

From Department of Oncology and Pathology
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FROM NATURAL TO EXIMIOUS: HARNESSING THE POWER OF NATURAL KILLER CELLS AGAINST SOLID TUMORS

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FROM NATURAL TO EXIMIOUS: HARNESSING THE POWER OF NATURAL KILLER CELLS AGAINST SOLID TUMORS

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What can change the nature of a man?

—Planescape: Torment

POPULAR SCIENCE SUMMARY OF THE THESIS

“Which system is the most important to maintain our country?” I asked myself before the Ph. D study. The answer varies between readers: economy, education, agriculture, transport system, culture, etc. My answer is “Military,” which protects the people from intense armed conflicts. A similar question happened to our body as well. “Which system is the most important to maintain your body?” The uncertainty such as viruses, bacteria, fungus, aged cells, stress, and cells with the mutation threatens our body. When I write my thesis, the COVID-19 has been spread worldwide for more than one year. Its spread has left national economies counting the costs. Travel plans have been put on hold and gatherings have transformed to online meetings. How can we survive this pandemic, and who can we ask for help? I believe the answer is ourselves, specifically, our immune system.

Most of us can still function properly and live life without constantly being sick due to our exquisite immune system, which works in a coordinated and synergistic way to exclude and clear those dangers. In brief, the innate assassins (Natural Killer cells) could sense and kill transformed “foreign” cells then subsequently send the smoke signal (inflammatory cytokines), which recruit special agents (myeloid cells), soldiers (T cells), and navy (B cells) to the battlefield. Through the release of tons of explosive bombs (perforin, granzymes) or precision-guided missiles (antibodies), we could kill those “foreign” invaders and collect the information (antigen presentation), which facilitates the army responses when the “foreign” invader comes again (memory formation).

NK cells are born to be at the forefront of the cancer-immunity war. Emerging evidence has been proved that higher intratumoral NK cell frequency correlated with better prognostic value in solid tumors. In contrast, NK cells could barely be detected from late-stage tumor patients. Tumors use various tricks to escape NK cell killing, like to beguile macrophage to immunosuppressive state and shed the surface identity, to create their unique immune-evade niche.

However, we found that “eximious” NK cells primed by cytokines could infiltrate tumors more than others. The chosen NK cells hold the promise to drive the cancer-immunity cycle from dysfunctional to normal.

The overall goal of this thesis is to understand how NK cell activity is regulated in solid tumors. Studies in this thesis focus on identifying “eximious” NK cells that are resistant to various immunosuppressive mechanisms, including prostaglandin E2 (PGE2)-**Study I**, reactive oxidative species (ROS) **Study II** and regulatory T cells (Treg) **Study III**.

ABSTRACT

Cancer heterogeneity, which enables clonal survival and treatment resistance, is shaped by active immune responses. Unchallenged results from clinical trials show the power of stimulating our immune system to attack tumor cells.

Engineered T cells and checkpoint blockade are at the forefront of current immunotherapy strategies. Whereas our immune system includes a diverse range of effector cells, which could directly or indirectly kill the target cells, and these immune cells must organize in a synergistic way to overcome multiple immune-evasion mechanisms and achieve complete tumor eradication.

An essential type of effector cell is natural killer (NK) cell. These are cytotoxic innate lymphocytes identified by their splendid capacity to kill virus-infected, stressed or transformed cells. *Ex vivo* expanded NK cells used for hematological malignancies showed promising results, associated with *in vivo* NK cells expansion after infusion. However, due to the limited growth factors in the tumor microenvironment (TME), infused NK cells undergo changes in their phenotype and ability to survive.

The type I cytokine family members IL-2 and IL-15 play a pivotal role to maintain homeostasis of the innate and adaptive immunity. Endogenous levels of IL-15 have been linked with sustained persistence of infused NK cells. Thus, the secret for NK cell resistance in the TME could be uncovered by investigating IL-15 primed NK cells under various forms of immunosuppression. **In study I**, we found that IL-15 primed NK cells acquire resistance against prostaglandin E2 (PGE2) mediated suppression by upregulation of phosphodiesterase 4A (PDE4A) in CD25⁺CD54⁺ NK cells. These CD25⁺CD54⁺ NK cells showed superior killing capacity under the suppression of PGE2 *in vitro* (2D and 3D culture) and *in vivo* (zebrafish model) experiments. **In study II**, we demonstrated that upregulated mTOR pathway primed by IL-15 lead to increased thiol density which protected not only NK cells but other lymphocytes against ROS in tumor microenvironment. **In study III**, we showed that upregulation of the IL-2 α receptor (CD25) in NK cells enables an immunometabolic competition of IL-2 in the TME between Treg and NK cells.

In summary, this thesis provides mechanistic insights for tumor-NK cell interaction and elucidates the potential therapeutic approach for harvesting "eximious" NK cells against solid tumors.

LIST OF SCIENTIFIC PAPERS

- I. **Chen Z**, Yang Y, Neo SY, Shi H, Chen Y, Wagner AK, Larsson K, Tong L, Jakobsson PJ, Alici E, Wu J, Cao Y, Wang K, Liu LL, Mao Y, Sarhan D, Lundqvist A.
Phosphodiesterase 4A confers resistance to PGE2-mediated suppression in CD25⁺/CD54⁺ NK cells.
EMBO Rep. 2021 Jan 22:e51329.
- II. Yang Y, Neo SY, **Chen Z**, Cui W, Chen Y, Guo M, Wang Y, Xu H, Kurzay A, Alici E, Holmgren L, Haglund F, Wang K, Lundqvist A.
Thioredoxin activity confers resistance against oxidative stress in tumor-infiltrating NK cells.
J Clin Invest. 2020 Oct 1;130(10):5508-5522.
- III. **Chen Z**, Tong L, Neo SY, Chen Y, Li SJ, Schlisio S, Lundqvist A.
CD25 bright NK cells display superior proliferative and metabolic activity and resist suppression by regulatory T cells.
Manuscript

RELATED PUBLICATIONS

Not included in this thesis

Journal Publications:

Huang WK, Gao J, **Chen Z**, Shi H, Yuan J, Cui HL, Yeh CN, Bränström R, Larsson C, Li S, Lui WO.

Heterogeneity of Metabolic Vulnerability in Imatinib-Resistant Gastrointestinal Stromal Tumor.
Cells. 2020 Jun;9(6):1333.

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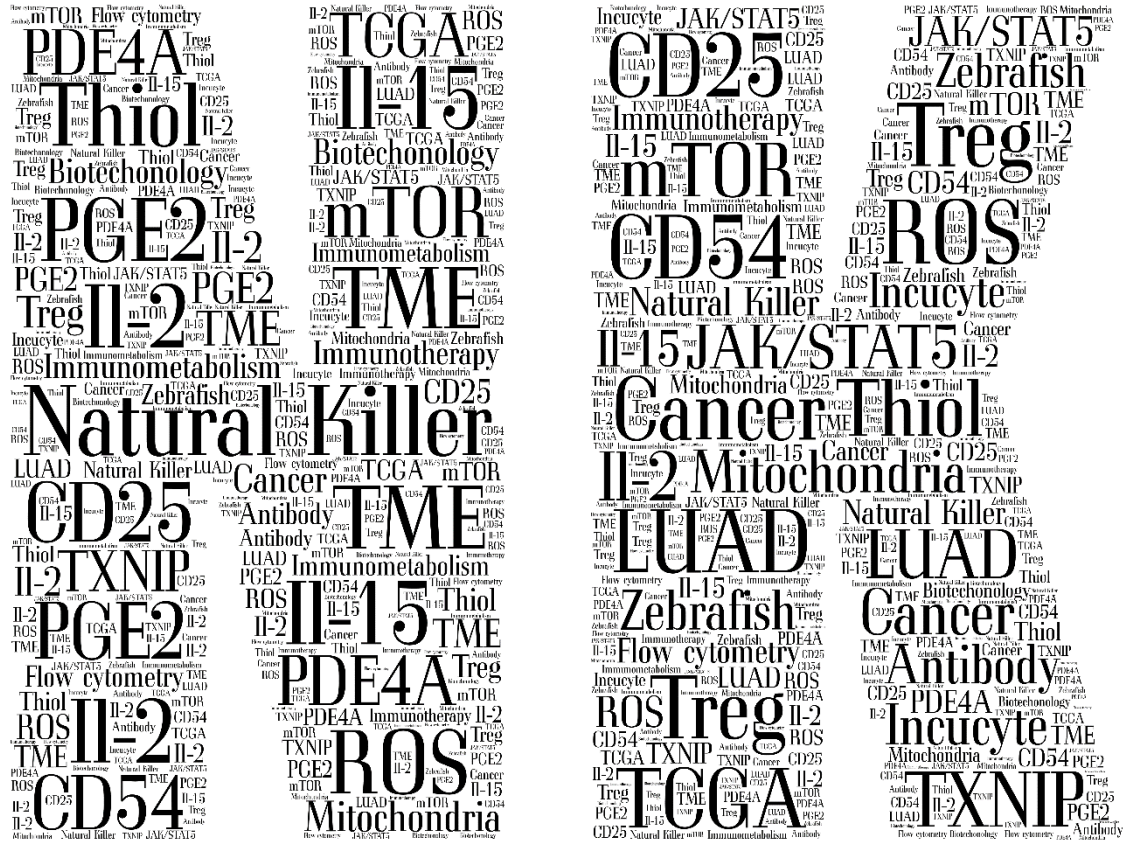
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LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ADCC	Antibody-dependent cell-mediated cytotoxicity
Bcl2	B-cell lymphoma 2
BiKE/TriKE	Bi- and Tri-specific killer engagers
cAMP	Cyclic adenosine monophosphate
CAR-T	Chimeric antigen receptor T cell
CISH	Cytokine-inducible SH2-containing protein
COX-2	Cyclooxygenase 2
CREB	cAMP response element-binding protein
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
EGFR	Epidermal growth factor receptor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HER2	Human epidermal growth factor receptor 2
HLA-E	Human leukocyte antigen-E
ICAM1	Intercellular adhesion molecule 1
iPSC	Induced pluripotent stem cells
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immune-receptor tyrosine-based inhibition motif
JAK	Janus kinase
KIR	Killer-cell immunoglobulin-like receptor
LAG-3	Lymphocyte activation gene 3
LFA1	Leukocyte function-associated molecule 1
LUAD	Lung adenocarcinoma
MDSCs	Myeloid-derived suppressor cells
MHC-I	Major histocompatibility complex 1
MICA/B	MHC class I polypeptide- related sequence A/B
mTOR	Mechanistic target of rapamycin
NCRs	Natural Cytotoxicity Triggering Receptors
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells

NKG2D	Natural killer group 2D
NSCLC	Non-small cell lung cancer
PD-1	Programmed cell-death protein 1
PD-L1/2	Programmed death-ligand 1/2
PDE4A	Phosphodiesterase 4A
PGE2	Prostaglandin E2
PTGES	Prostaglandin E synthase
RAET1	Retinoic acid early transcripts-1
ULBP	Unique long 16 (UL-16)-binding protein
RCC	Renal cell carcinoma
ROS	Reactive oxidative species
SCLC	Small cell lung cancer
SOX2	Sex determining region Y box 2
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TGF β	Transforming growth factor beta
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TILs	Tumor infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin 3
TME	Tumor microenvironment
TNF α	Tumor Necrosis Factor alpha
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
TXN1/2	Thioredoxin 1/2
TXNIP	Thioredoxin Interacting Protein
TXNRD1	Thioredoxin Reductase 1
VEGF	Vascular endothelial growth factor
VISTA	V-domain Ig-containing suppressor of T-cell activation



*The word cloud shows the keywords in this thesis. Created by wordart.com.

1 INTRODUCTION

1.1 Cancer

Cancer is characterized by the limitless proliferation of mutated cells with the ability to metastasize throughout the body. As a heterogeneous disease, cancer patients normally carries various genetic driver mutations which makes treating cancer extremely difficult and leads to resistance to traditional therapeutic agents (1).

The clonal selection model suggests that subsequential mutations gained by tumor cells over time lead to the selection of “fitter cells” that continue to grow and take over the tumor (2). With the help of modern RNA-sequence technology, the “Big Bang model” suggests that for some tumors, mutations occur in the initial stage when tumors are smaller, which could not be detected and target using traditional treatment (3, 4) (**Figure 1**).

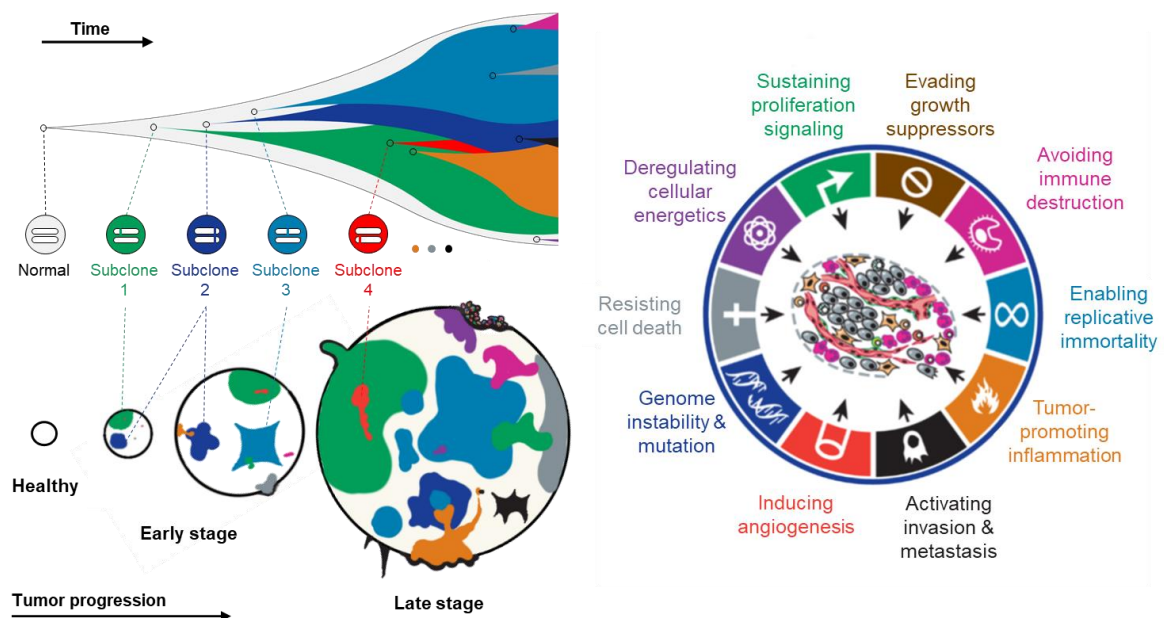


Figure 1. The Big Bang model of cancer development (left panel) and the ten hallmarks of cancer (right panel). Modified from Hanahan and Weinberg, *Cell*. 2011 Mar 4;144(5):646-74.

In 2011, Hanahan and Weinberg updated the hallmarks of cancer to include two additional immune-related features –"tumor-promoting inflammation" and "avoiding immune destruction" demonstrates the profound link between tumor cells and immune system (5) (**Figure 1**). In 2018, The Nobel Prize in Physiology or Medicine was honored to James P. Allison and Tasuku Honjo for “their discovery of cancer therapy by inhibition of negative immune regulation.” Based on their discovery, the checkpoint blockades proved to be strikingly effective in multiple clinical trials, which gives us confidence to clear the” enemy (tumor cells)” by using our own” army (immune cells).”

1.2 Immunotherapy

Cancer immunotherapy is a form of treatment that uses the ability of the immune system to fight cancer. Immunotherapy contains multiple strategies such as i) train the immune system to recognize and kill tumor cells ii) systematically stimulate the immune system to help them eliminate cancer iii) provide components to improve immune responses.

Various forms are included in cancer immunotherapy, such as: cancer vaccines, tumor-specific antibodies, oncolytic viruses, immune-modulatory cytokines, immune checkpoint inhibitors and adoptive cell transfer

(6, 7) (**Figure 2, Box 1**). Immunotherapies are a form of “living drug” since they take advantage from living organisms to fight cancer (8). Certain immunotherapies use gene editing method to enhance their cancer-fighting ability (9). Many immunotherapy treatments are also used in combination with conventional cancer therapies such as radiation, surgery, targeted therapies, or chemotherapy to improve their effectiveness.

Box. 1 Various categories immunotherapy

Cytokine: Cytokines are secreted proteins which provide signal to regulate cellular maturation, growth, and differentiation.

Oncolytic Virus: The antitumor effect of oncolytic viruses acts by directly infecting and lysing tumor cells, and simultaneously stimulate the immune system against the tumor.

Cancer Vaccine: Vaccines work by exposing individuals to a weakened or inactivated version of tumor specific antigen.

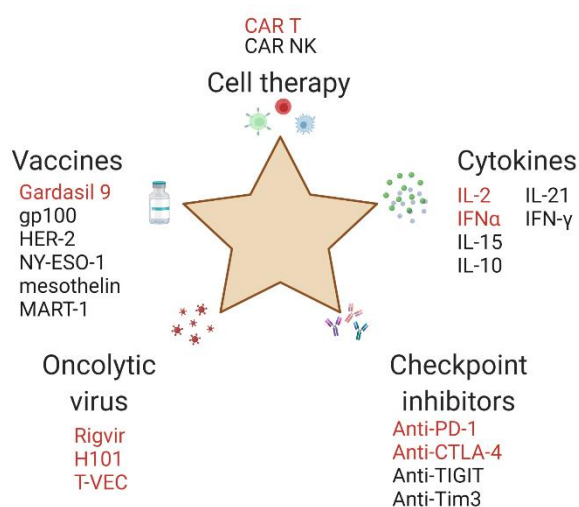


Figure 2. Five categories of cancer immunotherapy: Cell based therapy, cytokines, checkpoint inhibitor, oncolytic virus and cancer vaccine. Potential therapeutic targets or FDA approved treatments (labeled with red) are listed under each category. CAR T/NK- chimeric antigen receptor T/NK cell; IL-2, interleukin 2; IFN α , interferon alpha; PD-1, programmed cell-death protein 1; CTLA4, cytotoxic T-lymphocyte-associated protein 4; TIGIT, T cell immunoreceptor with Ig and ITIM domains; Tim3, T cell immunoglobulin and mucin domain-containing protein 3; gp100, glycoprotein 100; HER-2, human epidermal growth factor receptor 2; NY-ESO-1, New York esophageal squamous cell carcinoma 1; MART-1, melanoma antigen recognized by T cells 1. Created with BioRender.com

1.2.1 Checkpoint Inhibitors

The immune system with a fine-tuned function of its “machinery” has the ability to control the level of the immune response against foreign and self-antigens. “Overheating” immune reaction could be suppressed by immune checkpoint, which similar to “break” in our immune system (10). Antibodies targeted at these checkpoints can block the effector cells brake and unleash the immune system to fight against tumor cells.

One of the most well-studied immune checkpoint is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which is expressed at high levels on activated and regulatory T cells. Through binding to CD80/86 with a higher affinity compared with CD28, negative signal is transduced to prevent “overheating” T cells (11). Another checkpoint is called programmed cell-death protein 1 (PD-1) which is expressed on T or NK cells and its ligands programmed cell-death 1 ligand 1 (PD-L1) and/or PD-L2 which are normally expressed on tumor cells. The ligation of PD-L1/PD-L2 and PD-1 leads to inhibition of T and NK cell function. In 2019, there were 2975 active clinical trials to test PD-1/PD-L1 monoclonal antibody alone or in combination with other therapeutic reagents (12, 13).

The efficiency of checkpoint blockade is particularly documented in melanoma patients. The efficacy of single-agent PD-1 inhibitor in patients with advanced melanoma could reach 33% to 45% overall survival. By combining anti-PD-1 and anti-CTLA-4, the response rate could improve from 19% (single CTLA-4), or 43.7% (single PD-1) to 58% (14, 15).

Recently, the combination of the anti-PD-1 and anti-CTLA-4 therapy demonstrated durable and long-term clinical responses in NSCLC patients (phase III, Checkmate-227). At three years, the overall survival rate was 33 and 34 percent for patients with PD-L1-positive and PD-L1-negative tumors, respectively, compared with 22 and 15 percent for platinum-doublet chemotherapy (16). Furthermore, patients with advanced stages of bladder (NCT02603432), kidney (17), small-cell lung cancer (SCLC) (18), microsatellite instability (MSI)-high cancers (19) as well as melanoma have responded well to immunotherapy. Promising results from clinical trials leads to several checkpoint immunotherapies for multiple cancers become the standard of care in some cases (20).

However, two major questions for checkpoint inhibitors still need to be answered. One is that nearly approximately 50% of patients do not achieve significant clinical response; another is that a substantial proportion of responders will have a tumor relapse within two years (21-24). Collective efforts have been put to decipher the resistance mechanisms to immune checkpoint inhibitors. Tumor cells take advantage of TME to limit T-cell activation, tumor infiltration partly explained these resistance mechanisms (25). For instance, IFN- γ signaling plays a central role in T-cell mediated antitumor immunity. By upregulating MHC-I molecule, IFN- γ could promote tumor antigen presentation, which could further facilitate DCs and NK cells activation, and inhibit tumor cell proliferation. Decreased expression of IFN related genes have been identified in Ipilimumab-refractory melanoma patients. Specifically, loss of interferon-gamma receptor 1 (IFNGR1), IFNGR2 and interferon regulatory factor 1 (IRF1) in tumor cells leads to resistance to anti-CTLA-4 antibody (26).

A documented mechanism of acquired resistance to immune checkpoint therapy is the upregulation of other immune checkpoints on T cells. Upon the gained knowledge of tumor-resistance mechanism, antibodies targeting such alternative immune checkpoints have been developed including antibodies against: T-cell immunoglobulin and mucin 3 (TIM-3) (27, 28), lymphocyte activation gene 3 (LAG-3) (29, 30), V-domain Ig-containing suppressor of T-cell activation (VISTA) (31), CD47 (32, 33) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (34, 35).

Other resistance mechanisms of immune checkpoint inhibitor have also been identified such as the presence of immunosuppressive cytokines (TGF β , IL-10) and other immunoregulatory factors (e.g. adenosine, PGE2) present within TME (36). Putative therapeutic strategies will be boosted by re-gained understanding from ongoing clinical and basic onco-immunology studies.

1.2.2 Cell-based therapy

Cell-based immunotherapy is a treatment that builds on harvesting immune effector cells such as T or NK cells and stimulating these *ex vivo* and then transfer back to the cancer patients (**Figure 3**). The differences among categories depend on either source of the effector cells or the way to arm effector cells during *ex vivo* expansion.

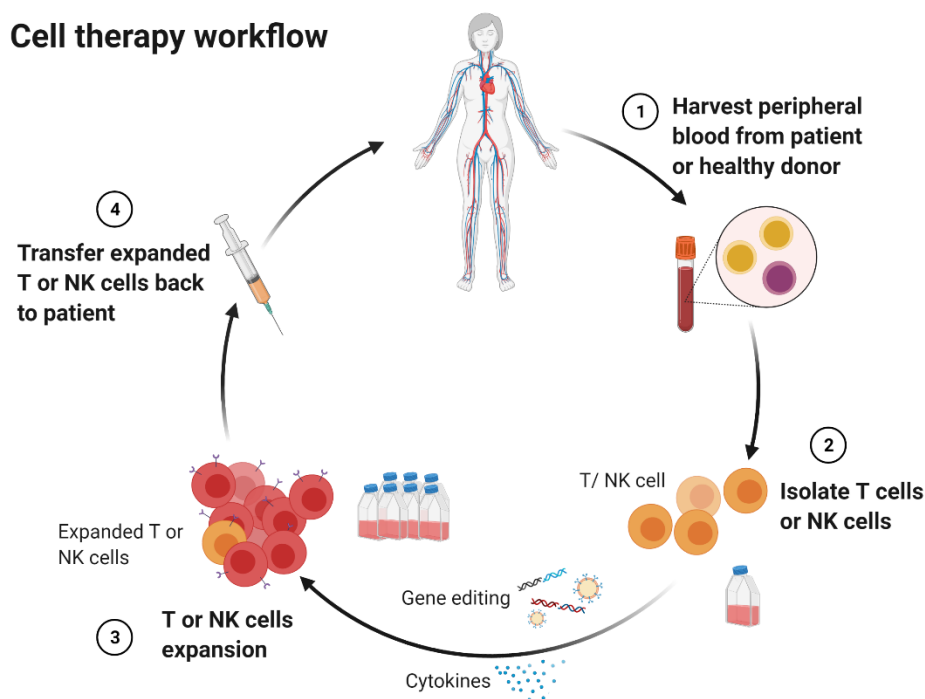


Figure 3. The general workflow for cell therapy includes 4 steps: harvest peripheral blood, isolate the effector cells, *ex vivo* manipulate effector cells expansion by either cytokines or gene editing method, and transfer expanded effector cells to patients. The graph is created by using Biorender.

1.2.2.1 Tumor-infiltrating lymphocyte therapy

The success of employing tumor-infiltrating lymphocytes (TILs) to treat metastatic melanoma was achieved by Rosenberg's team in the late 1980s (37). IL-2 was used not only to *ex vivo* expand TILs isolated from a cancer patient, but also as cytokine support of infused TILs. The objective response rate was 34% in 86 melanoma patients; however, the short median duration

Box. 2 Neoantigens

Unique antigens that are not expressed by self-tissues under normal conditions that manifest in the context of pathology. In tumor cells, these could be altered proteins/peptides encoded by mutated genes.

(only 4 months) and few complete responses lead to hesitation for using TILs as a therapeutic reagent. However, thanks to the next generation of high-throughput technologies the screening and enrichment of **neoantigen**-specific TILs (**Box 2**) is achieved in metastatic breast cancer patients (38). Furthermore, knockdown of a JAK/STAT signaling negative regulator of CISH shown to boost the anti-tumoral response of TILs therapy in a mouse model (39). Other

innovative targets to enhance effector cells activity may allow for a more promising treatments to be developed.

1.2.2.2 Chimeric antigen receptor cell therapy

Due to that synthetic chimeric antigen receptor (CAR) recognizes target molecule on malignant cells, CAR T cells could by pass MHC restriction and direct kill the target cells. The clinical success of CAR T cell therapy for the treatment of B cell acute lymphoblastic leukemia (ALL) (40), chronic lymphocytic leukemia (41), non-Hodgkin lymphoma (42) is due, in part, to targeting the CD19, a specific antigen that has high surface expression in certain B cell malignancies. In addition to directly kill target cells, CAR T cells can also reform the inhospitable TME and revive exhausted T cells (43). For instance, the suppression of myeloid cells and regulatory T cells in the TME could be overcome by CAR T cells engineered to produce IL-12, which could also promote CD8⁺ T cell cytolytic activity and enhance myeloid cell recruitment and antigen presentation (44, 45). Despite the hurdles within TME in solid tumor, with current successful CAR T cells immunotherapy for B cell malignancies, it will be interesting to continue and expand research on this new treatment strategy.

1.2.2.3 Engineered TCR T cell therapy

Not all patients have unique T cells that recognize tumor antigen. One of the reasons is that these T cells may not be able to be primed and expanded to sufficient numbers for adoptive cell transfer (46). To overcome this, engineered TCR T cells therapy has been developed to encode receptors that recognize tumor-specific antigens (47). Prolonged survival and migration to the tumor site could be achieved by encoding cytokines into engineered TCR T cells (48). TCR-T cells recognizing the tumor antigen NY-ESO-1 have been used to treat patients with advanced melanoma which can result in durable complete responses (49). Personal cancer medicine could be one of the future directions for TCR T cells. By allowing design an “right” target for each patient’s tumor and use distinct resources of T cells ($\gamma\delta$ T cells) to engineer, the therapeutic benefits could offer patients with greater hope.

1.2.2.4 Natural killer cell therapy

NK cells recognize tumor cells by mechanisms, that rely on a set of stimulatory and inhibitory receptors. These receptors can sense whether a nearby cell expresses a profile of corresponding ligands associated with oncogenic transformation leading to NK cells activation and killing (50). Due to the lack surface T cell receptors, NK cells have been shown to not cause graft-versus-host disease (**GvHD, Box 3**) (51). Thus, NK cells hold promise as an ‘off-the-shelf’ cell therapy product, which can be prepared in advance, and injected on demand to multiple recipients. Emerging data show an essential role of tumor-infiltrating NK cells to govern immunotherapy response (52).

According to the principle of “missing self” recognition (53-55), NK cells recognize target cells that do not express MHC class I molecules. They express a series of cell surface inhibitory receptors which is killer-cell immunoglobulin-like receptor (KIR) family that recognize major histocompatibility complex I (MHC-I) on target cells (56), and the NKG2A/CD94 heterodimer for HLA-E molecule (57).

The implementation of NK cell transfer was spurred on based on beneficial effects of NK cell alloreactivity in the setting of allogeneic hematopoietic cell transplantation (allo-HCT) (58). Alloreactivity of NK cells are triggered by mismatched KIRs on NK donor cells and MHC-I on recipient cells. Alloactions mediated by mismatched NK cells has been shown to eliminate leukemia through graft-versus-leukemia (**GvL, Box3**) effect. Furthermore, alloreactive NK cells can promote engraftment through depleting recipient T cells and protect against graft-versus-host disease (GvHD). Host NK cells can also target recipient antigen-presenting cells and thereby also limit GvHD reactions (59, 60). However, host Treg cells maintain and expand effectively when IL-2 is administered after NK cell transfer in ovarian cancer, breast cancer and refractory lymphoma (61, 62). The cytolytic ability of NK cells impaired by expanded Treg through TGFβ secretion and deprivation of local IL-2 (63, 64). Miller and colleagues employed a Treg depletion method using IL-2 diphtheria toxin together with adoptive NK cells transfer. This combination strategy improved complete response rate at day 28 (53% versus 21%; $P = 0.02$) and disease-free survival at 6 months (33% versus 5%; $P < 0.01$) for AML patients (65).

Low NK-cell infiltration in solid tumors reveals that the tumor microenvironment might grab the key to uncover how to increase NK cell persistence (66). The mechanism of primary and secondary resistance to cancer immunotherapy are manifold, deriving not only from the intrinsic heterogeneity of cancer cells but also from the intricate interplay between tumor cells and their surrounding TME (67).

1.3 Tumor microenvironment – The real battle field

As discussed previously, cancer progression is not only determined by driver mutation but also by the surrounding environment or cells. This environment provides critical factors to interfere with immune surveillance and thereby promote cancer progression and tumor dissemination (**Figure 4**).

Solid tumors comprise of malignant cells as well as vascular endothelial cells, mast cells, fibroblast cells, T cells, B cells, and several other cellular components of innate immune system including neutrophils, eosinophils, macrophage, NK cells. In addition, the TME constitutes of several extracellular soluble factors such as hormones, chemotactic factor, and cytokines. The TME also includes specialized cellular subsets including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T (Treg) cells (68). It is also characterized by altered pH levels, nutrient balance (glucose and fatty acids, etc.), metabolites,

Box. 3 GvHD, GvL

In Graft versus host disease (**GvHD**), the donated bone marrow or peripheral blood stem cells view the recipient’s body as foreign, and the donated cells/bone marrow attack the body.

Graft-versus-leukemia (**GvL**) reaction describes the ability of immune cells from the donor to attack host leukemic cells.

and oxygen levels (69-72). Interestingly, recent studies proved that bacterium and fungus could benefit tumor growth (73, 74). This unique TME provides essential nutrients, survival signals and simultaneously suppresses immune surveillance, together contributing to tumor progression and metastasis.

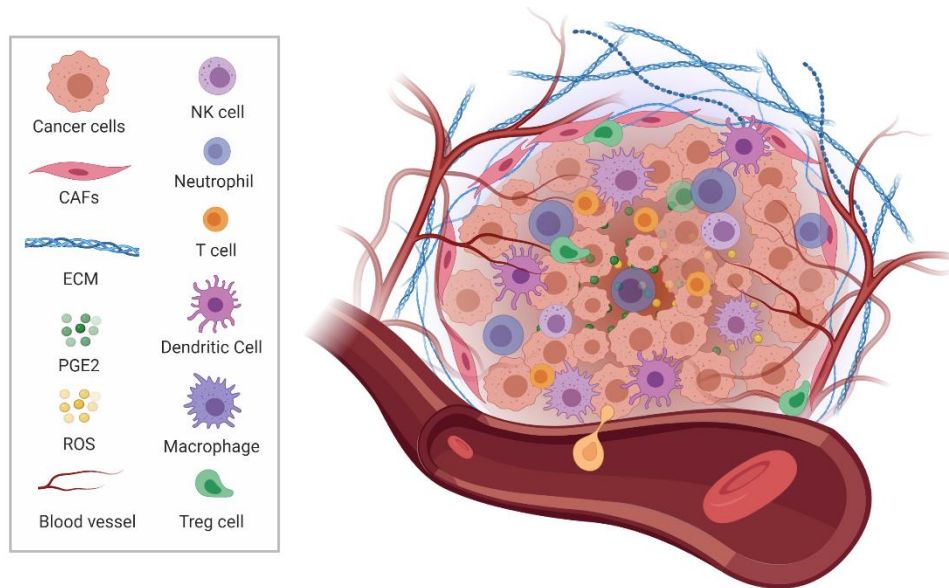


Figure 4. An overview of different cell types within the tumor microenvironment. Several immune cells together with cancer associated cells contained in TME surrounding by suppressive factors (PGE2 and ROS). CAF, cancer associated fibroblast, ECM, extracellular matrix. The graph is created by using Biorender.

“Hot” and “Cold” TME

The understanding of the differential composition of immune cells in TME is needed, which had a great impact on the responses of various immunotherapies. Moreover, the organization of immune cells in TME could change among different patients. Thus, mapping the distribution of immune cell infiltrates and their functional state is important in terms of evaluation and the design of therapies (75, 76). Here, I present a summary of recent novel technologies that might help us gain new insights for TME (**Box 4**).

The TME can be crudely classified as cold or hot, where a cold and hot TME is characterized by low and high frequency of T cell infiltration (77). Cold tumors are sometimes also described as “immune deserts.” (78). In general, patients with hot tumors has been found to respond better to immune checkpoint therapy with anti-programmed death ligand (PD-L)1/PD-1 (79).

Box. 4 Technologies for TME

Single cell RNA-seq, next-generation sequencing technologies applied to single cell level which provide high resolution of cellular differences within sample.

Spatially resolved single-cell RNA-seq, A new technology-driven field in which single-cell genomic data is derived from tissues by means to preserve spatial information.

Expansion microscopy, biological sample magnified smoothly and isotropically by swellable polyelectrolyte hydrogel where molecules in a diffraction-limited region are separated in space to greater distances, and can therefore be resolved by conventional diffraction-limited microscopes.

Assembloids, assembly of multiple organoid structures to gain deeper insights into tissue function.

There are several factors in the tumor site that drives the TME towards “cold,” which might contribute to the failure of immunotherapy, including but not limit to prostaglandin E2 (PGE2), reactive oxidative species (ROS), and regulatory T cells.

1.3.1 PGE2

Several soluble factors produced by tumor cells or tumor-associated cells shape the tumor microenvironment and inhibit the function of tumor-infiltrating cytotoxic lymphocytes. One such soluble factor is prostaglandin E2 (PGE2) known as a bioactive lipid that elicits multiple biological effects associated with inflammation (80, 81). PGE2 can be produced from different type of cells, for example, stressed neutrophils, fibroblasts, macrophage, MDSCs and Treg cells. The arachidonic acid (AA) mobilized by phospholipase A2 (PLA) family to cytoplasm, where cyclooxygenases take responsible to convert AA into prostaglandin H2. Finally, prostaglandin E synthase transfer PGH2 to the final formation --- PGE2 (82).

By binding to prostaglandin E2 receptors (EP 1-4), which belong to G protein-coupled receptor (GPCR) family, PGE2 turns the outside-in signals via cyclic adenosine monophosphate cAMP-CREB axis (83). As one of the major immunosuppressive factors, pro-inflammatory PGE2 is a critical mediator in the crosstalk between tumor epithelial cells and their surrounding immune cells in establishing an immunosuppressive tumor microenvironment (84).

Multifaceted roles of PGE2 has been discussed in cancer progression. As pro-inflammatory factors, PGE2 originally discovered to promote the tissue influx of macrophages and neutrophils from bloodstream leading to swelling at the site of infection or damaged tissue (85, 86). However, PGE2 also governs a number of mechanisms that regulate inflammation and subsequent tissue repair (87, 88). One important effect of PGE2 is to directly inhibit the synthesis of IL-2 and the expression of the IL-2 receptor in Th cells (89, 90). Moreover, PGE2 suppress anti-tumor activity of NK cells and cytotoxic T cells, partly by down-regulating cytokine receptor expression (91, 92). Our recent results showed that PGE2 can indirectly downregulate NK cell activity by increase TGF- β production in myeloid derived suppressor cells (MDSCs) (93).

1.3.2 ROS

The release of ROS by the host immune system is a natural mechanism for effector cells like macrophages and neutrophils to respond to pathogens (94). ROS function as important messenger molecules that can act intracellularly through the mitochondria (95). ROS contribute to tumorigenesis by affect multiple prospect such as cell proliferation, genomic instability, inflammation and metabolic reprogramming (96). Despite the intrinsic molecular mechanism, another way for ROS to achieve the promotor role in tumor progression is through immune suppression (97, 98). Due to their reactiveness, cells have multiple mechanisms to maintain the homeostasis of ROS such as scavenging systems of thioredoxin and glutathione (99).

The tumor microenvironment is known to be rich in ROS. Tumor beguiled cells, for example, tumor-associated macrophage, neutrophils and MDSCs can release massive amount of ROS (100, 101). Upon exposure to ROS, lymphocytes like T effector cells and NK cells loss their anti-tumor activity.

1.3.3 Treg-induced suppression

As a master regulator in our immune system, regulatory T cells (Tregs), identified as CD4⁺CD25⁺Foxp3⁺ cells, play crucial roles in maintaining homeostasis of tumor immunity (102). Tregs can also suppress the function of immune effector cells through i) cytokine deprivation ii) secretion of immunosuppressive cytokines such as TGFβ, IL-10, IL-35; iii) direct cytolysis; iv) cell-cell contact ligation (CTLA4-CD80/CD86)(103).

Lately, reinvigorated efforts have been made to describe the suppressive mechanisms through 'metabolic disruption.' A long-standing discussion in the Treg-cell field is if the high expression of CD25 enables Treg cells to take advantage of local IL-2 and thereby starve activated effector T cells or NK cells by consuming the IL-2 (104). A study showed cytokine (specifically IL-2)-deprivation-mediated apoptosis induced by Treg cells might contribute to a "cold" TME (105).

Promising results have been shown to combine checkpoint blockade with CD25-Treg-depleting antibody (106). By using a fucosylated anti-human CD25 antibody, efficient Treg depletion with no overt immune-related toxicities was observed in both nonhuman primates and humanized mouse model. Strikingly, single dose of anti-CD25 induced a 52% CR. Administration of a second dose led to a 70% CR in MCA205 bearing mice (107).

Depletion of metabolites in a hypoxic TME leads to dysfunction of infiltrated effector cells. McLane et al. showed Treg could upregulate the metabolism pathway related to lactic acid which make Treg more tolerated in lactic acid enriched TME. By knocking out the key lactate transporter gene, MCT1, they found that the MCT1 is required for maintaining Treg function in TME, but not in peripheral blood. Thus, the metabolic adaption of Treg could furthermore help tumor cells to avoid immune destruction(108).

1.4 NK cells – *The assassins*

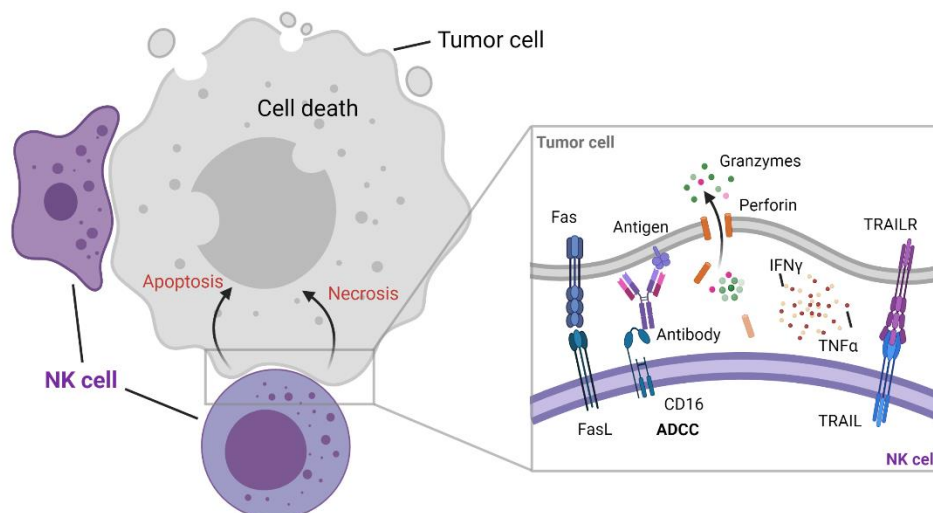


Figure 5. Different mechanisms of NK cell killing. ADCC, antibody-dependent cell-mediated cytotoxicity; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR, TRAIL receptor. The graph is created by using Biorender.

In the mid-1970s, NK cells were first identified as a lymphocyte subpopulation with the ability to kill transformed cells without prior sensitization (109, 110). NK cells and other lymphoid cells originate from the same common lymphoid progenitor cells. The type I cytokine, IL-15, has been found to be important to drive the development and maturation of NK cells (111).

Donna et al generate the high-resolution map of human tissue-driven NK cells across age. In blood, NK cells comprise approximately 2-18% of the total leucocyte pool. In other tissues such as BM, spleen, and lung, NK cell frequency can be as high as 50% of total lymphocytes. NK cells are broadly classified as CD56^{bright} or CD56^{dim} cells, where the CD56^{dim} NK cell population dominates in blood whereas the CD56^{bright} are often observed at higher frequencies within tissues (112).

NK cells as the frontline army, perform complementary roles in an earlier immune response against viruses and tumors. Approximately 90% of NK cells in the blood are CD56^{dim} which respond directly to infection or cancer through antibody-dependent cell-mediated cytotoxicity (ADCC), IFN γ , perforin, granzyme, FasL, or TRAIL (**Figure 5**) (113). CD56^{bright} NK cells occupy nearly 10% of blood NK cells, and they participate in cytokine secreting IFN γ , TNF α , G-CSF, GM-CSF, and IL-3, which are generally delivered in late (>16 hours) inflammatory response (114). Activation of NK cells are arranged by a suite of activating, co-stimulatory and inhibitory receptors. Analogous to an assassin pulling the trigger of a gun, target cell lysis occurs when the activating signal (kill) dominates the inhibitory signal (not kill) (115).

1.4.1 To kill or not to kill: NK cell recognition and signal balance

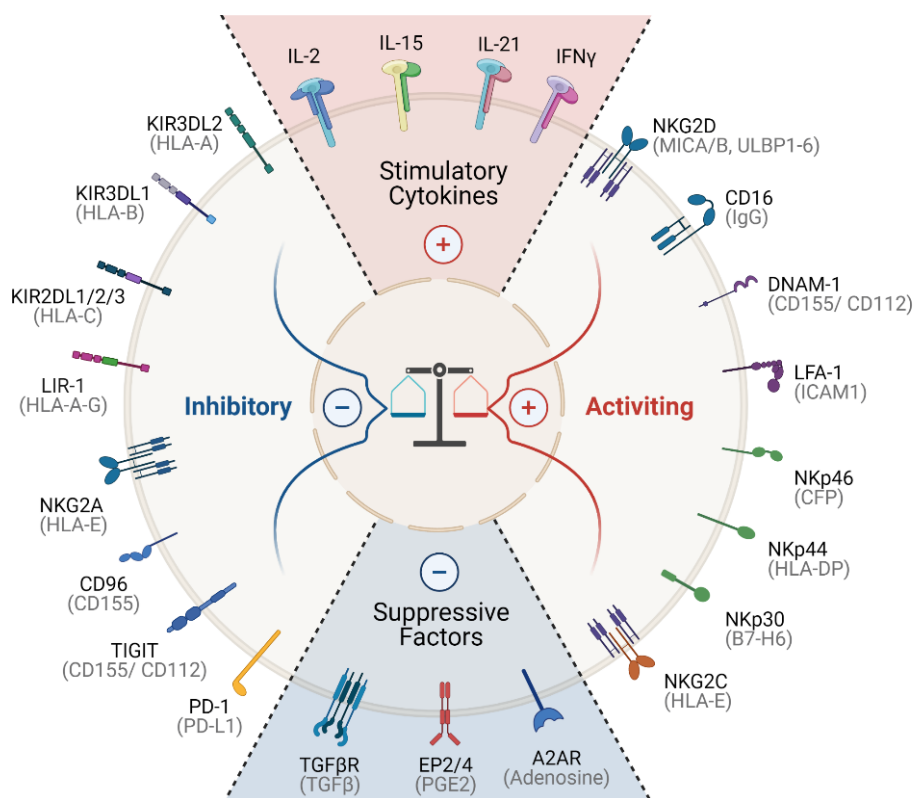


Figure 6. Examples of activating and inhibitory receptors and ligands in NK cells. Cytokine receptors (top) and suppressive factor receptors (bottom) are shown on human NK cells. Inhibitory receptors and activating receptors are shown on the left and right side respectively, which could transduce the signal “out-side-in”. The killing decision decided from various signals. The receptors and their ligands (in parentheses) are depicted in this graph. DNAM-1, DNAX accessory molecule-1, CFP, Complement factor P, LIR-1, leukocyte immunoglobulin-like receptor 1, A2AR, adenosine A2A receptor. The graph is created by using Biorender.

The joint signals from a suite of activating, co-stimulatory and inhibitory receptors determine whether an engaged cell is killed or not (**Figure 6**). The activation signal is transduced from engaged receptor via intracellular immunoreceptor tyrosine-based activation motifs (ITAMs)

which in turn initiate the phosphorylation cascades. CD16 is the one of the most essential activating receptors for NK cells. The Fc region of IgG antibodies could crosslink with CD16, as known as Fc region receptor III, which activate the ADCC process. Evidences showed through binding to same target with but different epitopes, enhanced ADCC effect through NK cells was observed in combining trastuzumab and pertuzumab (anti-HER2) (116).

Another important family members of NK cells are the natural cytotoxicity receptor family (NCRs), including NKp46 (NCR1), NKp44 (NCR2), NKp30 (NCR3), NKp40, NKp65 and NKp80 can initiate activation signals in NK cells; through binding to viral, bacterial, and tumor-associated ligands these receptor could enhance the production of cytokine and cell killing (117). Since NKG2D and NKG2C are activating receptors, antibodies developed to stimulate their downstream signals has gained more attention. By taking advantage of NKG2D-null mice, Guerra et al. proved a role for NKG2D in the initiation of spontaneous and transplantable tumor mouse models. These results suggested that the selection of lower NKG2D ligands could benefit tumor to escape from immunosurveillance at the beginning of immunoediting. (118). NKG2C forms a dimer with CD94 and its activation is dependent on binding to non-classical HLA- E.

Engagement of leukocyte function-associated molecule-1 (LFA1) has been shown to potentiate NK cell function *in vitro*, such as the production of TNF and IFN γ . The ligand for LFA1, intercellular adhesion molecule 1 (ICAM1) is an integrin that transduce a mechanical signal upon binding LFA1 (119-121). Recent studies have provided evidence that the function of ICAM1 in tumor cells instead of NK cells, which in our **study I** showed activating NK cells could increase the ICAM1 expression which form more immune cluster *in vitro*. The spatial organization of NK-NK bonds, via ICAM1-LFA1, could be interesting to explore of in tumor animal models.

NK cells also express a wide repertoire of inhibitory receptors, which provide negative-feedback that can counteract stimulatory signals (122). One of the most studied family of inhibitory receptors are the members of the killer cell immunoglobulin-like receptor (KIR) family (123). Each individual expresses a specific set of KIRs. 16 KIR genes have been described in human, the highly polymorphic of these genes constructed 1,110 variations (IPD-KIR Database, 2.10.0). Inhibitory KIRs contain immune-receptor tyrosine-based inhibition motif (ITIM) sequences in the intracytoplasmic tail responsible for the inhibitory signal. The canonical role for KIRs is provide inhibitory signals via ligation with MHC class I molecules. However, activating KIRs can associate with ITAM-bearing molecules to transmit an activating signal, which could associate with infectious diseases, pregnancy-associated disorders and cancers (124, 125). KIRs and other inhibitory receptors with their cognate ligands expressed in tumors is an interesting strategy for NK cell-based cancer therapy.

1.5 Cancer-NK cell immunity cycle

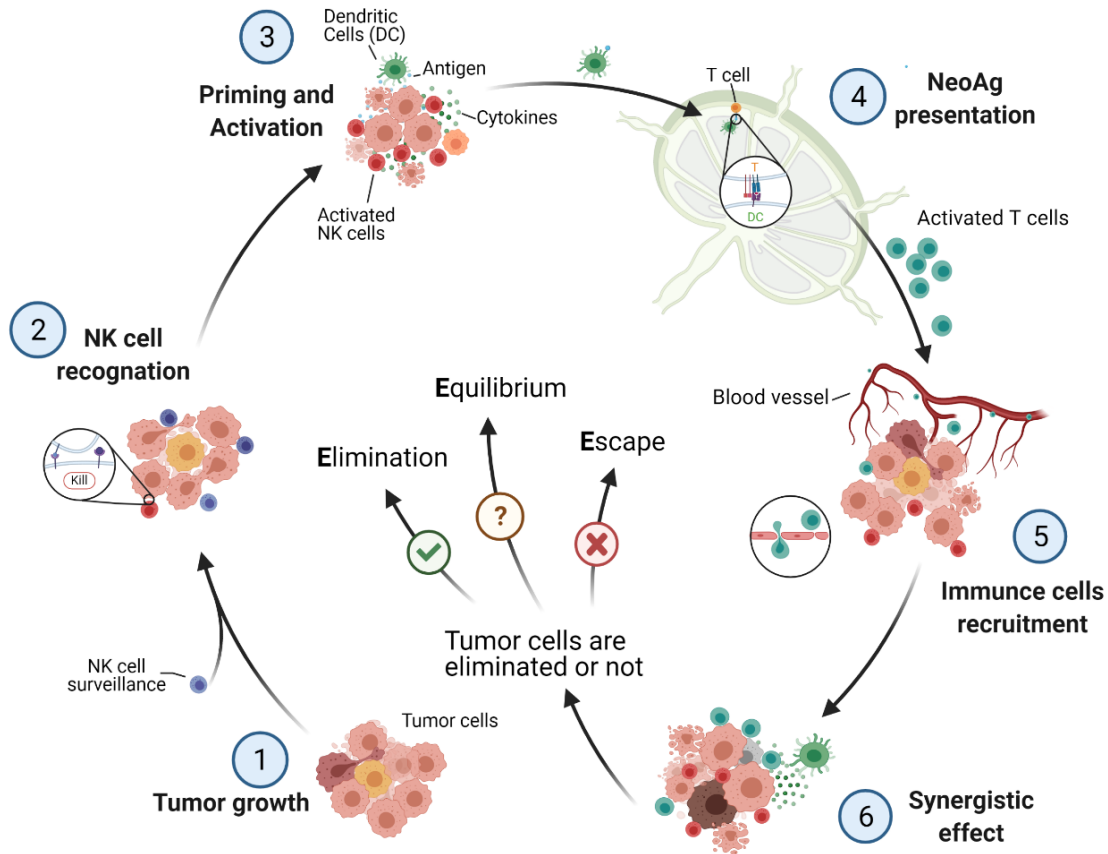


Figure 7. Cancer-NK cell immunity cycle. As primary tumor cells grow, NK cells could kill sensitive tumor cells, which released tumor-specific antigens that could recruit dendritic cells to the tumor lesion. Then, by homing to the drain lymph node, dendritic cells could further stimulate T cells, moving to the tumor site and coordinating with NK cells to kill the tumor cells. The results from the elimination determined the stage of immune editing. The graph is created by using Biorender.

The rationale to use NK cells in the clinic comes from result that NK cells can kill both autologous and allogeneic tumor cells (126). NK cell therapy was initially viewed as a strategy to debulk tumors. Emerging data suggest that this understanding is inadequate and that the full landscape of NK cell functional outcome needs to be reevaluated. Besides the quick release of lytic granules upon target recognition, NK cells are the main producer of $IFN\gamma$ in the early tumor recognition phase. The final decision of NK cell killing is controlled by the fine-tuned balance of a set of activating and inhibitory signals and is further regulated by its differentiation state and factors secreted from local TME. In light of the cancer immunity cycle, NK cells play a major role in multiple steps, which drives the cycle towards eliminating cancer cells (**Figure 7**).

1.5.1 “3E” principle for onco-immunology from an NK cell perspective

The three Es describe the interplay between the immune system and tumor cells. In the **Elimination** phase, the immune system controls tumor growth. Pressure from the immune system may shape the tumor to become less immunogenic. During this time, there is a constant battle between the immune system and the tumor cells, referred to the **Equilibrium** phase. During this phase, immune-mediated tumor cell killing may become weakened and novel mutations allow tumors to progress. Finally, the tumor may lose immunogenicity and attract immunosuppressive cell populations that it can ultimately **Escape** from the immune system.

1.5.1.1 Elimination

During cell transformation, danger signals are first expressed on the cell surface, which could be recognized by NK cells. This immune activation is further magnified by the cytokines like TNF α , IFN γ and IL-2, following by chemokines' production and other immune cells recruitment. The mechanisms by which endogenous NK cells can exert tumor immunosurveillance and influence tumor growth are largely unknown. But an increased abundance of NK cells in the TME has been linked with better prognosis value in multiple tumors (127, 128), pulmonary adenocarcinoma (129), breast cancer (130), gastric cancer (131), squamous cell lung cancer (132), non-small cell lung cancer (133, 134), and renal cell carcinoma (135).

In a liver carcinoma mouse model, NK cells were proved to eradicate senescent tumor cells in a manner that was dependent on tumor cell p53 expression. The senescent tumor cells, induced by p53, secreted various interleukins (IL-6, IL-15) and chemokines (CCL2) which recruited NK cells to tumor lesion (136). Mechanistically, tumor cells that express p53 could induce stress related NKG2D ligands such as ULBPs and MICA/B to stimulate NK cells in TME.

Since NK cells provided another option to tumors that can evade from CD8⁺ T cell-based elimination. Recently, Nicolai *et al.* used several mouse models to investigate the intratumoral STING signaling and tumor ejection. By injection of a STING agonist, cyclic dinucleotide (CDN), they showed that CDN induced type I interferons that directly primed NK cells and simultaneously enabled an indirect pathway of activation driven by IL-15/IL-15R α axis from dendritic cells (137). This study revealed the critical role for NK cells in tumor elimination phase Overall, if elimination of tumor cells is ineffective, progression towards equilibrium will slowly occur.

1.5.1.2 Equilibrium

The equilibrium phase involves the continuous elimination of tumor cells and generation of resistant variants (138). The Equilibrium phase is difficult to study, possibly due to that it can go on for extended periods of time (139). Koebel et al. used a mouse model to study the equilibrium phase where mice were injected with small doses of the carcinogen methylcholanthrene. The mice had small but stable masses at the injection site but developed into large cancers when specific immune cells were depleted (140).

Since its ambiguous definition and poorly understood molecular mechanisms it is difficult study the equilibrium phase. Few reports have been described only anecdotally in humans (141). Recent studies compared the cellular environment of tumors in equilibrium versus escape found that high proportions of effector cells (CD8⁺ T cells and NK cells) and a low amount of suppressive cells such as Treg cells and MDSCs existed in the equilibrium stage (142, 143). But the role of NK cells in this phase is not yet fully investigated. Hypoxia induced metabolism disruption could play a key role during this long-term interaction. Evidences showed that NK cells with conditional deletion of HIF-1 α resulted in reduced tumor growth, and enhanced anti-tumor activity based on NF- κ B activation. Furthermore, IL-18 produced by myeloid cells was the prerequisite for NF- κ B activation, and elevated NK-IL18-IFNG signature in melanoma patients associated with improved overall survival (144).

1.5.1.3 *Escape*

Tumor cells employ multiple tricks to escape the host immune system including; reduced immune recognition, upregulation of immune checkpoints, increased resistance or survival, development of an immunosuppressive TME, extensive review in (6, 145-147).

Several new insights from NK-tumor interaction in escape phrase could be interesting for development of therapeutic reagent. CD73 as a metabolic immune checkpoint orchestrates an essential role to maintain the homeostatic of extracellular adenosine, which is a negative feedback mechanism for immune system to control overactivated inflammatory responses (148). CD73⁺ NK cells could dampen the immune activation by increasing production of IL-10 via STAT3, simultaneously suppressing CD4 T cells proliferation and IFN γ production which induce local immune suppressive TME. Interestingly, the enriched frequency of CD73 positive NK cells associated with larger tumor size, which tumor tissue potentially experienced escape phrase (149). Thus, by targeting CD73⁺ NK cells in this later stage could thereby enhance current immunotherapies.

In the hypoxic environment of a solid tumor, NK cells show fragmented mitochondria in their cytoplasm, where normal liver NK cells had normal large, tubular mitochondria. This fragmentation in NK cells limits their cytotoxic activity and metabolism fitness. These data demonstrated an interesting metabolic immune escape mechanism from NK cells (150).

Recognized as key innate immune cells that limit tumor metastasis, the escape phrase, NK cell-mediated immune editing might have a substantial effect on the fate of circulating tumor cells (CTCs). Lo et al observed that CTCs clusters (polyclonal) metastasize better than single (single clonal) CTCs. Depletion of NK cells increase monoclonal but not polyclonal metastases, suggesting that CTC clusters may be less sensitive to NK-mediated suppression. Mechanistically, cell-cell adhesion and epithelial genes elevated in clustered CTCs which associated with decreased expression of NK cell activating signal (151). Interestingly, another study found that SOX2^{hi} tumor cells (stem-like features) were sensitive to NK cell-mediated killing, whereas SOX9^{hi} tumor cells (alveolar epithelial progenitor features) were resistant to NK cells-mediated killing(152). Thus, these two studies elucidate how NK cells construct the escape phrase by selecting modify the tumor subpopulation.

1.6 Strategies to augment NK cell activity

Antibodies targeting the immunological checkpoint axis have reformed present cancer treatment. Clinical phase III studies show a five-year survival of 15.3 to 34.2 % in patients with metastatic melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma (153). Encouragingly, patients that respond to initial treatment have long-lasting clinical responses. However, many patients who achieve an initial clinical response eventually develop resistance. Some of the mechanisms for acquired resistance to anti-PD1 therapy include defects in interferon- γ signaling or major histocompatibility complex presentation (154). These tumor cells can no longer be targeted by tumor-specific T cells, but instead become sensitive to targeting by NK cells.

The development of therapies based on activating NK cells, has emerged as a promising therapeutic option for patients with advanced cancer (155). Infusion of either allogeneic or autologous NK cells has in some patients resulted in long-lasting clinical responses (156). However, the majority of patients do not respond to NK cell adoptive cell therapy.

1.6.1 Cytokines

IL-2 and IL-15

The development and homeostasis of T and NK cells is governed by common γ -chain cytokine family, which includes interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21(157).

IL-2 and IL-15 play pivotal roles in controlling the survival and apoptosis of lymphocytes (**Figure 8**). In addition, the heterotrimeric receptors for IL-2 and IL-15 share another subunit - IL-2/IL-15R β (also known as IL-2R β , CD122). Furthermore, the high-affinity forms of IL-2R and IL-15R contain a third cytokine-specific receptor α subunit, IL-2R α (CD25) or IL-15R α (CD215), respectively (158).

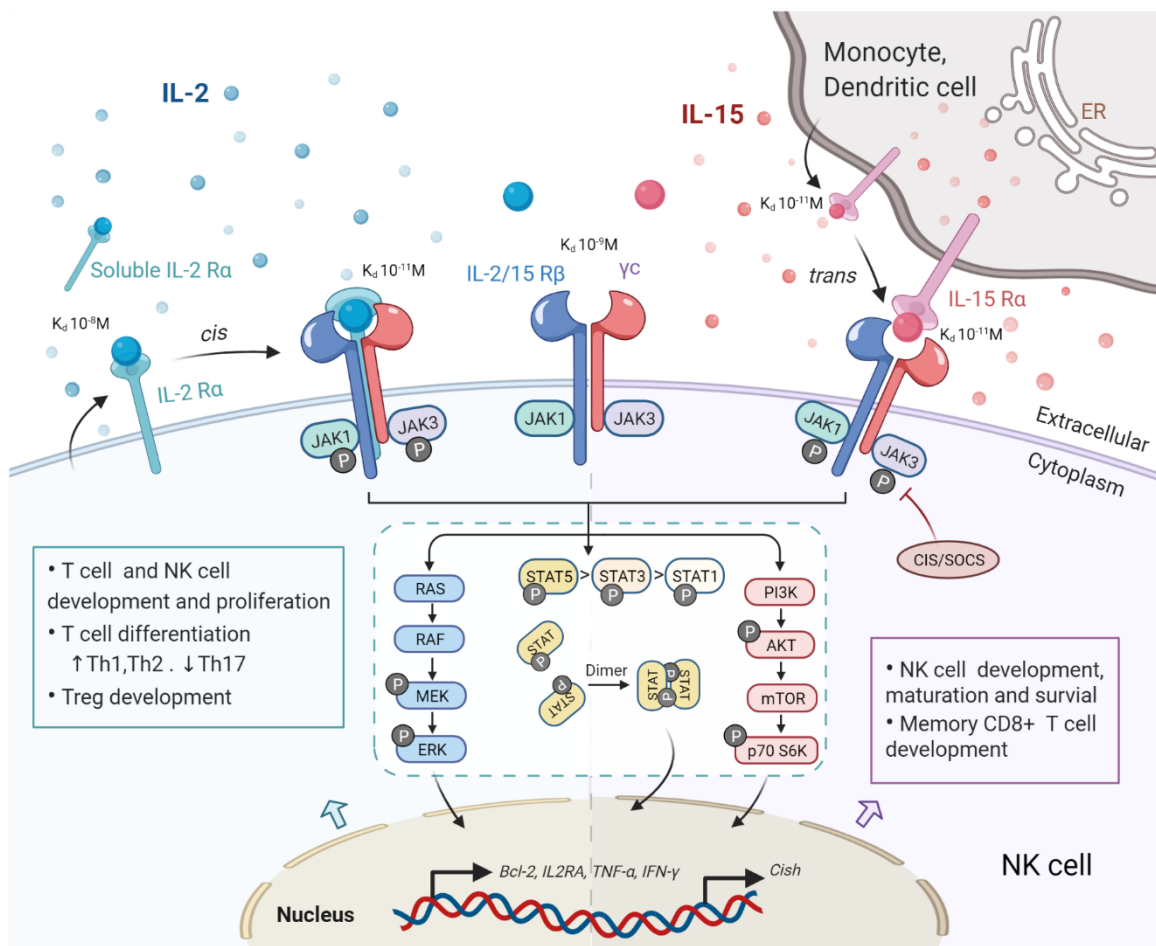


Figure 8. IL-2 and IL-15 signaling. By binding to different receptors combination, IL-2 and IL-15 showed great differences in their binding affinity. The downstream signaling transduced by JAK/STAT5 could alter the expression of Bcl-2, IL-2RA, TNF α , and IFN γ production. AICD Activation-induced cell death. SOCS Suppressor of cytokine signaling. Modified from Yang, Y., & Lundqvist, A. (2020). *Cancers*, 12(12), 3586.

In early clinical trial conducted by Rosenberg et al, patients with metastatic renal cell carcinoma and melanoma were treated with a high-dose IL-2 (600,000 or 720,000 IU/kg) therapy, resulting in a 14% overall objective response rate, with 5% complete responses and 9% partial responses (159). However, it caused significant toxicity and kept the maintenance of inhibitory CD25⁺Foxp3⁺ Treg cells instead(160). Therefore, due to the short half-life time and dose-limiting adverse event, the clinical outcome for IL-2 administration is unsatisfactory (161).

To improve the therapeutic potential of IL-2, Levin et al. engineered a "super-2", where the functional prerequisites for CD25 was excluded but simultaneously the binding affinity for IL-2R β was increased. Compared with native IL-2, super-2 induces T and NK cells' activation and thus improves anti-tumor responses *in vivo* with limited Treg expansion (162). NKTR-214 is PEGylated IL-2 preferentially activate CD8 T cells and NK cells through CD122 dependent IL-2 signaling. The well-tolerated and promising clinical activity (163) advance combination of NKTR-214 and Nivolumab toward phase III clinical trials in advanced solid tumors (NCT03635983).

Another type I cytokine that exhibits therapeutic potential is IL-15, which activates and expands NK cells. The IL-15 receptor complex is composed of IL-2R α / β / γ (164, 165). Despite sharing the common γ receptor and the same signaling subunits, the gene expression mark in lymphocytes are varied between IL-15 and IL-2 (166). Recently, it has been demonstrated that IL-15 treated NK cells are capable of maintaining anti-tumor effects in an immunosuppressive TME, while IL-2 treated NK cells are not (167-169). These findings suggest that IL-15 may induce a better anti-tumor effect than IL-2.

Unlike IL-2, IL-15 does not stimulate Treg cells, probably since IL-15 does not bind to CD25. In a study where IL-15 was applied to RCC, melanoma, squamous cell head and neck carcinoma and non-small cell lung cancer (NSCLC), the number of circulating NK cells increased in a dose-dependent fashion as IL-15 was administered. No objective clinical responses were observed in this trial, but disease stabilization occurred in several patients, including a patient with RCC whose disease was stable for over two years (170).

Considering the trans-present mechanism of IL-15, ALT-803, a novel IL-15N72D/IL-15R α -Fc superagonist complex was evaluated in hematologic malignancy. A 19% clinical response was observed including one complete remission lasting for 7 months. Furthermore, ALT-803 expands NK cells and CD8⁺ T cells without increasing regulatory T cells (171). Recently, de novo computational designed Neo-2/15 with hyper-stable and higher IL-2R β γ c receptor binding affinity showed promising *in vivo* results in melanoma and colon cancer (172).

Advancements in cytokine development has provoked a series of clinical efficacy in cancer patients. Besides, more and more type I cytokines are being investigated for clinical applications. For instance, IL-21 has been found to involve in the reversal of NK cell exhaustion (173). Additionally, combinations of various cytokines can further boost NK cell activity compared to the single cytokine. For example, the cocktail of IL-12, IL-15, and IL-18, which stimulates memory formation of NK cells, could enhance IFN- γ production, and targeting of leukemia cells *in vivo* (174-176).

1.6.2 NK cell engagers

Although monoclonal antibody-based therapy has been frequently improved, many patients do not benefit from it. In general, monoclonal antibodies usually prime several effector mechanisms, antibody dependent cellular cytotoxicity (ADCC) plays an important role to engage NK cells with target cells.

Optimization of the antibody molecule to improve the therapeutic potency is a main area in current translational research. For more than two decades, the mechanism of Fc glycosylation on ADCC was discovered by using CAMPATH-1H expressed in various tumor cell lines, with different glycosylation patterns (177). Since then, a variety of approaches have been developed to enhance the ADCC effect. Umana et al. revealed that the glycoengineering of an anti-neuroblastoma chimeric IgG1 mAb (chCE7) could increase NK cell-mediated ADCC by 20 times (178). Similar results have been found in rituximab (anti-CD20) and anti-CD19 antibody after glycol-modification (179, 180).

Apart from glycoengineering, amino acids replacement in Fc γ R binding site could strengthen ADCC as well. By using this method Lazar *et al.* produced an Fc variant anti-CD20 antibody with the improved Fc γ RII/IIIa binding affinity and ADCC effect. Strikingly, for this engineered rituximab, its depleted half of the circulating B cells at a nearly 50 times lower dose than the non-engineered rituximab (181). Furthermore, similar Fc variants enhancing ADCC activity were recent discovered on CD33 and CD133 antibodies against AML (182, 183).

Since the introduction of bispecific antibody to target CD30 on Hodgkin's lymphoma and CD16 on NK cells more than two decades ago (184), next-generation of the bi-specific antibody have been developed to engage NK cells and distinct tumor antigens. For example, target HER2 for breast cancer (185), CD30 for Hodgkin's lymphoma (186), CD19 and MHC-II for B cell malignancies (187, 188), CD33 for AML (189, 190), and EPCAM for carcinomas (191), and EGFR (192), which is overexpressed in several epithelial cancers.

In addition to the engagement of CD16, bispecific mAbs have been created to target other activating receptors such as NKp30 (193) and NKG2D (184, 194). Moreover, after fusing with a tumor-targeting variable fragment (Fv), the bispecific mAbs against the MICA or ULBP2 (NKG2D ligand) were found to induce NK cell-mediated killing (195-197). The link between syndecan-1 expressed on tumor cells, and syndecan-1 (BB4) could engage tumor cells with NK cells. Von Strandmann et al. showed that the bi-specific engager ULBP2-BB4 targeting NKG2D and Syndecan-1(CD138) could enhance NK cell antitumor activity against human multiple myeloma *in vitro* and *in vivo* (197).

NK cells' tri-specific engagement with dual targeting of tumor antigens has been explored to improve tumor selectivity further. Gantke et al. reported an enhanced *in vitro* potency of a tri-specific mAb targeting B-cell maturation antigen, CD16 and CD200, compared with bispecific engager targeting CD16 and B-cell maturation antigen or CD200 (198). Gauthier et al., described a similar approach where dual engagement of the NK cell receptors NKp46 and CD16 coupled with a CD19 targeting domain pointed to a significantly delayed tumor progression *in vivo* (199). A recent study conducted by Vallera et al. showed that IL-15 combined with a CD33 and CD16 bispecific mAb exhibited extended NK cell activity such as cytolytic ability, persistence, and activation *in vivo* (200). Notably, this tri-specific antibody also made NK cells less sensitive to suppression by MDSCs (201).

1.6.3 Immune checkpoints for NK cell

Blockade of inhibitory KIRs by IPH2101 has been shown to increase the killing potential by “arrested” NK cells. In preclinical mouse models in AML (202), B cell lymphoma (203) and multiple myeloma (204) is promising. However, a phase II trial of IPH2101 failed to show any clinical benefit in smoldering multiple myeloma (205). The increased frequency of hyporesponsiveness NK cells and decreased KIR2D+ NK subpopulation might contribute to the IPH2101 failure (205, 206).

The inhibitory cascade from PD-1 and CTLA-4 serves as a critical regulatory signal for NK cells to maintain homeostasis. A study in ovarian carcinoma identified a NK subset with abnormal higher levels of PD-1 (207). Extraordinary therapeutic effects have been showed in advanced cancer patients as well by using antibodies against CTLA-4 or PD-1 (15, 22), it is important to determine and explore the role for NK cells in this context. Interestingly, an *in vitro* study showed that through inhibition of PD-1/PD-L1 NK cells could restore the proliferation and antitumor activity in multiple myeloma (208). To refresh the classical immune checkpoint blockade with new insight, great efforts are needed for understanding the mechanism of NK cells during the anti-PD-1 and anti-CTLA-4 treatment.

Through engagement of HLA-E, NKG2A could suppress both T and NK cell activation signal (209). As a first-in-class blocking monoclonal antibody target NKG2A, Monalizumab (IPH2201), is currently being tested for the safety and antitumor activity in different types of cancers (210, 211). The combination of cetuximab (anti-EGFR) and monalizumab in phase II showed encouraging results. The objective response for the combination in squamous cell carcinoma of the head and neck is 31% (211). In 2020, this combination currently tested in phase III for recurrent or metastatic head and neck squamous cell carcinoma of the head and neck (NCT04590963).

A promising NK cell-specific immune checkpoint is the cytokine-inducible SH2-containing protein (CIS). CIS is encoded by the CISH gene and is a negative regulator of JAK/STAT5 signaling in NK cells. CIS knockout in murine NK cells could induce hypersensitivity to IL-15 and decreased metastasis burden (212). Moreover, CISH-depletion combined with immune checkpoint blockade (anti-PD-1, anti-CTLA-4, and anti-CD96) resulted in control of tumor metastasis (213). The humanized model using iPSC derived CISH knockout NK cells showed elevated antitumor activity and enhanced metabolic fitness (214).

Interleukin-1 receptor 8, is a member of IL-1 receptor family with unique negative regulatory function. Martina *et al.* showed that human NK cells express higher level IL-1R8 than other effectors. By blocking the IL-1R8, NK cells showed significant protection against liver carcinogenesis and metastasis (215).

2 RESEARCH AIMS

The overall aim of this thesis is to identify effective NK cells which are resistant to immunosuppressive factors within the TME, and to furthermore explore the underlying mechanisms of such resistance.

AIMS:

Paper I:

To uncover molecular mechanism of NK cells resistance against PGE2 suppression.

Paper II:

To investigate resistance mechanisms of tumor-infiltrating NK cells under oxidative stress.

Paper III:

To decipher the survival mechanism for NK cells under Treg-induced IL-2 deprivation.

3 MATERIALS AND METHODS

The detailed methods and materials are listed in publications. Here we describe selected methods that have been used in this thesis.

3.1 Real-time image-based assay.

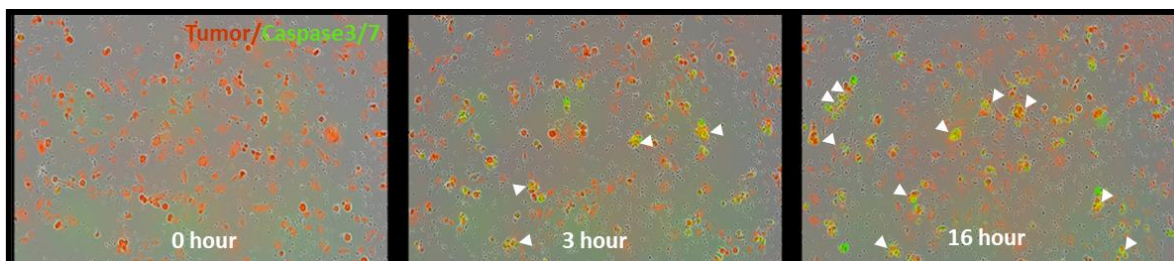


Figure 9. Real-time image for NK cells-Tumor cells interaction. The tumor cells labeled with red-fluorescent co-cultured with NK cells. The medium contained Caspase3/7 dye, which labels apoptosis cells in green. Arrows point out the dead tumor cells (yellow). Under help with Incucyte S3 we could we could observe and quantify the apoptosis tumor cells at real-time.

There is a needed to decipher the interaction between immune effector cells, like cytolytic T cells or NK cells and tumor cells, which could further refine gene and cell therapy which showed remarkable efficacy in the clinic against both liquid and solid tumors. By employing two color coded immune-tumor cells co-culture assay, the cytolytic activity of NK cells in contact with tumor cells in various conditions which could be continuously monitored.

In brief, NK cells were isolated and labeled by Cell tracker Red (Thermo). Labeled NK cells were cultured with target tumor cells as designed ratio. The medium contained Caspase-3/7 Green Dye, which enable for quantification of apoptotic cells by using green channel. Since the dead NK cells could be filtered as yellow, we could quantify the dead tumor cells by calculating green-only objects (**Figure 9**). This method was used in **Paper I** and **Paper II**.

3.2 3D tumor spheroid model

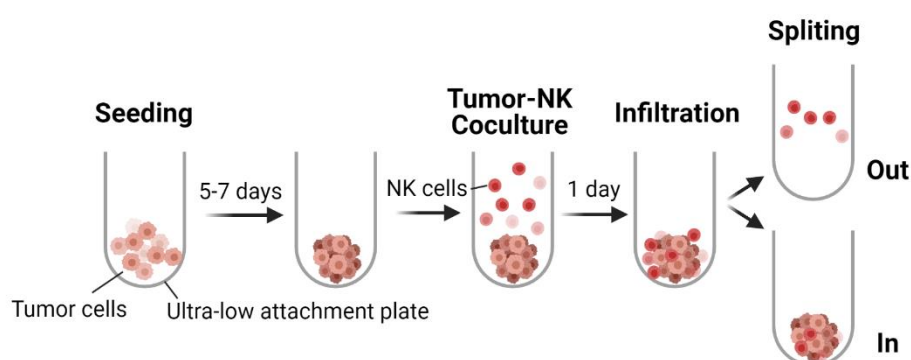


Figure 10. The workflow of tumor spheroid coculture assay. The graph is created by using Biorender.

Spheroids, or tumor cell aggregates, are more representative of *in vivo* conditions than cell monolayers, and tumor cells grown as spheroids exhibit several physiological traits including relevant morphology, increased cell survival, and a hypoxic core. By using tumor spheroid models the infiltrating lymphocytes could be monitored, and further verification could be done by flow cytometry. The general workflow is demonstrated in **Figure 10**. The Incucyte 2019B

was used for quantification and further movie generation. This method has been used in **Paper I** and **Paper II**.

3.3 Zebrafish model

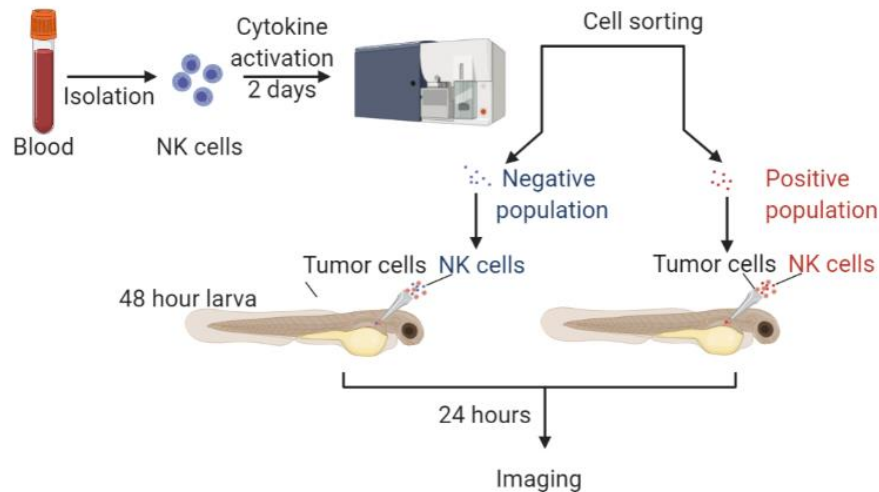


Figure 11. The workflow of zebrafish immune-tumor cells experiment. The graph is created by using Biorender.

Methods for phenotypic analysis of immune cell interactions with tumor cells have developed rapidly. The Zebrafish, as a non-mammalian vertebrate model of cancer, are not new to the field. The advantages of optically clear, small scale, less time and cost, the minimal amount of sample needed, multiplexing of conditions, and potential for automation bring zebrafish into the arena of phenotypic testing of cancer immunotherapy. Specifically, to further investigate the tumor-NK interaction, we developed the method using zebrafish larva with sorted NK cells and fluorescent-labeled tumor cells. The zebrafish larvae model was used in **Paper I**.

3.4 TCGA datasets analysis

The raw data for overall survival (OS) and progression free interval (PFI) together with clinical parameter: smoke history and normalized gene expression data, were exported from TCGA database through Xena (<http://xena.ucsc.edu>). CD160, PRF1, KLRB1, NCR1 and NCR3 were used to represent NK cell abundance in tumor samples, which has been used in previous studies (216, 217).

3.5 Statistical analysis

Unless stated otherwise, all statistical tests were performed using Prism 8 (Graphpad software). All results are presented as mean±SD and represented histogram or images were selected based on the average values, $p < 0.05$ was considered significant. Two-tailed unpaired or paired Student's t-tests between two groups. **In paper I**, the difference in overall survival was tested using log-rank tests. **In paper II**, using “survival” and survplot R packages, Kaplan-Meier analysis was performed with NK cell signature score or IL15 gene expression split into a binary (Low/High) variable based on the median value.

4 RESULTS AND DISCUSSION

Study I. CD25⁺/CD54⁺ NK cells resistant to PGE2 mediated suppression via PDE4A upregulation.

Our previous data shows that in comparison with IL-2, IL-15 provides NK cells with enhanced mTOR and JAK/STAT5 signals to maintain their anti-tumor activity *in vivo* (168). At the time I joined the group, our preliminary results showed inhibition of COX-2 restored the anti-tumor effect of IL-2 stimulated NK cells in co-culture experiments with melanoma cells. **We therefore hypothesized that IL-15 could render NK cells resistant against PGE2.**

We found that both proliferation and cytotoxicity were significantly higher in IL-15 NK cells compared with IL-2 stimulated NK cells under PGE2 suppression. While the expression of the EP2 and EP4 receptors did not change, but the expression of the intracellular phosphodiesterase 4A (PDE4As), which belongs to cAMP hydrolyzing enzyme family, was significantly upregulated in IL-15 primed NK cells. This increased expression was accompanied by reduced cAMP concentration upon PGE2 stimulation. cAMP as an intracellular second messenger from G_s-coupled receptors, prostaglandin E2 receptor 2 (EP2) and EP4, triggered cAMP/PKA/CREB pathway which drives the anti-inflammation response (218, 219). In T cells, overexpressed PDE4A renders CD4⁺ and CD8⁺ T cells to reverse PGE2 induced adverse effect on proliferation, cytokine production and cytotoxicity. Furthermore, the exhaustion markers between PDE4A overexpressed T cells and control did not show significant difference, which provides the opportunity for long time *ex vivo* expansion of PDE4A overexpressed T cells (220).

In colorectal cancer cells, inhibition of PDE4D, another PDE4 member, leads to repression of the mTOR pathway (168, 221, 222). Similarly, we found that the frequency of pS6 positive NK cells was maintained in IL-15 group, but significantly reduced in IL-2 group in the presence of PGE2. Furthermore, inhibition of mTOR activity in IL-15 activated NK cells revealed decreased expression of PDE4A. Thus, there is a reciprocal cross-talk between mTOR and PDE4 activity in IL-15 activated NK cells.

Analysis of RNA-sequencing data between IL-2 and IL-15 stimulated NK cells showed that CD25 and CD54 was significantly upregulated in IL-15 activated NK cells. Min-Oo et al. showed that IL-15 upregulates CD25 on NK cells to form memory-like NK cells (223). Several studies showed the LFA-1 activation in NK cells is an incipient identification signal for NK cell cytotoxicity (224, 225). Here we show that the LFA-1 ligand, CD54 (ICAM-1), is also important for NK cell function. Our results support those of Sun R et al. that IL-15 can indeed upregulate CD54 in NK cells (226). LFA-1 and ICAM-1 is an important receptor-ligand interaction to facilitate cellular clustering and activation (225, 227, 228). We observed similar pattern between IL-2 and IL-15 primed NK cells to form cell cluster, but in the presence of PGE2, IL-15 activated NK cells formed significantly more cell clusters. Upon blocking CD54 by antibody, cluster formation was impaired but the cytolytic activity did not change indicating that cell cluster does not contribute to the resistance to PGE2 in IL-15 primed NK cells.

To further validate the finding, we performed cell isolation experiment based on CD25 and CD54. Strikingly, purified CD25⁺/CD54⁺ NK cells exhibited superior killing activity against

K562 and A549 lung cancer cells in the presence of PGE2, regardless if they were stimulated with IL-2 or IL-15. This population of NK cells expressed significantly higher levels of perforin, TRAIL, CD107a and IFN γ .

Across 33 TCGA datasets, the prostaglandin E synthase (PTGES) expression was significantly higher in LUAD tissue compared with matched normal tissue. We furthermore found that NK cells level was significantly lower in tumors compared with normal tissue. However, the higher NK cell gene signature showed better survival only in stage I LUAD patients, but not other stages, suggesting that NK cells play a pivotal role in the immune surveillance in early stage of lung adenocarcinoma. Inflammatory-related pathway enriched in PTGES^{hi}NK^{hi} indicates the TME in those patients are more inflamed than PTGES^{hi}NK^{low}. Thus, the “hot” TME could potentially increase the tumor-infiltrating NK cells despite high levels of PTGES. These results further support the idea that impaired tumor growth and upregulation of inflammatory genes such as: *Ifng* and *Gzmb* could be achieved by genetic ablation of COX through PTGS2 knock-out (229). When tested for their ability to infiltrate tumors, CD25⁺/CD54⁺ NK cells showed increased infiltration compared with CD25⁻/CD54⁻ NK cells *in vitro* and *in vivo*. In patients with lung adenocarcinoma, the frequency of CD54 positive NK cells was significantly higher in the tumor central area compared with the invasive margin and normal tissue. These results are in line with Ni et al. who observed that activated NK cells under hypoxic condition express higher level of CD54 (144).

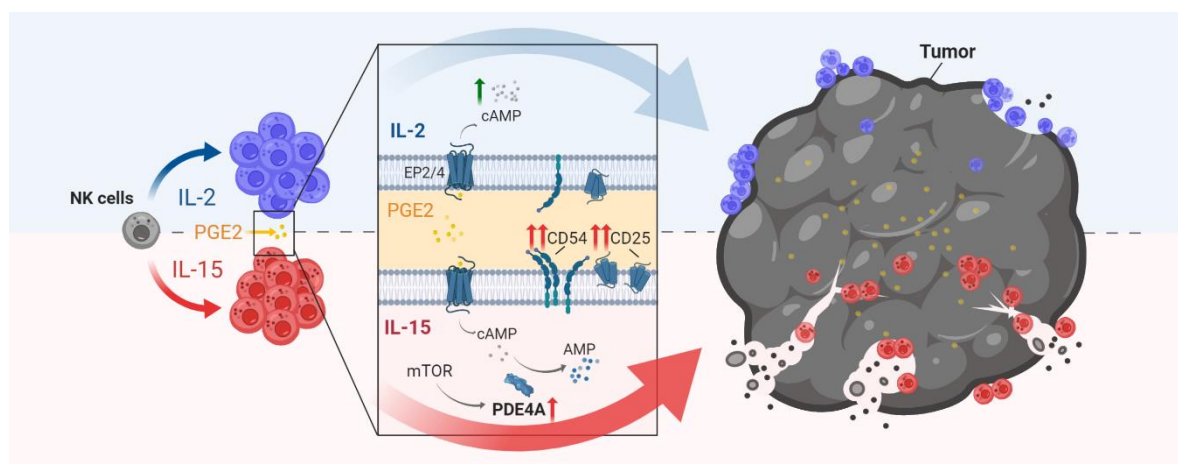


Figure 12. IL-15 promotes a subset of NK cells that resist PGE2-mediated suppression by mTOR-dependent upregulation of PDE4A. *Ex vivo* expansion of CD25⁺ CD54⁺ NK cells for adoptive cell therapy may be used to target tumors with high PGE2 levels. (Reprinted with permission from Chen, Ziqing, et al. *EMBO reports* (2021): e51329.)

In conclusion, we elucidate a potential mechanism behind IL-15 primed NK cell resistant against PGE2 inhibition, though upregulate a cAMP hydrolyzing enzyme PDE4 by enhancing mTOR signaling (**Figure 12**). Another interesting aspect of our study is the identification of two surface markers, CD25 and CD54, could be used to define “eximious” NK cells, which exhibits superior infiltrating and killing capacity. Approaches to selectively expand “eximious” NK cells for adoptive cell therapy or combination with checkpoint inhibitor could be potential therapeutic strategy for patients with high PGE2 produced tumor.

Study II. Thioredoxin activity confers resistance against oxidative stress in tumor-infiltrating NK cells

Reactive oxygen species (ROS) contain a diverse of radical species that have various roles depending on their location and concentration (230). ROS is produced by highly metabolic cancer cells as well as by activated immune cells like neutrophils, macrophages, regulatory T cells and myeloid-derived suppressor cells (MDSCs) (231). Thioredoxin, reduce oxidized cysteine residues and remove disulfide bonds. It serves as one of the essential antioxidants to keep the ROS homeostasis within cells (232, 233). Elevated levels of thioredoxin often associate with immune activation or survival regulation (234-236).

At the time I joined the group, our preliminary results showed that activation with IL-15 increase the expression of cell surface thiols compared with IL-2 primed NK cells. Cell surface thiols can act as a safety shield as they get oxidized by external free radicals (237, 238). It has been shown that oxidative stress has a durable and profound suppressive effect on NK cells (239-241) **We hypothesized that IL-15 may confer resistance against oxidative stress.**

We first found that NK cells primed by IL-15, instead of IL-2, revealed superior antitumor effect under oxidative stress and this was associated with reduced intracellular ROS in IL-15 primed NK cells. This result in agreement with another study that elevated thioredoxin and peroxiredoxin were observed in NK cells expanded with K562 feeders which express 4-1BBL and membrane IL-15 (242).

Through GSEA analysis, we identified several key elements regarding cellular ROS response, which including elevated thioredoxins (TXN1 and TXN2) and reduced TXNIP and TXNRD1 (inhibitory counterparts of thioredoxins). Indeed, flow cytometry analysis showed that TXNIP elevated in IL-2 stimulated NK cells compared with IL-15 stimulated NK cells. Regardless of stimulation with either IL-2 or IL-15, NK cells isolated based on high level of cell surface thiols revealed superior killing against K562 targets in the presence of H₂O₂. However, this result might somehow be limited by abnormal dose of H₂O₂ which could not represents the physical situation (243).

In co-culture of activated ROS-producing neutrophils and NK cells, the proliferation of NK cells was significantly suppressed. Furthermore, by using thioredoxin-1 inhibitor PX-12, the killing capacity of IL-15 primed NK cells were abrogated upon exposure to H₂O₂. Treatment with PX-12 reduced the proliferation of IL-15 primed NK cells to the same levels as PX-12 untreated NK cells (IL-2 primed). Finally, sorted NK cells with high surface thiol density displayed superior capability to infiltrate lung tumor spheroid. Interestingly, the infiltration of NK cells in 3D culture happened within hours, this fast, continuous mobility could link with the term ---“serial killer” (244), which cytolytic T cells or NK cells processed additional killing events after disengaged with dead target.

After the administration of mTOR inhibitor (Torin-1), the difference of thioredoxin expression between IL-2 and IL-15 NK cells diminished. It was previously shown that through inhibition of mTOR, cell death could be induced by dysfunctional TXNIP (245). Analysis of NSCLC patient samples showed that NK cells with higher surface thiol have the ability to infiltrate into the tumor core more frequently compared with those with lower surface thiol.

To further validated this result in more general clinical setting, LUAD dataset from TCGA was analyzed. As previously described smoking is one of the extrinsic factor to induce tissue ROS production (246). Next, we separated the LUAD cohort into smoker and non-smoker group. Interestingly, the distinguished overall survival and progression free survival interval only happened in smoker cohort. Whereas this trend was not observed in tissue-infiltrating T cells compared smoker and non-smoker. With growing evidence showed that NK cells might serve as a local “recruiter” for DCs or T cells by secreting inflammatory cytokines in TME, which turn the tumor from “Cold” to “Hot” (216, 221, 222).

In summary, this study provides another potential mechanism that activated NK cells employing thioredoxin system to neutralize oxidative stress in TME (**Figure 13**). By using IL-15 as adjuvant, which renders immune cells higher capability to higher levels of ROS, future investigations could study the combination of IL-15 with other novel cell therapy products especially under oxidative stress.

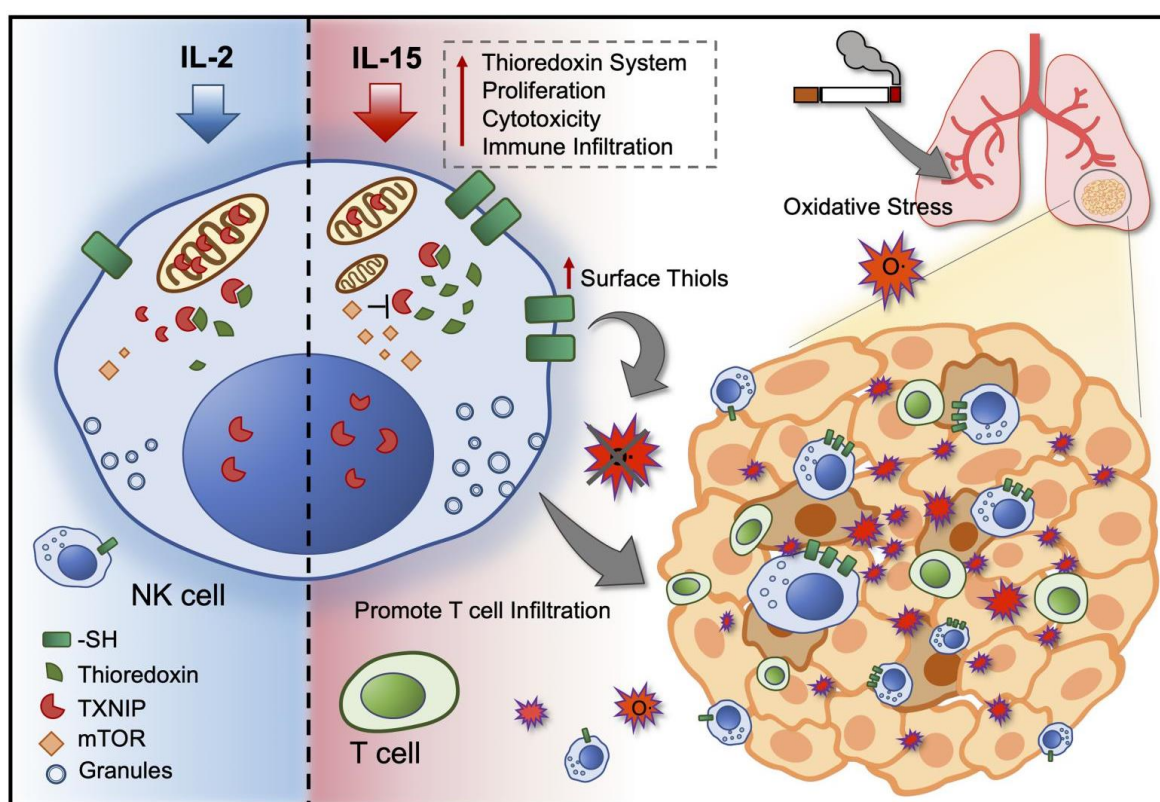


Figure 13. IL-15 renders NK cells resistance against oxidative stress through releasing the power of thioredoxin system by activated mTOR pathway. By providing extra thiols protection, IL-15 primed NK cells promote the T cells recruitment in TME which turn the tumor from “Cold” to “Hot”. Furthermore, the extrinsic factor for ROS production, smoking, could influence the IL15 and NK cells prognostic value in NSCLC. (Reprinted with permission from Yang, Ying, et al. *The Journal of clinical investigation* 130.10 (2020).)

Study III: CD25 bright NK cells display superior proliferative and metabolic activity and resist suppression by regulatory T cells

Regulatory T cells affect the NK cell immune response via the production of TGF β , IL-10 adenosine, ROS secretion and inhibitory signaling provided by CTLA4/CD28 ligation (102, 247). However, another poorly understood mechanism caused by Treg is metabolic disruption, especially by IL-2 deprivation. Since we observed that IL-15 increase the expression of CD25 on NK cells, **we hypothesized that these NK cells would survive better under Treg induced IL-2 deprivation.**

The upregulation of surface CD25 was correlated with increased level of phosphorylated STAT5(Y694). The frequency of pAKT+ pSTAT5+ NK cells was higher in IL-15 stimulated NK cells. Notably, phosphorylated AKT can directly activate the mTOR pathway which influence cell growth and survival (248, 249). We asked if IL-15 stimulated NK cells could survive better under cytokine competition. In co-culture of NK cells and Treg in the presence of 100U/ml of IL-2, NK cells treated with IL-15 showed significant higher level of membrane-bound IL-2. Similar results have been showed by comparing surface IL-2 of Treg cells with activated T effector cells (250). Moreover, flow cytometry results revealed significantly increased proliferation and IFN γ production in IL-15 primed NK cells. Notably, the viability and production of IL-10 by Treg maintained the same level in both groups. Additionally, comparable increased NK cell infiltration was observed in both A498 and 786O spheroid models. Taken together, IL-15 primed NK cells survival better than IL-2 primed NK cells under Treg induced cytokine deprivation. Due to the multiple ways of suppression induced by Treg cells, external blocking for TGF β and CTLA-4 could further validate the effect is only due to cytokine deprivation induced by Treg.

Based on these findings, we next sought to connect the intracellular signaling with surface marker which could facilitates *in vitro* evaluation by sorting cell based on the expression of CD25. Increased proliferation rate and phosphorylated S6 were observed in CD25 bright NK cells. Consistently, these data agree with our previous results which pAKT+pSTAT5+ NK cells may provide strong intracellular signal for IL-15 primed NK cells to maintain their antitumor activity. Thus, the increased intracellular AKT/STAT5 signal was correlated with surface CD25 expression on IL-15 stimulated NK cells. It was previously shown that the CD25 could be regulated by STAT5a and STAT5b, but no study has been showed that AKT is needed for this activation (251).

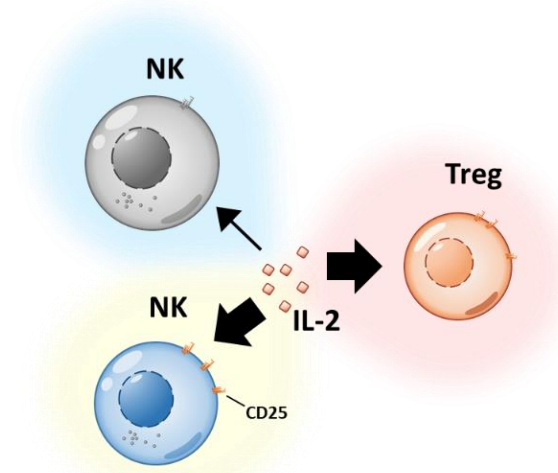


Figure 14. By upregulating the IL-2 receptor α , IL-15 primed NK cells have the ability to compete with Treg for limited IL-2 in TME, which IL-2 stimulated NK cells could not.

The mitochondria governs cellular metabolic process (252) and increased mitochondrial membrane potential is a hallmark of improved metabolic activity (253). Seahorse experiment revealed a superior basal respiration, maximal respiration, and ATP production in CD25bright

NK cells compared with CD25^{dim} and Cd25 negative NK cells. Interestingly, Huang et al. reported that CISH^{-/-} iPSC-derived NK cells display enhanced metabolic fitness and anti-tumor activity in mice model which confirmed that metabolic activity and mTOR pathway play an essential role in NK anti-tumor activity (214).

In summary, we report that increased CD25 expression on NK cells increases their ability to compete for IL-2 with Treg cells (**Figure 14**). Furthermore, upon IL-15 activation higher intracellular phosphorylated STAT5 and AKT provide signaling benefit for NK cell survival, together with improved metabolic fitness. These studies support clinical validation of enriched superior NK cells for adoptive immunotherapy.

5 CONCLUSIONS

In this thesis we provide new insights into the biology and therapeutic potential for harvesting the power of “eximious” NK cells against solid tumors. The key findings presented in three papers are summarized below.

Paper I, we identified the enzyme PDE4A to be enriched in CD54⁺/CD25⁺ NK cells and these markers may serve to select for NK cells with superior killing capacity against solid tumor under PGE2 suppression.

Paper II, we demonstrated that IL-15-primed NK cells acquired resistance against oxidative stress through the thioredoxin system activated by mTOR. Furthermore, the prognostic value of IL-15 and NK cell gene signature in tumors may be influenced by tobacco smoking history in NSCLC patients.

Paper III, we showed that CD25^{bright} NK cells have a higher ability to compete for IL-2 with Treg cells in the tumor microenvironment. The enhanced mitochondria activity in CD25^{bright} NK cells facilitates their survival and anti-tumor activity.

Taken together, the identification of surface markers and signaling pathways in activated NK cells could be essential for the clinical development of adoptive NK cell therapy.

6 FUTURE PERSPECTIVE

Historically, the interval between the implementation of cancer treatments has shortened (**Figure 15**). The unique “missing self” killing mechanism and “off the shelf” property set NK cells at the forefront of the next wave of immunotherapy.

Numerous strategies to develop novel therapeutics to augment the activity of NK cells are currently being investigated. In 2020, A phase I/II trial of 11 patients with relapsed or refractory CD19-positive cancers observe that most patients (8/11, 73%) respond to CD19-targeting CAR natural killer (NK) cells and show few major toxicity effects (254).

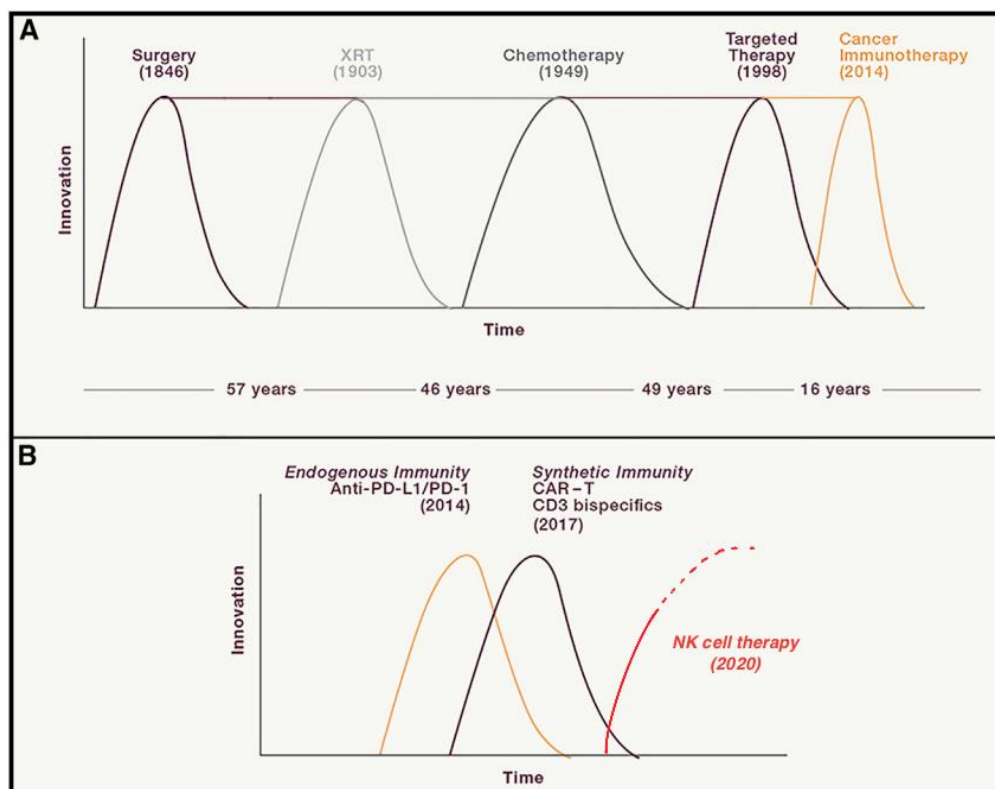


Figure 15. (A) The time of various innovations and impact in the treatment of cancer. (B) The new hope for NK cell therapy after the development of immune checkpoint inhibitors and synthetic immunity provides two overlapping and potentially disruptive treatment paradigm shifts. CAR, chimeric antigen receptor; CIT, cancer immunotherapy; PD-1, programmed death-1; PDL1, programmed death-ligand 1; XRT, external radiation. (modified from Hegde, P. S., & Chen, D. S. (2020). *Immunity*, 52(1), 17-35.)

Although the future of NK cell-based immunotherapy is promising, there are still hurdles that need to be overcome. Below I have listed questions I believe need to be carefully considered in order to develop NK cell-based immunotherapies in patients with cancer.

- How long do activated NK cells maintain their killing capacity after infusion?
- How can we generate a large amount of “eximious” NK cells?
- How will adoptively transferred NK cell communicate with other cells within the tumor microenvironment?
- How will an *ex vivo* expanded NK cell behave in an altered redox-balance situation such as an altered ROS balance and hypoxia?
- What other cancer therapies can synergize with adoptive transfer of NK cells?

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