

BIOENGINEERING OF HUMAN PLURIPOTENT STEM CELLS FOR CHIMERIC ANTIGEN RECEPTOR IMMUNOTHERAPY

by

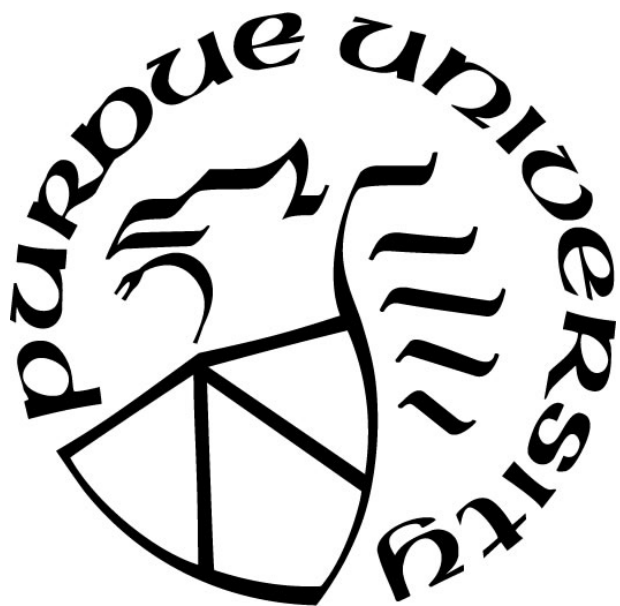
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This thesis is dedicated to my friends and family who inspire me to seek self-improvement, to improve the lives of others, and above all else, to be kind.

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LIST OF COMMON ABBREVIATIONS

AAVS1: Adeno associated virus integration site 1	LPS: Lipopolysaccharide
ADCC: Antigen dependent cellular toxicity	MHC: Major histocompatibility complex
ALL: Acute lymphoblastic leukemia	MiB: Minibody
CAR: Chimeric antigen receptor	MPSC: Multipotent stem cell
CD: Cluster of differentiation	NB: Nanobody
CRS: Cytokine release syndrome	NET: Neutrophil extracellular trap
DN: Double negative	NHL: Non-Hodgkin's lymphoma
DP: Double positive	NK: Natural killer cell
EDTA: Ethylenediaminetetraacetic acid	PCA: Principal component analysis
EHT: Endothelial to hematopoietic transition	PCa: Prostate cancer
ESC: Embryonic stem cell	PCR: Polymerase chain reaction
FDA: U.S Food and drug association	PD1: Programmed death ligand 1
G-CSF: Granulocyte colony stimulating factor	PSMA: Prostate specific membrane antigen
GM-CSF: Granulocyte/macrophage colony stimulating factor	RAG: Recombinase activation gene
HE: Hemogenic endothelium	ROS: Reactive oxygen species
HLA: Human leukocyte antigen	RRMM: Recurrent/relapsed multiple myeloma
hPSC: Human pluripotent stem cell	scFv: Single chain variable fragment
HSC: Hematopoietic stem cell	STAT: Signal transducer and activation of transcription
IL: Interleukin	TAM: Tumor associated macrophage
iPSC: Induced pluripotent stem cell	TAN: Tumor associated neutrophil
ITAM: Immunoreceptor tyrosine-based activation motif	TCR: T cell receptor
JAK: Janus kinase	TGF- β : Transforming growth factor beta
LNCaP: Lymph node carcinoma of the prostate	TME: Tumor microenvironment
	TNF: Tumor Necrosis Factor
	TRUCK: T cells redirected for universal cytokine killing
	VEGF: Vascular endothelial growth factor

ABSTRACT

Immunotherapy as a treatment for cancers that do not respond to surgery, chemotherapy, or radiotherapy is a powerful technique in which immune cells are modified to exert cytotoxic effects against a specified tissue. A classic technique in immunotherapy is the use of chimeric antigen receptor (CAR) expressing immune cells (typically T lymphocytes; referred to as CAR-T) to drive an immune response against cancerous tissue. The efficacy of CAR-T is reduced in solid tumors due to limitations of T lymphocytes as an effector cell in a tumor microenvironment. In this study we demonstrate that CAR-neutrophils differentiated from genetically-modified human pluripotent stem cells displayed a strong cytotoxic effect against prostate-specific membrane antigen expressing LNCaP cells as a model for prostate cancer *in vitro*. Additionally, we found that modification of the neutrophil differentiation scheme resulted in suspended, CD4⁺ cells, demonstrating potential to rapidly generate T lymphocytes under a feeder-free, xeno-free scheme *in vitro*.

1. IMMUNOTHERAPY AND CHIMERIC ANTIGEN RECEPTORS

1.1 Introduction

Immunotherapy is a branch of applied engineering of the immune system that acts as an additional method of care along with chemotherapy, radiotherapy, and surgery. Immune engineering is a joint field of immunology, systems biology, genetic engineering, and biological engineering. The core functionality of the immune system is to defend against foreign threats, and thus recruitment and improvement of this core function to target specific ailments is the main goal of immunotherapy. While the most common target of immunotherapy is cancer, a widely variable and dangerous disease, increasing research utilizing immune therapy against non-cancerous diseases such as auto-immune disorders and complex inflammatory states have recently been reported in the literature. Immunotherapy is a versatile option that works in combination with biological systems to treat the root of symptoms, and FDA-approved options for treating blood-borne malignancies using immune cells modified with a chimeric antigen receptor (CAR) have demonstrated strong treatment efficacy as a cancer treatment.

1.2 Literature Review

1.2.1 Chimeric Antigen Receptor T cells

Chimeric antigen receptor (CAR)-expressing T lymphocytes as a therapy against blood-borne tumors is a significant branch of immunotherapy and serve as a starting point for all CAR research¹⁻⁵. As a central hub of communication and response-coordination in the immune system, T cells make an excellent first choice for study into CAR technology.⁶ One of the main branches of blood cells, the leukocytes, are critical for protecting against foreign and domestic threats.

Leukocytes include the natural killer cells (NKs), macrophages, and granulocytes of the innate immune system as well as lymphocytes of the adaptive immune system: T, B, and their respective effector cells. Through a combination of chemical signals messengers such as interleukins, cytokines, and cell-to-cell contact mediated by surface receptors, the immune system creates a vast information network throughout the body to identify and eliminate threats, direct development of tissue, and ensure future protection. One of the key players in this information net are the T cells, which recognize peptide antigens, coordinate between innate cells and B lymphocytes, and retain immunological memory of past invaders.

To mimic the capability of T lymphocytes to recognize a wide variety of epitopes on different tissues, engineered receptor complexes are designed to recognize a specific antigen and stimulate an immune cell to target the tissue expressing the specified marker antigen. This receptor is a synthetic protein that fuses a unique extracellular receptor domain to invariable transmembrane and intracellular activation domains⁷. These chimeric antigen receptors (CARs) typically utilize a single chain antibody variable fragment (scFv) for the receptor domain due to the relatively simpler process of identifying a unique antibody compared to a *de-novo* approach⁸.

Due to this design, CARs can potentially recognize any manner of epitope provided an appropriate binding domain, in sharp contrast to the limitation of T cell receptor (TCR) antigens to short peptide sequences set by the requirement of TCR-HLA complex formation⁸. However, CARs are limited to recognize extracellular epitopes only, whereas MHCs recognized by lymphocytes can express antigens generated by threats on both sides of the cytoplasmic membrane⁸⁻¹⁰.

CAR structure can vary widely depending on desired target, employed immune cell, and desired level of activation sensitivity. A monoclonal antibody scFv is fused to a spacer and hinge

domain, followed by a transmembrane region, and finally a domain involved in providing an activation signal for a signal cascade¹¹, as shown in **Figure 1.1**.

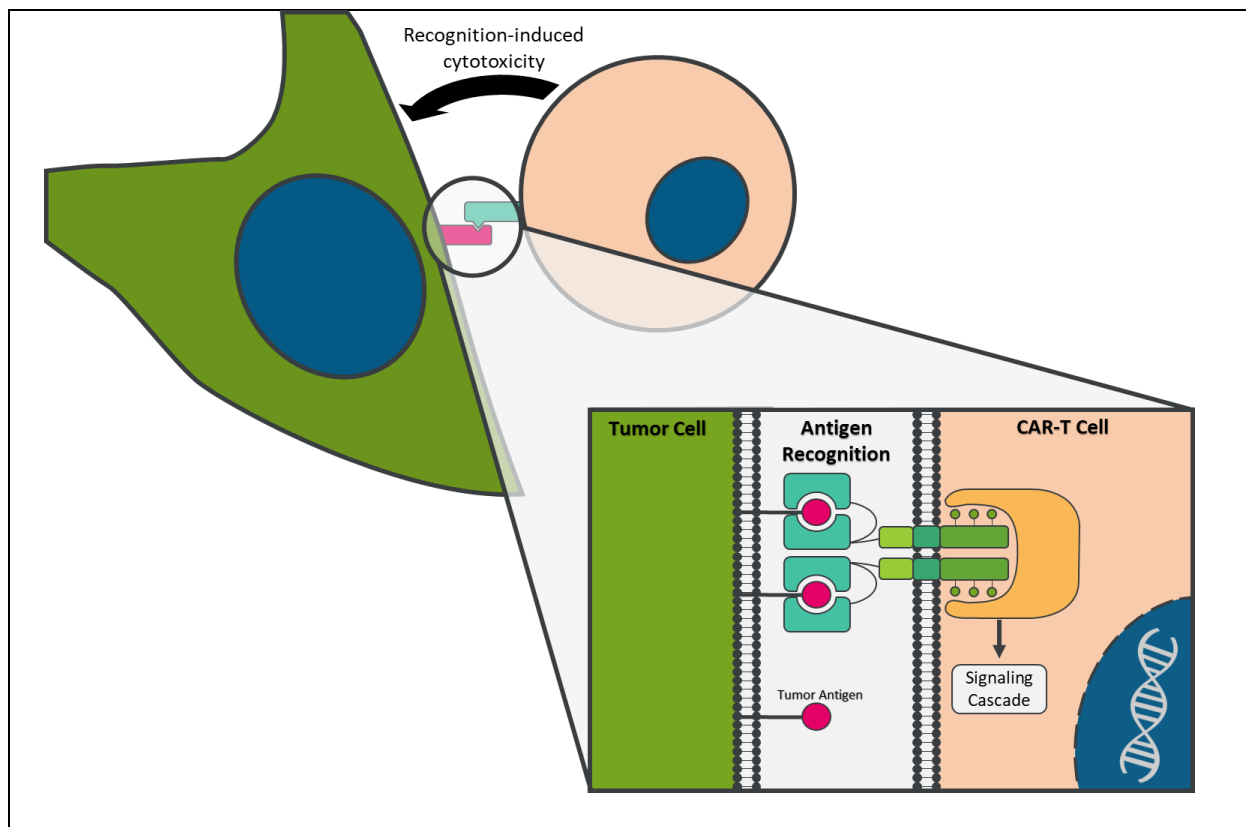


Figure 1.1: Chimeric antigen receptor (CAR) recognition of antigens

Various generations of CARs have been designed. The first area of development in CAR technology was in T lymphocytes. In nature, T cells are activated by CD3 ζ domains that conduct a signal as part of the TCR into the cell, while secondary signaling is conducted by accessory molecules such as CD28⁸. In 2nd and 3rd generation CARs, additional signaling domains are directly attached to the intracellular signaling region to improve the capability for a CAR to activate an immune cell and increase the degree to which said cell activates⁸ (**Fig. 1.2**). A very common signaling domain included in 2nd and 3rd generation CAR-T constructs is 4-1BB (CD137), a member of the tumor necrosis factor (TNF) receptor family that activates a signaling cascade

found natively in T lymphocytes and strongly upregulates pro-inflammatory pathways, induces cytokine release, and increases cell proliferation¹².

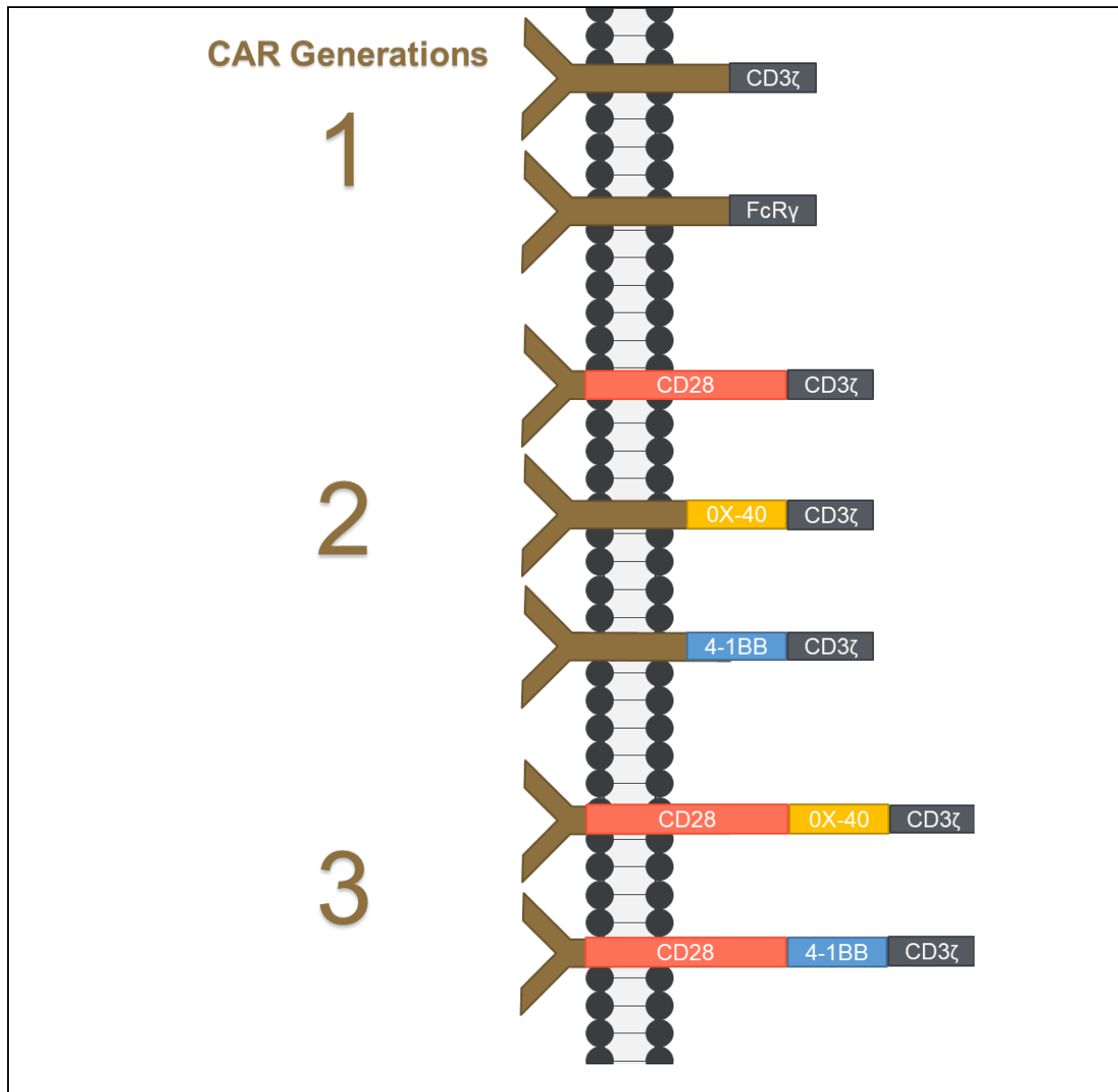


Figure 1.2: CAR designs: 1st, 2nd, and 3rd generations

By providing a highly sensitive CAR to a population of peripheral blood T cells by viral transfection/transduction, the immune system could be efficiently primed against a specific antigen.

This technology attracted attention through efficient targeting of cancers, most often blood-borne variants (lymphoma, leukemia, myeloma)⁸. A heavy focus was on B cell malignancies such as non-Hodgkin's lymphoma (NHL). CD19, CD20, and CD22 became widely used targets, as B cells would express this marker, clearly identifying them for removal by an anti-CD19/CD20/CD22 CAR-T cell. CD19 is the most common extracellular target due to the length and breadth of expression in B lymphocytes (**Fig. 1.3**).

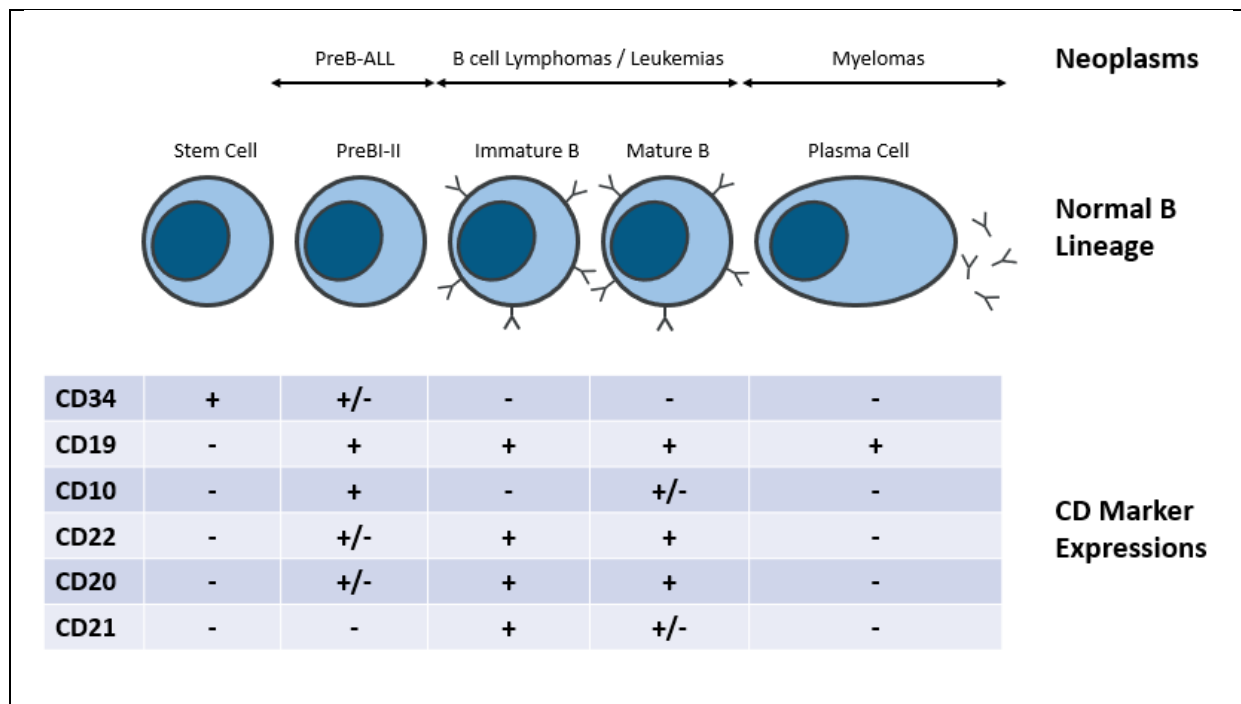


Figure 1.3: Expression profiles of common cluster of differentiation (CD) surface proteins on B-cell lineage. Adapted from Scheuermann *et al.*¹³

There are drawbacks to the use of CAR-T therapy in a clinical setting. Currently, CAR-T therapy cannot be performed using donor T cells. A patient must utilize their own cells, either primary T lymphocytes obtained by leukapheresis or somatic cells that are induced into a T cell phenotype (**Fig. 1.4**). Additionally, there is a significant time lag between lymphocyte collection from a patient and final infusion of CAR-Ts. This lag may be too long in certain clinical cases, where a patient cannot wait the several weeks needed.

There is a significant push to realize methods to utilize allogenic, or “off the shelf” cells that will avoid an undesired autoimmune reaction. One method considered to avoid a severe reaction against or by CAR-Ts is to preemptively generate several cell lines covering the spectrum of common HLA variants, such that they can be matched against a patient as needed¹⁴. On their own, T cells are difficult to proliferate in culture due to the complex and strict nature of biological control mechanisms that typically limit terminally differentiated immune cells. However, it is simpler to expand a culture of progenitor cells, and then differentiate them into T cells as needed.

Similarly, immune cells are much more resistant to genetic editing methods than other somatic cells. Thus, a significant improvement of the CAR-T process was through the editing of progenitor cells, such as induced pluripotent stem cells (iPSCs) from the patients’ own cells, and differentiation into CAR-T cells^{1,14,15}. Taken together, immortal lines from iPSCs, embryonic stem cells (ESCs), or lymphocyte progenitor stem cells can be preemptively edited to constitutively express a CAR and terminally differentiated as needed. This will also allow better quality control options for the CAR-Ts generated. This also avoids the challenges of low transfection efficiency and expansion of terminal cells in culture and enables CARs to maintain a longer presence without depletion due to the genetic editing performed.

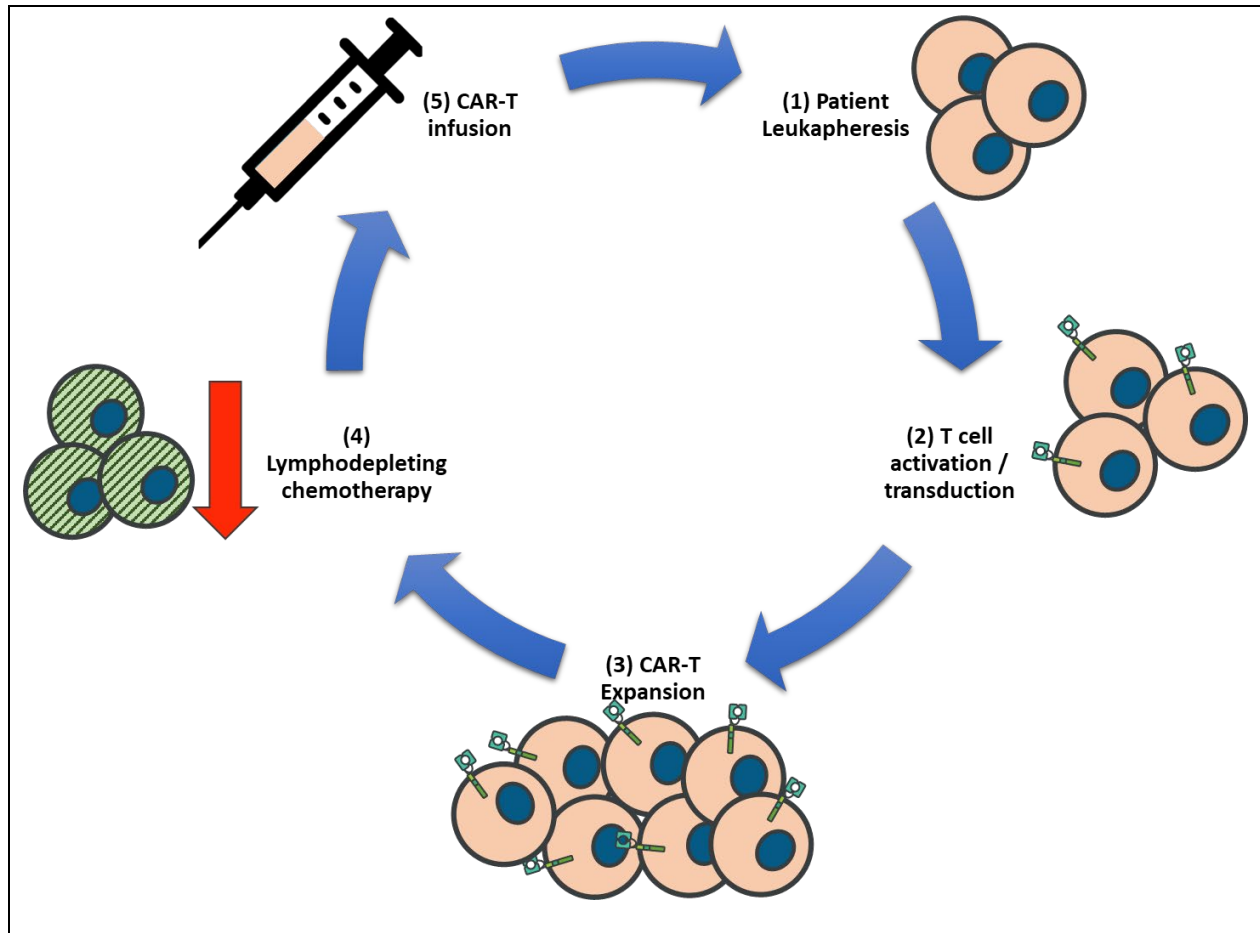


Figure 1.4: Typical CAR-T production pipeline.

Adapted from Tyagarajan *et al.*¹⁶

Another method considered to generate allogenic T cells is elimination of TCR expression in CAR-T cells. There is evidence that the lack of a TCR does not impact CAR-T cell performance¹⁷, and the removal of the TCR would further prevent CAR-Ts from recognizing the new host as foreign¹⁴.

A significant concern over the use of CAR-T therapy is cytokine release syndrome (CRS), where an overactive CAR-T reaction to its intended target can flood the body with inflammatory cytokines, leading to a host of adverse effects such as respiratory distress, various cytopenias, and global tissue damage¹⁸. CAR-Ts are designed to have a strong response to their target and can

easily overwhelm the homeostatic network of the immune system, leading to a host of complications such as inflammation and tissue damage^{4,18,19}. There have been many varied efforts to create a CAR with tunable properties. Tuning has been achieved in laboratory settings by spatial and temporal CAR activation. In one case, the intracellular CAR construct signaling domain is split into halves that only function when dimerized by a small molecule (**Fig. 1.5**). This effect can improve CAR-T safety primarily through preventing an autoimmune response.

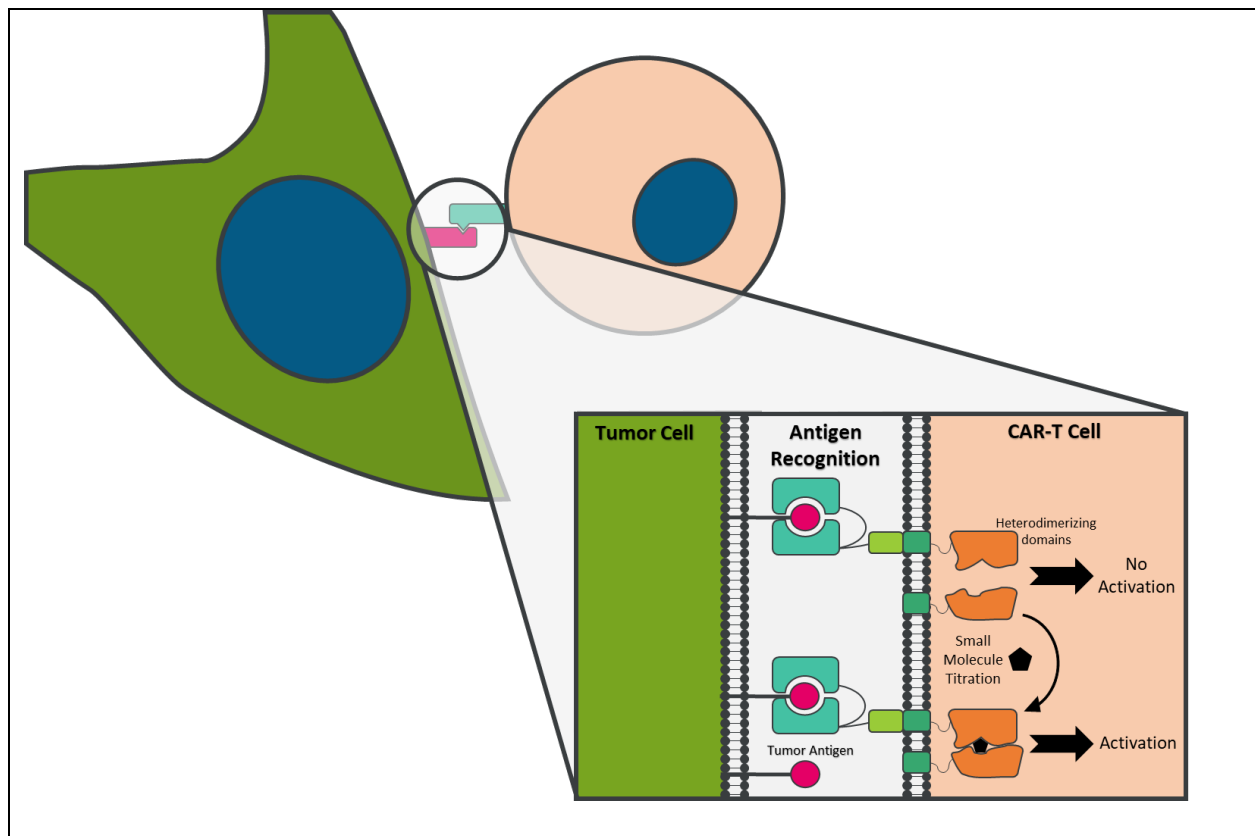


Figure 1.5: Small Molecule-Dimerizing CAR functionality. Adapted from Wu *et al.*²⁰

Solid tumor microenvironments (TME) remain a difficult task for CAR-T therapy. A combination of soluble factors, nutrient depletion, and hypoxic conditions pose a significant

challenge for long-term T cell activity. Additionally, tumor-associated immune cells, such as neutrophils, macrophages, dendritic cells, *etc.* committed to a non-inflammatory state by tumor interference, create negative feedback against T cell pro-inflammatory activity (**Fig. 1.6**). T cells can be recruited to the TME natively but have difficulties in penetrating the malignant tissue and maintaining a pro-inflammatory stance due to the overwhelming negative regulatory pressure^{9,10,21}. The adaptive immune must respond to a wide variety of signals from across the spectrum of cells, and as such are sensitive to both positive and negative regulatory signals.

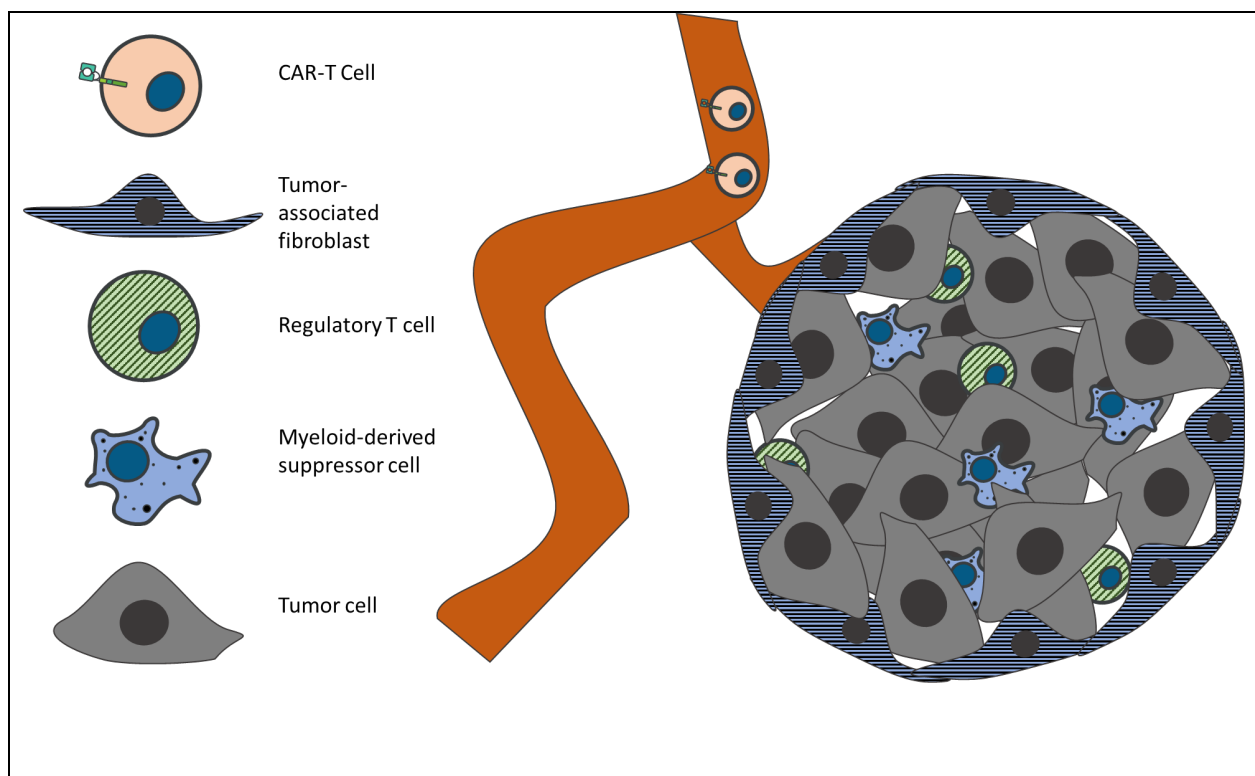


Figure 1.6: Solid tumor microenvironment: challenges for CAR-T. Adapted from Newick *et al*¹⁰

There are several lines of effort that attempt to improve anti-solid tumor action of CAR-T. The first line of reasoning is that T cells modified to ignore negative feedback signals from tumor associated immune cells will retain activity around the TME, which could be accomplished by addition of antibodies to block the programmed death protein 1 (PD-1) signal cascade used

between T lymphocytes and innate cells²². Alternatively, modification of T cells to silence expression of receptors for anti-inflammatory soluble factors such as adenosine, or inclusion of receptor antagonists has been shown to prevent T cell activity suppression²³. Additional T cell genetic modifications have been attempted with meaningful successes⁹. One group showed that phosphoenolpyruvate (PEP) is a critical limiting metabolite in CD4⁺ T cells infiltrating the TME, and overexpression of phosphoenolpyruvate carboxykinase 1 (PCK1) to generate more PEP from oxaloacetate allowed improved effector activity²⁴. A large amount of focus in the community has been towards modified autocrine signaling through the transgenic expression of several interleukins. A recent development in CAR constructs utilizes CAR T cells as directed cytokine factories for solid tumors. These 4th generation CARs, referred to as TRUCKs (T cells redirected for universal cytokine killing) utilize cytokine production by CAR-stimulated T cells to recruit innate immune cells to engage with the solid tumor²¹.

The use of cytokine signaling to generate an anti-tumor response is complicated, as many subclasses of immune cells react differently towards the same signal. As an example, while IL-2 is a potent activator of the inflammatory response in CD4⁺ helper and CD8⁺ effector T cells, it also strongly activates T regulatory cells. T regulatory cells have an anti-inflammatory role in the immune system, serving to induce immune cell apoptosis to prevent an immune overreaction. Thus, exogenous IL-2 addition or continuous production by modified CAR-T ultimately fails to induce anti-cancer activity in the long term²⁵. One promising interleukin is IL-15, which has been shown to improve proliferation of transgenic IL-15-producing CAR-Ts^{26,27}. These cells stay in circulation longer after initial infusion and thus improve the capacity and length of the treatment²⁸.

An interesting development in the field, potentially to be called the 5th generation of CARs, is antigen-dependent activation of the JAK-STAT signaling pathway²⁹. This is performed by

inclusion of an additional activation domain formed from a truncated cytoplasmic IL-2R β domain. Nominally, intracellular IL-2 and IL-15 signaling is performed using a common transmembrane heterodimer receptor (IL-2R β and IL-2R γ , or CD122 and CD132, respectively). This construct was designed primarily to avoid the inflammatory issues that arise from using IL-2 directly to stimulate immune cells, and instead activate IL-2 signaling pathways in a CAR activation-dependent manner.

Another explored modification to the CAR construct is the removal or addition of phosphorylation immunoreceptor tyrosine-based activation motif (ITAM) sites in the signaling and activation domains such as CD3 ζ and 4-1BB³⁰. Depending on the situation, it may be desired to have a less active, but more efficient and robust CAR construct. Highly active CARs may be of use in highly immunosuppressive cancers⁷, but for cases where long term, less-active treatments would be more beneficial. ITAM reduction can prevent overactivity, especially upon initial infusion, as well as T cell exhaustion. Activation of a T cell takes cellular resources, and a recurrent strong activation may lead to failure to recognize an antigen in the future. Overall, there are several engineering techniques that may lead to CAR-T efficacy improvements. In fact, many of the strategies addressed are not exclusive and can be combined to generate improved, allogenic CAR-Ts with enhanced solid-tumor cytotoxicity.

A significant reduction in mortality to blood-borne tumors is a direct result of clinical application of CAR-T therapy. This therapy is based around the native functionality of T cells to recognize threats and generate effector cells to activate humoral immunity, improve the innate response, or directly target the threat. CAR-T cells allow a direct line of communication to the complicated network of immune cells that functionalize and improve targeting against certain cancers and other diseases. The current efforts of the community can be summarized as improving

that line of communication such to expand the functionality of this treatment to be safer, more responsive, and increasingly effective.

1.2.2 CAR-Natural Killer Cells

Blood-based tumors are becoming easier to target by CAR-T methods, however the large hurdle of strong CAR efficacy against solid tumors is still an area of active research. An alternative to modification of T lymphocytes for improved CAR efficacy in solid tumors is the use of a different immune cell. The first alternative considered in the literature was natural killer (NK) cells, as their role in the immune system overlaps with T effector cells³¹. NK cells have a unique role in immunity to identify cells that evade recognition, by removal of HLA surface markers or other means, such as cancerous or virus-infected tissue. Due to their unique role, they do not self-recognize in the immune system, and as such allogenic NK cells can be introduced without inducing an immune reaction³². This capability has been exploited to create “off the shelf” solutions to various cancers³³, where NK cells engineered with CARs are injected without histocompatibility matching.

1.2.3 CAR-Macrophages

Another alternative immune cell for CAR therapy considered are macrophages. These innate cells differentiate from monocytes and serve as long-term monitors in the innate immune system^{34–37}. They circulate on longer time scales than granulocytes and take up residence in a variety of tissues such as the dermis, brain, bone marrow, lung alveoli, *etc.* They perform a variety of mediation tasks that would not be nominally attributed to a phagocytic immune cell, such as enucleation of erythrocyte progenitors in red blood cell generation, regulation of thermogenesis in brown fat, and maintaining alveoli surfactant levels in lung tissue all while simultaneously

watching for invading pathogens^{35,37–39}. As a poster child for professional antigen presenting cells, they crosstalk with the adaptive immune system through T lymphocytes to identify and eliminate pathogens that succeed against the barrage of the innate immune response. Macrophages are a multifaceted tool that wear many hats in homeostasis, and as such make an interesting choice of CAR-immune cells for solid tumors^{40–42}.

1.2.4 Current Clinical Applications of CAR Immunotherapy

CAR therapies have shown successful in a variety of clinical trials against several cancers. A representation of various clinical trials of CAR clinical trials is shown below in **Table 1.1**. Most CAR-based clinical trials utilize T cells as the host immune cell and utilize anti CD19 or anti CD269 CAR constructs. CD269 (B cell maturation antigen) is a well-described Tumor-associated antigen for relapsed or refractory multiple myeloma⁴³. Additionally, several CAR-NK studies are also ongoing, with increased focus in solid tumors, and generally targeting a wider variety of antigens including CD19, CD269, CD33, CD123, and NKG2DL. Only a single CAR-macrophage clinical trial was found for the treatment of HER2⁺ solid tumors. The US Food and Drug Association (FDA) has approved 5 CAR-T cell treatments: tisagenlecleucel (B-ALL; anti-CD19), axicabtagene ciloleucel (B cell NHL; anti-CD19), brexucabtagene autoleucel (B-ALL; anti-CD19), lisocabtagene maraleucel (B cell NHL; anti-CD19), and idecabtagene vicleucel (RRMM; anti-CD269). These drugs are very successful in eliminating cancers that did not respond well to traditional treatment strategies, indicating CAR technology is a novel and highly efficient addition to the clinical lineup.

Table 1.1: Clinical Trials of CAR-T, CAR-NK, and CAR-Macrophage Therapies

ID #	NCT03029338	NCT02975687	NCT03064269	NCT03076437	NCT02976857	NCT03173417
Target Antigen	CD19	CD19	CD19	CD19	CD19	CD19
Immune Cell	CAR-T	CAR-T	CAR-T	CAR-T	CAR-T	CAR-T
Disease	NHL	B cell ALL	CNS B cell acute lymphocytic leukemia	Leukemia, lymphoma	DLBCL	Leukemia
# Patients	20	20	3	28	10	83
Trial Phase	1	1	1	1,2	1	1,2
Region	China	China	China	China	China	China

ID #	NCT03097770	NCT02215967	NCT02658929	NCT03274219	NCT03090659	NCT03430011
Target Antigen	CD19/CD20	CD269	CD269	CD269	CD269, bi-epitope	CD269
Immune Cell	CAR-T	CAR-T	CAR-T	CAR-T	CAR-T	CAR-T
Disease	B cell leukemia and lymphoma	Multiple Myeloma	Multiple Myeloma	Multiple Myeloma	Multiple Myeloma	Multiple Myeloma
# Patients	99	30	67	72	57	169
Trial Phase	1,2	1	1	1	1,2	1,2
Region	China	US	US	US	N/A	US

ID #	NCT03915184	NCT03070327	ChiCTR1800018137	NCT02546167	NCT03288493	NCT03338972
Target Antigen	CD269	CD269	CD269	CD269	CD269	CD269
Immune Cell	CAR-T	CAR-T	CAR-T	CAR-T	CAR-T	CAR-T
Disease	Multiple Myeloma	Multiple Myeloma	Multiple Myeloma	Multiple Myeloma	Multiple Myeloma	Multiple Myeloma
# Patients	24	20	16	25	105	28
Trial Phase	1,2	1	1	1	1,2	1
Region	US, Canada	US	China	US	US	US

ID #	NCT05472558	NCT04887012	NCT05410717	NCT05574608	NCT05213195	NCT05528341
Target Antigen	CD19	CD19	CLDN6	CD123	NKD2DL	NKD2DL
Immune Cell	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK
Disease	B-cell Non-Hodgkin Lymphoma	B-cell Non-Hodgkin Lymphoma	Ovarian, Testicular, Endometrial cancer	AML	Metastatic colorectal cancer	Solid Tumors
# Patients	48	25	40	12	38	20
Trial Phase	1	1	1,2	1	1	1
Region	China	China	China	China	China	China

ID #	NCT05215015	NCT05194709	NCT05008575	NCT05507593	NCT05008536	NCT05410041
Target Antigen	CD33	5T4	CD33	DLL3	CD269	CD19
Immune Cell	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK
Disease	AML	Solid Tumors	AML	SCLC	Multiple Myeloma	Acute/chronic lymphocytic leukemia, NHL
# Patients	18	40	27	18	27	15
Trial Phase	1	1	1	1	1	1
Region	China	China	China	China	China	China

ID #	NCT05570188	NCT04847466	NCT04796675	NCT04623944	NCT05020678	NCT05563545
Target Antigen	CD19	PD-L1	CD19	NKD2DL	CD19	CD19
Immune Cell	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK
Disease	B cell leukemia and lymphoma	Gastroesophageal junction cancers, Advanced HNSCC	Acute/chronic lymphocytic leukemia, NHL	AML, MDS, Refractory myelodysplastic syndromes	B cell leukemia and lymphoma	acute lymphoblastic leukemia
# Patients	30	55	27	90	60	21
Trial Phase	1,2	2	1	1	1	1
Region	China	US	China	US	US	China

ID #	NCT03692663	NCT05248048	NCT04796688	NCT03056339	NCT05601466	NCT05379647
Target Antigen	PSMA	CD19	CD19	CD19	CD33	CD19
Immune Cell	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK	CAR-NK
Disease	mCRPC	Metastatic colorectal cancer	Acute/chronic lymphoblastic leukemia, B cell lymphoma	B cell leukemia and lymphoma	AML	B cell leukemia and lymphoma
# Patients	9	9	27	36	18	24
Trial Phase	1	1	1	1,2	1	1
Region	China	China	China	US	China	China

ID #	NCT05182073	NCT04660929
Target Antigen	CD269, CD38	HER2
Immune Cell	CAR-NK	CAR-M
Disease	Multiple Myeloma	HER2 positive solid tumors
# Patients	168	18
Trial Phase	1	1
Region	US	US

1.2.5 Treatment of Non-Cancerous disease states using CARs

Recent investigation into CAR-based therapies for non-cancer malignancies has shown interesting promise. One disease of interest is autoimmune disorders, such as pemphigus vulgaris⁴⁴, a chronic condition highlighted by blisters of the skin and mucous membranes. This disease is caused by an overproduction of antibodies against the endogenous B cell protein desmoglein 3 (DSG3). Treatment of this disease by depleting B cells via an anti-CD20 mAb rituximab was transiently successful but was plagued by relapse and safety concerns over non-specific immunosuppression. A CAR system designed to bind and attack DSG3-autoreactive B cells showed promise in mouse model and is currently under phase I clinical trial (NCT04422912; N=39). This application of the CAR technology, called a chimeric autoantibody receptor (CAAR) demonstrates a unique modification to the core functionality of retraining immune cells to achieve a specialized function. Additional methods to treat autoimmune disorders involves the use of CAR-T regulatory cells (CAR-Treg) to repress an excessive immune response locally, such as in ulcerative colitis⁴⁵, multiple sclerosis⁴⁶, and type 1 diabetes⁴⁷ are under active research. There is an increasing usage of CAR technology beyond oncology, demonstrating the potential uses of immune reprogramming in a wide variety of malignancies⁴⁴.

1.3 Conclusion

In summary, immunotherapy is a novel treatment option for disease states in addition to surgery, chemical, and radiologic methods. Improvement in this field will lead to a greater applicability in treatment of cancers, and further development of treatment options for non-cancerous malignancies states such as chronic inflammatory diseases, psoriasis, and immune

dysfunction disease-states. Improvement in CAR immunotherapy that is ongoing is the development of non-T cell CAR systems, such as CAR-NK, CAR-macrophage, and CAR-neutrophils, each with their own benefits over T cells. NK cells are of interest primarily for their allogenic potential, while macrophages are excellent choices for targeting solid tumors due to their tissue-infiltrating capabilities. Looking forward, one area of immunotherapy that still needs significant research is the interaction of CAR-modified immune cells with non-human factors such as the gut microbiome. Immune systems require beneficial microbes to properly develop and respond appropriately to future threats, while selectively ignoring helpful or non-pathogenic agents. Further understanding of immunology and the signaling pathways that mediate immune responses will allow improved knowledge of how disease-states form and how to better prevent and/or treat them.

2. HUMAN PLURIPOTENT STEM CELL DERIVED CAR NEUTROPHILS FOR PROSTATE CANCER

2.1 Introduction

Neutrophils are a powerful candidate for further CAR therapy improvement. Similar to macrophages and other innate cells, they readily migrate into local tissue in response to inflammatory chemokines and contribute to the innate defense. This effect in combination with a CAR construct would allow for efficient, local anti-tumor actions. Given the previous success of CAR-neutrophils in targeting glioblastoma in murine models, analysis of neutrophil-mediated cytotoxicity against other solid tumors, namely prostate cancer, was of significant interest. In this chapter, human pluripotent stem cell (hPSC) -derived neutrophils modified with a prostate-specific membrane antigen (PSMA)-targeting CAR construct is shown to recognize LNCaP cells *in vitro* and exert a dose-dependent cytotoxic effect at a greater efficacy than unmodified hPSC-neutrophils.

2.2 Review of Literature

2.2.1 Prostate Cancer

Prostate cancer (PCa) is the 2nd most common cancer in men worldwide, with 1.4 million cases in 2020, with an estimated number of deaths exceeding 320 thousand⁴⁸. It is the most common cancer-associated death for men, and commonly progresses into a castration-resistant variant with significant potential to metastasize. Metastatic, castration resistant PCa (mCRPC) is the most advanced form of the disease, and patient outcomes and quality of life are low⁴⁹. The discovery of prostate-specific membrane antigen (PSMA), which is an integral membrane protein expressed uniquely on in prostate tissue has been shown to be upregulated in PCa tissue^{50,51}. It was

also shown that monoclonal antibody treatment against PSMA can reduce PCa tumor advancement⁵². An excellent review of modern treatment methods and pre-clinical / clinical trials for prostate cancer is provided by Wang *et al*⁵³.

2.2.2 Tumor-Associated Immune Cells

Of interest in neutrophil research is their effect in the tumor microenvironment (TME). Solid tumors create a local anti-inflammatory zone using physical barriers, anti-inflammatory cytokines, small molecules, and tumor-recruited immune cells^{9,41,54}. Tumor-associated neutrophils (TANs) and macrophages (TAMs) are signaled by the cancerous tissue to adopt an immunosuppressive morphology which prevents an inflammatory response^{1,10}. TAMs and TANs have been shown to not be a population of traditional pro-tumor M2 or N2 type macrophages and neutrophils, respectively. Instead, the degree of activation of the innate cells is more akin to a spectrum, of which several different subclasses of macrophages, neutrophils, and other immune cells (T, NK, myeloid, Treg) interact and globally adopt an immunosuppressive stance. The result is a failure for the immune system to recognize the tumor as a threat and eliminate the cancerous tissue. TAMs/TANs can actively promote tumor survival through angiogenesis and metastasis, which worsens the tumor's burden on the surrounding tissue and body⁵⁴. It has been shown that typically M2/N2-like TAMs/TANs can transition to a M1/N1-like morphology upon presentation with proinflammatory cytokines such as IFN- γ ⁵⁵.

While general macrophage/neutrophil activity can be reduced through small molecule, miRNA, or antibody-based treatments, any treatment would not skew the global stance of the immunosuppressive TME and leads to severe health effects due to limited innate cell count or activity. To best alter the TME to adopt an anti-tumor, pro-inflammatory status, a change in balance is needed between pro- and anti-inflammatory cells. Addition of pro-inflammatory cells

into the TME would solve this problem. However, to ensure that a therapeutic dosage of M1/N1-like cells remain in the TME, introduced cells must be capable of targeting cancerous tissue, such as with a CAR-modified immune cell.

2.2.3 Neutrophil and Macrophage Differentiation

Both macrophage and neutrophil cells are terminally differentiated; they cannot be expanded from primary cells. Models of neutrophil/macrophage *in vitro* and *in vivo* must be isolated from peripheral mononuclear cells or differentiated from stem cell progenitors. Various studies have been conducted utilizing hematopoietic differentiation of hESCs and/or hiPSCs to generate neutrophils, macrophages, or monocytes granulocytes in general^{34,56–83}. These studies help better understand the factors leading to these disease states and the underlying biological principles governing the myeloid cell line. These models typically use ESCs or iPSCs to produce neutrophils *in vitro*. While monocytes/macrophages have been generated by a wide array of ESCs and iPSCs, neutrophils are typically restricted to iPSC differentiation in the literature (**Table 2.1**).

Table 2.1: Summary of Myeloid Cell Differentiation Studies

Cells Generated	Stem Cell Used	Study Purpose	Genetic Modification	Differentiation/ Model	Reference
Neutrophil/ Monocyte	hiPSC	Neutrophil differentiation	CRISPR/Cas9 knockouts	<i>In vitro</i>	7
Macrophage	hiPSC	Drug-Screening	Adenovirus infection; reporter lines	<i>In vitro</i>	56
Macrophage	hiPSC	Study of efferocytosis function in differentiated macrophages	No changes	<i>In vitro/vivo</i> / murine	57
Neutrophil	hiPSC	Generation of disease-state XCGD model	Ectopic gp91phox expression, retrovirus	<i>In vitro</i>	58
Macrophage	hiPSC	<i>Staphylococcus aureus</i> pulmonary model	No changes	<i>In vitro/vivo</i> / murine	59
Macrophage	hiPSC	Xenophagy evasion model	No changes	<i>In vitro</i>	60
Neutrophil	hiPSC	Neutrophil generation with activity against pathogenic bacteria	No changes	<i>In vitro</i> / murine	61
Neutrophil	Marmoset iPSC	Neutrophil differentiation, feeder- free	No changes	<i>In vitro</i>	63
Macrophage	hiPSC	Efficient Genetic modification of differentiated macrophages	CRISPR/Cas9 knockouts	<i>In vitro</i>	64
Neutrophil	hiPSC	Differentiated neutrophil with activity against pathogenic bacteria	No changes	<i>In vitro/vivo</i> / murine	65
Neutrophil	hiPSC	Differentiation by mmRNA treatment, feeder/xeno/serum free	No changes	<i>In vitro</i>	67
Monocyte/ Macrophage	hiPSC	Differentiation Protocol, feeder- free, serum-free	CRISPR/Cas9 knockouts	<i>In vitro</i>	68
Granulocyte/ Monocyte	hiPSC	Bulk differentiation of iPSC to myeloid cell types	No changes	<i>In vitro</i>	69

Neutrophil	hiPSC	Hematopoiesis process analysis	No changes	<i>In vitro</i>	70
Macrophage	hiPSC	Differentiation Protocol, defined medium, serum free	No changes	<i>In vitro</i>	71
Monocyte/ Macrophage/ Dendritic Cell	hESC, hiPSC	Efficient, high yield differentiation protocols	No changes	<i>In vitro</i>	72
Neutrophil/ Macrophage	hiPSC	Generation of disease-state XCGD model	No changes	<i>Ex vivo</i>	73
Monocyte/ Macrophage/ Dendritic Cell	hESC, hiPSC	Differentiation protocol, serum-free, feeder-free	No changes	<i>In vitro</i>	75
Monocyte/ Macrophage	hESC hiPSC	Long-term production of cell types for disease models	Lentivirus infection; reporter line	<i>In vitro</i>	76
Monocyte/ Granulocyte	hESC	Differentiation Protocol	No changes	<i>In vitro</i>	77
Monocyte/ Macrophage	hiPSC	Differentiation Protocol	No changes	<i>In vitro</i>	78
Macrophage	hiPSC	Disease model from cryopreserved PSCs	HDR	<i>In vitro</i>	79
Macrophage/ TAM	Immortalized Monocyte	Macrophage generation from Monocyte	No changes	<i>In vitro</i>	80
Neutrophil	hCMP	Label-free differentiation monitoring	No changes	<i>In vitro</i>	81
Neutrophil	hiPSC	Neutrophil Differentiation	No changes	<i>In vitro</i>	82
Neutrophil	hCMP	Comparison of neutrophil differentiation methods in gene expression	No changes	<i>In vitro</i>	84
Microglia	hESC, hiPSC	Brain disease model, protocol improvement	No changes	<i>In vitro/vivo</i> / murine	85
Neutrophil	hPSC	CAR Neutrophil for Immunotherapy	CRISPR/Cas9	<i>In vitro/vivo</i> / murine	86

Neutrophil differentiation is a less-developed area of study compared to macrophages. In studies of neutrophil proliferation, various protocols are employed to efficiently differentiate neutrophils from iPSCs, and most protocols rely on serum and/or feeder cell co-cultures. Additionally, the literature for neutrophil differentiation is still closely aligned towards production and efficiency. While there are examples of this in the macrophage literature space, there also exists a healthy number of studies that use an established protocol to study a specific aspect of macrophage function which is only possible due to the further advancement of the field. The most significant trend in the publications on neutrophil differentiation is the delineation of serum-free and/or feeder cell-free culture conditions, following a similar progression of the development of serum/feeder-free culture conditions for murine and human ESCs/iPSCs. **Table 2.2** summarizes several differentiation protocols for neutrophils / granulocytes in the literature.

Typical programs for differentiation of iPSCs into neutrophils are most broadly divided by their use of feeder-cells and serum. Feeder cells are typically murine cells (OP9, C3H10T1/2, MEF), and there is a preference to not use them at all, and as such feeder-free methods garner significant attention. Serum-free methods are also of interest, although recent literature only contains a small number of feeder & serum-free methods, indicating more research is necessary.

Table 2.2: Protocols of Neutrophil Differentiation

Starting Cell Type	Feeder Cells / Serum	Differentiation Protocol					Reference
hiPSC	C3H10T1/2 / FBS	D0-13 hiPSC	D14- HPC				58
		C3H10T1/2, IMEM FBS, insulin, transferrin, selenite, L-glutamine, MTG, ascorbate	C3H10T1/2, α MEM FBS, G-CSF, L-glutamine				
Monkey iPSC (<i>Callithrix jacchus</i>)	MEF / FBS	D0-12 Mo-iPSC	D13-37 Sort (CD34 ⁺ , VEGFR2 ⁻)	D38-48+			63
		MEF, DMEM VEGF	MEF, DMEM FBS, Glc, L-Glu, β ME, IL-3, TPO, SCF,	MEF, DMEM G-CSF, IL-3, TPO			
hiPSC	None / FBS	D0-4 hiPSC	D4-	5d Post harvest			65
		DMEM/F12 FBS, NEAA	APEL 2 IL-3, G-CSF	RPMI FBS, G-CSF			
hiPSC	None / None	D0-1 Transfect hiPSC	D1-2	D3-D(8-29) Myeloid prog.	D(14-37) [6-8d post]		67
		E8, Rock-i	Stemline II FGF2	Stemline II FGF2, GM-CSF, UM171	Stemspan H3000 Glutamax, ExCyte, G-CSF, Am580		

hiPSC	None / FBS	D0-4 hiPSC	D4- harvest	7-10d Post harvest			69
		ESC media bFGF, Rock-I	APEL IL-3, G-CSF	RPMI 1640 FBS, L-Glu, G-CSF			
hiPSC	OP9 / FBS, HS	D0-1 hiPSC	D2-18	D19-25	D26-32		70
		StemSpan Rock-i	IMDM FBS, NEAA, L-Glu, βMe, BMP4, SCF, FLT3L, IL-6, TPO	OP9, IMDM FBS, HS*, HDM**, βMe, SCF, FLT3L, IL-6, IL-3, TPO *Horse Serum **protein-free hybridoma media	OP9, IMDM FBS, βMe, G-CSF		
hiPCS	OP9 / FBS	D0-4	D5-10 hiPSC	D11-14 CD34 ⁺ MACS	D15-25		73
		OP9, α-MEM FBS	OP9, α-MEM FBS, MTG, Ascorbate	pHEMA, IMDM FBS, SCF, FLT3L, IL-6, IL-3, TPO	OP9, α-MEM FBS, G-CSF		
PLB-985	None / FBS	D0-6 PLB-985					81
		RPMI 1640, FBS, DMSO					
hiPSC	OP9 / FBS	D0-9 hiPSC	D10-29 TRA I-85 ⁺ , CD34 ⁺ , VEGFR2 ^{high}	D30-			82
		OP9, αMEM VEGF	OP9, αMEM FBS, β-ME, IL-3, SCF, TPO	OP9, αMEM IL-3, G-CSF			

hiPSC	None / None	D0-1 hiPSC EB		D2-3 TRA I-85 ⁺ , CD34 ⁺ , VEGFR2 ^{high}		D4-6		D7-32			83
		APEL Rock-I, FGF2		APEL FGF2, BMP4		APEL, Geltrex VEGF, SCF, IL-3		APEL IL-3, G-CSF			
hPSC	None / None	D-1 hPSC	D0-1	D2-3	D4-5		D6-8		D9-11	D12-20 Floating	86
		mTeSR+	DMEM/Vc CHIR99021	LaSR Basal VEGF	Stemline II SB431542, FLT3L, SCF,		Stemline II SCF, FLT3L, GM-CSF, IL-6, IL-3		Stemline II G-CSF, IL-6, IL-3	Stemline II GlutaMAX, G-CSF, AM580	

Several different variations of neutrophil differentiation schemes exist, although they all tend to rely on similar small molecules for key signaling steps. The most significant across the protocols is the use of granulocyte colony-stimulating factor (G-CSF). It was an early-discovered cytokine shown to be essential in stimulating neutrophil production, and G-CSF knockout mice have chronic neutropenia and low neutrophil mobilization^{87,88}. This cytokine ultimately serves to spur granulopoiesis, neutrophil maturation and mobilization, and can be used therapeutically as a direct injection in cases of neutropenia to fight infections^{88,89}. G-CSF normally circulates in the body at low levels, but during infection or other stresses, endothelial tissues release this cytokine triggering an increase in neutrophil production and distribution⁸⁸. Due to this, G-CSF is almost ubiquitous in end-stage granulocyte differentiation protocols, with the occasional use of granulocyte/macrophage-CSF (GM-CSF) being utilized at the mid-stage differentiation protocol to better transition myeloid progenitors into the monocyte/granulocyte lineage. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are occasionally used in differentiation schemes, which contributes to the proliferation of common progenitor cells to increase the number of terminally differentiated neutrophils⁹⁰, and thus are common in the beginning stages of differentiation from hPSC to hematopoietic stem cells (HSC) and then to common myeloid progenitor stem cells (CMP). Interleukin 3 (IL-3) is a multipotent colony stimulating factor that promotes proliferation of various hematopoietic and vascular cell types^{91,92}. Addition of IL-3 into neutrophil differentiation methods is performed in mid and late stage, as it will retain activity against very early and late progenitor cell types. Similarly, IL-6 is a cytokine secreted naturally by macrophages and T lymphocytes that can stimulate granulopoiesis, among other functions⁹³. Interestingly, there is a synergistic effect of IL-6 and IL-3 to stimulate the formation of granulocytes, and as such certain protocols use both to better generate neutrophils *in*

vitro. Stem cell factor (SCF) can be added to growth media to help promote hematopoietic stem cell entry into the cell cycle and differentiation, although it is incapable of doing this alone, and needs a combination of other cytokines such as IL-3 to make a meaningful contribution to hematopoiesis. SCF can additionally help prevent apoptosis of HSCs and enhance survivability, making it a useful and notable contribution to mid-stage differentiation media⁹⁴⁻⁹⁶. Finally, most early-stage differentiation media are a basal medium such as α -MEM, DMEM, or IMEM supplemented with serum, feeder cells, or both. Specific protocols are serum and feeder cell free, although they are in the minority. It is expected that these protocols will become more popular in the future in an aim to eliminate variability in differentiation batches.

2.2.4 Key Flow Cytometry Markers for Neutrophils

Following differentiation, flow cytometry of key neutrophil markers was performed. Key lineage markers tested included CD45, CD66b, CD16, CD18, CD11b, and intracellular myeloperoxidase (MPO). CD45, also known as leukocyte common antigen, is a transmembrane receptor expressed on a wide variety of hematopoietic lineages and helps verify HSC production through endothelial to hematopoietic transition (EHT). There are 8 major isoforms, each with a common cytoplasmic domain and unique extracellular domain. These isoforms are identified based on the presence of exons 4, 5, and 6 (A, B, and C respectively) to generate CD45R0, -RAC, -RC, -RB, -RA, -RBC, -RAB, and -RABC. In humans, only R0, RA, RB, RAB, and RABC have been identified⁹⁷. Different isoforms are common to different hematopoietic lineages, such as CD45R0 for activated and memory T cells, and myeloid cells while CD45RA and CD45RB isoforms are found primarily on naïve T cells, peripheral B cells, and thymocytes. In flow analysis for this research, a generic anti-CD45 antibody that recognizes all isoforms was utilized.

CD66b (CEACAM8 or CGM6) is a unique marker for eosinophils and neutrophils. It is a highly glycosylated, glycosylphosphatidylinositol (GPI) -anchored protein of the carcinoembryonic antigen immunoglobulin superfamily (including CD66a-d)⁹⁸. It serves roles in activation and adhesion and was found to be upregulated in rheumatoid arthritis⁹⁹. In eosinophils, it was shown that CD66b is localized on lipid rafts and induces strong cell adhesion during leukocyte rolling. It additionally recruits CD11b molecules and further activates the Src kinase Hck leading to degranulation and an inflammatory response¹⁰⁰.

CD16 (Fc- γ Receptor III) is a low affinity IgG Fc surface receptor found generally across innate cells that mediates binding and phagocytosis of opsonized targets by antibody-dependent cellular toxicity (ADCC)^{101,102}. There are two isotypes: Fc γ RIIIa and Fc γ RIIIb, or CD16a and CD16b respectively. CD16a is found generally across the innate cells while CD16b is alternatively called the neutrophil antigen system with two isoforms (NA1 and NA2). CD16b is found almost uniquely in neutrophils¹⁰³, although under some conditions mild CD16b expression or cytosolic CD16b mRNA has been observed in eosinophils and basophils¹⁰⁴. The A and B isotypes do not differ largely in the extracellular domains, with a sequence similarity of 97%¹⁰⁵; However, CD16b is restricted to the extracellular region only as a GPI-anchored peptide, while CD16a contains a transmembrane and cytoplasmic tail which allows for direct signal transduction by association with intracellular peptides such as CD3 ζ (CD247) and/or the intracellular Fc receptor γ subunit, FcR γ ^{106–108}. Despite the high sequence similarity in the extracellular domain, binding affinities for IgG immunoglobulins can differ greatly due to critical residue differences between the two isoforms¹⁰⁵. In fact, CD16b is considered a decoy receptor due to the lack of signaling domains and has been shown to negatively regulate ADCC in unstimulated peripheral blood neutrophils in a manner that was dependent on the copy number variation of the *FCGR3B* gene (*FCGR2/3* locus).

This effect disappeared after stimulation overnight with G-CSF and IFN- γ , indicating that the decoy CD16b might compete with Fc γ RIIa, another low affinity IgG Fc receptor, to prevent excessive toxicity in a resting state¹⁰³. As an aside, there is significant research into the specific roles and relations of Fc gamma receptors in inflammatory disease such as rheumatoid arthritis, of which certain IgG-binding affinity impacting polymorphisms of Fc γ RIIIa and Fc γ RIIa are associated with disease development and joint destruction, although there is conflicting evidence at the time of writing^{109,110}.

Beta-2 integrins are a class of leukocyte-unique, transmembrane heterodimer receptors named after their common β_2 integrin chain (CD18)^{111,112}. There are multiple alpha chains that can dimerize with CD18, including integrin alpha-L (ITGAL or CD11a), alpha-M (ITGAM or CD11b), and alpha-X (ITGAX or CD11c), and alpha-D (ITGAD or CD11d). The main role of integrins is binding to ECM glycoproteins to assist with cell adhesion. ECM proteins such as collagen and fibronectin express a generalized integrin binding domain consisting of a Arg-Gly-Asp (RGD) residue group¹¹³. Bacterial and viral glycoproteins are also known to bind to some integrins, with these receptors serving as an entry point for viruses in RGD-independent and -dependent manners¹¹⁴. Fibronectin-binding proteins are also shown to be used by bacteria such as *Staphylococcus aureus* for pathogenesis¹¹⁵.

The relative expressions of β_2 integrins are not identical across immune cells. $\alpha_L\beta_2$ is expressed throughout leukocytes, while $\alpha_M\beta_2$, and $\alpha_X\beta_2$ are found on phagocytotic cells, with $\alpha_D\beta_2$ being unique to macrophage lineage cells^{111,116,117}. Of interest to innate cell phagocytosis, the $\alpha_M\beta_2$ (CD11b/CD18) integrin receptor, also called Mac-1 or CR3, is expressed highly on myeloid and NK cells which mediates surface adhesion-based signaling to induce an inflammatory response and phagocytosis¹¹⁸. It is a critical regulator of the phagocytotic response of innate cells, and loss

or reduction of CR3 or β_2 integrin expression or function can result in leukocyte adhesion deficiency and result recurrent infection by normally harmless agents, reduced wound healing, and poor transmigration of leukocytes. CR3 is a highly promiscuous integrin, with binding partners in the ECM, ICAM1/2, microbial peptides (ex. LPS), non-peptide ligands, and complement C3bi^{118–121}. CR3 may also interact with CD14 in recognition and downstream signaling of LPS, although it is believed that this mechanism is in addition to the primary signal transduction performed by CR3 independently¹²². Given the many binding partners of CR3, it has high capacity to transduce signals to and from the cell. Neutrophil activation, transmigration, phagocytosis, and degranulation are all functions critically reliant on CR3 signaling¹²³. The complement system of innate cell threat-pattern recognition is additionally reliant on CR3 function, as CR3 binds C3bi in an RGD-dependent fashion¹¹⁹. C3bi is the inactive form of the opsonizing complement protein C3b that functions to degrade pathogen infection potential, improve phagocytosis, and initiate C5 convertase activation to form membrane attack complexes to directly destroy bacterial pathogens. Deeper reviews into the complement system are described elsewhere^{124,125}; However, CD11b and CD18 are critical for complement recognition and activation of myeloid cells, and thus differentiated neutrophils must express these surface receptors for full functionality.

2.3 Methods

2.3.1 hPSC Cell Culture Maintenance and Differentiation

Differentiation of neutrophils from H9 hPSCs was followed the Stemline II differentiation scheme (SII; **Scheme 2.1**). H9 hPSC line was obtained from WiCell and maintained on Matrigel-coated 6-well plates in mTeSR plus medium. For neutrophil differentiation, hPSCs were dissociated with 0.5 mM EDTA and seeded onto iMatrix 511-coated 12-well plate in mTeSR plus

medium with 5 μ M Y27632 for 24 h (day -1). At day 0, cells were treated with 6 μ M CHIR99021 (CHIR) in DMEM medium supplemented with 100 μ g/mL ascorbic acid (DMEM/Vc), followed by a medium change with LaSR basal medium (advanced DMEM/F12, 2.5 mM GlutaMAX and 100 μ g/mL ascorbic acid) from day 1 to day 4. 50 ng/mL VEGF was added to the medium from day 2 to day 4. At day 4, medium was replaced by Stemline II medium (Sigma) supplemented with 10 μ M SB431542, 25 ng/mL SCF and FLT3L (SII-SB). On day 6, SII-SB medium was aspirated, and cells were maintained in Stemline II medium with 50 ng/mL SCF and FLT3L, 25 ng/mL GM-CSF, 50 ng/mL IL-6, and 10 ng/mL IL-3. At day 9, the top half medium was aspirated and changed with 0.5 mL fresh Stemline II medium containing 50 ng/mL G-CSF, 50 ng/mL IL-6, and 10 ng/mL IL-3. On day 12, cells in suspension (floating) were gently harvested and filtered for terminal neutrophil differentiation in Stemline II medium supplemented with 1X GlutaMAX, 150 ng/mL G-CSF, and 2.5 μ M retinoic acid agonist AM580. Half medium change was performed every 3 days, and mature neutrophils could be harvested for analysis starting from day 20.

Scheme 2.1: Stemline II differentiation (SII) for hPSC neutrophils

D-1 hPSC	D0-1	D2-3	D4-5	D6-8	D9-11	D12-20 Floating
mTeSR+	DMEM/Vc CHIR99021	LaSR Basal VEGF	SB431542, FLT3L, SCF,	SCF, FLT3L, GM-CSF, IL-6, IL-3	G-CSF, IL-6, IL-3	GlutaMAX, G-CSF, AM580
StemLine II						

2.3.2 Nucleofection and Genotyping of hPSCs

To increase cell viability, 10 μ M Y27632 was used to treat hPSCs 3–4 hr or overnight before nucleofection. Cells were then singularized by Accutase for 8–10 min, and $1\text{--}2.5 \times 10^6$ hPSCs were nucleofected with 6 μ g SpCas9 AAVS1 gRNA T2 (Addgene; #79888) and 6 μ g CAR donor plasmids in 100 μ L human stem cell nucleofection solution (Lonza; #VAPH-5012) using program B-016 in a Nucleofector 2b. Nucleofected cells were seeded into one well of a Matrigel-coated 6-well plate in 3 mL pre-warmed mTeSR plus or mTeSR1 with 10 μ M Y27632. 24 hr later, the medium was changed with fresh mTeSR plus or mTeSR1 containing 5 μ M Y27632, followed by a daily medium change. When cells were more than 80% confluent, drug selection was performed with 1 μ g/mL puromycin (Puro) for 24 h. Once cells recovered, 1 μ g/mL Puro was applied for about 1 week. Individual clones were then picked using a microscope inside a tissue culture hood and expanded for 2–5 days in each well of a 96-well plate pre-coated with Matrigel, followed by a PCR genotyping. The genomic DNA of single clone-derived hPSCs was extracted in 40 μ L QuickExtract™ DNA Extraction Solution (Epicentre; #QE09050). 2 \times GoTaq Green Master Mix (Promega; #7123) was used to perform the genomic DNA PCR. For positive genotyping, the following primer pair with an annealing temperature T_m of 65°C was used: CTGTTTCCCCTTCCCAGGCAGGTCC and TCGTCGCGGGTGGCGAGGCGCACCG. For homozygous screening, we used the following set of primer sequences with an annealing temperature T_m of 60°C was used: CGGTTAATGTGGCTCTGGTT and GAGAGAGATGGCTCCAGGAA.

2.3.3 Flow Cytometry

Analysis of cell culture samples were performed using a BD Accuri C6 plus personal flow cytometer (Beckton Dickinson). Cell culture media was collected and filtered through a 70 μ m

mesh. Living cell flow cytometry was performed in cases where exterior surface-bound or transmembrane peptide expression were assessed, as well as for cell-permeating stains such as calcein-AM. For living cell flow analysis, growth culture was centrifuged at 0.5 x g for 5 min to pellet cells, which was washed with phosphate buffered saline (PBS) and resuspended in a small volume (~25% or original sample) of PBS containing 0.5% BSA (**Flow Buffer 1; FB1**). In cases where internally and/or cytosolically expressed peptides were analyzed, cell culture media was centrifuged at 0.5 x g for 5 min, washed with PBS, and resuspended in **FB1** additionally containing 0.1% TritonX100 as a fixing/permeabilizing agent (**Flow Buffer 2; FB2**). In all cases, primary conjugated antibodies were utilized for flow analysis following the manufacturer's recommended dilution. Staining was allowed to occur for 30 min at room temperature in the dark. Afterwards, samples were diluted 6-fold in **FB1** to reduce background signal. Samples were analyzed at a flow rate of 60 μ L/min at a core size of 16 μ m. Analysis was performed using FSCalyzer 0.9.22-alpha.

2.3.4 Bulk RNA Sequencing

Total RNA of sorted hPSC-derived CD16⁺ and peripheral blood neutrophils was prepared with Direct-zol RNA MiniPrep Plus kit (Zymo Research) according to the manufacturer's instructions. RNA samples were then prepared and performed in Illumina HiSeq 2500 by the Center for Medical Genomics at Indiana University. HISAT2 program¹²⁶ was employed to map the resulting sequencing reads to the human genome (hg 19), and the python script rpkmforgenes.py¹²⁷ was used to quantify the RefSeq transcript levels (RPKM). The original fastq files and processed RPKM text files were submitted to NCBI GEO (GSE188393). Principal component analysis (PCA) was performed in Perseus P2.0.6.0 and visualized in Mathematica 12.3. Gene expression data for each cell type were compared with that of hPSCs and significantly enriched gene ontology ($p < 0.05$) were considered for further analysis. MATLAB (Mathworks)

and Microsoft Excel were used to identify the unique and common pathways in different cell types. Heatmaps and hierarchical clustering analysis of selected gene subsets after normalization were then plotted using Morpheus (Broad Institute).

2.3.5 Phagocytosis Assay

Phagocytosis was assessed using pHrodo Green *E.coli* BioParticles Conjugate according to the manufacturer's protocol. In brief, pHrodo Green *E. coli* beads were resuspended in 2 mL of PBS and ultrasonicated 3 times. Beads per assay (100 μ L) were opsonized by mixing with opsonizing reagent at a ratio of 1:1 and incubated at 37°C for 1 h. Beads were washed 3 times with mHBSS buffer by centrifugation at 4°C, 1,500 RCF for 15 min, and resuspended in mHBSS buffer. Differentiated neutrophils were resuspended in 100 μ L of opsonized solution and incubated at 37°C for 1 h, followed by flow cytometry analysis using an Accuri C6 plus cytometer (Beckton Dickinson). Phagocytotic capacity was measured using the following index:

$$\text{Equation 2.1: Phagocytotic Index (P.I)} = \frac{CD45^+ \text{ Phagocytotic Cells}}{CD45^+ \text{ Cells}}$$

Where phagocytotic cells are positive for the fluorescent dye.

2.3.6 Immune Synapse Formation

To visualize immunological synapses, 100 μ L of LNCAP cells (1,000 cells/mL) were plated in a 96-well plate for 12 hours to adhere. Neutrophils (10,000 cells/mL) were added to LNCaP cells and incubated for 6 hours before fixation with 4% paraformaldehyde (in PBS). Cytoskeleton staining was then performed using an F-actin Visualization Biochem Kit

(Cytoskeleton Inc.). Additionally, CD45 antibody and Hoechst reagent (DAPI) was additionally added to clarify neutrophils and LNCaP cells.

2.3.7 Cytotoxic Assay

The viability of luciferase-expressing LNCaP was assessed by D-luciferin assay. 100 μ L of tumor cells (50,000 cells/mL) in RPMI medium containing 10% FBS were mixed with 100 μ L of 150,000, 250,000 and 500,000 cells/mL neutrophils in 96 well plates, and then incubated at 37°C and 5% CO₂ for 24 hr. After the incubation, the mixture was centrifuged at 1,000 rpm for 5 min, the suspension was removed and 100 μ L of culture medium containing 150 μ g mL⁻¹ D-luciferin was added into each well for 30 min of incubation at 37°C in a humidified 5% CO₂ incubator. The bioluminescence was measured by SpectraMax iD3. The cellular viability was calculated using the following equation:

$$\text{Equation 2.2: Cellular viability} = \frac{\text{Sample} - \text{Blank}}{\text{Negative} - \text{Blank}} * 100\%$$

2.4 Results and Discussion

2.4.1 Construction of anti-PSMA CAR knockin hPSCs

To investigate the anti-tumor effect of CAR-Neu on PCa cells, two CARs were designed (**Fig. 2.1.A**). Both CARs target PSMA, using either a J591 scFv minibody (MiB)¹²⁸ or an anti-PSMA nanobody (NB)¹²⁹, and share common domains including the GM-CSF-derived signal peptide (SP), IgG4 Fc domain (SmP), CD4 transmembrane domain, and intracellular CD3 ζ signaling domain containing tyrosine-based activation motif (ITAM) binding sites. CAR constructs were inserted into the *AAVS1* safe harbor locus via CRISPR/Cas9-mediated homologous recombination (**Fig. 2.1.B**). After nucleofection, single cell H9-hPSC clones were

genotyped by PCR (**Fig. 2.1.C**). MiB CAR construct insertion resulted in 12 successfully targeted clones of which 3 were homozygous, and NB CAR construct insertion resulted in 8 successfully targeted clones, of which 3 were homozygous.

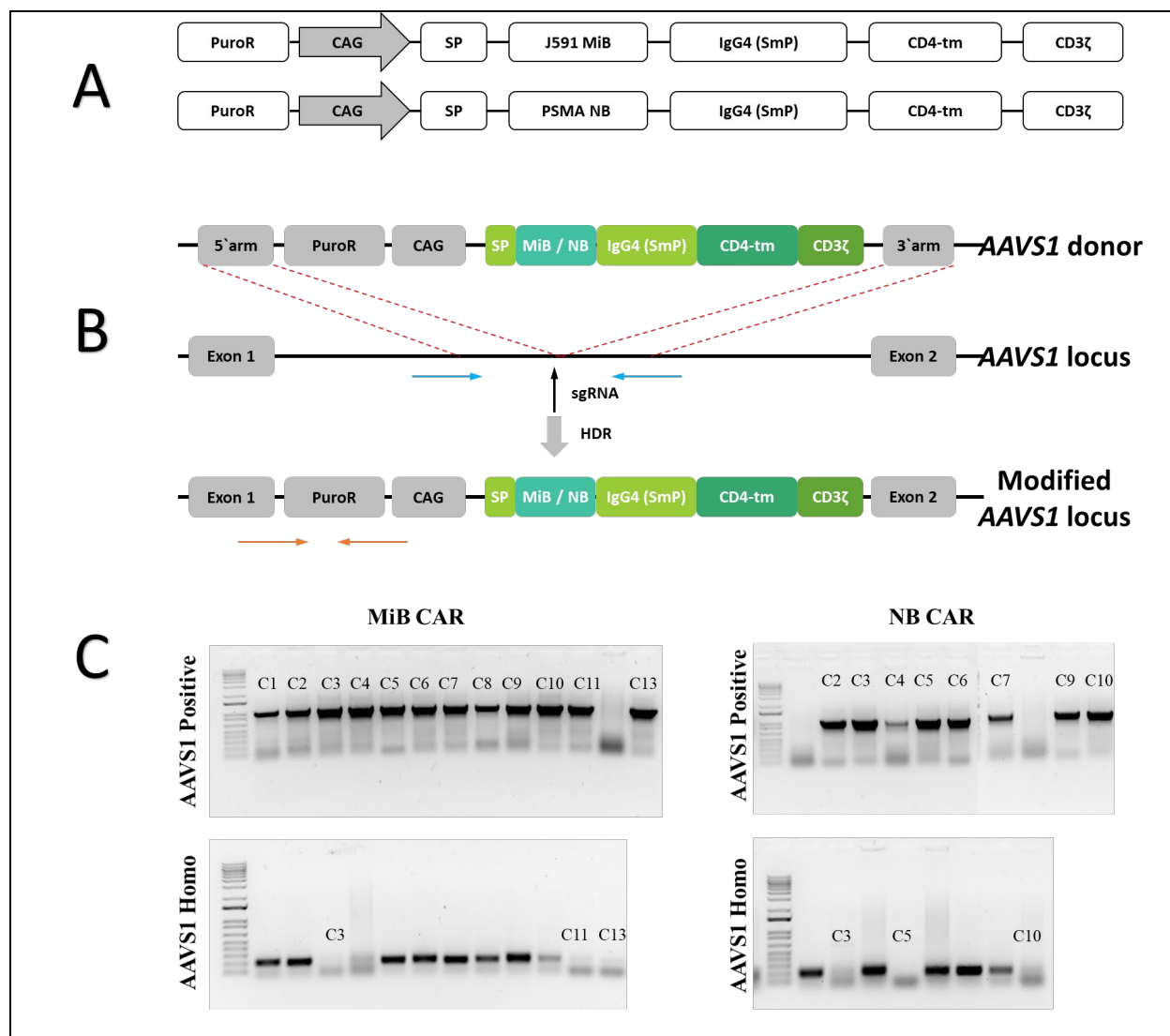


Figure 2.1: CAR construct design and engineering of H9 hPSCs. (A) Schematic of J591 Minibody CAR (MiB CAR), and the anti-PSMA nanobody CAR (NB CAR) donor plasmid containing a signal peptide (SP), an anti-PSMA binding domain (MiB or NB), the Fc domain of IgG4 (SmP), a CD4 transmembrane domain, and an intracellular CD3ζ signaling domain. (B) Knock-in strategy for the AAVS1 safe harbor locus. Vertical arrow represents a targeted sgRNA, and horizontal lines represent primer pairs for assay of insertion efficiency and homozygosity. (C) PCR of single hPSC clones for successful insertion of CAR constructs into the AAVS1 locus, and confirmation of homozygosity by lack of the ~240 bp band. MiB construct had 12 successful clones, of which 3 were homozygous. NB construct had 8 successful clones, of which 3 were homozygous. Heterozygous clones were utilized for further experiments.

2.4.2 Differentiation of CAR-hPSCs into CAR-neutrophils

Neutrophils were differentiated from hPSCs following our previously published protocol⁸⁶. Differentiation begins by generation of hematopoietic stem and progenitor cells (HSPCs) (**Fig. 2.2.A**). Activation of the canonical Wnt pathway with CHIR99021¹³⁰ and subsequent inhibition of the TGF- β signaling cascade with SB431542 differentiates hPSCs into a hemogenic endothelium and induces endothelial to hematopoietic transition (EHT)^{72,75}, respectively. Suspended (floating) myeloid progenitors are differentiated from HSPCs with granulocyte/macrophage colony stimulating factors (GM-CSF) and further specified into neutrophils with granulocyte colony stimulating factor (G-CSF) along with the retinoic acid receptor agonist AM580⁶⁷. The resulting cells present a typical morphology of neutrophils by Wright-Giemsa stain with pink-violet cytosol, violet granules, and nucleus segmentation (**Fig. 2.2.C**).

2.4.3 Flow Cytometry Analysis

Immunohistochemistry analysis of key surface markers and cytosolic proteins (CD45, CD11b, CD66b, MPO) by flow cytometry was performed on day 20 floating cells following the Stemline II (SII) differentiation scheme (**Fig 2.2.B**). Almost all cells were CD45 positive, demonstrating a non-erythroid hematopoietic lineage. Furthermore, a large majority of cells were CD11b⁺ CD66b⁺, indicating granulocyte cells were formed. Mirroring CD45, most cells expressed cytosolic MPO, identifying the major cell population as CD45⁺ CD11b⁺ CD66b⁺ MPO⁺ neutrophils. This additionally suggests hPSC-derived neutrophils are immature or early-stage, as MPO transcription occurs relatively early in a neutrophil's lifespan and is found in primary, azurophilic granules¹³¹.

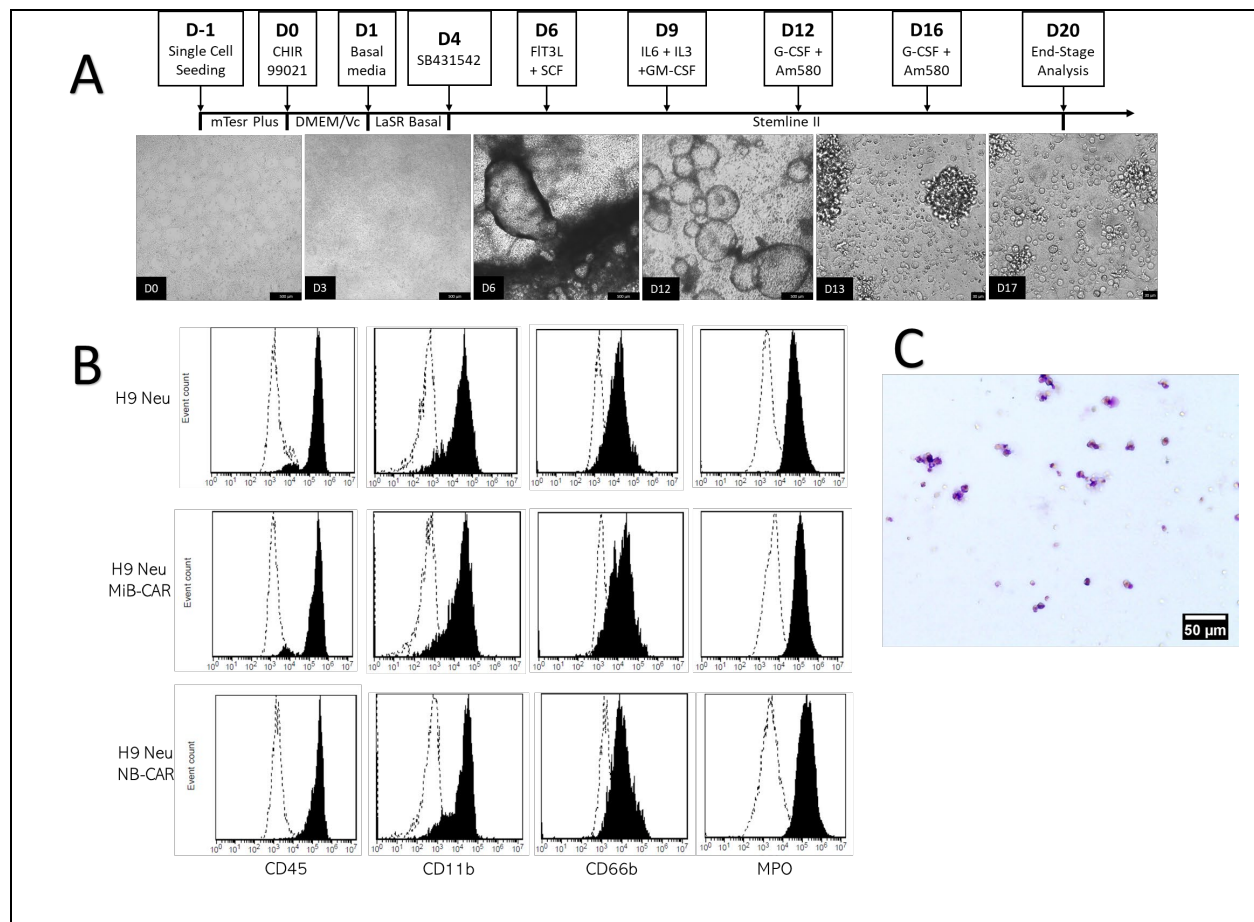


Figure 2.2: Differentiation of neutrophils from human pluripotent stem cells (hPSC). (A) Stemline II differentiation scheme with representative brightfield images. (B) Flow cytometry analysis of myeloid and neutrophil surface markers. Markers were assessed by addition of conjugated mAbs for CD45, CD11b, CD66b, and cytosolic MPO (Empty: unstained blank; Filled: Stained sample). Cells were permeabilized for MPO assessment.

2.4.4 Bulk RNA Sequence Analysis

To better understand the transcriptomic landscape of differentiated cells, bulk RNA sequencing was performed on hPSCs, hPSC-derived neutrophils with / without CAR modification, and peripheral blood neutrophils (**Fig 2.3**). Global hierarchical clustering demonstrated that differentiated cells clustered closest to PB neutrophils (**Fig 2.3.A**). Principal component analysis (PCA) of the global transcriptome identified two tight clusters of hPSCs and PB neutrophils separated by a wider cluster of differentiated neutrophils (**Fig. 2.3.B**). The expression of neutrophil

markers, including *CD45*, *CD11b*, *CD66b*, and *MPO*, in all cells confirmed their neutrophil identity. As compared to PB neutrophils, lower expression of selected genes that were related to neutrophil phenotype, migration, and other functions such as *PTPRC* (CD45), *ITGAM* (CD11b), *MME*, *CXCR1*, *CXCR2*, *NCF1*, *NCF2*, *CEBPB*, and *CEBPD* were observed, indicating the relative immaturity of hPSC-derived neutrophils. This was also confirmed by higher expression of neutrophil azurophilic granule markers, including *PRTN3* and *MPO*^{131,132}, in hPSC-derived neutrophils (**Fig 2.3.C**). This data suggested that the differentiation method results in a “neutrophil-like” cell that may retain functional and morphological qualities.

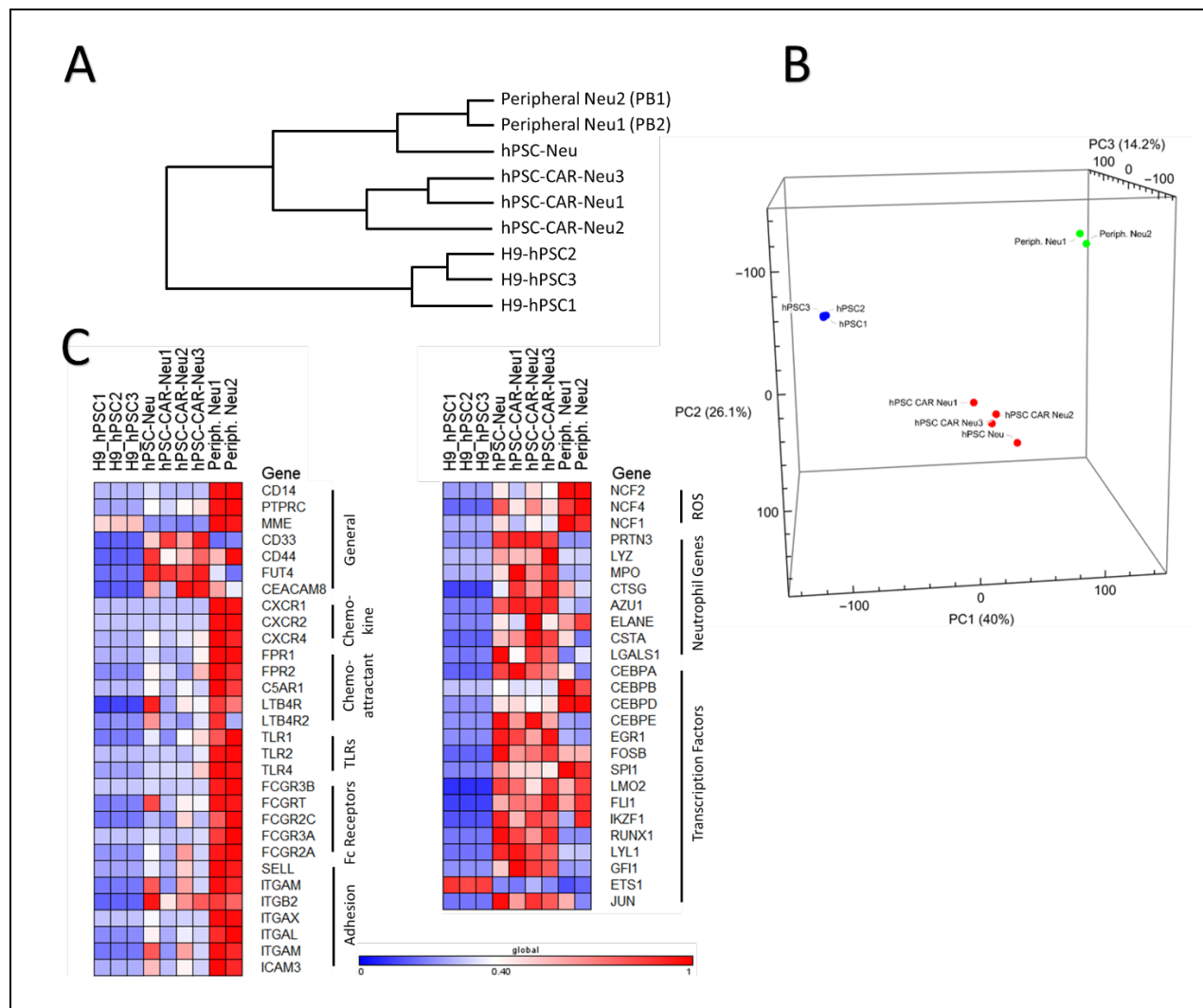


Figure 2.3: RNA-sequencing (RNA-seq) analysis of H9-hPSCs, hPSC-derived and peripheral blood (PB) neutrophils. (A) Hierarchical clustering of global transcriptome using a one minus Pearson correlation. (B) 3D Principal component analysis (PCA) of RNA-seq data. (C) Heatmaps of neutrophil-specific marker expression are shown with hierarchical clustering.

2.4.5 *In vitro* functional analysis of hPSC-derived CAR-neutrophils

To determine if differentiated neutrophils retain key capabilities of PB neutrophils, several functional assays were performed *in vitro*. An initial assessment of hPSC-differentiated-neutrophils (hPSC-Neu) to act as innate immune cells was performed using fluorescent marker conjugated *E. coli* bioparticles to measure phagocytosis (**Fig. 2.4.A-B**). Phagocytotic indices for

differentiated neutrophils measured from 80-100%, which demonstrates that hPSC-Neu replicate the primary functionality of innate immune cells.

Related to phagocytosis, a key function of immune cells is to recognize foreign targets for elimination. The formation of a tight hold between the target and effector cells is mediated by F-actin in response to recognition of extracellular epitopes by a TCR (T lymphocytes only) or CAR construct. Thus, formation of this synapse is a critical measure of neutrophil performance. To determine immunological synapse formation, differentiated neutrophils were co-cultured with the natively PSMA⁺ human prostate adenocarcinoma line, LNCaP. Imaging was performed by selective staining for F-actin. Synapse formation passes through several physical steps, resulting in an effector cell pressed up against the target. A local region of the neutrophil membrane recognized the target LNCaP cell and recruited F-actin to modify the interior cytoskeleton, as shown in (**Fig. 2.4.C**).

Finally, a cytotoxic assay was performed on hPSC-Neu in co-culture conditions with luciferase-expressing LNCaP (**Fig. 2.4.D**). A dose-dependent response of luciferase fluorescence to neutrophil concentration, indicating that hPSC-Neu phagocytize LNCaP. hPSC-derived CAR-neutrophils possess a marked increase in cytotoxicity than unmodified hPSC-Neu as indicated by a lower effector: target ratio necessary to achieve a similar cytotoxic effect.

These functional assays demonstrate that both MiB and NB CAR-expressing hPSC-derived neutrophils possess the capability to recognize LNCaP, form an immune synapse, and activate intracellular signaling cascades which result in potent anti-tumor cytotoxicity against LNCaP *in vitro* that is significantly improved compared to unmodified hPSC-Neu.

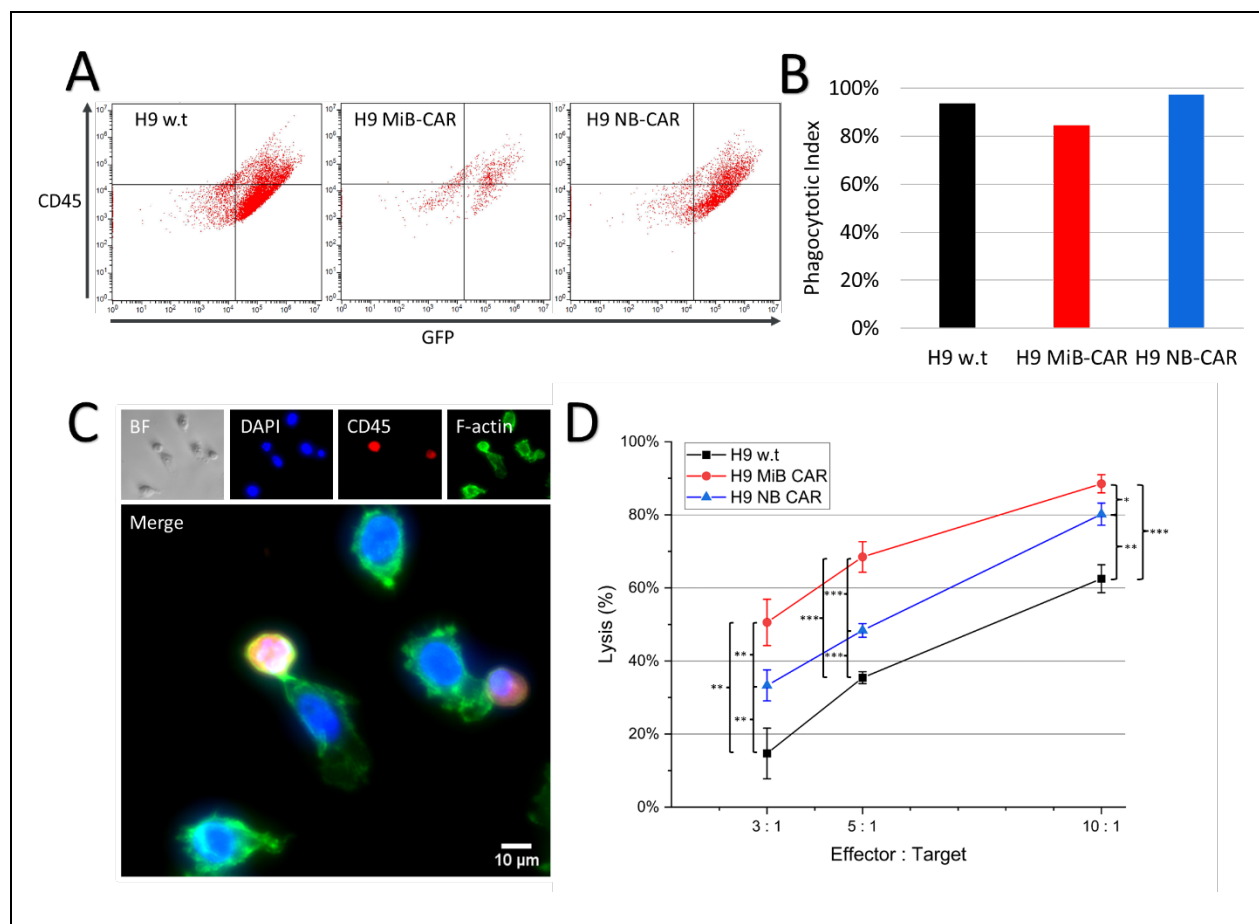


Figure 2.4: Functional analysis of hPSC-derived neutrophils. (A-B) Phagocytosis of pHrodo Green *E. coli* bioparticles. Representative flow cytometry analysis (A) and quantification (B) of phagocytosis of wild type H9 (H9 w.t), minibody (MiB) and nanobody (NB) CAR-modified neutrophils were shown. (C) Immunological synapse formation between hPSC-derived neutrophils and LNCaP cells were stained and shown. (D) Cytotoxicity of H9 hPSC-derived neutrophils (wild type, MiB CAR, and NB CAR) against luciferase⁺ LNCaP for 24 hrs at effector : target ratios (Neutrophil : LNCaP) of 3:1, 5:1, and 10:1 was measured. Significance values were measured by Student's T test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

2.5 Conclusion

The advent of CAR technology for immunotherapy has led to significant improvements in cancer treatments, especially those that do not respond well to chemotherapy, surgery, or castration in the case of PCa. Improving the capacity of immune cells to recognize cancers is the critical function of CAR-based therapies. Solid cancers such as PCa still has many challenges to solve before CAR treatments rival the efficacies in blood-borne tumors. One method to improve the

efficacies in solid tumors is the use of alternatives for cytotoxic T lymphocytes. There are several notable studies showing improved tumor cytotoxicity using CAR-NK or CAR-Macrophage models. In this study, we present a human pluripotent stem cell-derived CAR-neutrophil system that produces cytotoxic effects against a PCa model cancer cell LNCaP *in vitro*. Utilizing a hPSC differentiated cell allows for precise insertion of the CAR construct into a clinically applicable locus (AAVS1). hPSCs can be further engineered to ensure CAR-immune cell specificity, remove an allograft rejection response against the introduced cells, and to prevent over-activity by means of CRS or off-target activity. Furthermore, CAR-neutrophils can potentially be used in a cocktail with other CAR-immune cell systems or in combination with other treatments to produce synergistic effects. Further evaluation of PSMA-targeting hPSC-derived CAR neutrophils in an animal model of solid tumors will be critical to determine the therapeutic effects of this model *in vivo*.

3. IMPACT OF CULTURE MEDIA ON HPSC-NEUTROPHIL DIFFERENTIATION

3.1 Introduction

Choice of differentiation media for induction of EHT and differentiation of floating progenitor cells has a significant impact on the cell population formed. Limited supply of the traditional media used for neutrophil differentiation, Stemline II, led to an investigation of alternative differentiation media choices to generate neutrophils. Three alternatives were chosen based on their roles in cell differentiation: StemSpan H300, StemSpan SFEM II, and StemSpan-XF. All medium alternatives have been utilized in HSC / hPSC differentiation, although each claims different advantages. In this chapter, effects of differentiation medium alternatives from Day 4 to Day 20 of the established neutrophil differentiation scheme is investigated.

3.2 Additional Methods

3.2.1 Modified Differentiation Schemes

To investigate the effects of different medium alternatives for Day 4-20 of hPSC-neutrophil differentiation, four different schemes were chosen (**Scheme 3.1-4**). All differentiation batches were identical apart from the media replacement.

Scheme 3.1: Alternative differentiation schemes for neutrophils

D-1 hPSC	D0-1	D2-3	D4-5	D6-8	D9-11	D12-20 Floating
mTeSR+	DMEM/Vc CHIR99021	LaSR Basal VEGF	SB431542, FLT3L, SCF,	SCF, FLT3L, GM-CSF, IL-6, IL-3	G-CSF, IL-6, IL-3	GlutaMAX, G-CSF, AM580
			StemSpan H3000 (SP-H3)			
			StemSpan SFEM II (SP-SF)			
			StemSpan XF (SP-XF)			

3.3 Results and Discussion

3.3.1 Media Alternatives Impact Success and Reliability of Neutrophil Differentiation

To investigate the impact of differentiation media on neutrophil generation, lineage markers of neutrophils were assessed by flow cytometry (**Fig 3.1**). In all differentiation batches assessed, media usage from Day 4-Day 20 was either Stemline II (SII), StemSpan H3000 (SP-H3), StemSpan SFEM II (SP-SF), or StemSpan XF (SP-XF). These four schemes generated an observable quantity of floating, visually living cells 10-15 μm in size.

SII was the only differentiation scheme that reliably and routinely generated populations of cells positive for all neutrophil markers assessed. SP-H3 did reliably generate cell populations largely positive for CD45, CD11b, and MPO. However, a notable lack of CD16 and CD18 was seen under the SP-H3 scheme across samples, with intermittent expression of CD66b.

CD16 (Fc- γ RIII receptor) is a low affinity Fc receptor expressed on innate cells that serves roles in mediating phagocytosis. Cells not expressing CD16 would likely be unable to exert a cytotoxic effect upon target cells, making for a stunted or limited response to PSMA⁺ tumor cells.

CD18 (integrin $\beta 2$) is found on neutrophils, monocytes and macrophages, and dimerizes with CD11b to engage with selectins and permit cell migration, which is a critical signaling pathway in neutrophils for inducing phagocytosis and degranulation. Neutrophils deficient in these capacities would very likely be unfunctional. CD66b (CEACAM) is a surface glycosylated peptide associated with neutrophils and eosinophils that is involved in production of ROS and degranulation. Lack of CD66b may lead to lack of ROS generation in stimulated neutrophils, causing a diminished cytotoxic effect. To compound on the expression deficiencies, one batch under the SP-H3 scheme generated floating, living cells that visually resembled neutrophils in size, yet did not express representative surface markers. In this case, a single point of failure is not conclusive; However, it is indicative of potential reliability issues of the SP-H3 differentiation scheme. Collectively H3000 as a media choice was determined to be ineffective and potentially unreliable as a replacement for true CAR-neutrophils.

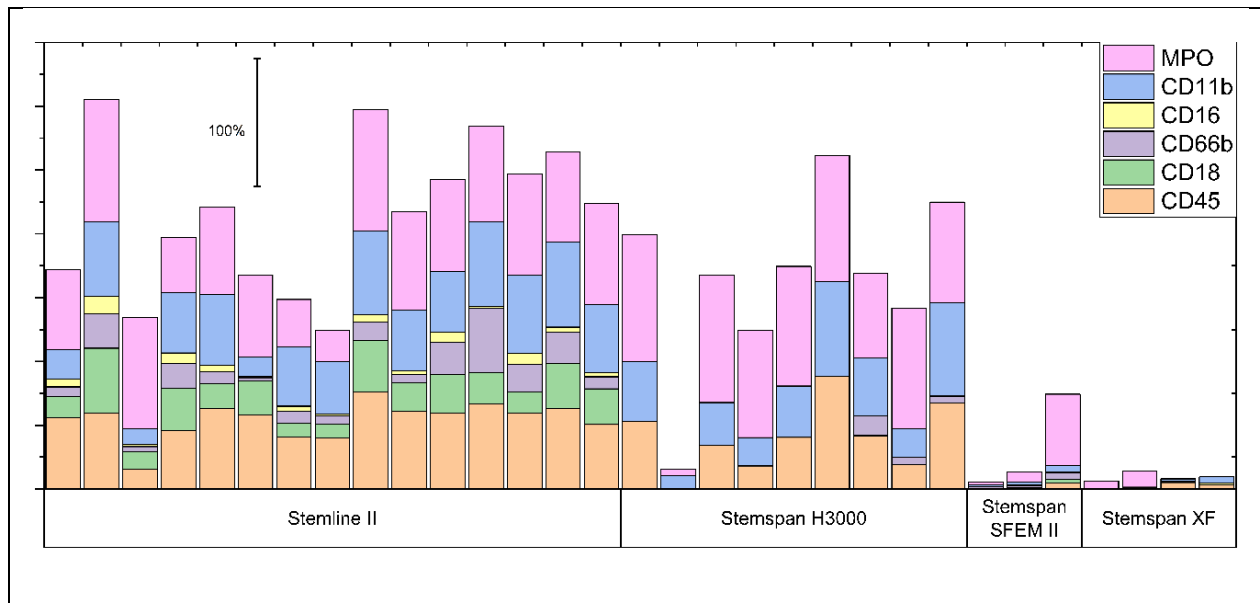


Figure 3.1: Flow cytometry analysis of neutrophil markers using alternatives to Stemline II as a late-stage differentiation medium. The SII differentiation scheme was modified with different late-stage media (Day 4-20). Day 20 cells were assessed for neutrophil lineage markers CD45, CD18, CD66b, CD16, CD11b, and MPO via flow cytometry. Total amount of positive cells for each marker by percent are represented in each batch.

Under a SP-SF differentiation scheme, extensive debris was observed throughout differentiation at relative abundances much greater than SII, SP-H3, and SP-XF schemes following floating cell formation. Resulting cells were of low viability and only a small subpopulation expressed neutrophil lineage markers in flow cytometry. As an alternative, this medium is not sufficient to produce CAR-neutrophils under our protocol. While this medium is claimed to be standalone for serum-free methods, addition of human serum albumin (HSA) may improve the results seen in this analysis.

Most interesting of the SII alternatives, the SP-XF differentiation scheme generated very few cells that were positive for neutrophil lineage markers despite a large amount of visually living, single floating cells. Additionally, the debris observed was only slightly greater than in the SII scheme. StemSpan XF is a xeno-free, serum-free media intended for expansion of hematopoietic cells, although there are additional supplements that can be included to expand cord blood CD34⁺ cells, or to generate hematopoietic lineage cells. No supplements were included in this study, indicating that potentially under the SP-XF scheme no terminally differentiated neutrophils were formed.

3.4 Conclusion

Basal medium is a critical component of a differentiation scheme. In this chapter it is shown that of the three potential alternatives for Stemline II in our neutrophil differentiation protocol, only SP-H3 generated meaningful amounts of CD45⁺MPO⁺ cells somewhat reliably, although other critical proteins such as CD16 or CD18 were not present, indicating that generated cells were likely unfunctional. Of interest was the peculiar results seen in the SP-XF scheme, as there was no clear result as to why neutrophils failed to form. Further investigation into the floating cells produced under this scheme is performed in Chapter 4.

4. GENERATION OF HELPER T LYMPHOCYTES *IN VITRO* UNDER A STEMSPAN-XF SCHEME

4.1 Introduction

The lack of neutrophil-presenting cells after differentiation under the SP-XF scheme led to a follow up question: What cells are present? These unidentified cells were small (10-20 μm in diameter), floating, and were observed to be moving under microscope. However, flow cytometry analysis clearly demonstrated lack of surface protein expression commonly associated with myeloid lineage cells such as monocytes, macrophages, and granulocytes. Thus, a follow-up analysis on the identity of the differentiated cells was performed. In this chapter, evidence is presented in favor of the conclusion of lymphocyte-lineage cell generation which are polarized towards the helper T lymphocyte morphology. In the field of hematopoietic and immune cell differentiation, T lymphocyte formation is a difficult challenge due to the unique requirements for T cell maturation *in vivo*. Whereas many hematopoietic lineage cells can now be differentiated *in vitro* under feeder-free, chemically defined conditions thanks to previous work, the biological role of the thymus in typical lymphopoiesis has yet to be replicated in a feeder-free manner. In this differentiation scheme, $\text{CD4}^+ \text{CD8}^- \text{CD3}^+$ expression is observed as early as day 6 of growth, suggesting early commitment to the T helper lymphoid lineage.

4.2 Literature Review

4.2.1 GM-CSF promotes IL-6 independent proliferation of T cells

Immune cell proliferation during hematopoiesis is critical to generate an effective number of effector and response cells from progenitors. Cytokines that polarize populations of innate cells such as macrophages and granulocytes to proliferate have been shown to also interact with other

immune cell lineages, such as NK, T, and B cells. One such cytokine, GM-CSF, has been shown to affect T cell proliferation through IL-6 signaling¹³³.

The differentiation scheme used to generate neutrophils throughout the course of this work utilizes GM-CSF on Day 9-12 to induce floating myeloid progenitors to expand and proliferate. This cytokine is naturally produced by many immune cells in response to LPS, IL-1, and TNF- α with the goal to stimulate an innate inflammatory response. However, investigation onto the mechanism of action behind this pluripotent cytokine has revealed a host of other functions. Early studies in mice deficient in GM-CSF indicated that myelopoiesis occurred normally, yet a pulmonary alveolar proteinosis (PAP) phenotype was seen, indicating GM-CSF was involved in pulmonary macrophage development and/or maintenance of lung surfactants. Recent studies into GM-CSF deficiency induction of PAP point to lipid metabolism as a culprit¹³⁴. GM-CSF has been shown to influence downstream signaling of various pathways including JAK2/STAT5 and ERK¹³⁵. In fact, it was due to the signaling of the ERK pathway that GM-CSF is a commonly included cytokine for myeloid cell differentiation *in vitro*. Interestingly, GM-CSF was proposed to impact CD4⁺ T cell proliferation, with one study showing that the cytokine was critical in inhibiting apoptosis and IL-17 production by CD4⁺ T cells in animal models of EAM (experimental autoimmune myositis) for human dilated cardiomyopathy¹³³. This study also showed that GM-CSF deficient mice had reduced production of IL-6 and IL-23 by dendritic cells *ex vivo*, and specifically helper T cells are induced to proliferate and differentiate in response to GM-CSF in an IL-6 dependent manner¹³³. This effect was seen despite CD4⁺ T cells not expressing a receptor for GM-CSF, indicating engagement by other immune cells such as activated macrophages and dendritic cells.

4.2.2 Current Differentiation Methods of T cells

T cells remain a unique challenge for *in vitro* differentiation. T cells mature in the thymus from early progenitors, while simultaneously losing capacity to form other hematopoietic lineages¹³⁶. A critical pathway for inducing T cell development is Notch¹³⁷. Various Notch ligands are present in thymus tissue to support the differentiation of early thymocytes, although it was found that DL4 and DL1 are critical ligands, and mouse bone marrow derived stromal feeder cell lines (OP9) edited to express either DL1 or DL4 are alone capable of inducing T cell differentiation. This area of hematopoiesis research is rapidly improving. In 2000, the most advanced method to form T cells involved a 3D thymus organoid^{138,139}. In 2002 the use of OP9-DL1/4 was described¹⁴⁰. Currently, T cells are capable of being generated from a variety of progenitor cells (hPSCs, HSPCs, iPSCs, ESCs)¹⁴¹, yet the use of DL/4-expressing cells are still heavily relied on feeder system in either 2D or 3D culture systems.^{142–147}. One group published a protocol on a feeder-free system to differentiate T cells, although notably this protocol involves a recombinant Fc-DL4-coated growth plate¹⁴⁸. Also, protocols to generate T cells mostly form T effector cells instead of T helper cells¹⁴⁹.

4.2.3 T Helper Lymphocyte Subclasses

The primary distinction between mature T cells is the surface expression of either CD8 or CD4. The former indicates a cytotoxic, effector functionality while the latter delineates a T helper variety. Among the CD4⁺ T helper lymphocytes, there exist several subtypes, classified primarily on their cytokine production capacities. The classical subtypes are T_H1 and T_H2 (T helper 1 and 2 lymphocytes) which produce either IL-2, TNF- α , and IFN- γ to assist in mediating cell-mediated immunity or IL-4, IL-5, and IL-13 to regulate humoral immunity. Other variants of T cells have been identified^{136,150,151} such as T_H3 which produce TGF- β to induce immunosuppression, T_H9

which produce IL-9 to serve similar but distinct roles as T_H2 cells, T_H22 which exclusively excrete IL-22, and T_H17 which are identified by IL-17 production. Both T_H17 and T_H22 serve similar inflammatory roles. Additionally, T_{FH} cells (follicular helper variety) are uniquely involved in B cell activation and secrete IL-21. Another key T cell subvariant are the T regulatory cells (Treg) which act upon other immune cells to limit overactivation and prevent autoimmune responses. Treg serve roles to consume thus lower IL-2 by high expression of CD25, the high-affinity IL-2 receptor, and express immunosuppressive cytokines TGF- β , IL-10, and IL-35.

Interestingly, there exists a high plasticity among T helper lymphocyte subtypes (**Fig. 4.1**)^{152,153}. Subtype selection from $CD4^+$ progenitors is not truly terminal, as further cytokine signaling can retrain a T cell into a new subtype. Thus, T_H lymphocyte populations are dynamic in response to the local cytokine environment, and the relative abundances of specific subtypes in relation to each other orchestrate a global response¹⁵².

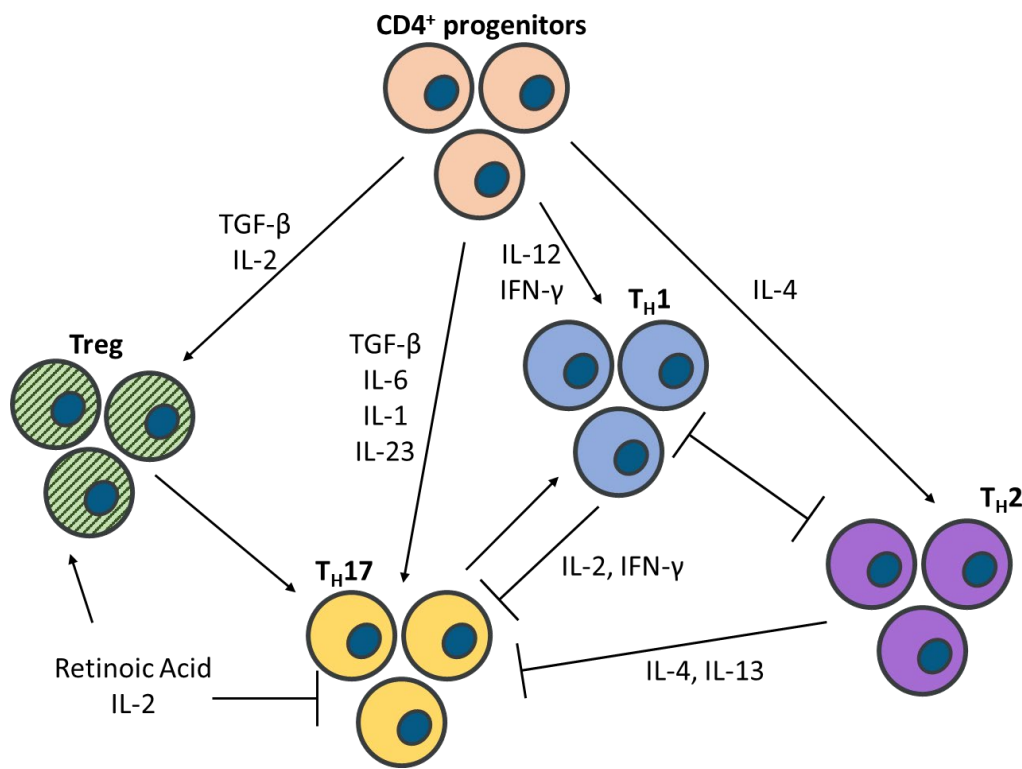


Figure 4.1: Plasticity of T Cell Phenotypes. Adapted from *Peck et al.*¹⁵²

4.2.4 Transcriptional Regulators of T_H17 and Treg Phenotypes

A key transcriptional regulator of T cell differentiation and development is RORC2 (retinoic acid-related orphan receptor variant 2; ROR γ t in mouse)^{154,155}. This transcription factor is found highly expressed in T_H17 cells and is considered the master regulator/inducer of the T_H17 morphology^{155,156}. In ROR γ t knockout mouse models, lower IL-17 secreting cells were observed which were additionally unresponsive to IL-23 known to stimulate T_H17 cells. Additionally, forced expression of ROR γ t in CD4⁺ murine T cells resulted in IL-17 production under TCR stimulation, indicating that ROR γ t is a critical regulator in mouse T_H17 development. Similar experiments in human Th cells showed that forced expression of RORC2 resulted in a cytokine and protein expression profile consistent with T_H17 cells: IL-17A, IL-17F, and IL-26 secretion, CCR6⁺, and loss of IFN- γ secretion¹⁵⁵. Also, reduced RORC2 mRNA was observed in human neonatal T cells which results in defective T_H17 production, which was explained as a potential mechanism to prevent reactions against self or colonizing beneficial microbes¹⁵⁷. Later study into T_H17, and other IL-17 T lymphocytes identified CD161 as a key marker of the IL-17 secreting phenotype, whose expression on CD4⁺ T cells is controlled by RORC2¹⁵⁸. Additionally, CD4⁺ CD161⁻ T lymphocytes were transduced by lentivirus to force expression of RORC2 which resulted in IL-17 secretion and CD161 expression, indicating a direct impact of the RORC2 transcription factor on CD161 and establishment of the T_H17 subtype.

Interestingly, differentiation of Treg and Th17 are very similar, yet result in T lymphocytes with very different roles¹⁵⁹. For example, TGF- β is required for both subtypes to form, yet high concentrations result in Treg fate determination while medium to low concentrations result in T_H17 varieties. One of the key differences in Treg vs. T_H17 phenotype selection is RORC2 or FOXP3 transcription factor activation, where FOXP3⁺ CD4⁺ T cells result in a Treg phenotype¹⁶⁰. It was shown that all-*trans* retinoic acids (ATRA) interact with retinoic acid receptor alpha (RAR α) and

result in FOXP3 activation in a STAT3/STAT5 independent manner^{161,162}. This effect is also seen when RAR α antagonist LE540 reduced Treg phenotype cells by 30%, and RAR α agonist Am580 increased the abundance of Treg cells by 40% in coculture conditions of CD4⁺ naïve T cells with CD14⁺ myeloid derived suppressor cells (MDSC)¹⁵³.

4.3 Results and Discussion

4.3.1 Hematopoietic Lineages in SP-XF Differentiation Scheme

CAR modified H9 cells were assessed for hematopoietic lineage markers after differentiation under the StemSpan-XF differentiation method (**Fig 4.2**). Leukocyte common antigen (CD45) expression was low across both batches, at no more than 15% and 5% for the NB and MiB construct, respectively. Interestingly there was a wide variability in CD34 expression across the two batches (70% and <5%). Given the traditional role of CD34 as a HSC marker, this suggested the MiB-CAR batch contains cells more polarized towards HSCs. More cells in the MiB CAR batch were positive in expression for CD10, CD56, and CD90, which are used as earlier markers for T/B, NK, and mesenchymal stem cells, respectively. Conversely, more cells in the NB CAR batch were positive for CD31, CD33, CD11b, and CD235, all of which exist on more terminally differentiated cells. This may indicate that the variability between batches may result in different ratios of early and late progenitor cells. Given the high expression of CD90 (>85%), it is likely that a large amount of the cells after the 20-day SP-XF scheme retain qualities of stem cells and/or early progenitors.

It is additionally clear that SP-XF differentiated cells contain various subpopulations (**Fig 4.2**). Platelet endothelial cell adhesion molecule (PEACAM1 or CD31) expression is found across a wide variety of immune cell types, including monocytes, T, NK, and granulocyte cells. The

highly glycosylated transmembrane protein has roles in cell-cell mediated signaling, transmigration of leukocytes, and angiogenesis. Additionally, only a small population of cells are positive for Siglec-3 (CD33), which is typically a marker for myeloid lineage cells, although there is evidence that additional isoforms can be found on cells in the lymphoid lineage. Interestingly, CD235 is a unique marker of erythroid lineage cells, and constituted 30-55% of ungated cells. Differentiated cells were not visually red, suggesting that CD235⁺ cells may be erythroid progenitor cells. Notably, little to no expression was seen for common myeloid lineage markers CD14, CD11b, and CD16. Additionally, no expression for CD123 was seen, which is a common marker for dendritic cells. This indicates that very few cells are of the myeloid lineage. Expression of CD10 and CD56 was unexpected, as CD10 is found largely on cells of the lymphoid lineage (T/B/NK), and CD56 is a common marker for NK cells. Taken together, it appeared that differentiation under the XF-scheme generated various hematopoietic lineage progenitors, despite the addition of cytokines that produce uniquely myeloid lineage cells in other differentiation schemes. Given the use of StemSpan-XF in maintaining HSCs, it is possible that conflicting signals to both terminally differentiate and remain multipotent caused this effect. From this initial collection, it appeared that largely erythroid and lymphoid lineage progenitor cells were produced. The remainder of this chapter will delve into the lymphoid lineage specifically.

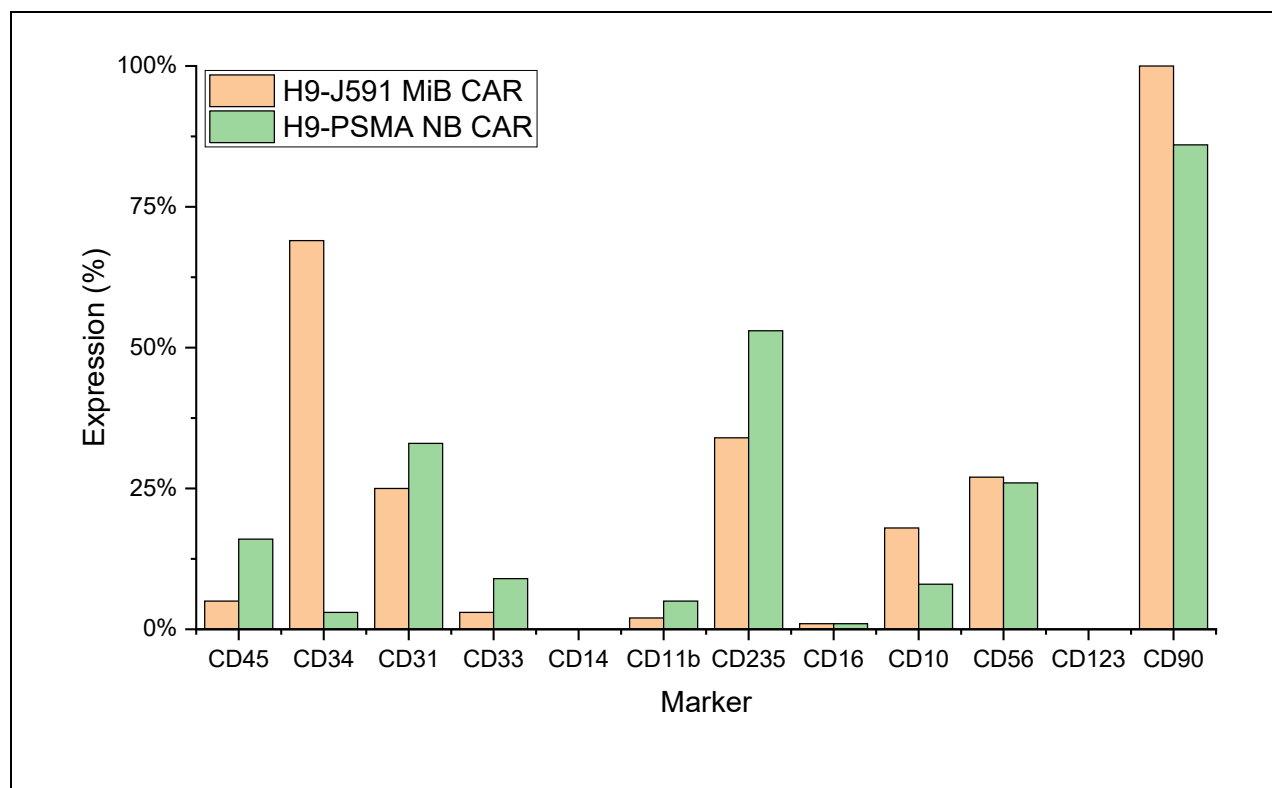


Figure 4.2: Flow Cytometry of Day 20 CAR-modified H9 derived cells. Cells were differentiated for 20 days following the SP-XF differentiation scheme and assessed for surface markers by live-cell flow cytometry.

4.3.2 CD4 and CD10 Sharply Increase in Expression in SP-XF Scheme Floating Cells

To better understand the lineages formed under the XF-scheme, day 20 cells were assessed for CD10 expression across multiple batches (**Fig 4.3.A**). CD10 (common acute lymphoblastic leukemia antigen [CALLA] or neutral endopeptidase) sharply increased from 10% CD10⁺ on day 6 to 50% CD10⁺ on day 14. This trend continues until a maximum at day 25, when a drop in CD10⁺ cells is observed. This is potentially due to increasing amounts of cell debris passing through the Calcein⁺ living cell gate, as the gate was established by back-gating, not through sequential cytometry or FACS. Cell debris increased throughout all differentiation protocols, and increasing debris overlap with the living cell gate may account for the drop in expression of CD10 seen.

However, given that CD90 expression remained at ~100% throughout the differentiation scheme, including after day 25, increasing debris does not fully explain the drop in CD10 expression.

CALLA is a zinc endopeptidase that interacts and degrades components of the extracellular matrix (ECM) and serves a role for immune cells to degrade proinflammatory cytokines such as IL-1 β . It plays roles in the immune system, mammary epithelial tissue, and the neural system and is associated with sphere-forming stem cell progenitor clusters¹⁶³. One study found that CD10 is also expressed on post-thymic T cells undergoing apoptosis induced by either HIV infection or by introduction of CD95 (apoptosis antigen ligand) monoclonal antibodies, or defined apoptosis inducers such as etoposide or staurosporine¹⁶⁴. An additional study of childhood acute lymphoblastic leukemia (ALL) demonstrated CD10 is a marker of cycling B cells with a tendency to perform apoptosis¹⁶⁵. A study into neoplastic T cells in angioimmunoblastic lymphomas also express CD10 (27 of 30 cases), yet no cases of peripheral T cell lymphomas (10) or reactive lymphoid hyperplasia (10) contained CD10⁺ cells¹⁶⁶. Another study claimed that blocking CD10 in developing T lymphocytes inhibits the formation of a double positive (CD4⁺ CD8⁺) morphology required for maturation and induces a triple negative (CD4⁻ CD8⁻ TCR $\alpha\beta$ ⁻) morphology¹⁶⁷. Furthermore, CD10 is also expressed on neutrophils, and recently has been shown as a discriminatory marker between immunosuppressive, mature neutrophils (CD10⁺) and immature, inflammatory neutrophil populations in patients treated with G-CSF¹⁶⁸. Clearly, CD10 performs a variety of roles in modifying the ECM and impacts cell signaling pathways, and more research is needed to fully elucidate its functions. In this study, the significant increase in CD10 expression over time (10% to 100% in MiB CAR batches) under the StemSpan-XF scheme could suggest that a form of neutrophil is being produced, however the lack of CD66b⁺ expression seen in the previous study with CD10⁺ neutrophils¹⁶⁸ suggested that lymphoid cells are more likely.

To better understand the types of cells formed, and in turn the effect of StemSpan-XF on the differentiation scheme, cells were assessed for CD90 (THY-1) expression throughout differentiation (**Fig 4.3.A**). THY-1 is another GPI-anchored glycosylated protein that is found on hematopoietic stem cells, mesenchymal stem cells, T/B lymphocytes¹⁶⁹, as well as differentiated cells such as NK cells, endothelia, neurons, and fibroblasts¹⁷⁰. It contains a single immunoglobulin V-set domain¹⁷¹ that similarly to CD10 can interact with the ECM and has several roles. It has been shown to interact with integrin $\alpha_v\beta_3$ (CD51/CD61), $\alpha_x\beta_2$ (CD11c/CD18), syndecan-4, and CD97¹⁷¹. These ligands are associated with wound healing¹⁷², activated endothelial cells¹⁷³, cell adhesion¹⁷⁴, engaging complement proteins¹⁷⁵, and cancer¹⁷⁶. One group even claimed that THY-1 is an indicator for T_H17 (CD4⁺) or T_C17 (CD8⁺) T cells¹⁷⁷. CD90 was strongly expressed in virtually all living cells throughout differentiation (Day 6 to Day 31) regardless of wild type H9 or CAR-modified batches. With the potential of T_H17 or Treg as the major cell type subpopulation in mind, further investigation into T cell markers were performed.

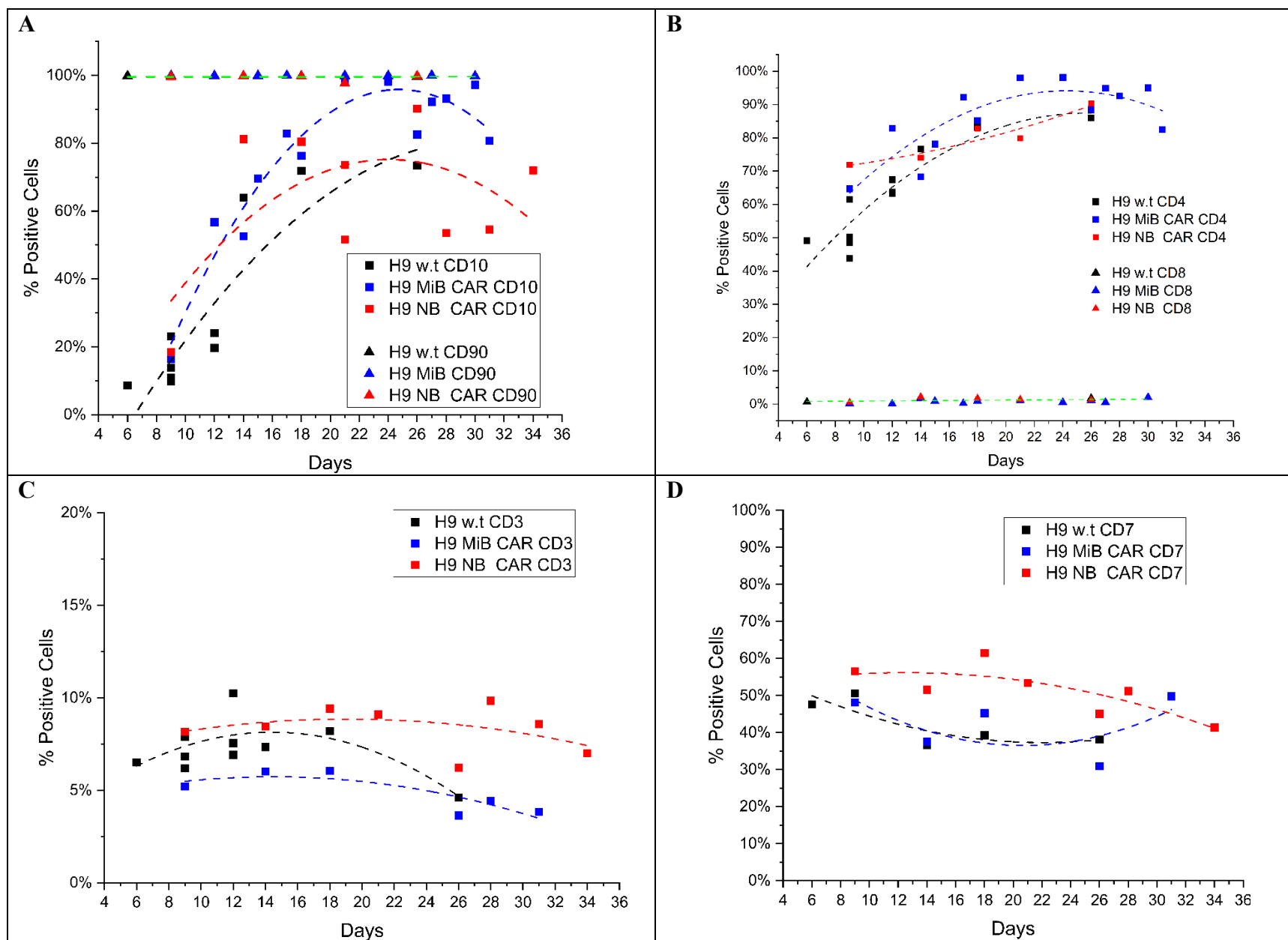
The increasing amount of CD10⁺ CD90⁺ double positive cells in the SP-XF differentiation scheme led to an interest in lymphoid lineage cells, as a strong overlap in expression of CD10 and CD90 is found in T/B lymphocytes. Assessment of CD4 and CD8 was performed on floating cells across multiple SP-XF scheme differentiation batches (**Fig 4.3.B**). An interesting result was seen in which CD4 increased strongly in wild type and MiB CAR construct batches, with NB CAR construct batches demonstrating a high but varied amount of CD4⁺ cells that did not follow a clear pattern over time, which could be attributed to lack of sufficient data points or higher variability because of the NB CAR construct. The increase in expression of CD4 mirrored the trend of CD10, in which a peak was seen by day 25 followed by a decline. CD8 expression was not meaningful in any batch, regardless of CAR construct, from day 6 to day 30.

It was surprising that CD4⁺ CD8⁻ cells were detected as early as day 6, the same time as the formation of floating cells from the hemogenic endothelium. Additionally, at no point was a CD4⁺ CD8⁺ (DP) morphology observed. The lack of an early double positive morphology opposes the typical pathway taken by T lymphocytes *in vivo*. This data in combination with CD10 and CD90 expression curiously suggested that helper T cell progenitor cells were formed almost immediately. There was not enough time for a traditional route of lymphopoiesis from floating hematopoietic progenitors.

Recently, rapid T cell generation from hemogenic endothelium (HE) was described in which day 8 HE rapidly generated floating cells with pro-T morphologies (RAG1⁺CD7⁺) which later formed CD4⁺CD8⁺ DP T lymphocytes¹⁷⁸. This same group previously reported generation of aorta-gonad-mesonephros (AGM)-like organoids that express Notch ligands that are required for T cell development¹⁷⁹, further justifying their own conclusion of rapid T cell development.

Early fetal hematopoietic lineage cells (myeloid, lymphoid, erythroid) do not come from established HSCs, and are directly formed from early endothelial tissue before establishment of the classic, permanent hematopoietic lineage hierarchy. These early cells are formed throughout various waves, with HSCs only appearing in the third wave of definitive hematopoiesis that emerges from the AGM. One study showing that immune-restricted (T/B/GM) lineages before definitive hematopoiesis in murine fetal liver¹⁸⁰. Another claimed that zebrafish produce two waves of T lymphopoiesis: an early, HSC-independent wave consisted largely of CD4⁺ TCRαβ⁺ cells followed by a later HSC-dependent wave that was maintained until adulthood¹⁸¹. This result was also seen in mouse models, where T cell progenitors were observed to form directly from the HE before definite HSC formation¹⁸². To investigate early T lymphocyte generation from hematopoietic progenitor *in vitro*, one group created an inducible Notch system that during a

critical 72-hour window of early mesoderm differentiation greatly increases T cell capable multipotent progenitors, ending with CD8⁺ SP T cells¹⁸³. Under the SP-XF scheme, further investigation into the presence of Notch ligands in Day 4-6 HE and activation of RAG in early progenitors may result in a similar conclusion.



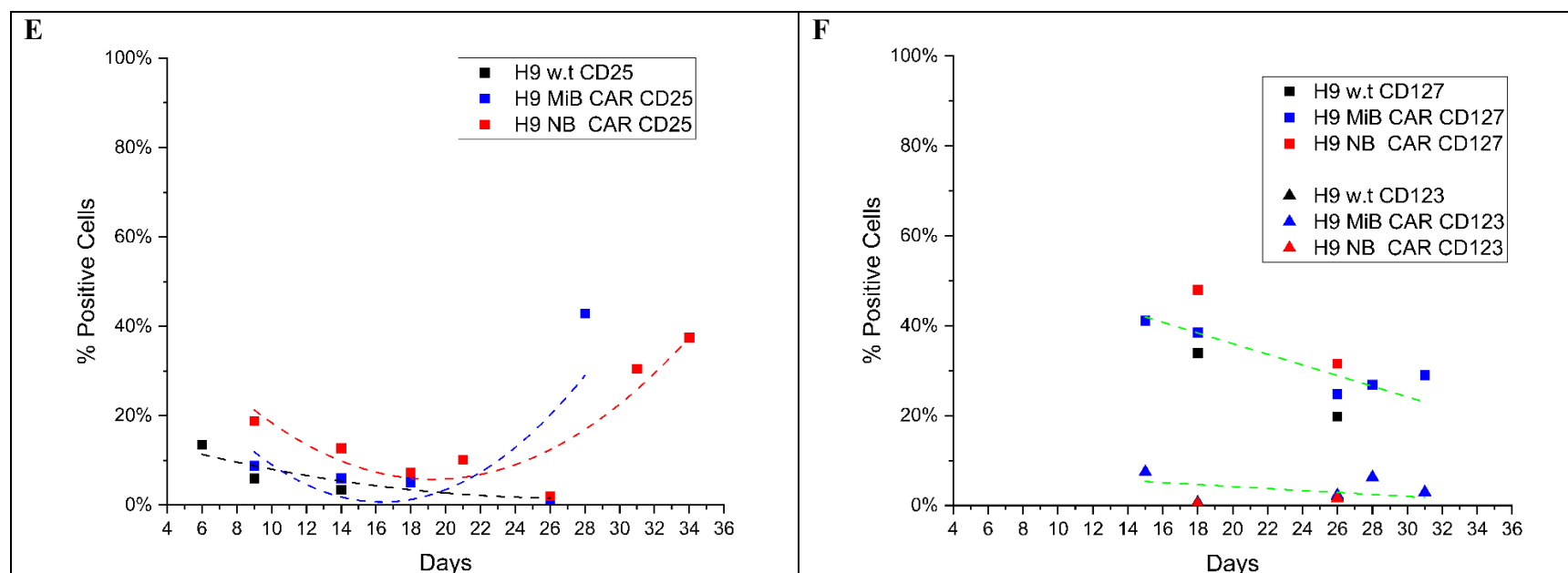


Figure 4.3: Time-based assessment of T cell markers throughout XF-scheme differentiation. Floating cells were collected for flow cytometry analysis starting at day 6 and stained for (A) CD10 and CD90, (B) CD4 and CD8, (C) CD3, (D) CD7, (E) CD25, and (F) CD127 and CD123. FSC/SSC gate was established by back gating for calcein positive cells (not shown). Second order polynomial fits are shown for visualizing general trends in wild type (Black), MiB CAR modified (Blue), and NB CAR modified (Red) cells. In analysis with near-identical trends, a global fit (green) was applied.

4.3.3 Floating Cells Formed by SP-XF Scheme Exhibit Additional T Cell Markers

Additional flow analysis for T cell flow markers was performed. CD3 expression was assessed in several differentiation batches (**Fig 4.3.C**). CD3 is a critical transmembrane peptide in the TCR and a unique marker for T cells. In the SP-XF differentiation scheme, a small but notable population of cells were positive for CD3 across H9 wild type and CAR modified (MiB and NB) cells (5-10%). This itself is strong evidence for T lymphocyte lineage under the SP-XF scheme. However, the low amount of expression of CD3 was an interesting result, and one study found that low TCR expression ultimately leads to T cell population decay¹⁸⁴. In the literature, evaluation of 14 patients with angioimmunoblastic lymphadenopathy (AILD)-T cell lymphoma revealed a small population of CD4⁺ CD2⁺ CD5⁺ CD7⁺ CD3⁻ T cells, suggesting that low expression of CD3 with positive expression for other T cell markers may correspond to an aberrant T cell population¹⁸⁵. In the context of the SP-XF scheme, this may indicate the environment is not completely suitable for T cell development. Given that this protocol was primarily designed to produce neutrophils, this is not a surprising conclusion.

CD7 (LEU 9) is a critical surface protein for early T lymphocyte progenitors and vanishes after thymic development along with CD5. Analysis of CD7 in SP-XF scheme differentiation over several batches (**Fig 4.3.D**) revealed that regardless of CAR modification LEU9 remained largely unchanged from day 6 to day 28, with slight decrease in expression from day 28 to day 34.

Further analysis of SP-XF derived cells was performed over select batches assessing key interleukin receptors: IL-2R (CD25), IL-7R (CD127), and IL-3R (CD123) (**Fig 4.2.E**). CD123 expression was low across batches tested from day 15 to day 31. In contrast to CD127, CD123 is not readily preset in T cells, and is most commonly associated with overexpression in various non-T leukemia and lymphomas¹⁸⁶. Normal hematopoietic stem and progenitor cells do express CD123

in low amounts, further suggesting that SP-XF scheme differentiation results in progenitor cells, not true terminally differentiated cells.

Expression of CD25 and CD127 fluctuate in double negative (DN) ($CD4^- CD8^-$) T lymphocyte progenitors, starting from a $CD25^- CD127^{low}$ early thymic progenitor (DN1) to a $CD25^+ CD127^+$ morphology (DN2-3), and finally a $CD25^- CD127^{+/-}$ DN4 progenitor¹⁵¹. Under the SP-XF scheme, regardless of CAR modification, a decrease in CD25 positive cells from day 6 to day 15 was observed, followed by an increase until day 28-34, while CD127 decreased in expression from day 15 to day 31. The expression pattern of SP-XF cells do not cleanly align to the expected pattern of early thymocytes due to a lack of $CD25^+ CD127^+ CD4^- CD8^-$ phenotype cells. Day 12 marked a transition in CD25 expression, which correlated with a change in cytokines added to culture media under the SP-XF scheme. Interestingly, starting day 12, RAR α agonist AM580 is included, which is known to induce Treg phenotype by induction of the FOXP3 transcription factor. In a study of Treg populations in PB, it was found that $CD4^+ CD25^+ CD127^{low/-}$ T cells most readily express FOXP3, had the strongest suppressive capability against stimulated $CD25^-$ T responder cells, and may be the best definition of the Treg morphology¹⁸⁷. Collectively, the decrease in CD127 expression and increase in CD25 expression in $CD4^+$ T cells under the SP-XF scheme following AM580 addition resulted in the conclusion that the primary cell formed by SP-XF are T regulatory cells.

4.4 Conclusion

The cells produced in the SP-XF scheme appear to not cleanly follow typical lymphopoiesis, as cells positive for early lymphoid progenitor marker CD7 remained constant throughout differentiation, all while terminal markers such as CD4 and CD3 are also present. CD90 may

indicate early T lymphocyte or a T_H17 variety formed as a result of the SP-XF scheme. More research into the steps taken in differentiation by the lymphoid cells is required to better understand if the differentiation progression better mirrors early non-hematopoietic lymphopoiesis, or if the differentiation scheme induces a hybrid morphology in the cells, in which both terminal and progenitor markers are present. However, the most significant conclusion is the formation of cells with clear markers for T helper lineage, despite the lack of OP9 feeder cells expressing Notch ligands.

5. FUTURE DIRECTIONS

5.1 CAR Neutrophil for Prostate Cancer Treatment

5.1.1 Future analyses of CAR-Neutrophils

CAR-modified neutrophils formed from the SteSII differentiation scheme demonstrate increased cytotoxicity against LNCaP. Further investigation into the impacts of anti-PSMA CAR neutrophils on PCa models will be required.

An additional function of phagocytotic cells is the production of reactive oxygen species (ROS) within lysosomes¹⁸⁸. It was found in bulk RNA sequencing analysis that ROS genes *NCF1*, *NCF2*, and *NCF4* are upregulated in hPSC-neutrophils. To verify functional activity, phorbol 12-myristate 13-acetate (PMA) can be added to Day 20 hPSC-neutrophils to stimulate ROS production, which can be assessed by H₂DCFDA fluorescence. To determine the effect of CAR-expression on ROS generation, hPSC-neutrophils can be co-cultured with LNCaP and ROS production measured. It is expected that significantly more ROS will be produced in CAR-neutrophils compared to the wild type. This would serve to verify a major method of neutrophil-mediated cytotoxicity against targeted cells and provide a reasoning for the observed cytotoxicity increase against LNCaP when CAR-neutrophils are co-cultured, as the CAR construct would serve to activate pro-inflammatory signaling cascades including ROS production and release.

A unique function of neutrophils is the release of genetic material containing bound lytic enzymes, ROS, and other cytotoxic agents that ravage the local area, referred to as neutrophil extracellular traps (NETs)^{189,190}. hPSC-neutrophil NET formation can be assessed under LNCaP co-culture conditions by analysis of double-stranded DNA in the culture media. For a similar

reasoning to ROS production, it is expected that CAR-modified neutrophils will generate more NETs upon co-culture with LNCaP.

5.1.2 Improved *In Vitro* and *In Vivo* models

All cytotoxic analyses of hPSC-neutrophils in this study were performed under 2D co-culture conditions with LNCaP. Future assessment of LNCaP specific cytotoxicity using an improved solid tumor model could include a 3D luciferase-expressing LNCaP cluster suspended in Matrigel (representative TME) followed by introduction of CAR-neutrophils. This model can be assessed for tumor size, neutrophil migration into the TME, and ROS/NET generation. Additionally, assessment of CAR-neutrophil cytotoxicity using an *in vivo* model with mouse or zebrafish will allow for determination of tumor-burden reduction and increased survivability.

5.1.3 Combination Treatments

Anti-tumor activity may be improved using a combination of CAR-N with CAR-T, CAR-NK, CAR-M, or mAb treatments. It stands to reason that as the immune system engages threats through a variety of cell types, cytokines, complement proteins, and antibodies that a combination of CAR modified cells and/or anti-tumor drugs may also elicit a synergistic response. This would be a relatively simple effect to determine using the models described above, although optimization and translation to PCa treatments would likely be a difficult endeavor.

5.1.4 Investigation into NK and erythroid lineage differentiation

This study focused on the generation of lymphoid cells, however, there appear to be populations of NK and erythroid -capable progenitor cells also formed under the SP-XF scheme. This provides an additional point of investigation for the future. As an initial consideration, a

reliable method to form blood cells for clinical research and infusions would serve as a novel research pathway. Also, considering the recent advances in NK differentiation, investigation into the NK lineage cells potentially formed under the SP-XF scheme may be of interest.

5.1.5 Optimizing T cell differentiation

This study focused on a single modification of a neutrophil differentiation scheme that generated floating T cell progenitors, potentially of the Treg lineage. Assessment of generated cells by bulk or single-cell RNA sequencing will better elucidate to what degree SP-XF scheme cells are terminally differentiated or progenitors. It is of significant interest to optimize this protocol for T cells. Substituting StemSpan-XF differentiation media after initial CD4⁺ floating cell formation may allow for terminal differentiation. Also, modifying the cytokines provided to the differentiation media to better select for T cell differentiation may allow for subtype specification. Under the SP-XF scheme, it is likely that a Treg subtype is specified using GM-CSF, IL-6, and Am580. Introduction of IL-2, IL-12, TGF- β , or IFN- γ may change this specification.

REFERENCES

1. Lee, J. M. When CAR Meets Stem Cells. *International Journal of Molecular Sciences* 2019, Vol. 20, Page 1825 **20**, 1825 (2019).
2. June, C. H. & Sadelain, M. Chimeric antigen receptor therapy. *New England Journal of Medicine* **379**, 64–73 (2018).
3. Porter, D. L., Levine, B. L., Kalos, M., Bagg, A. & June, C. H. Chimeric antigen receptor–modified T cells in chronic lymphoid leukemia. *N engl j Med* **365**, 725–733 (2011).
4. Barrett, D. M., Singh, N., Porter, D. L., Grupp, S. A. & June, C. H. Chimeric antigen receptor therapy for cancer. *Annu Rev Med* **65**, 333–347 (2014).
5. Grupp, S. A. *et al.* Chimeric Antigen Receptor–Modified T Cells for Acute Lymphoid Leukemia. *New England Journal of Medicine* **368**, 1509–1518 (2013).
6. Sterner, R. C. & Sterner, R. M. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J* **11**, 69 (2021).
7. Lee, Y.-H., Chan, • & Kim, H. Evolution of chimeric antigen receptor (CAR) T cell therapy: current status and future perspectives. doi:10.1007/s12272-019-01136-x.
8. Sadelain, M., Brentjens, R. & Rivière, I. The basic principles of chimeric antigen receptor design. *Cancer Discov* **3**, 388–398 (2013).
9. Fuca, G., Reppel, L., Landoni, E., Savoldo, B. & Dotti, G. Enhancing Chimeric Antigen Receptor T-Cell Efficacy in Solid Tumors. *Clinical Cancer Research* **26**, 2444–2451 (2020).
10. Newick, K., Moon, E. & Albelda, S. M. Chimeric antigen receptor T-cell therapy for solid tumors. *Molecular therapy-oncolytics* **3**, 16006 (2016).
11. Larson, R. C. & Maus, M. v. Recent advances and discoveries in the mechanisms and functions of CAR T cells. doi:10.1038/s41568-020-00323-z.
12. Campana, D., Schwarz, H. & Imai, C. 4-1BB chimeric antigen receptors. *Cancer Journal (United States)* **20**, 134–140 (2014).
13. Scheuermann, R. H. & Racila, E. CD19 antigen in leukemia and lymphoma diagnosis and immunotherapy. *Leuk Lymphoma* **18**, 385–397 (1995).
14. Depil, S., Duchateau, P., Grupp, S. A., Mufti, G. & Poirot, L. ‘Off-the-shelf’ allogeneic CAR T cells: development and challenges. doi:10.1038/s41573-019-0051-2.

15. Gill, S., Maus, M. v. & Porter, D. L. Chimeric antigen receptor T cell therapy: 25 years in the making. *Blood Rev* **30**, 157–167 (2016).
16. Tyagarajan, S., Spencer, T. & Smith, J. Optimizing CAR-T Cell Manufacturing Processes during Pivotal Clinical Trials. *Mol Ther Methods Clin Dev* **16**, 136–144 (2020).
17. Stenger, D. *et al.* Endogenous TCR promotes in vivo persistence of CD19-CAR-T cells compared to a CRISPR/Cas9-mediated TCR knockout CAR. *Blood* **136**, 1407–1418 (2020).
18. Shimabukuro-Vornhagen, A. *et al.* Cytokine release syndrome. *J Immunother Cancer* **6**, 1–14 (2018).
19. Brudno, J. N. & Kochenderfer, J. N. Toxicities of chimeric antigen receptor T cells: recognition and management. *Blood, The Journal of the American Society of Hematology* **127**, 3321–3330 (2016).
20. Wu, C. Y., Roybal, K. T., Puchner, E. M., Onuffer, J. & Lim, W. A. Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. *Science* **350**, (2015).
21. Yu, S. *et al.* Chimeric antigen receptor T cells: a novel therapy for solid tumors. *J Hematol Oncol* **10**, 1–13 (2017).
22. Alsaab, H. O. *et al.* PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. *Front Pharmacol* **8**, 1–15 (2017).
23. Beavis, P. A. *et al.* Targeting the adenosine 2A receptor enhances chimeric antigen receptor T cell efficacy. *Journal of Clinical Investigation* **127**, 929–941 (2017).
24. Ho, P. C. *et al.* Phosphoenolpyruvate Is a Metabolic Checkpoint of Anti-tumor T Cell Responses. *Cell* **162**, 1217–1228 (2015).
25. Bell, M. & Gottschalk, S. Engineered Cytokine Signaling to Improve CAR T Cell Effector Function. *Front Immunol* **12**, 1–16 (2021).
26. Alizadeh, D. *et al.* IL15 Enhances CAR-T Cell Antitumor Activity by Reducing mTORC1 Activity and Preserving Their Stem Cell Memory Phenotype. *Cancer Immunol Res* **7**, 759–772 (2019).
27. Johnston, J. A. *et al.* Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15. *Proc Natl Acad Sci U S A* **92**, 8705–8709 (1995).

28. Chen, Y. *et al.* Eradication of neuroblastoma by T cells redirected with an optimized GD2-specific chimeric antigen receptor and interleukin-15. *Clinical Cancer Research* **25**, 2915–2924 (2019).
29. Kagoya, Y. *et al.* A novel chimeric antigen receptor containing a JAK-STAT signaling domain mediates superior antitumor effects. *Nat Med* **24**, 352–359 (2018).
30. Majzner, R. G. *et al.* Tuning the Antigen Density Requirement for CAR T-cell Activity. *Cancer Discov* **10**, 702–723 (2020).
31. Liu, S. *et al.* NK cell-based cancer immunotherapy: from basic biology to clinical development. *J Hematol Oncol* **14**, (2021).
32. Du, N., Guo, F., Wang, Y. & Cui, J. NK Cell Therapy: A Rising Star in Cancer Treatment. *Cancers (Basel)* **13**, (2021).
33. Montagner, I. M. *et al.* Anti-PSMA CAR-engineered NK-92 Cells: An Off-the-shelf Cell Therapy for Prostate Cancer. *Cells* **9**, (2020).
34. Lee, C. Z. W., Kozaki, T. & Ginhoux, F. Studying tissue macrophages in vitro: are iPSC-derived cells the answer? *Nature Reviews Immunology* 2018 18:11 **18**, 716–725 (2018).
35. Franklin, R. A. Fibroblasts and macrophages: Collaborators in tissue homeostasis. *Immunol Rev* **302**, 86–103 (2021).
36. Snyder, R. J. *et al.* Macrophages: A review of their role in wound healing and their therapeutic use. *Wound Repair and Regeneration* **24**, 613–629 (2016).
37. Gordon, S. & Martinez-Pomares, L. Physiological roles of macrophages. *Pflugers Archiv* **469**, 365 (2017).
38. Wynn, T. A., Chawla, A. & Pollard, J. W. Macrophage biology in development, homeostasis and disease. *Nature* 2013 496:7446 **496**, 445–455 (2013).
39. Driscoll, K. E. Macrophage inflammatory proteins: biology and role in pulmonary inflammation. *Exp Lung Res* **20**, 473–490 (1994).
40. Chen, Y. *et al.* CAR-macrophage: A new immunotherapy candidate against solid tumors. *Biomedicine & Pharmacotherapy* **139**, 111605 (2021).
41. Klichinsky, M. *et al.* Human chimeric antigen receptor macrophages for cancer immunotherapy. *Nature Biotechnology* 2020 38:8 **38**, 947–953 (2020).

42. Zhang, W. *et al.* Chimeric antigen receptor macrophage therapy for breast tumours mediated by targeting the tumour extracellular matrix. *British Journal of Cancer* 2019 121:10 **121**, 837–845 (2019).
43. Chen, K. H. *et al.* A compound chimeric antigen receptor strategy for targeting multiple myeloma. *Leukemia* **32**, 402–412 (2018).
44. Aghajanian, H., Rurik, J. G. & Epstein, J. A. CAR-based therapies: opportunities for immuno-medicine beyond cancer. *Nat Metab* **4**, 163–169 (2022).
45. Blat, D., Zigmond, E., Alteber, Z., Waks, T. & Eshhar, Z. Suppression of Murine Colitis and its Associated Cancer by Carcinoembryonic Antigen-Specific Regulatory T Cells. *Molecular Therapy* **22**, 1018–1028 (2014).
46. Fransson, M. *et al.* CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J Neuroinflammation* **9**, 576 (2012).
47. Tenspolde, M. *et al.* Regulatory T cells engineered with a novel insulin-specific chimeric antigen receptor as a candidate immunotherapy for type 1 diabetes. *J Autoimmun* **103**, 102289 (2019).
48. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **71**, 209–249 (2021).
49. Henríquez, I. *et al.* Current and emerging therapies for metastatic castration-resistant prostate cancer (Mcrpc). *Biomedicines* **9**, 1–13 (2021).
50. Chang, S. S. *et al.* Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res* **59**, 3192–3198 (1999).
51. Liu, H. *et al.* Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. *Cancer Res* **57**, 3629–3634 (1997).
52. Kuratsukuri, K. *et al.* Inhibition of prostate-specific membrane antigen (PSMA)-positive tumor growth by vaccination with either full-length or the C-terminal end of PSMA. *Int J Cancer* **102**, 244–249 (2002).
53. Wang, F., Li, Z., Feng, X., Yang, D. & Lin, M. Advances in PSMA-targeted therapy for prostate cancer. *Prostate Cancer Prostatic Dis* **25**, 11–26 (2022).

54. Kim, J. & Bae, J. S. Tumor-associated macrophages and neutrophils in tumor microenvironment. *Mediators Inflamm* **2016**, (2016).
55. Wang, N., Liang, H. & Zen, K. Molecular Mechanisms That Influence the Macrophage M1-M2 Polarization Balance. *Front Immunol* **5**, (2014).
56. Gutbier, S. *et al.* Large-Scale Production of Human iPSC-Derived Macrophages for Drug Screening. *International Journal of Molecular Sciences* 2020, Vol. 21, Page 4808 **21**, 4808 (2020).
57. Wanke, F. *et al.* Ligand-dependent kinase activity of MERTK drives efferocytosis in human iPSC-derived macrophages. *Cell Death & Disease* 2021 12:6 **12**, 1–13 (2021).
58. Lin, H.-T. *et al.* An assessment of the effects of ectopic gp91phox expression in XCGD iPSC-derived neutrophils. *Molecular Therapy-Methods & Clinical Development* **2**, 15046 (2015).
59. Rafiei Hashtchin, A. *et al.* Human iPSC-derived macrophages for efficient *Staphylococcus aureus* clearance in a murine pulmonary infection model. *Blood Adv* (2021) doi:10.1182/BLOODADVANCES.2021004853.
60. Bernard, E. M. *et al.* M. tuberculosis infection of human iPSC-derived macrophages reveals complex membrane dynamics during xenophagy evasion. *J Cell Sci* **134**, (2021).
61. Miyauchi, M. *et al.* Efficient production of human neutrophils from iPSCs that prevent murine lethal infection with immune cell recruitment. *Blood* (2021) doi:10.1182/BLOOD.2021011576.
62. Lim, W. F., Inoue-Yokoo, T., Tan, K. S., Lai, M. I. & Sugiyama, D. Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells. *Stem Cell Res Ther* **4**, 71 (2013).
63. Schrimpf, C. *et al.* Differentiation of induced pluripotent stem cell–derived neutrophil granulocytes from common marmoset monkey (*Callithrix jacchus*). *Transfusion (Paris)* **57**, 60–69 (2017).
64. Navarro-Guerrero, E. *et al.* Genome-wide CRISPR/Cas9-knockout in human induced Pluripotent Stem Cell (iPSC)-derived macrophages. *Scientific Reports* 2021 11:1 **11**, 1–11 (2021).

65. Trump, L. R. *et al.* Neutrophils derived from genetically modified human induced pluripotent stem cells circulate and phagocytose bacteria in vivo. *Stem Cells Transl Med* **8**, 557–567 (2019).
66. Hansen, M., von Lindern, M., van den Akker, E. & Varga, E. Human-induced pluripotent stem cell-derived blood products: state of the art and future directions. *FEBS Letters* vol. 593 3288–3303 Preprint at <https://doi.org/10.1002/1873-3468.13599> (2019).
67. Brok-Volchanskaya, V. S. *et al.* Effective and Rapid Generation of Functional Neutrophils from Induced Pluripotent Stem Cells Using ETV2-Modified mRNA. *Stem Cell Reports* **13**, 1099–1110 (2019).
68. Cui, D. *et al.* High-Yield Human Induced Pluripotent Stem Cell-Derived Monocytes and Macrophages Are Functionally Comparable With Primary Cells. *Front Cell Dev Biol* **9**, 754 (2021).
69. Lachmann, N. *et al.* Large-Scale Hematopoietic Differentiation of Human Induced Pluripotent Stem Cells Provides Granulocytes or Macrophages for Cell Replacement Therapies. *Stem Cell Reports* **4**, 282–296 (2015).
70. Sweeney, C. L. *et al.* Molecular Analysis of Neutrophil Differentiation from Human Induced Pluripotent Stem Cells Delineates the Kinetics of Key Regulators of Hematopoiesis. *Stem Cells* **34**, 1513–1526 (2016).
71. Vaughan-Jackson, A. *et al.* Differentiation of human induced pluripotent stem cells to authentic macrophages using a defined, serum-free, open-source medium. *Stem Cell Reports* **16**, 1735–1748 (2021).
72. Armitage, L. H. *et al.* High-Yield Monocyte, Macrophage, and Dendritic Cell Differentiation from Induced Pluripotent Stem Cells Running Title: High-Yield Monocyte Differentiation from hiPSC. doi:10.1101/2021.04.29.441947.
73. Brault, J. *et al.* Optimized generation of functional neutrophils and macrophages from patient-specific induced pluripotent stem cells: Ex vivo models of X0-Linked, AR220- and AR470-chronic granulomatous diseases. *Biores Open Access* **3**, 311–326 (2014).
74. Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E. & Weissman, I. L. Identification of a lineage of multipotent hematopoietic progenitors. *Development* **124**, 1929–1939 (1997).

75. Yanagimachi, M. D. *et al.* Robust and highly-efficient differentiation of functional monocytic cells from human pluripotent stem cells under serum- and feeder cell-free conditions. *PLoS One* **8**, (2013).
76. van Wilgenburg, B., Browne, C., Vowles, J. & Cowley, S. A. Efficient, Long Term Production of Monocyte-Derived Macrophages from Human Pluripotent Stem Cells under Partly-Defined and Fully-Defined Conditions. *PLoS One* **8**, 71098 (2013).
77. Choi, K. D., Vodyanik, M. A. & Slukvin, I. I. Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors. *J Clin Invest* **119**, 2818–2829 (2009).
78. Cao, X. *et al.* Differentiation and Functional Comparison of Monocytes and Macrophages from hiPSCs with Peripheral Blood Derivatives. *Stem Cell Reports* **12**, 1282–1297 (2019).
79. Munn, C. *et al.* Generation of cryopreserved macrophages from normal and genetically engineered human pluripotent stem cells for disease modelling. *PLoS One* **16**, e0250107 (2021).
80. Heideveld, E. *et al.* Methods for macrophage differentiation and in vitro generation of human tumor associated-like macrophages. *Methods Enzymol* **632**, 113–131 (2020).
81. Maria, M. A. & Cojoc, D. Monitoring Human Neutrophil Differentiation by Digital Holographic Microscopy. *Front Phys* **9**, 190 (2021).
82. Morishima, T. *et al.* Neutrophil differentiation from human-induced pluripotent stem cells. *J Cell Physiol* **226**, 1283–1291 (2011).
83. Prykhozhij, S. v., Cordeiro-Santanach, A., Caceres, L. & Berman, J. N. Genome Editing in Zebrafish Using High-Fidelity Cas9 Nucleases: Choosing the Right Nuclease for the Task. in *Methods in Molecular Biology* vol. 2115 385–405 (Humana Press Inc., 2020).
84. Rincón, E., Rocha-Gregg, B. L. & Collins, S. R. A map of gene expression in neutrophil-like cell lines. *BMC Genomics* **19**, (2018).
85. Fattorelli, N. *et al.* Stem-cell-derived human microglia transplanted into mouse brain to study human disease. *Nature Protocols* **2021 16:2** **16**, 1013–1033 (2021).
86. Chang, Y. *et al.* Engineering chimeric antigen receptor neutrophils from human pluripotent stem cells for targeted cancer immunotherapy. *Cell Rep* **40**, 111128 (2022).

87. Lieschke, G. J. *et al.* Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood* **84**, 1737–1746 (1994).
88. Boettcher, S. *et al.* Endothelial cells translate pathogen signals into G-CSF-driven emergency granulopoiesis. *Blood* **124**, 1393–1403 (2014).
89. Bendall, L. J. & Bradstock, K. F. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine Growth Factor Rev* **25**, 355–367 (2014).
90. Theilgaard-Mönch, K. *et al.* The transcriptional program of terminal granulocytic differentiation. *Blood* **105**, 1785–1796 (2005).
91. Yang, Y. C. *et al.* Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* **47**, 3–10 (1986).
92. Korpelainen, E. I., Gamble, J. R., Vadas, M. A. & Lopez, A. F. IL-3 receptor expression, regulation and function in cells of the Vasculature. *Immunol Cell Biol* **74**, 1–7 (1996).
93. Liu, F., Poursine-Laurent, J., Wu, H. Y. & Link, D. C. Interleukin-6 and the Granulocyte Colony-Stimulating Factor Receptor Are Major Independent Regulators of Granulopoiesis In Vivo But Are Not Required for Lineage Commitment or Terminal Differentiation. *Blood* **90**, 2583–2590 (1997).
94. Broudy, V. C. Stem Cell Factor and Hematopoiesis. *Blood* **90**, 1345–1364 (1997).
95. Hassan, H. T. & Zander, A. R. Stem Cell Factor as a Survival and Growth Factor in Human Normal and Malignant Hematopoiesis. *Molecular Biology of Hematopoiesis* **5** 549–558 (1996) doi:10.1007/978-1-4613-0391-6_66.
96. Kolls Oliver, J. K. *et al.* for IL-17-Mediated Granulopoiesis and Granulocyte-Colony-Stimulating Factor Requirement of Endogenous Stem Cell Factor. *J Immunol References* **164**, 4783–4789 (2000).
97. Craig, W., Poppema, S., Little, M.-T., Dragowska, W. & Lansdorp, P. M. *CD4 5 isoform expression on human haemopoietic cells at different stages of development. British Journal of Haematology* vol. 88 (1994).
98. Hammarström, S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin Cancer Biol* **9**, 67–81 (1999).

99. Torsteinsdottir, I., Arvidson, N.-G., Hallgren, R. & Hakansson, L. Enhanced Expression of Integrins and CD66b on Peripheral Blood Neutrophils and Eosinophils in Patients with Rheumatoid Arthritis, and the Effect of Glucocorticoids. *Scand J Immunol* **50**, 433–439 (1999).
100. Yoon, J., Terada, A. & Kita, H. CD66b Regulates Adhesion and Activation of Human Eosinophils. *The Journal of Immunology* **179**, 8454–8462 (2007).
101. Bredius, R. G. M. & Medical, A. Role of neutrophil FcγRIIa (CD32) and FcγRIIIb (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. *Transfus Med Rev* **9**, 343 (1995).
102. Junker, F., Gordon, J. & Qureshi, O. Fc Gamma Receptors and Their Role in Antigen Uptake, Presentation, and T Cell Activation. *Front Immunol* **11**, 1–13 (2020).
103. Treffers, L. W. *et al.* FcγRIIIb restricts antibody-dependent destruction of cancer cells by human neutrophils. *Front Immunol* **10**, 1–13 (2019).
104. Davoine, F. *et al.* Expression of FcγRIII (CD16) on human peripheral blood eosinophils increases in allergic conditions. *Journal of Allergy and Clinical Immunology* **109**, 463–469 (2002).
105. Roberts, J. T. & Barb, A. W. A single amino acid distorts the Fc γ receptor IIIb/CD16b structure upon binding immunoglobulin G1 and reduces affinity relative to CD16a. *Journal of Biological Chemistry* **293**, 19899–19908 (2018).
106. Hamdan, T. A., Lang, P. A. & Lang, K. S. The diverse functions of the ubiquitous fcγ receptors and their unique constituent, fcγ subunit. *Pathogens* **9**, (2020).
107. Blázquez-Moreno, A. *et al.* Transmembrane features governing Fc receptor CD16A assembly with CD16A signaling adaptor molecules. *Proc Natl Acad Sci U S A* **114**, E5645–E5654 (2017).
108. Wirthmueller, U., Kurosaki, T., Murakami, M. S. & Ravetch, J. v. Signal transduction by Fc gamma RIII (CD16) is mediated through the gamma chain. *J Exp Med* **175**, 1381–90 (1992).
109. Radstake, T. R. D. J. *et al.* Role of Fcγ receptors IIA, IIIA, and IIIB in susceptibility to rheumatoid arthritis. *Journal of Rheumatology* **30**, 926–933 (2003).

110. Magnusson, S. E., Engström, M., Jacob, U., Ulfgren, A. K. & Kleinau, S. High synovial expression of the inhibitory FcγRIIb in rheumatoid arthritis. *Arthritis Res Ther* **9**, 1–11 (2007).
111. Fagerholm, S. C., Guenther, C., Lloret Asens, M., Savinko, T. & Uotila, L. M. Beta2-Integrins and Interacting Proteins in Leukocyte Trafficking, Immune Suppression, and Immunodeficiency Disease. *Front Immunol* **10**, (2019).
112. van Spruiel, A. B. *et al.* Mac-1 (CD11b/CD18) is essential for Fc receptor-mediated neutrophil cytotoxicity and immunologic synapse formation. *Blood* **97**, 2478–86 (2001).
113. Nieberler, M. *et al.* Exploring the Role of RGD-Recognizing Integrins in Cancer. *Cancers (Basel)* **9**, 116 (2017).
114. Hussein, H. A. M. *et al.* Beyond RGD: virus interactions with integrins. *Arch Virol* **160**, 2669–2681 (2015).
115. Agerer, F., Michel, A., Ohlsen, K. & Hauck, C. R. Integrin-mediated invasion of *Staphylococcus aureus* into human cells requires Src family protein-tyrosine kinases. *J Biol Chem* **278**, 42524–31 (2003).
116. Ding, Z. M. *et al.* Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J Immunol* **163**, 5029–38 (1999).
117. Bednarska, K. *et al.* Heterogeneity of the Mac-1 expression on peripheral blood neutrophils in patients with different types of epithelial ovarian cancer. *Immunobiology* **221**, 323–332 (2016).
118. Ehlers, M. R. CR3: a general purpose adhesion-recognition receptor essential for innate immunity. *Microbes Infect* **2**, 289–94 (2000).
119. Wright, S. D., Reddy, P. A., Jong, M. T. & Erickson, B. W. C3bi receptor (complement receptor type 3) recognizes a region of complement protein C3 containing the sequence Arg-Gly-Asp. *Proceedings of the National Academy of Sciences* **84**, 1965–1968 (1987).
120. Tanaka, T. Leukocyte Adhesion Molecules. in *Encyclopedia of Immunobiology* vol. 3 505–511 (Elsevier, 2016).
121. Zhou, H. *et al.* CD11b/CD18 (Mac-1) Is a Novel Surface Receptor for Extracellular Double-Stranded RNA To Mediate Cellular Inflammatory Responses. *The Journal of Immunology* **190**, 115–125 (2013).

122. Zarewych, D. M., Kindzelskii, A. L., Todd, R. F. & Petty, H. R. LPS induces CD14 association with complement receptor type 3, which is reversed by neutrophil adhesion. *J Immunol* **156**, 430–3 (1996).
123. Bouti, P. *et al.* β 2 Integrin Signaling Cascade in Neutrophils: More Than a Single Function. *Front Immunol* **11**, (2021).
124. Gadjeva, M. Complement System - Overview. in *Methods in Molecular Biology* vol. 1100 1–9 (Humana Press Inc., 2014).
125. Noris, M. & Remuzzi, G. Overview of Complement Activation and Regulation. *Semin Nephrol* **33**, 479–492 (2013).
126. Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* **37**, 907–915 (2019).
127. Ramsköld, D., Kavak, E. & Sandberg, R. How to Analyze Gene Expression Using RNA-Sequencing Data. in *Methods* (eds. Wang, J., Tan, A. C. & Tian, T.) vol. 802 259–274 (Humana Press, 2012).
128. Parker, S. A., Diaz, I. L. C., Anderson, K. A. & Batt, C. A. Design, production, and characterization of a single-chain variable fragment (ScFv) derived from the prostate specific membrane antigen (PSMA) monoclonal antibody J591. *Protein Expr Purif* **89**, 136–145 (2013).
129. Hassani, M. *et al.* Construction of a chimeric antigen receptor bearing a nanobody against prostate specific membrane antigen in prostate cancer. *J Cell Biochem* **120**, 10787–10795 (2018).
130. Lian, X. *et al.* Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. *Stem Cell Reports* **3**, 804–816 (2014).
131. Dalton, J. A., Higgins, M. K., Miller, A. H., Keefe, F. J. & Khuri, F. R. Myeloperoxidase: A new player in autoimmunity.HHS Public Access. *Cell Immunol* **38**, 457–464 (2016).
132. FALLOON, J. & GALLIN, J. Neutrophil granules in health and disease. *Journal of Allergy and Clinical Immunology* **77**, 653–662 (1986).
133. Sonderegger, I. *et al.* GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. *Journal of Experimental Medicine* **205**, 2281–2294 (2008).

134. Carey, B. & Trapnell, B. C. The molecular basis of pulmonary alveolar proteinosis. *Clinical Immunology* **135**, 223–235 (2010).
135. Hamilton, J. A. GM-CSF in inflammation. *Journal of Experimental Medicine* **217**, 1–16 (2020).
136. Zúñiga-Pflücker, J. C. T-cell development made simple. *Nat Rev Immunol* **4**, 67–72 (2004).
137. Shah, D. K. & Zúñiga-Pflücker, J. C. An Overview of the Intrathymic Intricacies of T Cell Development. *The Journal of Immunology* **192**, 4017–4023 (2014).
138. Poznansky, M. C. *et al.* Efficient generation of human T cells from a tissue-engineered thymic organoid. *Nat Biotechnol* **18**, 729–734 (2000).
139. Mohtashami, M. & Zúñiga-Pflücker, J. C. Cutting Edge: Three-Dimensional Architecture of the Thymus Is Required to Maintain Delta-Like Expression Necessary for Inducing T Cell Development. *The Journal of Immunology* **176**, 730–734 (2006).
140. Schmitt, T. M. & Zúñiga-Pflücker, J. C. Induction of T Cell Development from Hematopoietic Progenitor Cells by Delta-like-1 In Vitro. *Immunity* **17**, 749–756 (2002).
141. Smith, M. J. *et al.* In Vitro T-Cell Generation From Adult, Embryonic, and Induced Pluripotent Stem Cells: Many Roads to One Destination. *Stem Cells* **33**, 3174–3180 (2015).
142. Mohtashami, M., Brauer, P. M. & Zúñiga-Pflücker, J. C. Induction of Human T Cell Development In Vitro with OP9-DL4-7FS Cells Expressing Human Cytokines. in 249–260 (2023). doi:10.1007/978-1-0716-2740-2_15.
143. Mohtashami, M., Zarin, P. & Zúñiga-Pflücker, J. C. Induction of T cell development in vitro by delta-like (Dl1)-expressing stromal cells. in *T-Cell Development: Methods and Protocols* 159–167 (Springer New York, 2015). doi:10.1007/978-1-4939-2809-5_14.
144. Mohtashami, M., Shah, D. K., Kianizad, K., Awong, G. & Zúñiga-Pflücker, J. C. Induction of T-cell development by Delta-like 4-expressing fibroblasts. *Int Immunol* **25**, 601–611 (2013).
145. Reimann, C. *et al.* Human T-Lymphoid Progenitors Generated in a Feeder-Cell-Free Delta-Like-4 Culture System Promote T-Cell Reconstitution in NOD/SCID/ γ c^{-/-} Mice. *Stem Cells* **30**, 1771–1780 (2012).
146. de Smedt, M., Hoebeke, I. & Plum, J. Human bone marrow CD34⁺ progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment. *Blood Cells Mol Dis* **33**, 227–232 (2004).

147. Suraiya, A. B., Hun, M. L., Truong, V. X., Forsythe, J. S. & Chidgey, A. P. Gelatin-Based 3D Microgels for In Vitro T Lineage Cell Generation. *ACS Biomater Sci Eng* **6**, 2198–2208 (2020).
148. Iriguchi, S. *et al.* Feeder-free differentiation and expansion for T cells from induced pluripotent stem cells. (2021) doi:10.21203/rs.3.pex-1271.
149. Iriguchi, S. *et al.* A clinically applicable and scalable method to regenerate T-cells from iPSCs for off-the-shelf T-cell immunotherapy. *Nat Commun* **12**, 430 (2021).
150. Saravia, J., Chapman, N. M. & Chi, H. Helper T cell differentiation. *Cell Mol Immunol* **16**, 634–643 (2019).
151. Yang, Q., Jeremiah Bell, J. & Bhandoola, A. T-cell lineage determination. *Immunol Rev* **238**, 12–22 (2010).
152. Peck, A. & Mellins, E. D. Plasticity of T-cell phenotype and function: the T helper type 17 example. *Immunology* **129**, 147–153 (2010).
153. Hoechst, B., Gamrekashvili, J., Manns, M. P., Greten, T. F. & Korangy, F. Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. *Blood* **117**, 6532–6541 (2011).
154. Park, B. v. & Pan, F. The role of nuclear receptors in regulation of Th17/Treg biology and its implications for diseases. *Cell Mol Immunol* **12**, 533–542 (2015).
155. Unutmaz, D. RORC2: The master of human Th17 cell programming. *Eur J Immunol* **39**, 1452–1455 (2009).
156. Mazzoni, A. *et al.* Demethylation of the RORC2 and IL17A in human CD4⁺ T lymphocytes defines Th17 origin of nonclassic Th1 cells. *J Immunol* **194**, 3116–26 (2015).
157. de Roock, S. *et al.* Defective TH17 development in human neonatal T cells involves reduced RORC2 mRNA content. *Journal of Allergy and Clinical Immunology* **132**, 754-756.e3 (2013).
158. Maggi, L. *et al.* CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. *Eur J Immunol* **40**, 2174–2181 (2010).
159. Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–238 (2006).
160. Ohue, Y. & Nishikawa, H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Sci* **110**, 2080–2089 (2019).

161. Elias, K. M. *et al.* Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* **111**, 1013–1020 (2008).
162. Crome, S. Q., Wang, A. Y., Kang, C. Y. & Levings, M. K. The role of retinoic acid‐related orphan receptor variant 2 and IL‐17 in the development and function of human CD4⁺ T cells. *Eur J Immunol* **39**, 1480–1493 (2009).
163. Maguer-Satta, V., Besançon, R. & Bachelard-Cascales, E. Concise review: Neutral endopeptidase (CD10): A multifaceted environment actor in stem cells, physiological mechanisms, and cancer. *Stem Cells* **29**, 389–396 (2011).
164. Cutrona, G. & Ferrarini, M. Expression of CD10 by human T cells that undergo apoptosis both in vitro and in vivo [5]. *Blood* **97**, 2528 (2001).
165. Cutrona, G. *et al.* CD10 is a marker for cycling cells with propensity to apoptosis in childhood aLL. *Br J Cancer* **86**, 1776–1785 (2002).
166. Attygalle, A. *et al.* Neoplastic T cells in angioimmunoblastic T-cell lymphoma express CD10. *Blood* **99**, 627–633 (2002).
167. Guérin, S. *et al.* CD10 plays a specific role in early thymic development. *The FASEB Journal* **11**, 376–381 (1997).
168. Marini, O. *et al.* Mature CD10⁺ and immature CD10⁻ neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood* **129**, 1343–1356 (2017).
169. Ritter, M. A., suavage, C. A. & Delia, D. Human Thy-1 antigen: Cell surface expression on early T and B lymphocytes. *Immunology* **49**, (1983).
170. Kumar, A., Bhanja, A., Bhattacharyya, J. & Jaganathan, B. G. Multiple roles of CD90 in cancer. *Tumor Biology* **37**, 11611–11622 (2016).
171. Sauzay, C., Voutetakis, K., Chatziioannou, A. A., Chevet, E. & Avril, T. CD90/Thy-1, a cancer-associated cell surface signaling molecule. *Front Cell Dev Biol* **7**, 1–11 (2019).
172. Pérez, L. A., Leyton, L. & Valdivia, A. Thy-1 (CD90), Integrins and Syndecan 4 are Key Regulators of Skin Wound Healing. *Front Cell Dev Biol* **10**, 1–22 (2022).
173. Wandel, E., Saalbach, A., Sittig, D., Gebhardt, C. & Aust, G. Thy-1 (CD90) Is an Interacting Partner for CD97 on Activated Endothelial Cells. *The Journal of Immunology* **188**, 1442–1450 (2012).

174. Saalbach, A. *et al.* Interaction of human Thy-1 (CD 90) with the integrin $\alpha\beta 3$ (CD51/CD61): An important mechanism mediating melanoma cell adhesion to activated endothelium. *Oncogene* **24**, 4710–4720 (2005).
175. Chen, X., Yu, Y., Mi, L. Z., Walz, T. & Springer, T. A. Molecular basis for complement recognition by integrin $\alpha X\beta 2$. *Proc Natl Acad Sci U S A* **109**, 4586–4591 (2012).
176. Danhier, F., Breton, A. le & Pr  at, V. RGD-based strategies to target $\alpha(v)\beta(3)$ integrin in cancer therapy and diagnosis. *Mol Pharm* **9**, 2961–2973 (2012).
177. Guillot-Delost, M. *et al.* Human CD90 Identifies Th17/Tc17 T Cell Subsets That Are Depleted in HIV-Infected Patients. *The Journal of Immunology* **188**, 981–991 (2012).
178. Motazedian, A. *et al.* Multipotent RAG1⁺ progenitors emerge directly from haemogenic endothelium in human pluripotent stem cell-derived haematopoietic organoids. *Nat Cell Biol* **22**, 60–73 (2020).
179. Ng, E. S. *et al.* Differentiation of human embryonic stem cells to HOXA + hemogenic vasculature that resembles the aorta-gonad-mesonephros. *Nat Biotechnol* **34**, 1168–1179 (2016).
180. B  iers, C. *et al.* Lymphomyeloid Contribution of an Immune-Restricted Progenitor Emerging Prior to Definitive Hematopoietic Stem Cells. *Cell Stem Cell* **13**, 535–548 (2013).
181. Tian, Y. *et al.* The first wave of T lymphopoiesis in zebrafish arises from aorta endothelium independent of hematopoietic stem cells. *Journal of Experimental Medicine* **214**, 3347–3360 (2017).
182. Yoshimoto, M. *et al.* Autonomous murine T-cell progenitor production in the extra-embryonic yolk sac before HSC emergence. *Blood* **119**, 5706–5714 (2012).
183. Heinze, D. *et al.* Notch activation during early mesoderm induction modulates emergence of the T/NK cell lineage from human iPSCs. *Stem Cell Reports* (2022) doi:10.1016/j.stemcr.2022.10.007.
184. Labrecque, N. *et al.* How much TCR does a T cell need? *Immunity* **15**, 71–82 (2001).
185. Singh, A. *et al.* CD3-Negative CD4⁺ T-Cells: A Useful Diagnostic Tool with High Specificity in Angioimmunoblastic Lymphadenopathy (AILD)-Type T-Cell Lymphoma. *Blood* **112**, 5311–5311 (2008).

186. Angelova, E. *et al.* CD123 expression patterns and selective targeting with a CD123-targeted antibody-drug conjugate (IMGN632) in acute lymphoblastic leukemia. *Haematologica* **104**, 749–755 (2019).
187. Yu, N. *et al.* CD4+CD25+CD127^{low/-} T Cells: A More Specific Treg Population in Human Peripheral Blood. *Inflammation* **35**, 1773–1780 (2012).
188. Blanter, M., Gouwy, M. & Struyf, S. Studying Neutrophil Function in vitro: Cell Models and Environmental Factors. *J Inflamm Res* **14**, 141 (2021).
189. Volkov, D. V, Tetz, G. V, Rubtsov, Y. P., Stepanov, A. V & Gabibov, A. G. Neutrophil Extracellular Traps (NETs): Opportunities for Targeted Therapy. *Acta Naturae* **13**, 15–23 (2021).
190. Kaplan, M. J. & Radic, M. Neutrophil Extracellular Traps: Double-Edged Swords of Innate Immunity. *The Journal of Immunology* **189**, 2689–2695 (2012).