bone marrow, and lungs [70] (**Figure 4**). However, T_{EMRA} cells display a low proliferative capacity and they are short-lived [68, 69].

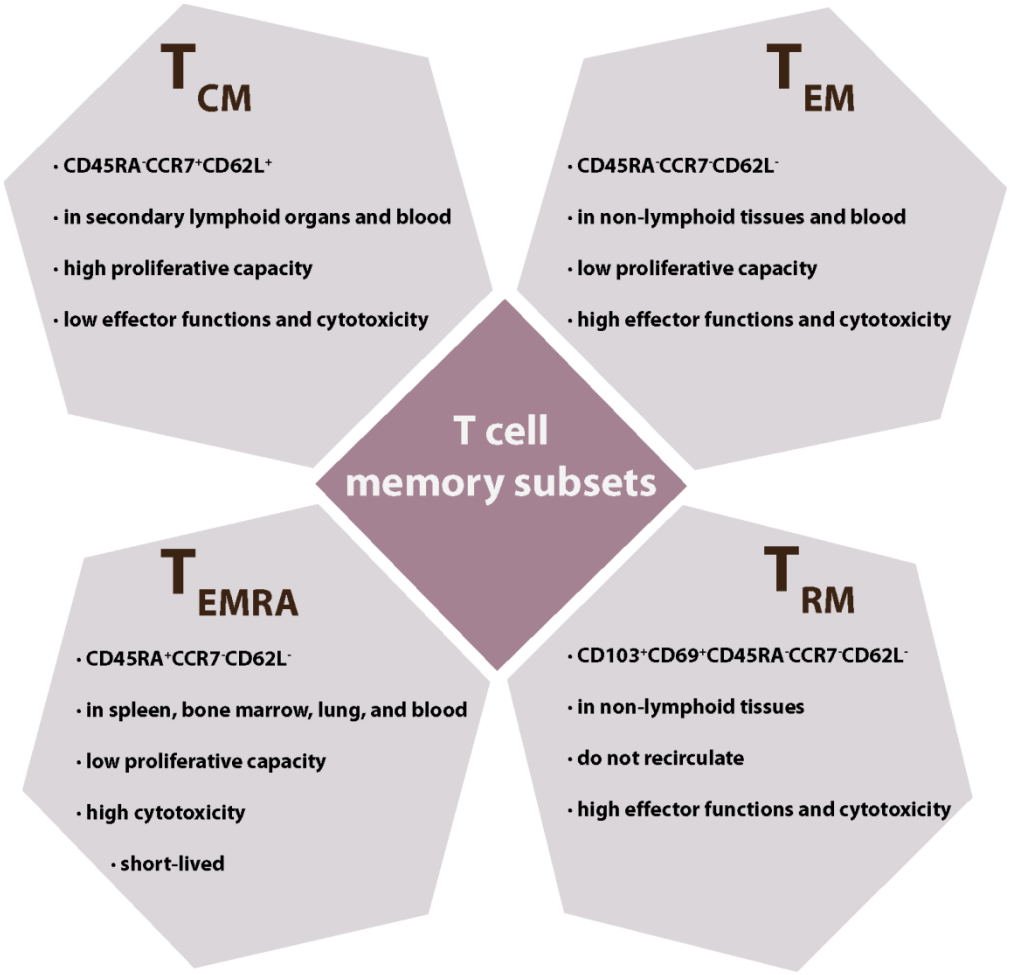


Figure 4. The T cell memory subsets. Following contraction phase, the remaining effector T cells differentiate into memory T cells with different subsets. Each memory subset has different phenotypes, localizations, and functions. T_{CM}: central memory T cells, T_{EM}: effector memory T cells, T_{EMRA}: effector memory T cells with CD45RA upregulation, T_{RM}: tissue-resident memory T cells.

Emerging evidence in this field further answers the question of whether T_{EM} cells would keep recirculating for surveillance or that they remain in the non-lymphoid tissues. This question was answered by the work done using infection models, which led to the discovery of tissue-resident memory T (T_{RM}) cells [71, 72]. T_{RM} cells, which are well-described in $CD8^+$ T cell compartment, have a specific feature of not recirculating in the peripheral blood and instead they localize in the non-lymphoid tissues, such as intestines, skin, brain,

lungs, and female reproductive tract [73]. The differentiation, maintenance, and residency of T_{RM} cells are controlled by Runx3 [74]. These cells have two key surface markers, which are CD69 and CD103 [75] (**Figure 4**). CD69 causes T cell retention in the tissue due to its antagonistic effect towards sphingosine-1-phosphate receptor 1 (S1PR1) that mediates egress from tissues [76]. Meanwhile, CD103 that is an αE integrin, will pair to the β7 integrin and bind to E-cadherin expressed on epithelial cells, contributing to their persistence in the non-lymphoid tissues [63, 73, 77]. In addition, T_{RM} cells in the skin are characterized by their expression of CD49a [78].

Accordingly, T_{RM} cells harbor effector-like properties, they are localized in the non-lymphoid tissues, and they provide a *bona fide* immediate protection and elimination following secondary antigenic exposure [63] (**Figure 4**). Moreover, T_{RM} cells in the non-lymphoid tissues are recently reported to give rise to the T_{RM} cells in the draining lymph nodes following secondary antigenic challenge. This indicates the possibility of immunosurveillance by the T_{RM} cells in the regional lymph nodes [79].

1.4 EPIGENETIC REGULATION OF THE T CELL PHENOTYPE

1.4.1 Epigenetics – the concept

The term 'epigenetics' is defined as the inherited pattern of gene expression without the changes in the DNA sequence. Epigenetics act by stabilizing the gene expression programming, which thus results in the determination of a cell identity [80]. The two extensively studied mechanisms in the field of epigenetics are the DNA methylation and histone modifications.

DNA methylation occurs in the cytosine residue (5-methylcytosine) of a cytosine-phosphate-guanine (CpG) dinucleotide and most studies concentrate on the methylation profile of the CpG-rich regions (CpG islands) [81]. Commonly, CpG islands are found in the promoter regions of genes, however they can also be located in the exons [81]. DNA methylation allows the binding of the methyl-CpG-binding domain (MBD) protein to the methylated CpG, which makes it impossible for transcription factors to bind to the methylated CpG. Consequently, this event results in a transcriptional repression [82].

Another epigenetic regulation occurs in the form of histone modifications. As widely known, DNA is packed into nucleosomes with eight histone components (two copies of H2A, H2B, H3, and H4). Generally, the acetylation of the histones promotes transcription, whereas methylation may have a positive or negative effects on transcription [83].

As mentioned above, T cells have the capacity to differentiate into different effector and memory subsets following naïve T cell activation. This phenotypical plasticity of T cells which have an identical underlying genome may be partly accounted by epigenetics. In T cell differentiation, epigenetics regulate a cell lineage-specific transcriptional program

leading to different cell-lineage-specific phenotypes [84]. Therefore, epigenetic regulation is responsible to maintain a heritable cell lineage commitment.

1.4.2 Epigenetic regulation in CD8$^+$ T cells

Upon external stimulation on CD8$^+$ T cells that promotes cell differentiation, epigenetic regulation in terms of DNA methylation and histone modifications was demonstrated to serve the signature transcriptional programing of each cell lineage [85]. A study using a single-cell RNA sequencing approach revealed that after CD8$^+$ T cells are antigen-stimulated, the changes of epigenetic and transcriptional regulation within the cells occur since the first cell division, resulting in distinct gene profiles between effector and memory cell subsets [86]. Accordingly, DNA methylation profile of signature genes differs among differentiated memory CD8$^+$ T cell subsets, such as stem cell memory T (T_{SCM}) cells, T_{CM} cells, and T_{EM} cells.

Notably, the DNA methylation status of the signature loci remains unchanged even after three consecutive rounds of cell division using homeostatic cytokines IL-7 and IL-15 stimulation [87]. For instance, it was seen in the effector-associated genes *PRF1* and *IFNG* that there is no change in the methylation pattern of undivided and divided cells [87], marking a crucial role of epigenetics in maintaining effector cell lineage commitment. In contrast, selected genes responsible for homing (*CCR7*) and self-renewal regulation (*TCF7*) can be reprogrammed in long-lived T_{SCM} and T_{CM} cells following homeostatic *ex vivo* stimulation [87]. This indicates that the epigenetic regulation may be flexible on selected genes in order to rapidly differentiate from memory into effector cells.

Furthermore, it was demonstrated that during differentiation, CD8$^+$ T cells have highly dynamic [88] and distinct enhancer repertoires as determined by the intensity of H3K4me1 signal [89]. Correspondingly, these different sets of enhancers regulate the expression of lineage-specific signature genes [89]. In addition, epigenetic modification of a lineage-specific enhancer will lead to a lineage-specific gene transcription [84]. Therefore, it indicates that the epigenetic regulation of the lineage-specific enhancers is crucial for CD8$^+$ T cell subset generation and maintenance.

1.4.3 Epigenetic regulation in regulatory T cells

Major discovery in the field has appointed FOXP3 as the marker for regulatory T cells (Tregs) [90]. However, since conventional CD4$^+$ T cells also transiently express FOXP3 upon activation [91], further quest was to find an answer whether FOXP3 served as a specific lineage marker for human Tregs. To this end, investigation of the epigenetic regulation by means of DNA methylation in the regulatory regions of the *FOXP3* gene may aid us in identifying truly committed Tregs.

Studies over the years have identified specific DNA methylation patterns in the *FOXP3* gene that reflects a specific Treg lineage. Our group has discovered that hypomethylation in the CpG position -77 located in the promoter, to be specific for a stable FOXP3 expression

in Tregs [92]. This methylation pattern is not observed in the conventional T cells, even after activation [92].

In addition, investigation done by other groups demonstrated that demethylation of CpGs located in the conserved non-coding sequence (CNS) of the *FOXP3* gene is specific for Tregs and is not displayed in the activated conventional T cells [93, 94].

In conclusion, epigenetic modifications of the *FOXP3* gene help us in identifying the committed Tregs with stable phenotypes. Treg identity is important to be apprehended, especially in the arena of cancer immunology research where tumor Treg infiltration may lead to either positive or negative prognosis [95].

1.5 THE TUMOR MODELS

In this thesis, we used two human urological tumor models which derived from urinary bladder cancer and renal cancer. The disease background of each tumor is further explained below.

1.5.1 Urinary bladder cancer

Urinary bladder cancer (UBC) is the fourth most common cancer in men and the ninth in women in the western world [96], with 429.000 estimated new cases worldwide [97]. Some risk factors have been identified for UBC, including tobacco smoking and occupational irritants [98]. In the majority of the cases, UBC shows the histopathological feature of urothelial type (90%) [99]. Moreover, about 25% of the cases displays muscle invasiveness, which contributes to the higher probability of developing metastasis and worse survival outcome [96].

The non-muscle invasive form of the UBC (75% of the cases) is diagnosed by transurethral resection of the bladder (TUR-B), which aims to secure the diagnosis and remove the visible lesion [100]. Adjuvant treatment following TUR-B, such as intravesical chemotherapy or intravesical Baccilus Calmette Guérin (BCG) treatments may prevent recurrences of the non-muscle invasive UBC [100]. The BCG treatment acts by inducing the anti-tumor immune activation resulting in tumor regression [101].

For the muscle invasive UBC, TUR-B is also performed for the diagnostic and therapeutic purposes. Patients proven to have a muscle invasive UBC by the histopathological examination, will be managed by radical cystectomy [102]. In addition, for fit patients, cisplatin-based chemotherapy prior to surgery (neoadjuvant chemotherapy) is the standard treatment for the muscle invasive UBC [98, 102]. The neoadjuvant chemotherapy has been shown to reduce micrometastasis and improve patient survival [102]. Moreover, the advantages of providing neoadjuvant chemotherapy prior to radical cystectomy include: the delivery is done when the micrometastatic disease burden is still low, potential *in vivo* chemo-sensitivity, better tolerability of chemotherapy regimen prior to cystectomy compared

to after it, and favorable histopathological examination with negative lymph nodes and surgical margins can be determined following radical cystectomy operation [103]. Consequently, the inclusion of neoadjuvant chemotherapy in the treatment regimen will give 5-8% overall survival advantage to the patients [104-106], in which only 50% of the 5-year survival rate is displayed when radical cystectomy alone was provided [107].

Furthermore, cancer immunotherapy involving immune checkpoint blockade has been demonstrated to provide promising results for UBC. Atezolizumab, which is a PD-L1 inhibitor, is now approved for UBC treatment [108]. Additionally, more studies investigating other targets for immune checkpoint blockade are currently ongoing in clinical trials.

1.5.2 Renal cancer

Renal cancer or renal cell carcinoma (RCC) is one of the most common cancers with 338.000 new cases worldwide in 2012 [109]. The incidence of RCC increases with age and is more common in males [109]. Some identified risk factors include high body weight, hypertension, and cigarette smoking [110]. This cancer has a broad spectrum of histopathological entities, in which clear cell type is named as the most common (75% of incidence), followed by papillary type (10%) and chromophobe type (5%) [111].

The prognosis of RCC can be unpredictable. It was demonstrated that 4.2-7.1% of patients presented with tumor mass ≤4 cm may have a metastasis. On the contrary, 40% of the patients with lymph node metastasis are alive after five years of being diagnosed with RCC [112]. This emphasizes that proper diagnosis is critical, especially in establishing the presence of metastasis in RCC. Accordingly, cytological biomarker such as carbonic anhydrase IX (CA9) has been studied and considered as a tool in predicting the diagnosis and prognosis of clear cell RCC [113]. This further aids in solving the intricate problem of patient prognosis prediction in RCC.

The gold standard diagnostic procedure for RCC is the histopathological examination of the tumor biopsy. This tool will determine the next treatment modalities for the patients, which may involve partial or radical nephrectomy [114]. Moreover, as the era of cancer immunotherapy progresses in the past decades, RCC has also been treated using this treatment modality. Cytokines such as IFN-α and IL-2 have been used as RCC treatments [115], however they give a substantial toxicity. Furthermore, immune checkpoint blockade agents against PD-1, PD-L1, or CTLA-4 are currently investigated for their benefit and patient tolerability for treating RCC [110].

1.6 TUMOR MICROENVIRONMENT

The field of research in tumor microenvironment was pioneered by Stephen Paget over a hundred years ago. Based on his observation in breast cancer metastasis, Paget proposed the concept of ‘seed and soil’, in which tumor cells are like seed of plants that are carried around

until they find the favorable soil (organs) to live and grow [116]. This hypothesis underlies an understanding of an existing relationship between tumor cells and the microenvironment that supports the tumor growth.

As the research expands, the tumor microenvironment composition is discovered to be beyond the earlier proposed concept. Tumor microenvironment is revealed to be consisting of the tumor cells, residing or recruited cells, extracellular matrix (ECM), and environmental conditions like hypoxia, acidosis, and increased interstitial pressure. Correspondingly, all these components will have active interactions with the tumor cells that may support or oppose the tumor growth [117].

Mounting evidence has demonstrated the impact of ECM remodeling in the tumor microenvironment to the tumor invasion and metastasis. The remodeling of ECM is correlated with the production of fibronectin, lysyl oxidase, and type I collagen which are associated with the transition from cellular dormancy in tumor to the metastatic growth by creating a permissive environment [118] (**Figure 5**). Additionally, matrix metalloproteinases (MMPs) are also part of the ECM remodeling (**Figure 5**). Originally, MMP is proposed to cause the degradation of the basement membrane which facilitates the tumor cell invasion [119]. However, it turns out that MMPs also induce TGF-β production, resulting in the establishment of the metastatic-permissive niche and the expression of vascular endothelial growth factor (VEGF) that promotes angiogenesis and lymphangiogenesis. In addition, MMPs drive inflammation in cancer [119]. All this mechanisms contribute in the promotion of tumor progression.

One notable condition observed in the tumor microenvironment is hypoxia. This condition will result in the expression of hypoxia-inducible factors (HIF-1α, HIF-2α, HIF-3α, or HIF-1β) (**Figure 5**). HIF-1α is displayed to promote expression of transcription factors responsible for tumor growth, vascularization, and metastasis [120]. In addition, the elevated HIF-1α and HIF-2α will in turn induce glycolysis [121]. Consequently, as the glycolytic metabolism increases, lactic acid production will be elevated, posing to the acidosis condition (**Figure 5**). This acidosis condition is further driven by the pH-regulating systems that maintain the level of intracellular pH in order to support cell survival in the tumor microenvironment [122].

As mentioned above, tumor microenvironment also consists of recruited cells such as the immune cells. Paradoxically, the immune cells in the tumor microenvironment may possess pro-tumorigenic features, as proposed in the concept of inflammation-induced cancer [123]. It was demonstrated that the innate myeloid cells produce sets of pro-inflammatory cytokines that lead to the induction of STAT3 and nuclear factor-κB (NF-κB) in the tumor cells (**Figure 5**). This signal activation will promote survival and proliferation of the tumor cells, DNA damage, and epithelial-mesenchymal transition (EMT) that facilitates the tumor growth [123]. In addition, other chronically-activated innate immune cells, such as macrophages, mast cells, and granulocytes present in this microenvironment are also linked in supporting tumor development [124].

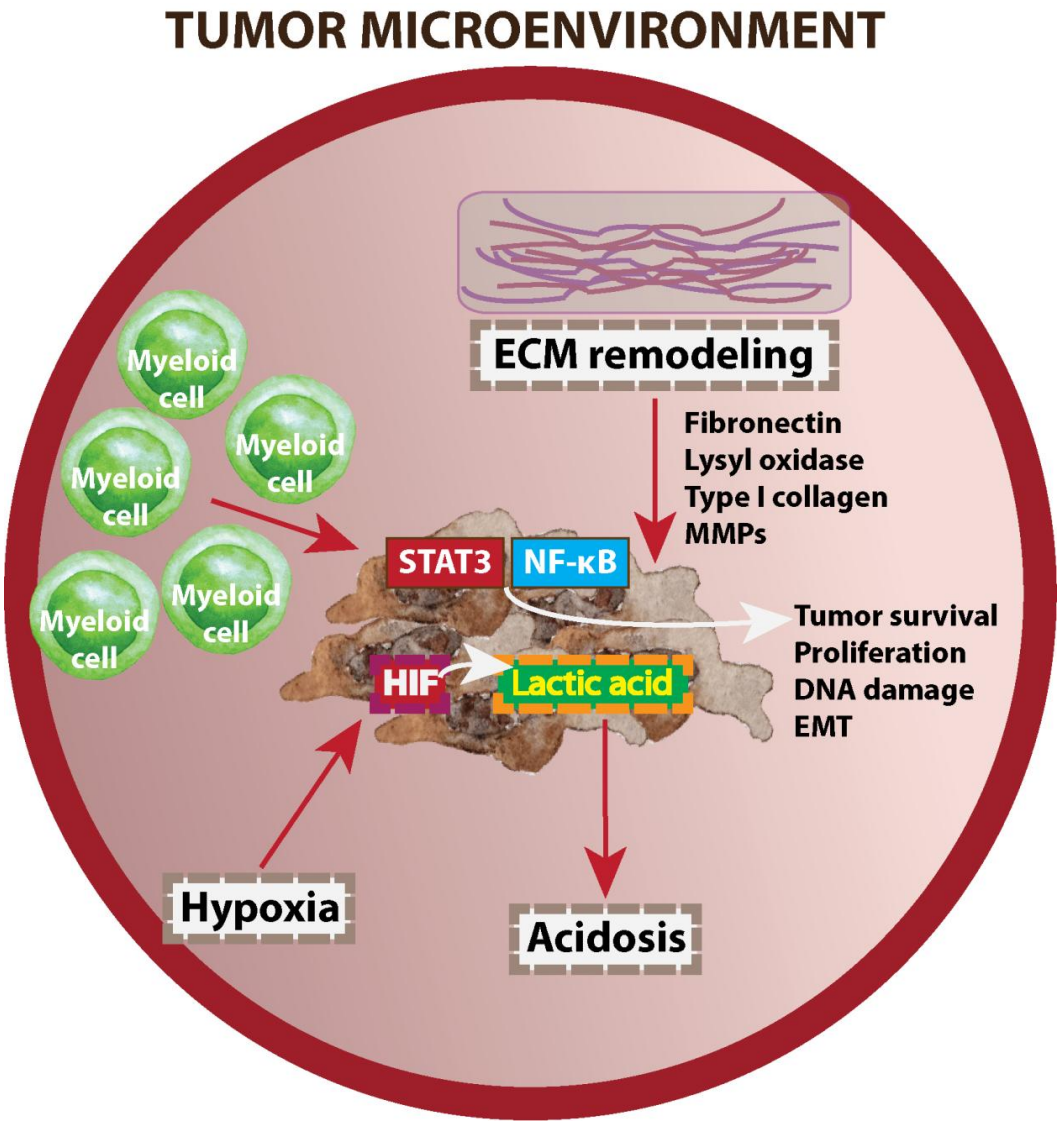


Figure 5. The tumor microenvironment. The tumor is supported by the microenvironment which is composed of the ECM, residing or recruited cells, and conditions like hypoxia and acidosis. The complex interaction among the components of the microenvironment will induce the upregulation of factors inside the tumor cells that promote the tumor cell survival and progression. ECM: extracellular matrix, EMT: epithelial-mesenchymal transition, HIF: hypoxia-inducible factor, MMP: matrix metalloproteinase, NF-κB: nuclear factor-κB, STAT3: signal transducer and activator of transcription 3.

Besides pro-tumoral activities, immune cells elicit anti-tumor responses in the tumor microenvironment. However, the success of the anti-tumor immune response depends on how well the adaptive immune cells can infiltrate the tumor microenvironment. There are three models of immune cell infiltration in the tumor microenvironment. The first model is when there is a high infiltration of leukocytes but the cytotoxic lymphocytes (also with low expression of activation markers granzyme B and IFNγ) do not enter the tumor core; this is called the infiltrated-excluded microenvironment [125]. This condition is indicative of lack immune recognition to the tumor [126]. The second model is termed infiltrated-inflamed which shows that the cytotoxic lymphocytes infiltrate the tumor core through PD-1 and PD-L1 ligation, expressed by the lymphocytes and tumor cells, respectively [125]. The last model is the extension of the infiltrated-inflamed microenvironment, in which the leukocyte

infiltration will form a tertiary lymphoid structure similar to a lymph node [125]. The tertiary lymphoid structure contains other immune cell types beside cytotoxic lymphocytes and may correlate with better prognosis for its anti-tumor property [127].

Taken together, the dynamics inside the tumor microenvironment resemble a chronic inflammation, with its different constituents that may provide positive or negative signals for tumor growth. By having a deep understanding of the complex interplay within this environment, new targets for cancer immunotherapy may be unveiled.

1.7 TUMOR IMMUNITY

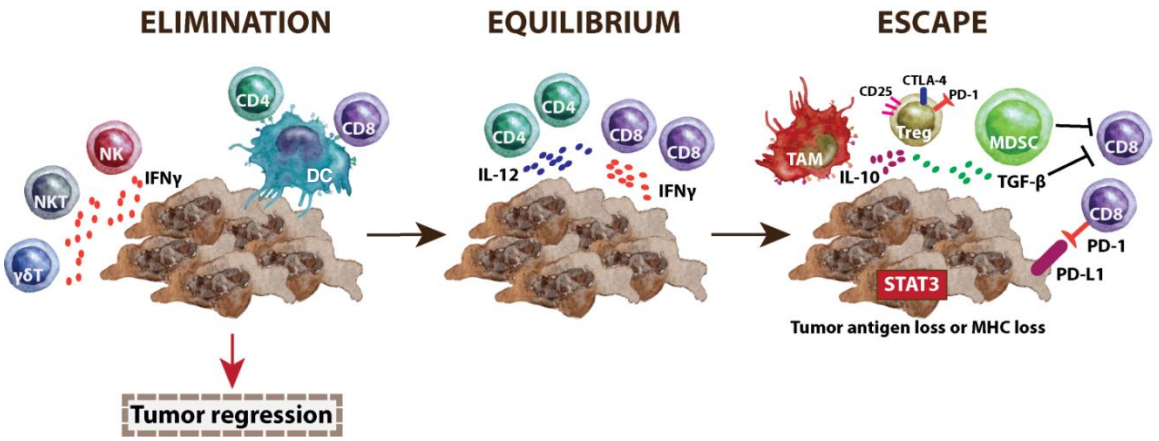
The concept of tumor immunity has come to be widely acknowledged over the past decades. It is believed that the tumor cells are targeted by the immune cells through the recognition of two categories of antigens: tumor-specific antigens (antigens that are unique to the tumor cells) or tumor-associated antigens (antigens that are not exclusively expressed by the particular tumor cells) [128]. This concept gave rise to the tumor immunosurveillance hypothesis proposed by Frank M. Burnet, in which the pre-cancerous and cancerous cells are recognized and eliminated by the immune cells before causing any harm [129, 130]. However, despite the fully functioning immune cells within the body, tumors are still capable to grow. Therefore, the concept of immunoediting is better in explaining the interaction between tumor and immune cells.

The immunoediting concept comprises of three phases: 1) elimination, in which the immune cells eliminate tumor cells as what has been proposed in the tumor immunosurveillance hypothesis, 2) equilibrium, when there is a temporary state of equilibrium between the immune cells and the growing tumor, resulting in tumor dormancy, and 3) escape, where the tumor is capable to avoid or even suppress the immune cell-mediated tumor destruction, leading to further tumor growth [131]. More detailed explanation of each phase is described below.

1.7.1 The elimination phase

The tumor immunosurveillance has taught us that elimination of the developing tumor cells may be optimally executed by the immune cells. This process requires both the innate and adaptive immune responses. The elimination phase starts when the innate immune cells such as NK cells, NK T cells, γδ T cells, and macrophages are recruited to the tumor site due to pro-inflammatory molecules and chemokines produced by the tumor microenvironment (**Figure 6**). Once these cells reach the tumor site, they will recognize the ligands for NKG2D expressed on the tumor cells and they start to secrete IFNγ [132]. The major result of IFNγ secretion is the destruction of some tumor parts by the innate cells. Furthermore, recruited DCs may obtain the tumor antigens by ingesting the tumor cell debris. Subsequently, they will migrate to the draining lymph nodes to present the tumor antigens to $CD8^+$ and $CD4^+$ T

cells (**Figure 6**). This event will result in the homing of tumor-specific T cells to the tumor which will completely eliminate the remaining tumor cells [131].



Adapted from *Schreiber RD, et al. Science. 2011*

Figure 6. The tumor immunoediting. The concept of tumor immunoediting consists of three phases. In the elimination phase, the innate and adaptive immune cells can eliminate the tumor cells, leading to the tumor regression. The next phase is the equilibrium in which the tumor cell growth is controlled by the adaptive immune cells, resulting in tumor dormancy. Lastly, the tumors can escape the immune destruction by different mechanisms that enable the tumors to grow further. CTLA-4: cytotoxic T lymphocyte-associated protein 4, DC: dendritic cell, IFNγ: interferon-γ, IL: interleukin, MDSC: myeloid-derived suppressor cell, NK: natural killer cells, NKT: natural killer T cells, PD-1: programmed cell death 1, PD-L1: programmed death-ligand 1, STAT3: signal transducer and activator of transcription 3, TAM: tumor-associated macrophage, TGF-β: transforming growth factor-β, Treg: regulatory T cell.

1.7.2 The equilibrium phase

The phase of the equilibrium is described to be the longest among the three phases. In here, only the adaptive arm of the immune system that plays a significant role to control the outgrowth of the tumor cells which survive from the elimination phase. The major control by the adaptive immune system is demonstrated to rely on constant IL-12 and IFNγ secretion by T cells (**Figure 6**). The result of this process is the tumor dormancy within the host [133].

1.7.3 The tumor immune escape

In the immune escape phase, the tumor cells manage to surpass the equilibrium phase and breach the immune defense. Consequently, the tumors can grow further and become clinically visible. The capacity of tumors in evading immune destruction is now part of the Hallmarks of cancer: the next generation [5]. This condition is the result of the evolving cellular and molecular changes within the tumor cells which are heterogeneous among the patients and tumor types [134]. Multiple mechanisms utilized by the tumors in avoiding the innate and adaptive immune destruction have been discovered in the past few years. This heterogeneity of the tumor immune escape will have an impact in the selection of immunotherapeutic strategies to target the right escape mechanisms [135]. In here, we will discuss some known and newly emerging tumor immune escape mechanisms.

1.7.3.1 Escape through resistance to immune recognition and destruction

The tumor cells are displayed to possess an elevated rate of somatic mutations, resulting in a high generation of neo-antigens [136]. This capacity results in the potential recognition of the neo-antigens by T cells that leads to a high T cell infiltration in the tumor tissue during the elimination and equilibrium phases. As the tumor evolves, the tumor immune recognition may be reduced due to the loss of tumor antigen expression. In this case, the tumor cells can stop expressing the antigens that are recognized by T cells [137]. Additionally, the tumor cells can also downregulate MHC class I expression on the cell surface, causing the impairment of antigen presentation to the $CD8^+$ T cells [138] (**Figure 6**). Another possible mechanism that leads to the loss of tumor antigen expression may be due to disturbed antigenic processing inside the tumor cells, such as defected proteosomal unit and peptide transporter associated with antigen processing 1 and 2 (TAP-1 and TAP-2) [139]. Consequently, the tumor cells are invisible to the T cells and are capable to grow extensively.

Furthermore, tumor cells can develop insensitivity towards IFNγ due to the abnormality of the IFNγ receptor signaling pathway in the tumor cells [140]. Another way of avoiding immune destruction can be reached by expressing STAT3, which induces anti-apoptotic signals in tumor cells [141] and also mediates immunosuppression [142] (**Figure 6**). Moreover, under glycolytic metabolism in the tumor microenvironment, lactate is produced and selectively inhibits FAK family-interacting protein of 200 kDa (FIP200). The loss of FIP200 results in the naïve T cell apoptosis which will dampen anti-tumor immune response [143]. Recently, it was demonstrated that tumor can also escape immune destruction by suppressing TNF cytokine signaling in $CD8^+$ T cells, which further reduces the T cell-mediated tumor immunosurveillance [144].

1.7.3.2 Escape through tumor-induced chronic inflammation

The other immune escape model displays a chronic inflammation induced by the tumors which may lead to the expression of tolerogenic molecules and infiltration of suppressive cells causing impairment of anti-tumor immune responses [134]. Tumor cells are capable of secreting suppressive cytokine TGF-β, which can inhibit the transcription of granzymes and perforin encoding-genes in $CD8^+$ T cells [145] (**Figure 6**). Another tumor-produced molecule involved includes VEGF. VEGF can recruit and activate immunosuppressive Tregs and myeloid-derived suppressor cells (MDSCs) in the tumor [146].

Accordingly, Tregs suppress tumor-specific T cells through mechanisms like IL-2 over-consumption, expression of co-inhibitory molecules CTLA-4, PD-1, and PD-L1, as well as secretion of IL-10 and TGF-β [147] (**Figure 6**). However, evidences indicate that the infiltration of Tregs in the tumor has a heterogeneous outcome among tumor types and may be linked to a favorable prognosis [95]. MDSCs also inhibit T cells through several mechanisms, such as depletion of amino acids (arginine, tryptophan, and cysteine) required by T cells to function, TGF-β secretion, and Tregs induction [148] (**Figure 6**).

Another tumor immune escape mechanism is contributed by tumor-associated macrophages (TAMs) (**Figure 6**). Under chronic inflammatory condition, macrophages can be found infiltrating the tumors. Correspondingly, infiltrated macrophages are stimulated by IL-4, IL-13, IL-10, and macrophage colony stimulating factor (M-CSF) from the tumor microenvironment, resulting in the polarization towards M2 macrophages instead of M1 macrophages [135]. TAMs in M2 macrophage phenotype own anti-inflammatory and pro-tumorigenic properties, as seen by their poor antigen-presenting capacity towards $CD8^+$ T cells and their secretion of immunosuppressive cytokines IL-10 and TGF-β [149]. Additionally, TAMs express CCL22 that attracts CCR4-expressing Tregs into the tumors [150].

It was also demonstrated that IFNγ produced by tumor-infiltrating $CD8^+$ T cells promotes the upregulation of PD-L1 and indoleamine-2,3-dioxygenase (IDO) expression in the tumors. These two molecules are responsible for T cell suppression. Moreover, the Treg recruitment to the tumors may also be driven by $CD8^+$ T cells which is mediated through CCL22/CCR4 axis [151].

Furthermore, IFNγ-driven PD-L1 expression on tumor cells is utilized by the tumors to escape immune destruction through ligation to its ligand PD-1 on T cells, resulting in inhibitory signaling pathway (**Figure 6**). PD-L1 expression is maintained on the cell surface of tumor cells by CKLF-like MARVEL trans-membrane domain-containing protein 6 (CMTM6). CMTM6 acts as a key regulator of PD-L1 that inhibits its degradation in a lysosome-mediated fashion, resulting in a persistent PD-L1 expression [152, 153].

Chronic inflammatory environment together with continuous antigenic exposure inside the tumors will also cause the alteration in the T cell programming that can lead to exhaustion. Additionally, it was demonstrated that IL-10 and TGF-β may also regulate T cell exhaustion [154]. Exhausted T cells express markers that are distinct to the effector, memory, and naïve T cells [155]. Typically, exhausted T cells express multiple inhibitory receptors such as PD-1, Tim-3, LAG3, 2B4, CD160, and CTLA-4. The higher co-expression of these receptors indicates the more severe exhaustion of T cells [156]. Functionally, exhausted T cells are lack of effector functions like proliferation, cytokine production, and cytotoxicity [154, 155]. Therefore, even with the possibility of T cells in infiltrating the tumor tissue, as suggested by the infiltrated-inflamed tumor model [125], components from the tumor-induced chronic inflammation can manage to dampen effector T cell activities by inducing exhausted and non-functional T cells.

In summary, tumor cells utilize various approaches in order to avoid the immune destruction (**Figure 6**) as suggested by Hanahan and Weinberg in the Hallmarks of cancer: the next generation [5]. Consequently, the tumors can grow and give a clinical consequence to the patients, even though our immune system has a potent element of immunosurveillance. The heterogeneous nature of tumor immune escape mechanisms among patients and cancer types may be used as targets in pursuing tailored cancer immunotherapy in the future.

2 AIMS OF THE THESIS

The general aim of this thesis was to investigate the modulation of T cells in the tumor microenvironment of solid tumors, in order to find potential targets in cancer immunotherapy. The specific aims of each paper were as follows:

Paper I. To investigate whether $FOXP3^+$ $CD4^+$ T cells in the tumors were committed regulatory T cells (Tregs) and to examine the functions of the tumor-infiltrating Tregs.

Paper II. To characterize the cytotoxic capacity of the tissue-resident memory T (T_{RM}) cells in the tumors by DNA methylation profiling and correlate it with their phenotypes.

Paper III. To identify the tumor immune escape mechanisms on $CD8^+$ T cells from the tumor-draining sentinel lymph nodes.

Paper IV. To evaluate the effects of neoadjuvant chemotherapy (NAC) on the phenotypes and functions of T cells from tumor-draining sentinel lymph nodes.

Paper V. To explore an alternative method to detect micrometastases in the sentinel lymph nodes using flow cytometry.

3 METHODOLOGICAL APPROACH

This chapter contains a summary of materials and underlying methodologies used in the studies included in this thesis. A detailed description of this account is displayed in the materials and methods section of each paper (I-V).

3.1 PATIENTS

3.1.1 Cancer patients

3.1.1.1 Urinary bladder cancer (Paper I, II, III, and IV)

In total, 99 patients diagnosed with urothelial urinary bladder cancer (UBC) were recruited in the four prospective studies (Paper I-IV) from participating hospitals in Sweden between the years 2013-2018. Paper I included 46 patients, paper II included 53 patients, paper III included 42 patients, and paper IV included 40 patients. The mean age of the patients was 70 years old (range 50-87). 29 patients were female and 70 patients were male. Following clinical diagnosis, the patients underwent TUR-B and the patients diagnosed with muscle invasive tumors would proceed to radical cystectomy. Neoadjuvant chemotherapy (1-4 cycles) was administered to eligible patients prior to radical cystectomy, consisting of Methotrexate, Vinblastine, Adriamycin, and Cisplatin (MVAC).

3.1.1.2 Renal cancer (Paper V)

Five patients (three females and two males) diagnosed with renal tumors were included in this study from two participating hospitals in Sweden. The mean age of the patients was 61 years old (range 47-69). Four patients were diagnosed with malignant tumors and one patient with benign tumor. All the patients underwent nephrectomy, in which removal of tumor and tumor-surrounding lymph nodes took place.

3.1.1.3 Cancer patient samples

From UBC patients, we received peripheral blood and tumor tissues at the time of TUR-B. Meanwhile, at radical cystectomy, we obtained peripheral blood, lymph nodes, and in some cases, tumor tissues. In renal cancer patients, we received peripheral blood, lymph nodes, and tumor tissues at the time of nephrectomy.

Lymph nodes were identified as tumor-draining sentinel nodes (SNs) using radioactive tracer ^{99m}Technetium, injected during cystectomy or nephrectomy [157]. The injection was performed in the area surrounding the tumor and the radioactivity of each lymph node was measured using a handheld Geiger counter. Nodes with positive radioactive signal were classified as tumor-draining SNs.

All the samples were processed to acquire single cell suspensions. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using a density gradient

centrifugation (Ficoll-Paque Plus). The tumor-infiltrating immune cells were extracted from the tumor tissues by a mechanical disruption using the GentleMACS instrument together with an enzymatic digestion by collagenase/hyaluronidase and DNAse I. The cells from lymph nodes were isolated by gentle pressure homogenization through a 40 μM cell strainer.

The cells isolated from all the samples were subjected to T cell phenotype investigations at steady state and after *in vitro*-stimulated conditions by epigenetic analysis (Paper I, II, and IV), gene expression analysis (Paper III), and protein expression analysis (Paper I, II, III, and IV). Finally, single cell suspensions from lymph nodes were investigated for the presence of micrometastatic tumor cells (Paper V).

3.1.2 Healthy donors (Paper I, II, III, and V)

We received leukocytes-enriched whole blood (buffy coat) of healthy donors from Karolinska University Hospital Blood Bank. PBMCs were isolated from the buffy coat by a density gradient centrifugation (Ficoll-Paque Plus) and were used in experimental setups and *in vitro* studies. For experimental setups, healthy donor PBMCs were used for the identification of the perforin gene (*PRF1*) reporter CpG site (Paper II) and for the optimization of the micrometastasic detection method (Paper V). Meanwhile, *in vitro* studies were carried out in the investigation of Treg effect on the MMP2 suppression (Paper I), stimulation of sorted $CD8^+$ T cells using 5-Azacytidine (Paper II) and UBC cell line supernatant (Paper III), and in the validation of proteomic analysis (Paper III).

3.2 CELL LINES (PAPER I, III, AND V)

In paper I and III, we used two UBC cell lines, RT4 and 5637. RT4 is a non-muscle invasive cancer cell line, whereas 5637 is a muscle invasive cancer cell line. The expression of MMP2 was measured on 5637 cells after co-culture with Tregs (Paper I). Furthermore, the supernatant from RT4 and 5637 cell line cultures were used to stimulate $CD8^+$ T cells from healthy donor PBMCs *in vitro* to demonstrate the effect of tumor immune escape mechanism from muscle invasive UBC (Paper III).

In paper V, we worked with three kinds of renal cancer cell lines, which were RCC4, ACHN-3, and CAKI-6. The cell lines were used for the optimization of micrometastatic detection by flow cytometry since they express tumor cell markers cytokeratin 18 (CK18), CA9, and Cadherin 6 (Cad6). The optimization was performed by spiking these three cell lines into PBMCs from healthy donors in known concentrations. The amount of positive cells was measured by flow cytometry.

3.3 CELL SORTING (PAPER I, II, III, AND IV)

Cells isolated from peripheral blood, lymph nodes, and tumors were sorted for different cell subsets. Initially, the cells were magnetically sorted using CD3, CD8, CD4, or CD14 EasySep positive selection kits. Positively selected cells were then subjected to sorting by flow cytometry after staining with fluorescence-conjugated antibodies.

Throughout the studies, we sorted for bulk $CD8^+$ T cells (Paper II, III, and IV), different memory $CD8^+$ T cell subsets such as T_{RM} ($CD8^+$ $CD103^+$), T_{CM} ($CD8^+$ $CD45RA^-$ $CCR7^+$), T_{EM} ($CD8^+$ $CD45RA^-$ $CCR7^-$), and T_{EMRA} ($CD8^+$ $CD45RA^+$ $CCR7^-$) cells (Paper II), as well as $perforin^+$ or $perforin^-$ $CD8^+$ T cells (Paper II). Additionally, $CD4^+$ T cells were sorted into effector T cells (Teffs) ($CD4^+$ $CD25^-$ $CD127^+$) or Tregs ($CD4^+$ $CD25^+$ $CD127^-$) (Paper I).

For sorting based on an intracellular marker like perforin (Paper II), magnetically selected $CD8^+$ T cells were firstly stained for surface markers by fluorescence-conjugated antibodies. Next, the cells were fixated using HOPE I Fixation solution (DCS Innovative Diagnostik-Systeme) overnight in 0-4^{o}C. This fixation protocol has the advantage of not causing any degradation of the nucleic acids as our readouts, compared to formaldehyde-based fixation solution. Following fixation, the cells were permeabilized using 0.1% saponin and intracellularly stained using anti-perforin antibody for sorting by flow cytometry.

3.4 *IN VITRO* CELL STIMULATION

All *in vitro* T cell stimulation assays throughout the studies were performed in RPMI medium supplemented with 10% fetal calf serum (FCS), 1% l-glutamine, and 1% penicillin/streptomycin (Paper I) or AIM-V medium (Paper II, III, and IV). The cells were incubated at 37^{o}C with 5% CO_2.

3.4.1 Regulatory T cell suppression assay (Paper I)

In order to investigate the suppressive capacity of Tregs, sorted Tregs ($CD4^+$ $CD25^+$ $CD127^-$) from PBMCs and tumors of UBC patients were co-cultured with Teffs ($CD4^+$ $CD25^-$ $CD127^+$) as responder cells. The co-culture was performed in the presence of anti-Biotin beads loaded with biotinylated CD2, CD3, and CD28 antibodies to stimulate responder Teffs. Teffs were pre-labeled using carboxyfluorescein succinimidyl ester (CFSE) and the proliferation following 72 hours of co-culture, measured as the dye dilution, was used as the readout.

3.4.2 Treg suppression on MMP2 expression assay (Paper I)

Isolated Tregs were co-cultured with either differentiated M2-like macrophages or 5637 UBC cell line in order to investigate the suppressive effect of Tregs on MMP2 expressed by TAMs and UBC tumor cells. M2-like macrophages were differentiated from healthy donor PBMC $CD14^+$ monocytes by providing M-CSF stimulation for six days [158]. In parallel, isolated Tregs were stimulated using anti-Biotin beads loaded with biotinylated CD2, CD3, and CD28

antibodies for six days. Activated Tregs were then added at different ratios to the M2-macrophage or 5637 cell cultures. At different time point, culture supernatants were collected and adhered M2-macrophages and 5637 cells were acquired after Treg removal for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

3.4.3 5-Azacytidine (Paper II)

We used the demethylating agent 5-Azacytidine (5-aza) to treat cultured $CD8^+$ T cells and investigate the effect of DNA methylation on perforin transcriptional regulation. 5-aza acts by binding irreversibly to the DNA methyltransferase (DNMT), resulting in loss of methylation of the daughter cells [159]. In this experiment, isolated $CD8^+$ T cells from healthy donor PBMCs were activated by TCR stimulation, using plate-coated anti-CD3 and soluble anti-CD28 stimulating antibodies, in the presence of recombinant human IL-2 for 48 hours. The cells were then treated with 5-aza for 48 hours, with additional 48 hours of culture without the presence of 5-aza. Perforin expression was assessed by flow cytometry at the end of the culture.

3.4.4 Tissue-resident memory T cell activation assay (Paper II)

Isolated T_{RM} cells ($CD8^+$ $CD103^+$) from PBMCs and tumors of UBC patients were activated using recombinant human IL-15 and TCR stimulation using plate-coated anti-CD3 and soluble anti-CD28 stimulating antibodies for 48 hours. At the end of culture, the cells were phenotyped by flow cytometry and investigated for their *PRF1* DNA methylation status by pyrosequencing.

3.4.5 Tumor homogenate stimulation (Paper III and IV)

In order to evaluate the response of SN-derived T cells from UBC patients towards tumor antigen, we stimulated these cells using autologous tumor homogenate for seven days. Tumor homogenate was used as the tumor antigenic source and it was prepared as described in paper III. Following seven days of culture, flow cytometry was performed to phenotype T cells (Paper III and IV). In addition, stimulation index (SI) was counted from the ratio of $CD4^+$ lymphoblasts to $CD4^+$ lymphocytes, as adapted from Flow cytometric Assay for Specific Cell-mediated Immune response in Activated whole blood (FASCIA) protocol (Paper IV) [160].

3.4.6 Tc1-promoting stimulation (Paper III)

To investigate the feasibility in restoring perforin expression in $CD8^+$ T cells from SNs of UBC patients, we stimulated these cells in Tc1-promoting conditions. Isolated $CD8^+$ T cells from SNs were cultured in the presence of recombinant human IL-12 and IL-2, neutralizing anti-IL-4 antibody, and TCR stimulation using plate-coated anti-CD3 and soluble anti-CD28 stimulating antibodies. After seven days, cells were phenotyped by flow cytometry and RT-qPCR.

3.4.7 UBC cell line supernatant stimulation and proteomic analysis validation (Paper III)

For evaluating the tumor immune escape mechanism by tumor cells, we stimulated healthy donor $CD8^+$ T cells from peripheral blood using the culture supernatant of RT4 and 5637 cell lines for five days. The culture supernatants were collected from the cell line cultures, in which we used RPMI-Serum and phenol red free medium (SFM) in the last 24 hours of culture, so that we did not have the contamination of serum-derived proteins in the downstream mass spectrometry (MS) analysis. The stimulated $CD8^+$ T cells were then harvested after five days of UBC cell line supernatant stimulation and phenotyped by flow cytometry and RT-qPCR. Meanwhile, the culture supernatants from both cell lines were analyzed for their proteomic profile using mass spectrometry (LC – MS/MS).

Following MS analysis, we validated the effects exerted by the identified proteins exclusively expressed by the muscle invasive 5637 cell lines (ICAM-1 and TGFβ2) on $CD8^+$ T cells. Healthy donor PBMC $CD8^+$ T cells were stimulated using plate-coated ICAM-1 Fc chimera and soluble TGFβ2, in the presence of anti-CD3 stimulating antibodies. After five days of culture, we analyzed the cells using flow cytometry and RT-qPCR.

3.5 EPIGENETIC – DNA METHYLATION ANALYSIS

3.5.1 Genomic DNA extraction and bisulfite conversion (Paper I, II, and IV)

We investigated the DNA methylation status in the regulatory regions of FOXP3 encoding gene *FOXP3* (Paper I), perforin encoding gene *PRF1* (Paper II and IV), and IFNγ encoding gene *IFNG* (Paper IV). Genomic DNA (gDNA) from Tregs and $CD4^+$ Teffs (Paper I), as well as $CD8^+$ T cells (Paper II and IV) was extracted and bisulfite-converted using the EZ DNA Methylation Direct kit. Bisulfite treatment will convert unmethylated cytosine residues into uracil and subsequent polymerase chain reaction (PCR) will convert uracil into thymine residues. Conversely, methylated cytosine residues will remain unchanged after bisulfite treatment.

3.5.2 TA cloning and bisulfite sequencing (Paper II)

In order to identify the *PRF1* reporter CpG site, we used the TA cloning method, followed by bisulfite sequencing. The advantage of using this method is the possibility to evaluate DNA methylation status from a single cell, which is essential when predicting the CpG site responsible to regulate *PRF1* transcription. In here, we assessed the DNA methylation status of *PRF1* enhancer region from $perforin^+$ and $perforin^-$ PBMC-derived $CD8^+$ T cells. The enhancer region of *PRF1* from the bisulfite-converted DNA was PCR-amplified, TA-cloned into pCR4-TOPO vector, and transformed into TOP10 *E. coli*. Next, DNA from each colony representing a single cell was Sanger sequenced to determine the DNA methylation status from each cell subset.

3.5.3 Pyrosequencing (Paper I, II, and IV)

The benefit of using pyrosequencing method is the possibility to sequence the bisulfite-converted DNA directly on the PCR amplicons without TA-cloning. However, the result of this sequencing protocol will be an accumulative DNA methylation percentage of each CpG site from all cells. The reporter CpG sites of *FOXP3* [92, 93], *PRF1,* and *IFNG* [161] were pyrosequenced from PCR amplicons, in which one of the PCR primer pairs was biotinylated to ensure specificity of the amplicons to be sequenced.

3.6 GENE EXPRESSION ANALYSIS

3.6.1 Microarray analysis (Paper I)

Gene expression analysis by microarray was performed from messenger RNA (mRNA) isolated from tumor tissues of UBC patients. Isolated mRNA was amplified and labeled using GeneChip WT PLUS Reagent kit resulting in cDNA product that was hybridized to Affymetrix GeneChip Human Transcriptome 2.0 arrays. The microarray data were deposited in the EMBL-EBI database.

3.6.2 Reverse transcription – quantitative PCR (RT-qPCR) (Paper I and III)

RT-qPCR was used to measure the gene expression coded in mRNA. Isolated mRNA was reverse-transcribed into cDNA and run under RT-qPCR protocols using SYBR Select. We evaluated the expression of *MMP2* gene in M2-macrophages and 5637 UBC cell line after co-culture with Tregs (Paper I), as well as *GZMB*, *PRF1*, *TBX21*, and *GATA3* genes in $CD8^+$ T cells from PBMCs, SNs, and tumors of UBC patients (Paper III). *GAPDH* or *RPII* were used as the housekeeping genes. The gene expression level was calculated using the $\Delta\Delta$Ct formula.

3.7 PROTEIN ANALYSIS

3.7.1 Flow cytometry (Paper I, II, III, IV, and V)

Multiparameter flow cytometry method enables us in measuring the expression of the surface and intracellular cell markers. Fluorescence-conjugated antibodies were used to label the specific epitopes of the cell markers. For intracellular marker staining, the cells were first fixed and permeabilized before staining with the antibodies.

In paper I, II, III, and IV, we used flow cytometry to phenotype T cells from samples obtained from the UBC patients. Meanwhile, in paper V, we used flow cytometry as a method to detect micrometastases in lymph nodes of patients with renal tumors based on the tumor cell marker expression. All the flow cytometry data were acquired using LSRFortessa II instrument and analyzed using FlowJo software.

3.7.2 Enzyme-linked immunosorbent assay (ELISA) (Paper I and III)

The ELISA method was used to measure the amount of secreted soluble MMP2 from M2-macrophages and 5637 UBC cell line after co-culture with Tregs (Paper I), as well as perforin and granzyme B from $CD8^+$ T cells derived from PBMCs and SNs of UBC patients after tumor homogenate stimulation (Paper III). The culture supernatants were obtained at the given time points and analyzed using ELISA. Sandwich ELISA was used in these studies with capture antibodies and secondary antibodies against MMP2, perforin, and granzyme B. The concentration of proteins in the culture supernatants were calculated based on the standard curve for each protein.

3.7.3 Liquid chromatography – mass spectrometry (LC – MS/MS) (Paper III)

We used LC – MS/MS for our proteomic analysis in identifying the secreted proteins from the culture supernatants of UBC tumor cell lines (RT4 and 5637). The LC – MS/MS was performed using a Dionex UltiMate™ 3000 RSLCnano System coupled to a Q-Exactive mass spectrometer. The LC system separates the proteins, while MS provides the structural identity of the analytes. The acquired raw data from the LC – MS/MS run were analyzed using Sequest-Percolator or Target Decoy PSM Validator under the Proteome Discoverer 1.4 software platform against human Uniprot database.

3.8 BIOINFORMATIC DATA ANALYSIS

3.8.1 KEGG pathway analysis (Paper I)

Transcriptomic data from the microarray analysis were calculated using partial least squares (PLS) analysis for standardized coefficients of Treg influence on individual gene expression. The calculations (loadings) were then plotted against KEGG pathway for UBC using the R platform.

3.8.2 ViSNE (Paper II)

ViSNE was used to analyze and visualize high dimensional multiparameter data from flow cytometry. In this study, we exported manually gated $CD8^+$ T cell data with compensated parameters using FlowJo software. The exported data were analyzed using CYT tool [162] on the MATLAB platform. The measured fluorescence intensity of the parameters used in the analyses was transformed using the arcsin function with a cofactor of 150. All groups of the samples were adjusted to have a comparable number of events. ViSNE analysis was performed using the Barnes-Hut Stochastic Neighbor Embedding (bh-SNE) algorithm to map the events.

3.8.3 Network analysis using STRING database (Paper III)

The proteins identified from LC – MS/MS analysis were analyzed for their interaction using the STRING network analysis. First, proteins categorized under the Gene Ontology (GO)

term “immune system process” were selected and the known protein-protein interactions from the STRING database were used to produce a network graph using igraph [163] on the R platform. The size was used to represent the differential relative expression between RT4 and 5637 proteomic components and the color indicators represented the influence of the protein position in the network (betweenness). Davidson Harel algorithm was used for the layout of the network graph.

4 RESULTS AND DISCUSSION

This thesis aims to investigate the modulation of T cell phenotypes in the solid tumor microenvironment. We build our fundamental investigation starting from the molecular to cellular level, in the hope to identify novel strategies for cancer immunotherapy in the future.

There are two important sites of the tumor microenvironment: the primary tumor tissues and the tumor draining sentinel nodes. In **paper I** and **paper II**, we focus on the primary tumor site. Here, we investigated how the Tregs played a role in regulating the tumor invasiveness (**Paper I**) and how T_{RM} cells, as the most dominant subset of $CD8^+$ T cells in the tumors, had a protective effect as displayed by their epigenetic commitment of cytotoxicity (**Paper II**).

We next sought to understand the microenvironment in the sentinel nodes. In here, we first demonstrated how tumor immune escape caused the cytotoxicity of $CD8^+$ T cells to be compromised (**Paper III**). Moreover, we described that the standard neoadjuvant chemotherapy could promote the anti-tumor T cell responses (**Paper IV**), which was discovered to be suppressed by the tumors in **paper III**. Furthermore, the implication of the tumor immune escape mechanisms is the opportunity for tumors to progress and metastasize into sentinel nodes. We here revealed that the micrometastatic cells in the sentinel nodes could be detected using flow cytometry (**Paper V**), allowing for an objective, time-efficient, and standardized method for staging metastatic dissemination in the lymph nodes.

In this section, all the key findings are explained and discussed further. The detailed account for each study can be acquired from the original constituent papers.

4.1 REGULATORY T CELLS MAY REGULATE TUMOR INVASIVENESS BY SUPPRESSING MMP2 (PAPER I)

Tregs have been known to suppress anti-tumor immune response [164]. However, this paradigm starts to be shifted, since not all of the cancers display a poor prognosis when having a high infiltration of Tregs in the tumors [165]. Previously, we have demonstrated that in UBC, a high number of $FOXP3^+$ tumor infiltrating lymphocytes (TILs) correlates with an improved patient survival [166]. From this surprising finding, we first argued that FOXP3 is not only a marker of Tregs, but also can be expressed by activated conventional Teffs transiently [91], which accordingly would explain the positive prognosis.

Nevertheless, we further asked the question whether tumor $FOXP3^+$ T cells conferred a committed Treg phenotype. To answer this, we performed an epigenetic analysis by means of DNA methylation to determine the lineage commitment of $FOXP3^+$ $CD4^+$ T cells as Tregs. We investigated the CpGs at the two regulatory regions of the *FOXP3* gene: the promoter [92] and the conserved non-coding sequence 2 (CNS2) [93] regions. CpG hypomethylation of these two regions are previously proven to mark a specific and stable Treg status. Our finding displayed that sorted Tregs ($CD4^+CD25^+CD127^-$) from the UBC tumors were hypomethylated in both promoter and CNS2 CpG sites compared to the Teffs ($CD4^+CD25^-CD127^+$) (**Figure 7**). This methylation status was most pronounced at the CpG position -77 in the promoter region, as described before [92]. Additionally, we validated the Treg stability, as indicated by this epigenetic profile, by performing a long-term culture of tumor Tregs using IL-2 stimulation alone and assessed the inheritability of FOXP3 expression [167]. We observed that at the end of the culture (day 14), FOXP3 was stably expressed by the Tregs. Hence, it indicates that tumor $FOXP3^+$ $CD4^+$ T cells are committed Tregs and not activated conventional Teffs.

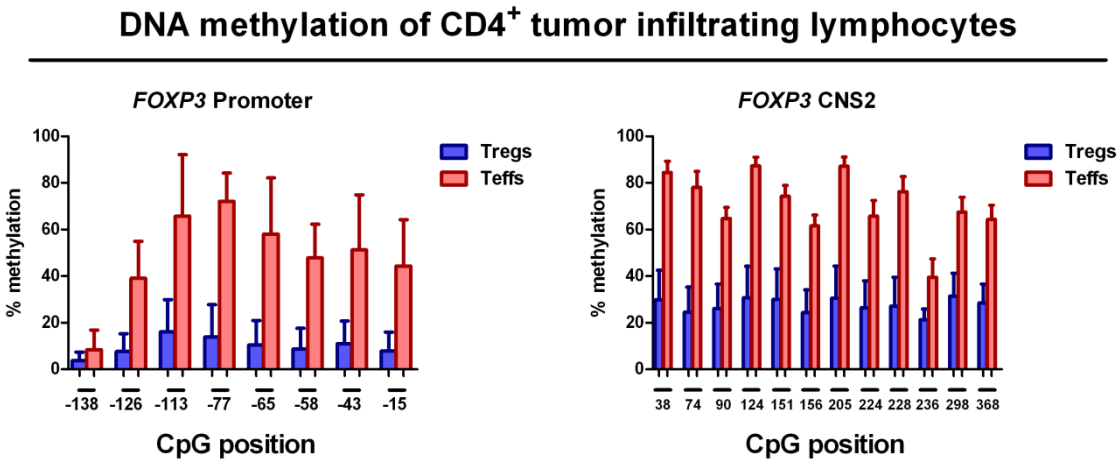


Figure 7. Lower DNA methylation of *FOXP3* regulatory regions in Tregs. The DNA methylation status of CpGs located in the promoter and CNS2 regions of the *FOXP3* gene is compared between Tregs ($CD4^+CD25^+CD127^-$) and Teffs ($CD4^+CD25^-CD127^+$) infiltrating the UBC tumors. The mean methylation percentage of each CpG site is displayed with the error bars indicating SEM. CNS2: conserved non-coding sequence 2, CpG: cytosine-phosphate-guanine, SEM: standard error of the mean, Teffs: effector T cells, Tregs: regulatory T cells, UBC: urinary bladder cancer.

Furthermore, we phenotyped tumor FOXP3$^+$ CD4$^+$ T cells and demonstrated that these cells significantly expressed activated Treg markers, such as CD45RO, HLA-DR, CD69, CTLA-4, and CD39. In addition, FOXP3$^+$ CD4$^+$ T cells from the tumors worked functionally as Tregs, as exhibited by their capacity to increase pSTAT5 expression following IL-2 stimulation, their suppressive effect towards the Teffs, and their low expression of IFNγ and IL-2. Taken together, we have proven that FOXP3$^+$ CD4$^+$ T cells infiltrating the tumors are epigenetically, phenotypically, and functionally Tregs.

In the later part of the study, we prompted to investigate on how committed Tregs in the tumors had a positive effect to the patient survival. First, we observed that the Treg frequency was lower in the invasive front of the tumors compared to the central part in patients with muscle invasive UBC. Second, a lower number of Tregs in the invasive front of the tumors displayed a worse survival outcome of the patients. Consequently, we started to understand that Tregs may have a protective role in inhibiting the tumor invasion. Furthermore, we performed a transcriptomic analysis demonstrating that *MMP2* was the most influenced gene by the Tregs, as shown in the KEGG pathways. Accordingly, we validated that Tregs suppressed the expression of MMP2 both in TAMs and tumor cells.

MMP2 has been identified to support the UBC tumor invasiveness [168-170]. The MMPs are expressed by the tumor cells and the surrounding stroma [171, 172]. MMPs function by degrading the extracellular matrix [173], as well as modulating neo-angiogenesis and cell motility when highly expressed in the invasive front of the tumor tissues [174]. These processes will facilitate the tumor invasion. Altogether, as our data suggest that MMP2 expression in both TAMs and tumor cells is suppressed by the Tregs, it therefore answers our question regarding the paradoxical protective effect of Tregs in UBC.

Although the conclusion to draw this causal relationship was based on *in vitro* analysis using cell line, we somehow could predict how Tregs, in the UBC setting, had a protective role by regulating tumor invasiveness. Moreover, this data was supported by our *ex vivo* analysis that made the conclusion stronger.

In conclusion, we have answered our previous questions of why and how FOXP3$^+$ T cells infiltrating the tumors provided a beneficial factor for survival of UBC patients. We discover that tumor FOXP3$^+$ CD4$^+$ T cells are committed Tregs which regulate tumor invasiveness by suppressing MMP2 expression in TAMs and tumor cells. This novel understanding may assist us in finding a new strategy of cancer immunotherapy by targeting this axis in the future.

4.2 TISSUE-RESIDENT MEMORY T CELLS IN THE TUMORS ARE EPIGENETICALLY CYTOTOXIC AND NOT TERMINALLY EXHAUSTED (PAPER II)

One of the valuable assets of the T_{RM} cells is their location in non-lymphoid tissues [65]. Consequently, T_{RM} cells are the first barrier of T cells to respond rapidly against tumor cells in the tissues, compared to the other $CD8^+$ T cell subsets [73]. Upon antigen encounter, T_{RM} cells have been reported to provide recruiting signals, such as IFNγ, IL-2, and TNF [175]. T_{RM} cells also promote vascular cell adhesion molecule 1 (VCAM1) upregulation in blood vessel endothelial cells [176]. These will result in the recruitment of immune cells including NK cells, DCs, and other memory $CD8^+$ T cells [177, 178].

However, less is known whether T_{RM} cells are effective killers against the tumor cells. The study performed in human lung cancer reveals that T_{RM} cells residing in the tumor correlate with cytotoxic features, such as granzymes and perforin expression [179]. On the contrary, T_{RM} cells are demonstrated to express exhaustion markers PD-1, Tim-3, and LAG3 [180], which makes it paradoxical to link T_{RM} cells to cytotoxic activity. Taking this into account, an analysis of T_{RM} cell commitment with regards to the cytotoxic capacity from an epigenetic angle, despite having signs of exhaustion needs to be investigated.

In this study, we identified the reporter CpG site (-1053bp upstream of transcription start site/TSS) for the perforin gene (*PRF1*) transcription which was located in the enhancer region. Furthermore, we discovered that tumor T_{RM} cells in the UBC were epigenetically available for perforin transcription. This was marked by a lower DNA methylation of the *PRF1* reporter CpG site in tumor T_{RM} cells (mean methylation=32.9%) when compared to T_{RM} cells found in the PBMC (mean methylation= 66%) (**Figure 8A**). Correspondingly, tumor T_{RM} cells displayed a higher expression of perforin protein (**Figure 8B**). As suggested previously [180], we observed that 79% of tumor T_{RM} cells expressed the exhaustion marker PD-1, which came with a low number of T_{RM} cells that expressed T cell activation marker T-bet (mean=11.4%). This high number of PD-1^+ T_{RM} cells was not seen in PBMC.

Knowing that tumor T_{RM} cells were epigenetically regulated to be cytotoxic despite having signs of exhaustion, we attempted to assess the functional capacity of T_{RM} cells residing in the tumors. Isolated T_{RM} cells from tumor tissues were activated *in vitro* using IL-15 and TCR stimulation (stimulating anti-CD3 and anti-CD28 antibodies) for 48 hours. Following *in vitro* stimulation, the number of perforin-expressing tumor T_{RM} cells increased by two-fold compared to the non-exhausted T_{RM} cells from PBMC and the unstimulated tumor T_{RM} cells (**Figure 8C**). Additionally, the frequency of PD-1^+ T-bet^+ T_{RM} cells from the tumors increased together with the number of Ki-67-expressing tumor T_{RM} cells when compared to the unstimulated condition. This is in line with a previous study using a viral infection in a skin model [178]. Therefore, we suggest that T_{RM} cells from the tumors are not terminally exhausted despite of their phenotype, which consequently allows their functional cytotoxic capacity. Further study to assess activated T_{RM} cell cytotoxic function in killing the autologous tumor cells can be addressed using an *in vitro* killing assay in the future.

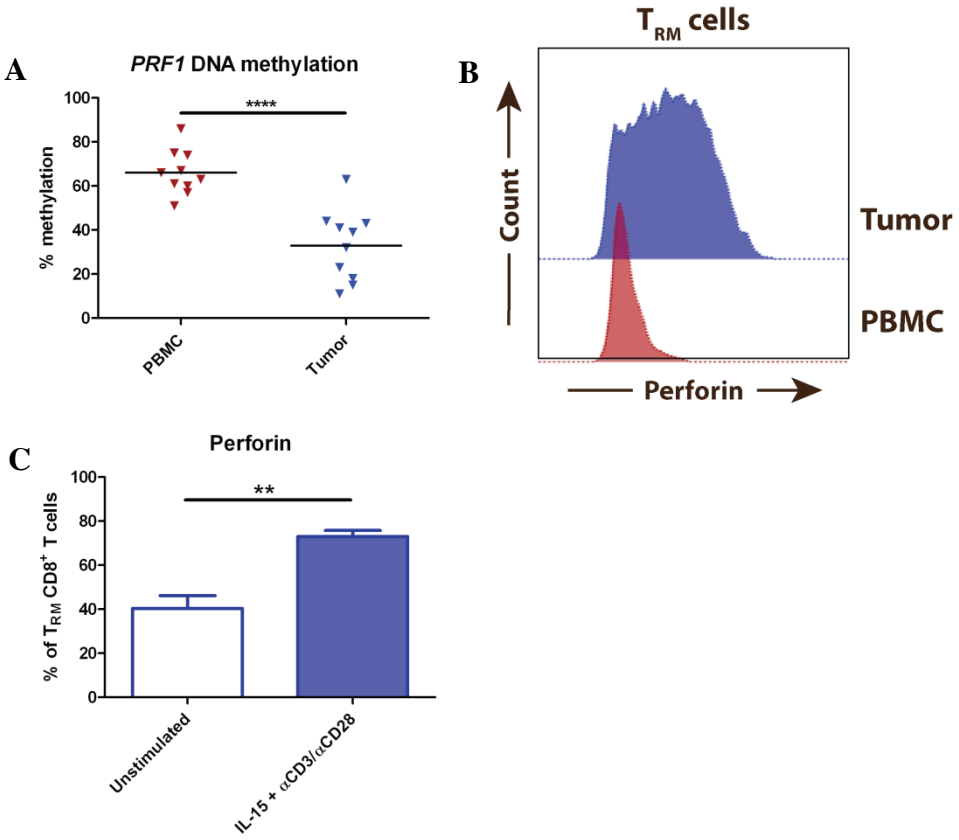


Figure 8. Tumor T_{RM} cells are epigenetically cytotoxic and not terminally exhausted. (**A**) The mean DNA methylation of *PRF1* reporter CpG site (-1053bp upstream TSS) is compared between T_{RM} cells from PBMC and tumor (n=10). Independent t-test was used as the statistical test. (**B**) The expression of perforin in T_{RM} cells from PBMC and tumor was measured by flow cytometry. (**C**) Tumor T_{RM} cells were stimulated *in vitro* using IL-15, anti-CD3 and anti-CD28 stimulating antibodies for 48 hours. The frequency of perforin-expressing tumor T_{RM} cells was measured by flow cytometry and compared to the unstimulated control (n=3). Independent t-test was used as the statistical test. CpG: cytosine-phosphate-guanine, IL-15: interleukin-15, PBMC: peripheral blood mononuclear cell, T_{RM}: tissue-resident memory T cells, TSS: transcription start site. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

The reason of why cytotoxic T_{RM} cells expressed the exhaustion marker PD-1 needs further studies beyond what is known to be a result of a chronic antigenic exposure in the tumor microenvironment [154]. We can nevertheless speculate that PD-1 is a mere T_{RM} cell marker that does not affect the cell functionality, since permanent PD-1 expression is epigenetically imprinted [181] and antigen-independent [182], as seen in the chronic infection. Accordingly, our data also suggested that the frequency of PD-1^+ T_{RM} cells was not different between tumor stages.

Furthermore, we demonstrated that T_{RM} cells were the most dominant and cytotoxic memory subset of $CD8^+$ T cells in the tumor tissue, accounting for 56.8% population. Importantly, the higher number of T_{RM} cells residing within the tumor correlated with a lower tumor stage of the UBC patients (n=10). This further validates the protective role of T_{RM} cells, which has previously been linked with an improved survival in various cancer patients [183-186].

The limiting factor of this study was the low number of patients (n=10) that we could correlate between the tumor stages and the frequency of T_{RM} cells. The reason was due to a

restricted number of UBC patients recruited to this study, which fell into the category of stage I-III. However, we could recapitulate the trend in which the frequency of T_{RM} cells was linked to a lower tumor stage and further demonstrated the protective anti-tumor property of T_{RM} cells.

To conclude, epigenetic profiling of the perforin locus by means of DNA methylation adds to our current understanding that T_{RM} cells guarding the tumor tissues are committed to be cytotoxic and helps us to find a clue that they are not terminally exhausted. Since a high infiltration of T_{RM} cells is linked to a better prognosis, it implicates to the possibility to target T_{RM} cells for future translational applications of cancer immunotherapy.

4.3 TUMOR MAY ESCAPE IMMUNE DESTRUCTION BY SUPPRESSING PERFORIN EXPRESSION IN CD8$^+$ T CELLS (PAPER III)

$CD8^+$ T cell has a significant role in the anti-tumor immunity. It is part of the adaptive immune response and it has the capacity to kill tumor cells using its cytotoxic constituents, such as perforin and granzymes [33]. However, as tumors develop, they have the capability to escape the immune destruction, which is part of the cancer immunoediting process [133]. This immune attack avoidance by the tumor cells is now integrated in the Hallmark of cancer: the next generation, proposed by Hanahan and Weinberg [5].

There are several tumor immune escape mechanisms described in solid tumors [134, 136, 138, 154, 187]. However, the mechanism of tumor escape that affects $CD8^+$ T cell cytotoxicity residing in the tumor-draining SNs has not been investigated. SN is an interesting site to study $CD8^+$ T cells since SNs receive a high lymphatic flow from the tumors due to the high pressure of interstitial fluid and the neo-lymphangiogenesis [188]. Therefore, SNs have a high reception of tumor signals and cytokines from the primary tumor, which may modify SN $CD8^+$ T cells to be tolerogenic as an escape mechanism [189].

In this study, we unveiled a novel mechanism on how UBC tumors may suppress the cytotoxicity of SN-derived $CD8^+$ T cells. Our finding displayed that perforin was not expressed in SN $CD8^+$ T cells, meanwhile granzyme B expression was preserved (**Figure 9A**). We discovered that the low perforin expression was originated from the transcript (*PRF1* gene) level, which was also seen to be downregulated in the tumor-derived $CD8^+$ T cells. Similarly, this phenotype was previously reported in the lung adenocarcinoma [190]. Hence, this discovery implies that the cytotoxicity of $CD8^+$ T cells from SNs is compromised.

Next, we prompted to investigate the phenotype of perforin-deficient (granzyme B^+/perforin$^-$) $CD8^+$ T cells from the SNs. Perforin-deficient $CD8^+$ T cells were 85% T_{EM} cells, where the majority of these cells were PD-1^+ (mean=65%), suggesting exhaustion. Consequently, perforin-deficient $CD8^+$ T cells had a low expression of the Tc1 transcription factor T-bet (mean=25.5%). Furthermore, we demonstrated that in SN $CD8^+$ T cells, the Tbet encoding gene *TBX21* transcript was downregulated, alongside the upregulation of the Tc2

transcription factor *GATA3* transcript. Altogether, this indicates that perforin-deficient $CD8^+$ T cells from SNs are exhausted T_{EM} cells with a Tc2 anti-inflammatory phenotype.

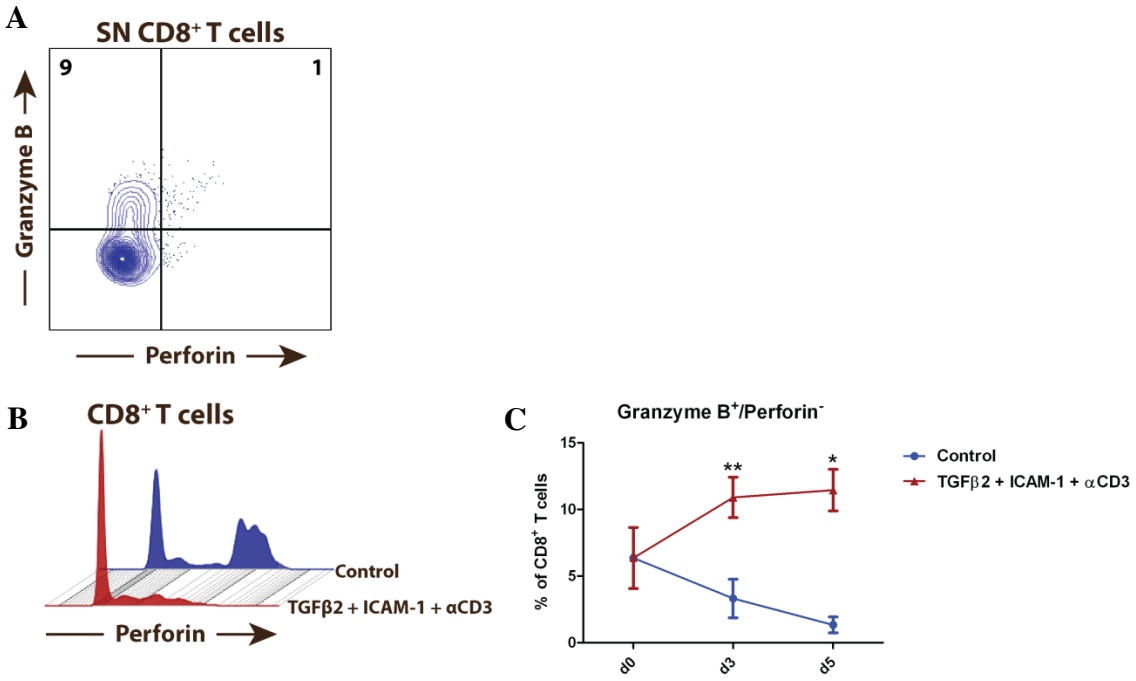


Figure 9. UBC tumors may suppress perforin expression in $CD8^+$ T cells via ICAM-1/TGFβ2-mediated pathway. (**A**) The expression of perforin and granzyme B in SN $CD8^+$ T cells was measured by flow cytometry. The data from a representative patient is displayed in a density plot with the frequency of perforin- and granzyme B-expressing $CD8^+$ T cells shown. (**B**)(**C**) $CD8^+$ T cells from healthy donor PBMCs (n=3) were stimulated *in vitro* using TGFβ2, ICAM-1, and anti-CD3 stimulating antibody for five days. At day 3 and day 5, *in vitro* stimulated cells were measured by flow cytometry. The expression of perforin (B) and the mean frequency of granzyme B^+/perforin$^-$ $CD8^+$ T cells (C) were compared between stimulated cells and unstimulated control. The error bars in (C) indicate SEM and paired t-test was used as the statistical test. ICAM-1: intercellular adhesion molecule 1, PBMC: peripheral blood mononuclear cell, SEM: standard error of the mean, SN: sentinel node, TGFβ2: transforming growth factor β2. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Exhaustion in $CD8^+$ T cells is caused by a chronic antigenic exposure formed by the tumor microenvironment [191, 192]. The exhausted T cells will have lower effector capacities, due to the inhibition of the TCR-induced phosphorylation of ZAP70/CD3ζ [193] and CD28 [194], as well as the suppression of the PI3K, AKT, and RAS pathways [195]. These events may lead to the restriction of Tbet expression [196], as seen in our data. Consequently, since Tbet expression was decreased, it may have an impact in the low perforin expression, since Tbet regulates perforin transcription by binding to its promoter region [197].

Further investigation was performed to ascertain the mechanism exerted by the UBC tumor cells that caused perforin suppression. We set up an *in vitro* assay where we cultured sorted $CD8^+$ T cells in the presence of culture supernatants from the UBC cell lines: RT4 (non-muscle invasive) and 5637 (muscle invasive). Muscle invasive UBC supernatant was

displayed to downregulate perforin gene and protein expression, compared to its counterpart. Subsequently, we conducted a proteomic analysis using MS, in which we discovered that ICAM-1 and TGFβ2 were the two proteins that were present only in the culture supernatant of muscle invasive UBC cell line. We validated this finding *in vitro* and observed that both ICAM-1 and TGFβ2 suppressed the expression of perforin in $CD8^+$ T cells (**Figure 9B-C**). Therefore, we may speculate that ICAM-1 and TGFβ2 are utilized by the muscle invasive UBC in order to suppress the cytotoxicity of SN $CD8^+$ T cells. One issue to be addressed in the future is whether the ICAM-1 signaling is mediated in a contact-dependent or contact-independent fashion.

ICAM-1 is known to provide signal 2 upon TCR-dependent T cell activation [198]. When ICAM-1 synergistically works with TGFβ2, they both can elicit a potent perforin-deficient $CD8^+$ T cell. Furthermore, TGFβ2 has the capability to regulate tumor invasiveness. As TGFβ2 was secreted by the muscle invasive UBC, there is a possibility that the TGFβ receptor 2 is downregulated by the tumor cells, which will lead to the elevation of CXCL1/CXCL5 – CXCR2 chemokine receptor signaling. This will in turn enhance the tumor recruitment of MDSCs that can produce MMPs, which supports tumor invasion [145]. Our STRING network analysis of the proteomic data supported this notion in which TGFβ2 were displayed to interact with CXCL1, CXCL5, and MMP1.

The limitation of this study relied on the fact that we used an *in vitro* assay in order to seek the causal mechanism behind our *ex vivo* phenotype. Additionally, due to the limitation in isolating viable primary tumor cells form the patient samples, we used UBC cell lines that are not entirely representative of the tumor microenvironment. However, we could describe an immunosuppressive mechanism that may be used by the muscle invasive UBC tumors to dampen $CD8^+$ T cell cytotoxicity.

Taken together, synergistic ICAM-1 and TGFβ2-mediated pathway may be used as an escape mechanism by the muscle invasive UBC tumors to cause perforin suppression in SN $CD8^+$ T cells, together with exhaustion and Tc2-skewed environment. This finding may open up a possible translational application towards the clinic by targeting ICAM-1 and TGFβ2 in combination as a new strategy of cancer immunotherapy.

4.4 NEOADJUVANT CHEMOTHERAPY REINFORCES THE ANTI-TUMOR T CELL RESPONSES IN THE SENTINEL NODES (PAPER IV)

Chemotherapy is one of the standard treatments in solid tumors. The widely known mechanism of action of chemotherapy is by direct cytotoxicity towards the tumor cells [199]. Surprisingly, evidences have demonstrated that chemotherapy can promote anti-tumor immune responses [199, 200]. There are two mechanisms on how chemotherapy induces anti-tumor immune responses. First, chemotherapy increases the immunogenicity of the tumor cells and second, it reinforces the activation of the immune cells [200, 201]. In this study, we focused on the latter mechanism in the human UBC model.

In UBC, the standard chemotherapy regimen is to be administered prior to the radical cystectomy (neoadjuvant). This regimen consists of Methotrexate, Vinblastine, Adriamycin, and Cisplatin (MVAC). Similarly, MVAC is reported to induce the modulation of the immune cell responses [202, 203]. In here, we focused on the immunomodulation of T cells inside the SNs. SN is the site where APCs present tumor antigens to $CD8^+$ and $CD4^+$ T cells [204], resulting in the activation of tumor-specific T cells. Accordingly, SN is an attractive site to investigate the T cell immunomodulation by neoadjuvant chemotherapy (NAC).

In this study, we observed that NAC reduced the exhaustion of $CD8^+$ T cells, marked by a lower fraction of $CD8^+$ T cells expressing PD-1 in NAC patients (mean=20%) compared to NAC-naïve patients (mean=43.4%). Consequently, the cytotoxic capacity of $CD8^+$ T cells increased, as seen by the elevated expression of T-bet, granzyme B, and perforin, in a dose-dependent manner. Furthermore, this effect was pronounced in patients with complete response towards NAC (pT0N0M0 stage) (**Figure 10**). Additionally, the complete responder patients displayed a CpG hypomethylation in the *PRF1* and *IFNG* genes, suggesting that $CD8^+$ T cells in these patients are functionally committed cytotoxic cells.

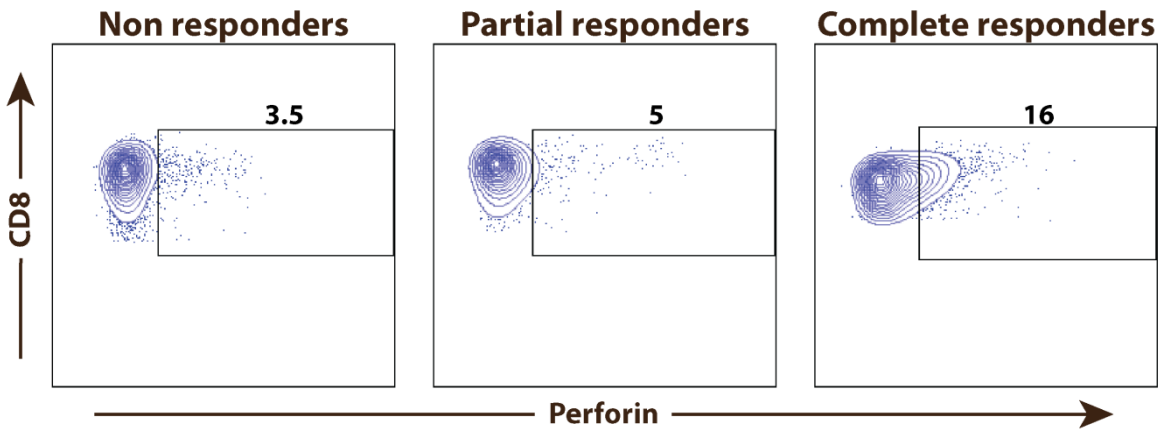


Figure 10. Patients with complete response to NAC have a better $CD8^+$ T cell cytotoxicity. The frequency of $perforin^+$ $CD8^+$ T cells from SNs was compared among non responders (≥pT2-N0M0 stage), partial responders (pTa/pTis/pT1-N0M0 stage), and complete responders (pT0N0M0 stage) of NAC. The data from representative UBC patients are displayed in density plots. NAC: neoadjuvant chemotherapy, pTNM: pathological tumor-node-metastasis stage, SN: sentinel node, UBC: urinary bladder cancer.

Similarly, NAC reduced the exhaustion of $CD4^+$ effector T cells, as demonstrated by the lower frequency of $PD\text{-}1^+$ $CD4^+$ effector T cells in NAC patients (mean=12.2%) compared to NAC-naïve patients (mean=24.9%). This resulted in a better clonal expansion of $CD4^+$ effector T cells following *ex vivo* activation using autologous tumor homogenate stimulation. Hence, it implies that NAC reduces the exhaustion of the effector T cells in the SNs and consequently restores their functions.

Surprisingly, Tregs which are known to suppress effector T cells [164], had a lower frequency in the SNs of patients receiving NAC, in a dose-dependent manner. In addition, SN-Tregs were less activated after NAC treatment, marked by a lower expression of CD39, HLA-DR, and PD-1 in these cells. Moreover, complete responders to NAC had a decreased number of Tregs expressing CD39 and CD69. This finding is in line with a previous study on breast cancer [205]. Taken together, we find that NAC promotes activation of $CD8^+$ and $CD4^+$ effector T cells and reduces the suppressive effects of Tregs. As a consequence, patients with complete response to NAC had a higher ratio of $CD4^+$ effector T cell number to $CD39^+$ or $CD69^+$ Treg frequency.

Overall, we observe that patients with a better NAC response have better anti-tumor T cell responses. This may be partly explained due to the diversity in the mutational signatures among individual tumors, which may affect the susceptibility of each tumor towards the cytotoxicity of chemotherapy. Accordingly, in the tumor that is sensitive to the cell-death induction by chemotherapy, tumor antigen uptake by APCs i.e. DCs and B cells is enhanced [206, 207], resulting in an improved T cell activation in the SNs. Additionally, since the histopathological stage of the tumor is downgraded, there will be less chronic inflammatory activities in the tumor microenvironment that suppress the T cell functions. Thus, better anti-tumor $CD8^+$ and $CD4^+$ T cell responses may be incited, alongside the lower recruitment of Tregs.

This study was limited by the fact that the NAC-naïve group that we used as the control was not clinically eligible for receiving NAC. Therefore, there was a risk of introducing confounding factors when comparing them with patients that clinically were eligible for NAC. However, this was the best control that we could obtain, since lymph node resection prior to NAC is not possible. Moreover, we could see that the immunomodulatory effects exerted by NAC were dose-dependent with significant differences between NAC responders.

We conclude that NAC reinforces protective anti-tumor T cell responses, which implicates that we can optimize the utilization of NAC in the clinic. One possible instance is to use NAC in combination with the cancer immunotherapy, such as checkpoint blockade agents [208]. The selection of the immunotherapy strategy may be done after the analysis of the T cell phenotype following the reinforcement by NAC, as performed in this study. Moreover, future clinical application to predict patients that will benefit from NAC treatment by correlating the molecular signature subtypes of the tumor to the chemotherapy responses may be needed [209]. This will lead to a tailored approach for the patient treatment, since we could see that there was a variability of immunomodulation among patients with different NAC responses. In other words, we may be able to treat the patients with no response to NAC to have a better prognosis in the future.

4.5 FLOW CYTOMETRY CAN DETECT MICROMETASTASES IN THE SENTINEL NODES OF CANCER PATIENTS (PAPER V)

One of the outcomes of the immunomodulation by the tumor microenvironment is the tumor immune escape acquired during tumor genesis [5]. The impact of such condition is the possibility of the tumor cells to progress and metastasize into adjacent lymph nodes or tissues and even into distant locations. The presence of metastasis in the lymph nodes itself, has a major implication that worsens the prognosis of cancer patients [210, 211]. Hence, a sensitive, objective, time- and cost-effective method for detecting metastasis in the lymph nodes is critical to be established in light of the increase in cancer incidence.

In fact, there are several diagnostic methods for detecting metastatic cells in the lymph nodes. Microscopic methods to visually examine hematoxylin and eosin (H&E)-stained lymph node sections or immunohistochemistry (IHC) are standard histopathological examinations that are routinely used in the clinic as a gold standard diagnostic approach in renal cancer [110]. However, the weakness of these methods is the possibility to miss micrometastases in the lymph nodes since only ~0.1% of the node is sliced into sections, and those sections may not contain the metastatic cell deposits [212]. Moreover, these diagnostic procedures are labor-intensive, subjective, and costly since they are carried out by pathologists.

Flow cytometry, which is regularly used to phenotype immune cells, offers a practical solution due to a possibility of detecting multi parameters of a single cell. Flow cytometry is also quick to run and it is an objective method compared to histopathological examination. In addition, as we analyzed single-cell suspension derived from the lymph nodes using flow cytometry, the chance of missing micrometastases is lower compared to the microscopic examination, since all of the metastatic cells are obtained inside the cell suspension [213].

In this study, we established that cancer cells could be reliably detected using flow cytometry. This assay detected three markers of renal cancer cells, which are routinely used for IHC in the clinical practice. The markers examined were CA9 and Cad6, both expressed on the cell surface, as well as intracellular CK18. Notably, we demonstrated that the intra-assay and inter-assay variability were low, which proved the stability of the assay. Furthermore, we adopted the International Union against Cancer consensus that defined micrometastasis as a tumor deposit between 0.2 to 2 mm [214]. Taking this into account, we assumed that a lymph node is ~10 x 5 x 5 mm in size and converted 0.2 to 2 mm of tumor deposit into 0.032% to 1.6% positive cells out of the total cell number from the lymph nodes.

Subsequently, we applied our assay to detect metastasis in the lymph nodes from renal cancer patients. We demonstrated that four out of five patients initially diagnosed with no metastases in their lymph nodes by the histopathological examination, turned out to have metastases detected by our flow cytometry method (**Table 1**). Additionally, six of the metastases-positive nodes were displayed to be micrometastases (0.032% - 1.6% positive cells) (**Table 1**). Thus, it implies that these four patients are restaged from pN0 to pN1, which results in different standard clinical treatment and prognosis [114]. One important thing to note is that the three markers did not detect the metastatic cells concomitantly. Therefore, it is

recommended to detect these markers simultaneously when using this flow cytometry method.

Table 1. The results of metastatic cell detection by flow cytometry are compared to the histopathological examination in each node from five renal cancer patients.

Patients	Samples	Histopathological examination	Flow cytometry (CK18, Cadherin 6, CA9)
Patient 1	SN1	Negative	**Micrometastasis**
	SN2	Negative	Metastasis
	SN3	Negative	**Micrometastasis**
Patient 2	nSN1	Negative	Negative
	nSN2	Negative	**Micrometastasis**
Patient 3	SN1	Negative	Negative
	SN2	Negative	Metastasis
	SN3	Negative	Negative
	SN4	Negative	**Micrometastasis**
	nSN1	Negative	**Micrometastasis**
Patient 4	SN2	Negative	**Micrometastasis**
	nSN1	Negative	Negative
Patient 5	nSN1	Negative	Negative
	nSN2	Negative	Negative
	nSN3	Negative	Negative

CA9: carbonic anhydrase IX, CK18: cytokeratin 18, nSN: non-sentinel node, SN: sentinel node

This study was limited due to a fact that it was performed prospectively, resulting in a short follow-up period to obtain the patient clinical information. Therefore, we could not correlate the benefit of our metastatic detection method to the patient survival. In the future, it will be appealing to do this study longitudinally for a longer period of time, so that it is possible to correlate our finding from flow cytometry with relapse free survival, cause-specific survival, and overall survival of the patients.

We conclude that flow cytometry allows detection of a very low number of cancer cells, i.e. micrometastases, when present in the lymph nodes. By having a high reliability and stability in the metastatic detection, flow cytometry can potentially have a promising future as a diagnostic tool. Moreover, we believe that it can be applied to various types of solid tumors for a better clinical practice in the future.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Hippocrates once said: "The natural forces within us are the true healers of diseases." Accordingly, over two millennia later, through major discoveries in biology and medicine, especially in the field of immunology, this proposed concept is revealed to be partly true. Our immune system protects us from infectious diseases and most importantly, from life-threatening tumors.

Nonetheless, the nature of the solid tumor microenvironment resembles a labyrinth. Such complexity of this microenvironment can modulate the immune cell phenotypes and functions in order for the tumors to grow. Correspondingly, the immunomodulation in the tumor microenvironment needs to be deciphered, in order to pave the road towards a successful cancer immunotherapy. In this thesis, we focus on the modulation of T cells, since they are part of the adaptive immune response and they play a central role in tumor immunity.

Our observations from the TILs in human UBC model indicate the unexpected protective function of Tregs in preventing tumor invasiveness. The data suggest that the Tregs residing in the invasive front of the tumors suppress MMP2 expression in the tumor microenvironment (**Figure 11**). Consequently, a higher frequency of Tregs in the invasive front provides a better survival. However, we still encounter the dual characters of Tregs in which they are also suppressive. Overall, Tregs in the UBC tumors express CD39 and CTLA-4, and they functionally suppress effector T cells. Therefore, prior to designing a cancer immunotherapy to target Tregs in UBC, a proper phenotyping of these cells should be taken into high consideration.

Furthermore, the exploration into the effector $CD8^+$ TIL population reveals that T_{RM} cells are the most dominant subset in UBC tumors. Tumor T_{RM} cells exhibit an epigenetic commitment of cytotoxicity (to transcriptionally express perforin) and they are not terminally exhausted, even in the tumor-induced chronic inflammatory microenvironment (**Figure 11**). As a result, their protective roles are seen, in which a lower tumor T_{RM} cell infiltration is linked with muscle invasiveness in UBC tumors. Moreover, we discover that the muscle invasive UBC tumors may escape immune destruction by suppressing perforin expression in $CD8^+$ T_{EM} cells from the SNs via ICAM-1/TGFβ2-mediated signal (**Figure 11**). Thus, we speculate that the muscle invasiveness of the UBC tumors may partly occur due to the faulty protection from tumor T_{RM} cells and SN T_{EM} cells. A low frequency of tumor T_{RM} cells results in a decreased production of IFNγ which leads to a reduction of chemokines and vascular adhesion molecule expression [73]. Consequently, the recruitment and activation of DCs and other memory $CD8^+$ T cells will be dampened. The lack of DC recruitment may result in a diminished antigenic uptake from the tumors and subsequently may cause a lower tumor-specific T cell priming in the SNs [215]. Accordingly, there will be a deficiency of new T_{RM} cells and other memory T cells that traffic to the tumor, which may liberate the tumors to escape the immune system and grow further, resulting in tumor invasiveness.

Nonetheless, it is still an open question regarding which mechanisms that caused the original reduction of T_{RM} cell frequency in the muscle invasive tumors. We may argue that the mutation and differentiation of tumor cells, including the modification of tumor microenvironment over the course of tumor development may make it harder for the T_{RM} cells to be retained in the tumor. In addition, it is unknown whether T_{RM} cells are tumor-antigen specific or that they have already been present in the tissues prior to tumor development. In this case, deep sequencing of the recombined TCR genes from the tumor T_{RM} cells may provide us a better clue regarding the T cell repertoire [216].

Furthermore, the promising checkpoint blockade towards CTLA-4, PD-1, and PD-L1 has been introduced clinically as cancer treatments. However, not all of the treated patients display durable responses [217]. From the studies of the T cell immunomodulation in this thesis, we have unveiled several candidates to target for cancer immunotherapy. Correspondingly, we may propose to target the Treg suppression and the tumor immune escape on $CD8^+$ T cells. In addition, we may expand tumor T_{RM} cells *in vitro* for an adoptive cellular therapy. However, when expanding the tumor T_{RM} cells, initial screening of the cell reactivity against the mutations identified by whole-exome sequencing and RNA sequencing of the tumors is important. Therefore, the expansion of cells with mutation-reactive TCR clonotypes can be secured [218].

In addition, as we have demonstrated, the use of chemotherapy regimen can promote the anti-tumor effector T cell responses and reduce the suppressive activity of Tregs (**Figure 11**). As a result, it is reasonable to propose the combination of chemotherapy and immunotherapy for more effective therapeutic approaches against cancer and its metastasis [219]. Moreover, further studies to investigate the effect of chemotherapy on the cytotoxic T_{RM} cells inside the solid tumors can be addressed in the future.

On the other hand, we still need to remember that the durability of positive clinical responses from each cancer immunotherapy is crucial. Thus, in the future era of an unbiased approach, it is compelling to broadly characterize the immune cell signatures in the tumor microenvironment from each patient prior to deciding the cancer treatment. The immune cells from the tumor microenvironment can be evaluated using single-cell RNA sequencing or whole genome DNA methylation arrays. Moreover, an approach using CRISPR-Cas9, which can identify how genetic manipulation of either T cells or tumor cells can affect the complex interplay between them, is an attractive method to be applied [220].

In summary, the intricacy of the T cell immunomodulation in the solid tumor microenvironment needs a thorough exploration. Indeed, the discoveries revealed in the tumor-immune interactions have been translationally applied to various groundbreaking cancer immunotherapy strategies. But, there are still some drawbacks. Therefore, an extensive investigation beyond the current evidences is fundamental to provide a strong foundation for cancer immunotherapy, so that we can treat cancer patients optimally.

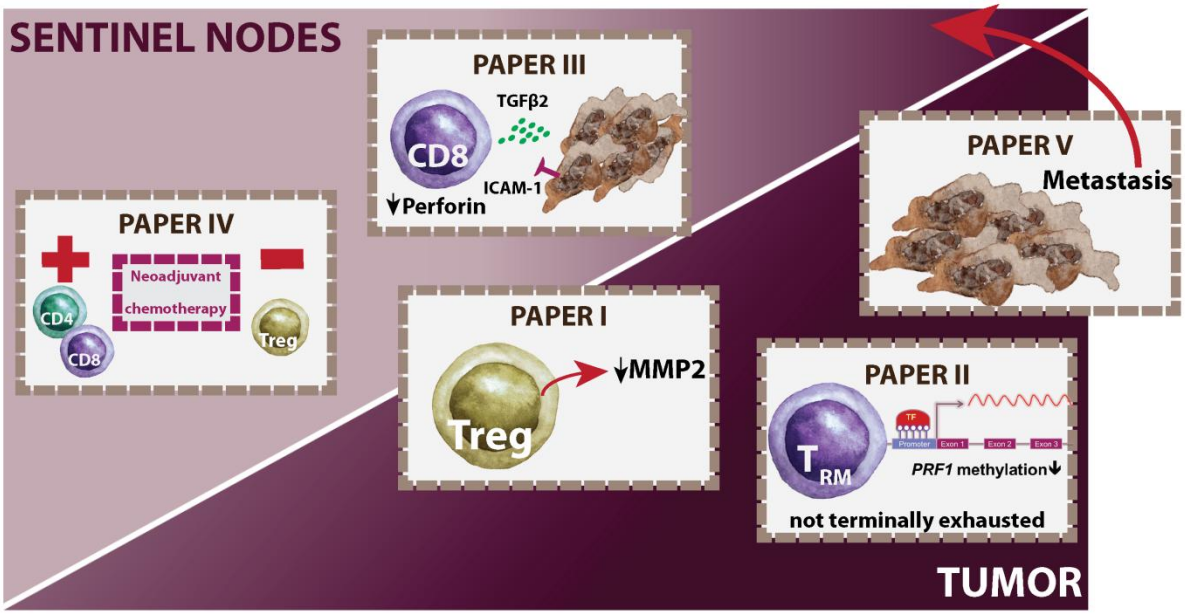


Figure 11. The summary of our findings in this thesis. The major findings from each paper in regards of the T cell immunomodulation in the solid tumor microenvironment are illustrated. Paper I demonstrates that Tregs regulate tumor invasiveness by suppressing MMP2 in the tumors. Paper II reveals that tumor T_{RM} cells are epigenetically cytotoxic and not terminally exhausted. Paper III identifies the tumor immune escape mechanism that suppresses perforin in SN $CD8^+$ T cells via an ICAM-1/TGFβ2-mediated pathway. Paper IV demonstrates that NAC promotes effector T cell functions while suppressing Treg activity in SNs. Paper V shows the possibility of detecting micrometastases in SNs by flow cytometry. ICAM-1: intercellular adhesion molecule 1, MMP2: matrix metalloproteinase 2, NAC: neoadjuvant chemotherapy, SN: sentinel node, TGFβ2: transforming growth factor β2, Treg: regulatory T cell, T_{RM}: tissue-resident memory T cells.

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7 REFERENCES

1. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010; **140**(6): 805-820.
2. Neefjes J, Jongsma MLM, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nature Reviews Immunology 2011; **11**(12): 823-836.
3. Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, MacIntyre MF, Allen C, Hansen G *et al*. The Global Burden of Cancer 2013. JAMA Oncol 2015; **1**(4): 505-527.
4. Vineis P, Wild CP. Global cancer patterns: causes and prevention. Lancet 2014; **383**(9916): 549-557.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; **144**(5): 646-674.
6. Koch U, Radtke F. Mechanisms of T cell development and transformation. Annu Rev Cell Dev Biol 2011; **27**: 539-562.
7. Bell JJ, Bhandoola A. The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature 2008; **452**(7188): 764-767.
8. von Boehmer H. Unique features of the pre-T-cell receptor alpha-chain: not just a surrogate. Nat Rev Immunol 2005; **5**(7): 571-577.
9. Klein L, Hinterberger M, Wirnsberger G, Kyewski B. Antigen presentation in the thymus for positive selection and central tolerance induction. Nat Rev Immunol 2009; **9**(12): 833-844.
10. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T Cell Antigen Receptor Recognition of Antigen-Presenting Molecules. Annual Review of Immunology Vol 33 2015; **33**: 169-200.
11. van der Merwe PA, Dushek O. Mechanisms for T cell receptor triggering. Nature Reviews Immunology 2011; **11**(1): 47-55.
12. Smith-Garvin JE, Koretzky GA, Jordan MS. T Cell Activation. Annu Rev Immunol 2009; **27**: 591-619.
13. Pages F, Ragueneau M, Rottapel R, Truneh A, Nunes J, Imbert J, Olive D. Binding of Phosphatidylinositol-3-Oh Kinase to Cd28 Is Required for T-Cell Signaling. Nature 1994; **369**(6478): 327-329.
14. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 1999; **397**(6716): 263-266.
15. Watts TH. Tnf/tnfr family members in costimulation of T cell responses. Annu Rev Immunol 2005; **23**: 23-68.
16. Teft WA, Kirchhof MG, Madrenas J. A molecular perspective of CTLA-4 function. Annu Rev Immunol 2006; **24**: 65-97.
17. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1

upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. Journal of Immunology 2004; **173**(2): 945-954.

18. Woodland DL, Dutton RW. Heterogeneity of CD4(+) and CD8(+) T cells. Curr Opin Immunol 2003; **15**(3): 336-342.

19. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996; **383**(6603): 787-793.

20. Cerwenka A, Carter LL, Reome JB, Swain SL, Dutton RW. In vivo persistence of CD8 polarized T cell subsets producing type 1 or type 2 cytokines. Journal of Immunology 1998; **161**(1): 97-105.

21. Seder RA, Paul WE. Acquisition of Lymphokine-Producing Phenotype by Cd4+ T-Cells. Annu Rev Immunol 1994; **12**: 635-673.

22. Mittrucker HW, Visekruna A, Huber M. Heterogeneity in the differentiation and function of CD8(+) T cells. Arch Immunol Ther Exp (Warsz) 2014; **62**(6): 449-458.

23. Zhu JF, Yamane H, Paul WE. Differentiation of Effector CD4 T Cell Populations. Annual Review of Immunology, Vol 28 2010; **28**: 445-489.

24. Lee YK, Turner H, Maynard CL, Oliver JR, Chen DQ, Elson CO, Weaver CT. Late Developmental Plasticity in the T Helper 17 Lineage. Immunity 2009; **30**(1): 92-107.

25. Wei G, Wei L, Zhu JF, Zang CZ, Hu-Li J, Yao ZJ, Cui KR, Kanno Y *et al*. Global Mapping of H3K4me3 and H3K27me3 Reveals Specificity and Plasticity in Lineage Fate Determination of Differentiating CD4(+) T Cells. Immunity 2009; **30**(1): 155-167.

26. Vizler C, Bercovici N, Heurtier A, Pardigon N, Goude K, Bailly K, Combadiere C, Liblau RS. Relative diabetogenic properties of islet-specific Tc1 and Tc2 cells in immunocompetent hosts. Journal of Immunology 2000; **165**(11): 6314-6321.

27. Hinrichs CS, Kaiser A, Paulos CM, Cassard L, Sanchez-Perez L, Heemskerk B, Wrzesinski C, Borman ZA *et al*. Type 17 CD8(+) T cells display enhanced antitumor immunity. Blood 2009; **114**(3): 596-599.

28. Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair DA, Waite J, Sacristan C, Victora GD *et al*. Functional anatomy of T cell activation and synapse formation. Annu Rev Immunol 2010; **28**: 79-105.

29. Stinchcombe JC, Bossi G, Booth S, Griffiths GM. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity 2001; **15**(5): 751-761.

30. Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. Nature 2006; **443**(7110): 462-465.

31. Thiery J, Lieberman J. Perforin: a key pore-forming protein for immune control of viruses and cancer. Subcell Biochem 2014; **80**: 197-220.

32. Cullen SP, Brunet M, Martin SJ. Granzymes in cancer and immunity. Cell Death Differ 2010; **17**(4): 616-623.

33. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. Nature Reviews Immunology 2015; **15**(6): 388-400.

34. Martinvalet D, Zhu PC, Lieberman J. Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. Immunity 2005; **22**(3): 355-370.

35. Trapani JA, Thia KY, Andrews M, Davis ID, Gedye C, Parente P, Svobodova S, Chia J *et al*. Human perforin mutations and susceptibility to multiple primary cancers. Oncoimmunology 2013; **2**(4): e24185.

36. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM *et al*. Cytotoxicity Mediated by T-Cells and Natural-Killer-Cells Is Greatly Impaired in Perforin Deficient Mice. Nature 1994; **369**(6475): 31-37.

37. Castellino F, Germain RN. Cooperation between CD4(+) and CD8(+) T cells: When, where, and how. Annu Rev Immunol 2006; **24**: 519-540.

38. Wagner H, Kronke M, Solbach W, Scheurich P, Rollinghoff M, Pfizenmaier K. Murine T-Cell Subsets and Interleukins - Relationships between Cyto-Toxic T-Cells, Helper T-Cells and Accessory Cells. Clinics in Haematology 1982; **11**(3): 607-630.

39. Schulz O, Edwards AD, Schito M, Aliberti J, Manickasingham S, Sher A, Sousa CRE. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. Immunity 2000; **13**(4): 453-462.

40. Ahrends T, Spanjaard A, Pilzecker B, Babala N, Bovens A, Xiao YL, Jacobs H, Borst J. CD4(+) T Cell Help Confers a Cytotoxic T Cell Effector Program Including Coinhibitory Receptor Downregulation and Increased Tissue Invasiveness. Immunity 2017; **47**(5): 848-+.

41. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunological Self-Tolerance Maintained by Activated T-Cells Expressing Il-2 Receptor Alpha-Chains (Cd25) - Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune-Diseases. Journal of Immunology 1995; **155**(3): 1151-1164.

42. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T Cells: Mechanisms of Differentiation and Function. Annual Review of Immunology, Vol 30 2012; **30**: 531-564.

43. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT *et al*. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001; **27**(1): 20-21.

44. Pandiyan P, Zheng LX, Ishihara S, Reed J, Lenardo MJ. CD4(+) CD25(+) Foxp3(+) regulatory T cells induce cytokine deprivation -mediated apoptosis of effector CD4(+) T cells. Nat Immunol 2007; **8**(12): 1353-1362.

45. Bachmann MF, Kohler G, Ecabert B, Mak TW, Kopf M. Cutting edge: Lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. Journal of Immunology 1999; **163**(3): 1128-1131.

46. Murakami N, Riella LV. Co-Inhibitory Pathways and Their Importance in Immune Regulation. Transplantation 2014; **98**(1): 3-14.

47. Kumar S, Malik S, Singh UP, Ponnazhagan S, Scissum-Gunn K, Manne U, Mishra MK. PD-1 expression on Foxp3(+) Treg cells modulates CD8(+) T cell function in prostatic tumor microenvironment. Journal of Immunology 2017; **198**(1).

48. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K *et al*. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. Journal of Experimental Medicine 2007; **204**(6): 1257-1265.

49. von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat Immunol 2005; **6**(4): 338-344.

50. Li MO, Wan YSY, Flavell RA. T cell-produced transforming growth factor-beta 1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity 2007; **26**(5): 579-591.

51. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: Contact-mediated suppression by CD4(+)-CD25(+) regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. Journal of Immunology 2005; **174**(4): 1783-1786.

52. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nat Rev Immunol 2012; **12**(11): 749-761.

53. Kumar BV, Connors TJ, Farber DL. Human T Cell Development, Localization, and Function throughout Life. Immunity 2018; **48**(2): 202-213.

54. Herndler-Brandstetter D, Ishigame H, Shinnakasu R, Plajer V, Stecher C, Zhao J, Lietzenmayer M, Kroehling L *et al*. KLRG1(+) Effector CD8(+) T Cells Lose KLRG1, Differentiate into All Memory T Cell Lineages, and Convey Enhanced Protective Immunity. Immunity 2018; **48**(4): 716-+.

55. Joshi NS, Cui W, Dominguez CX, Chen JH, Hand TW, Kaech SM. Increased numbers of preexisting memory CD8 T cells and decreased T-bet expression can restrain terminal differentiation of secondary effector and memory CD8 T cells. J Immunol 2011; **187**(8): 4068-4076.

56. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. Nat Immunol 2010; **11**(2): 114-120.

57. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, Li HYS, Rivera RR *et al*. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8(+) T cell subsets. Nat Immunol 2011; **12**(12): 1221-U1117.

58. Pearce EL. Metabolism in T cell activation and differentiation. Curr Opin Immunol 2010; **22**(3): 314-320.

59. Michalek RD, Rathmell JC. The metabolic life and times of a T-cell. Immunol Rev 2010; **236**: 190-202.

60. Araki K, Morita M, Bederman AG, Konieczny BT, Kissick HT, Sonenberg N, Ahmed R. Translation is actively regulated during the differentiation of CD8(+) effector T cells. Nat Immunol 2017; **18**(9): 1046-+.

61. Tyrakis PA, Palazon A, Macias D, Lee KL, Phan AT, Velica P, You J, Chia GS *et al*. S-2-hydroxyglutarate regulates CD8(+) T-lymphocyte fate. Nature 2016; **540**(7632): 236-+.

62. Surh CD, Sprent J. Homeostasis of Naive and Memory T Cells. Immunity 2008; **29**(6): 848-862.

63. Jameson SC, Masopust D. Understanding Subset Diversity in T Cell Memory. Immunity 2018; **48**(2): 214-226.

64. Purton JF, Tan JT, Rubinstein MP, Kim DM, Sprent J, Surh CD. Antiviral CD4(+) memory T cells are IL-15 dependent. Journal of Experimental Medicine 2007; **204**(4): 951-961.

65. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, Bickham KL, Lerner H *et al*. Distribution and Compartmentalization of Human Circulating and Tissue-Resident Memory T Cell Subsets. Immunity 2013; **38**(1): 187-197.

66. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. Nature Reviews Immunology 2014; **14**(1): 24-35.

67. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. Science 2001; **291**(5512): 2413-2417.

68. Geginat J, Lanzavecchia A, Sallusto F. Proliferation and differentiation potential of human CD8(+) memory T-cell subsets in response to antigen or homeostatic cytokines. Blood 2003; **101**(11): 4260-4266.

69. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: Function, generation, and maintenance. Annu Rev Immunol 2004; **22**: 745-763.

70. Thome JJC, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, Kato T, Lerner H *et al*. Spatial Map of Human T Cell Compartmentalization and Maintenance over Decades of Life. Cell 2014; **159**(4): 814-828.

71. Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat Immunol 2009; **10**(5): 524-530.

72. Masopust D, Choo D, Vezys V, Wherry EJ, Duraiswamy J, Akondy R, Wang J, Casey KA *et al*. Dynamic T cell migration program provides resident memory within intestinal epithelium. Journal of Experimental Medicine 2010; **207**(3): 553-564.

73. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. Nat Rev Immunol 2016; **16**(2): 79-89.

74. Milner JJ, Toma C, Yu BF, Zhang K, Omilusik K, Phan AT, Wang DP, Getzler AJ *et al*. Runx3 programs CD8(+) T cell residency in non-lymphoid tissues and tumours. Nature 2017; **552**(7684): 253-+.

75. Watanabe R, Gehad A, Yang C, Scott LL, Teague JE, Schlapbach C, Elco CP, Huang V *et al*. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. Science Translational Medicine 2015; **7**(279).

76. Mackay LK, Braun A, Macleod BL, Collins N, Tebartz C, Bedoui S, Carbone FR, Gebhardt T. Cutting Edge: CD69 Interference with Sphingosine-1-Phosphate Receptor Function Regulates Peripheral T Cell Retention. Journal of Immunology 2015; **194**(5): 2059-2063.

77. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon ML, Vega-Ramos J, Lauzurica P *et al*. The developmental pathway for CD103(+)CD8(+) tissue-resident memory T cells of skin. Nat Immunol 2013; **14**(12): 1294-+.

78. Cheuk S, Schlums H, Serezal IG, Martini E, Chiang SC, Marquardt N, Gibbs A, Detlofsson E *et al*. CD49a Expression Defines Tissue-Resident CD8(+) T Cells Poised for Cytotoxic Function in Human Skin. Immunity 2017; **46**(2): 287-300.

79. Beura LK, Wijeyesinghe S, Thompson EA, Macchietto MG, Rosato PC, Pierson MJ, Schenkel JM, Mitchell JS *et al*. T Cells in Nonlymphoid Tissues Give Rise to Lymph-Node-Resident Memory T Cells. Immunity 2018; **48**(2): 327-+.

80. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nature Reviews Genetics 2016; **17**(8): 487-500.

81. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001; **293**(5532): 1068-1070.

82. Schubeler D. Function and information content of DNA methylation. Nature 2015; **517**(7534): 321-326.

83. Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000; **403**(6765): 41-45.

84. Henning AN, Roychoudhuri R, Restifo NP. Epigenetic control of CD8(+) T cell differentiation. Nature Reviews Immunology 2018; **18**(5): 340-356.

85. Rodriguez RM, Suarez-Alvarez B, Lavin JL, Mosen-Ansorena D, Raneros AB, Marquez-Kisinousky L, Aransay AM, Lopez-Larrea C. Epigenetic Networks Regulate the Transcriptional Program in Memory and Terminally Differentiated CD8(+) T Cells. Journal of Immunology 2017; **198**(2): 937-949.

86. Kakaradov B, Arsenio J, Widjaja CE, He ZR, Aigner S, Metz PJ, Yu BF, Wehrens EJ *et al*. Early transcriptional and epigenetic regulation of CD8(+) T cell differentiation revealed by single-cell RNA sequencing. Nat Immunol 2017; **18**(4): 422-+.

87. Abdelsamed HA, Moustaki A, Fan YP, Dogra P, Ghoneim HE, Zebley CC, Triplett BM, Sekaly RP *et al*. Human memory CD8 T cell effector potential is epigenetically preserved during in vivo homeostasis. Journal of Experimental Medicine 2017; **214**(6): 1593-1606.

88. He B, Xing SJ, Chen CY, Gao P, Teng L, Shan Q, Gullicksrud JA, Martin MD *et al*. CD8(+) T Cells Utilize Highly Dynamic Enhancer Repertoires and Regulatory Circuitry in Response to Infections. Immunity 2016; **45**(6): 1341-1354.

89. Yu BF, Zhang K, Milner JJ, Toma C, Chen RQ, Scott-Browne JP, Pereira RM, Crotty S *et al*. Epigenetic landscapes reveal transcription factors that regulate CD8(+) T cell differentiation. Nat Immunol 2017; **18**(5): 573-582.

90. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3(+) regulatory T cells in the human immune system. Nature Reviews Immunology 2010; **10**(7): 490-500.

91. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4(+)CD25(-) T cells. Journal of Clinical Investigation 2003; **112**(9): 1437-1443.

92. Janson PCJ, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 Promoter Demethylation Reveals the Committed Treg Population in Humans. PLoS One 2008; **3**(2).

93. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang HD *et al*. Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol 2007; **5**(2): 169-178.

94. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, Thiel A, Boeld TJ *et al*. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. Eur J Immunol 2007; **37**(9): 2378-2389.

95. deLeeuw RJ, Kost SE, Kakal JA, Nelson BH. The Prognostic Value of FoxP3+ Tumor-Infiltrating Lymphocytes in Cancer: A Critical Review of the Literature. Clinical Cancer Research 2012; **18**(11): 3022-3029.

96. Burger M, Catto JWF, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, Kassouf W, Kiemeney LA *et al*. Epidemiology and Risk Factors of Urothelial Bladder Cancer. Eur Urol 2013; **63**(2): 234-241.

97. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global Cancer Statistics, 2012. Ca-a Cancer Journal for Clinicians 2015; **65**(2): 87-108.

98. Sanli O, Dobruch J, Knowles MA, Burger M, Alemozaffar M, Nielsen ME, Lotan Y. Bladder cancer. Nature Reviews Disease Primers 2017; **3**.

99. Humphrey PA, Moch H, Cubilla AL, Ulbright TM, Reuter VE. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part B: Prostate and Bladder Tumours. Eur Urol 2016; **70**(1): 106-119.

100. Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Bohle A, Palou-Redorta J, Roupret M. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. Eur Urol 2011; **59**(6): 997-1008.

101. Kawai K, Miyazaki J, Joraku A, Nishiyama H, Akaza H. Bacillus Calmette-Guerin (BCG) immunotherapy for bladder cancer: current understanding and perspectives on engineered BCG vaccine. Cancer Sci 2013; **104**(1): 22-27.

102. Witjes JA, Comperat E, Cowan NC, De Santis M, Gakis G, Lebret T, Ribal MJ, Van der Heijden AG *et al*. EAU Guidelines on Muscle-invasive and Metastatic Bladder Cancer: Summary of the 2013 Guidelines. Eur Urol 2014; **65**(4): 778-792.

103. Stenzl A, Cowan NC, De Santis M, Kuczyk MA, Merseburger AS, Ribal MJ, Sherif A, Witjes JA. Treatment of muscle-invasive and metastatic bladder cancer: update of the EAU guidelines. Eur Urol 2011; **59**(6): 1009-1018.

104. Abol-Enein H, Bassi P, Boyer M, Coppin CML, Cortesi E, Grossman HB, Hall RR, Horwich A *et al*. Neoadjuvant chemotherapy in invasive bladder cancer: Update of a systematic review and meta-analysis of individual patient data. Eur Urol 2005; **48**(2): 202-206.

105. Sherif A, Holmberg L, Rintala E, Mestad O, Nilsson J, Nilsson S, Malmstrom PU. Neoadjuvant cisplatinum based combination chemotherapy in patients with invasive bladder cancer: A combined analysis of two Nordic studies. Eur Urol 2004; **45**(3): 297-303.

106. Bono AV, Goebell PJ, Groshen S, Lehmann J, Studer U, Torti FM, Abol-Enein H, Bassi P *et al*. Adjuvant chemotherapy in invasive bladder cancer: A systematic review and meta-analysis of individual patient data. Eur Urol 2005; **48**(2): 189-201.

107. Stein JP, Skinner DG. Radical cystectomy for invasive bladder cancer: long-term results of a standard procedure. World J Urol 2006; **24**(3): 296-304.

108. Donin NM, Lenis AT, Holden S, Drakaki A, Pantuck A, Belldegrun A, Chamie K. Immunotherapy for the Treatment of Urothelial Carcinoma. Journal of Urology 2017; **197**(1): 14-22.

109. Znaor A, Lortet-Tieulent J, Laversanne M, Jemal A, Bray F. International Variations and Trends in Renal Cell Carcinoma Incidence and Mortality. Eur Urol 2015; **67**(3): 519-530.

110. Hsieh JJ, Purdue MP, Signoretti S, Swanton C, Albiges L, Schmidinger M, Heng DY, Larkin J *et al*. Renal cell carcinoma. Nature Reviews Disease Primers 2017; **3**.

111. Lopez-Beltran A, Scarpelli M, Montironi R, Kirkali Z. 2004 WHO classification of the renal tumors of the adults. Eur Urol 2006; **49**(5): 798-805.

112. Sun M, Shariat SF, Cheng C, Ficarra V, Murai M, Oudard S, Pantuck AJ, Zigeuner R *et al*. Prognostic factors and predictive models in renal cell carcinoma: a contemporary review. Eur Urol 2011; **60**(4): 644-661.

113. Tunuguntla HS, Jorda M. Diagnostic and prognostic molecular markers in renal cell carcinoma. J Urol 2008; **179**(6): 2096-2102.

114. Ljungberg B, Bensalah K, Canfield S, Dabestani S, Hofmann F, Hora M, Kuczyk MA, Lam T *et al*. EAU Guidelines on Renal Cell Carcinoma: 2014 Update. Eur Urol 2015; **67**(5): 913-924.

115. McDermott DF, Regan MM, Clark JI, Flaherty LE, Weiss GR, Logan TF, Kirkwood JM, Gordon MS *et al*. Randomized phase III trial of high-dose interleukin-2 versus subcutaneous interleukin-2 and interferon in patients with metastatic renal cell carcinoma. Journal of Clinical Oncology 2005; **23**(1): 133-141.

116. Paget S. THE DISTRIBUTION OF SECONDARY GROWTHS IN CANCER OF THE BREAST. The Lancet 1889; **133**(3421): 571-573.

117. Maman S, Witz IP. A history of exploring cancer in context. Nature Reviews Cancer 2018; **18**(6): 359-376.

118. Barkan D, Green JE, Chambers AF. Extracellular matrix: A gatekeeper in the transition from dormancy to metastatic growth. European Journal of Cancer 2010; **46**(7): 1181-1188.

119. Kessenbrock K, Plaks V, Werb Z. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. Cell 2010; **141**(1): 52-67.

120. Semenza GL. The hypoxic tumor microenvironment: A driving force for breast cancer progression. Biochimica Et Biophysica Acta-Molecular Cell Research 2016; **1863**(3): 382-391.

121. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev 2010; **20**(1): 51-56.

122. Parks SK, Chiche J, Pouyssegur J. Disrupting proton dynamics and energy metabolism for cancer therapy. Nature Reviews Cancer 2013; **13**(9): 611-623.

123. Elinav E, Nowarski R, Thaiss CA, Hu B, Jin CC, Flavell RA. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nature Reviews Cancer 2013; **13**(11): 759-771.

124. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nature Reviews Cancer 2006; **6**(1): 24-37.

125. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, Coussens LM, Gabrilovich DI *et al*. Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat Med 2018; **24**(5): 541-550.

126. Evans RA, Diamond MS, Rech AJ, Chao T, Richardson MW, Lin JH, Bajor DL, Byrne KT *et al*. Lack of immunoediting in murine pancreatic cancer reversed with neoantigen. Jci Insight 2016; **1**(14).

127. Sautes-Fridman C, Lawand M, Giraldo NA, Kaplon H, Germain C, Fridman WH, Dieu-Nosjean MC. Tertiary Lymphoid Structures in Cancers: Prognostic Value, Regulation, and Manipulation for Therapeutic Intervention. Front Immunol 2016; **7**.

128. Graziano DF, Finn OJ. Tumor antigens and tumor antigen discovery. Cancer Treat Res 2005; **123**: 89-111.

129. Burnet FM. Immunological surveillance in neoplasia. Transplant Rev 1971; **7**: 3-25.

130. Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest 2007; **117**(5): 1137-1146.

131. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004; **22**: 329-360.

132. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, Knoblaugh S, Cado D *et al*. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 2008; **28**(4): 571-580.

133. Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. Science 2011; **331**(6024): 1565-1570.

134. Becht E, Goc J, Germain C, Giraldo NA, Dieu-Nosjean MC, Sautes-Fridman C, Fridman WH. Shaping of an effective immune microenvironment to and by cancer cells. Cancer Immunol Immunother 2014; **63**(10): 991-997.

135. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 2013; **14**(10): 1014-1022.

136. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW. Cancer genome landscapes. Science 2013; **339**(6127): 1546-1558.

137. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002; **3**(11): 991-998.

138. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 2007; **25**: 267-296.

139. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002; **3**(11): 999-1005.

140. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A 1998; **95**(13): 7556-7561.

141. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L *et al*. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999; **10**(1): 105-115.

142. Wellenstein MD, de Visser KE. Cancer-Cell-Intrinsic Mechanisms Shaping the Tumor Immune Landscape. Immunity 2018; **48**(3): 399-416.

143. Xia HJ, Wang W, Crespo J, Kryczek I, Li W, Wei S, Bian ZQ, Maj T *et al*. Suppression of FIP200 and autophagy by tumor-derived lactate promotes naive T cell apoptosis and affects tumor immunity. Science Immunology 2017; **2**(17).

144. Kearney CJ, Vervoort SJ, Hogg SJ, Ramsbottom KM, Freeman AJ, Lalaoui N, Pijpers L, Michie J *et al*. Tumor immune evasion arises through loss of TNF sensitivity. Science Immunology 2018; **3**(23).

145. Yang L, Pang Y, Moses HL. TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. Trends Immunol 2010; **31**(6): 220-227.

146. Terme M, Colussi O, Marcheteau E, Tanchot C, Tartour E, Taieb J. Modulation of Immunity by Antiangiogenic Molecules in Cancer. Clin Dev Immunol 2012.

147. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv Cancer Res 2010; **107**: 57-117.

148. Keskinov AA, Shurin MR. Myeloid regulatory cells in tumor spreading and metastasis. Immunobiology 2015; **220**(2): 236-242.

149. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. Cancers (Basel) 2014; **6**(3): 1670-1690.

150. Curiel TJ, Coukos G, Zou LH, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR *et al*. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004; **10**(9): 942-949.

151. Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, Gajewski TF. Up-Regulation of PD-L1, IDO, and T-regs in the Melanoma Tumor Microenvironment Is Driven by CD8(+) T Cells. Science Translational Medicine 2013; **5**(200).

152. Burr ML, Sparbier CE, Chan YC, Williamson JC, Woods K, Beavis PA, Lam EYN, Henderson MA *et al*. CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity. Nature 2017; **549**(7670): 101-105.

153. Mezzadra R, Sun C, Jae LT, Gomez-Eerland R, de Vries E, Wu W, Logtenberg MEW, Slagter M *et al*. Identification of CMTM6 and CMTM4 as PD-L1 protein regulators. Nature 2017; **549**(7670): 106-+.

154. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol 2015; **15**(8): 486-499.

155. Bengsch B, Ohtani T, Khan O, Setty M, Manne S, O'Brien S, Gherardini PF, Herati RS *et al*. Epigenomic-Guided Mass Cytometry Profiling Reveals Disease-Specific Features of Exhausted CD8 T Cells. Immunity 2018; **48**(5): 1029-+.

156. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, Betts MR, Freeman GJ *et al*. Coregulation of CD8(+) T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 2009; **10**(1): 29-37.

157. Rosenblatt R, Johansson M, Alamdari F, Sidiki A, Holmstrom B, Hansson J, Vasko J, Marits P *et al*. Sentinel node detection in muscle-invasive urothelial bladder cancer is feasible after neoadjuvant chemotherapy in all pT stages, a prospective multicenter report. World J Urol 2017; **35**(6): 921-927.

158. Mia S, Warnecke A, Zhang XM, Malmstrom V, Harris RA. An optimized Protocol for Human M2 Macrophages using M-CSF and IL-4/IL-10/TGF-beta Yields a Dominant Immunosuppressive Phenotype. Scand J Immunol 2014; **79**(5): 305-314.

159. Christman JK. 5-Azacytidine and 5-aza-2 '-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene 2002; **21**(35): 5483-5495.

160. Svahn A, Linde A, Thorstensson R, Karlen K, Andersson L, Gaines H. Development and evaluation of a flow-cytometric assay of specific cell-mediated immune response in activated whole blood for the detection of cell-mediated immunity against varicella-zoster virus. J Immunol Methods 2003; **277**(1-2): 17-25.

161. Janson PCJ, Marits P, Thorn M, Ohlsson R, Winqvist O. CpG methylation of the IFNG gene as a mechanism to induce immunosupression in tumor-infiltrating lymphocytes. Journal of Immunology 2008; **181**(4): 2878-2886.

162. Amir ED, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, Shenfeld DK, Krishnaswamy S *et al*. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nature Biotechnology 2013; **31**(6): 545-+.

163. Csardi G, Nepusz T. The igraph software package for complex network research. InterJournal 2006; **Complex Systems**: 1695.

164. Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. International Journal of Cancer 2010; **127**(4): 759-767.

165. Shang B, Liu Y, Jiang SJ, Liu Y. Prognostic value of tumor-infiltrating FoxP3(+) regulatory T cells in cancers: a systematic review and meta-analysis. Sci Rep 2015; **5**.

166. Winerdal ME, Marits P, Winerdal M, Hasan M, Rosenblatt R, Tolf A, Selling K, Sherif A *et al*. FOXP3 and survival in urinary bladder cancer. BJU Int 2011; **108**(10): 1672-1678.

167. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature 2010; **463**(7282): 808-U120.

168. Blaveri E, Simko JP, Korkola JE, Brewer JL, Baehner F, Mehta K, DeVries S, Koppie T *et al*. Bladder cancer outcome and subtype classification by gene expression. Clinical Cancer Research 2005; **11**(11): 4044-4055.

169. Stransky N, Vallot C, Reyal F, Bernard-Pierrot I, de Medina SGD, Segraves R, de Rycke Y, Elvin P *et al*. Regional copy number-independent deregulation of transcription in cancer. Nat Genet 2006; **38**(12): 1386-1396.

170. Sanchez-Carbayo M, Socci ND, Lozano J, Saint F, Cordon-Cardo C. Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. Journal of Clinical Oncology 2006; **24**(5): 778-789.

171. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. Metastatic Potential Correlates with Enzymatic Degradation of Basement-Membrane Collagen. Nature 1980; **284**(5751): 67-68.

172. Jodele S, Blavier L, Yoon JM, DeClerck YA. Modifying the soil to affect the seed: role of stromal-derived matrix metalloproteinases in cancer progression. Cancer and Metastasis Reviews 2006; **25**(1): 35-43.

173. Brown GT, Murray GI. Current mechanistic insights into the roles of matrix metalloproteinases in tumour invasion and metastasis. Journal of Pathology 2015; **237**(3): 273-281.

174. Rowe RG, Weiss SJ. Navigating ECM Barriers at the Invasive Front: The Cancer Cell-Stroma Interface. Annu Rev Cell Dev Biol 2009; **25**: 567-595.

175. Schenkel JM, Fraser KA, Vezys V, Masopust D. Sensing and alarm function of resident memory CD8(+) T cells. Nat Immunol 2013; **14**(5): 509-+.

176. Schenkel JM, Fraser KA, Beura LK, Pauken KE, Vezys V, Masopust D. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. Science 2014; **346**(6205): 98-101.

177. Beura LK, Mitchell JS, Thompson EA, Schenkel JM, Mohammed J, Wijeyesinghe S, Fonseca R, Burbach BJ *et al*. Intravital mucosal imaging of CD8(+) resident memory T cells shows tissue-autonomous recall responses that amplify secondary memory. Nat Immunol 2018; **19**(2): 173-+.

178. Park SL, Zaid A, Hor JL, Christo SN, Prier JE, Davies B, Alexandre YO, Gregory JL *et al*. Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses. Nat Immunol 2018; **19**(2): 183-+.

179. Ganesan AP, Clarke J, Wood O, Garrido-Martin EM, Chee SJ, Mellows T, Samaniego-Castruita D, Singh D *et al*. Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer. Nat Immunol 2017; **18**(8): 940-+.

180. Webb JR, Milne K, Nelson BH. PD-1 and CD103 Are Widely Coexpressed on Prognostically Favorable Intraepithelial CD8 T Cells in Human Ovarian Cancer. Cancer Immunol Res 2015; **3**(8): 926-935.

181. Youngblood B, Oestreich KJ, Ha SJ, Duraiswamy J, Akondy RS, West EE, Wei ZY, Lu PY *et al*. Chronic Virus Infection Enforces Demethylation of the Locus that Encodes PD-1 in Antigen-Specific CD8(+) T Cells. Immunity 2011; **35**(3): 400-412.

182. Shwetank, Abdelsamed HA, Frost EL, Schmitz HM, Mockus TE, Youngblood BA, Lukacher AE. Maintenance of PD-1 on brain-resident memory CD8 T cells is antigen independent. Immunology and Cell Biology 2017; **95**(10): 953-959.

183. Wang B, Wu SX, Zeng H, Liu ZW, Dong W, He W, Chen X, Dong XL *et al*. CD103(+) Tumor Infiltrating Lymphocytes Predict a Favorable Prognosis in Urothelial Cell Carcinoma of the Bladder. Journal of Urology 2015; **194**(2): 556-562.

184. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, de Montpreville V, Validire P, Besse B *et al*. CD8(+) CD103(+) Tumor-Infiltrating Lymphocytes Are Tumor-Specific Tissue-Resident Memory T Cells and a Prognostic Factor for Survival in Lung Cancer Patients. Journal of Immunology 2015; **194**(7): 3475-3486.

185. Komdeur FL, Prins TM, van de Wall S, Plat A, Wisman GBA, Hollema H, Daemen T, Church DN *et al*. CD103+tumor-infiltrating lymphocytes are tumor-reactive intraepithelial CD8+T cells associated with prognostic benefit and therapy response in cervical cancer. Oncoimmunology 2017; **6**(9).

186. Webb JR, Milne K, Watson P, deLeeuw RJ, Nelson BH. Tumor-Infiltrating Lymphocytes Expressing the Tissue Resident Memory Marker CD103 Are Associated with Increased Survival in High-Grade Serous Ovarian Cancer. Clinical Cancer Research 2014; **20**(2): 434-444.

187. Ruffell B, DeNardo DG, Affara NI, Coussens LM. Lymphocytes in cancer development: Polarization towards pro-tumor immunity. Cytokine Growth Factor Rev 2010; **21**(1): 3-10.

188. Swartz MA, Lund AW. OPINION Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. Nature Reviews Cancer 2012; **12**(3): 210-219.

189. Preynat-Seauve O, Contassot E, Schuler P, Piguet V, French LE, Huard B. Extralymphatic tumors prepare draining lymph nodes to invasion via a T-cell cross-tolerance process. Cancer Res 2007; **67**(10): 5009-5016.

190. Andreev K, Trufa ID, Siegemund R, Rieker R, Hartmann A, Schmidt J, Sirbu H, Finotto S. Impaired T-bet-pSTAT1alpha and perforin-mediated immune responses in the tumoral region of lung adenocarcinoma. Br J Cancer 2015; **113**(6): 902-913.

191. Wu X, Zhang H, Xing Q, Cui J, Li J, Li Y, Tan Y, Wang S. PD-1(+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. Br J Cancer 2014; **111**(7): 1391-1399.

192. Baitsch L, Baumgaertner P, Devevre E, Raghav SK, Legat A, Barba L, Wieckowski S, Bouzourene H *et al*. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. Journal of Clinical Investigation 2011; **121**(6): 2350-2360.

193. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, Qiu Y, Jussif JM *et al*. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. Febs Letters 2004; **574**(1-3): 37-41.

194. Hui EF, Cheung J, Zhu J, Su XL, Taylor MJ, Wallweber HA, Sasmal DK, Huang J *et al*. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science 2017; **355**(6332): 1428-+.

195. Patsoukis N, Brown J, Petkova V, Liu F, Li LQ, Boussiotis VA. Selective Effects of PD-1 on Akt and Ras Pathways Regulate Molecular Components of the Cell Cycle and Inhibit T Cell Proliferation. Science Signaling 2012; **5**(230).

196. Thaventhiran JE, Fearon DT, Gattinoni L. Transcriptional regulation of effector and memory CD8+ T cell fates. Curr Opin Immunol 2013; **25**(3): 321-328.

197. Northrop JK, Wells AD, Shen H. Cutting edge: chromatin remodeling as a molecular basis for the enhanced functionality of memory CD8 T cells. J Immunol 2008; **181**(2): 865-868.

198. Franciszkiewicz K, Le Floc'h A, Boutet M, Vergnon I, Schmitt A, Mami-Chouaib F. CD103 or LFA-1 Engagement at the Immune Synapse between Cytotoxic T Cells and Tumor Cells Promotes Maturation and Regulates T-cell Effector Functions. Cancer Res 2013; **73**(2): 617-628.

199. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. Cell Death Differ 2014; **21**(1): 15-25.

200. Galluzzi L, Buque A, Kepp O, Zitvogel L, Kroemer G. Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents. Cancer Cell 2015; **28**(6): 690-714.

201. Fridman WH, Zitvogel L, Sautes-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. Nature Reviews Clinical Oncology 2017; **14**(12): 717-734.

202. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of Action of Conventional and Targeted Anticancer Therapies: Reinstating Immunosurveillance. Immunity 2013; **39**(1): 74-88.

203. Chen G, Emens LA. Chemoimmunotherapy: reengineering tumor immunity. Cancer Immunology Immunotherapy 2013; **62**(2): 203-216.

204. Fransen MF, Arens R, Melief CJM. Local targets for immune therapy to cancer: Tumor draining lymph nodes and tumor microenvironment. International Journal of Cancer 2013; **132**(9): 1971-1976.

205. Roselli M, Cereda V, di Bari MG, Formica V, Spila A, Jochems C, Farsaci B, Donahue R *et al*. Effects of conventional therapeutic interventions on the number and function of regulatory T cells. Oncoimmunology 2013; **2**(10).

206. Hu J, Kinn J, Zirakzadeh AA, Sherif A, Norstedt G, Wikstrom AC, Winqvist O. The effects of chemotherapeutic drugs on human monocyte-derived dendritic cell differentiation and antigen presentation. Clin Exp Immunol 2013; **172**(3): 490-499.

207. Zirakzadeh AA, Kinn J, Krantz D, Rosenblatt R, Winerdal ME, Hu J, Hartana CA, Lundgren C *et al*. Doxorubicin enhances the capacity of B cells to activate T cells in urothelial urinary bladder cancer. Clinical Immunology 2017; **176**: 63-70.

208. Pfirschke C, Engblom C, Rickelt S, Cortez-Retamozo V, Garris C, Pucci F, Yamazaki T, Poirier-Colame V *et al*. Immunogenic Chemotherapy Sensitizes Tumors to Checkpoint Blockade Therapy. Immunity 2016; **44**(2): 343-354.

209. Seiler R, Ashab HA, Erho N, van Rhijn BWG, Winters B, Douglas J, Van Kessel KE, van de Putte EEF *et al*. Impact of Molecular Subtypes in Muscle-invasive Bladder Cancer on Predicting Response and Survival after Neoadjuvant Chemotherapy. Eur Urol 2017; **72**(4): 544-554.

210. Vasselli JR, Yang JC, Linehan WM, White DE, Rosenberg SA, Walther MM. Lack of retroperitoneal lymphadenopathy predicts survival of patients with metastatic renal cell carcinoma. J Urol 2001; **166**(1): 68-72.

211. Pantuck AJ, Zisman A, Dorey F, Chao DH, Han KR, Said J, Gitlitz B, Belldegrun AS *et al*. Renal cell carcinoma with retroperitoneal lymph nodes. Impact on survival and benefits of immunotherapy. Cancer 2003; **97**(12): 2995-3002.

212. Tschmelitsch J, Klimstra DS, Cohen AM. Lymph node micrometastases do not predict relapse in stage II colon cancer. Ann Surg Oncol 2000; **7**(8): 601-608.

213. Karlsson M, Nilsson O, Thorn M, Winqvist O. Detection of metastatic colon cancer cells in sentinel nodes by flow cytometry. J Immunol Methods 2008; **334**(1-2): 122-133.

214. Hermanek P, Hutter RV, Sobin LH, Wittekind C. International Union Against Cancer. Classification of isolated tumor cells and micrometastasis. Cancer 1999; **86**(12): 2668-2673.

215. Amsen D, van Gisbergen KPJM, Hombrink P, van Lier RAW. Tissue-resident memory T cells at the center of immunity to solid tumors. Nat Immunol 2018; **19**(6): 538-546.

216. Brown SD, Raeburn LA, Holt RA. Profiling tissue-resident T cell repertoires by RNA sequencing. Genome Med 2015; **7**.

217. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature 2017; **541**(7637): 321-330.

218. Zacharakis N, Chinnasamy H, Black M, Xu H, Lu YC, Zheng ZL, Pasetto A, Langhan M *et al*. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. Nat Med 2018; **24**(6): 724-+.

219. Medler TR, Cotechini T, Coussens LM. Immune Response to Cancer Therapy: Mounting an Effective Antitumor Response and Mechanisms of Resistance. Trends in Cancer 2015; **1**(1): 66-75.

220. Patel SJ, Sanjana NE, Kishton RJ, Eidizadeh A, Vodnala SK, Cam M, Gartner JJ, Jia L *et al*. Identification of essential genes for cancer immunotherapy. Nature 2017; **548**(7669): 537-+.