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IMMUNOREGULATORY PROPERTIES OF CD137-CD137 LIGAND AXIS IN TUMOURS AND CHRONIC INFLAMMATORY CONDITIONS

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Cover illustration: CD137L (red) forms a jaw-like structure that engulfs CD137 (green) in the trogocytosis process and internalizes the resulting yellow complex into the cell.
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THE CD137-CD137L AXIS CAN REGULATE IMMUNE RESPONSES AND BE EXPLOITED BY TUMOUR AND CHRONIC INFLAMMATORY CONDITIONS
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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~To my wife and my family, who are always there to inspire me.~

POPULAR SCIENCE SUMMARY OF THE THESIS

Our immune system uses T cells and Natural Killer (NK) cells to eliminate viral infection and malignancies. To enhance those functions, antigen-presenting cells (APC) interact closely with T cells and NK cells via many costimulatory pathways, one of which is CD137. CD137 is a receptor molecule found mainly on activated T cells and NK cells. Its natural ligand is CD137 ligand (CD137L), mainly expressed on APC. Once CD137L engages with CD137, a stimulatory signal triggers both interacting APC and the T cell or NK cell bearing CD137. Hence, antibodies activating CD137 have been tested in many clinical trials, either alone or in combinations, as a form of cancer immunotherapy.

On the other hand, our immune system must maintain a balance so that immune responses do not go out of control and damage healthy tissues. One central aspect of immune regulation is exerted by regulatory T cells (Treg). Treg have many mechanisms of action to dampen an immune response. Given the fact the tumours often escape immune recognition and killing, it is not surprising that tumours regularly recruit Treg into the tumour microenvironment. Tumours themselves also develop several immunosuppressive barriers to escape immune killing. Curiously, we observe that both tumours and tumour-infiltrating Treg express CD137, suggesting that CD137 expression dysregulates immune responses and confers the tumours with survival advantages. Hence, we set to investigate the immunosuppressive function of the CD137-CD137L interaction in cancers and chronic inflammatory conditions.

We observe that tumours and Treg produce a form of CD137 that is soluble and secreted into the surrounding environment. Hence, in many tumours and chronic inflammatory conditions such as liver cirrhosis, we can detect an elevated level of soluble CD137. We observe that soluble CD137 interferes with CD137L-CD137 interaction on the cell surface, blocks type-1 and type-2 T cells immune response but does not impact type-17 and regulatory T cell response. This helps to maintain the inflammatory condition (usually seen in chronic inflammatory conditions and cancer), while potentially decrease the ability of the immune system to clear infections and to target tumours.

Furthermore, we observe that at resting state, Treg express more CD137 than other T cells. In a process termed “trogocytosis”, CD137 bind to CD137L, form a complex, and internalize this CD137-CD137L complex. Using fluorescent CD137 and CD137L, we reveal that this complex can be transferred to either of the two interacting cells. Additionally, intracellular signaling is not necessary for trogocytosis process, because our modified CD137 that lacks the cytoplasmic region can still internalize CD137L. Because of this, APC lose CD137L on their surface, and are less able to stimulate T cells.

In addition to that, stimulation of CD137 on normal T cells can make them exhausted and even regulatory. We reveal that physiological stimulation of T cells enhances the expression of a molecule named B7-H7. B7-H7 is typically detected on tumours and associated with a worse disease outcome. Stimulation of CD137 on T cells increases B7-H7 expression, and B7-H7 can impair the further proliferation and activation of T cells, as negative feedback. Simultaneously,

T cells that express B7-H7 have characteristic of exhausted T cells: they produce less inflammatory cytokines and express more markers of exhaustion.

Learning from how CD137 suppresses the immune system to help tumours survive, we create a strategy to counter that effect. We know that tumours rely on coinhibitory molecule such as PD-L1 to inhibit T cell functions via PD-1. We devise a PD-1-CD137L fusion protein. The PD-1 component will bind to and block PD-L1 function. At the opposite domain of the protein, the CD137L component will bring T cells close to the tumour cells and activate T cell immune functions to clear the tumour. On its own, the CD137L domain of this fusion protein can trap soluble CD137 in the environment and bind to CD137 on tumour to block trogocytosis.

In conclusion, our studies elucidate mechanisms by which tumour cells and Treg in the tumour can exploit to escape immune killing. This understanding prepares us to improve existing immunotherapy.

ABSTRACT

CD137 (4-1BB/TNFRSF9) is a member of the TNF receptor superfamily. CD137 expression is activation-dependent, and usually reported on T cells and NK cells. Its natural ligand is CD137 ligand (CD137L), mainly found on antigen-presenting cells (APC) such as monocytes, or dendritic cells. CD137-CD137L interaction will send a stimulatory signal to activate both interacting cells, thus enhancing the immune response. Hence, it is curious to detect CD137 on tumour cells and regulatory T cells (Treg), who mainly aim to suppress the immune functions. Hence, we aim to investigate how the CD137-CD137L can confer immunosuppression which is beneficial for tumour growth, or the maintenance of a chronic inflammatory condition.

Because of RNA alternative splicing, soluble isoforms of CD137 (sCD137) that lack the cytoplasmic domain can be produced and secreted to the surrounding environment. In **Paper I**, we reveal that sCD137 is a marker of liver cirrhosis in patients suffering hepatitis C and alcohol-associated disease. sCD137 impairs type-1 and type-2 T cell response but maintain type-17 and regulatory T cell response. Thus, it helps maintain the chronic inflammation associated with cirrhosis.

In **paper II**, we reveal how Tregs express more CD137 than conventional T cells (Tcon), use CD137 to form a complex with CD137L, internalize the complex and deplete CD137L on APC, in a process termed "trogocytosis". This process does not require CD137 signaling because our modified truncated CD137 that lacks the cytoplasmic domain can still deplete CD137L. APC that have their CD137L depleted in the process are less able to co-stimulate T cells.

In **paper III**, we study how the stimulation of CD137 on T cells can induce B7-H7 expression. B7-H7 is a ligand usually upregulated in cancers and associated with worse prognosis for a wide variety of cancers. On immune cells, B7-H7 is usually reported on APC. Our study reveals that B7-H7 can be induced on T cells to regulate T cell function and serve as a biomarker for exhausted T cells.

In **paper IV**, using the nasopharyngeal carcinoma (NPC) model, we study why tumours are selected to express CD137. Besides using CD137 for trogocytosis to deplete CD137L from APC, tumour can also receive CD137 signaling to upregulate p38 MAPK pathway, secrete more Interleukin (IL)-8, and express LAMC2 to enhance their survival, invasion, and metastasis. IL-8 has been reported to be important to recruit other immunosuppressive cells into the tumour microenvironment, and LAMC2 is involved in the Epithelial-Mesenchymal Transformation (EMT) process.

Learning from these mechanisms, in **paper V**, we devise a fusion protein PD-1-CD137L that masks PD-L1, a coinhibitory molecule, with CD137L, a costimulatory molecule. In effect, this converts the tumour microenvironment from cold to hot. Not only blocking PD-L1/PD-1 pathway, but this fusion protein also presents more CD137L in the tumour microenvironment,

sequesters sCD137, inhibits trogocytosis and liberates existing CD137L to activate tumour-specific T cells.

In conclusion, while CD137 has been exploited as target for immunotherapy (as seen in several clinical trials where agonistic anti-CD137 antibodies are used for cancer treatment), it can induce immunosuppression beneficial to tumour and chronic inflammation. Studying these mechanisms of action elucidate how we can improve future immunotherapy involving CD137 and similar receptors.

LIST OF SCIENTIFIC PAPERS

Paper I. Soluble CD137 is a novel serum marker of liver cirrhosis in patients with hepatitis C and alcohol-associated disease etiology
Kilian Weigand, Georg Peschel, Jonathan Grimm, **Khang Luu**, Doris Schacherer, Reiner Wiest, Martina Müller, Herbert Schwarz, Christa Buechler
European journal of immunology, 10.1002/eji.202149488. 16 Dec. 2021.

Paper II. Regulatory T Cells Inhibit T Cell Activity by Downregulating CD137 Ligand via CD137 Trogocytosis
Khang Luu, Mugdha Vijay Patwardhan, Qun Zeng, Stina Wickström, Andreas Lundqvist, Herbert Schwarz
Cells 10.2 (2021): 353.

Paper III. B7-H7 is inducible on T cells to regulate their immune response and serves as a marker for exhaustion
Khang Luu, Herbert Schwarz, Andreas Lundqvist
Frontiers in Immunology 12 (2021): 1997.

Paper IV. Epstein-Barr Virus-Induced Ectopic CD137 Expression Helps Nasopharyngeal Carcinoma to Escape Immune Surveillance and Enables Targeting by Chimeric Antigen Receptors
Mukul Prasad, Sashigala Ponnalagu, Qun Zeng, **Khang Luu**, Si Min Lang, Hiu Yi Wong, Man Si Cheng, Meihui Wu, Karthik Mallilankaraman, Radoslaw Mikolaj Sobota, Yan Ting Lim, Loo Chien Wang, Chuan Keng Goh, Kai Xun Joshua Tay, Kwok Seng Loh, Cheng-I Wang, Wen-Hsien Lee, Boon Cher Goh, Chwee Ming Lim, Herbert Schwarz
Accepted in Cancer Immunology, Immunotherapy.

Paper V. Fusion PD-1-CD137L reverses the immunosuppressive CD137 effect and invigorates T cells against cancer
Khang Luu, Herbert Schwarz
Manuscript

Publications not included in thesis

The relevance of soluble CD137 in the regulation of immune responses and for immunotherapeutic intervention.
Khang Luu, Shao Zhe, Herbert Schwarz.
Journal of leukocyte biology 107.5 (2020): 731-738.

Destroy, what destroys you.
Khang Luu, Emily Nickles, Herbert Schwarz.
Oncoimmunology 9.1 (2020): 1685301.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AML	Acute myeloid leukaemia
ANOVA	Analysis of Variance
APC	antigen presenting cells
CAF	cancer-associated fibroblasts
CAR	chimeric antigen receptor
CD	cluster of differentiation
CD28H	CD28 Homolog
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CRISPR	clustered regularly interspaced short palindromic repeats
CTA	cancer/testis antigen
CTLA-4	cytotoxic T-lymphocyte–associated antigen 4
CXCL1	The chemokine (C-X-C motif) ligand 1
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-assisted cell sorting
FBS	foetal bovine serum
FcR	crystallizable fragment receptor
FDA	Food and Drug Administration
FOXP3	Forkhead box P3
GATA-3	GATA binding protein 3
GFP	green fluorescent protein
HCV	hepatitis C virus
HGF	hepatocyte growth factor
HHLA-2	HERV-HLTR-associating 2
HPV	Human papillomavirus
HRP	horse-radish peroxidase

HTLV-1	Human T-lymphotropic virus type 1
IDO	Indoleamine 2,3-dioxygenase
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide synthase
IU	International Unit
Iv IgG	intravenous Immunoglobulin G
JNK	c-Jun N-terminal Kinase
KIR3DL3	Killer Cell Immunoglobulin Like Receptor, Three Ig Domains And Long Cytoplasmic Tail 3
KO	knockout
KRAS	Kirsten rat sarcoma virus
kyn	kynurenine
LAG-3	Lymphocyte-activation gene 3
LMP-1	Latent Membrane Protein 1
LPS	lipopolysaccharide
MACS	magnetism-assisted cell sorting
MAGE	Melanoma Antigen Gene
MAPK	mitogen-activated protein kinase
mo-DC	monocyte-derived dendritic cell
MDSC	myeloid derived suppressor cell
MHC	major histocompatibility complex
MMP	Matrix Metalloprotease
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NPC	nasal pharyngeal carcinoma
NY-ESO-1	New York esophageal squamous cell carcinoma 1
OFP	orange fluorescent protein

PBMC	peripheral blood mononuclear cell
PBS	protein buffer saline
PD-1	Program cell death protein 1
PD-L1/2	Program cell death protein 1- Ligand 1/2
PEI	Polyethyleneimine
PGE-2	Prostaglandin E2
PMA	Phorbol 12-myristate 13-acetate
PVDF	Polyvinylidene difluoride
rhCD28H	recombinant human CD28 homolog
ROR-γT	Retinoic acid receptor-related orphan receptor- gamma T
sCD137	soluble CD137
TAA	tumour associated antigen
TAM	tumour-associated macrophages
Tbet	T-box protein expressed in T cells
Tc1	cytotoxic T cells 1
Tcon	conventional T cell
TCR	T cell receptor
TGF	tumour
Th1/2/17	helper T cell 1/2/17
TIL	tumour infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TMB	3,3',5,5'-Tetramethylbenzidine
TME	tumour microenvironment
TNF	tumour necrosis factor
TNFRSF9	tumour necrosis factor receptor super family 9
Treg	regulatory T cell
TSA	tumour specific antigen
WT	wild type

1 INTRODUCTION

At the moment of this writing, it has become cliché to mention that cancer is the disease of our time. According to World Health Organization, cancer is the leading cause of death, robbing us 10 million lives in 2020 alone. The classic treatment modalities consist of chemotherapy, radiotherapy, and surgery. Compared to those methods, immunotherapy has been dismissed as a less desirable treatment for an extended period. Dr. Coley's concoction, arguably the first form of immunotherapy, has received a healthy amount of criticism. Understandably, the idea of treating tumours by infecting the already-ill patient with a mixture of bacteria, albeit heat-killed, could be seen as too radical at the time[1]. Starting from when Dr. Coley devised the much ridiculed "Coley's toxin", immunotherapy has been in the shadow for almost a hundred years, before bursting into the scene again with the discovery of immune checkpoint inhibitors[2]. With immunotherapy, cancer patients who have failed all other treatments finally have a chance to put one foot onto the land of disease remission. Given their impressive efficacy, immune checkpoint inhibitors are FDA-approved for an expanding spectrum of malignancies. All this recognition culminates in the award of the 2018 Physiology Nobel Prize for Dr. James Allison and Dr. Takusu Honjo, for their work on the two immune checkpoints CTLA-4 and PD-1, respectively. The role of our immune system in the fight against cancer has become indisputable. Despite the impressive success, immunotherapy still has a long way to go to combat the disease of our time. Only a fraction of treated patients shows response, and many patients are still not responding to immunotherapy (inherent resistance)[3, 4]. Among those who respond, many later suffer a relapse that is usually more malicious than the original condition (acquired resistance). More than ever, now is the time to advance the field of immunology, and cancer immunotherapy.

1.1 Cancer

The disease of our time, cancer is a much-simplified basket term for a heterogenous group of diseases. Cancer can refer to solid tumours arising from our own various organs, or to hematological tumours stemming from the lymphocytes flowing in our veins. To complicate matters, within the very same organ, different cell types lead to different cancer types. As the tumour mass progresses, several features develop and different cancer into even more subtypes.

As complicated as this disease is, several common characteristics can be observed across tumours, and beautifully summarized by Hanahan and Weinberg as "Hallmarks of Cancers"[5]. In short, cancer cells are normal cells transformed to proliferate even in the absence of external growth signal. They can evade inherent growth suppressors, resist cell death, replicate to reproduce indefinitely, induce the formation of blood vessels to themselves to obtain nutrients, and transform to invade and metastasize to other tissues. These are the initial six hallmarks proposed. Over time, four new hallmarks are observed and supplemented. To exist in the tumour microenvironment, those cells must have irregular metabolism to consume nutrients in an unorthodox manner, evolve to escape immune attacks, possess an instable genome that allow them to evolve and adapt at a faster rate, and sustain an inflammatory state to support tumour growth.

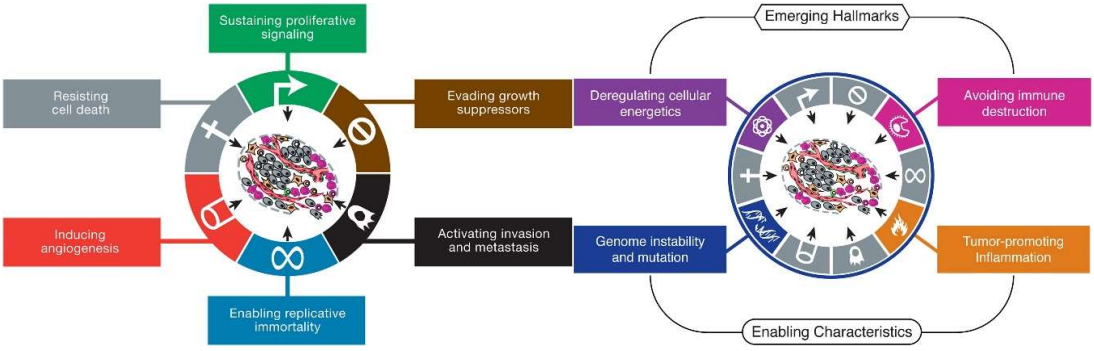

Fig.1 The six initial hallmarks of cancers (left panel) and four new hallmarks of cancer (right panel), adapted from Hanahan and Weinberg, Cell. 2011 Mar 4;144(5):646-74.

The recognition of cancer's ability to evade immunosurveillance and its preference for an inflammatory environment further testifies the arising importance of tumour immunology.

1.2 T cell activation and inhibition

1.2.1 The two-signal model of T cell activation

Responsible for the killing of malignant cells and virus-infected cells, the T cell plays a key role in the adaptive immune system. According to the two-signal model of activation, T cells require stimulation via two signals to become optimally activated[6]. Signal one is through T cell Receptor (TCR). As APC engulfs pathogenic organism/ virus-infected cells/ malignant cells, APC processes and presents small peptide remnants on its major histocompatibility complex (MHC) molecule. Only T cells with TCR binding with high affinity to the MHC-protein complex found on the surface of that APC can be activated. This ensures that the response is specific to a target. Signal two is via costimulatory pathways, where costimulatory ligands bind to specific costimulatory receptors on T cells and enhance T cell activation further. After the binding to the right T cells, APC can activate T cells further with costimulatory ligands. The most common costimulatory receptor is CD28, which binds to the costimulatory ligands CD80 and CD86. As T cells undergo the activation process, several additional costimulatory receptors are induced to receive even more stimulatory signals. A non-exhausted list of several relevant costimulatory receptors can be found in Figure 2.

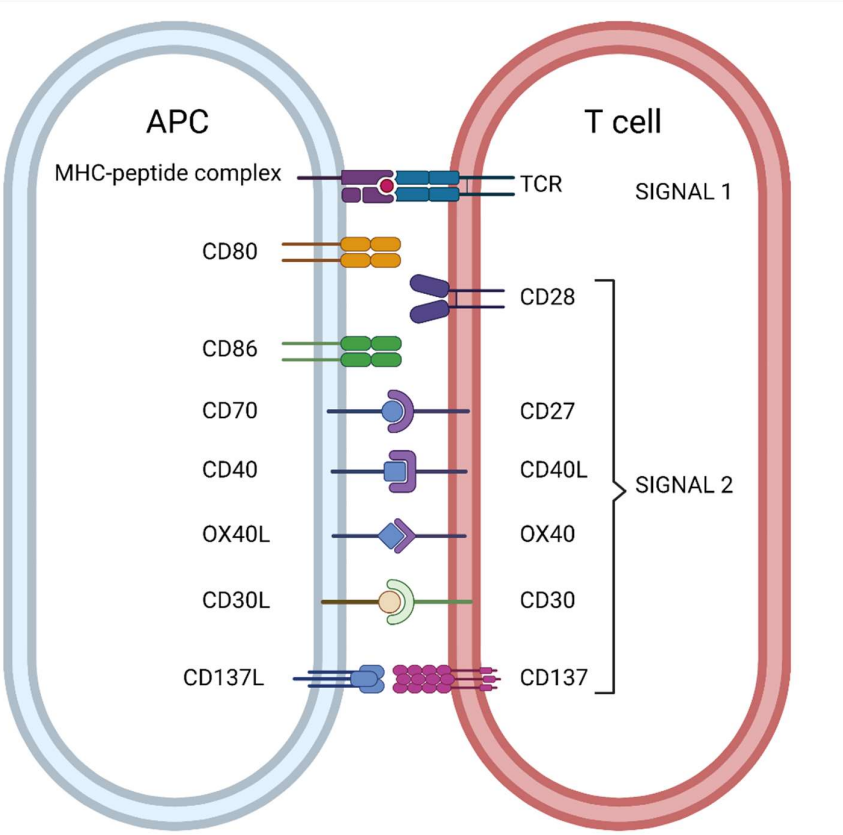

Figure 2. The two-signal model of T cell activation. Besides showing signal 1 through TCR binding, the figure also shows several other costimulatory receptors which deliver signal 2. This thesis focuses on the interaction between costimulatory ligand CD137L and costimulatory receptor CD137. This figure is created based on the review by Chen et al[6]. Created with Biorender.

1.2.2 T cell co-inhibition

As immune responses can be intense, our immune system must have different checks in place to prevent them from getting out of control and damaging other healthy tissues in the vicinity. Hence, T cells can increase the expression of several coinhibitory receptors upon activation.

When coinhibitory receptors bind to coinhibitory ligands (commonly expressed on APC), T cells receive an inhibitory signal that can fine tune their functions, not unlike a fire being put out by extinguisher. The two famous examples of these receptors are CTLA-4 and PD-1, for which their discoverers earn the Nobel Prize in 2018. For instance, upon TCR stimulation, T cells start to express PD-1. When activated T cells secrete the inflammatory cytokine IFN-γ, this cytokine can induce the expression of PD-L1 (a PD-1 ligand) on APC or tumour cells. PD-L1 and PD-1 then interact to ascertain that the activated T cells do not overreact. Several other important coinhibitory receptor-ligand pairs can be found in Figure 3.

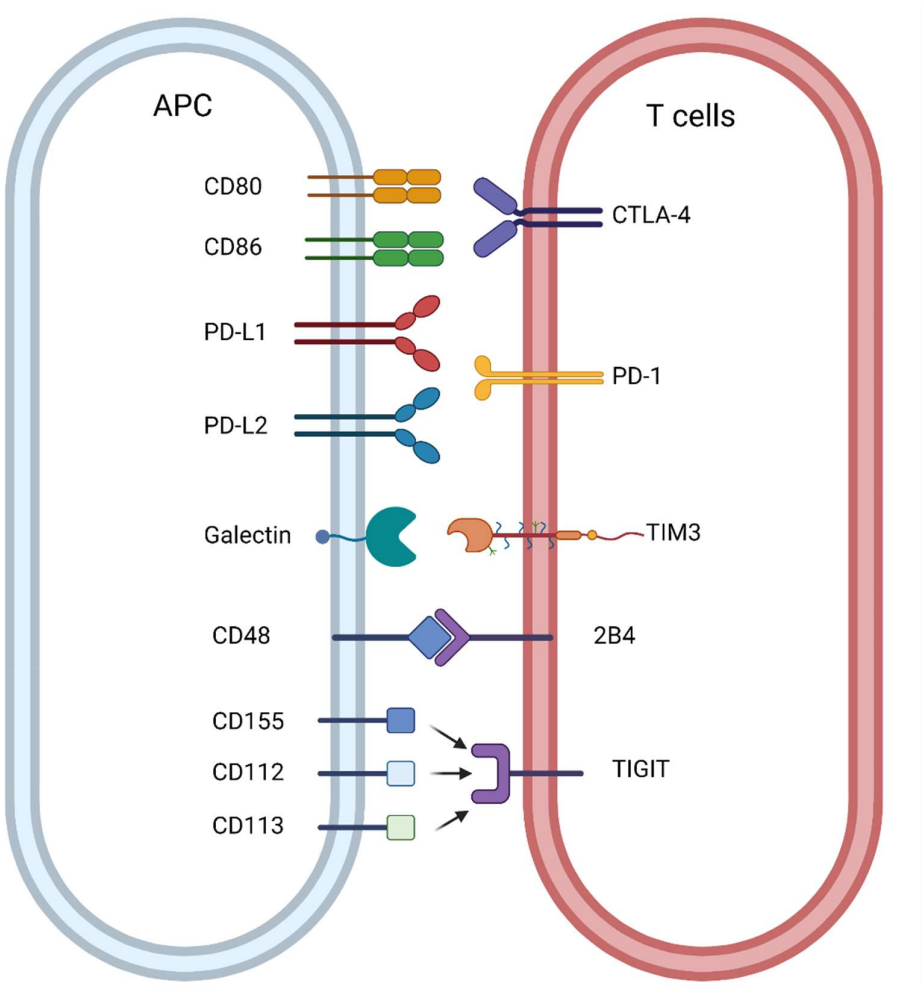

Figure 3. Besides delivering costimulatory signals, APCs are capable of presenting coinhibitory signal via a variety of coinhibitory ligands. This figure is created based on the review by Chen et al[6]. Created with Biorender.

1.2.3 Cytokines – the soluble modulator

While the two-signal model of T cell activation is widely accepted, many also consider a third signal: cytokines. Cytokines are small protein molecules acting as messengers to deliver stimulatory or inhibitory signals to cells via binding to specific cytokine receptors on the cell surface. They carry many roles. Cytokines such as IL-10 and TGF-β may help dampen an immune response to prevent damage to healthy tissues and promote healing[7, 8]. On the other hand, many cytokines play an essential role in the survival, maintenance, and activation of T cells.

For instance, IL-7 is strongly associated with T cell survival and development from thymocytes[9-11]. Cytokines such as IL-2, IL-4, and IL-15 even prevent the death of activated T cells in vitro[9].

Certain cytokines are stimulatory to T cells, hence earning the name as the third signal. IL-2 initiates and enhances the proliferation of naïve T cells[12]. Upon initial activation, these cells

express more receptors for IL-2 which further drive their differentiation into either memory T cells or effector T cells[13]. Upon activation, T cells may secrete more cytokines to differentiate further into different polarization states. Among those, cytokines such as TNF-α and IFN-γ are pro-inflammatory and enhance T cell functions[14, 15].

1.3 Tumour immunology

1.3.1 Tumour immunosurveillance

The immune system has one overarching objective: to rid our body of harmful foreign/ "non-self" elements. Thus, the concept of immunosurveillance arises: the immunological resistance from the host to fight against the progression of cancer[16]. While a malignant cell is arguably still a "self" element, it possesses certain qualities that potentially distinguish itself from normal cells and can trigger an immune response. For a sizeable portion of patients, their endogenous T cells have TCR that can recognize peptides displayed on the MHC molecule of tumour cells[17]. Such interactions provide T cells with the signal 1 stimulation they need to ignite T cell activation. Those peptides are called antigens and generally divided into three classes: tumour-associated antigens (TAA), tumour-specific antigens (TSA) and cancer/testis antigens (CTA)[18].

TAA are non-mutated peptides that exist naturally in the human genome. They are expressed at a low level on normal cells, but at a much higher level on tumour cells due to genetic alterations. Examples include CD19 on B-cell tumour[19, 20], or mesothelin on pancreatic cancer[21, 22].

Like TAA, CTA are also present in the human genome. However, they are absent on normal adult cells, and present on reproductive tissues and various tumours. MAGE and NY-ESO-1 antigen are some antigens of this class[23, 24].

Among the three, TSA generate the most interest, because they are completely absent on normal cells, and ideal targets to selectively destroy tumour cells. Malignant cells, due to their highly instable genome and abnormal replicative rate, can have multiple spontaneous mutations that can translate to peptides entirely absent in the human genome, such as mutated KRAS[25]. On the other hand, they can also arise from oncogenic viral proteins, such as HPV[26, 27].

Without an active immune system to remove abnormal malignant cells displaying these antigens, cancer would have happened at a much higher rate.

1.3.2 The immunosuppressive tumour microenvironment

The tumour mass is a heterogeneous population of cancer cells, resident host cells and infiltrating cells. They communicate with each other in a complicated network of signals, in the form of secreted factors and extracellular matrix proteins. Altogether, they form the tumour microenvironment (TME). The interactions that take place in this intricate environment have a profound impact on the fate of the tumour, whether it withers or prospers, or achieves a stalemate with the oncoming immune system. In addition to that, the TME has a considerable

influence on how the tumour resists or responds to treatment. Given that one hallmark of cancer is the ability to escape immune destruction, unsurprisingly, the tumour microenvironment has evolved to possess a plethora of mechanisms to suppress the immune functions. Many of those are currently targeted by existing and emerging immunotherapies.

1.3.2.1 Cell surface protein

The TME expresses an abundance of immune checkpoint ligands that can send a coinhibitory signal, weaken TIL and promote tumour progression. A majority of tumour expresses PD-L1 while certain subsets of lymphomas express PD-L2 (which binds PD-1 three times as strongly as PD-L1) to engage PD-1 on TIL to induce tolerance and promote tumour growth[28, 29]. CEACAM1 expression on tumour has been associated with a worse prognosis[30] and tumour may also express CEACAM1 to bind to TIM-3, an inhibitory receptor on T cells, to regulate T cell functions[31]. To escape TCR recognition, some tumours evolve to downregulate surface MHC-I expression, albeit at the risk of being targeted by NK cells[32]. Several tumours also choose to express CD73, a surface protein that can convert extracellular immunostimulatory adenosine triphosphate to the immunosuppressive counterpart adenosine[33].

1.3.2.2 Immunosuppressive factors

The tumour and associated cells within the microenvironment can secrete a wide variety of soluble factors to dampen the host's immune response. The tumour can secrete or recruit cells to secrete anti-inflammatory cytokines such as TGF-β and IL-10[34, 35]. TGF-β interferes with T cell activation and infiltration into the tumour, inducing T cell exhaustion[36]. On the other hand, IL-10 downregulate the expression of MHC-II complex on APC to interfere with T cell priming[37], suppress pro-inflammatory cytokine secretion[38], and induce T cell anergy[39].

The altered energetics of tumour, how they consume nutrients, also leads to the upregulation of various metabolic factors that can suppress immune functions, such as IDO, lactate, PGE-2, adenosine, and arginase. IDO facilitates the conversion of Tryptophan into toxic kynurenine (kyn) derivatives. Kyn derivatives have been shown to downregulate TCR and suppress T cell activation[40]. The Warburg effect and altered genome also lead to an accumulation of lactate, which can regulate T cell functions[41]. On the other hand, PGE-2, an essential homeostatic factor, has the natural role of controlling the extent of immune response. It can inhibit T cell functions[42], suppress pro-inflammatory cytokine secretion[43], and recruit more suppressor cells into the tumour[44]. Adenosine, the product of CD73, has been well-established to inhibit T cell activation and proliferation[33, 45, 46]. Arginase, most notably arginase 1, and iNOS are regularly upregulated in tumour[47-50]. They deplete arginine and accumulate nitric oxide inside the tumour microenvironment. The increased level of nitric oxide leads to immunosuppression, metastasis and angiogenesis[51].

1.3.2.3 Tumour-associated cells

Certain host cell populations can assist tumour growth. Thus, tumour not only put up their own defense system, but also recruit such specific host cell populations to their site to help them

grow. Those recruited cells are not necessarily transformed or malignant, yet they play a significant role in helping the tumour progress, most of the time at the expense of the immune response. Hence, they are usually termed tumour-associated cells.

Tumour-associated macrophages (TAM) play a crucial role. High density of such cells correlates to a worse overall survival in several cancers[52, 53]. While in the tumour, TAM can differentiate into either the pro-inflammatory M1 macrophage or the anti-inflammatory M2 macrophage, based on the existing polarization signals present in the tumour. M2 TAMs release multiple immunosuppressive factors and upregulate surface molecules such as PD-L1 to help suppress the immune response, build blood vessels into the tumours, and allow them to grow and metastasize[54].

In addition to TAM, myeloid-derived suppressor cells hold an influential role. They are similar to granulocytes and monocytes in terms of morphology. The tumour secretes chemokines such as CXCL1 to proactively recruit MDSC into their site[55]. Activated in the TME by many factors, MDSC upregulate Arg, iNOS, IDO and ROS to reduce antibody-dependent cellular cytotoxicity (ADCC) in NK cells, suppress T cells infiltration, inhibit T cell funcfunctions, induce T cell apoptosis[55-58]. Furthermore, the cytokines secreted from MDSC further stimulate Treg[56].

Treg is another population that actively infiltrates the tumour, and its presence is associated with a worse outcome[59]. Inside the tumour, Treg secrete a variety of mentioned immunosuppressive factors, such as TGF-β and IL-10. They inhibit the maturation of DC and impair the function of DC as an APC by downregulating their MHC-II complex[60]. Treg also express a high level of CD39 and CD73 to accumulate adenosine[61]. Due to their high CD25 expression, Treg can also act as a sink for IL-2, which is necessary for the activation of effector T cells. By sequestering IL-2 in the TME, Treg also deprive other effector T cells of their essential nutrient[62].

Recently, cancer-associated fibroblasts (CAF) have been studied extensively for their roles in tumour progression. Fibroblast refers to a heterogenous group of cells that have specific functions in different tissues (such as wound healing), interact with stromal cells, and immune cells[63, 64]. CAF in TME also originate from different cell types based on different activating signals[65]. However, they are in constant interaction with tumour cells in the TME network. Tumour induces CAF to express the anti-apoptotic, anti-inflammatory hepatocyte growth factor (HGF), which can help with tumour angiogenesis, growth agrowth,astasis[66]. CAF also secretes MMPs (to help digest tissue for tumour invasion), immunosuppressive factors and CAF-derived exosomes[65, 67, 68]. CAF-derived exosomes can empower tumours to make them more aggressive, promote motility and metastasis[69, 70].

In short, many cancer-associated cells exist in the TME. Together, they form an intricate communication network that empowers each other and present a significant barrier for the immune system to carry out its functions to combat the tumour.

1.4 CD137 - CD137 ligand interaction

1.4.1 CD137 and CD137L biology

CD137 (also known as 4-1BB, or TNFRSF9) is a costimulatory receptor, and a member of TNF receptor superfamily. The human gene for CD137 lies on chromosome 1p36, which also contains several other members of the TNF receptor family[71]. CD137 was discovered in a screen for molecules that are inducible in human and murine T cells after stimulation[72, 73]. In healthy tissue, its expression is activation dependent. However, in several tumours, CD137 can be expressed constitutively[74, 75]. In our immune system, CD137 is commonly observed on activated T cells or NK cells[76]. In T cells, $CD8^+$ T cells have a higher level of expression of CD137 than that of $CD4^+$ T cells[77].

CD137's native ligand is aptly named CD137 ligand, or CD137L. CD137L is found on professional APC and the expression is enhanced after activation[78]. It has been well characterized to implicate many processes including inflammation, hematopoiesis, and bone formation[79].

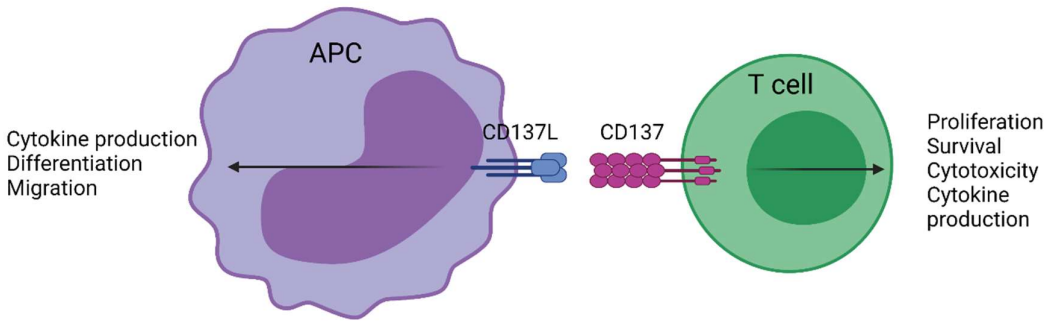

Figure 4. The CD137-CD137L systems is bidirectional and stimulatory. Created with Biorender.

1.4.2 CD137-CD137L function

When CD137 is stimulated with CD137L, a bidirectional stimulation occurs. The ability to signal in both directions of the CD137-CD137L system is a property commonly seen in tumour necrosis factor receptor-ligand family. Crosslinking of CD137 on T cells strongly enhances T cell proliferation, inflammatory cytokine production and cytotoxicity[80]. In physiological conditions, only antigen-experienced T cells express CD137. Hence an activation through CD137 enhances an antigen-specific response.

CD137L on APC, upon crosslinking CD137, sends a stimulatory signal back into the engaging APC. This process is called CD137L reverse signaling[79]. Reverse signaling into monocytes, for instance, enhances their ability to secrete inflammatory cytokines such as IL-6, IL-8 and, TNF[81]. In a different scenario, CD137L reverse signaling can induce monocytes to

differentiate into CD137L-DC, which is more effective than classical mo-DC in activating T cells[82].

Overall, CD137-CD137L interaction causes the activation of type-1 immune responses, which are important for the clearance of virus-infected cells and malignant cells. Because of that, this interaction is recently exploited for cancer immunotherapy. Several agonistic anti-CD137 antibodies are currently used in clinical trials to stimulate CD137 on T cells to invigorate them against malignant cells[83-85]. The costimulatory ability of CD137 is validated further when its cytoplasmic domain is incorporated in the design of new generations of CAR-T cells[86-88].

1.4.3 CD137 in cancer

In the case of cancer, CD137 is a reliable marker for tumour-specific tumour-infiltrating lymphocytes[89]. Human tumours contain many bystander T cells that are not capable of recognizing malignant cells. As CD137 expression on T cells is dependent on activation, CD137 is regularly used to identify those that have been activated by tumour-associated-antigens.

Given the anti-tumour and anti-viral effect of the CD137L-CD137 axis, it is surprising to observe that CD137 can be found in many immunosuppressive environments. Several viruses such as EBV and HTLV-1 use their viral proteins (LMP-1 in EBV and Tax-1 in HTLV-1) to induce CD137 expression on T cells[90]. In addition to that, EBV-related NPC tumours can also be induced to express CD137. Several non-Hodgkin lymphoma cell lines such as KM-H2 or HDLM-2 also express CD137 constitutively[91]. CD137 is also preferably expressed on Treg inside the tumours, as compared to peripheral Treg of the same patient[92]. Several studies have reported that $CD137^+$ Treg is more immunosuppressive than their $CD137^-$ counterpart[93]. In addition to that, CD137 also can exist as a soluble isoform without cytoplasmic domain and transmembrane domain, due to alternative splicing. This soluble form can bind to CD137L and function as a decoy receptor that can interfere with normal CD137L-CD137 interaction[94]. Thus, it is reasonable to assume that the CD137-CD137L axis may also play a role to suppress the immune response in some scenarios.

1.5 T cell exhaustion

T cells can be stimulated via the TCR, via costimulatory receptors, and via inflammatory cytokines secreted from other immune cell populations and even T cells themselves. While T cells can be activated by a plethora of stimulatory signals, there is a limit to how active they can be. Like many physiological processes in our body, an immune response needs to be kept in check by various feedback mechanisms. One such mechanism is T cell exhaustion, when T cells become too activated and diminish their functions.

1.5.1 Origin of exhausted T cells

The definition of T cell exhaustion remains controversial. However, experts generally agree on a few points. Exhausted T cells are in a functional state that is distinct from naïve, effector and memory T cells. When naïve T cells are activated to become effector T cells, a small subset of those effector T cells ($CD127^{+}KLRG1^{-}$) will be at a crossroad. If the antigen is cleared and the inflammation subsides, such cells will become memory cells. If the antigen persists and cannot be cleared, chronic inflammation ensues and those T cells become exhausted[95]. Thus, T cells become exhausted if they are exposed to persistent antigen presentation, or inflammatory signals, as in the case of chronic viral infection or cancer[96, 97].

1.5.2 Exhausted T cell characteristics

Exhausted T cells are in a dysfunctional state. They have a diminished (but not absent) ability to produce cytokines, proliferate, and kill their targets [98-100] . Exhausted T cells can exert weak immune response against tumour or chronic infection, and this effect is only minimal and temporary.

Additionally, exhausted T cells express several coinhibitory receptors simultaneously that can act together to dampen their function: such as PD-1, TIM-3, and LAG-3[101]. The level of co-expression of these inhibitory receptors are associated with the extent of the dysfunctional state of exhausted T cells. Checkpoint inhibitors have been shown to reverse exhaustion and lead to tumour regression[102].

1.5.3 The relevance of T cell exhaustion

While T cell exhaustion is generally viewed as a dysfunctional state under which T cells cannot function to its fullest extent, it is not deemed as inherently good or bad. In fact, it is a necessary mechanism, conserved through evolution and across species, to allow the activated T cells to persist and partially subdue malignant cells or virally infected cells. At the same time, T cell exhaustion tunes down the immune response to limit the damage to neighboring tissues and prevent autoreactivity[103]. In the case of autoimmunity, T cell exhaustion is sometimes correlated with better prognosis for various autoimmune conditions, and the induction of exhaustion can even become a therapeutic strategy[104].

The study of T cell exhaustion provides insight on how to reinvigorate exhausted tumour-infiltrating T cells to treat cancer. It also potentially helps to identify those T cells most suitable for adoptive T cell transfer therapy. The exclusion of exhausted T cells means that only prime T cells are injected into the patients.

1.6 HHLA-2 – B7-H7

HHLA-2, also known as B7-H7, was first discovered in a screening for new members of the Immunoglobulin (Ig) super family[105]. B7-H7 is found in human and many other species including fish, frog, panda, and monkey. However, B7-H7 homologs are not found on lab mice and rats[106].

In human, B7-H7 can be found on GI tract, the kidney, and the placenta[107]. In the immune system, it is found mostly on APC like monocytes and dendritic cells[108]. Interestingly, it is also detected on a variety of cancers such as breast, lung, and melanoma. Its expression has been associated with a worst disease outcome[107, 109-111].

B7-H7 share amino acid sequence and structure similarities with other members of the B7 family members, including B7.1 (CD80), B7.2 (CD86), B7-H1 (PD-L1) and B7-DC (PD-L2)[106]. It is reasonable to assume that B7-H7 can affect T cell functions like other members of the B7 family. However, the influence of B7-H7 on T cells has been debatable. Several groups have reported that B7-H7 can co-stimulate T and NK cells via CD28H[108, 112]. Nevertheless, recently more groups have reported that B7-H7 can regulate T cells function, potentially by interacting with an inhibitory receptor KIR3DL3[106, 113-115]. It is not unusual for B7 members to have dual functions. CD80 and CD86 can bind to both CD28 to stimulate T cells or to CTLA-4 to inhibit T cells[116]. Such intricate network of interactions allows our immune system to fine-tune its response to the many dynamic situations it can face.

1.7 PD-1 immune checkpoint and its cognate ligand PD-L1 and PD-L2

1.7.1 PD-1 pathway

Although PD-1 was originally discovered as an apoptosis-related gene, it was later revealed that PD-1 expression is not essential for apoptosis[117, 118]. However, PD-1 expression is upregulated on T cells and B cells after activation[119], and PD-1 plays an essential role to regulate the immune response, promote tolerance toward “self”, cause the death of antigen-experienced T cells and stop the death of regulatory T cells[120-122]. PD-1 ligands include PD-L1 and PD-L2. PD-L1 is expressed on both stimulated APC and T cells. While PD-L2 has a three-fold affinity to PD-1 as compared to PD-L1, it is expressed on far less cell types, mostly on stimulated APC. Both are transmembrane proteins and considered to be coinhibitory molecules[118].

As tumour clearance closely depends on the infiltration of tumour-reactive T cells, PD-1 and its ligands play vital role in dampening the host’s immune response against tumour. Thus, many tumours have evolved to express PD-L1 and PD-L2 to activate this pathway, escape immune surveillance and promote tumour progression[120, 123]. PD-1, together with CTLA-4, have been identified as the stop-signal tumours regularly employ to survive.

1.7.2. PD-1/PD-L1 immune checkpoint inhibitors

Given the involvement of PD-1 in tumour progression, inhibitors for PD-1/PD-L1 pathway have been developed, proven a success, and approved for treatment in a plethora of late-stage cancer conditions.

Nivolumab is a fully humanized monoclonal antibody that binds to PD-1. As demonstrated in the Checkmate studies, compared to standard chemotherapy, nivolumab confers a better long-term benefit, with better overall survival rate, for many cancer patients, including those

suffering from advance non-small-cell lung cancer[124] head and neck squamous cell carcinoma[125], renal cell carcinoma[126].

Pembrolizumab, another PD-1-targeted antibody, also demonstrates superior overall survival, compared to standard chemotherapy in a variety of cancer patients, including those suffering from non-small-cell lung cancer [127], advanced esophageal cancer[128], head and neck squamous cell carcinoma[129], urothelial carcinoma[130].

Atezolizumab and avelumab are two monoclonal antibodies targeting PD-L1 and inhibiting its binding to PD-1. While avelumab is still undergoing trials, atezolizumab has been shown to confer superior overall survival compared to standard chemotherapy for patients suffering from non-small-cell lung cancer[131], and metastatic urothelial cancer[132].

Despite recent success making a paradigm shift in cancer treatment, immune checkpoint inhibitors in general, or anti-PD1/PD-L1 inhibitors in particular, only prove effective for a subset of patients, for which many are partial responders ("primary resistance" to therapy). Even responders who showed initial response may succumb to disease progression later ("acquired resistance")[133]. For non-small-cell lung cancer patients, the overall response rate for nivolumab and pembrolizumab is approximately 17.1% and 19%, respectively[134]. Biomarkers such as PD-L1 expression and microsatellite instability have been developed to identify responder with moderate success[135, 136]. Hence, immune checkpoint inhibitors leave room for improvement.

2 RESEARCH AIMS

The overall aim of the thesis is to explore how the well-established immunostimulatory CD137L-CD137 can be exploited by malignant cells and chronic infection scenarios to become immunoinhibitory. At the same time, we would like to characterize those immunosuppressive mechanisms, in order to suggest improvement in future immunotherapy.

The specific aims of the papers are:

I. To investigate the influence of soluble CD137 on the response of T cells, in terms of cytokine secretion and helper T cell polarization. (Paper I)

II. To investigate how regulatory T cells use CD137 to downregulate CD137L on APC during trogocytosis, and how those APC co-stimulate other T cells. (Paper II)

III. To investigate how B7-H7 can be expressed on T cells, enhanced by CD137 stimulation, enhanced by CD137 stimulation, and used as a marker of exhausted T cells. (Paper III)

IV. To investigate why certain tumours evolve to express CD137, and how CD137 stimulation confers the tumour growth and survival advantage. (Paper IV)

V. To develop an immunotherapy that simultaneously upgrades current immune checkpoint inhibitors and neutralizes the immunosuppressive functions of CD137 expressed on tumour. (Paper V)

3 MATERIALS AND METHODS

Details on specific material and methods can be found in respective constituent papers and manuscripts. Here we provide general information on several methods commonly used.

3.1 CELL CULTURE

3.1.1 Primary cell culture and preparation

Primary immune cells were extracted from mouse or human and isolated into subpopulations, by Fluorescence-assisted cell sorting (FACS) or Magnetic-assisted cell sorting (MACS).

For murine immune cell preparation, murine spleens were extracted, pulverized, and filtered through cell strainers to produce suspension of splenocytes. Fluorochrome-tagged antibodies against CD4 and CD25 were used to identify murine Treg ($CD4^+CD25^+$) from Tcon ($CD4^+CD25^-$) before they were separated into two distinct populations during FACS.

For human immune cell populations, PBMC were isolated from healthy donors' blood, by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Chicago, USA). From PBMC, $CD3^+$ T cells, $CD8^+$ T cells, NK cells or monocytes were isolated by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells and NK cells were isolated via negative selection while monocytes were isolated via positive selection.

Monocyte-derived dendritic cells (moDC) were derived from monocytes. Isolated monocytes were seeded at 10^6 cells/ml, supplemented with 100 ng/ml IL-4 at and 80 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ, USA) for five days. Mature moDC (mat-moDC) were moDC matured with LPS treatment. LPS (Sigma-Aldrich, St. Louis, MO, USA) was added on day five at 5 µg/ml. On day six, mat-moDC were harvested.

All primary cells were cultured in RPMI1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Biowest, Riverside, MO, USA) and 50 µg/ml streptomycin and 50 IU/ml penicillin.

3.1.2 Cell line culture

Suspension cell lines were cultured in RPMI1640 medium (Gibco) supplemented with 10% FBS (Biowest). Adherent cell lines were cultured in DMEM medium (Biowest) supplemented with 10% FBS. All cells were cultured in 37ºC and 5% CO_2 in an incubator and passaged every two to three days. HEK293-6E cells, used for recombinant protein production, were cultured in FreeStyle™ 293 Expression medium (Gibco), cultured in 37ºC and 5% CO_2 in a shaking incubator and passaged every two to three days.

3.1.3 Generation of PD-L1 KO Rd18 cell line

CD274 CRISPR guide RNA was cloned into pLentiCRISPR v2 plasmid and ordered from GenScript (Piscataway, NJ, USA). The plasmid was transfected into wild type (WT) Rd18 cells with Turbofect transfection agent (Thermo Fisher Scientific). After 48 hours, cells were

washed and incubated in DMEM (Biowest) supplemented with 10% FBS, and 0.5 µg/ml puromycin for seven days. Cells were then washed and transferred to fresh DMEM medium (Biowest) supplemented with 10% FBS, without puromycin, and cultured to confluency. Resultant cells were then stained with anti-PD-L1 antibody conjugated with APC fluorophore and sorted for APC-negative cells by FACS, to ascertain complete knockout status.

3.2. FLOW CYTOMETRY

In general, cells were first washed with cold phosphate buffer saline (PBS) solution. Afterward, cells were stained with dead cell marker (Near Infra-Red, or AQUA) diluted in PBS for at least 30 minutes at room temperature. Either FcR blocker agent (Miltenyi Biotec) or Iv IgG (Privigen, Germany) was added for 5 minutes at room temperature to inhibit non-specific binding between fluorophore-conjugated antibody with the FcR. After incubation with fluorophore-conjugated antibodies (30 minutes at 4ºC, or 10 minutes at room temperature), cells were washed twice and resuspended in FACS buffer before flow cytometry analysis. If DAPI (Thermo Fisher Scientific) was the dead cell marker, it was added at the final step after cells were resuspended in FACS buffer. For intracellular staining, eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) was used. Briefly, after extracellular staining, cells were washed and treated with fixing solution for one hour, then washed with permeation and fixing buffer. After staining with the relevant antibodies for at least 30 minutes at room temperature, cells were wash again with permeation and fixing buffer before running flow cytometry. When staining for intracellular cytokines, the cells were incubated with GolgiStop™ Protein Transport Inhibitor (BD Bioscience, Franklin Lakes, NJ, USA) (4 µl in 6 ml of culture) for six hours prior to staining. All flow cytometry data was analyzed by TreeStar FlowJo program.

3.3. GENE EXPRESSION ANALYSIS

To analyse HHLA-2/B7-H7 mRNA expression for primary immune cells, we employed In Silico Transcriptomic (IST) Online by Medisapiens (https://ist.medisapiens.com/). We employed the Human Protein Atlas project (https://www.proteinatlas.org/).

Published databases were obtained and visualized via the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl).

3.4. PLASMID TRANSFECTION AND ELECTROPORATION

3.4.1. Transfection

For the transfection of HEK-293 and HEK293T, Turbofect (Thermo Fisher Scientific) was used as the transfection reagent, according to the manufacturer's protocol. For the transfection of HEK-293-6E cells for recombinant protein production, cells were seeded in 72 ml of medium at a concentration of 0.48 x 10^6 cells/ml overnight. On the day of transfection, 8 ml of NaCl 150 mM was mixed with 80 µg of plasmid and 240 ug of PEI, and the mixture was added directly to the cell culture, making up approximately 80 ml. The culture was

incubated for one day and then supplemented with Trypton-N1 (Organotechnie Sas, India) (final concentration: 0.5%). The culture was incubated for another six days before the supernatant was harvested for protein purification.

3.4.2. Electroporation

Electroporation was done with the recommended Nucleofector™ kit and recommended protocols, in the 4-D Nucleofactor™ X-unit (Lonza, Basel, Switzerland).

3.5 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Supernatant was collected after centrifugation. IL-8, IL-10, IL-13, IL-17a, and TNF-α were measured by the respective ELISA kits (Thermo Fisher Scientific). IFN-γ was measured by ELISA kit either from Mabtech (Nacka, Sweden) or R&D systems (Minneapolis, MN, USA).

Briefly, capture antibody was diluted in PBS and added onto Nunc MaxiSorp™ flat-bottom 96-well plate (Thermo Fisher Scientific), at 50 µl per well, overnight. Between steps, wells were washed with PBS supplemented with 0.05% Tween20 (PBST). The plate was then blocked with 100 µl of ELISA diluent for 1 hour. After washing, standards and samples were added and incubated for two hours. Then, diluted detection antibody was added at 50 µl per well and incubated for one hour. Afterward, diluted Streptavidin-HRP was added at 50 µl per well and incubated for 30 minutes. In the final step, TMB susbstrate was added at 50 µl per well, incubated for 10 to 15 minutes until sufficient color development was observed, and stopped with 25 µl Stop solution (2N H_2SO_4). The plate was then read for absorption at 450nm and 595 nm wavelength.

3.6 WESTERN BLOT

Western blot was done for the verification of purified recombinant fusion PD1-CD137L protein. Purified protein samples were mixed with 5x Laemli WB loading buffer supplemented with β-mercapto ethanol (Sigma-Aldrich), boiled at 99ºC for at least five minutes, chilled to room temperature and ran on 8% resolving SDS-PAGE gel. The proteins on the gel were transferred to PVDF membrane by semi-dry transfer. Between steps, the blot was washed with Tris buffer saline-Tween (TBST, TBS +0.1% Tween20) three times. The blot was then blocked with 5% skim milk for one hour at room temperature. Afterward, the blot was probed with primary antibody solution (anti-CD137L antibody, clone 5F4, 1 ug/ml in TBST), for 1 hour at room temperature or overnight at 4ºC. The blot was then probed with the secondary antibody (anti-mouse IgG H+L chain, AF488), washed and view with ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA).

3.7 RECOMBINANT PROTEIN PURIFICATION

3.7.1 Purification via His-Tag

The transfected HEK-293-6E culture was centrifuged at 4000 rpm for 15 minutes. The cell pellet was discarded and the supernatant containing the target protein is collected. 80 ml of

the supernatant was mixed with 0.6 ml of slurry of Smart Ni beads 6FF (Bio Basic, Singapore) overnight at 4ºC. A 5 ml purification column was packed with a disc to stop the Ni beads from flowing through. The column was washed and equilibrated with buffer A (20 mM Tris, 500mM NaCl, pH 8.0). The supernatant mixed with Ni beads was then passed through the column. The column was washed with 20ml of buffer A, and then 10ml of buffer C (20 mM Tris, 500mM NaCl, 5mM Imidazole, pH 8.0). The protein of interest bound to the column was eluted with buffer B (20 mM Tris, 500mM NaCl, 500mM Imidazole, pH 8.0) into five fractions, 0.5 ml each. The fractions with the highest and second highest amount of protein were pooled together, and buffer-exchanged to PBS with Vivaspin 6 10000 MWCO column (Merck Millipore, Burlington, MA, USA).

3.7.2 Purification via Strep-Tag

The transfected HEK-293-6E culture was centrifuged at 4000 rpm for 15 minutes. The cell pellet was discarded and the supernatant containing the target protein is collected. 856 µl of BioLock solution (IBA Life Sciences, Göttingen, Germany) and 8 ml of 10x PBS were added to 80ml of supernatant and incubated for 10 minutes at room temperature. A purification column like that in the His-tag purification protocol was packed and equilibrated with buffer W (100mM Tris, 150 mM NaCl, pH 8.0). 0.8 ml of Strep-Tactin®XT 4Flow® resin (IBA Life Sciences) was added to the column and washed with 5 ml of buffer W. The total volume of the supernatant was added to the column, and wash with 10 ml of buffer W twice. The protein of interest was eluted with BXT solution (IBA Life Sciences) in fractions of 0.5 ml. The fractions with the highest and second highest amount of protein were pooled together, and buffer-exchanged to PBS with Vivaspin 6 10000 MWCO column (Merck Millipore).

3.7.3 Coomassie-blue staining

InstantBlue ® Coomassie Protein Stain (Abcam, Cambridge, UK) was used determine purified protein purity and size verification. The SDS-PAGE gel, after samples were run, was washed with water three times, and stained with 10 ml of InstantBlue ® Coomassie Protein Stain for 10 minutes, before being washed with water for another three times.

3.8 CONFOCAL MICROSCOPY

Confocal dishes were obtained from SPL (Gyeonggi-do, Korea). Cell surface markers were stained in the same method as in flow cytometry staining. Images were captured with ZEISS LSM710 Confocal microscope (Zeiss, Oberkochen, Germany).

3.9 STATISTICAL ANALYSIS

Statistical analyses were completed with Prism Graphpad software. Survival analysis was completed using R2 platform. Student's t test was employed to compare the difference between two groups, and one-way ANOVA were done to compare multiple groups. Overall survival probability was calculated by the log-rank test.

4 RESULTS

4.1 Paper I. Soluble CD137 interferes with T cell activation and polarization

Background

Persistent chronic inflammation leads to dysfunctional T cells, which in turn, allows further chronic inflammation to persist because the T cells cannot perform properly and fail to clear the pathogens completely[137, 138]. This is also true for chronic hepatitis C virus (HCV) infection.

CD137 is a costimulatory receptor on T cells that, upon stimulation, can empower T cells to clear viral infection. However, given a strong or persistent stimulus, T cell can secrete soluble CD137, an isoform that lacks the transmembrane and cytoplasmic domains, but still can compete with surface CD137 to bind to CD137L. We detect an increased level of soluble CD137 (sCD137) in the serum of HCV patients with liver cirrhosis complication. Given that sCD137 potentially interferes with T cell functions, we explore how sCD137 alters T cell polarization, changes cytokine secretion, and plays a role in the establishment of chronic inflammation.

Aim

We aim to determine if the level sCD137 in the serum of HCV patients is correlated to cirrhosis-associated complications. As CD137 is a major component of the immune system, we would like to assess how the increased level sCD137 would affect the immune response, potentially shedding light on how the associated chronic inflammatory state is maintained.

Results

1. sCD137 interferes with the secretion of type-1 and type-2 related cytokines.

In order to evaluate the effect of sCD137, recombinant human CD137 extracellular domain is obtained from CHO, tagged with a His-tag. This recombinant protein retains the ability to bind to CD137L and compete with normal transmembrane CD137. Hence it is an ideal substitute for the endogenous sCD137.

Supplemented with a suboptimal dose of anti-CD3 agonistic antibody, healthy donors' peripheral blood mononuclear cells (PBMC) are cocultured with Daudi, a Burkitt lymphoma cell line that expresses elevated levels of CD137L, and that can co-stimulate CD137-expressing T cells. We hypothesize that if sCD137 competes with full-length CD137 on the surface of T cells for binding to CD137L, the presence of sCD137 would dampen the T cell activity. Indeed, sCD137 decreases the secretion of IFN-γ, TNF-α and IL-13. However, the secretion of IL-17a and IL-10 remains unchanged, indicating that sCD137 does not affect Th17 polarization or a Treg induction.

2. sCD137 affect the polarization of T helper cells to become Th1 and Th2 subpopulations

IFN-γ and TNF-α are characteristic cytokines of by Th1 cells, and IL-13 is the characteristic cytokine secreted by Th2 cells. Because the production of these cytokines is affected by sCD137, we speculate that the lack of CD137L stimulation caused by sCD137 leads to diminished polarization of CD4 T cells to become Th1 and Th2. Concurrently, since the level of IL-17 and IL-10 are not affected, we hypothesize that the level of Th17 cells and regulatory T cell subtypes remain unchanged with the presence of sCD137. The determining transcriptional factor for helper T cell subpopulations are T-bet, GATA-3, ROR-γT, and FOXP3 for Th1, Th2, Th17 and Treg, respectively.

Upon the addition of sCD137 into PBMC-Daudi cell coculture, we observe that the proportions of $CD4^+$ROR-γT^+ T cells and $CD4^+FOXP3^+$ T cells remain unchanged. This indicates that Th17 and Treg populations are maintained. However, there is a reduction in the proportion of $CD4^+$T-bet^+ T cells (Th1) and $CD4^+GATA\text{-}3^+$ T cells (Th2). This reduction is consistent with the reduction of IFN-γ, TNF-α, and IL-13.

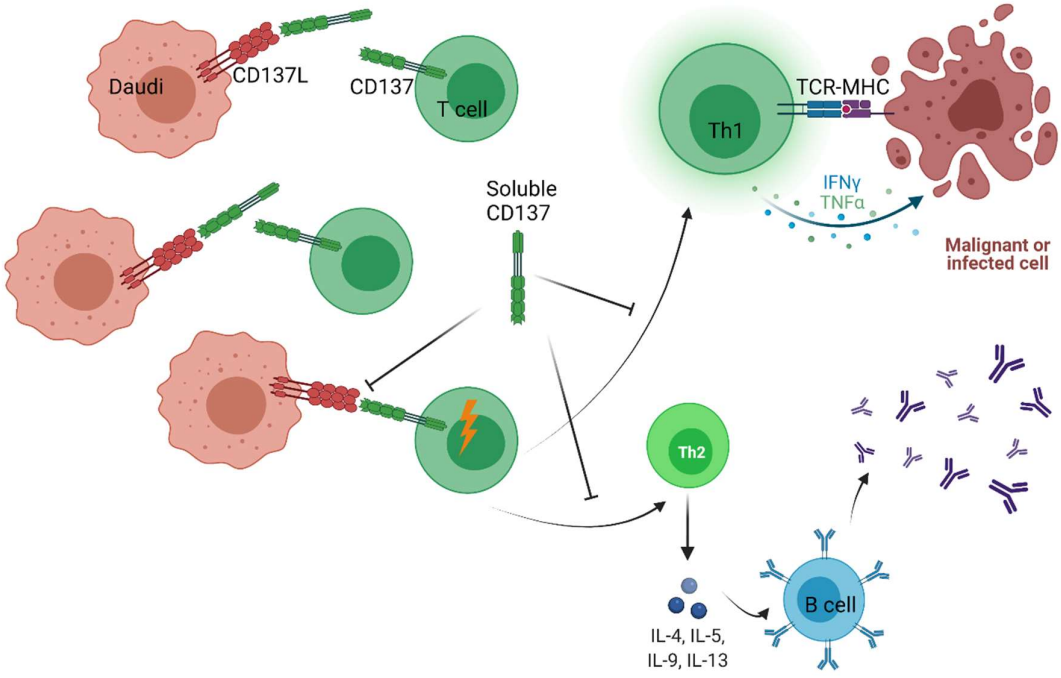

Figure 5. sCD137 competes with normal CD137 to bind to CD137L and interferes with T cell activation and polarization to Th1 and Th2 subpopulations. Created with Biorender.

4.2 Paper II. Treg uses CD137 to downregulate CD137L during trogocytosis

Background

Although CD137 expression is inducible on T cells upon activation, CD137 is preferentially expressed on Treg. In human, given the same stimulus, $CD4^+CD25^+$ T cells (Treg) express CD137 much more readily as compared to $CD4^+CD25^-$ T cells (Tcon) [139]. CD137 is also a marker for stably activated Treg, and $CD137^+$ Treg is more immunosuppressive than its $CD137^-$ counterpart[93, 140]. Consistent with that, Treg in the tumour expresses more CD137 than peripheral Treg in the same patients[92]. Hence, it is reasonable to assume that CD137 plays a significant role in the immunosuppressive function of Treg.

On the other hand, Treg also expresses a prominent level of CTLA-4[141]. Treg's CTLA-4 can suppress the immune response via multiple mechanisms of action. Firstly, CTLA-4 competes with costimulatory receptor CD28 to bind to the costimulatory ligands CD80 and CD86, in the process depriving T cells of costimulatory signals. Additionally, Treg also uses CTLA-4 to internalize CD80 and CD86 on APC, in a process named trans-endocytosis. This deprives APC of surface CD80 and CD86, rendering them less able to co-stimulate T cells[142]. Thus, we hypothesize that Treg can use CD137 to downregulate CD137L in a similar process, to deprive APC of the costimulatory CD137L molecule.

Aim

We aim to assess whether $CD137^+$ Treg can use trogocytosis to internalize and deplete CD137L on APC cells. We plan to explore if intracellular signaling is required for this process, and what happen to the internalized complex. We also assess the co-stimulation ability of APC after their surface CD137L is depleted by trogocytosis.

Results

1. CD137 and CD137L form a complex that is internalized toward both directions

We hypothesize that trogocytosis is conserved across species, hence we test in both human and mouse. To visualize the movement of CD137 and CD137L in flow cytometry and confocal imaging, we modify and attach a fluorescent tag to each molecule. Human and murine CD137 are fused to GFP. Human and murine CD137L are fused to mCherry and OFP, respectively. We then coculture cells that are transfected with fluorescent CD137 only and cells that are transfected with fluorescent CD137L only. Cells that are transfected with fluorescent CD137L only are labelled with Cell Trace Violet. Upon one hour of coculture, we observe that some single cells have both the fluorescent CD137 and CD137L. Among these cells, some are positive for Cell Trace Violet, and some are not. This indicates that CD137 and CD137L can form a complex that could be internalized in both directions. The existence of these complexes is further verified with confocal imaging, by which yellow dots can be

observed in the cytoplasm of the cells. We obtain comparable results in both murine and human system. This indicates that this mechanism is conserved between species.

2. CD137 does not require intracellular signaling to form a complex with CD137L

In both the murine and human system, we also create a modified version of CD137 where the cytoplasmic domain is truncated from the protein structure, while the extracellular domain and the transmembrane domain remain intact. Both truncated CD137 species are also fused to GFP to facilitate observation, similar to their full-length CD137 counterpart.

Like full-length CD137, truncated murine and human CD137 can also downregulate CD137L on other cells and the complex formed can be transferred in both directions.

3. Murine and human Treg use CD137 to downregulate CD137L on APC

At resting state, only murine Treg expresses CD137 while Tcon does not. Hence, upon coculture with RAW264.7 cells (a murine macrophage cell line that expresses high level of CD137L), only Treg can downregulate CD137L on RAW264.7 surface. At the same time, we also notice that the CD137 on Treg disappears after such coculture. Because the CD137-CD137L interaction can produce many downstream effects, the coculture duration is one hour to prevent any effect seen due to activation. In such a short amount of time, any changes in the expression level of CD137L on RAW264.7 cells, and CD137 on Treg, will mostly be due to trogocytosis.

To ascertain that the downregulation of CD137L on RAW264.7 cells are due to CD137, we also sort Tregs from $CD137^{-/-}$ mice and coculture them with RAW264.7 cells. As expected, WT Treg can downregulate CD137L on RAW264.7 cells more than $CD137^{-/-}$ Tregs do.

Human T cells generally do not express CD137 at the resting state and need to be activated to induce CD137 expression. We observe that activated human Tregs can express CD137 and downregulate CD137L in a variety of APC cell line such as Raji and THP-1. While in T cells, the internalized CD137-CD137L complex would be disintegrated by proteasome activity, rather than autophagy (as is the case with CTLA-4 – CD80/CD86 complex). The presence of bortezomib (a proteasome inhibitor) but not Bafilomycin A (an autophagy inhibitor) allows for the accumulation of such fluorescent complex.

4. APC with CD137L downregulated by trogocytosis are less able to co-stimulate T cells

Using THP-1 (a monocytic AML cell line) as accessories cells in a T cell costimulation assay, we examine the costimulatory ability of APC after trogocytosis. THP-1 cells constitutively express elevated level of CD137L at the surface. Upon culturing THP-1 with HEK293 cells transfected with either full-length CD137-GFP or truncated CD137-GFP, we consistently observe a downregulation of surface CD137L on gated single, live, $CD45^{+}$ THP-1 cells. The THP-1 cells that come out of the coculture also possess GFP fluorescence, indicating the internalization of CD137-GFP into their cytoplasm.

We then isolate THP-1 cells from the coculture based on CD45 expression, fix these isolated THP-1 cells to lock in their CD137L expression, and use them to co-stimulate freshly isolated $CD8^+$ T cells from healthy donors, in the presence of sub-optimal anti-CD3 stimulation. THP-1 that are cocultured with WT HEK293 cells could retain their high CD137L expression and understandably perform the best at co-stimulating T cells, inducing higher expression of CD25 and secretion of IFN-γ.

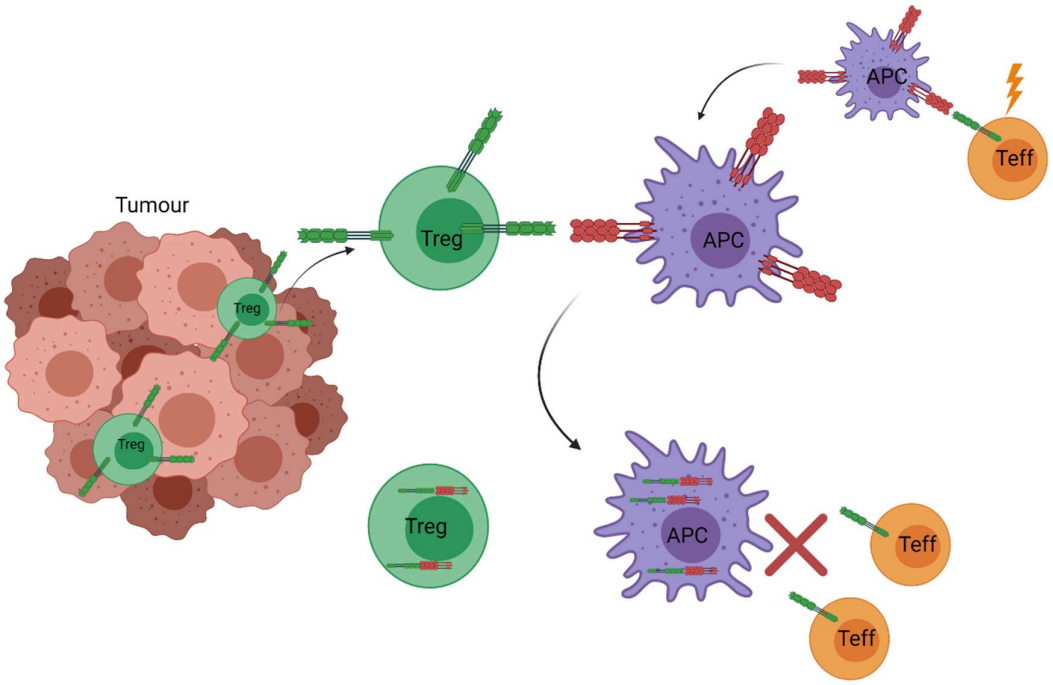

Figure 6. APC contact $CD137^+$ Treg after tumour infiltration, lose surface CD137L and become less able to costimulate other effector T cell population. Created with Biorender.

4.3 Paper III. B7-H7 is inducible on T cells to regulate their immune response and serves as a marker for exhaustion

Background

B7-H7 is a member of the B7 family, like CD80, CD86, PD-L1 and PD-L2. It is regularly reported to be expressed by APC and can affect T cell immune response[106, 108]. Its function has been controversial ever since its discovery. While many studies claimed that it can stimulate T cells and NK cells via its receptor CD28H, many other studies also showed that B7-H7 can profoundly inhibits T cells' function[108, 113].

Recently, one study suggests that agonistic anti-CD137 antibody treatment can upregulate B7-H7 expression at the mRNA level in T cells[143].

Aim

We investigate if T cells can express B7-H7 at a protein level. We also assess its influence on other T cells by blocking it with rhCD28H. At the same time, we study the phenotypes of T cells that do express B7-H7.

Results

1. T-cell hematological cancers and cancer cell lines of T cell origin can express B7-H7

To investigate the relevance of B7-H7 in cancer setting, we study and detect the expression of B7-H7 on a variety of cancer cell line of T-cell origin, as well as T-cell related tumours. We observe that the level of B7-H7 mRNA expression is correlated with overall survival probability for patients suffering from Peripheral T-cell lymphoma. Unsurprisingly, we can detect B7-H7 at the protein level on T-cell related tumour cell line such as HDLM-2 or MAC-1. Examining primary cells RNASeq data, we also notice that T cells can express B7-H7 mRNA as much as, if not more than, other immune cell subpopulations such as monocytes and dendritic cells. Hence, it is plausible that primary T cells can express B7-H7 at the protein level to regulate immune functions.

2. B7-H7 expression on T cell is dependent on activation, CD137 co-stimulation, and mTOR

Consistent with other studies, we do not detect B7-H7 on resting T cells. However, upon activating T cells with CD3 and CD28 agonists, we detect B7-H7 expression on both $CD4^+$ and $CD8^+$ T cells. The presence of the stimulus is important because when we remove it and let T cells to rest in growth media containing only IL-2, the expression level rapidly drops. Other costimulatory signal, such as recombinant CD137L stimulating via the CD137 pathway, can enhance B7-H7 expression level. Curiously, a strong mitogenic stimulation, PMA and ionomycin, cannot induce any B7-H7 expression.

Because other members of the B7 family such as PD-1 depends on the mTOR pathway to maintain expression, we postulate that B7-H7 expression is also mTOR-dependent. Indeed, mTOR inhibitor Torin-1 significantly impairs B7-H7 expression on T cells.

3. T cells use B7-H7 to regulate immune functions

The function of B7-H7 had been controversial because it could bind to both a costimulatory receptor and a coinhibitory receptor. Different experimental settings in which B7-H7 is studied would likely yield different conclusions. In our hand, we use purified T cells alone and attempt to block it with recombinant CD28H, one of its receptors. In the presence of rhCD28H, T cells proliferate more, and more of them express markers of activation such as CD25 and PD-1. We also observed a consistent, albeit not statistically significant increase in IFN-γ secretion. Intracellular staining revealed that $B7\text{-}H7^+$ T cells produce less IFN-γ and TNF-α, which further corroborates the hypothesis that $B7\text{-}H7^+$ T cells regulate rather than bolster the immune response.

4. B7-H7 is a marker for exhausted T cells

Because B7-H7^{+} T cells express minimal IFN-γ and TNF-α, we hypothesize that they were either exhausted or from subpopulations other than Th1 or Tc1. In our hand, most T cells commit to the Th1/Tc1 subsets after activation with CD3 and CD28 agonists, consistent with previous reports. In addition to that, compared to B7-H7^{-} T cells, more B7-H7^{+} T cells express CXCR3, suggesting a more dominant Th1/Tc1 phenotypes. Concurrently, we also detected that B7-H7^{+} T cells are enriched for markers of exhaustion, such as PD-1, LAG-3, CTLA-4 and TIM-3. In summary, B7-H7 could serve as a marker for exhausted Th1 or Tc1 cells that produce only few inflammatory cytokines.

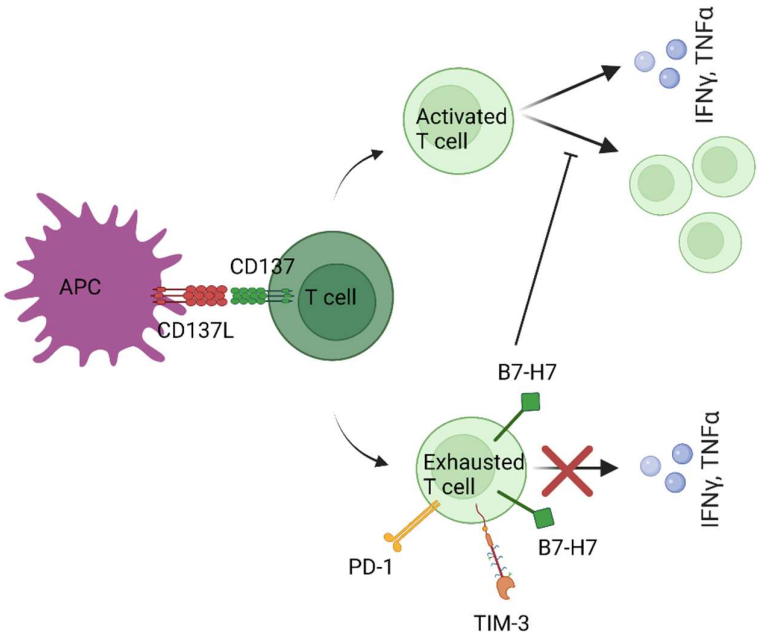

Figure 7. B7-H7 expression depends on activation, marks exhausted T cells and regulates T cell immune response. Created with Biorender.

4.4 Paper IV. Epstein-Barr Virus-Induced Ectopic CD137 Expression Helps Nasopharyngeal Carcinoma to Escape Immune Surveillance

Background

Head and neck cancer has become one of the most common cancers. Each year, there are approximately 550,000 new cases, and 380,000 new deaths[144]. A specific type of head and neck cancer is nasopharyngeal carcinoma (NPC). One remarkable thing about NPC is how distorted the distribution of this malignancy is across geographical regions and races. Across

the world, NPC can be considered a rare malignancy, with an incidence rate below 1 per 100,000 persons every year[145]. However, the exceptions are the Chinese population and Southeast Asians, where the rate is much higher. For instance, the incidence rate for a Vietnamese male in Hanoi was 10.3 per 100000 persons per year, and that for a Chinese male in Hong Kong was 24.3 per 100000 persons per year. There is also a bias toward the gender of the patients. Across populations, the incident rate ratio between males and females is approximate 2-3 to 1[146].

There are many potential risk factors associated with NPC, such as latent EBV infection, the consumption of EBV-activating substances in food, the consumption of local preserved food, smoking or alcohol[146]. However, one common theme has been EBV. Interestingly, EBV-related viral proteins have been shown to upregulate CD137 expression on tumour cells[147]. Our preliminary data also detect a substantial proportion of NPC tissue expressing CD137, upon immunohistochemistry (IHC) staining.

Given that CD137 is commonly known as a costimulatory receptor by which T cells are activated to be anti-tumour, it is surprising to observe that NPC evolved to induce CD137 expression. One reason is that NPC can emulate Treg and use CD137 to downregulate CD137L on APC cell by trogocytosis. However, NPC expresses the full-length membrane protein that can also send downstream signaling into the tumours. If the well-established stimulatory role of CD137 on T cell is any indication, it is logical to assume that tumour can also receive activation signals upon CD137L ligation onto their surface CD137.

Aim

We aim to investigate the effect of CD137 signaling into NPC tumour, the downstream pathways involved, and whether this signaling upregulate or downregulate any significant proteins by genomics and proteomics assays.

Results

1. CD137 stimulation activates the p38-MAPK pathway

CD137 downstream signaling can activate immune cells via three pathways: p38 MAPK[148], JNK[149] and NF-κB[150]. Thus, we examine the phosphorylation status of these three signal transduction factors. As expected of transformed malignant cells, all three pathways' factors are well phosphorylated even before any CD137L stimulation is provided. Although it is difficult to activate such stimulated cells further, we notice that CD137L can enhance the phosphorylation status of p38-MAPK, while not affecting the status of JNK and NF-κB. This upregulation of p38-MAPK phosphorylation is observed in both transfected NPC cell lines C666-CD137 and HK1-CD137.

2. p38-MAPK inhibition reverses the upregulation of IL-8 production in NPC

Similar to rhabdomyosarcoma cell lines, NPC cell line C666-CD137 and HK1-CD137 also produce higher level of IL-8 upon CD137 stimulation. Because CD137 stimulation activates

p38-MAPK pathway, we test if an MAPK inhibitor, VX-702, can reverse the upregulated IL-8 production. Indeed, VX-702 reduces IL-8 production even in the presence of rhCD137L stimulation, further confirming the important role of p38-MAPK pathway in the activation of NPC cells.

3. CD137 stimulation enhances LAMC2 expression, a protein implicated in the EMT process

Using RNASeq, we identify several leads that are upregulated or downregulated in the three NPC cell lines C666, HK-1, HONE-1 after CD137 stimulation. The most prominent upregulation in RNASeq was IL-1 receptor 2, while LAMC2 mRNA was upregulated to a smaller extent.

Afterward, we repeatedly stimulate $CD137^+$ C666 cells with recombinant CD137L and analyzed the changes in protein expression level, via Mass-Spectrophotometry. The most prominent upregulation is LAMC2 protein. Hence, we decide to validate RNASeq and Mass-Spec findings with flow cytometry. While $CD137^+$ C666 does not show any upregulation in IL-1 receptor 2 expression at the protein level, we consistently observe an upregulation in LAMC-2 protein during flow cytometry.

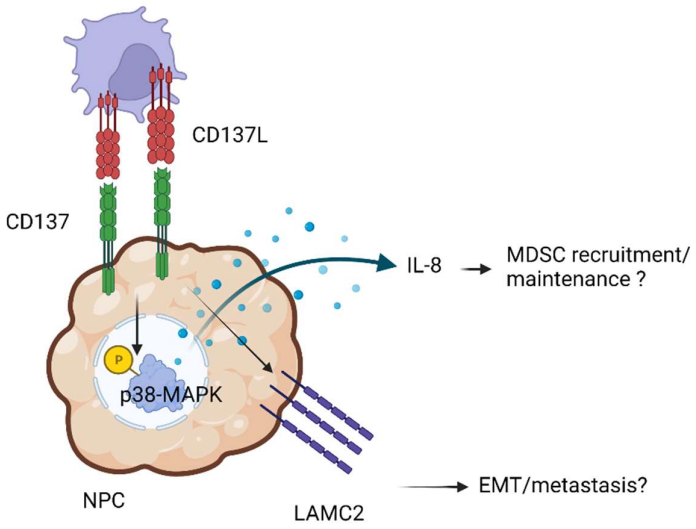

Figure 8. CD137 stimulation enhances p38 MAPK phosphorylation, IL-8 secretion and LAMC2 expression on NPC. Created with Biorender.

4.5 Paper IV. Fusion PD-1-CD137L inhibits the immunosuppressive effect of CD137, blocks PD-1 checkpoint signal and reinvigorates CD137 on activated T cells

Background

Malignant cells have been reported to successfully trigger immunosuppressive molecules, like PD-1 and CTLA-4 immune checkpoints, to evade immune destructions[151, 152]. These molecules originate from physiological pathways commonly employed by our body to regulate and limit damaging immune reactions to the host. Exploiting PD-L1/PD-1 pathway, tumour can upregulate PD-L1 expression, ligate PD-1 on infiltrating T cells, and transmit immunoinhibitory signals into these T cells.

In previous papers, we assess how tumours use CD137, a T-cell costimulatory receptor, to regulate our immune response to its advantage. Tumours secrete soluble CD137 to compete with CD137 on T cells for costimulatory CD137L signaling. Tumours also express CD137 to extract and downregulate CD137L from APC via trogocytosis. In addition to that, tumours receive signaling via CD137 to secrete inflammatory cytokines and upregulate activation pathways.

Hence, we would like to bring the fight against cancer, and neutralize its CD137, as well as PD-L1, killing two birds with one stone.

Aim

We aim to design and produce a recombinant fusion protein that combines the extracellular domain of PD-1 and CD137L. The PD-1 domain would bind to PD-L1 and PD-L2 on tumour surface and stop these two ligands from transmitting immunoregulatory signals via PD-1 to TIL. On the other end, the CD137L domain would bind to CD137 on tumour-specific T cells and bring those T cells close to the tumour and activate them. Effectively, fusion PD-1-CD137L would convert an inhibitory signal (PD-L1) to become a stimulatory signal (CD137L). Additionally, the free CD137L domain is able to capture free floating soluble CD137 and interfere with CD137-mediated trogocytosis.

Results

1. Designing PD-1-CD137L

As a control, a recombinant human CD137L protein with a TwinStrep tag (rhCD137L) that is previously produced in our lab is also included. For PD-1-CD137L construction, the extracellular domain of PD-1 (including the signal sequence) and that of CD137L are joined together by two linkers sequences flanking a stretch of 6 Histidine amino acids (6xHis tag). Being a type-1 transmembrane protein, the PD-1 extracellular domain is located at the N-terminus while the extracellular domain of CD137L, being a type-2 transmembrane protein, is located at the C-terminus. The 6x His tag is in the middle of the protein so that it does not

interfere with the PD-1 domain binding to PD-L1/L2 or CD137L domain binding to CD137. Although a 3D simulation shows that the 6xHis tag is accessible, its location may be masked during protein folding and impair protein purification. Thus, as a backup, one more version of PD-1-CD137L is also built with one Strep tag at the C-terminus, thus named PD-1-CD137L-Strep. Although a TwinStrep tag confers superior affinity to the purification column, a single Strep tag is selected for the fusion protein to minimize any interference to the binding affinity of CD137L. The purification tag should not be located at the N-terminus (where PD-1 is) because it will be cleaved together with the signal sequence of PD-1 during post-translational modifications.

The PD-1 signal sequence is essential to produce the PD-1 domain of the fusion protein. The lack of the PD-1 signal sequence leads to the production of deformed fusion protein for which the CD137L domain is still probably formed and recognized by anti-CD137L antibody, but PD-1 cannot be detected by anti-PD-1 antibody. When the signal sequence is included, both domains of PD-1-CD137 are observed.

2. PD-1-CD137L binds to both PD-L1 and CD137

Many cell lines express PD-L1. The rhabdomyosarcoma cell line Rd18 is chosen to test the binding of PD-1-CD137L because it expresses elevated level of PD-L1 and minimal CD137 or CD137L. The growth medium of HEK-293-6E cells transfected with PD-1-CD137L, is diluted 10-fold and used to incubate Rd18 cells for 10 minutes at room temperature. This allows the PD-1-CD137L fusion protein produced into the medium to bind to PD-L1 on Rd18, coat the cells and display CD137L instead. The undiluted supernatant of untransfected HEK-293-6E is used as the negative control. Indeed, we observe that most of the Rd18 cells become positive for CD137L. This also means that the PD-1 domain of PD-1-CD137L can bind to PD-L1 and thus, is functional.

We reason that if the CD137L domain of PD-1-CD137L is functional, it should bind to CD137, coat the $CD137^+$ cells and present PD-1 on the cell surface. Hence, we employ two NPC cell lines, C666 and HK1, and their $CD137^+$ variants (for which we have transfected CD137 so that the cells express CD137 constitutively). C666, C666-CD137, HK1, and HK1-CD137 are then incubated with supernatant of HEK293-6E cells (either untransfected or transfected with PD-1-CD137L) for 10 minutes and tested for PD-1 detection on the cell surface.

In general, the presence of PD-1-CD137L allows for higher level of PD-1 detected, even on WT C666 or HK1 cells, which do not express CD137. This is because both WT C666 and HK1 cells express PD-L1, which binds to PD-1-CD137L. Even when the PD-1 domain of PD-1-CD137L is bound to PD-L1, the anti-PD-1 antibody can still bind PD-1 and render the cells as PD-1 positive in flow cytometry analysis. For C666-CD137 and HK1-CD137, the CD137L domain of the fusion protein binds to CD137 and displays additional PD-1 on the cell surface for detection. We can also observe a positive correlation: the higher the CD137 expression level, the more PD-1 detected on the cell surface.

The superior binding capacity of C666-CD137 and HK1-CD137, as compared to that of C666 and HK1, respectively, is due to the expression of CD137, not due to expression of PD-L1 and PD-L2. In fact, C666-CD137 and HK1-CD137 have even lower levels pf PD-L1 and PD-L2 expression levels, as compared to that of their WT counterparts.

3. PD-1-CD137L and PD-1-CD137L-Strep purification

As seen in Western blot, both versions of the fusion protein can be purified, and both contain the CD137L domain. The expected size of the fusion protein is approximately 38 kDa. The bands being at slightly larger size 45 kDa could be due to protein glycosylation. Both PD-1-CD137L and PD-1-CD137L-Strep are then used to coat C666-CD137 cells. As expected, the CD137L domain of both fusion proteins binds to CD137 and renders the cells positive for PD-1 at the surface, while rhCD137L is used as a negative control. There is also a positive correlation like when we use the supernatant containing the fusion protein: the higher the level of CD137 expression, the higher the level of PD-1 captured on the surface.

4. PD-1-CD137L sequesters soluble CD137

sCD137 is secreted by tumours or Treg to interfere with membrane CD137 and disrupt T cell activation. The same tumour cells that secrete sCD137 can also express PD-L1 and can be targeted by PD-1-CD137L. As soon as sCD137 is released from the cell, PD-1-CD137L immobilized on the surface of the cell will use the free CD137L domain to capture sCD137 and prevent it from reaching out to the surrounding environment. Indeed, PD-1-CD137L can bind to PD-L1 on KM-H2 and act as a sink to trap secreted sCD137.

PD-L1 needs not come from the tumour. Tumours can evolve to downregulate their PD-L1 to escape our PD-1-CD137L. However, as long as there are cells in the TME expressing PD-L1, such as MDSC, those become agents to clear sCD137. As expected, the growth medium of the KM-H2 cell line passed through Rd18 cells coated with PD-1-CD137L shows a lower level of sCD137 as compared to the growth medium that was passed through uncoated Rd18 cells

5. PD-1-CD137L inhibits trogocytosis

As reported previously, tumours exploit an immunoregulatory mechanisms from Treg, CD137 trogocytosis. CD137 on the membrane of tumour cells or Treg can extract, or be extracted by, CD137L on APC. The outcome is that APC has less costimulatory CD137L available on the surface and hence, is less able to co-stimulate other T cells. Hence, we attempt to block this process by PD-1-CD137L fusion protein.

The CD137L domain of PD-1-CD137L can bind to CD137 on tumour cells and inhibit its interaction with CD137L on APC, thereby blocking trogocytosis. Indeed, while HEK-CD137-GFP cells can extract CD137L-mCherry from HEK-CD137L-mCherry cells during coculture, the presence of PD-1-CD137L inhibits this process and decreases the mCherry signal extracted into HEK-CD137-GFP cells.

6. PD-1-CD137L enhances T cell function

We hypothesize that once PD-1-CD137L infiltrates the TME, it will bind to PD-L1, mask this inhibitory signal, and instead present the costimulatory CD137L to activate T cells. Indeed, our preliminary data show that PD-1-CD137L reinvigorates T cells against the tumour. The presence of PD-1-CD137L enhances both IFN-γ and TNF-α secretion on activated T cells, in coculture with KM-H2 cells, a tumour cell line that readily expresses PD-L1.

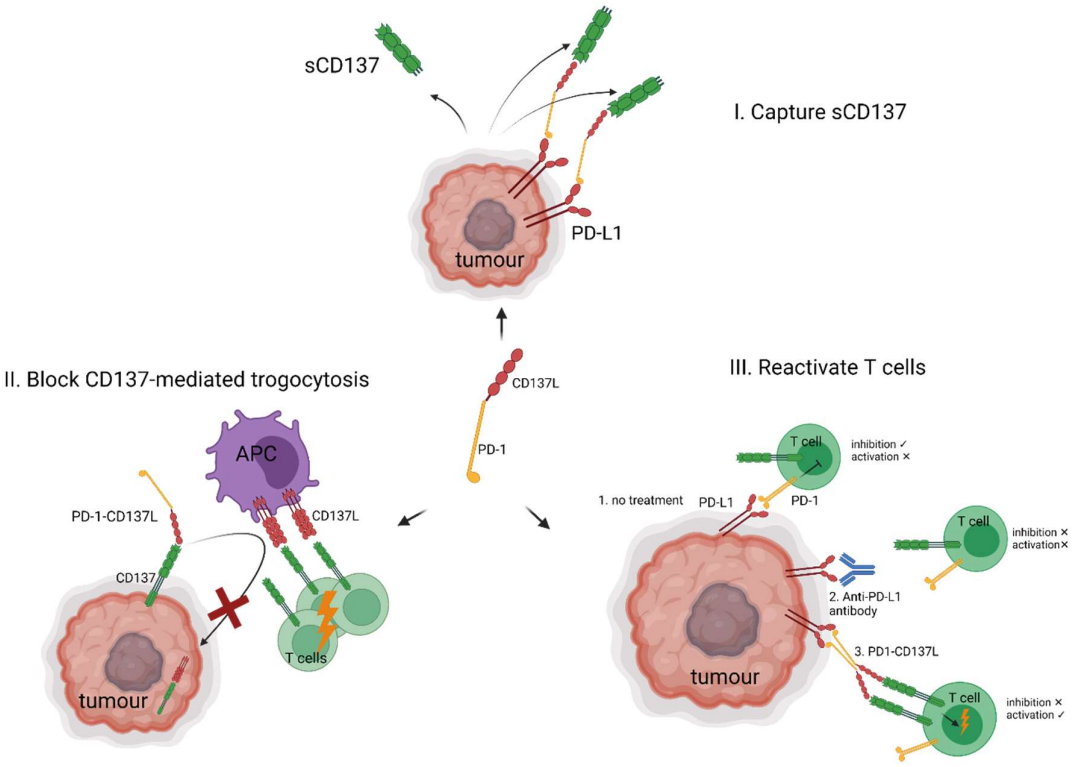

Figure 9. PD-1-CD137L sequesters sCD137, blocks trogocytosis and reactivates tumour-infiltrating T cells. Created with Biorender.

5 DISCUSSION

To evade immunosurveillance, rather than devising novel pathways, tumour generally exploits existing immunoregulatory pathways. These systems, evolutionarily conserved between species, keep our immune reactions in check and maintain self-tolerance. For instance, both CTLA-4 and PD-1 play an important role to limit autoimmunity, while still allows for protective immunity. Mice with CTLA-4 knocked out suffer and die from aggressive autoimmune diseases in as soon as three weeks of birth[153, 154]. Mice with PD-1 deficiency can survive to a later stage but they have a higher risk of autoimmune dilated cardiomyopathy and encephalomyelitis[155]. Because these checkpoints are so effective, tumours have successfully adapted these molecules for their survival advantage[156, 157]. Hence, although CD137 is usually involved in an anti-tumour response, it is plausible that tumours may adapt CD137 and exploit this pathway for its immunosuppressive properties.

5.1 Soluble CD137 interferes with membrane CD137 and disrupts immune responses

Tumours and chronic inflammatory conditions may tune down the immune response by secreting a soluble isoform of the receptor in the pathway, as is the case with soluble CD137. This approach by tumours is not new. Our immune systems occasionally secrete decoy receptors/ligands in the soluble form to regulate immune function, such as soluble CTLA-4[158, 159], soluble CD80[160], or soluble PD-L1[161]. The soluble isoforms could arise due to mRNA splicing or cleavage of the normal membrane-bound molecules[162]. These soluble isoforms can still bind to its target and compete with their membrane-bound counterparts.

For CD137, soluble CD137 is actively secreted by overactivated PBMC as a negative feedback mechanism, that prevents the immune response to get out of hand. This mechanism is exploited by tumour, and tumour cell lines such as KM-H2 and HDLM-2 actively secrete soluble CD137. Its secretion is also reported to be enhanced in the hypoxic environment, which is characteristic of the tumour microenvironment[163]. Soluble CD137 competes with membrane bound CD137 and interferes with T cell immunity functions, a fate shared by both tumours and chronic inflammatory conditions. Hence, it is plausible to detect soluble CD137 not only in the tumour microenvironment, but also serum of patients suffering from chronic hepatitis C infection. As documented in **paper I**, we also observe a correlation between serum soluble CD137 level and liver cirrhosis status. In these patients, higher level of soluble CD137 is associated with spleen length, extent of liver injury, and model of end-stage liver disease (MELD) score. We observe that during an immune response, soluble CD137 can interfere with helper T cell polarization into type 1 and 2, while maintaining type 17 population. Such T cell dysfunctions are consistent with the phenotype of the disease. The lack of type 1 and 2 polarizations may hinder the clearance of infected cells, while type 17 population can maintain the low basal level of inflammation, causing disease progression.

Thus, it is plausible that chronic inflammatory conditions and tumours prefer to express soluble CD137 to perpetuate their existence.

5.2 CD137 downregulates co-stimulatory CD137L via trogocytosis

In addition to secreting a decoy receptor in the form of soluble CD137, tumours also evolve to express CD137 on the cell membrane, not unlike how they usurp PD-L1 expression. It is logical that they prefer to express CD137 but not CD137L because CD137L would stimulate tumour-specific $CD137^+$ T cells. In fact, we rarely observe CD137L expression on solid tumour cell lines. We may find them on leukemia and lymphoma cell lines, possibly due to their origin being myeloid cells capable of expressing CD137L. Thus, we believe that tumour, especially solid tumours, purposely exclude CD137L from their microenvironment. Past publications from our group report that tumours with a virus infection history tend to express CD137. Certain viral protein such as LMP-1 or Tax-1 can induce CD137 expression in cells[147]. If tumours express CD137, they can downregulate CD137L through trogocytosis, as described in **paper II**. This function does not require any intracellular signaling because we show that a truncated version of CD137 lacking the cytoplasmic domain can also carry out the trogocytosis process. APC with their CD137L downregulated have less ability to co-stimulate T cells.

5.3 Tumour uses CD137 signaling for survival advantage

While intracellular signaling is not required for CD137 trogocytosis to remove CD137L and impair the immune response, it does not mean that CD137 intracellular signaling does not play a role. CD137 signaling typically activates three pathways in immune cells: JNK, p38 MAPK and NF-κB[149, 150, 164]. We observe that in NPC tumour cells, all three pathways are constitutively activated, as determined by phosphorylation status. However, CD137L can still stimulate CD137 on tumour cells and upregulate p38 MAPK phosphorylation levels, increase IL-8 secretion and LAMC2 expression, as shown in **paper IV**.

IL-8 has been well reported to be a chemokine to attract myeloid cells to the tumour microenvironment, which then can differentiate to become MDSC[165]. IL-8 can also attract MDSC themselves[166-168]. The presence of IL-8 in the microenvironment maintains the population of MDSC and help tumour avoid immune destruction. IL-8 can also promote tumour progression by activating the EMT process[169].

On the other hand, LAMC2 role is less clear. CD137 signaling consistently enhances LAMC2 expression across the three NPC cell lines tested. LAMC2 gene decodes Laminin gamma 2 subunit, an epithelial basement membrane protein. Recently, LAMC2 has been reported to promote migration and invasion via EMT and play a role in metastasis in cancers[170, 171]. Moreover, LAMC2 is also reported to be a biomarker to profile cancer[172] and a potential therapeutic target[173].

5.4 CD137 stimulation induces B7-H7 to regulate T cell immunity

As we study how CD137 stimulation can lead to immunosuppression in T cells, we notice the induction of B7-H7 expression (encoded by the gene HHLA-2), at the mRNA level [143]. This finding is peculiar because B7-H7 is usually detected on the APC population instead.

In **paper III**, after activating T cells, we also detect B7-H7 upregulation at the protein level which is dynamic and dependent on the stimulus. Blocking B7-H7 on T cells with recombinant CD28H significantly enhances T cell proliferation marker and activation marker. However, it should be noted that B7-H7 can have a dual function depending on the receptor it binds to, CD28H or KIR3DL3. CD28H is reported to be a costimulatory receptor and KIR3DL3 is a coinhibitory receptor[114]. Hence, targeting KIR3DL3, or at least the region on B7-H7 that binds to KIR3DL3, is a better therapy.

Our finding also identifies that B7-H7$^+$ T cells lack the ability to secret inflammatory cytokines like IFN-γ and TNF-α. Joining the ranks of PD-1, TIM-3, and LAG-3, B7-H7 could also further identify the population of exhausted T cells. In applications where functional T cells are selected for, such as adoptive T cell transfer therapy, B7-H7$^+$ T cells can be removed to improve the growth and function of the other T cells.

5.5 Fusion PD-1-CD137L presents a multi-prong immunotherapy

In **paper V**, we hypothesize that the fusion protein PD-1-CD137L can counter most of the immunosuppressive aspects of CD137 that tumours have adapted from us. Not only does it block PD-L1, the CD137L portion can inhibit CD137 on tumour to prevent trogocytosis process. Bound PD-L1 on tumour surface, it can also act as a trap to capture soluble CD137, so that any soluble CD137 secreted by the tumour cannot travel freely and far from its source. While anti-PD-L1 antibodies block PD-L1 coinhibitory signal, PD-1-CD137L provides an additional costimulatory CD137L signal. It brings the tumour cells closer to the tumour specific CD137$^+$ T cells for more effective anti-tumour immunity.

Many tumours express PD-L1. Even when their baseline PD-L1 expression level is low, upon effective immunotherapy, IFN-γ will be secreted and may induce higher level of PD-L1[174]. The more tumour relies on and expresses PD-L1, the more effective the treatment will be, the more IFN-γ secretion, and again more PD-L1 expression. This treatment has the potential to create a powerful positive feedback loop. Even when some tumour cells evolve to stop PD-L1 expression, other MDSC in the TME can still express PD-L1 and attract CD137$^+$ T cells over.

6 CONCLUSIONS

Using CD137 as a case study, we learn that tumours not only can benefit from adapting immunoinhibitory pathways such as PD-1 and CTLA-4, but they also can exploit seemingly immunostimulatory pathways. As our immune response can regularly become too strong and potentially harmful to nearby cells, even immunostimulatory pathways have negative feedbacks mechanisms in place to keep a check on themselves. Hence, tumours can exploit such loopholes to escape immunosurveillance. Our studies on CD137 could be an example for further studies on other immunostimulatory pathways.

As the PD-1-CD137L fusion protein counteracts tumours immunosuppressive mechanisms, we believe it has the potential to be the next generation of immune checkpoint inhibitors. The smaller size also means a simpler method of production and purification, and better penetration into the TME. Most importantly, this multi-target approach can be used for other targets, such as immunoinhibitory CTLA-4, TIM-3, LAG-3 and immunostimulatory CD40L, OX40, CD27. For a better outcome, we may even consider administering a cocktail of fusion proteins to patients.

7 ACKNOWLEDGEMENTS

This journey would not have been possible if not for the help of so many individuals, to whom I would like to take this opportunity to show how grateful I am.

First, I am deeply grateful for my family members, who have supported me wholeheartedly. My parents have not seen their son for two years and always say that everything is fine at home, so that I could concentrate on research. Despite my protest, there are hardships they shield me away from so that my work takes priority. They deserve my utmost gratitude.

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