Université de Montréal

Improving Anti-Viral T Cell Therapies by Knockout of the NR4A Family of Transcription Factors

Par

Lorne Schweitzer

Université de Montréal

Faculté de médecine

Mémoire présenté en vue de l'obtention du grade de Maîtrise

en Sciences biomédicales option Médecine expérimentale

Août 2023

Université de Montréal

Université de Montréal, Faculté de médecine

Ce mémoire intitulé

Improving Anti-Viral T Cell Therapies by Knockout of the NR4A Family of Transcription Factors

Présenté par

Lorne Schweitzer

A été évalué(e) par un jury composé des personnes suivantes

Marie-Claude Bourgeois-Daigneault

Président-rapporteur

Jean-Sébastien Delisle

Directeur de recherche

Nathalie Labrecque

Codirecteur

Michel Duval

Membre du jury

Résumé

Les infections virales peuvent demeurer latentes pendant plusieurs décennies et se réactiver pendant des périodes d'immunosuppression. Les receveurs de greffes hématopoïétiques sont particulièrement susceptibles compte tenu de l'immunosuppression importante qui est nécessaire pour prévenir le rejet ou la maladie du greffon contre l'hôte, souvent pendant des périodes prolongées. La plupart de ces infections ne peuvent pas être traitées avec des médicaments antiviraux, et lorsque c'est possible, les traitements peuvent amener de la résistance. L'injection de cellules T spécifiques contre les virus provenant de donneurs sains est un traitement efficace pour traiter ces infections virales potentiellement mortelles ou les cancers qu'elles causent. Cependant, la persistance de ces cellules est limitée en partie par la stimulation antigénique chronique qui cause l'épuisement des cellules T. En éliminant les membres de la famille de récepteurs orphelins NR4A, qui favorisent l'épuisement et limitent la différenciation en cellules mémoires durables, notre but est de rendre ces cellules transférées plus persistantes et efficaces. Nos données à ce jour montrent que l'élimination du récepteur NR4A3 n' altère pas la différenciation mémoire ni la production de cytokines effectrices. Cependant, l'absence de NR4A3tend à amener une diminution le l'expression du marqueur d'épuisement Tim-3, ce qui suggère que l'on peut prévenir l'épuisement et ainsi améliorer les thérapies cellulaires en ciblant les membres de la famille des récepteurs NR4A.

Mots-clés : infections opportunistes, virus Epstein-Barr, cellules T spécifiques aux virus, NR4A3, épuisement des cellules T, cellules T mémoires, thérapie cellulaire.

Abstract

Viral infections can lay dormant for decades only to reactivate in periods of immune suppression. Transplant recipients are particularly susceptible to these infections as they require intensive immunosuppression to prevent rejection or graft-versus-host-disease, often for the rest of their life. Most of these infections cannot be treated with currently available antiviral medications and those that do can develop resistance. Virus-specific T cells (VSTs) are a treatment that uses expanded T cells to treat these infections by infusing donor cells into patients with life-threatening viral infections and cancers. However, these cells have a limited lifespan in part due to chronic antigen stimulation causing T cell exhaustion and lack of persistence. By knocking out members of the NR4A family of orphan receptors, which favour exhaustion and limit differentiation into long-lasting memory cells, we aim to make these transferred cells more persistent and effective. NR4A3 knockout did not alter memory differentiation or effector cytokine production but did result in a trend towards decreased expression of the exhaustion marker Tim-3, which indicates that targeting members of this family may improve clinically translatable cellular therapies.

Keywords : opportunistic infection, Epstein-Barr virus, virus-specific T cells, NR4A3, T cell exhaustion, memory T cell, cellular therapy.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAV: adeno-associated virus

ADCC: antibody-dependent cellular cytotoxicity

ACMR: acute cell mediated rejection

ADV: adenovirus

AIRE: autoimmune regulator

Allo-HSCT: allogenic hematopoietic stem cell transplant

AP-1: activator protein-1

APC: antigen-presenting cell

ATG: antithymocyte globulin

BCR: B cell receptor

BL: Burkett's lymphoma

CAR-T : chimeric antigen receptor T cell

Cas: CRISPR-associated protein

spCas9: *Streptococcus pyogenes* Cas9

saCas9: *Staphylococcus aureus* Cas9

ChIP : chromatin immunoprecipitation

CLIP: class II-associated invariant chain peptide

CLP: common lymphoid progenitor

CMV: cytomegalovirus

CNI: calcineurin inhibitor

CRISPR: clustered regularly interspaced short palindromic repeats

crRNA: CRISPR RNA

cSMAC: central supramolecular activation complex

cTEC: cortical thymic epithelial cell

CypA: cyclophilin A

DAG: diacylglycerol

DC: dendritic cell

DLBCL: diffuse large B cell lymphoma

DN: Double-negative

DP: Double-positive

DSB: double-stranded break

dsODN: double stranded oligodeoxynucleotide

dsRNA: double-stranded RNA

EBER: EBV-encoded small RNA

EBNA: EBV nuclear antigen

EBV: Epstein-Barr virus

Eomes: eomesodermin

ER: endoplasmic reticulum

FKBP12: FK-binding protein 12

G-Rex: gas permeable reactor

GVHD: graft-versus-host disease

HDR: homology directed repair

HHV: human herpesvirus

HIV: human immunodeficiency virus

HPV: human papillomavirus

HSV: herpes simplex virus

ICAM: intercellular adhesion molecule-1

ICE: Inference of CRISPR edits

IgA: immunoglobulin A

IgD: immunoglobulin D

IgE: immunoglobulin E

IgG: immunoglobulin G

IgM: immunoglobulin M

ILC: innate lymphoid cell

Indel: insertion or deletion

IP_3: inositol-1,4,5-trisphosphate

IR: inhibitory receptor

IRF: interferon regulatory factor

ITAM: immunoreceptor tyrosine-based activation motif

KI: knock-in

KO: knockout

KS: Kaposi's sarcoma

KSHV: Kaposi sarcoma herpesvirus

LCL: lymphoblastoid cell line

LCMV: lymphocytic choriomeningitis virus

LFA-1: lymphocyte function-associated antigen-1

LMP: latency-associated membrane protein

LRS: leukocyte reduction system

MAC: membrane attack complex

MAMP: microbial-associated molecular pattern

MAPK: mitogen-activated protein kinase

MHC: major histocompatibility complex

MMF: mycophenolate mofetil

mTEC: medullary thymic epithelial cell

mTOR: mammalian target of rapamycin

M-VST: multivirus specific T cell

NAbs: naturally occurring antibodies

NFAT: nuclear factor of activated T cells

NGS: next-generation sequencing

NHEJ: non-homologous end-joining

NK cell: natural killer cell

NTM: non-tuberculous mycobacteria

OI: opportunistic infection

PALE: post-transplant acute limbic encephalitis

PAM: protospacer associated motif

PBMC: peripheral blood mononuclear cell

PCNSL: primary central nervous system lymphoma

PI3K: phosphoinositide 3-kinase

PKC-θ: protein kinase C- θ

PLC: peptide-loading complex

PLC-γ: phospholipase C-γ

PML: progressive multifocal leukoencephalopathy

pSMAC: peripheral supramolecular activation complex

pTα: pre-T-cell α chain

PTLD: post-transplant lymphoproliferative disease

RIS: reduction of immunosuppression

RNP: ribonucleoprotein

ROS: reactive oxygen species

S1PR: spingosine-1-phosphate receptor

SCID: severe combined immunodeficiency

sgRNA: small guide RNA

shRNA: short hairpin RNA

siRNA: short interfering RNA

SNX5: sorting nexin 5

SOT: solid organ transplant

SPF: specific pathogen free

TALEN: transcription activator-like effector nuclease

TAP: transporter associated with antigen processing

T_{CM}: T central memory cell

TCR: T cell receptor

TdT: terminal deoxynucleotidyl transferase

T_{EM}: T effector memory cell

T_{EMRA}: T effector memory cell re-expressing CD45RA

TGF-β: transforming growth factor-β

T_{EX}: exhausted T cell

TF: transcription factor

TIDE: tracking of indels by decomposition

TIL: tumour infiltrating lymphocyte

Th: T helper cell

TKO: triple knockout

TLR: Toll-like receptor

TNF: tumour necrosis factor

tracrRNA: trans-activating crRNA

T_{RM}: T resident memory cell

T_{SCM}: T memory stem cell

VST: virus specific T cell

VZV: Varicella-Zoster virus

WASp: Wiskott-Aldrich syndrome protein

WT: wild-type

ZFN: zinc finger nuclease

To DT, MZ, FV, JB, and HH, the patients who inspired me to pursue a research career.

Acknowledgements

I must begin by acknowledging my supervisors Drs Jean-Sébastien Delisle and Nathalie Labrecque. Their guidance and mentorship were instrumental to my development as a scientist, and I am truly grateful. I must also acknowledge my laboratory colleagues. From the Delisle laboratory: Dr. Sabrina Guettouche, who provided me with my initial training in the laboratory, and Marie-France Aubin, Cedric Carli, Gabrielle Boudreau, and Valerie Janelle who helped me immensely with the technical aspects of my project. I am also thankful to Ali S'maani, Caroline Rulleau, Leila Jafarzadeh, and Fateme Shahbeiki for their ideas and thoughtful discussions. Semester student Elisa Bchara and stagiaire Lisa Weir performed some of the experiments contained in this work under my supervision.

From the Labrecque laboratory: Jean-François Daudelin who provided me with much assistance with designing my molecular assays, Livia Odiagiu who helped me understand NR4A family biology, Dave De Souza who taught me how to work with mice, Salix Boulet, Laure Le Corre, Sarah Mezrag, Moustafa Nouh, Eric Sonounometo, Roxann Hetu-Arbour, and Mathilde Dumas who provided many excellent ideas to improve my project. I am also thankful to Fred Duval, Anne-Marie Aubin, and Martine Dupuis from the flow cytometry platform.

I also wish to thank my mentors and supporters from McGill, especially Drs. Marcel Behr, Jerry Zaharatos, Makeda Semret, Vivian Loo, and Nathalie Johnson. I am also fortunate to count on the support of my wife, Dr. Victoria Korsos, and parents, Dr. Morris Schweitzer and Diana Schweitzer.

Finally, I must acknowledge the financial support that I received for this project from the *Fonds de recherche du Québec – Santé* (salary support through the *Programme de formation en recherche pour les résidents en médecine spécialisé intéressés par une carrière en recherche*) and the Canadian Institutes of Health Research and Richard and Edith Strauss Foundation (funding of operating costs of research).

Chapter 1 – T Cell Development, Memory Differentiation, and Exhaustion

All organisms face constant threats from outside and within. The immune system is often seen uniquely as a constant sentinel against the threat of external microorganisms invading the host, with the most severe primary immunodeficiencies such as SCID resulting in infant mortality from normally innocuous commensal organisms without rapid diagnosis and treatment (1). However, the immune system is being increasingly recognized as vital to protecting the host from internal threats, especially cells undergoing neoplastic transformation. Importantly, the immune system itself can go rogue and cause inflammatory, autoimmune, and allergic diseases itself, thus it must be subject to stringent safeguards to prevent both overexuberant responses to threats and inappropriate responses against healthy tissues. The intricacies of this system lead us to study the science of immunology.

At its most basic, the skin and mucosal barriers are the organism's first line of defense, establishing a physical barrier between contaminated surfaces exposed to the environment and sterile tissues. Breakdowns in these barriers due to trauma, disease, or treatments can allow translocation of organisms into tissues and organ spaces resulting in localized or extensive infections and even life-threatening infections (2, 3). The next line of defense is the innate immune system. This arm of the immune system targets patterns common to many invading microorganisms without necessarily adapting to specific antigens. It has both humoral and cellular components. Among the humoral components, the complement system is the best studied, but also include natural occurring antibodies (NAbs), low affinity antibodies produced independently of antigen exposure that bind pathogens, providing additional opsonization and complement activation, and pentraxins, serum proteins that bind to a wide range of pathogens providing similar functions to NAbs (4). Finally, defensins are peptides produced within mucosae with multiple antimicrobial functions (5).

The cellular component of innate immunity primarily consists of phagocytes, cells that endocytose and destroy microbes and cellular debris. Importantly, they also bridge the innate and adaptive responses with some acting as antigen presenting cells (APCs) and expressing co-stimulatory molecules. These cells are of myeloid origin and recognize their targets through receptors that recognize microbial-associated molecular patterns (MAMPs) and opsonins. Macrophages reside in tissues and can be augmented by circulating monocytes that transpose into the tissues and derive into macrophages during an inflammatory response. These produce inflammatory cytokines that augment the immune response. Dendritic cells are tissue resident cells that act as specialized APCs; transporting internalized antigens to secondary lymphoid tissues to present to the adaptive immune system. Circulating neutrophils infiltrate massively into tissues during an inflammatory response due to the secretion of pro-inflammatory chemokines and cytokines to provide effective phagocytosis of pathogens on a large scale (6). After internalization, phagosomes bind to lysosomes to form the phagolysosome, which acquires digestive enzymes, acidifies, and produces reactive oxygen species (ROS) to destroy ingested microbes (6).

The other cellular component are the innate lymphoid cells (ILCs). Of these, the most studied are the natural killer (NK) cells. These recognize a balance of activating and inhibitory receptors on the surface of cells that can suggest a stress state such as malignancy or infection. One of the key inhibitory receptors is major histocompatibility complex (MHC)-I, whose absence is referred to as missing self and induces the NK cell to kill the target cell via apoptosis (6). This mechanism is key for host defense against intracellular infections that downregulate MHC-I to avoid antigen recognition by the adaptive arm of the immune system; patients with inherited NK cell deficiencies are particularly susceptible to herpes- and papillomavirus infections that exploit this mechanism (7). Other ILCs include the group 1, 2, and 3 ILCs that are tissue resident and produce cytokines to steer T helper cells towards type 1 (Th1), Th2, or Th17 differentiation, respectively (6).

While the innate arm recognizes patterns associated with microbes or cell stress, the adaptive immune system can specifically differentiate self from non-self-antigens. Rather than being stably encoded, the antigen-recognition (variable) domains of its receptors are produced by random recombination of an individual's unique genetic elements, resulting in a much broader antigen-recognition capacity. Small numbers of cells specific for a specific antigen circulate in a naïve state, which undergo massive clonal expansion if they encounter said antigen. The adaptive immune system is also characterized by the development of immunological memory, long-lasting cells that will produce a stronger and accelerated response if the antigen is reencountered. Central and peripheral tolerance mechanisms are critical to avoid autoimmunity, an inappropriate response to self-antigens that can cause disease. The principal actors of the adaptive immune system are of lymphoid origin: B cells (derived from the bone marrow) that produce antibodies and T cells (that undergo a thymic stage of development) with diverse functions depending on the subset. Initial antigen recognition primarily occurs in secondary lymphoid organs which are histologically organized so that APCs, B cells, and T cells can encounter each other in a way that effectively initiates the adaptive response.

Both B cells and T cells originate from a common lymphoid progenitor (CLP). B cells are so named because they complete their development in the bone marrow, as opposed to T cells that have a thymic stage in their development. Their primary role is to produce antibodies. Antibodies neutralize microbial exotoxins, adhesion factors, and receptor ligands that allow entry into host cells, provide opsonisation facilitating recognition and endocytosis by phagocytes via Fc receptors, and activate the complement cascade. On the other hand, T cells undergo much of their development within the thymus. These cells are characterized by the presence of the T cell receptor (TCR) on the surface, a heterodimeric structure that recognizes peptides presented by compatible MHC. Most mature T cells possess a TCR composed of an α and a β chain (so called αβ T cells) associated with a CD4 or CD8 coreceptor, which are functionally distinguished as T helper (Th) cells or cytotoxic T cells. Other populations of mature T cells remain positive for both CD4 and CD8 (so called double-positive T cells) or possess a TCR composed of a γ and a δ chain (so called γδ T cells) (6). This is explored in detail below.

T Cell Development

Like all lymphocytes, T cells originate from a CLP within the bone marrow early in hematopoiesis. However, T cell progenitors leave the bone marrow and migrate hematogenously to the thymus. The thymus is composed of a stroma of epithelial origin, developing T cells (thymocytes) and dendritic cells derived from hematopoietic stem cells. At this early stage, T cells are known as double negative (DN) cells as they have not yet developed and do not display their TCR, and thus do not display the coreceptors CD4 or CD8. Developing T cells enter at the junction of the outer cortex and inner medulla of the thymus, undergo TCR gene rearrangement within the cortex, then complete their development in the medulla before being released to the periphery. The thymus is a larger, well-developed organ during childhood but undergoes significant atrophy during puberty; how T cells continue to be produced in adults to maintain homeostasis despite this atrophy remains unclear, but is likely dependent on the existing pool of mature T cells (6).

The TCR is composed of a heterodimer of two chains, α and β. The β is the first to develop in DN thymocytes through rearrangement of the T cell receptor β chain (TRBC) gene through the action of the RAG recombinase and non-homologous end-joining (NHEJ). A TCR is expressed on the surface by paring this β chain with an invariant placeholder pre-TCR α (pTα). At this stage, the thymocyte acquires surface expression of both CD4 and CD8 coreceptors, becoming a double-positive (DP) thymocyte. The α chain is then synthesized and replaces pTα so that the cell expresses a complete TCR. Thymocytes then interact with MHC molecules displayed by thymic cortical epithelial cells to establish whether the TCR can interact with self MHC; if it successfully binds self MHC, T cell receptor α chain (TRAC) gene rearrangement also ceases, and the cell receives survival signals. If not, α chain rearrangement continues until TCR:MHC interaction occurs. If no TCR that binds self MHC is produced, the thymocyte dies by neglect. This process of positive selection ensures that despite the random nature of TCR recombination, T cells are nonetheless able to bind self MHC and recognize the peptides that they display. The MHC molecule that the TCR binds to also influences the single coreceptor that it will express; thymocytes that successfully bind MHC-I become CD8 single-positive cells, while those that bind MHC-II become CD4+ cells (6).

After positive selection, thymocytes undergo negative selection to eliminate cells strongly recognizing autoantigens presented by self MHC molecules. This process is critical to central tolerance to avoid autoimmunity. First, double-positive cells that too strongly recognize self MHC are eliminated in the cortex. Once thymocytes become single-positive, they migrate to the medulla where self-antigens are displayed by medullary thymic epithelial cells (mTECs) and dendritic cells (DCs). However, since many proteins are tissue-specific, not all self-antigens would be expected to be expressed within the thymus and thus potentially autoreactive T cells would escape negative selection. mTECs express the autoimmune regulator (AIRE) gene that induces expression of tissue-specific antigens that would otherwise not be found within the thymus, thus allowing thymocytes to be exposed to these antigens and autoreactive cells to be eliminated (6). A recently described alternative mechanism is the expression of lineage-defining transcription factors of extrathymic tissue cells to produce cellular mimics within the thymus (8).

The fate of thymocytes during positive and negative selection depends essentially on the strength with which they recognize self MHC and peptides. If the recognition is too weak, they fail positive selection, while if it is too strong, they undergo negative selection, both resulting in death through apoptosis. Cells that survive are those that recognize self MHC with just the right amount of TCR signaling. Cells that bind self-peptide with higher affinity, but not such strong affinity that they are eliminated, may become thymic T regulatory (Treg) cells that play a role in peripheral tolerance (6).

At the end of their thymic stage, mature naïve T cells that have survived positive and negative selection express the chemokine receptors sphingosine-1-phosphate receptor (S1PR), that attracts them to leave the thymus through lymphatic vessels and venules, and CD62 ligand that guides them towards lymphatic tissues to begin patrolling for foreign antigen (6).

T Cell Activation

Naïve T cells continue to patrol secondary lymphoid tissues, interacting with APCs and searching for the antigen they recognize. DCs are particularly efficient at antigen uptake, transport to

secondary lymphoid tissues, and presentation to T cells. Upon recognition of its cognate antigen, the T cell undergoes extensive phenotypic and functional changes known as activation (6). Bystander CD4+ T cells can also proliferate and produce effector cytokines through TCR-independent activation in the setting of active infection and an inflammatory cytokine milieu, but these will not undergo complete activation will not produce immunological memory (9).

Antigen Presentation

Essentially, two types of MHC present antigen: MHC-I is present on all cells and presents intracellular antigen to CD8+ T cells, while MHC-II is present on APCs and thymic epithelial cells and presents antigens derived from phagocytosis to CD4+ T cells. During periods of stress and inflammation, surface MHC is upregulated to ensure efficient antigen presentation to defend against infection. The structure of MHC is highly polymorphic, but carried on the same chromosome, so one MHC haplotype is inherited from each parent. Recognition of non-self MHC-I is the principal reason for alloreactivity or T cell mediated rejection of organ transplantation or graft versus host disease against cells or tissues harvested from a non-compatible donor (6).

All cells present peptides produced by degradation of cytosolic proteins via MHC-I to ensure that cells infected with intracellular microbes are efficiently detected and destroyed. Small peptides that result from protein degradation are transported to the endoplasmic reticulum (ER) where they are loaded onto MHC-I molecules for surface display to CD8+ T cells, thus allowing immune detection of virally infected cells (6(6).

Professional APCs, on the other hand, display via both MHC-I and MHC-II. While DCs are the most efficient APCs, macrophages and B cells also display MHC-II to CD4+ T cells. After an organism or cellular debris is phagocytosed, peptides resulting from proteinolysis are loaded onto MHC-II and displayed on the surface. These cells also perform cross-presentation, transferring peptides from the phagolysosome to the cytosol to be presented on MHC-I. This ensures that uninfected DCs can still activate CD8+ T cells, while simultaneously activating CD4+ T cells to support the response. Furthermore, this ensures that organisms that survive endosomal uptake and escape to the cytosol cannot fully escape immune recognition (6).

TCR Activation and Post-TCR Signaling: the "First Signal"

Effective TCR interactions with MHC require the coreceptor CD4 or CD8, which bind to MHC-II or MHC-I, respectively, and stabilize the interaction. Most interactions will, of course, not result in T cell activation, but help maintain survival. However, if the TCR stably binds to MHC displaying its cognate antigen, it unleashes a signalling cascade within the cell leading to activation (6).

TCR exists in a complex with CD3, which itself is a complex of three dimers: δε and γε heterodimers on the surface, and a ζ homodimer sitting internally below the TCR. These possess cytoplasmic immunoreceptor tyrosine-based activation motif tails (ITAMs) that act as a scaffold for post-TCR signalling. TCR binding to its cognate antigen results in a conformational change in CD3 components that exposes the ITAMs, which undergo phosphorylation by the kinase Lck. Phosphorylated ITAMs then bind to the kinase ZAP-70, bringing it into proximity with Lck as well, which phosphorylates it and activates its kinase domain to phosphorylate downstream kinases. This cascade then results in the activation of PI3K and multiple downstream signalling pathways which lead to release sequestered calcium from the ER and influx of extracellular calcium. This increased intracellular calcium is sensed by calmodulin, which binds to and activates calcineurin, which in turn dephosphorylates the nuclear factor of activated T cells (NFAT). By dephosphorylating NFAT, calcineurin releases it from the cytoplasm and allows it to be transported into the nucleus to act as a transcription factor, transcribing genes that are essential to T cell activation (6).

A second signalling pathway activated by TCR binding is the Ras-mitogen-associated protein kinase (MAPK) pathway. This pathway leads to the transcription of the transcription factor Fos, which then binds to Jun to form the heterodimeric transcription factor activator protein 1 (AP-1), which itself is activated through the MAPK cascade through Jun phosphorylation by Jun kinase (JNK). JNK can also be phosphorylated through the protein kinase C-θ (PKC-θ). AP-1 also transcribes genes that are essential to T cell activation (6).

The PKC-θ pathway also activates the NFκB family of transcription factors, which along with NFAT and AP-1 is required for T cell activation. Essentially, membrane-associated PKC-θ assembles and activates a polyubiquitination scaffold called IKK that ubiquitinates the inhibitor IκB, which is associated with NFκB in resting T cells. This ubiquitination causes the degradation of IκB, thus releasing NFκB from the cytoplasm and allowing it as well to travel to the nucleus to participate in transcription and thus T cell activation. In fact, unless the three transcription factors NFAT, AP-1, and NFκB are all active, IL-2 transcription will not occur (6).

Finally, the post-TCR cascade recruits Akt, which has multiple effects to reprogramme cellular processes to support T cell activation. Firstly, it promotes T cell survival by freeing Bcl-2 to inhibit apoptosis. Secondly, it modifies surface receptors to promote T cell migration to sites where effector functions are required. Finally, it modifies cellular metabolic processes to support the significant increase in energy requirements required for clonal proliferation and effector functions that result from activation. It both increases glycolytic pathways to provide energy to the cell and activates the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway increases synthesis of lipids, nucleotides, and amino acids which are consumed during the increase in transcription, translation, and eventual cell division that occur during T cell activation (6).

It is vital to understand these pathways given their implications in multiple facets of this work. The NR4A family interacts with some of the transcription factors discussed previously in this section and their transcription is thought to be activated by post-TCR signalling (see below for a detailed description). Similarly, the opportunistic infections that occur in the post-transplantation setting result mainly from iatrogenic T cell immunocompromised from pharmacological inhibition of these activation pathways. The calcineurin inhibitors tacrolimus and cyclosporin inhibit NFAT-activation and thus transcription of NFAT-target genes, while mTOR inhibitors impede the metabolic reprogramming that occurs through the Akt pathway. This will be further explored in chapter 3.

Co-Stimulation: the "Second Signal"

APCs that are activated by pro-inflammatory cytokines produced by components of the innate and adaptive immune systems, as well as by recognition of PAMPs by TLRs, upregulate co-stimulatory ligands on their cell surface. These co-stimulatory ligands then bind to receptors on the T cell, leading to productive T cell activation. Naïve T cells must bind their cognate antigen and a co-stimulatory molecule on the same APC to be activated. CD28 signalling is indispensable for T cell activation. TCR activation in the absence of co-stimulation, however, leads to anergy, an important component of peripheral tolerance. A T cell in an anergic state is no longer able to respond to TCR stimulation, thus avoiding inappropriate activation to self-antigens in the absence of an appropriate inflammatory response (6).

CD28 is a co-stimulatory receptor that is constitutively expressed by resting T cells and binds to B7.1 or B7.2 (CD80 or CD86) on APCs. It is then phosphorylated by Lck and increases PI3K activation and thus PIP_3 production. Thus, as discussed previously, it reinforces NFAT-mediated and NFκB-mediated effects, as well as the anti-apoptotic and metabolic changes that occur through the Akt pathway. It also increases IL-2 production by stabilizing its mRNA and reorganization of cellular polarity by reorganizing the cytoplasm (discussed further below). Additionally, CD28 co-stimulation induces expression of the high-affinity IL-2 receptor, which in turn allows the cell to enter mitosis, thus allowing clonal expansion in response to IL-2 signalling. Another co-stimulatory receptor in the CD28 family, inducible co-stimulator (ICOS), binds to ICOS ligand (ICOSL), and promotes proliferation, expression of non-IL-2 cytokines, and differentiation of Th cells into T follicular helper (Tfh) cells that are necessary for B cell activation and class switching (6).

Tumour necrosis factor (TNF) family receptors also act as co-stimulatory receptors, but their role and function are less well understood than that of the CD28 family. These are not expressed on naïve cells and are thus not required for activation, but reinforce the effector response. These receptors seem to exert their effect through the non-canonical NFκB pathway, in which they interact with TNF receptor-associated factors (TRAFs) to ubiquitinate and degrade IκB, thus

activating NFκB. CD40L is upregulated on activated T cells and binds to CD40 on APCs, which in turn upregulates co-stimulatory ligands, thus providing a positive feedback loop for T cell activation. 4-1BB and OX40 are both co-stimulatory receptors of the TNF family that are present on T cells and which respond to ligands displayed by APCs; these both increase T cell proliferation (6).

Formation of the Immunological Synapse

T cell activation results in cellular polarization. The actin cytoskeleton reorganizes itself through the influence of Vav and the Wiskott-Aldrich syndrome protein (WASp) to establish T cell polarity towards the APC or target cell. It also results in upregulation of adhesion molecules, particularly lymphocyte function-associated antigen (LFA)-1 that binds to the intercellular adhesion molecule (ICAM)-1 to maintain cell-cell contact and allow for stable MHC:TCR and co-stimulator ligand:receptor interactions. The synapse is further organized into the peripheral supramolecular activation complex (pSMAC), where LFA-1 is concentrated, and central SMAC (cSMAC), where TCR, coreceptors, and co-stimulatory receptors are concentrated. TCR signalling is further strengthened by the organization of MHC-bound TCRs into microclusters, thus concentrating their effects on downstream signalling pathways. Furthermore, CD45, a regulatory dephosphorylase that inhibits TCR signalling, is excluded from the cSMAC. That said, TCR is endocytosed and degraded within the cSMAC to avoid overstimulation. The polarization of activated T cells also plays a role in effector functions as it directs cytokine and cytotoxic granule secretion towards the target cell (6).

Cytokines: the "Third Signal"

Cytokines are protein messengers produced by diverse cell types and which can act locally or at a distance. Locally acting cytokines act either on the cell itself (autocrine signalling) or on adjacent cells (paracrine signalling); they are often secreted in a polarized fashion across the immunological synapse. IL-2 reinforces T cell survival and proliferation after activation, with both IL-2 production and upregulation of its receptor being early and robust markers of T cell activation. Other cytokines influence T cell differentiation after activation (discussed in detail in the subsequent sections). Still yet, cytokines can have distant effects on hematopoiesis, resulting

in increased production of precursor cells, production of acute phase reactants, fever, and vascular permeability (6).

T Cell Effector Functions and Differentiation

Productive activation of a naïve T cell leads to both clonal expansion and differentiation into an effector phenotype. The changes that occur transform the T cell from a quiescent, patrolling cell to a cell that can rapidly hone towards and combat infection. Clonal expansion further increases the number of T cells available to accomplish effector functions. While most effector T cells will die by apoptosis in the contraction phase once the infection is cleared, a minority will differentiate to long-lasting memory cells that continue to provide protection in case the antigen is reencountered in the future (6).

Phenotypic Changes During Early T Cell Activation

Effector T cells must leave the secondary lymphoid organs to migrate to infected tissues. Many of the surface phenotypic changes occur to permit the T cell to accomplish this transition of tissue niche. Activated T cells first upregulate CD69, which retains them within secondary lymphoid tissue to complete their activation, then downregulate the sphingosine-1-phosphate receptor (S1PR), trapping them within lymphoid tissues to complete effector cell differentiation and undergo an initial clonal expansion. These cells further lose expression of CD62L and CCR7, two chemokine receptors that lead naïve cells to hone to lymph nodes, thus allowing them to traffic to and remain in non-lymphoid tissues. Thus, presence or absence of these markers can be used to differentiate naïve from effector T cells. Activated T cells also upregulate expression of integrins that allow them to bind to activated vascular endothelium and exit the bloodstream to enter inflamed tissues (6).

Cytotoxic (CD8+) T Cell Activation and Effector Functions

Cytotoxic T cells are responsible for killing cells infected with intracellular infections, particularly viruses. Upon recognizing foreign antigen displayed on MHC-I, these cells are activated to destroy the target cell. As a result, they have great potential for tissue damage if inappropriately activated by autoantigens. Thus, these cells must undergo an initial activation event with strong co-stimulation by a professional APC and differentiate into effectors before being able to perform

this task. This is aided by T helper (CD4+) cells that interact with APCs through the immunological synapse and cytokine production to activate them and thus increase their expression of co-stimulatory ligands. While some pathogens can evoke a robust CD8+ effector response in the absence of reinforcement by CD4+ cells, the majority do require both to elicit at least an effective initial response (6).

Effector cytotoxic T cells limit tissue inflammatory damage by killing targets through apoptosis rather than necrosis. Rather than releasing intracellular contents into the cytosol, thus potentially causing greater inflammation, and disseminating intracellular infections, this results in orderly and contained cell death with cellular fragments and debris that can be cleared by phagocytosis. Apoptosis also involves the activation of nucleases that degrade microbial nucleic acids, thus further minimizing spread of viral infections (6).

Cytotoxic T cells induce apoptosis through one of two methods: either expression of Fas ligand (FasL), which engages Fas on target cells and activates the extrinsic pathway of apoptosis, or secretion of pre-formed cytotoxic granules that activate the intrinsic pathway. These granules contain perforin, which permeabilizes the vesicle membrane to allow granule contents to escape towards the cell, and granzymes that enter the target cell via mannose receptor endocytosis and activate caspases and release cytochrome c from the mitochondria, thus activating the intrinsic pathway of apoptosis. Also, cytotoxic T cells also produce effector cytokines, namely IFN-γ, TNF-α, and IL-2, that activate innate intracellular defenses against viruses, activate surrounding macrophages, induce apoptosis, and enhance T cell expansion and activation (6).

Understanding cytotoxic T cell effector functions is essential to understanding this work. The common principle of cell therapies is to redirect cytotoxic T cells to destroy target cells, be they cancerous or infected, through this highly effective killing process.

T Helper (CD4+) Cell Subsets and Effector Functions

T helper cells accomplish their effector functions by recruiting and activating other components of the immune system, thus tailoring the immune response to the infection at hand. This is

primarily accomplished through direct interaction between ligands and receptors across the immunological synapse, as well as secretion of cytokines. The cytokines produced, and thus the effects that they have on other effector cells of the immune system, depend on the subset of the T helper cell. These subsets differentiate based on the cytokines, produced by components of the innate immune system, that provide signals of the type of pathogen that has been encountered (see table 1) (6).

Subset	**Transcription factor**	**Stimulatory cytokine(s)**	**Effector cytokine(s)**	**Function**
T_H1	T-bet	IL-12, IFN-γ	IFN-γ	Defense against intracellular organisms
T_H2	GATA3	IL-4	IL-4, IL-5, IL-13	Defense against helminths Allergies
T_H17	RORγT	TGF-β, IL-6	IL-17, IL-22	Mucosal defense
iTreg	FoxP3	TGF-β	TGF-β, IL-10	Peripheral tolerance
T_{FH}	Bcl-2	IL-6?	IFN-γ, IL-4, IL-17, IL-21	B cell co-stimulation and class switching

Tableau 1. – CD4+ T cell subsets

Naïve CD4+ T cells differentiate into T_H1 under the influence of IFN-γ and IL-12. Their role is to activate macrophages to more effectively kill intracellular pathogens that persist within phagocytes by inducing classical (M1) activation; if pathogens cannot be eliminated, they coordinate the formation of granulomas to wall off viable organisms (6). In their absence, the organism is susceptible to multiple infections; this became apparent during the emergence of the HIV/AIDS pandemic and in the context of iatrogenic immunosuppression to treat autoimmune disease and prevent rejection of organ transplants (discussed in detail in chapter 3).

T_H2 differentiation is favoured by IL-4, produced by type II ILCs, eosinophils, basophils, mast cells, and other T_H2 cells. These cells programme the immune response to combat multicellular helminths, but also cause allergic disorders. They also favour class switching towards IgE in activated B cells. These cells produce IL-4, which recruits and activates mast cells and basophils, IL-5, which recruits eosinophils, and IL-13, which stimulates mucus production and smooth muscle contraction. Together, these effector functions provide an effective antihelminitic response and favour expulsion of the worm. IL-4 and IL-13 further influence macrophages to differentiate into an alternatively activated (M2) state that helps to repair tissue after the inflammatory insult and worm transmigration (6).

T_H17 cells require both IL-6 and transforming growth factor (TGF)-β signalling to develop. Cells also upregulate the receptor for IL-23, which is also required for continued development. These cells coordinate defense against extracellular bacteria and fungi, particularly at mucosal barriers. These cells are so named because they produce IL-17, as well as IL-22. These cytokines induce mucosae to produce antimicrobial peptides and increase turnover to decrease microbial adhesion. IL-17 also drives production and release of neutrophils from the bone marrow and recruitment to sites of infection by inducing production of chemoattractant chemokines. They also directly produce the chemokine CCL20 that recruits further T_H17 cells to sites of infection. (6).

T cells that receive TGF-β stimulation without IL-6 differentiate into induced T regulatory (iTreg) cells rather than T_H17 cells. These cells constitutively express the transcription factor FoxP3 and surface IL-2R and have an anti-inflammatory role by producing the suppressive cytokine IL-10. They also produce TGF-β to further induce iTreg differentiation. Finally, T follicular helper (T_{FH}) cells hone to B cell follicules of secondary lymphoid tissues and provide co-stimulatory and cytokine stimulation of B cells to allow for class switching and an effective B cell response (6).

Some pathogens need cooperation between T cell subsets or even conversion of cells from one subset to another to respond to different phases of infection. For example, a pathogen may transit from an extracellular phase to one that survives within phagocytes, thus requiring an initial T_H17 response and a subsequent T_H1 response. Thus, some CD4+ T cell subsets can demonstrate plasticity in which one cell type can differentiate into another. As in the example above, this is best described in T_H17 to T_H1 reprogramming of the same clonotype. Some antigens may also elicit different T cell subsets, with antigens associated with mucosal adhesion eliciting a more T_H17 response, while those associated with persistence within phagocytes eliciting a more T_H1 response (6).

T Cell Memory

During an acute infection, there is a significant clonal proliferation of effector cells targeting the pathogen. If these cells continued to proliferate unabated after the infection has resolved, this would lead to chronic infection and eventually clonal lymphocyte disorders such as leukemia and lymphoma. This would also continue to use valuable resources that would not allow the organism to thrive, or the immune system to respond to future threats. Thus, once the infection has resolved, most cells must die by apoptosis during the contraction phase of the response, usually within 30 days of the initial infection. This is, of course, different in chronic infections and cancer, and this will be discussed in detail in the following section on T cell exhaustion (6).

Despite this contraction, it is advantageous for antigen-specific cells to persist to defend against a future encounter with the pathogen. This produces a higher relative frequency of antigen-specific T cells compared to naïve cells, thus increasing the surveillance for pathogen-specific antigens. These cells no longer require co-stimulation as they have already encountered antigen, and thus can provide far more rapid and robust effector responses than naïve cells, which must go through the activation process described above, with the first effector responses taking at least 4 days to appear. This immunological memory is a distinguishing characteristic of the adaptive immune system and will be explored in detail (6).

Models of Memory Cell Differentiation

Much of what is known about memory T cell differentiation after a primary immune response has been learned from studies of mouse models. These provide many advantages to the *in vivo* study of immunological memory: mice are mammals with significant homology to humans, are small and relatively inexpensive to house, have a short gestation period and rapidly reach sexual maturity, can be experimentally infected with well-studied model infections, and tissue samples are easy to obtain after euthanasia (10). Inbred mouse lines with genetic homogeny are readily available, as are genetically-modified mice with gene knockouts (KO) or knock-ins (KI). (11).

Despite these advantages, data from mouse experiments can be difficult to extrapolate to human biology. While there is significant homology between mice and humans, there are still enough phenotypic differences such that what occurs in mice may not necessarily occur in humans. As well, mouse experimental models are often not physiologic in terms of the effect of a KO or overexpression of a protein (rather than physiologic up and down regulation) and the use of artificial experimental models. Caution is particularly necessary when studying immunological memory, as laboratory strains of mice lack genetic diversity and are raised in specific pathogen free (SPF) facilities, leading to a more naïve T cell repertoire. In fact, when laboratory mouse strains are raised with “dirty” mice purchased from pet stores, results from mice raised in SPF facilities may not be reproducible (10, 12).

Bearing in mind the limitations above, many of the most significant discoveries in mammalian immunology were made using murine models (10). An important model for studying memory is the adoptive transfer of T cells bearing a transgenic TCR into syngeneic mouse strains (made possible by the availability of inbred laboratory mouse strains). Donor mice are engineered to have a single transgenic TCR so that the entire T cell repertoire recognizes a single epitope. Adoptively transferred cells can then be differentiated from native donor T cells using tracking dyes or through differences in surface markers, such as isoforms of CD45, that can be detected using antibodies and analyzed with flow cytometry (13).

CD8+ memory T cell differentiation can be studied using experimental infection with intracellular pathogens. One of the most used models is adoptive transfer of OT-I cells, with a transgenic TCR that recognizes the ovalbumin peptide SIINFEKL presented by compatible MHC-I, into recipients infected with *Listeria monocytogenes* engineered to express ovalbumin. Acute infection with *L. monocytogenes* induces a robust CD8+ T cell response and resulting memory cells can be followed longitudinally. Recipient mice can also be rechallenged after initial infection to study CD8+ T cell secondary responses. Another frequently used model is infection with lymphocytic choriomeningitis virus (LCMV), an arenavirus and common mouse pathogen. Mice, known as P14 mice, have been bred with a transgenic TCR that recognizes the LCMV glycoprotein peptide KAVYNFATM (gp_{33-41}) (11). Polyclonal responses to LCMV infection can also be studied with tetramers. An advantage of using LCMV as a model organism is that it exists in two strains, Armstrong and clone 13, that differ by only 2 amino acids, yet cause acute and chronic infection, respectively (14). Thus, this experimental model can be used to study the different memory response that occurs between acute and chronic viral infections.

Using these models, two main different populations of effector cells have been identified in mice during the acute phase of an infection. Short lived effector cells (SLECs) comprise most antigen-specific T cells during the acute response but die by apoptosis by day 30. Memory precursor effector cells (MPECs) only represent approximately 5% of cells during the acute response but go on to differentiate into long-lived memory cells. These can be differentiated using KLRG1 and CD127 expression. KLRG1, a marker of activation, is expressed by SLECs but not MPECs, whereas CD127, a component of the IL-7 receptor, is expressed by MPECs but not SLECs (15-17). While IL-7 is required for maintenance of memory T cell populations, IL-7 receptor expression does not rescue KLRG1 cells from apoptosis (18). Also, some $KLRG1^{lo}/CD127^{hi}$ cells may still die during the contraction phase, while some $KLRG1^{hi}/CD127^{hi}$ cells can persist. This suggests that there is more to cell fate than surface marker expression, and that there may be some plasticity during the acute phase of the primary immune response. Other surface markers can also be seen, with MPECs expressing CD27 and CXCR3 (19).

The fate decision that skews differentiation towards a SLEC or MPEC phenotype seems to occur from the first TCR engagement of a naïve T cell (20). Stronger TCR signalling, ratio of co-stimulatory to inhibitory receptor (notably PD-1 and Tim-3, described in detail below) engagement, and continued antigen exposure later during the course of the infection favour SLEC rather than MPEC differentiation (15, 20-22). During early activation, transcription factors (TFs) known as pioneer transcription factors, such as, interferon regulatory factor (IRF) 4, BATF, Runx3, T-bet, and BLIMP-1 induce epigenetic changes to allow for subsequent transcription of transcription factors that programme effector or memory differentiation (15, 19, 23-27). Stronger TCR signalling induces stronger IRF4 activity, which increases transcription of transcription factors favouring effector functions and SLEC differentiation, while weaker IRF4 activity favours transcription of eomesodermin (Eomes) and TCF1 that skew towards MPEC differentiation (25). Transcription factors of the AP-1 family are also induced by stronger TCR signalling and favour SLEC differentiation; BACH2 blocks transcription of AP-1-targeted genes and thus favours MPEC differentiation (24). BLIMP-1 serves as a master regulator of terminally differentiated effector cells, and is essential for the epigenetic changes associated with this fate (23).

The cytokine milieu also influences memory differentiation, with more inflammatory signalling favouring SLEC differentiation. IL-12 most consistently, but also IFN-γ, and type I interferons, upregulate expression of T-bet and Blimp1, which favour SLEC differentiation, and downregulate Bcl6, Eomes, TCF-1 and IL-17Rα, which favour survival and MPEC differentiation (15, 28-31). In contrast, IL-10 and IL-21 upregulate Bcl6, Eomes, and SOCS3 to promote memory differentiation. IL-15 signalling is also required for optimal early memory cell proliferation (32). The effect of IL-2 on memory differentiation is more complex, with IL-2 administration during clonal expansion decreasing memory cell formation but increasing memory cell persistence when administered during the contraction phase (33, 34).

The fate decision for a given early effector T cell thus depends on a balance of opposing transcription factors, with T-bet, Blimp1, ID2, and ZEB2 favouring SLEC differentiation, and Eomes, TCF1, ID3, and ZEB1 favouring MPEC differentiation (23, 35-37). Epigenetic changes in both SLECs

and MPECs downregulate pro-survival and anti-apoptotic factors that are expressed in resting naïve T cells, but memory T cells can reverse these epigenetic changes and reacquire expression of these transcription factors. Specifically, memory T cells reacquire expression of TCF1 and LEF1, which both can epigenetically silence the expression of Blimp-1, and FoxO1, which acts as a promotor for *IL7r*, *Bcl2*, *Sell*, *Ccr7*, *Eomes*, *Tcf7*, *Bach2*, *Zeb1*, and *Socs3* (37-39). Thus, MPECs are epigenetically reprogrammed towards a resting and pro-survival phenotype as they become memory cells, but SLECs are not and die by apoptosis (40). However, resting memory CD8+ T cells differ from naïve cells as genes encoding elements required for rapid effector function such as IFN-γ, granzyme B, and perforin remain available, allowing them to more quickly accomplish their effector function in case the antigen is reencountered (19).

While much of the fate of a given effector T cell depends on the strength of TCR signalling, transgenic TCR models allow for all the adoptively transferred TCRs to be identical and thus bind the same epitope with the same strength. Despite this, some cells will differentiate into SLECs and others into MPECs. How and why this occurs in single cells with identical TCRs is an area of active investigation, but two principal models have been proposed for both murine and human memory cell differentiation. The model of asymmetric division driven differentiation holds that cytokine receptors and signalling pathways are unequally divided among daughter cells during the first cellular division, and that this may favour pathways that bias towards SLEC or MPEC differentiation among the descendent generations of cells (41). The model of progressive differentiation, on the other hand, holds that cells progressively differentiate through MPEC states and that, based on the strength of TCR signalling and external stimuli like cytokines and co-stimulation, the majority become terminally differentiated SLECs (42). A more recent third model posits that naïve T cells may already be predestined to differentiate into SLECs or MPECs depending on their developmental origin (43). Using technologies that allow longitudinal tracking and observation of single cells, such as barcoding, single cell RNA sequencing, and single cell ATAC sequencing (to study the epigenetic landscape of a single cell), these models of single cell fate continue to be refined.

Human Memory T Cell Subsets

As memory precursors differentiate further into memory cells, they display the CD45 isoform CD45RO rather than CD45RA, as in naïve and effector T cells (with the notable exceptions of T_{SCM} and T_{EMRA} discussed further below). They can be further divided into subsets with distinct functional phenotypes. These subsets differ in their surface markers, tissue localization, and capacity for effector function (44). A key characteristic needed for persistent memory T cell pools in the long term with the capacity for effector functions is "stemness," meaning the ability to proliferate, undergo self-renewal, and retain multiple effector functions (45). This concept will be further developed below when discussing T cell exhaustion.

The main subsets of circulating T cells are T central memory cells (T_{CM}) and T effector memory cells (T_{EM}). T_{CM} act more like naïve T cells, displaying CCR7 and CD62L on their surface, patrolling through secondary lymphoid tissues, and, although they have less killing capacity and produce less effector cytokines on restimulation, they have greater proliferative capacity and polyfunctionality, hence more stemness (46). T_{EM}, on the other hand, act more like effector T cells, not displaying CCR7 and CD62L, but possessing integrins that allow them to enter and patrol through peripheral tissues. These also have higher levels of pre-formed perforin and granzyme, more cytotoxic capacity, and produce greater levels of cytokines (47, 48). Thus, T_{EM} are more effective at rapidly killing pathogens upon re-exposure in peripheral tissues but have less long-term persistence compared to T_{CM} and T resident memory cells (T_{RM}), which remain in tissues (49).

Two less common subsets of circulating memory T cells that display CD45RA also exist. T memory stem cells (T_{SCM}) retain many naïve-like characteristics (50). They display CCR7, CD62L, and CD27 like naïve cells, but also express CD95, CD58, CD11α, and CXCR3 in humans (51). They maintain the most stemness of T memory cell subsets, and therefore have much potential to be long-lasting and maintain polyfunctionality and cytotoxic potential. Like T_{CM}, they also circulate through secondary lymphoid organs rather than peripheral tissues (51). T_{EMRA}, however, are at the other end of the stemness spectrum. These cells regain expression of CD45RA but are $CD27^-$ and $CD62L^-$ /$CCR7^-$. This population is associated with aging and chronic viral infection, particularly

cytomegalovirus (CMV), and appears to be a more divided, senescent cell type. These cells retain the ability to produce IFN-γ on restimulation, but poorly proliferate (52, 53).

Based on DNA methylation analysis and adoptive transfer experiments, circulating T memory subset differentiation appears to occur linearly from the most stem-like to the least stem-like, going from T_{SCM} to T_{CM}, then T_{EM} (46, 54, 55). Given the telomere length and markers of senescence in T_{EMRA}, these are thought to be the final stage of T memory cell differentiation (56). This progression in differentiation is thought to be due to antigen exposure; with antigen reexposure, memory T cells continue to divide and differentiate, from more stem-like forms that hone to lymphoid tissues to more effector forms that migrate to inflamed peripheral tissues (46). This makes sense when one considers the different roles that T_{CM} and T_{EM} play. T_{CM} may be memory cells, but they patrol secondary lymphoid tissues like naïve cells waiting to encounter antigen (57). As soon as they do, like naïve cells differentiate into effector cells, they differentiate into T_{EM}, which are programmed to migrate to sites of infection and clear the infection (46). However, unlike naïve cells, they require neither co-stimulation nor time to differentiate into full-fledged effectors, thus fulfilling their memory role.

The prevalence of these subsets depends on the amount of time that has passed after the primary immune response. T_{EM} initially outnumber T_{CM} among circulating antigen-specific memory cells, but over the long term T_{CM} become more numerous as they retain better proliferative and autorenewal capacity (58). This provides both long-lasting protection against future antigen encounter and continued activity against chronic infections. Recurrent exposure to acute infection, however, boosts both numbers and diversity of T memory cells and skews the ratio back towards T_{EM} predominance in the short-term after re-exposure; this also leads to more effective protection against re-infection and is the rationale behind a booster vaccination strategy (59). A particular phenomenon seen in CMV-seropositive individuals is memory inflation, in which persistent low-level reactivation of latent virus throughout the lifetime leads to an increase in CMV-specific CD8+ memory cells, which can constitute up to 20% of the circulating CD8+ T cell

population in the elderly (60). Furthermore, T_{EMRA} cells occur in older CMV-seropositive individuals and are also thought to be due to chronic low-level reactivation of latent CMV (52).

T_{RM} are non-circulating memory T cells that reside in peripheral tissues. These exist in barrier tissues such as skin, tonsils, intestinal and vaginal mucosa, and the lungs, but also internal organs like the liver, spleen, lymph nodes, salivary glands, and brain (12). The existence of this cell type was demonstrated using parabiosis models, in which littermates are surgically attached to each other allowing non-circulating T cells found in one to migrate to the other (61). T_{RM} provide local effector responses against recurrent infection and, thus, their localization against specific antigens depends on the tissue tropism of the infection. For example, skin T_{RM} are important for protection against herpes simplex virus (HSV), while influenza-specific T_{RM} are found in the lungs, EBV-specific T_{RM} are found in the spleen and tonsils, and hepatitis B virus (HBV)-specific T_{RM} are found in the liver (62-67). Many T_{RM} express CD69 and CD103, although $CD69^-$ is dispensable for T_{RM} formation depending on the tissue, and lack S1PR expression, which retains them within tissues (68, 69). In mice, the transcription factor Hobit is required for T_{RM} differentiation but is not strongly expressed in human T_{RM} (70-72). Other transcriptional factors identified as favouring a T_{RM} phenotype are Blimp-1, Runx3, and the Notch signaling pathway; differentiation and maintenance of the T_{RM} repertoire in both mice and humans remains an area of active investigation (73-75).

T Cell Exhaustion

The sequence of memory T cell development as described previously primarily occurs in the context of acute infection or vaccination. However, studies of chronic infection of mice using specific LCMV strains (such as clone 13) allowed the study of what occurs in the context of chronic viral infection. Instead of memory cells, a population of exhausted T cells (T_{ex}) develops, with their own unique functional, transcriptomic, and epigenetic profile (76, 77). Further study identified several inhibitory receptors that serve to restrain post-TCR signaling and oppose costimulatory receptors and can be used to differentiate T_{ex} from other T cell subsets (78).

Despite three decades of studying T_{ex}, the precise definition of T cell exhaustion continues to divide experts in the field. Essentially, it is a hypofunctional state of decreased effector cytokine production and cytotoxicity that occurs after prolonged stimulation via the TCR in the setting of chronic antigen exposure. This is an adaptive response to reduce inflammatory tissue damage during a chronic immune response, limit autoimmunity, and maintain a pool of precursor effector cells so that the immune response can persist over time (79). However, T cell exhaustion is also exploited by immunogenic cancers to limit cytotoxic responses and promote their growth and metastasis, thus becoming a threat to the organism's survival, and limits the efficacy of cellular therapies (80-84).

Inhibitory Receptors

Inhibitory receptors (IRs) are the functional equivalent of co-stimulatory receptors, but instead of potentiating the T cell response they restrain it. Productive TCR engagement with an APC results in upregulation of both co-stimulatory and inhibitory ligand-receptor dyads across the immunological synapse. What results depends on the overall balance of ligand-receptor interactions. After primary activation, T cells rapidly increase their surface expression of IRs to avoid overexuberant activation that could result in inflammatory damage, then downregulate them as the process of T cell activation continues. However, in the setting of chronic antigen exposure, IRs reappear on the surface and outnumber co-stimulatory receptors as T_{ex} develop (78).

CTLA-4 is one of the most well-described IRs and competes with CD28 for the same ligands, B7.1 and B7.2 (CD80/86), on APCs. Beyond direct competition, CTLA-4 is able to capture B7.1 and B7.2 ligands and pull them into the T cell via trans-endocytosis, thus reducing the density of co-stimulatory ligands on the APC (85). It is clearly a critical component of peripheral tolerance as KO mice rapidly succumb to autoimmunity (86, 87). CTLA-4 is further constitutively expressed on the surface of T_{reg}, unlike other T cell subsets, and plays an important role in T_{reg} homeostasis (85). However, CTLA-4 blockade alone is unable to functionally rescue T_{ex} on its own and has been rather ineffective as an anti-cancer immunotherapy (88).

The IR that has come to define T_{ex} is programmed death (PD)-1, encoded by the gene *PDCD1*, which binds its ligands PD-L1 and -L2. PD-L1 is widely expressed including on non-immune cells (notably cancer cells), while PD-L2 is restricted to APCs. The importance of PD-1 was noted when it was found that not only is it transcriptionally upregulated in T_{ex} but blocking antibody treatment could functionally rescue these cells in chronic LCMV infection (88). This crucial discovery also demonstrated that not all T_{ex} are terminally exhausted, paving the way for immunotherapies targeting T cell exhaustion. Functionally, PD-1 recruits the phosphatase SHP-2, which interferes with post-TCR signalling, and dephosphorylates CD28, restraining co-stimulation; while SHP-2 is not essential for PD-1 effects, CD28 is (89, 90). The essential tolerogenic function of PD-1 signalling was demonstrated by using PD-1 and PD-L1 KO mice who eventually develop autoimmunity or die of overwhelming septic shock in the early phase of chronic LCMV infection due to an overwhelming inflammatory response (91).

Another key IR is the T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), encoded by the gene *HAVCR2*, which is a marker of a later and more dysfunctional subset of T_{ex} and often co-expressed with PD-1, although it is also upregulated on initial T cell engagement like other IRs. The intracellular domain of Tim-3 is bound by HLA-B-associated transcript 3 (BAT3), which impairs its catalytic function in the resting state (92). However, on engaging its ligand, the cytoplasmic tyrosine tail is phosphorylated and BAT3 is released, allowing it to exert its inhibitory effects through inhibitory phosphorylation of Lck, disrupting TCR signalling, disruption of the immunological synapse by interacting with CD45, recruitment of phosphatases, and induction of T cell apoptosis. Multiple ligands have been identified including galectin-9, phosphatidylserine, high motility group protein B1 (HMGB1), and carcinoembryonic antigen-related adhesion molecule 1 (CEACAM1) (93).

Lymphocyte activation gene-3 (LAG-3) has been most studied in CD4+ T cells, but also appears to act as an inhibitory receptor in CD8+ T cells. It is expressed on both cell types in response to chronic TCR stimulation. Molecularly, LAG-3 strongly binds to stable peptide-MHC-II complexes and produces inhibitory signals that interfere with post-TCR-signalling. However, the exact

mechanism by which it inhibits signalling and how it acts in CD8+ T cells is unclear. Blockade of LAG3 alone has little effect on T cell function, however it does act synergistically with PD-1 inhibition (94).

T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) has several inhibitory functions. It binds to CD155 on DCs, competing with CD226 which acts as a co-stimulatory receptor, which in turn interferes with formation of the immunological synapse, and inducing DCs to produce more IL-10. It also increases T_{reg} numbers and production of inhibitory cytokines. KO mice do not necessarily develop autoimmunity but have more severe phenotypes in models of autoimmune disease. TIGIT blockade is also synergistic with anti-PD1 therapy in animal models of cancer (95).

Development of T Cell Exhaustion

T_{ex} develop progressively, however the exact order and mechanisms of this progression are a matter of much debate. What is known is that persistent antigen exposure, stimulation of inhibitory receptors by their ligands, and an appropriate cytokine milieu all contribute to the development of exhaustion. Lack of CD4+ T cell help has itself been shown to contribute to exhaustion of CD8+ T cells, with mice lacking CD4+ T cells developing exhaustion more quickly (78).

Both the magnitude and duration of antigen exposure have a strong influence on T_{ex}, with both higher antigen density (i.e. a higher viral load in a chronic viral infection) and longer durations driving more severe exhaustion (78, 96). Molecularly, persistent TCR engagement leads to an imbalance of NFAT and AP-1, such that NFAT is present at higher levels than AP-1 family transcription factors Fos and Jun and enters the nucleus without forming a heterodimer (97). This downregulation of Fos and Jun may be due to another AP-1 family transcription factor, basic leucine zipper ATF-like transcription factor (BATF) (98). This “partnerless” NFAT then triggers the transcription of exhaustion-associated genes, particularly *PDCD1* (PD-1), *HAVCR2* (Tim-3), and *LAG3* (LAG-3) (99).

The anti-inflammatory cytokine IL-10 also plays a role in producing T cell exhaustion and increased IL-10 levels are often encountered in chronic viral infections and cancers (78). IL-10 blockade decreases T_{ex} and viral load in chronic LCMV infection, can reverse T cell exhaustion, and improves vaccine response (100, 101). Similarly, TGF-β also favours a T_{ex} phenotype and blocking it results in better T cell polyfunctionality and viral control (102). On the other hand, IL-2 signalling opposes T_{ex} development and improves control of chronic LCMV, however it can also expand T_{reg} cells and clinical results in cancer and chronic viral infection have been mixed. IL-21 also maintains effector CD8+ cells and may oppose T_{ex} development during chronic infection (78). Finally, type I and type II interferons have complex roles in exhaustion, although recent data demonstrated that the interferon response factor 2 (IRF2) which is stimulated by persistent interferon signalling favours a T_{ex} phenotype and blunts the response to immune checkpoint inhibitors (103).

Functional, transcriptomic (RNA-seq), and epigenetic (ATAC-seq) profiling in recent years has provided insights into how T_{ex} develop from precursors and which populations can be rescued by PD-1 blockade, and which have passed the point of no return. These are summarized in figure 1. The Rafi Ahmed group defined three T_{ex} subsets: a stem-like precursor defined by PD-1+/TCF-1+, a transitory stage defined by PD1+/Tim-3+/CX3CR1+, and a terminally exhausted stage defined by PD1+/Tim-3+/CD101+ (104). The stem-like and transitory stages were found to have high proliferative potential, while the exhausted stage did not. Further, anti-PD-1 therapy could differentiate stem-like T_{ex} into transitory T_{ex} with high levels of granzyme and perforin, and thus cytotoxicity. Stem-like T_{ex} were found to produce the most IFN-γ in response to antigen, followed by transitory then exhausted subtypes. Sorting and adoptive transfer experiments demonstrated that stem-like T_{ex} could differentiate into both transitory and exhausted subsets, while the transitory could differentiate into only the exhausted subset, and the exhausted could not produce the other two, thus demonstrating linear progression between subsets (104). Importantly, terminally exhausted T_{ex} have little response to immune checkpoint blockade, thus demonstrating that the earlier subsets are those that are rescued during anti-PD-1 therapy. Furthermore, TCF-1 was found to be highly present in stem-like precursors, but not in later stages,

while the transcription factor TOX was found to be most present in the transitory stage, and Eomes in both stem-like and exhausted subtypes (104).

The group of E. John Wherry subsequently published similar findings but divided the stem-like subset into two precursor subsets: progenitor 1 (T_{ex}^{prog1}) and progenitor 2 (T_{ex}^{prog2}). T_{ex}^{prog1} was defined as a quiescent, non-circulating subset that is $Ly108^+$ (*SlamF6*, representative of TCF-1 expression)/CD69+. This then transitions into a proliferative, circulating subset that is $Ly108^+/CD69^-$ (T_{ex}^{prog2}), however this subset can regain CD69 expression and cycle back to T_{ex}^{prog1}. T_{ex}^{prog2} can also irreversibly differentiate into an intermediate subset (T_{ex}^{int}) that loses TCF-1 (and thus Ly108) expression, but gains T-bet, and is thus $Ly108^-/CD69^-$, which appears analogous to the transitory stage as defined by the Ahmed group. Finally, these lose T-bet and regain Tox and Eomes expression, which mediate re-expression of CD69, thus becoming $Ly108^-/CD69^+$ terminally differentiated T_{ex} (T_{ex}^{term}), which appear analogous to the exhausted stage of the Ahmed group. Again, PD-1 blockade expanded T_{ex}^{prog2} and T_{ex}^{int}, but not T_{ex}^{term}, demonstrating once again that immune checkpoint inhibitor therapy appears to recruit and activate earlier precursors, but cannot rescue T_{ex}^{term} (105).

Finally, the group of Weiguo Cui proposed a third model of T_{ex} development that holds that there is a bifurcation in fate that depends on CD4+ T cell help rather than a linear progression. They propose that there is a single $Ly108^+$ (TCF1-expressing) progenitor, that under the influence of IL-21 provided by CD4+ T cells differentiate into a cytotoxic subset that is CX_3CR1^+ and expresses both T-bet and the transcription factor Zeb2, or in the absence of CD4+ T cell help differentiate into a PD-1-expressing exhausted subset expressing Eomes and NR4A2. In addition, they showed that PD-1 blockade cannot rescue the $Eomes^+/NR4A2^+$ subtype without CD4+ T cell help (106). Thus, while these models differ in the number of precursors and the linearity of progression, they all demonstrate that there is a terminally differentiated exhausted subtype that cannot be rescued by PD-1 blockade, providing important insights into how immune checkpoint inhibitor therapy works.

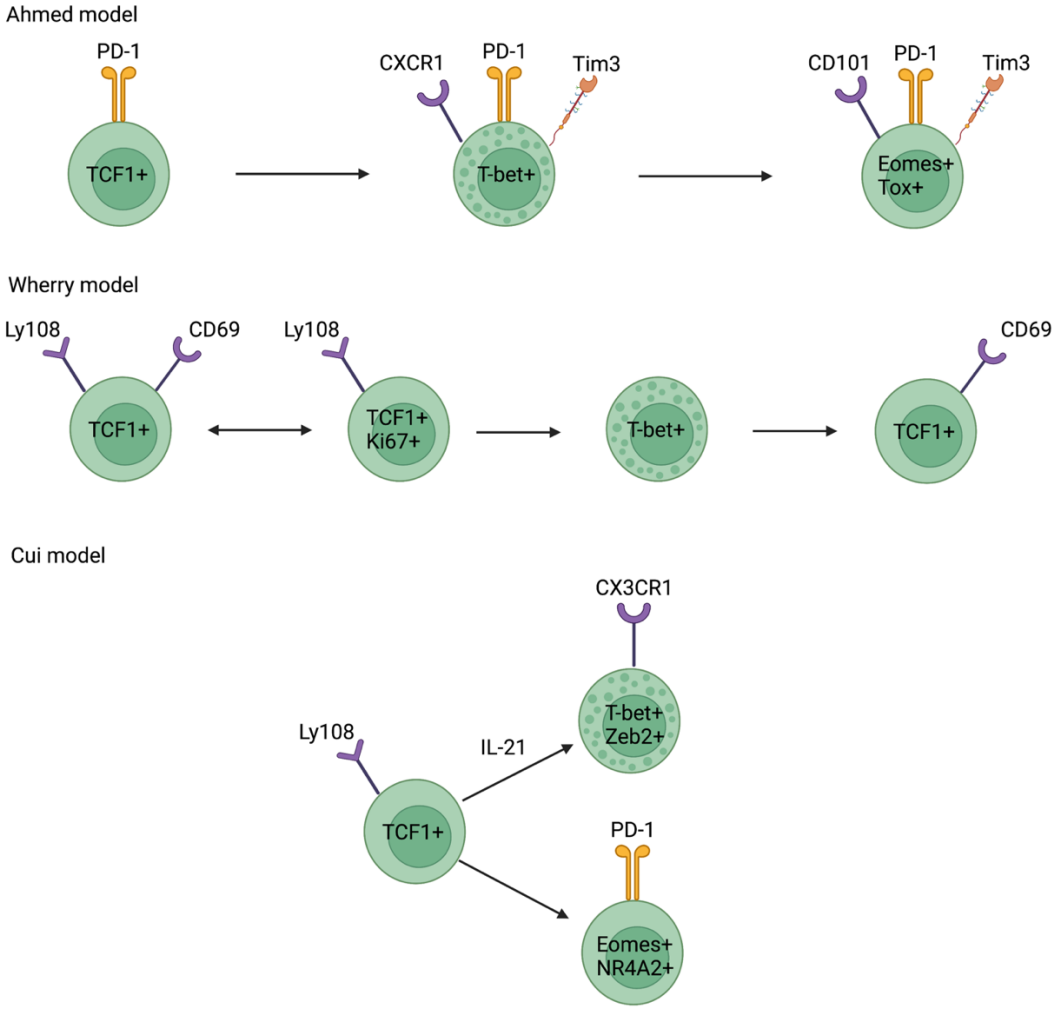


Figure 1. – Models of development of T cell exhaustion (adapted from Dave Maurice-De Sousa, created with BioRender)

Role of the NR4A Family in T Cell Differentiation and Function

The NR4A family belong to the orphan nuclear receptors, receptors that act as transcription factors but whose ligands are unknown. It consists of three receptors: NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (Nor1), which are highly inducible and present in multiple cell types, including in nerve cells where they are induced by growth factors, and cancer cells in which they play a role in apoptosis. Their ligand and DNA binding domains are well-conserved between the three members of the family, but their N-terminal domains differ greatly. On DNA, they act as transcription factors by binding to the NGFI-β-response element (NBRE) and Nur-response element (NuRE) (107).

In T cells, NR4A family transcription factors are induced as immediate early genes, meaning that they are rapidly expressed following TCR engagement (108). In fact, TCR engagement is required for NR4A1 expression as demonstrated using a green fluorescent protein (GFP) reporter cell model and stronger TCR engagement induces more NR4A1 expression (109, 110). Transcripts of all three NR4A family members are easily detectable 1 hour after stimulation and have already begun to decline after 6 hours, then return to basal levels by 48 hours, demonstrating that their activity occurs very early after activation via the TCR and declines rapidly as the cell differentiates and divides (110). Thus, interventions targeting any NR4A members must occur before the initial TCR-mediated activation.

Role in Thymic T Cell Development

NR4A1 and 3 are both expressed in developing thymocytes, while NR4A2 is not (111). Both mediate negative selection of autoreactive thymocytes through apoptosis in the cortex. KO models have demonstrated at least some redundancy between NR4A1 and NR4A3, however their individual expression may depend on the strength of the TCR signal, with NR4A3 expression requiring stronger TCR engagement during negative selection (112). However, the role that NR4A3 plays in negative selection to tissue-restricted antigens in the medulla is not known. The role of the NR4As in positive selection is even less clear, with NR4A1 possibly increasing positive selection of either CD4+ or CD8+ cells (113, 114). What is clear is that NR4A1 and 3 are required for the development of Tregs with both triple KO of all three family members and double KO of

NR4A1 and 3 resulting in a lack of Tregs and severe autoimmunity (108). Thus, the overall contribution of the NR4A family in T cell development appears to be tolerogenic for autoantigens.

Role in Acute CD8+ T Cell Response and Memory Differentiation

NR4A-targeted genes are rapidly upregulated and genes containing NBRE motifs are more accessible following TCR stimulation, but this disappears more rapidly in effector cells than memory cells (108). Studies of KO mice, both germline and conditional KO in T cells, have allowed for further understanding of their role in the acute CD8+ T cell response. NR4A1 KO improves antigen-specific T cell expansion during the acute phase, which appears to be related to its repression of IRF4 (115). It also increases production of IFN-γ and granzyme B, indicative of greater effector functions. The effect on memory differentiation, however, is less clear, with one study showing preferential differentiation into SLECs, while another showed no effect on the SLEC/MPEC ratio (108, 116). NR4A1 has been found to block the effects of AP-1 within the nucleus, which possibly explains why its deficiency improves cellular expansion and effector cytokine production (116).

A recent study comprehensively examined the role of NR4A3 during acute infection using the adoptive transfer of NR4A3 KO OT-I cells and infection with an ovalbumin-producing *Listeria monocytogenes* (Lm-OVA) model bacterium. NR4A3 KO did not affect T cell expansion, but did skew differentiation towards an MPEC phenotype, and later towards the T_{CM} subtype, and increased production of effector cytokines IFN-γ, TNF-α, and IL-2 after restimulation via the TCR using ovalbumin. However, NR4A3 KO did not lead to better control of infection. This was validated with transcriptomic and phenotypic studies that demonstrated increased levels of MPEC-associated transcription factors (*Tcf7*, *Eomes*, *Id3*, *Bcl6*, and *Bach2*) and decreased levels of SLEC-associated transcription factors (*Id2*, *Prdm1*, *Zeb2*, and *Rbpj*). CD25 expression, associated with SLEC differentiation, was also decreased in NR4A3 KO. However, unlike in NR4A1 KO, *Irf4* transcription was not affected. However, ATAC-seq demonstrated increased chromatin accessibility of AP-1-targeted genes in NR4A3 KO, thus supporting the notion that NR4A3 antagonizes AP-1 activity like NR4A1 (117).

Interestingly, NR4As are stably expressed in T_{RM}, rather than being transiently expressed as in other T cell subsets (108). In fact, antagonism of all three by short hairpin (sh)-RNAs was negatively associated with T_{RM} development (26). NR4A1 has been shown to be particularly important with KO resulting in a significant reduction in T_{RM} in the liver, Peyer's patches, and intestinal epithelial lymphocytes in an CD8 adoptive transfer and influenza infection model (118). Similarly, NR4A2 knockdown by shRNA was shown to reduce T_{RM} differentiation in a P14 adoptive transfer and acute LCMV infection model (119). The effect of NR4A3 on T_{RM} differentiation is less clear.

Role in Chronic CD8+ T Cell Response and Exhaustion

The NR4A family has mostly been studied in mouse models of chronic infection and cancer, with some limited human data. The first signal that suggested a role in exhaustion came from a model that transduced murine T cells with constitutively active NFAT that could not interact with AP-1. This resulted in severely exhausted T cells and transcriptomic studies indicated that the *Nr4a3* transcript was the most differentially expressed in both CD4+ and CD8+ T cells, while *Nr4a2* was the most differentially expressed transcript that was increased in only CD8+ T cells. NBRE motifs were also enriched in the reads obtained by chromatin immunoprecipitation sequencing (ChIP-Seq) (99). This suggested that unopposed NFAT strongly induces NR4A2 and 3 and that these may play an important role in inducing an exhaustion programme. Both these transcripts were also increased in exhausted tumour infiltrating lymphocytes (TILs) in a murine model of melanoma (120).

Another study that adoptively transferred OT-I cells in a murine cancer model demonstrated that PD-1 blockade reduced NR4A1 upregulation in exhausted T cells and that NR4A1 KO cells resulted in better T cell infiltration of tumours, greater tumour shrinkage, less PD-1 and Tim-3 expression, and increased IFN-γ and TNF-α expression (116). In contrast, a simultaneously published paper that transferred murine CAR-T cells targeting human CD19 into mice inoculated subcutaneously with a transgenic B16 melanoma cell line expressing human CD19 demonstrated significant reduction of tumour size, increased tumour infiltration, and decreased PD-1 and Tim-3 expression only when all three members of the family were knocked out, but not with single knockout cells,

suggesting redundancy (121). Importantly, however, they did not evaluate double KO cells, so it is unclear which individual factors are redundant and which, if any, may be dispensable. This is a critical question for translational applications that is raised by this work.

Far less data is available in human T cells. A study of TILs in samples from colorectal cancer patients demonstrated increased *Nr4a1* transcript and greater availability of NRBE binding sites in tumour-reactive lymphocytes compared to bystanders (122). Similarly, the previously mentioned paper using murine CAR-T cells examined TILs in human melanoma samples and found that PD-1^{high}Tim-3^{high} highly exhausted T cells upregulated all three NR4A transcripts (121).

A very recent paper identified *Nr4a3* as being the most upregulated transcript in *Prdm1* (encoding the pro-SLEC/exhaustion TF BLIMP-1) KO prostate-specific membrane antigen (PSMA)-targeted human CAR-T cells that were serially stimulated with a PSMA-expressing cell line *in vitro*. Intriguingly, double CRISPR KO of both *Nr4a3* and *Prdm1* resulted in a significant resistance to exhaustion, increased cytotoxic function, and improved persistence in an *in vivo* murine model compared to single KO of either gene (which had little to no effect) or an irrelevant KO control. It is important to note that *Nr4a1* expression was also found to be induced to a lesser extent in *Prdm1* single KO, while *Nr4a2* expression was not (123). This vital work provides the first data examining NR4A3 KO in human cells, but also suggests redundancy with and even compensation for other TFs, providing a further rationale for multiplex KO.

The molecular interactions that induce NR4A factor expression and by which they programme exhaustion are less clear and have mostly been studied in mice. Models in which NFAT is overexpressed but cannot interact with AP-1 induce both the pro-exhaustion TFs TOX and TOX2 and all three members of the NR4A family. As well, NR4A family members and TOX/TOX2 reciprocally upregulate each other in a positive feedback loop. All of these TFs then act on the loci *PDCD1*, *HAVCR2*, and *LAG3* to induce expression of the IRs PD-1, Tim-3, and LAG3, respectively (19). Furthermore, ChIP seq data suggested that *PDCD1*, *HAVCR2*, and *LAG3* recruit NR4A1 to them, demonstrating a direct interaction between it and these loci, further supporting the notion

that NR4A TFs increase IR expression (116). Overall, positive interactions between TOX and NR4A family TFs lead to a terminally exhausted state in CD8+ T cells under chronic antigen stimulation.

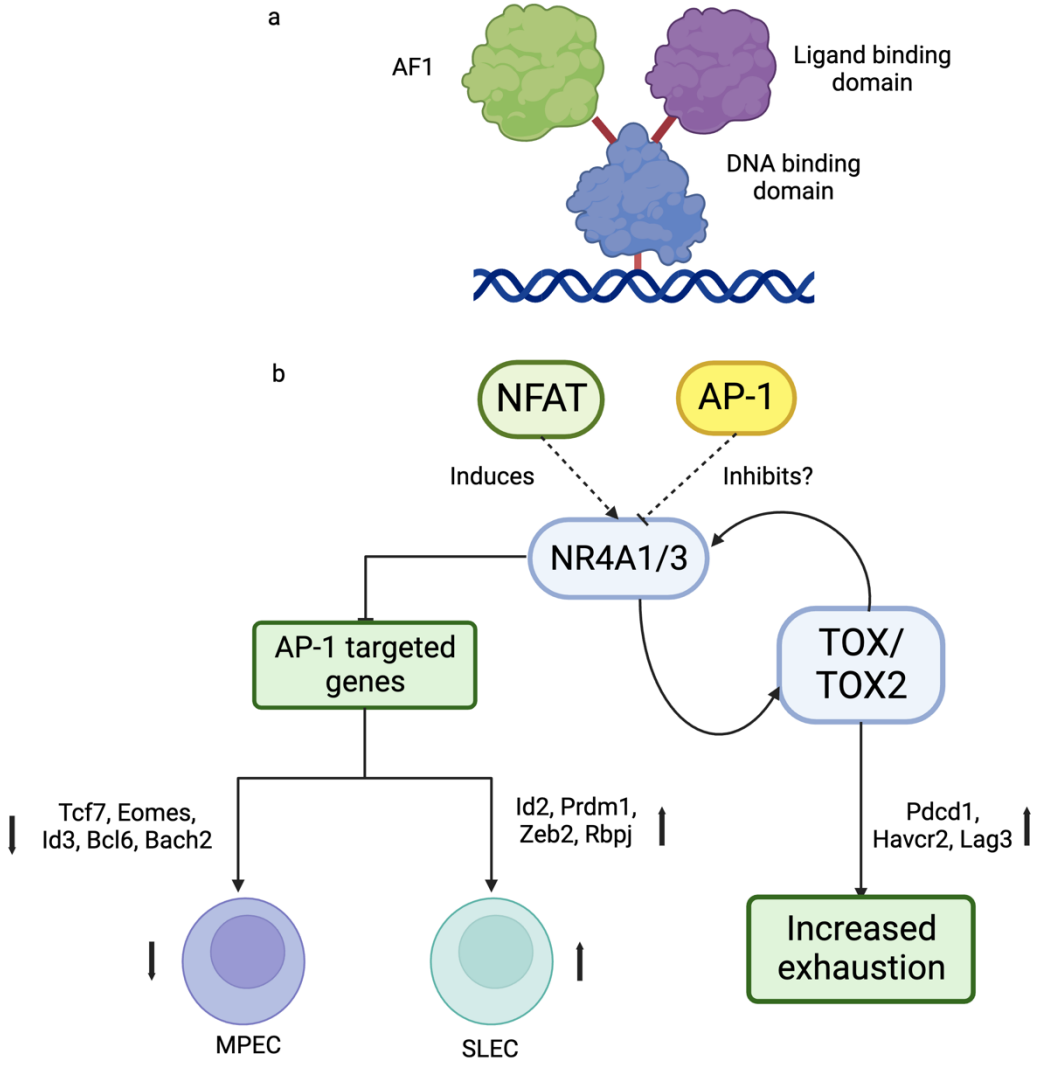


Figure 2. – Structure and functional effects of NR4A1/3 in T cells a) Conserved structure of the NR4A family, showing the N-terminus AF1 domain, the DNA binding domain (DBD), and the C-terminus ligand binding domain (LBD) b) Hypothesized interactions between NR4A1/3 and other transcription factors within T cells. NR4A family transcription is induced by unopposed NFAT that is unable to interact with AP-1. NR4A1 and 3 inhibit transcription of AP-1 target genes, reducing pro-MPEC transcription factors and increasing pro-SLEC transcription factors, thus leading to preferential SLEC differentiation. NR4A family

members also induce TOX and TOX2, which then increase NR4A family transcription through a positive feedback loop, and induce inhibitory receptor gene transcription, leading to T cell exhaustion.

Role in Peripheral CD4+ T Cell Response

The role of the NR4A family is more mixed in CD4+ T cells. NR4A2 increases IFN-γ and IL-17 production by CD4+ T cells and NR4A2 knockdown blocks Th17 differentiation and decreases severity of experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis, using both murine and human cells (124, 125). NR4A1 KO, on the other hand, increases CD4+ T cell expansion and effector cytokine production and EAE, contact dermatitis, and collagen-induced arthritis in mice, indicating that NR4A1 restricts rather than promotes CD4+ T cell mediated autoimmune disease in mice, in contrast to NR4A2 (126). NR4A1 also induces T cell exhaustion in CD4+ T cells exposed to NFAT overexpression without AP-1 interaction analogous to chronic CD8+ T cell activation (116). The role of NR4A3 in CD4-mediated autoimmune disease, however, is unknown. NR4A family members were also found to be required to maintain Tregs in the periphery, with TKO leading to less FOXP3 expression and favouring a Th2 or Tfh phenotype (127). It is less clear, however, which of the individual members is required to maintain peripheral Tregs as data have been contradictory.

In summary, the NR4A family plays a mostly tolerogenic role throughout the life of the T cell by facilitating the deletion of autoreactive clones during thymic development, restricting effector functions during acute infection, inducing T cell exhaustion in the context of chronic antigen stimulation, limiting CD4+ mediated autoimmunity (except NR4A2), and facilitating the development and peripheral maintenance of Tregs. As such, this family is of interest to the immune and cellular therapy community, as taking the brakes off engineered T cells targeting cancer or chronic infection could lead to deeper and more durable patient responses. Indeed, groups working with both murine and human CAR-T cells have been able to demonstrate improved effector functions, resistance to exhaustion, and persistence with KO of NR4A family members, albeit most studies requiring at least dual KO, although one study did show improved T cell effector function with NR4A1 single KO (116, 121, 123). Furthermore, NR4A3 single KO has

been demonstrated to programme effector CD8+ T cells towards a memory fate, and further towards T_{CM} differentiation, in acute infection (117). Thus, manipulation of the NR4As may lead to engineered T cell therapies with greater efficacy and persistence.

Chapter 2 – CRISPR/Cas9 Editing of Primary T Cells

Clustered regularly interspersed short palindromic repeats (CRISPR) are genetic elements found in the genome of prokaryotes. Essentially, they are integrated fragments of bacteriophage genomes that serve as a form of adaptive immune system for bacteria to defend themselves against repeat infection with the bacteriophage. These code for RNAs complementary to viral sequences, and when complexed with a CRISPR-associated protein (Cas) target viral mRNA or integrated DNA for inactivation or destruction. Thus, they directly inhibit viral replication or inactivate integrated viral genomes altogether (128). This bacterial defense system has been harnessed for precision eukaryotic gene editing, resulting in the awarding of the 2020 Nobel Prize in Chemistry to Jennifer Doudna and Emmanuelle Charpentier (129).

CRISPR/Cas technology represents a significant advance in gene editing because of the precision and durability of the gene edits, while also being quickly adaptable. Previous gene editing enzymes, zinc finger nucleases (ZFNs) and transcription effector-like-nucleases (TALENs) required extensive engineering to target a specific DNA sequence, making engineering time-consuming, laborious, and poorly adaptable (130). RNA interference strategies, such as short-interfering RNA (siRNA) or short-hairpin RNA (shRNA) can knockdown gene products, but their effects only last as long as the interfering RNA does and are thus less durable. The same can be said for introducing genes using plasmids (131). Retroviral transduction can introduce genes permanently into the genome, but the location and number of copies integrated cannot be controlled (132). CRISPR/Cas technology is an answer to all of these disadvantages and challenges, which is why it represents such a leap forward in gene editing.

Guide RNA and Cas Structure and Function

The CRISPR/Cas system works by introducing precision double-stranded breaks (DSB) in genomic DNA. The CRISPR RNA (crRNA) is responsible for guiding the CRISPR/Cas complex to the correct complementary DNA where the DSB is intended to be made. In the bacterial CRISPR/Cas9 system, this crRNA associates with a trans-activating RNA (tracrRNA) by forming a partial double-stranded

RNA (dsRNA) via complementary base pairing; most Cas12 systems only use a single crRNA (133). The tracrRNA also associates with the Cas, thus forming an adaptor between the gRNA and Cas to form a complete CRISPR/Cas unit. In engineered CRISPR systems, the gRNA and tracrRNA are often produced as a single linear RNA molecule, called a small guide RNA (sgRNA), eliminating the need for complementary base pairing (130).

Cas9 and Cas12 both act as endonucleases, producing precise double-stranded breaks on genomic DNA at exactly three base pairs proximal to a protospacer associated motif (PAM), a DNA sequence that must immediately follow the sequence targeted by the crRNA. Cas9 produces blunt ends, meaning that it cuts straight across the DNA molecule, while Cas12 produces staggered cuts. Cas13, on the other hand, targets mRNA for degradation. Eukaryotic cells poorly tolerate DSBs, thus the DNA repair machinery is rapidly activated to repair them. If there is no DNA repair template, this repair machinery defaults to the non-homologous end joining (NHEJ) pathway, which directly ligates the ends of the DSB. However, this repair process is imperfect and often results in insertions or deletions of nucleotides (indels). This can result in a premature stop codon, or more frequently a frameshift mutation if the number of nucleotides gained or lost is not a multiple of 3. This results in either a truncated protein or a complete change in the subsequent sequence of amino acids, which may affect protein function, folding, or anchoring depending on the target site. If the DSB is restored to the original sequence by NHEJ, it is left vulnerable to repeat attack by the CRISPR/Cas system until an indel develops. Thus, Cas9 or Cas12-based systems eventually result in a knockout of the protein (130).

If DNA repair template is introduced along with the CRISPR/Cas system, repair can occur through either NHEJ or homology-directed repair (HDR). Through HDR, CRISPR/Cas can result in a precise knock-in of a DNA sequence (130). This has many advantages: the knock-in sequence can precisely replace a dysfunctional sequence, can simultaneously knockout the original gene that is being replaced, and can place transcription under the control of proximal promotors, thus making its expression more physiologic. It also has the advantage of controlling the number of copies of the

gene product that are integrated (as it can only integrate them to the 2 loci of the targeted genes), as over integration of constructs has led to neoplastic transformation (134).

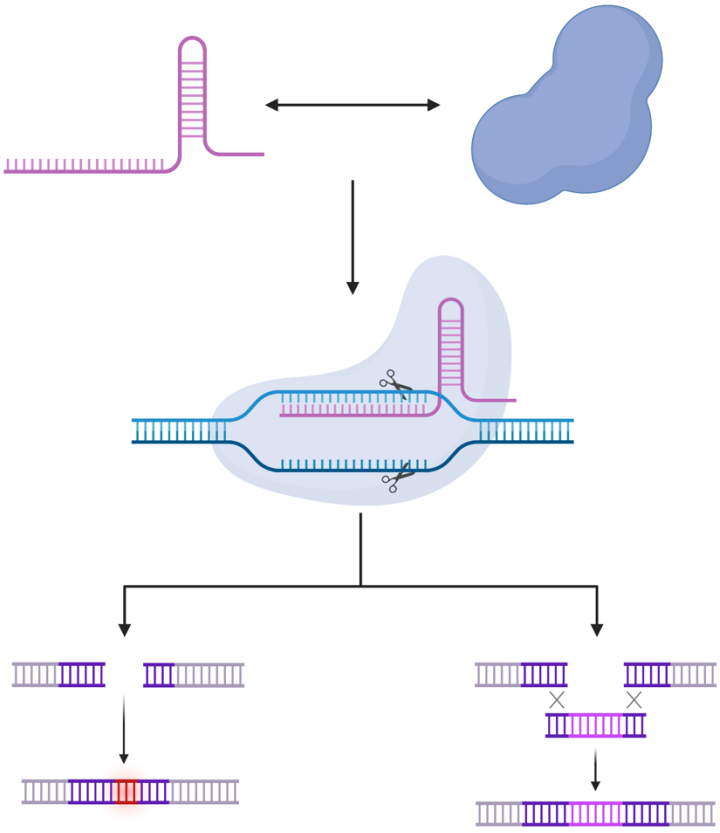


Figure 3. – Function of CRISPR/Cas9 system for precise eukaryotic editing. A sgRNA is complexed with Cas9 to form a ribonucleoprotein (RNP). This then creates a double-stranded break (DSB) three base pairs proximal to the protospacer-associated motif (PAM). This is then repaired either through non-homologous end-joining (NHEJ), resulting in insertions and deletions (indels), or homology-directed repair (HDR), resulting in a precise knock-in (KI) of the repair template. Created with BioRender.

The choice of Cas depends on several factors. Cas9 is often used for knockouts using NHEJ since it creates blunt ends, while Cas12 is used more for knock-ins since the repair template can be designed to be homologous to the staggered ends. An alternative approach is to use Cas9 nickases, in which one cleavage domain is catalytically dead, with two different sgRNAs to make different staggered cuts on each strand of the target DNA sequence. This may also reduce off-target editing (see below).

Different bacterial species' Cas9 and Cas12 also have different advantages. Each has a different PAM specificity, so using a Cas from a different species may help to find a targetable sequence. For example, the PAM for the most commonly used *Streptococcus pyogenes* Cas9 (SpCas9) is NGG, where N is any nucleotide, while the PAM for the *Staphylococcus aureus* Cas9 (SaCas9) NNGRRT, where R is an adenine or guanine (135). Also, different species' Cas have different molecular weights, which may become relevant if the delivery system (see below) has a limited payload capacity (136).

Guide RNA and Cas Delivery

For a CRISPR/Cas gene edit to take place in a eukaryotic cell, an exogenously designed ribonucleoprotein (RNP) complex must enter the cell and localize to the nucleus. As the cell membrane is hydrophobic and thus impermeable to nucleic acids and proteins, a delivery system must be used to safely shepherd them across. One of the most used delivery systems to introduce DNA to eukaryotic cells is viral transfection. This may employ an integrating viral vector such as a lentivirus, which integrates a gene coding for the sgRNA and the Cas enzyme, or a non-integrating vector such as an adenovirus (ADV) or adeno-associated virus (AAV) that carries the sgRNA and Cas directly. However, viral vectors are difficult and expensive to produce, can be immunogenic *in vivo*, can have toxic effects on cells, and have a very limited payload which can make it difficult to package all the necessary components into one vector. Furthermore, certain cell types are not amenable to viral transfection, notably resting primary T cells (137).

For these reasons, non-viral delivery options are quite attractive. One of the most used methods for editing primary T cells is electroporation. A programmed electric current is run through the cell to create pores in the cell membrane, allowing the RNP to enter the cytoplasm. The current can be further programmed for nucleofection, guiding the RNP to the nucleus so that it can perform the edit. While this method is cheap, easy to perform, and other than an electroporator requires no specialized equipment, it causes significant cellular trauma and death that affects the final cell yield. Another non-viral method is the use of lipid or gold nanoparticles to physically transport the cargo across the hydrophobic cellular membrane. However, these techniques have a lower transduction efficiency in primary T cells (137). A similar approach uses modified

amphiphilic peptides, which associate with the RNP and allow it to escape into the cytosol after endocytosis. This results in high efficiency transduction in primary human T cells without the toxic effects of viral vectors or electroporation, which improves the viability and thus yield of edited cells (138). A final non-viral delivery method is direct microinjection into individual cells however this is not practical or scalable for experiments that require millions of edited T cells (137).

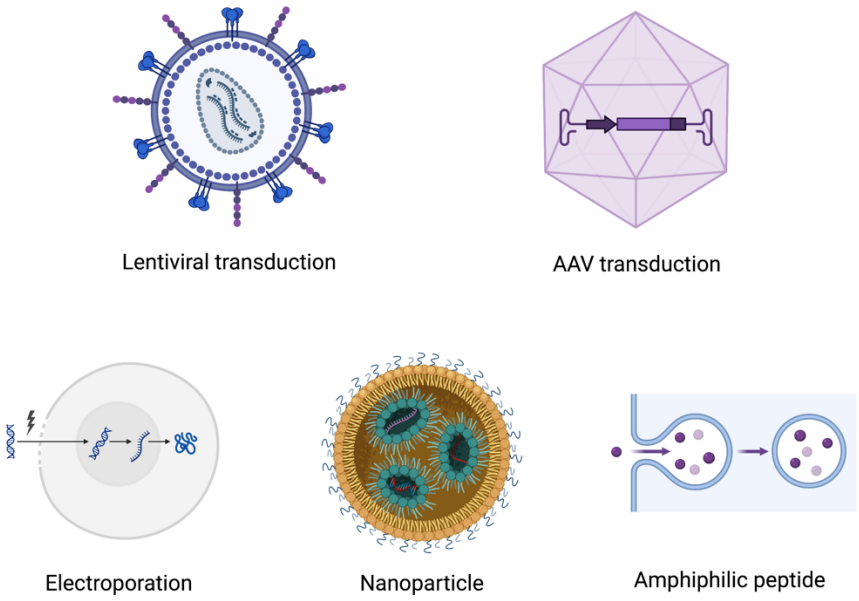


Figure 4. – Methods of RNP delivery to eukaryotic cells. Shown are viral (integrating and non-integrating) and non-viral methods. Not shown is microinjection. Created with BioRender.

Editing of Non-Dividing Cells

The cellular repair machinery responsible for NHEJ or HDR is only expressed in cells undergoing active division. In non-dividing cells, such as neurons, muscle, or resting T cells, creation of DSBs alone will not result in the desired edit. T cells may be induced to divide without TCR stimulation using cytokines such as IL-2, IL-7, and IL-15; low concentrations of IL-7 and IL-15 have been successfully used to induce successful CRISPR-based editing of primary human CD4 T cells without TCR stimulation (139). Other CRISPR-based methods that do not rely on DNA repair machinery

are guided base editors that can repair single nucleotide mutations and prime editing, which uses a CRISPR-guided reverse-transcriptase and RNA template to introduce a precise edit (140).

Multiplex Editing

Because of the targeted nature of CRISPR, multiple edits can be made simultaneously by introducing multiple sgRNAs. This can result in multiple simultaneous gene knockouts, or a knock-in and a knockout. An example of the latter strategy in T cells is CRISPR-based introduction of a CAR or transgenic TCR to the TCR locus. Essentially, a sgRNA targeting the TRAC locus (coding for the TCR α chain) is used to introduce the new construct (bearing homology at its extremities to the overlapping ends of a staggered cut) in the middle of the TRAC gene, thus simultaneously accomplishing a KO of the TCR α chain and putting the construct under the influence of TCR promotors, hence resulting in more physiologic expression. However, if a transgenic TCR is introduced in this fashion with the TRBC locus (encoding the β chain) left intact, mispairing may occur between the transgenic α chain and the endogenous β chain, carrying the risk of off-target autoimmune effects. This mispairing is eliminated by simultaneously introducing a sgRNA targeting the TRBC locus that knocks out the endogenous β chain (141).

One concern with multiplex editing by creating DSBs is the potential for chromosomal translocations if the targeted genes are on different chromosomes, or microdeletions if they are on the same chromosome. The effects of such chromosomal abnormalities are unknown but could potentially be oncogenic, thus this is of paramount importance for clinical applications. This could be mitigated by performing sequential editing by introducing the sgRNAs one at a time, however this would need to be done using a non-toxic delivery method such as an amphiphilic peptide (138). Another way to mitigate these concerns is to use a method that does not introduce DSBs, such as base or prime editing.

Validation of On- and Off-Target Editing

On-Target Editing

For knock-in and knockout of specific proteins, phenotypic validation using flow cytometry or Western blot can definitively demonstrate successful editing. However, an antibody may not be

available or practical for validating the edit, so it may be necessary to employ a genotypic method. The simplest methods use restriction endonucleases, such as T7E1 or Surveyor (142). Essentially, the target sequence is amplified using PCR with the predicted cut site asymmetrically located on the amplicon. The amplicon is subsequently purified, denatured, and reannealed by slowly cooling the sample in a ThermoCycler. If indels are present, this will create heteroduplexes with strands containing different indels reannealing to each other. Finally, the sample is incubated with one of the above endonucleases which cleaves any mismatches. If editing has occurred and heteroduplexes have formed, two daughter bands will be seen when the product is run on an agarose gel. The size of these daughter bands can be precisely calculated since the cut site is precisely known (see figure 7). If editing has not been successful, only one band will be visible representing the original amplicon (143). While these techniques are inexpensive and simple to implement, they lack sensitivity and are unable to precisely quantify the editing efficiency.

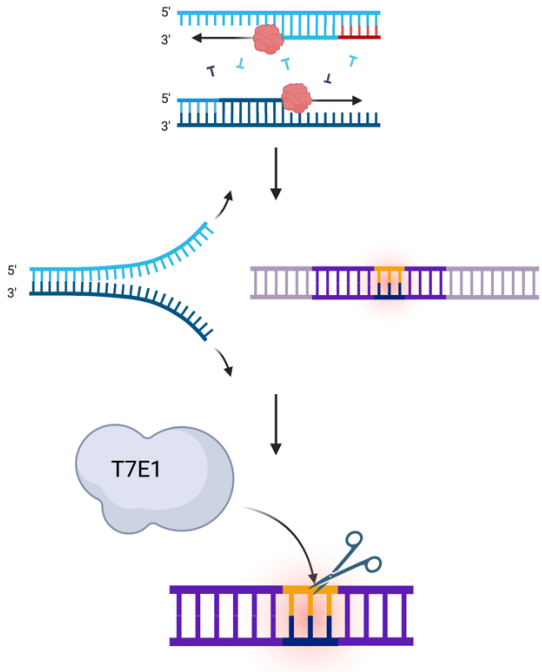


Figure 5. – Function of T7E1 endonuclease assay to validate CRISPR/Cas9 genomic editing. The target sequence is amplified, denatured, and reannealed, then incubated with the T7E1 enzyme. Sites where indels have occurred will be cut, validating the edit. Created with BioRender.

The other methods for validating on-target editing are sequencing-based. The sequence containing the cut site is amplified, then sequenced using either Sanger or next generation sequencing (NGS). Sanger sequencing data then needs to be analyzed using bioinformatic programmes such as tracking of indels by decomposition (TIDE) or interference of CRISPR edits (ICE), which compare the obtained sequences to the reference sequence to detect the frequency of indels, and thus calculate editing efficiency (144, 145). Otherwise, NGS can also be used to calculate the editing efficiency, however this is more expensive and higher depth of sequencing is required to obtain a precise measurement.

Off-Target Editing

A key safety concern with all gene editing technologies is unintentional, off-target editing. This is of particular concern if CRISPR is used for germline editing as any unwanted edits could be passed down to future generations. Nonetheless, off-target effects are equally concerning in editing primary T cells as it could result in neoplastic transformation. The risk of chromosomal translocations was introduced previously when discussing multiplex editing, but even simplex edits can result in off-target effects if a similar sequence is found elsewhere in the genome (146).

The most important step to mitigate off-target effects is careful sgRNA design. Several bioinformatic programmes, both commercial and academic-based, can be used to screen potential sgRNAs for complementary sequences elsewhere in the genome. The more mismatches between the sgRNA and the potential off-target site, the lower the risk that off-target editing will occur. As well, potential off-target sites within known coding regions or adjacent to the PAM of the Cas that is used are generally avoided. If this is not possible, the Cas can be changed to that of another species recognizing a different PAM and the sgRNAs can be redesigned. Finally, high-fidelity Cas or nickases can be used to mitigate off-target effects (147).

Even if an sgRNA is not predicted to produce off-target effects, this must be validated before a treatment can be translated for human or animal use. This validation can use methods that are biased or unbiased. A biased validation uses bioinformatic tools to identify the most high-risk sites for off-target editing, followed by targeted amplification of those sequences and evaluation using

one of the techniques used for validation of on-targeting editing mentioned previously. While this provides good sensitivity for off-target edits on those sequences due to the amplification step, it is limited only to sequences that the bioinformatic analysis identifies.

An unbiased validation, on the other hand, performs a wide-ranging evaluation of the entire genome looking for off-target effects that may not have been predicted. The simplest way to do this is by performing whole genome sequencing via NGS, however this may be cost prohibitive due to the depth of sequencing required to reasonably detect low-frequency edits. NGS-based modifications use *in vitro* CRISPR/Cas digestion followed by tagging of cut sites by various means to improve detection sensitivity without requiring the same depth of sequencing as traditional NGS, as these tags can be more easily detected (148).

Utility in Cellular Immunotherapy

The ability to use CRISPR/Cas technology to perform targeted non-viral KI of CARs and transgenic TCRs has already been discussed above. Another potential use is for selective KOs of immune checkpoints or transcription factors to influence memory differentiation towards more stem-like, and thus persistent, phenotypes, and to make these therapies more resistant to exhaustion in the setting of chronic antigen stimulation. PD-1 KO anti-cancer cellular immunotherapies were found to be slightly more effective in mouse models without any noted toxicity, oncogenic potential, or reduced persistence (149, 150). Other checkpoint KOs or multiplex editing combining multiple KOs or receptor and transcription factor KOs may lead to more impressive effects. An attempt to KO BLIMP-1 in CAR-T cells by targeting the *PRDM1* gene did not result in the desired resistance to exhaustion, however this was found to have caused NR4A3 upregulation, and the desired increase in efficacy was achieved with dual KO of both *PRDM1* and *NR4A3* by multiplex CRISPR/Cas9 editing (123). Another recent publication identified sorting nexin-9 (SNX9) as a pro-exhaustion and anti-memory differentiation protein using a multiplex CRISPR/Cas9 screen; single KO of SNX9 was found to improve memory differentiation and effector functions in human CAR-T cells, and notably reduced expression of *Nr4a1*, *Nr4a3*, and *Tox* mRNA (151).

In summary, CRISPR/Cas editing provides the ability to precisely replace the native TCR with a CAR or a transgenic TCR to effectively redirect the T cell to attack infection or cancer, but also to reprogramme these cells to take on more persistent and stem-like memory phenotypes and be more resistant to exhaustion, thus more effective. However, these efforts face both technical and biological challenges before they are ready for clinical translation. A high level of editing efficiency must be achieved before these products can be produced at clinical scale, which is particularly challenging for KI using HDR. As well, currently available delivery systems, notably viral transduction, and electroporation, are quite toxic for T cells and significantly reduce their viability. Cas9 and dsDNA templates are also themselves toxic to T cells and thus must be transiently expressed or modified to reduce this toxicity (152). Off-target effects must also be carefully considered and excluded using highly sensitive methods to ensure the safety of these products. On a biological level, simplex KO to manipulate memory differentiation and exhaustion have been quite disappointing due to redundancy and induction of other pro-exhaustion genes, but multiplex KO appear to compensate for this. While these challenges are significant, they are not insurmountable, and CRISPR/Cas technology is likely to be extensively employed for manufacturing T cell therapeutics in the future.

Chapter 3 – Opportunistic Viral Infections and Virus Specific T Cell Therapy

Iatrogenic Immunosuppression

T cell immunosuppression occurs in patients for many reasons. In a minority, this is due to an inborn error of T cell immunity or a direct effect of a disease, such as circulating dysfunctional T cells associated with T cell leukemia/lymphoma or CD4 immunodeficiency due to advanced HIV. However, most cases are seen in patients receiving immunosuppressive treatments to avoid auto- or alloimmunity. Solid organ transplant (SOT) and allogenic hematopoietic stem cell transplant (allo-HSCT) recipients are the most deeply T cell immunosuppressed and are thus particularly susceptible to opportunistic infections (OIs).

While there is considerable overlap, SOT and allo-HSCT recipients differ in the causes and duration of susceptibility to OIs. In both cases, conditioning regimens are given at or shortly before the transplant, meant to rapidly induce immunosuppression and prevent rejection. HSCT recipients receive myeloablative conditioning chemotherapy, while SOT, and some HSCT recipients, recipients receive direct T cell lymphodepleting treatments and high dose corticosteroids. Some HSCT recipients will receive a T cell depleted graft or cord blood transplant, putting them at even greater risk of OIs (153).

In general, most infections that occur in the first 30 days after induction are related to myelosuppression or surgical and intravenous access complications given the longer half-life of T cells than other blood cell lines. However, from 30 days on patients enter the period of maximal T cell immunosuppression with the highest risk of OIs. For HSCT recipients, assuming successful engraftment, this period of high risk lasts until 100 days after the transplant, when T cell immunosuppressive agents can be stopped in most patients, but full T cell reconstitution does not occur for up to two years (153). For SOT recipients, however, this high-risk period lasts from 6-12 months after immunosuppression dependent on the organ and depth of

immunosuppression, and full T cell reconstitution never occurs due to the need for lifelong therapy to prevent acute cell mediated rejection (ACMR) (154). Immunosuppression must once again be intensified, however, if the patient develops GVHD or rejection, leading to increased depth and duration of OI risk.

Induction Therapy

HSCT induction rapidly destroys the cellular component of the bone marrow, creating tolerogenic immune environment within the bone marrow for the donor's stem cells to engraft. Modern induction regimens use myeloablative chemotherapy, with total body irradiation more seldom used due to toxicity and risk of secondary cancers. The other type of induction, used in both HSCT and SOT, directly targets T cells to rapidly reduce their numbers and function, thus preventing GVHD or ACMR, respectively. While T cell directed induction therapy is universal in SOT, in HSCT it is used in patients with higher GVHD risk as judged by the treating Hematologist (154, 155).

Antiproliferative Chemotherapy

Some chemotherapies are considered particularly T cell depleting and are thus used primarily for this purpose, almost exclusively in the HSCT and cell therapy world. Cyclophosphamide is an alkylating chemotherapy that directly attacks and cross links DNA in rapidly dividing cells, functionally impairing alloreactive T cells while relatively sparing Tregs (156). Fludarabine is a purine analogue that directly inhibits eukaryotic DNA polymerase, thus preventing DNA synthesis and mitosis (157). The combination of these chemotherapies is used for lymphocyte depletion to improve engraftment in chimeric antigen receptor T cell (CAR-T) and expanded tumour infiltrating lymphocyte (TIL) recipients, while post-transplant cyclophosphamide alone can be used as GVHD prophylaxis in higher risk HSCT recipients (155, 158).

T Cell Depleting Antibodies

Antibodies directly targeting circulating T cells can rapidly reduce their numbers via antibody-dependent cellular cytotoxicity (ADCC). Both polyclonal and monoclonal products exist. The most common is antithymocyte globulin (ATG), a polyclonal product generated by alloimmunizing a rabbit or a horse with human thymocytes. When administered to a human, this product results in rapid T cell depletion, but may also result in serum sickness from pre-formed antibodies against

the animal Fc domain of the antibodies (159). A monoclonal product performing the same function is alemtuzumab, which targets the pan-lymphocyte marker CD52 and causes even more profound and longer lasting B and T cell depletion and a particularly high infectious risk. Thus, this product is only used after careful consideration of the individual risk of rejection and OIs (160).

IL-2R Targeted Antibodies

Basiliximab and daclizimab (no longer marketed) are both monoclonal antibodies targeting the IL-2 receptor (CD25) and are used for SOT induction, particularly in renal transplantation (161). By targeting CD25, which is only expressed by activated T lymphocytes, they selectively deplete these cells while sparing resting T lymphocytes, except notably Tregs (162). While their short-term effects are comparable to other induction agents, they carry a lower long-term risk of injection and cancer, which makes them desirable from a toxicity standpoint (163).

Maintenance Therapy

Calcineurin Inhibitors

Calcineurin inhibitors (CNIs) are the mainstay of maintenance therapy after SOT and for GVHD prevention in the first 100 days post-HSCT. These directly inhibit dephosphorylation of NFAT by calcineurin in response to increased cellular calcium after TCR. This prevents transcription of NFAT-targeted genes, particularly IL-2, and thus T cell activation. Cyclosporine A binds to cyclophilin A (CypA), while tacrolimus (FK506) binds to FK binding protein 12 (FKBP12), both of which then bind with greater affinity to and inhibit calcineurin. Thus, both these drugs prevent ACMR by inhibiting the activation of alloreactive T cells (164).

Antiproliferative Agents

Mycophenolate mofetil (MMF) is a potent T and B lymphocyte inhibitor and carries a high risk of OIs. It is a prodrug of mycophenolic acid, which inhibits synthesis of guanosine nucleotides in lymphocytes. By depleting purines, MMF significantly inhibits the ability of lymphocytes to proliferate upon activation, thus preventing clonal expansion of alloreactive T lymphocytes (165). Leflunomide prevents pyramidine synthesis and can also be used as an immunosuppressant post-transplant, but is less used due to toxicity (166).

mTOR Inhibitors

Sirolimus (rapamycin) targets the aptly named mammalian target of rapamycin (mTOR) thus inhibiting the Akt pathway. mTOR also forms a complex with FKBP12, the target of tacrolimus, thus leading to synergy between these agents. Akt inhibition impairs cellular metabolism and entry into the growth cycle, thus inhibiting clonal proliferation. A significant advantage is that sirolimus does not carry a significant infection risk itself, and can reduce the required dosage of tacrolimus due to this synergy, thus it is attractive for long-term maintenance in SOT recipients (167).

Glucocorticoids

Glucocorticoids are broad spectrum anti-inflammatory molecules targeting the glucocorticoid receptor, an intracellular steroid hormone receptor that acts as a transcription factor upon binding its ligand. They are used at low doses for maintenance therapy post-transplantation, but in much higher doses for induction or as first-line treatment of rejection and GVHD. This often requires higher-than-normal doses for a prolonged duration, inhibiting not only T and B cell function, but also phagocytes. Thus, the associated infection risk is broad and profound, and is dose- and duration-dependent. The non-infectious, particularly metabolic, adverse effects of prolonged glucocorticoid treatment are also particularly important, thus the previously mentioned non-steroid immunosuppressive drugs are used as much as possible as steroid-sparing agents (168).

Acquisition or Reactivation of Latent Viruses

Most viral infections are acute, with the virus disappearing from the host once it is cleared by the immune system. However, some DNA viruses and retroviruses can establish latency, persisting in a non-replicative state in host cells, but maintaining the ability to re-enter the lytic cycle and resume active replication. However, this comes at a cost, as display of viral antigens attracts attention from an immune system already primed through immunological memory to unleash a robust cytotoxic attack. If there is a state of severe T cell deficiency, as in HSCT and SOT, this immunological response does not occur, and the virus can replicate with impunity. This explains why latent viruses are so problematic in these patients (169).

The risk of active infection with latent viruses depends largely on the donor/recipient serostatus for the virus in question. In SOT recipients, donor positive, recipient negative (D+/R-) are at highest risk, as the host is completely naïve to the virus while the transplanted organ carries it latently. Thus, these patients will develop acute infection at the period of highest T cell immunosuppression post-transplant and will be unable to mount an adaptive response. If an antiviral prophylaxis agent exists for the virus in question, these recipients are almost universally provided with prophylaxis during the highest risk period. D+/R+ and D-/R+ are at intermediate risk of infection, while D-/R- are at lowest risk since the recipient would need to acquire the infection through a third-party contact for it to be introduced. The transplanted organ also matters greatly, with those that carry the most lymphoid and vascular tissue being more likely to transmit the virus in D+/R- dyads. Lung and small bowel transplants carry the highest risk, followed by heart, liver, and composite tissue (face and hands), and kidney transplants carry the lowest risk (154).

In HSCT, the D/R paradigm is reversed. The patients at highest risk of reactivation are D-/R+, recipients carrying latent infection being transplanted from a donor who is naïve for the virus in question. Patients receiving cord blood transplants are particularly at risk of viral reactivation since these are completely antigen naïve. D+/R+ and D+/R- are at intermediate risk since there is a lower risk of viral transmission via HSCT than SOT, while D-/R- are again at lowest risk for the same reason as in SOT (153).

Another important risk factor for viral infection is the age of the transplant recipient. Respiratory viruses, particularly adenoviral infection (discussed further below), are a particular problem in pediatric HSCT because of the high baseline circulation of these viruses in their siblings and peers. SOT recipients are immunosuppressed for life, thus pediatric SOT recipients, who are more likely to be seronegative to most latent viruses at baseline, are more likely to develop primary infection from an exposure later in life, especially in their teens and twenties. Thus, late primoinfection years after the transplant is a more frequent challenge in this population compared to adult SOT

recipients, many of whom have already been infected and, if not, are at a lower epidemiologic risk of acquiring these viruses (170).

Herpesviruses

Herpesviruses are DNA viruses that establish lifelong latency in the host after primary infection. Rather than integrating themselves into the host genome, they develop an episome, a small DNA inclusion in the nucleus that replicates along with the host cell. They are further classified into three subgenera: the alphaherpesviruses, herpes simplex virus (HSV) 1 and 2 and varicella-zoster virus, the betaherpesviruses, CMV and human herpesvirus (HHV)-6 and 7, and the gammaherpesviruses, EBV and HHV-8 (also known as the Kaposi sarcoma herpes virus, KSHV). These viruses can reactivate in both immunocompetent and immunocompromised individuals but are much more common and severe in patients with T cell immunocompromise (169). Much of the clinical need for virus-specific T cell (VSTs) therapies in HSCT and SOT recipients is to treat these viruses.

Alphaherpesviruses

The alphaherpesviruses are the best known to the public, with much of the human population having experienced HSV-1 infection (normally orolabial herpes or “cold sores/feux sauvages”) or VZV infection, known as chicken pox (“varicelle”) in its acute form and shingles (“zona”) when it reactivates. HSV-2, which primarily causes recurrent genital herpes, is less common but just as well known. These viruses maintain their latency within sensory nervous tissue; cutaneous sensory neurons for HSV 1 and 2 and dorsal root ganglia for VZV (169). While they can cause severe, recurrent, and disseminated disease in patients with T cell immunocompromise, they are readily treated and prevented using targeted DNA polymerase inhibitors: acyclovir, pencicliovir. and their derivatives. Rare resistant infections do occur, but multiple lines of antiviral therapy exist, so these viruses are not important candidates for VST therapy.

Betaherpesviruses

Cytomegalovirus

CMV is one of the biggest challenges in transplant medicine. Its seroprevalence depends on socioeconomic status and country of residence; in high income countries it is 50-60% while in low- and middle-income countries it is 90-95%. Primoinfection may range from asymptomatic to a mononucleosis-like infection, that is differentiated by EBV by the absence of heterophile antibodies (so called "Monospot-negative mononucleosis"). Its primary site of latency is controversial but appears to be primarily vascular endothelium with a minority population in CD34+ hematopoietic stem cells and monocytes. There is frequent recurrence throughout life, which helps to shape the memory cell repertoire through memory inflation, and which may be a cause of immunosenescence. These recurrences are almost always asymptomatic in immunocompetent hosts but can be severe or even deadly in SOT and HSCT recipients (171).

CMV manifestations can be organ-limited or multisystemic. The "CMV syndrome" which is most often encountered in HSCT recipients is a multisystemic manifestation with fever, malaise, hepatitis, lymphadenopathy, and myelosuppression mimicking acute mononucleosis. End organ manifestations include encephalitis, pneumonitis, hepatitis, and gastrointestinal disease. Pneumonitis is particularly deadly and mostly encountered in HSCT, while gastrointestinal disease is common in SOT and can manifest with ulcerative mucosal inflammation anywhere from mouth to anus and can lead to gastrointestinal perforation (171). While 90% of cases respond to therapy, 10% progress to resistant and refractory disease, which is a major potential indication for VSTs (172).

Disease prevention in transplant recipients relies on primary prophylaxis and pre-emptive therapy. Primary prophylaxis involves administration of anti-CMV treatment to high-risk recipients to delay infection until after the period of highest immunosuppression. The only agent available for many years was valganciclovir, the oral prodrug of the DNA polymerase inhibitor ganciclovir, however this could not be used in HSCT recipients because it is myelosuppresive and led to higher mortality due to delayed engraftment (171). The new CMV terminase inhibitor,

letermovir, is indicated for primary prophylaxis in HSCT recipients as it does not cause myelosuppression, however its cost has precluded its widespread adoption in Canada (173). It was also recently approved in the United States for CMV prophylaxis in high-risk renal transplant recipients (174). Ganciclovir can also be used for treatment of CMV disease, with the more toxic agent foscarnet (which targets another site on the DNA polymerase molecule) being reserved for patients who develop high-level ganciclovir resistance on therapy. The newest anti-CMV agent, maribavir, targets the viral kinase UL97 and is approved for resistant and refractory CMV, however in the largest clinical trial to date only 50% of treatment experienced patients had a durable response to maribavir (175). Thus, VST development against CMV is a priority to treat patients with resistant and refractory disease. Most VSTs target the immunodominant antigens pp65 and IE1.

HHV-6

HHV-6 normally causes roseola in children can frequently reactivate in T cell immunocompromised HSCT recipients, usually of little clinical consequence, but can very rarely cause a neurological disease called post-transplant limbic encephalitis (PALE) (176). This is most common in cord transplant recipients. PALE is often treated with ganciclovir, but outcomes are quite poor, which has led to HHV-6 being identified as a target for VST therapy and being included as a target in the Allovir product posoleucel (177).

Gammaherpesviruses

Epstein Barr virus

EBV is the type virus of this subgenera and the main focus of this work, thus it will be most extensively discussed. Approximately 95% of the human population is infected with EBV, making it one of history's most successful viruses. The primary infection can be asymptomatic, but in most people causes infectious mononucleosis, with fever, fatigue, lymphadenopathy, splenomegaly, atypical lymphocytosis, and hepatitis. EBV then establishes latency mainly within resting memory B cells, with a smaller reservoir in epithelial cells. It can reactivate throughout life during periods of stress or relative immunosuppression but is typically rapidly controlled. In immunocompromised and some immunocompetent people, EBV can cause various cancers (see

table 2) (178). Interestingly, unlike the other viruses discussed in this chapter, most of the clinical manifestations of EBV reactivation are related to more active latency programmes, rather than lytic infection.

Origin Cell	**Cancer**
B cell	Post-transplant lymphoproliferative disorder (PTLD) Diffuse large B cell lymphoma (DLBCL) Burkitt's lymphoma Primary CNS lymphoma (PCNSL) Primary angioimmunoblastic lymphoma Hodgkin's lymphoma Primary effusion lymphoma
NK-T cell	NK-T cell lymphoma
Epithelial cell	Nasopharyngeal carcinoma Gastric carcinoma
Smooth muscle cell	Pediatric leiomyosarcoma

Tableau 2. – EBV-related cancers

Lytic infection does cause the benign condition oral hairy leukoplakia, which is readily treated with acyclovir. However, most of the EBV manifestations in immunocompromised patients, particularly the cancers mentioned above, are related to accelerated latency programmes. Latent EBV needs to maintain B cells and induce them to divide to maintain long-term latency in humans. Thus, it produces proteins that encourage B cell proliferation (by stimulating post-BCR signalling) and block apoptosis, both of which predispose to malignant transformation. However, EBV must be cautious about how many antigens it displays, as viral antigens will be recognized by the adaptive immune system which will destroy infected B cells. Primary infection of tonsillar B cells involves extensive latent antigen expression (latency III), which then progresses to latency IIa in the germinal centres (where it can access memory B cells), then to latency 0 once in long-lasting memory B cells (their primary latency reservoir). To maintain stealth, the latency 0 state expresses two small RNAs (EBERs), with occasional expression of EBNA1 during B cell replication (latency I).

However, in states of T cell immunodeficiency, EBV can display many more antigens than in an immunocompetent host, thus it no longer requires stealth and can push B cells into unchecked clonal proliferation. In these states, it can reactivate in any of the latency programmes, including latency III (179).

Latency programme	Antigens	Associated Transformation
0	EBERs	Persistence in memory B cells
I	EBERs EBNA1	Burkitt's lymphoma PTLD
IIa	EBERs EBNA1 LMP1/2	Hodgkin's lymphoma PTLD
IIb	EBERs EBNA1 EBNA2/3/LP	NK-T cell lymphoma Nasopharyngeal carcinoma PTLD
III	EBERs EBNA1 LMP1/2 EBNA2/3/LP	DLBCL PTLD Primary angioimmunoblastic lymphoma Lymphoblastoid cell lines

Tableau 3. – EBV latency programmes and associated antigens. Adapted from (180, 181)

EBV-encoded small RNAs (EBERs) are two non-translated RNAs that are expressed in all latency programmes. They interact with RNA regulatory proteins in the nucleus to establish and maintain latency (179). The Epstein-Barr virus nuclear antigen 1 (EBNA1) is expressed transiently during cellular division to replicate and maintain the episome, ensuring that it is transmitted to daughter cells. It also binds to host DNA and drives neoplastic transformation, as evidenced by the latency I programme's ability to transform B cells into highly aggressive Burkitt's lymphoma (BL) (179, 181). EBNA2 induces expression of the host protein MYC which acts as a powerful oncogene and restrains lytic EBV replication. EBNA leader protein (EBNA-LP) induces expression of the viral latency-associated membrane proteins 1 and 2 (LMP1 and 2). These proteins in turn drive B cell

replication by mimicking BCR (LMP2) and CD40 (LMP1) engagement, thus providing signals 1 and 2 to the B cell independent of antigen recognition and T cell help and driving proliferation. EBNA 3A and 3C inhibit pro-apoptotic proteins, while EBNA3B actually inhibits lymphoma transformation by attracting a T cell infiltrate, thus ensuring host and thus virus survival. Lytic proteins BHRF1 and BALF1 also act as anti-apoptotic proteins by acting as Bcl2 homologues, thus preventing apoptosis in primary infection and contributing to oncogenesis (179).

Immunocompromised patients can develop any of these cancers, but one of the most feared complications of EBV reactivation after transplant is PTLD. Notably, PTLD can use any of the above latency programmes and take on many of the aggressive lymphoma subtypes listed above. Early PTLD, occurring within one year of transplant, tends to be EBV positive, as evidenced by positive EBER immunostaining, while late PTLD is often EBV negative. The highest risk is in D+/R- SOT dyads in which PTLD often develops within the transplanted organ from donor lymphoid tissue and in pediatric SOT recipients who acquire primary EBV infection in adolescence or young adulthood. PTLD progresses through linear stages, with early disease involving mononucleosis like symptoms with adenopathy, particularly within tonsils and associated tissue, then progressing to polymorphic disease, with a polyclonal B cell infiltrate that has yet to transform into a recognizable lymphoma histology, to monomorphic disease that often resembles a highly aggressive BL. A fourth type of disease is Hodgkin's lymphoma-type PTLD that uses a latency IIa programme. As noted above, however, PTLD at its various stages can take on any of the latency programmes, with early and polymorphic disease often using a latency III programme and monomorphic disease taking on the latency programme associated with the lymphoma subtype (182).

Given the above pathophysiology, it is vital to recognize and treat PTLD early to avoid progression to highly aggressive monomorphic disease. This involves monitoring high-risk patients with regular blood EBV PCR, although no clear viral load associated with PTLD has been established. However, since it is associated with latent rather than lytic infection, antiviral treatments that target viral replication are not useful. The mainstays of treatment of early PTLD are reduction of

immunosuppression (RIS) to the minimal tolerated levels and rituximab to reduce the latent B cell pool. RIS is limited of course by the onset of rejection or GVHD, which often prevents complete cessation of immunosuppression, although this is sometimes required resulting in sacrifice of a non-vital organ such as a kidney. Should these fail or should monomorphic PTLD be detected on biopsy, aggressive combination chemotherapy must be initiated, but outcomes are often quite poor. Therefore, VST treatment is considered most promising for treating PTLD, as it strikes at the direct pathophysiology of the disease: lack of T cell virus-specific immunity (182). The viral antigens used to produce EBV-targeted VSTs, EBNA1 and LMP2, make perfect sense when one understands the latency programmes. While these are both immunogenic antigens, they are also expressed by almost the latency programmes; EBNA1 is expressed by latency I, IIa, IIb, and III, while LMP2 is expressed by latency IIa and III.

HHV-8

HHV-8 is also known as the Kaposi's sarcoma herpesvirus (KSHV) because of its associated with Kaposi's sarcoma (KS), a vascular tumour of the skin that can metastasize to the lungs and gastrointestinal tract. It also interacts with lytic EBV to cause primary effusion lymphoma, a plasma cell lymphoma of body cavity linings, and causes Castleman's disease, a multisystemic inflammatory disease. HHV-8 seroprevalence is most common in Africa, followed by the Mediterranean basin, and uncommon in Northern Europe and North America. This, combined with the HIV pandemic has caused significant morbidity from the endemic (HIV-negative African) and epidemic (HIV-positive) forms of KS. KS is also encountered in older Ashkenazi Jewish and Mediterranean men, likely associated with immunosenescence (classic), and transplant recipients (iatrogenic) due to T cell immunodeficiency (183). While no VSTs against HHV-8-related diseases exist at this time, it could be a logical target for future work.

Polyoma and papillomaviruses

Polyoma and papillomaviruses are so closely related that they were previously considered part of the same family, the papovaviridae, before being split off into their own families. Both are non-enveloped, DNA viruses with very simple genomes that have co-evolved with their hosts. Neither has its own DNA polymerase, but rather uses the host's own DNA replication machinery to

reproduce itself (184). To do so, these viruses must push the cell into its replicative cycle to induce expression and assembly of these proteins. This makes these viruses, especially the papillomaviruses, associated with benign proliferations (warts) and cancers including cervical, vulvar, penile, anorectal, and oropharyngeal squamous cell carcinomas (185).

This simple structure and reliance on the host replisome makes these viruses difficult to treat with small-molecule antivirals due to a lack of targets. That, combined with the fact that polyomavirus associated diseases occur almost exclusively in T cell immunocompromised individuals and immunodominant antigens are easily identified given their simple structure makes them highly amenable to VST treatment. Due to significant homology between the two, the pathogenic polyomaviruses BK and JC are often treated with the same VSTs targeting BK large T (LT) antigen and the major structural protein VP1. These have been used to treat the BK-associated diseases hemorrhagic cystitis and ureteral stenosis in HSCT recipients and BK nephropathy in renal transplants. Anti-BK VSTs have also been used to treat the JC virus associated demyelinating neurological disease PML with limited success (186-189). Similarly, HPV-reactivity was found to correlated with response to TILs in cervical cancer, which raises the possibility of using HPV-targeted VSTs to treat its associated cancers (190)

Adenoviruses

Adenoviruses normally cause self-limited conjunctivitis and upper respiratory tract and gastrointestinal infections in children. However, in immunocompromised patients, particularly HSCT recipients, they can cause life-threatening, disseminated disease including severe pneumonitis, hepatitis, and hemorrhagic cystitis (along with BK virus, causing severe bleeding from the bladder). A single antiviral agent, cidofovir, is effective against adenoviruses, but its use is limited by severe nephrotoxicity. Thus, adenoviruses are also excellent targets for VSTs, with most products targeting the penton and hexon-derived epitopes (191).

Virus Specific T cells

VST Production Methods

Ex vivo expansion of VSTs relies on exposing donor cells to immunodominant antigens of the virus(es) being targeted by the cellular therapy product. Most products under clinical evaluation are produced using peptide mixes (pepmixes). These are synthetic, overlapping, 15-mer fragments of the full antigenic protein sequence that are loaded into APCs and presented to T cells present within the sample (192). Since there is significant interindividual variability in immunodominant epitopes for a given antigen, this overlapping arrangement is essential to ensuring that there is optimal VST expansion from different donors. Another key advantage is the ability to easily modify the antigen-specificity by changing the pepmix, while keeping the same production method, which is practical for large-scale clinical applications. Pepmixes for multiple viruses can also be used in the same production lot to produce multivirus-specific T cells (M-VSTs) to target multiple infections using a single clinical protocol and production method.

The cell that is used as an APC depends on whether the donor is seropositive or seronegative for the infection being treated. Naïve T cells can only be expanded using DCs, which adds time and manufacturing complexity (and thus expense, equipment, and regulatory hurdles) to generate autologous DCs before performing VST expansion. Time is an especially valuable commodity when treating highly immunocompromised patients with severe viral OIs, who need an effective product as soon as possible. Thus, rapid expansion protocols using seropositive donors, who have more easily expanded memory cells targeting viral antigens, are now largely used to produce VSTs. In this technique, first described by Gerdemann *et al* in 2012, donor PBMCs are pelleted with a centrifuge, loaded with pepmixes for the target antigens, then placed in a G-Rex bioreactor along with IL-4 and IL-7 for 12-14 days (see figure 3). These pepmixes are taken up by APCs present within the sample, particularly circulating B cells and monocytes, and presented to T cells also present within the mix. Thus, without a need for any enrichment or further manipulation, antigen-specific memory cells within the sample can be greatly expanded to target any antigen for which a pepmix exists within a clinically reasonable timeframe (192).

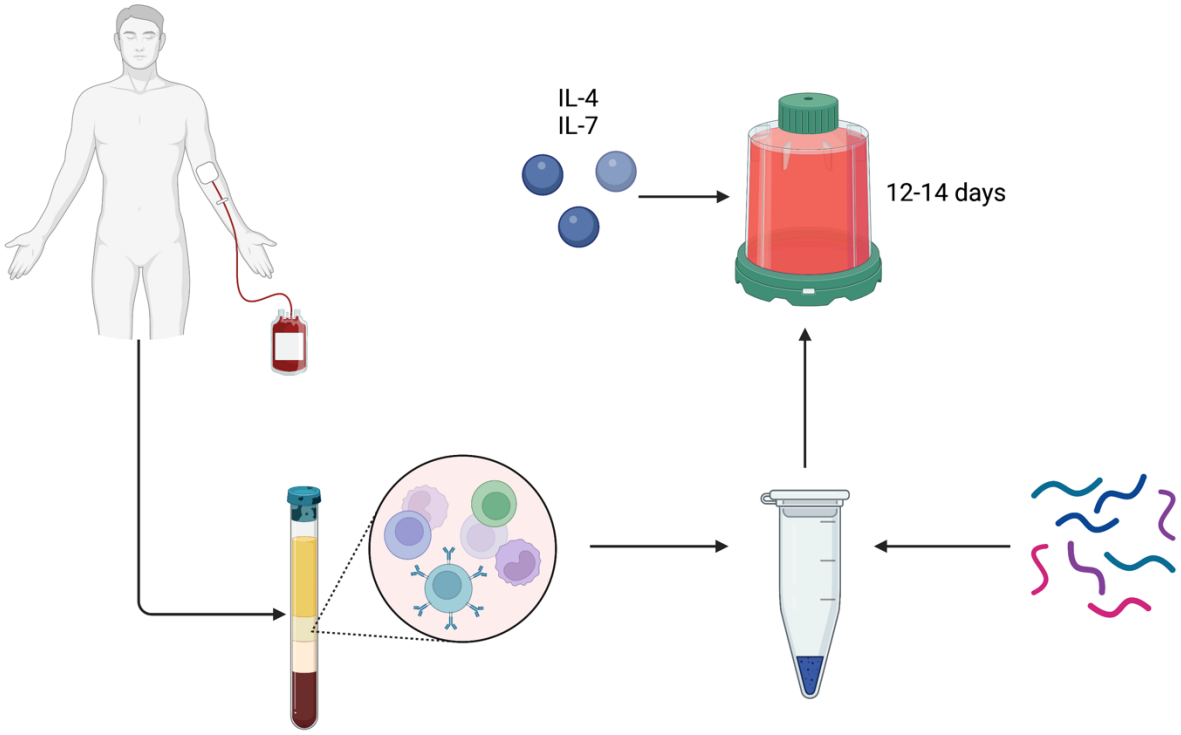


Figure 6. – VST rapid expansion method (Produced in Biorender)

From a translational perspective, this technique has the advantages of being scalable and can be completely accomplished within a closed system. The G-Rex (gas permeable rapid expansion) bioreactor is a gas permeable culture system that allows in flow of oxygen and outflow of metabolic waste products, thus optimizing cell expansion (see figure 4). It exists in sizes ranging from 24 well (6mL/well) plates for research and development to 500cm^2 flasks able to expand 250 million cells to 10-20 billion cells (Wilson Wolf Inc.). Thus, processes can be optimized at small scale during research and development and rapidly expanded to clinically useful quantities using the same protocol. Closed systems allow for media changes, cytokine addition, and cell harvesting to occur without direct manipulation, thus minimizing the risk of contamination and the material resources required to produce current good manufacturing practices (cGMP)-compliant products for clinical applications.

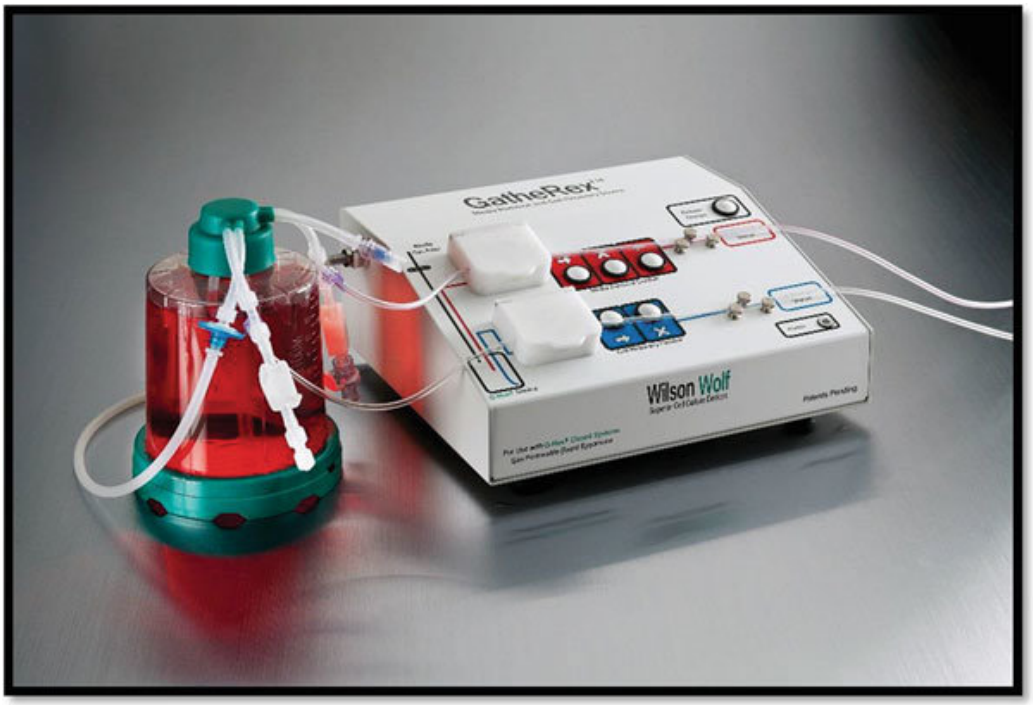


Figure 7. – G-Rex closed system for rapid cellular expansion with gas permeable bioreactor and closed pump allowing for media changes and cell harvesting without direct technician interaction with the product (source Wilson Wolf Inc.)

The source of PBMCs is also an important aspect of VST production. While many studies have used first-degree relatives, others have used unrelated, "third-party" donors. This has the advantage of producing and banking VSTs proactively, thus permitting rapid treatment of patients without needing to identify a suitable donor and produce a custom product. The disadvantage of this approach, however, is that foreign HLA or minor antigens on leukocytes may lead to alloreactivity and rejection of the cells, hence much decreased persistence (193).

One convenient source of cells is leukocyte reduction system (LRS) chambers, which are used to remove white blood cells from fractionated blood products to avoid transfusion associated GVHD and transmission of latent infections. LRS chambers are a potentially inexhaustible source of third-party donor cells for cellular therapy production. LRS-sourced PBMCs were found to be equivalent to phlebotomy for producing CMV-specific T cells and are used for the experiments performed in this work (194).

Apart from rapid expansion using pepmixes, other methods have been published to produce VSTs with more limited success. One of the simplest methods uses pull down of cells that react to viral antigens by producing IFN-γ. Essentially, donor PBMCs are incubated with peptide mixes for the target antigen and then labelled using a conjugated IFN-γ detection antibody. Magnetic beads are then added which bind to IFN-γ-producing cells that are separated from the rest of the donor pool using a magnet. This positive enrichment technique is extremely rapid, taking approximately 12 hours, and can be performed in a fully automated closed system called CliniMACS (Miltenyi Biotec), thus greatly reducing manufacturing complexity and expense (195). A similar method uses streptamers specific for specific TCR clonotypes targeting immunodominant antigens to enrich virus-specific T cells (196). However, as there is no *ex vivo* cell expansion using these techniques, they require a pre-sensitized donor with a high frequency of circulating antigen-specific memory cells, which limits available donors.

Another method for producing EBV-specific T cells uses lymphoblastoid cell lines (LCLs), EBV-immortalized B cells that are frequently used in immunology research. Essentially, autologous PBMCs are infected with a laboratory strain of EBV, then irradiated and co-cultured with non-irradiated PBMCs in the presence of acyclovir (to prevent lytic replication of EBV) and IL-2. This is a slow process that takes 2-3 months to produce a clinically-useful dose of VSTs, thus it must be done ahead of time using third-party donors that are stored until needed (197). Other than production time, another disadvantage is a lack of flexibility, as this technique can only be used to produce cells targeting EBV, as opposed to pepmix-based rapid expansion protocols that can easily be used to target other pathogens by simply changing the pepmix. A further disadvantage specific to EBV is that LCLs, without the selective pressure of a cytotoxic T cell response, EBV continues in the latency III programme with display of antigens that are rarely encountered *in vivo* (198). Thus, expanded VSTs using this method may target antigens that are not displayed in the cells being targeted by the therapy, which is a significant disadvantage compared to pepmixes that can be selected to represent antigens that are found in target cells.

VST Clinical Experience

Development of the pepmix-based rapid expansion protocol at the Baylor College of Medicine led to the product posoleucel (previously known as Viralym-M, Allovir), a multivirus specific third-party product targeting six viruses: adenovirus, BK and JC polyomaviruses, CMV, EBV, and human herpesvirus (HHV)-6. The largest clinical trial using this product, a phase II trial in HSCT recipients, was published in 2017. In this study, posoleucel was administered to 38 patients to treat 45 infections (some patients had 2 simultaneous viral infections). All patients derived some clinical benefit and 92% had a complete or partial viral response. The results were particularly impressive for patients with BK virus-associated hemorrhagic cystitis (a common complication of HSCT), with 100% of patients treated for BK virus having a virological response and 13 out of 14 patients with cystitis having complete resolution of hematuria. 94% of patients with refractory CMV infection had virological improvement, while only 71% of patients with adenovirus responded. Both patients treated for EBV had a viral response, while two out of three patients with HHV-6 responded, although this is more complicated to assess as HHV-6 can be chromosomally integrated. Importantly, these third-party, off the shelf cells persisted up to 12 weeks after infusion (177). This promising study led the company to initiate phase III studies for hemorrhagic cystitis in allo-HSCT recipients (NCT04390113), multivirus prevention in allo-HSCT recipients (NCT05305040), and BK nephropathy in renal transplant recipients (NCT04605484).

Other studies have used similarly produced VSTs targeting single viruses. One study of off-the-shelf BK VSTs to treat hemorrhagic cystitis in 59 patients post allo-HSCT with 81.6% of evaluable patients responding at day 45 and cells persisting 3 months after infusion (189). Another ongoing study had administered first-degree relative or allo-HSCT donor-derived EBV-specific VSTs (targeting EBNA and LMP2) to seven HSCT and SOT recipients with PTLD (NCT02580539). A single-patient study of anti-Merkel cell polyomavirus-specific cells to treat the virus-associated Merkel cell carcinoma resulted in significant regression of tumours (199).

Another OI for which VSTs could be a promising treatment is PML. Two case reports from Italy, one in an HSCT recipient and one in a lung transplant recipient, demonstrated sustained remission

of PML using JC-specific VSTs produced from related donors (187, 200). Two American case series used BK-specific VSTs to treat PML given the significant homology of their two immunodominant antigens, VP1 and large T. In one series, two out of three patients had a durable clinical and virological remission, while a third had a temporary improvement before dying of the disease 8 months later (201). In the largest trial to date, 12 PML patients were infused with BK-specific VSTs generated using pepmixes from healthy first-degree relatives, with seven patients disease free at one year, and five patients dying of PML (186). While this therapy is promising, many unknowns remain such as whether BK-specific cells really are equivalent in efficacy to JC-specific cells, whether off-the-shelf donor cells are equivalent to cells produced from a first-degree relative, and at what point in the disease course therapy remains effective.

Enriched peripheral T cells have also been in clinical trials in allo-HSCT recipients in Germany. Hexon-specific T cells enriched using the CliniMACS system were administered to 30 HSCT recipients, mostly children, with adenoviral disease. 21 patients (72.4%) responded at least partially, eight patients did not respond, all of whom died, and one was lost to follow-up (202). In another phase I/II trial, allo-HSCT recipients with refractory CMV disease received streptamer-enriched anti-CMV cells. Of eight patients with a CMV seropositive stem cell donor, CMV-specific T cells were successfully enriched from all of them, and 7/8 patients responded. However, of eight patients with a CMV seronegative donor who received third-party cells, seven never had detectable donor cells and none responded (196).

Despite the notable disadvantages noted above, the LCL-derived anti-EBV product tabelecleucel (tab-cel, Atara Biotherapeutics Inc.), is currently being studied in multiple clinical trials to treat EBV-associated conditions. A phase III trial to treat post-transplant lymphoproliferative disorder (PTLD) (ALLELE, NCT03394635) in SOT and HSCT recipients had an overall response rate of 50% at interim analysis. Other earlier phase trials are underway to treat other EBV-associated diseases (NCT04554914) and multiple sclerosis (NCT03283826). An early-phase study of tab-cel combined with pembrolizumab for EBV+ nasopharyngeal carcinoma was recently terminated by the sponsor for toxicity (NCT03769467).

Beyond Viruses

Ex vivo expanded cells have been produced *in vitro* to target non-viral OIs. One group produced cells targeting five different mycobacterial antigens using pepmixes; each donor responded to on average three out of five antigens and the expanded cells produced effector cytokines on exposure to both *M. tuberculosis* and *M. avium* lysates (203). Another group successfully expanded Mucorales-specific cells using *Rhizopus oryzae* lysates to treat these highly destructive invasive fungal infections (204). However, the efficacy of these T cell products against active infection has not yet been tested *in vivo*.

Effect of Iatrogenic Immunosuppression on VSTs

A major challenge in cellular therapy of transplant recipients is the fact that administered cells remain as susceptible to systemic immunosuppressants as host cells. This is obviously a barrier in patients being treated for GVHD or acute rejection, in whom immunosuppression often cannot be tapered. Thus, several groups have investigated using gene editing to knockout the drug targets to "armour" these cells against ongoing immunosuppressive drugs. One group used TALEN to knockout the glucocorticoid receptor from CMV-specific T cells before the wide availability of CRISPR; a CRISPR-based method could also be envisioned (205). Another group used CRISPR to knockout FKBP12 from CMV-specific cells to render them resistant to tacrolimus (206). Our group has also successfully performed this knockout in multivirus specific and CAR-T cells (S. Guettouche and J.S. Delisle, personal communication). CRISPR-edited TRAC and CD52 KO anti-CD19 CAR-T cells have been administered to humans in a phase 1 trial; this technology could also be used to produce alemtuzumab-resistant anti-viral T cells (207). In fact, multiplex editing could create a highly engineered cellular therapy product resistant to tacrolimus, glucocorticoids, and alemtuzumab for use in patients requiring extensive immunosuppression, however this would need to be carefully considered given the potential safety implications of administering cells resistant to so many clinically available immunosuppressants.

Treatment of PTLD Using NR4A Family Knockout EBV-Specific VSTs

Viral infections in immunocompromised patients are clearly an important clinical challenge, with PTLD associated with EBV reactivation being particularly challenging to manage given the aggressiveness of the associated B cell lymphoma and the lack of antiviral options. As previously stated, the principal treatment is RIS, but this is often not possible if life- or organ-threatening rejection or GVHD occurs and does not always result in immediate T cell reconstitution particularly in HSCT recipients who may suffer from delayed engraftment and reconstitution. Rituximab can reduce latent B cells and combination chemotherapy is used to treat monomorphic disease, but most of these patients nonetheless succumb to it. Thus, it is critical to develop novel therapies, with VSTs being some of the most promising.

That said, both the production process and chronic antigen exposure once adoptively transferred into the patient can lead to a lack of stemness and T cell exhaustion, thus limiting *in vivo* persistence and efficacy of the VST product. That leads to the interest in developing genetically modified cellular therapy products that will be permanently reprogrammed to take on a more stem-like and less exhausted phenotype. Indeed, a critical mouse study published in 2019 demonstrated that NR4A family triple KO (TKO) CD19-targeted CAR-T cells had a dramatic improvement in anti-tumour efficacy and persistence when adoptively transferred into congenic mice subcutaneously injected with a B16 melanoma model transgenically expressing CD19. These mice had a statistically significantly greater reduction in tumour size at 21 days compared to those transferred wild-type (WT) cells, with 75% of mice living to 90 days compared to the WT in which all mice died by 40 days, demonstrating persistence and efficacy of the transferred cells. TKO TILs in this study expressed less Tim-3 and produced more effector cytokines compared to WT (121).

A similar experiment using adoptive transfer of NR4A3 single KO OT-I cells in mice with ovalbumin-expressing melanomas by our group also demonstrated better tumour control, better survival, and cells that expressed less PD-1 and Tim-3 than WT. Notably, NR4A3$^{-/-}$ was better than anti-PD-L1 therapy in WT cells but was also found to synergize with anti-PD-L1 therapy to provide better tumour control and survival than KO alone. These cells took on a transcriptional profile associated

with anti-PD-L1 therapy and expressed fewer markers of terminal exhaustion. Thus, this study suggests that NR4A3 KO alone can result in similar improvements in efficacy and persistence as the TKO cells previously reported (208). That said, all these data have been obtained using completing antigen-naïve, murine KO cells in mice raised in SPF facilities, which may not necessarily represent real-world cellular therapy outcomes using adult human donors.

Hypotheses and Objectives

We aim to validate the above murine findings in a scalable, clinically translatable, human model. We hypothesize based on previous murine work that NR4A3 KO (via CRISPR-Cas9) human EBV-targeted VSTs will differentiate preferentially into a more stem-like T_{CM} memory subtype, will be more resistant to exhaustion, and will express more effector cytokines and have greater antigen-specific cytotoxic capacity than unedited cells. We further hypothesize that these *in vitro* findings will translate into more persistent and effective anti-EBV VST therapies to treat PTLD and to multivirus specific VSTs to treat other OIs in immunocompromised patients.

Our specific objectives were to 1) validate a CRISPR-Cas9 mediated NR4A3 KO in primary human T cells, 2) perform serial non-specific stimulation of NR4A3 KO primary human T cells and evaluate their memory phenotype, exhaustion markers, and effector cytokine production and 3) produce NR4A3 KO EBV-specific human T cells and test them in an antigen-specific serial stimulation model.

Chapter 4 – Materials and Methods

Isolation of Primary Human PBMCs

Leukocyte reduction system chambers (LRSCs) from healthy volunteer blood donors were obtained from Hema Québec. The plastic tubing was disinfected and cut with scissors then the chamber was flushed in a retrograde fashion with 35mL Hank's Balanced Salt Solution (HBSS) 1:10 anticoagulant citrate-dextrose (ACD) into a 50mL polystyrene tube. A long sterile glass Pasteur pipette was inserted all the way to the bottom of the tube and 15mL of Ficoll solution (StemCell Technologies Inc.) was slowly added through the pipette. The tubes were then centrifuged at 1000g for 20 minutes at room temperature and allowed to slowly decelerate without braking. The intermediate layer containing PBMCs was then carefully aspirated and transferred to a new 50mL polystyrene tube. The cells were counted using a hemacytometer. 50mL of sterile phosphate buffered saline (PBS) was then added and the tube was centrifuged 300g for 10 minutes at room temperature. This was repeated twice. Cells were then resuspended for a final concentration of 5 x 10^7 cells/mL first in 70% RPMI and 30% FBS, then 50% RPMI, 30% FBS, and 20% DMSO, for a final ratio of 1:1. These were then transferred to cryovials at 5 x 10^7 cells (1mL) per tube, which were placed in a Mr. Frosty container precooled to 4°C and placed at -80°C overnight, then transferred to liquid nitrogen for long-term storage.

Thawing of Stored PBMCs

Microtubes containing PBMCs were removed from liquid nitrogen and placed immediately on ice for transport. These were then placed in a 37°C water bath until thawing and transferred to a 50mL polystyrene tube containing 9mL of CTL 10% media (advanced RPMI, 10% human serum, 1% L-glutamate). Viable cells were then counted using trypan blue staining and a Countess Cell Counter (Beckman Coulter, Inc.). The cells were then centrifuged at 300g for 10 minutes at room temperature, the media decanted, and resuspended in CTL 10% at a concentration of 2-3 x 10^6 cells/mL. These were then transferred to a 25cm^3 suspension flask and placed in an incubator at 37°C/5% CO_2 overnight.

T Cell Enrichment

T cells were enriched by negative selection using the EasySep Human T Cell Enrichment Kit (StemCell Technologies Inc.) according to the manufacturer's instructions. Briefly, cells were collected from the suspension flasks, counted, and centrifuged at 300g for 10 minutes at room temperature. The media was decanted, and they were resuspended in PBS with 2% fetal bovine serum (FBS) and 100uM ethylenediaminetetraacetic acid (EDTA) at a concentration of 5 x 10^7 cells/mL. This suspension was transferred to a sterile 5mL polystyrene tube and 50uL/mL of enrichment cocktail was added followed by 10 minutes of incubation. 50uL/mL of magnetic beads were added followed by 5 minutes of incubation. The tube was then topped off with the same media up to 2.5mL and placed uncovered in a magnetic separator for 5 minutes. The enriched T cells were then poured off and counted.

Nucleofection

Enriched T cells were divided into clean tubes at 2 x 10^6 T cells per tube. The cells were centrifuged 300g for 10 minutes at room temperature then resuspended in 1mL of warm CTL 10% and placed in the incubator at 37°C/5% CO_2 until ready for use. The culture hood was then wiped down with ethanol and RNaseZap (ThermoFischer Scientific, Inc.) decontamination solution. A mix was then made of 250pmol of the appropriate sgRNA and 16.1µg Alt-R spCas9 (Integrated DNA Technologies, Inc), or 16.1µg Cas9 alone for the negative control. For NR4A3 KO, the sequence of the sgRNA that was used was CGCTTGAGAGCCACCCGTAC. This was heated at 37°C for 15 minutes to form RNP complexes then placed on ice. The cells were then centrifuged 300g for 10 minutes at room temperature and resuspended in 100 µL Lonza electroporation solution (82µL solution and 18µL supplement, Lonza Group AG) per condition. The RNP was then added, and the cell suspension was transferred to a cuvette (Lonza Group AG). The solution was then nucleoporated using programme T-020 in a Lonza 2b electroporator (Lonza Group AG), 250µL of CTL 10% was added, and the solution was transferred using a Pasteur pipette to a well of a 24 well suspension plate containing 750µL of CTL 10%. Non-nucleoporated controls were prepared by resuspending in 350µL of CTL 10% and transferring to the same 24 well plate. 10ng/mL of recombinant IL-7 was added to each well. Plates were incubated overnight at 37°C/5% CO_2 and 1mL of CTL 10% and an additional 10ng/mL recombinant IL-7 were added to each well the next day.

Non-Specific Serial Stimulation

A 96-well U-shaped suspension plate was coated with anti-CD3 (clone HIT3a, BioLegend) activating antibody as follows: 50µL of a 2.5µg/mL solution of anti-CD3 antibody in PBS was added per well and the plate was incubated at 37°C for 90 minutes, then the solution was removed by shakedown and each well was rinsed twice with 100µL of PBS. Cells were collected from the 24-well suspension plate by pipetting up and down and washing once with 1mL PBS then counted. The cell solution was centrifuged at 300g for 10 minutes at room temperature, the supernatant was decanted, and the cells were resuspended in CTL 10% at a concentration of 1 x 10^6 cells/mL. 100µL of a solution containing 2µg/mL activating anti-human CD28 antibody (clone CD28.2, BioLegend) in CTL 10% was added per well, followed by 100µL (1 x 10^5 cells) of cell solution per well, for a total of 200µL or 1µg/mL anti-CD28 antibody. The plates were incubated at 37°C/5% CO_2 and this process was repeated every 5-7 days for a total of three stimulations.

After each stimulation, 2.5 x 10^5 cells per condition were collected for flow cytometry staining and 1 x 10^6 cells per condition were collected for restimulation and intracellular cytokine staining. After the first stimulation, cells were also collected to be stored as a dry pellet for later use in genotypic and phenotypic knockout validation. Briefly, cell suspension was placed in a sterile 1.7mL microtube with 1mL of sterile PBS and centrifuged at 1000g for 10 minutes at 4°C. The supernatant was removed using a micropipettor and the dry pellet was stored at -80°C until needed.

Flow Cytometry

Cells were collected and placed in a sterile 5mL polystyrene tube. 2-3mL of PBS was added and the tube was centrifuged at 300g for 5 minutes at room temperature. The supernatant was decanted and 100µL of a 1:1000 dilution of LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFischer Scientific) in PBS was added. The tube was incubated at room temperature in the dark for 15 minutes. 2-3mL of FACS Flow solution (PBS with 2% FBS) was added to the tube and it was centrifuged 300g for 5 minutes at room temperature, then the supernatant was decanted. The surface antibodies were then added to the tube (see table 5) and incubated at 4°C in the dark

for 30 minutes. 2-3mL of FACS Flow solution was again added and the tube was centrifuged at 300g for 5 minutes at room temperature, then the supernatant was decanted. 100µL of 2% paraformaldehyde in PBS was added and the tube was incubated 15 minutes in the dark at room temperature. 2-3mL of FACS Flow solution was again added and the tube was centrifuged at 300g for 5 minutes at room temperature, then the supernatant was decanted. The tube was kept at 4°C in the dark until acquisition on a LSR Fortessa X-20 platform (BD Biosciences). Data were analyzed using FlowJo version 10.8 (FlowJo LLC) and gated using fluorescence minus one (FMO) controls.

Marker	**Fluorochrome**	**Company**	**Cat. No.**	**Volume added (µL) per tube**
CD3	BUV395	BD Horizon	564001	3
CD4	PE-Cy5	BD Pharmingen	555348	1
CD8	APC-Cy7	BD Pharmingen	557834	2
CD45RO	BV711	BioLegend	304236	2
CD62L	Pacific Blue	BioLegend	304826	2
PD-1	BV605	BioLegend	329924	2
Tim-3	PE	BioLegend	345006	2
LAG3	APC	Invitrogen	17-2239-42	5
KLRG1	FITC	BioLegend	138410	5

Tableau 4. – Antibody panel for surface phenotyping and exhaustion markers

Intracellular Staining for Cytokines

Cells were stimulated for four hours in a 96-well suspension plate in the presence of Brifeldin A before being stained. Briefly, collected cells were centrifuged at 300g for 10 minutes at room temperature then resuspended in CTL 10% at a concentration of 1 x 10^7 cells/mL. 100µL of a solution containing 15µg/mL Brifeldin A were added per well, followed by 100µL of the cell suspension (1 x 10^6 cells). 2µL 100x PMA/ionomycin mix (for a final concentration of 5µg/mL PMA and 50µg/mL ionomycin) were added per well. The plate was then incubated at 37°C/5% CO_2 for 4 hours.

Cells were collected by pipetting up and down and transferred to a 5mL polystyrene tube. 2-3mL of PBS was added and the tube was centrifuged at 300g for 5 minutes at room temperature. The supernatant was decanted and 100µL of a 1:1000 dilution of LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFischer Scientific) in PBS was added. The tube was incubated at room temperature in the dark for 15 minutes. 2-3mL of FACS Flow solution (PBS with 2% FBS) was added to the tube and it was centrifuged 300g for 5 minutes at room temperature, then the supernatant was decanted. The surface antibodies were then added to the tube (see table 6) and incubated at 4°C in the dark for 20 minutes. 2-3mL of FACS Flow solution was again added and the tube was centrifuged at 300g for 5 minutes at room temperature, then the supernatant was decanted. Cells were then fixed using the eBioscience FOXP3/Transcription Factor Staining Buffer Set. Briefly, a 1:4 dilution of fixation/permeabilization concentrate in fixation/permeabilization diluent was made. 100µL per sample was then added and the tube was incubated for 30 minutes at room temperature in the dark. 1mL per tube of permeabilization buffer was then added, the tube was centrifuged at 300g for 5 minutes at room temperature then the supernatant was decanted. Intracellular antibodies were then added (see table 6) and the tube was incubated at 4°C in the dark for 20 minutes. 1mL per tube of permeabilization buffer was then added, the tube was centrifuged at 300g for 5 minutes at room temperature then the supernatant was decanted. The tube was then kept at 4°C in the dark until acquisition on a LSR Fortessa X-20 platform (BD Biosciences).

Marker	**Fluorochrome**	**Company**	**Cat. No.**	**Volume added (µL) per tube**
CD3	BUV395	BD Horizon	564001	3
CD4	PE-Cy5	BD Pharmingen	555348	1
CD8	APC-Cy7	BD Pharmingen	557834	2
IFN-γ	FITC	BD Pharmingen	554551	2
TNF-α	APC	BD Pharmingen	551384	2
IL-2	PE	BD Pharmingen	559334	5

Tableau 5. – Antibody panel for intracellular cytokine staining

Genotypic Validation by T7E1 endonuclease Digestion

The dry pellet was thawed, and genomic DNA (gDNA) was extracted using the PureLink Genomic DNA MiniKit (Invitrogen) as per the kit's instructions. gDNA was then quantified using the Tecan Infinite M1000 Pro (Tecan Trading AG). 100ng of gDNA was then amplified by PCR for the *NR4A3* gene containing the cut site with forward primer CCAGCACCTCCATGTACTTCAA and reverse primer GGTTCGCACGCCGTAGT and Q5 Hot Start Master Mix (New England Biolabs). Each sample was amplified in quintuplicate. ThermoCycler settings were 98°C initial denaturation for 30 seconds, then 35 cycles of 98°C denaturation, 60°C annealing for 30 seconds, and 72°C elongation for 30 seconds, then initial elongation at 72°C for 2 minutes. Amplicons were then run on a 1% agarose gel containing 0.01% SYBR Safe Gel Stain (Invitrogen) at 100V for 30 minutes and the band at 452bp (the predicted size) was excised. The band was then extracted from the gel using the PureLink Quick Gel Extraction kit (Invitrogen) as per the kit's instructions. DNA was once again quantified and 200ng of DNA was placed with nuclease-free water and 2µL NEB buffer 2 10x for a total volume of 19µL. This was then denatured and reannealed in a ThermoCycler by heating at 95°C for 5 minutes, cooling to 85°C over 5 seconds, then slowly cooling to 25°C over 10 minutes. The resulting amplicon was then incubated with 1µL of T7E1 endonuclease I (New England Biolabs) or H_2O for the undigested control at 37°C for 15 minutes. The product was then run on a 1% agarose gel containing 0.01% SYBR Safe Gel Stain (Invitrogen) at 100V for 30 minutes and captured under ultraviolet light.

Phenotypic Validation Using Western Blot

Dry pellets stored at -80°C were thawed at room temperature. 30µL RIPA lysis buffer with 1:100 PMSF was added, and cells were incubated on ice for 15 minutes, vortexed 15 seconds, then incubated again on ice for 15 minutes. Samples were then centrifuged at 13000g for 5 minutes at 4°C and 15µL of supernatant (representing 5 x 10^5 cells) was transferred to a clean microtube. 3µL of 6x sample buffer was then added and the samples were incubated at 98°C for 10 minutes and immediately placed on ice. Samples were then run on a 10% SDS-PAGE gel at 80V for 20 minutes then 120V for 1 hour. The protein lysate was then transferred onto a PVDF membrane

at 100V for 1 hour in cold conditions. The membrane was then rinsed in tris-buffered saline with 1% tween (TBS-tween) for 5 minutes twice then blocked in 5% bovine serum albumin (BSA) in TBS-tween for 1 hour. The membrane was then incubated in primary antibody (see table 7) in 10mL 5% bovine serum albumin (BSA) in TBS-tween at 4°C overnight. The membrane was rinsed 3 times for 5 minutes in TBS-tween then incubated for 1 hour at room temperature with the secondary antibody protected from light. The membrane was rinsed 3 times for 10 minutes in TBS-tween, blotted on paper, and incubated for one minute with Western Lightning enhanced chemiluminescence (ECL) substrate, blotted again, and revealed using an Azure 600 imager (Azure Biosystems, Inc.). The membrane was then rinsed twice for 5 minutes with TBS-tween, incubated for 1 hour at room temperature with anti-actin primary antibody, then rinsed and incubated with secondary antibody as above.

Target	**Animal**	**Company**	**Cat. No.**	**Dilution**
NR4A1	Rabbit	Cell Signalling Technology	3960	1:3000
NR4A2	Mouse	Santa Cruz Biotechnology	sc-376984	1:1000
Actin	Mouse	BD Biosciences	612657	1:3000
Rabbit (HRP conjugated)	Goat	Bio-Rad	1706515	1:3000
Mouse (HRP conjugated)	Horse	Cell Signalling Technology	7076	1:3000

Tableau 6. – Antibodies used for Western blotting

Quantitative PCR

RNA Extraction

T cells were collected and stimulated with PMA/ionomycin as described above. These were then collected and transferred to a DNAse/RNAse free microtube, centrifuged 1000g for 10 minutes at 4°C, and the media was removed with a filtered pipette. The pellets were then resuspended in 1mL of TRIzol reagent (Ambien by Life Technologies, Inc) and frozen at -80°C until use. Samples were removed from the freezer and allowed to completely thaw at room temperature. 200µL of chloroform was then added and the tube was mixed by inversion then incubated for 2 minutes at room temperature. It was then centrifuged 12000g for 15 minutes at 4°C. The aqueous phase was

then transferred to a new tube and 0.5mL of isopropanol was added. This was incubated on ice for 10 minutes, then centrifuged at 12000g for 10 minutes at 4°C. The supernatant was discarded with a micropipettor and the pellet resuspended in 1mL of 75% ethanol. The sample was vortexed then centrifuged at 7500g for 5 minutes at 4°C. The supernatant was discarded with a micropipettor then the samples were allowed to airdry. The pellet was resuspended in 20µL of RNAse-free water and incubated in a heat block at 55°C for 10 minutes. The RNA was then quantified using the Tecan Infinite M1000 Pro. It was then frozen at -80°C until use.

cDNA synthesis

Complementary DNA (cDNA) was synthesized from RNA as follows: 1µg of RNA was placed in a PCR tube and diluted with RNAse-free water to a final volume of 10µL, unless 1µg would be a larger volume in which case 10µL of RNA-containing solution was added. This was then heated to 65°C in a heat block and put directly on ice. 10µL of the following master mix in table 8 was added to the tube:

Reagent	**Volume (µL)**
RNAse-free water	2.25
5X RT Buffer	4
2.5mM dNTPs	1
OligodT	1 (20ng)
DTT 0.1M	1
RNAse OUT	0.25 (10 units)
RT Superscript II	0.5 (200 units)

Tableau 7. – Master mix for cDNA synthesis

This was then incubated at 42°C for 1 hour in the ThermoCycler, then 70°C for 10 minutes to inactivate the reverse transcriptase. The tubes were then frozen at -20°C until use.

RT-PCR

Real-time PCR (RT-PCR) was performed on a Life Technologies QuantStudio 12K Flex instrument (ThermoFischer Scientific Inc.) using SyBr Green dye in a 96 well plate. Reagents were added to each well as shown in table 9:

Reagent	Volume (µL)
RNAse-free water	4.5
SyBr Green 2x Master Mix	12.5
Forward primer	2.5
Reverse primer	2.5
cDNA	3

Tableau 8. – Master mix for RT-PCR

Primers that were used are shown in table 10. Hypoxanthine phosphoribosyltransferase 1 (HPRT) is used as a sample quantity control to normalize the starting cDNA during analysis.

Primer	Sequence
NR4A1 forward	GTACATCTGCCTGGCTAAC
NR4A1 reverse	TTCGGACAACTTCCTTCAC
NR4A2 forward	CGACACTGTCCACCTTTAAT
NR4A2 reverse	TTCTCCCGAAGAGTGGTAA
NR4A3 forward	CAGAGCCTGAACCTTGAT
NR4A3 reverse	AGCTCTTCGACTCTCTCTTTG
HPRT forward	CCCTGGCGTCGTGATTAGTG
HPRT reverse	CACCCTTTCCAAATCCTCAGC

Tableau 9. – Primers used for RT-PCR

All samples were run in triplicate at 95°C for 10 minutes of initial denaturation, then 40 cycles of 95°C for 15 seconds, then 60°C for 1 minute, then analyzed using the QFlex software, normalized for HPRT expression and using the Cas9 condition as the comparator.

Antigen Specific Expansion

PBMCs were thawed and rested overnight as described above. Cells were collected and counted, then 3 x 10^6 PBMCs were aliquoted in a 15mL polystyrene tube. The tube was centrifuged at 300g for 10 minutes at room temperature then the media was decanted, and the pellet was agitated to resuspend it in remaining media. 100ng of a 15-mer overlapping peptide pool for EBNA1 (PM-

EBV-EBNA1 P03211) and LMP2 (PM-EBV-LMP2 P13285) (JPT Peptide Technologies GmbH) were added, and the tube was incubated for 30 minutes at 37°C. The cells were then diluted in 6mL of CTL 10% and transferred to a 24-well G-Rex (Wilson Wolf, Inc). IL-4 (research grade, Miltenyi Biotec) was added for a final concentration of 1666 units/mL and IL-7 (research grade, Miltenyi Biotec) was added for a final concentration of 10ng/mL. The cells were incubated for 5 days at 37°C, then a half media change was performed with 3mL of media removed, 3mL of fresh CTL 10% added, and the above cytokines readded for the same final concentration. After 8 days of total incubation, the cells were collected and counted. If there were more than 1 x 10^7 cells per well, they were divided between 2 wells. The cells were centrifuged and resuspended in 6mL of fresh CTL 10% then returned to the G-Rex. IL-4 and IL-7 were added as above. If there were more than 1 x 10^7 cells per well at day 12, the cells were collected and counted, if not, a half media change as above was performed and the culture extended to day 14, when the cells were collected and counted.

ELISpot

ELISpot was performed using the ELISpot PLUS Human IFN-γ (HRP) kit (Mabtech). 4 wells were cut from the supplied strip, placed in the supplied plate, and rinsed 5 times by adding 200µL of PBS and removing by shakedown. 200µL CTL 10% were then added and the plate was incubated at 37°C for 30 minutes. This was then removed by shakedown and 100µL warm CTL 10% was added. Cells were centrifuged 300g for 10 minutes at room temperature then resuspended at a concentration of 1 x 10^6 cells/mL in warm CTL 10%. 100uL of the cell suspension (1 x 10^5 cells) was then added per well. Peptides were then added as follows: 2µL of a 1:10 dilution of DMSO (negative control), 2µL of a 1:10 dilution of EBNA1 pepmix, 2µL of a 1:10 dilution of LMP2 pepmix, then 2µL of a 1:5 dilution of anti-CD3 antibody (positive control, supplied in the kit). The plate was incubated for 18 hours at 37°C/5% CO_2. The media was removed by shakedown and the wells were washed 5 times with 200µL of sterile PBS. 100µL of primary anti-IFN-γ antibody (1:1000 in PBS + 0.5% FBS) was added and the plate was incubated at room temperature for 2 hours. The solution was removed by shakedown and the wells washed 5 times with PBS. 100µL of biotinolated secondary antibody conjugated with streptavidin-HRP (1:1000 in PBS + 0.5% FBS) was added and the plate incubated at room temperature in the dark for 1 hour. The solution was

removed by shakedown and the wells washed 5 times with PBS. 100µL 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was then added, and the plate closely observed for appearance of spots. The wells were then immediately flooded with distilled water and left to air-dry overnight. The plate was then captured and analyzed using AID-Vspot Spectrum (AID Autoimmun Diagnostika GmbH) device and software.

Restimulation Using Phytohemagglutinin (PHA) Blasts

At day 11 of culture (3 days before restimulation), autologous PBMCs were thawed as described above and resuspended in CTL 10% at 3 x 10^6 cells/mL. Phytohemagglutinin-P (Millipore Sigma, Inc.) was added at a concentration of 20µg/mL, and the resulting suspension was transferred to a T25 suspension flask and incubated at 37°C/5% CO_2 for 3 days. After 3 days, the suspension was collected, and cells were counted. The cells were then irradiated with 4000Gy of gamma radiation, centrifuged at 300g for 10 minutes at room temperature, and the supernatant was decanted. The tube was centrifuged at 300g for 10 minutes at room temperature then the media was decanted, and the pellet was agitated to resuspend it in remaining media. 100ng of a 15-mer overlapping peptide pool for EBNA1 (PM-EBV-EBNA1 P03211) and LMP2 (PM-EBV-LMP2 P13285) (JPT Peptide Technologies GmbH) were added, and the tube was incubated for 30 minutes at 37°C. The cells were then resuspended in CTL 10% for a concentration of 1 x 10^7 cells per mL. Remaining antigen specific T cells were then centrifuged at 300g for 10 minutes at room temperature then the media was decanted, and they were resuspended in 1mL of CTL 10%. These were then added to a 24-well G-Rex and peptide-loaded PHA blasts were added at an antigen-specific T cell:PHA blast ratio of 1:1 or 3:1. The media was topped up to 6mL with warm CTL 10% and IL-4 was added for a final concentration of 1666 units/mL and IL-7 for a final concentration of 10ng/mL. The G-Rex was incubated at 37°C/5% CO_2. A half media change was performed at day 3 with the addition of IL-4 and IL-7 for the same concentration, and cells were collected after 7 days of incubation.

Statistical analyses

All comparisons were performed using pairwise, two-tailed, non-parametric tests given a non-normal distribution and a sample size below 20. Paired Mann-Witney U test and Wilcoxon

matched-pairs signed rank test were used to determine statistical significance of between group differences with significance pre-defined as $p < 0.05$.

Chapter 6 – Results

Nr4a3 is Transiently Expressed After T Cell Activation

Nr4a3 mRNA was previously found not to be expressed in resting murine T cells, is rapidly transcribed after stimulation of murine T cells, and falls back to baseline within 72 hours of activation (117). We aimed first to determine whether these kinetics were similar in human T cells. RT-PCR was used to quantitatively evaluate *Nr4a3* mRNA transcription in magnetically enriched human T cells at serial time points after pharmacological activation with PMA/ionomycin. In keeping with its status as an immediate early gene, *Nr4a3* mRNA was rapidly upregulated up to seven-fold 4 hours after stimulation. It remained elevated at 12 and 24 hours after stimulation, then returned to baseline levels within 72 hours, demonstrating that its expression is transient, and the kinetics are similar to what was previously observed in murine T cells (see figure 8).

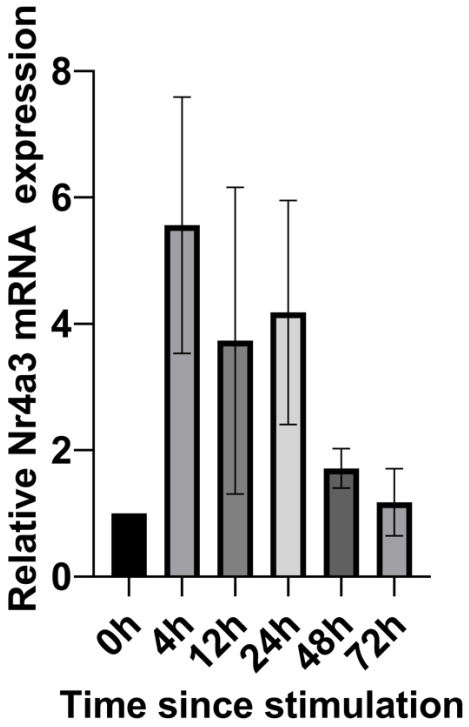


Figure 8. – Nr4a3 expression kinetics after PMA/ionomycin stimulation. Magnetically enriched human T cells were harvested in their resting state then at 4, 12, 24, 48, and 72 hours after pharmacological activation with PMA/ionomycin in 96 well plates. T cells were collected at

each time point and stored in Trizol until measurement of *Nr4a3* expression by RT-PCR. This was calculated as relative *Nr4a3* expression compared to the resting state and normalized to HPRT expression (n=3).

NR4A3 is Successfully Knocked Out by CRISPR/Cas9 Editing

Next, successful NR4A3 KO of primary human T cells using CRISPR-Cas9 needed to be validated. We first attempted to do so using a phenotypic technique demonstrating loss of the protein, however this was unsuccessful due to a lack of a specific antibody for western blot or flow cytometry. The KO was thus genotypically validated using the T7E1 endonuclease assay to demonstrate successful introduction of indels in the Nr4a3 gene at the sgRNA target site, which can be accurately predicted due to the precision of CRISPR-Cas9 gene editing. Magnetically enriched human T cells underwent nucleoporation with an RNP consisting of spCas9 and a sgRNA targeting a segement of *NR4A3* exon 4 (CGCTTGAGAGCCACCCGTAC), were incubated for 3 days with IL-7 supplementation, and then stimulated with activating anti-CD3 and -CD28 antibodies. After a further seven days of incubation, the cells were collected, gDNA was extracted, and PCR was performed to produce an amplicon containing the predicted cut site. This amplicon was then incubated with T7E1 endonuclease and DNA was separated using electrophoresis on an agarose gel.

Specific KO of edited samples was successfully demonstrated in triplicate during the validation by observing daughter bands of the predicted molecular weight, thus proving that indels had been introduced at the predicted cut site for the selected sgRNA, without any digestion of the Cas9 nucleofected or non-nucleofected negative controls (see figure 9). While T7E1 is a qualitative assay, thus an editing efficiency cannot be precisely calculated, it requires an editing efficiency of at least 50% to demonstrate a positive result, thus we estimate the efficiency to be above this threshold. Note the smaller mother bands in the edited samples (on the left of the image), which may indicate a larger deletion associated with CRISPR/Cas9 editing of the gene. This could be confirmed by a sequencing-based technique.

a

CCAGCACCTCCATGTACTTCAAGCAGTCCCCACCGTCCACCCCCACCACGCCGGCCTTCCCCCCGCAGGC
GGGGGCGTTATGGGACGAGGCACTGCCCTCGGCGCCCGGCTGCATCGCACCCGGCCCGCTGCTGGACC
CGCCGATGAAGGCGGTCCCCACGGTGGCCGGCGCGCGCTTCCCGCTCTTCCACTTCAAGCCCTCGCCGC
CGCATCCCCCCGCGCCCAGCCCGGCCGGCGGCCACCACCTCGGCTACGACCCGACGGCCGCTGCCGCGC
TCAGCCTGCCGCTGGGAGCCGCAGCCGCCGCGGGCAGCCAGGCCGCCGCGCTTGAGAGCCACCCG|TA
CGGGCTGCCGCTGGCCAAGAGGGCGGCCCCGCTGGCCTTCCCGCCTCTCGGCCTCACGCCCTCCCCTAC
CGCGTCCAGCCTGCTGGGCGAGAGTCCCAGCCTGCCGTCGCCGCCCAGCAGGAGCTCGTCGTCTGGCG
AGGGCACGTGTGCCGTGTGCGGGGACAACGCCGCCTGCCAGCACTACGGCGTGCGAACC

b

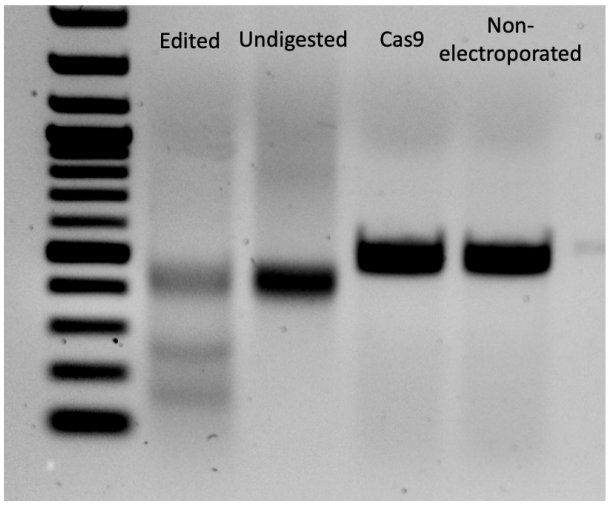
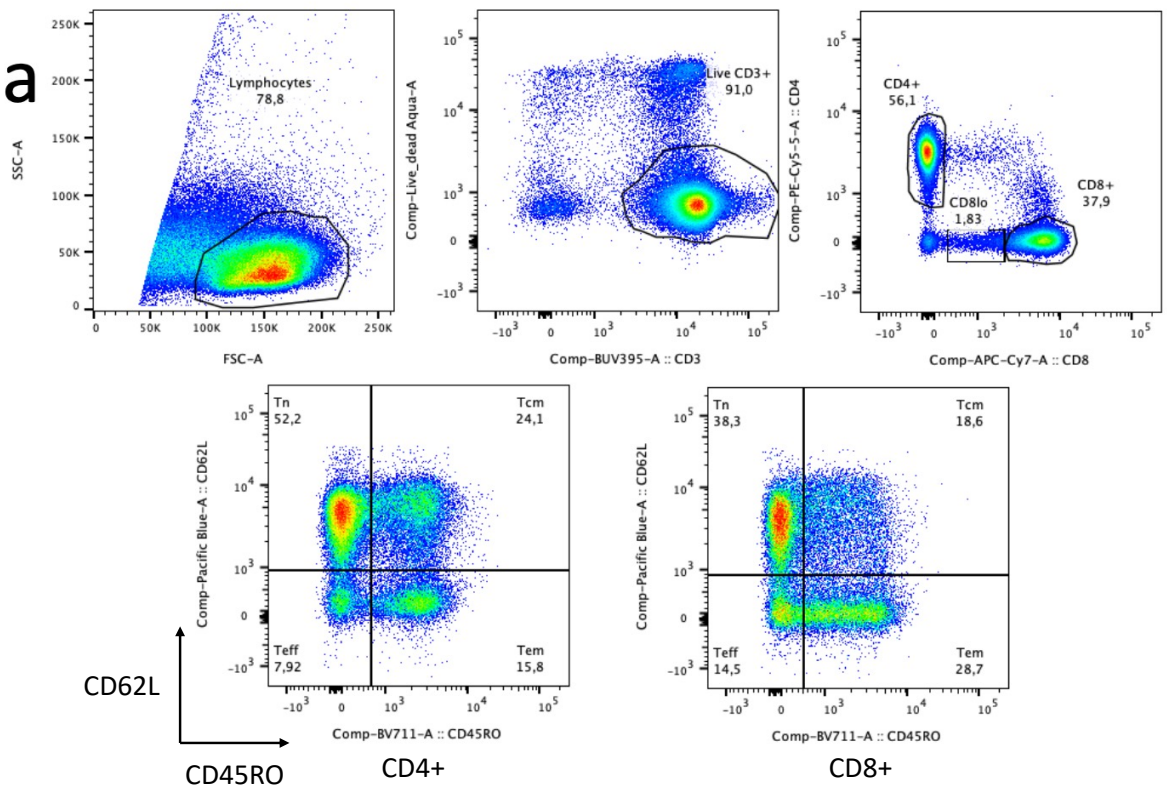


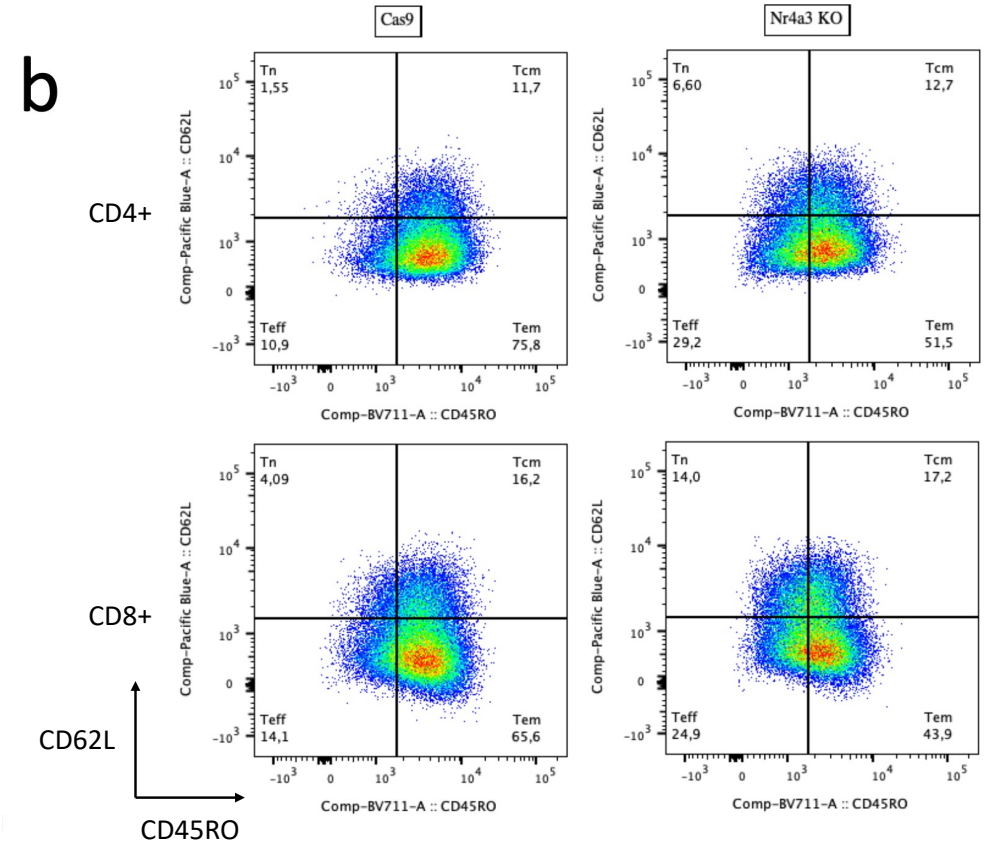
Figure 9. – T7E1 assay for validation of successful CRIPSR-Cas9 KO of NR4A3 a) Sequence of Nr4a3 exon 4 gDNA amplicon used for T7E1 assay, with sequence targeted by sgRNA highlighted in teal and cut site marked with a bold bar b) Representative image of T7E1 endonuclease assay demonstrating successful Nr4a3 editing. Enriched human T cells were nucleoporated with Cas9/sgRNA RNPs targeting the Nr4a3 locus, stimulated using activating anti-CD3/CD28 antibodies, then collected and gDNA amplified for DNA containing the sgRNA target site. Resulting amplicons were then incubated with T7E1 endonuclease, which cleaves dsDNA at sites of base mismatches, indicating the presence of indels. The edited sample (column 1) demonstrates cleavage of the amplicon with daughter bands of the predicted sizes, indicating that indels occurred at the predicted site, while unedited controls (columns 3 and 4) are not digested, indicating that digestion is specific to the edited sample. An undigested amplicon of the edited sample (column 2) is smaller than the

unedited samples, which may indicate a larger deletion occurred in addition to smaller indels.

NR4A3 KO in Human T Cells is Not Associated with an Alteration in Memory Differentiation

Next, we aimed to examine the memory differentiation of human T cells under conditions of serial non-specific stimulation using activating anti-CD3 and -CD28 antibodies. Briefly, magnetically enriched T cells from healthy human blood donors were nucleoporated with a complete RNP containing Cas9 and the selected sgRNA targeting Nr4a3, mock nucleoporated with Cas9 alone, or resuspended in media without nucleoporation as a non-electroporated control. These were rested for three days in IL-7 containing media then stimulated activating anti-CD3 and -CD28 antibodies in 96 well plates. Restimulation was performed every five to seven days for a total of three stimulations. Cells were collected and stained for memory subsets before each restimulation. CD45RO and CD62L were used to define memory subsets, with T_n being $CD45RO^-/CD62L^+$, T_{eff} being $CD45RO^-/CD62L^-$, T_{CM} being $CD45RO^+/CD62L^+$, and T_{EM} being $CD45RO^+/CD62L^-$ (see figure 10a for gating strategy). No difference in memory subset differentiation was observed between Cas9 electroporated negative controls and NR4A3 KO cells (see figure 10b-d) at any of the time points (n=5).


a
SSC-A
FSC-A
Lymphocytes
78,8
Comp-Live_dead Aqua-A
Comp-BUV395-A :: CD3
Live CD3+
91,0
Comp-PE-Cy5-5-A :: CD4
Comp-APC-Cy7-A :: CD8
CD4+
56,1
CD8lo
1,83
CD8+
37,9
Comp-Pacific Blue-A :: CD62L
Comp-BV711-A :: CD45RO
Tn
52,2
Tcm
24,1
Teff
7,92
Tem
15,8
CD62L
CD45RO
CD4+
Tn
38,3
Tcm
18,6
Teff
14,5
Tem
28,7
CD8+


b
Cas9
Nr4a3 KO
CD4+
CD8+
CD62L
CD45RO
Tn
1,55
Tcm
11,7
Teff
10,9
Tem
75,8
Tn
6,60
Tcm
12,7
Teff
29,2
Tem
51,5
Tn
4,09
Tcm
16,2
Teff
14,1
Tem
65,6
Tn
14,0
Tcm
17,2
Teff
24,9
Tem
43,9
Comp-Pacific Blue-A :: CD62L
Comp-BV711-A :: CD45RO

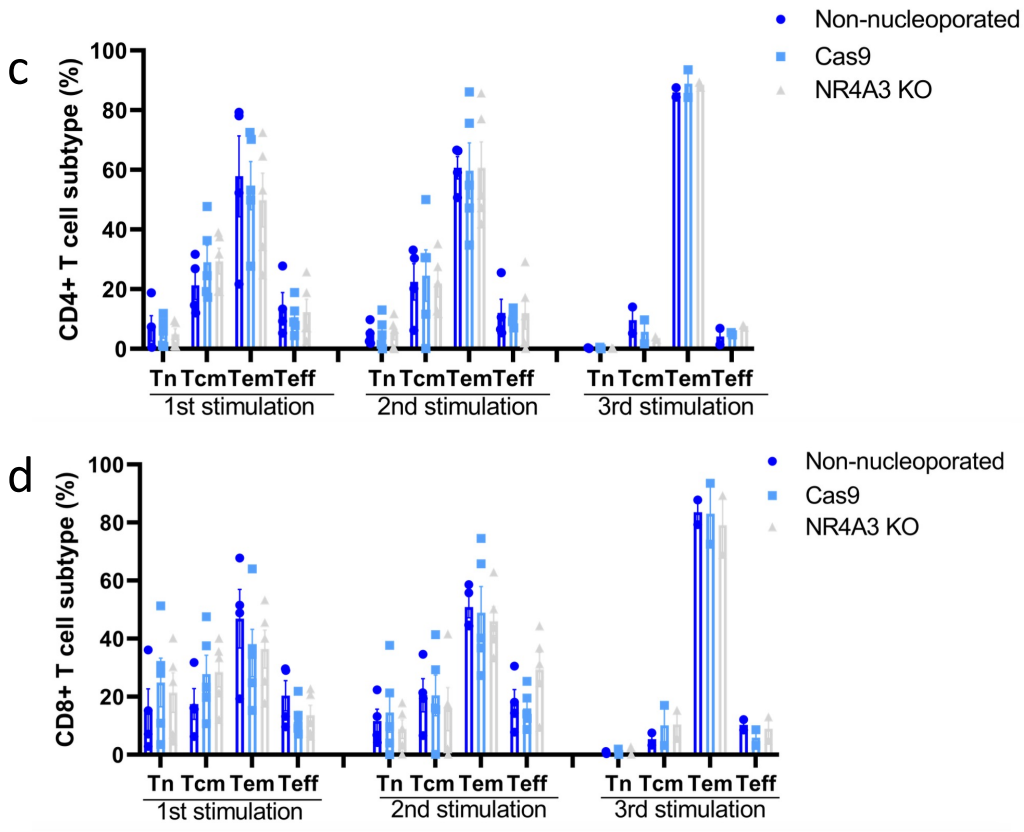
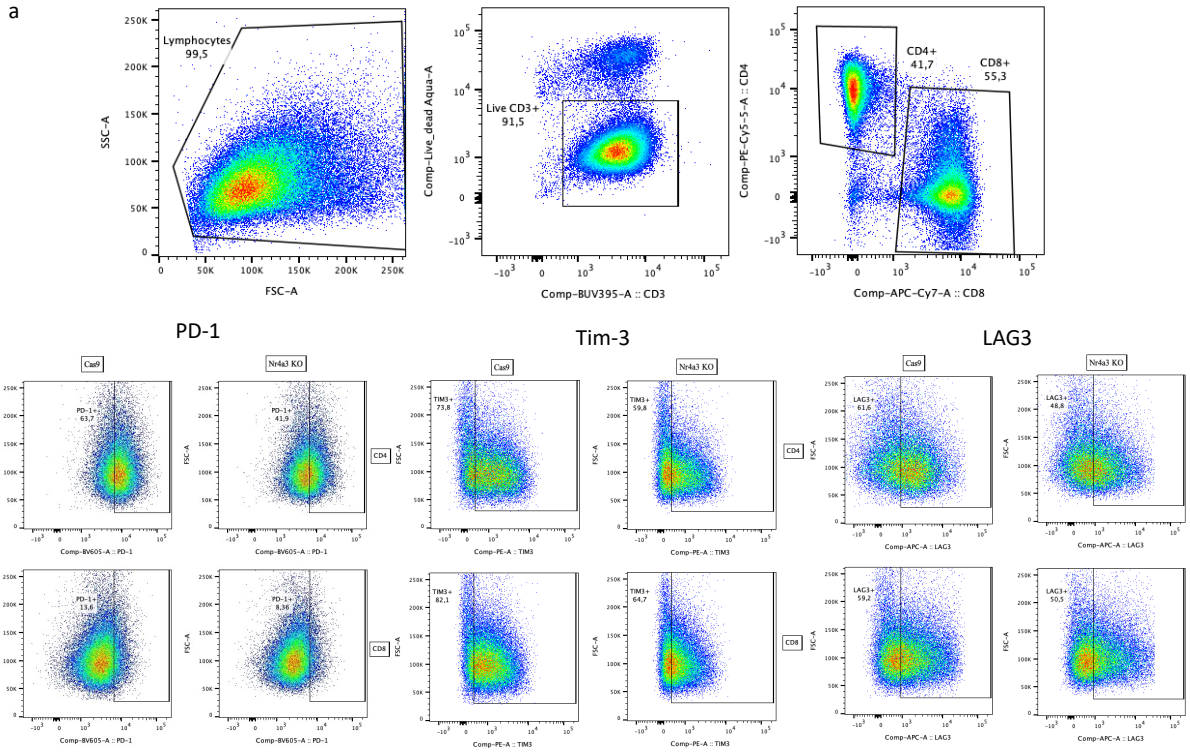


Figure 10. – Memory subsets after serial anti-CD3/CD28 stimulation (n=5). Enriched T cells from healthy human blood donors were nucleoporated with a complete RNP containing Cas9 and sgRNA targeting Nr4a3, mock nucleoporated with Cas9 alone, or resuspended in media as a non-nucleoporated control. These were rested for 3 days then serially stimulated with activating anti-CD3 and -CD28 antibodies every 5-7 days for a total of 3 stimulations. a) Representative gating strategy for memory subsets, showing memory subsets from both $CD3^+CD4^+$ and $CD3^+CD8^+$ gated cells. The $CD62L^+CD45RO^-$ quadrant is defined as T_n, the $CD62L^-CD45RO^-$ quadrant is defined as T_{eff}, the $CD62L^+CD45RO^+$ quadrant is defined as T_{CM}, and the $CD62L^-CD45RO^+$ quadrant is defined as T_{EM} b) Comparison of flow cytometry plots for memory differentiation between Cas9 nucleoporated and Nr4a3 KO of both $CD3^+CD4^+$ (upper plot) and $CD3^+CD8^+$ (lower plot) gated cells after 2 anti-CD3/CD28 stimulations, showing no difference in memory subsets c-d) Graphical compilation of memory differentiation after serial stimulation for live $CD3^+CD4^+$ (c) and live $CD3^+CD8^+$ (d) gated human T cells, again showing no difference in memory subsets after the first,

second, or third stimulations between the NR4A3 KO condition and the Cas9 and non-nucleoporated negative controls.

NR4A3 KO is Associated with a Decrease in Tim-3 Expression in Both CD4+ and CD8+ Human T Cells

Next, we aimed to examine the effect of NR4A3 KO on human T cell exhaustion after serial stimulation. The same serially stimulated cells as described above for memory differentiation were simultaneously stained for the exhaustion markers PD-1, Tim-3, and LAG3 after each stimulation (after the first, second, and third anti-CD3/28 stimulation every 5-7 days). While there was no change in PD-1 or LAG3 expression, there was a consistent decrease in the percentage of Tim-3^{+} CD3^{+}CD4^{+} and CD3^{+}CD8^{+} human T cells after 2 stimulations (see figure 11). This was done after 2 stimulations as surface expression of exhaustion markers may represent activation rather than exhaustion after 1 stimulation but will more accurately represent exhaustion after 2 stimulations as this better simulates conditions of chronic TCR stimulation that lead to exhaustion *in vivo*. While this was not statistically significant (p=0.0625 by Wilcoxon matched pair ranking for both CD4 and CD8 percentage), the trend is consistent between replicates.



b

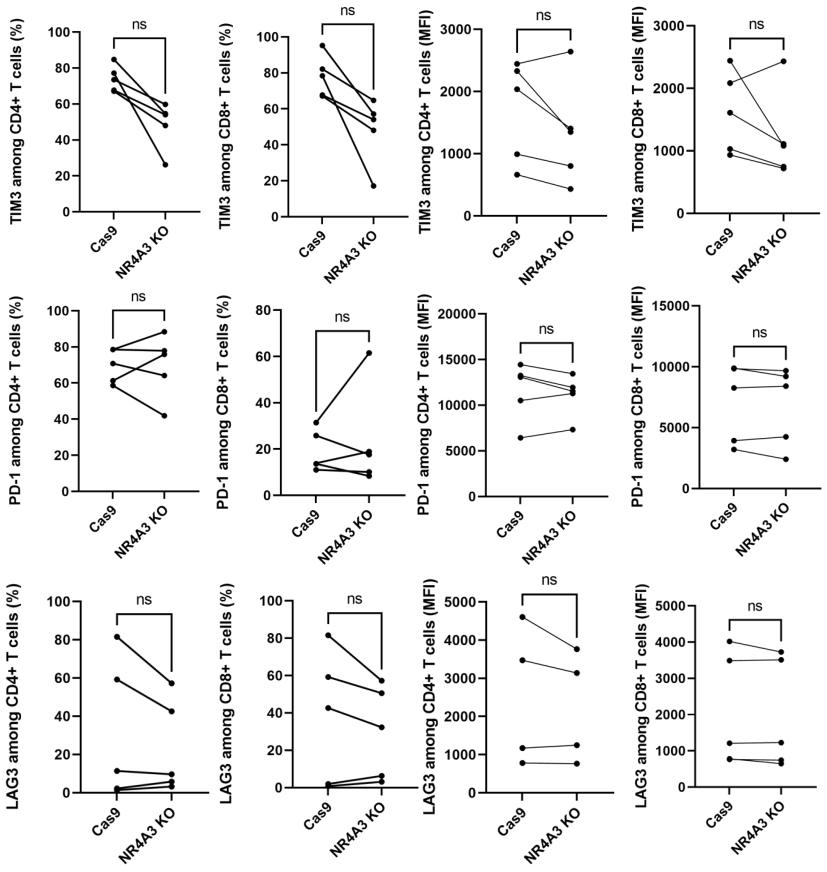


Figure 11. – Exhaustion markers after serial anti-CD3/CD28 stimulation (n=5). a) Representative gating strategy. Live $CD3^+CD4^+$ and $CD3^+CD8^+$ were surface stained for the exhaustion markers PD-1, Tim-3, and LAG3 after the first, second and third anti-CD3/28 stimulations. Shown here is a representative plot after the second stimulation. Both the percentage of cells staining positive and mean fluorescence intensity (MFI) were calculated. Positive and negative populations were gated using fluorescence minus one (FMO) controls b) Graphical representation of PD-1, Tim-3, and LAG3 surface expression as percent positive and MFI after two anti-CD3/28 stimulations for live $CD3^+CD4^+$ and $CD3^+CD8^+$ human T cells.

NR4A3 KO is not Associated with a Change in Effector Cytokine Production after PMA/Ionomycin Stimulation

Effector cytokine production in serially stimulated human T cells was also measured after each CD3/CD28 stimulation as a measure of predicted effector function. Briefly, cells were collected,

counted, and activated by a four-hour restimulation with PMA/ionomycin in the presence of brefeldin A, followed by intracellular staining for IFN-γ, TNF-α, and IL-2, then captured by flow cytometry. No difference in IFN-γ, TNF-α, and IL-2 production was noted between Cas9 nucleoporated and NR4A3 KO live $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells as measured using the percentage of positive cells (gated using FMO controls) (see figure 12). Note that cytotoxicity could not be directly measured given the antigen independent nature of CD3/CD28 T cell stimulation and expansion, although in the future granzyme B and perforin could be measured.

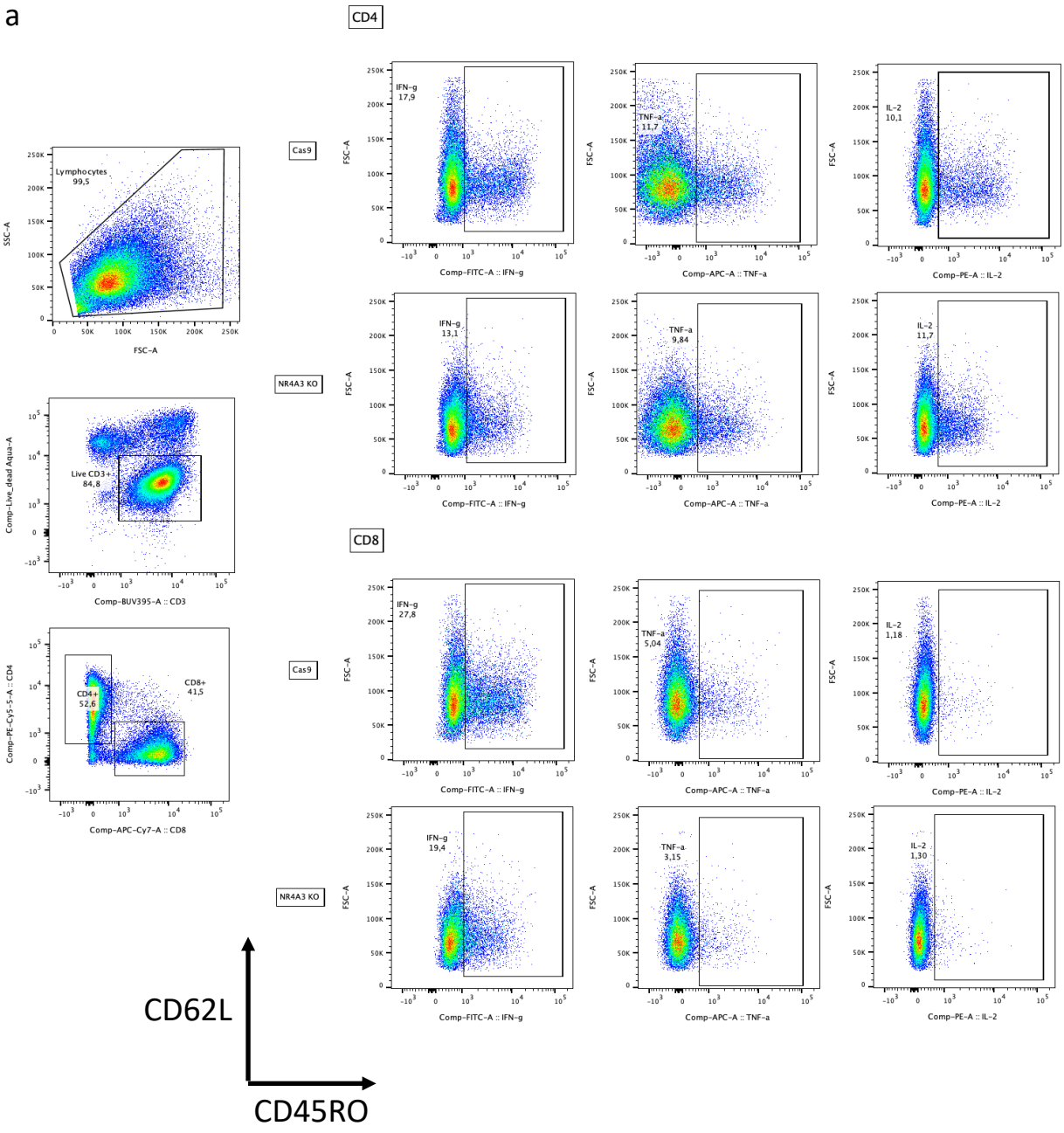

a
CD4
CD8
Cas9
NR4A3 KO
Lymphocytes
99,5
SSC-A
FSC-A
Live CD3+
84,8
Comp-Live_dead Aqua-A
Comp-BUV395-A :: CD3
CD4+
52,6
CD8+
41,5
Comp-PE-Cy5-5-A :: CD4
Comp-APC-Cy7-A :: CD8
IFN-g
17,9
TNF-a
11,7
IL-2
10,1
IFN-g
13,1
TNF-a
9,84
IL-2
11,7
IFN-g
27,8
TNF-a
5,04
IL-2
1,18
IFN-g
19,4
TNF-a
3,15
IL-2
1,30
Comp-FITC-A :: IFN-g
Comp-APC-A :: TNF-a
Comp-PE-A :: IL-2
FSC-A
CD62L
CD45RO
b
Cytokines in CD4+ T cells (%)
Cytokines in CD8+ T cells (%)
IFN-γ
TNF-α
IL-2
1st stimulation
2nd stimulation
3rd stimulation
Non-nucleoporated
Cas9
NR4A3 KO

Figure 12. – Effector cytokine production in Cas9 mock nucleoporated and NR4A3 KO human T cells after first anti-CD3/28 stimulation after four-hour restimulation with PMA/ionomycin (n=4) a) Gating strategy shown for IFN-γ, TNF-α, and IL-2 secretion in live $CD3^+CD4^+$ (upper panel) and $CD3^+CD8^+$ (lower panel) cells, as measured using percentage positive by intracellular flow cytometry (gated using FMO controls). Pre-gated to remove doublets (not shown) b) Graphical representation of cytokine secretion by serially stimulated non-nucleofected, Cas9 mock nucleofected, and NR4A3 KO T cells after 1, 2, and 3 anti-CD3/CD28 stimulations and four-hour restimulation with PMA/ionomycin, as measured using percentage positive by intracellular flow cytometry. The left panel represents $CD3^+CD4^+$ human T cells, while the right panel represents $CD3^+CD8^+$ human T cells.

EBV-Specific T cells Were Successfully Expanded using a Rapid Expansion Protocol

Donor antigen-specific T cells were successfully expanded using PBMCs pulsed with EBNA1 and LMP2 peptide libraries and were found to be reactive to these peptide libraries by EliSpot, starting with 4 x 10^6 PBMC in a 24-well G-Rex (see figure 14). However, attempts to expand antigen-specific T cells incorporating nucleofection of unenriched PBMCs and same-day stimulation by pulsing PBMCs with the same EBNA1 and LMP2 peptide libraries immediately after nucleofection were unsuccessful (see figure 13). Thus, no comparison of EBV-specific NR4A3 KO and wildtype cells could be performed. However, non-nucleoporated expanded T cells were successfully restimulated using peptide loaded, irradiated, autologous PHA blasts, with a 6.7-fold expansion obtained one week after restimulation with these blasts. The successfully expanded non-nucleoporated T cells were further found to produce IFN-γ on reexposure to the EBNA1 peptide library, but not LMP2, by EliSpot at day 14, confirming that the expanded cells were responsive to at least one of the peptide libraries used to expand cells in the G-Rex (see figure 14).

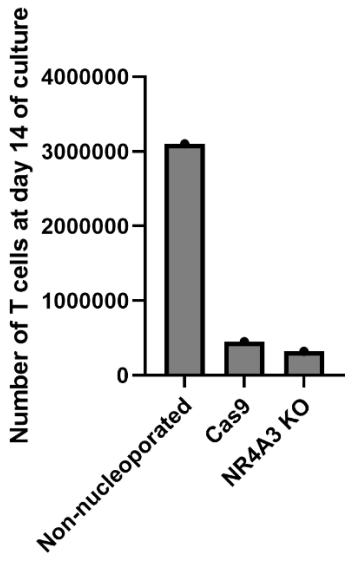


Figure 13. – Growth of EBV-specific T cells after 14 days of culture in a 24-well G-Rex following same-day nucleofection and pulsing with EBNA1 and LMP2 peptide libraries (starting number of cells 4 x 10^6 PBMC, n=1)

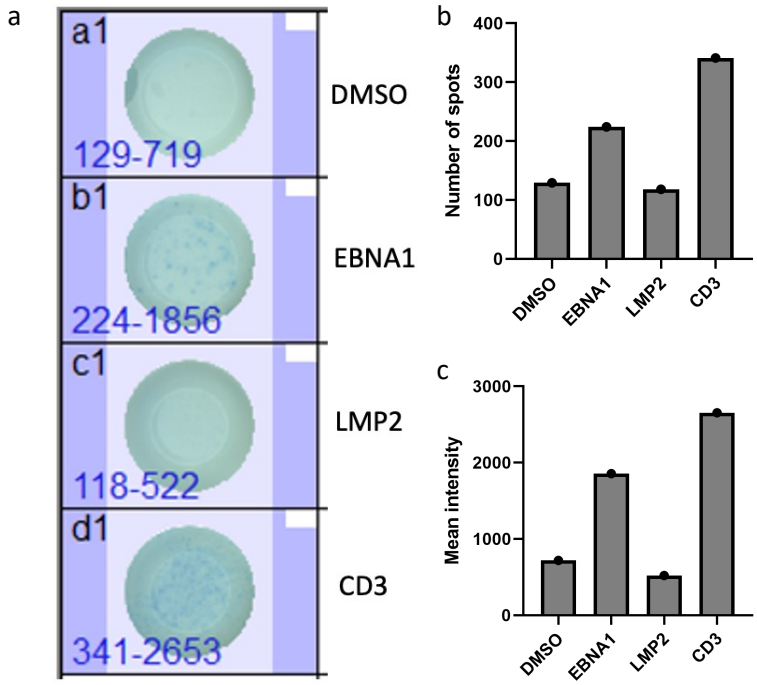


Figure 14. – EliSpot of EBV-specific T cells obtained after 14 days of culture in G-Rex demonstrating reactivity to EBNA1, but not LMP2 (a) and graphical representation of

number of spots (b) and mean intensity (c) obtained by EliSpot (n=1). DMSO is a negative control with the peptide library diluent, EBNA1 and LMP2 are the wells containing the respective peptide libraries, and CD3 is an anti-CD3 positive control.

NR4A1 and NR4A2 KO Were Successfully Performed on Activated T Cells

To test candidate sgRNAs targeting *NR4A1* and *NR4A2*, donor PBMCs were stimulated with anti-CD3/28 antibodies for three days to activate the contained T cells followed by nucleofection with candidate guides and Western blot after an additional four days of incubation. For NR4A1, the candidate sgRNAs were AA: ACCTTCATGGACGGCTACAC; AB: TCGGTGCTGGTGTCCCATAT; and AC: TCGGTGCTGGTGTCCCATAT. For NR4A2, the candidate sgRNAs were AA: GATCCCGGGTCGTCCCACAT and AB: GGGCTTGTAGTAAACCGACC.This was performed on activated rather than resting T cells to ensure optimal nucleofection and knockout for sgRNA validation and selection purposes. Evaluation with Western blot was performed after four-hour restimulation with anti-CD3 antibody (required as both proteins are poorly expressed at rest but rapidly induced after TCR activation) at day 7 since reliable antibodies were available for both NR4A1 and NR4A2 (see figure 15). Based on these results guide AC was chosen for NR4A1 and AA for NR4A2.

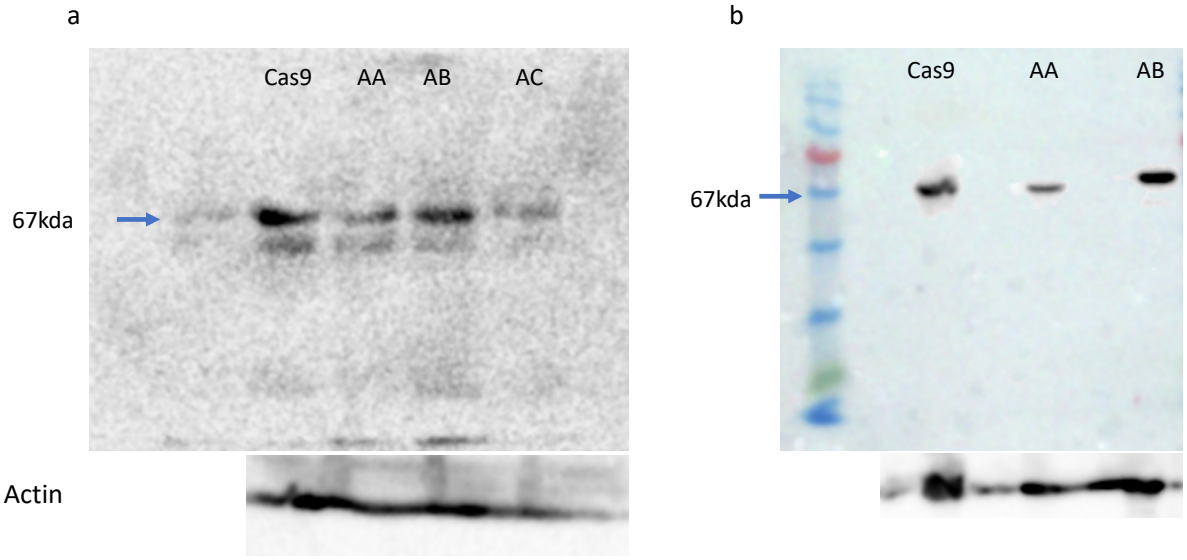


Figure 15. – Western blot images used for validation of candidate sgRNAs for a) NR4A1 KO with the Cas9 mock nucleoporated control on the left, followed by RNPs containing Cas9 and each sgRNA being validated (AA, AB, and AC) and b) NR4A2 KO with Cas9 mock

nucleoporated control on the left, followed by RNPs containing Cas9 and each sgRNA being validated (AA and AB). Actin loading control is shown below each blot. 2 x 10^6 enriched human T cells were activated with anti-CD3/CD28 antibodies then nucleofected after 3 days with either Cas9 alone or RNP containing Cas9 and one the sgRNAs being tested. After 4 more days these cells were collected, stimulated for 4 hours with anti-CD3 antibody, then protein lysates were collected and run on a SDS-PAGE gel for Western blotting.

Chapter 7 – Discussion and Conclusion

NR4A3 KO in human T cells after non-specific serial stimulation with anti-CD3/28 activating antibodies did not result in preferential differentiation into T_{CM} over T_{EM} or increase effector cytokine production as expected. What was noted was a consistent decrease in Tim-3 among all replicates; while this was not statistically significant, this was likely due to sample size, and adding replicates will likely lead to a statistically significant result. Unfortunately, these hypotheses could not be evaluated in antigen-specific T cells due to technical difficulties with integrating nucleofection into the rapid expansion process, so antigen-specific cytotoxicity could not be assessed. While not antigen specific, cytotoxic potential could be indirectly measured in the future by measuring granzyme B and perforin. This is in contrast to what was previously observed after adoptive transfer of NR4A3 KO OT-I cells in an acute Lm-OVA infection model, which differentiated preferentially into T_{CM} and produced more effector cytokines (117).

The consistent decrease in Tim-3 expression after two serial stimulations in NR4A3 KO is quite intriguing in the context of more recent models of the development of exhaustion in the setting of chronic antigen stimulation. This is similar to what was previously described in mice by Odagiu et al, who observed downregulation of *Havcr2* (encoding Tim-3) in NR4A3 KO OT-I cells in an acute Lm-OVA infection model (117). Chen *et al* also reported a decrease in both PD-1 and Tim-3 expression in NR4A triple KO tumour infiltrating murine CAR-T cells, but Tim-3 expression was more significantly decreased. A concurrent decrease in both *Pdcd1* (encoding PD-1) and *Havcr2* transcripts was also observed (121). Finally, Liu *et al* reported a decrease in the proportion PD-1^{+}Tim-3^{+} OT-II ($CD4^{+}$) cells with NR4A1 KO during chronic LCMV (clone 13) infection in mice (116). Taken together, the decrease in Tim-3 that we observed is consistent with the previously published murine data supporting a role for NR4A family members in the development of exhaustion, and particularly their association with Tim-3 expression.

Tim-3 expression is known to be particularly associated with the development of intermediate and terminal T_{ex}, while PD-1 is expressed on both precursor T_{ex} and terminal T_{ex} (104). Thus, it is

possible that NR4A3 KO restricts T_{ex} differentiation into terminally exhausted cells, which may mean that there is a larger pool of precursor T_{ex} that could be rescued by checkpoint blockade. Indeed, Odagiu et al observed preferential differentiation of murine NR4A3 KO OT-I cells infiltrating into an ovalbumin expressing tumour into SLAMF6^{hi}Tim-3^{lo}, while competitively transferred NR4A3 WT OT-I cells differentiated into SLAMF6^{lo}Tim-3^{hi} terminally exhausted T cells (208). This could be further explored by adding SLAMF6 as a surface marker in future human experiments, by performing intracellular flow cytometry for TCF-1, or by measuring *Tcf1* expression by RT-PCR or RNA sequencing (either bulk or single cell) to better characterize potential precursors (105).

Possible Biological Explanations for Observed Results

Differences Between Murine Models and Donor Human T Lymphocytes

Although the basic principles of T cell development, memory differentiation, and exhaustion are conserved between mice and humans, the overall phenotypic differences between mice and humans can be quite striking. While mice are convenient models for mammalian physiology and pathophysiology, data must be interpreted with caution when it is extrapolated to humans (10). Thus, NR4A3 may simply have different effects in mouse T lymphocytes than in humans, although limited human data indeed suggests that the NR4A family indeed plays a similar role in human T lymphocytes (120, 125). This is the crux of why we pursued this project, as findings in mice must be rigorously validated in humans.

Another key difference is the genetic homogeny of inbred mouse strains, compared to the genetic heterogeneity of human donors. Laboratory mouse strains have been extensively backcrossed to ensure genetic homogeny and increase consistency of results. Indeed, it is possible that the same mouse experiments performed on a different strain may have produced different results. However, human blood donors are a diverse group that are taken from the general population without the artificial selection that occurs in mouse models. Thus, human samples often demonstrate significant inter-donor variability that can make data difficult to interpret and can dilute the effect of a given intervention.

An additional important element is the use of murine T cells with a single TCR. In human donors, genetic differences in HLA types can produce variable response to the same epitope or result in different responses to different epitopes in different hosts from the same immunodominant antigen. Indeed, some antigens may be MHC-I dominant in some hosts resulting in a predominantly CD8+ T cell response, and MHC-II dominant in others resulting in a predominantly CD4+ T cell response. This may lead to more heterogenous responses in humans, particularly if the effect of NR4A3 KO is differential between $CD8^+$ and $CD4^+$ cells. This is largely unknown the previous murine studies involving NR4A3 KO were performed using adoptive transfer of only $CD8^+$ T cells, with only Liu *et al* studying $CD4^+$ OT-II cells (116, 117, 121, 208).

There are also significant phenotypic differences between mouse models and adult human donors. Laboratory mice spend their entire lives in SPF facilities without significant exposure to environmental organisms or pathogens, which is particularly important for mice with transgenic TCRs that are extremely immunosuppressed. However, adult human donors have an entire lifetime of pathogen exposures behind them, which influences their memory repertoire. While transgenic mouse models can be assumed to have an entirely naïve T cell repertoire, human donors have significant circulating memory cells that reflect their heterogenous exposures. Since the type and magnitude early childhood exposures can significantly alter T cell development, and this cannot be controlled in human donors as in mice, this may lead to further donor heterogeneity that makes human results more difficult to interpret than those of mice raised in SPF facilities. Indeed, “dirty” pet store mice can produce a very different immune repertoire compared to those raised in SPF facilities, let alone the much longer lifetime of exposures that an adult human has experienced (10). It is important to note that rapid expansion protocols used to produce antigen-specific T cells from human donors will preferentially expand memory cells, so these factors become especially relevant when considering the translational implications of this work. Interindividual differences in the intestinal microbiome may also significantly impact the immune repertoire. Thus, the uncontrollable heterogeneity of exposures may also have partially

impacted our results compared to the homogenous, sheltered, controlled environment present in SPF animal facilities.

An important related concept is the difference between germline edits in knockout mouse models and somatic gene editing in adult human T cells. Every cell of an NR4A3 knockout mouse lacks NR4A3 activity from birth. Thus, every T cell is NR4A3 deficient the moment it sees antigen for the first time upon encountering its cogent antigen, for example during a model infection or cancer. However, in our experimental model, T cells are only knocked out after having fully developed into mature T cells in an adult immune system. Importantly, T cells may be committed to memory differentiation or to differentiate into a particular memory subtype from the first TCR engagement, so it is possible that circulating memory cells that are already antigen experienced will react differently to NR4A3 KO compared to naïve cells. This may explain why we did not see the same effect on memory subset differentiation as Odagiu *et al* working with an entirely naïve T cell population (117). This could be further explored by enriching donor T cells for naïve or memory cells using magnetic enrichment or cell sorting and performing KO on the enriched populations. However, this would again have significant translational disadvantages, as not only do antigen-specific rapid expansion protocols selectively expand memory cells, but current commercial CAR-T production processes also require T cell activation to facilitate CAR transduction. That said, Jung *et al* were able to produce significant functional improvement with *PRDM1* and *NR4A3* double KO even with activation and viral transduction of the CAR construct before CRISPR-mediated gene editing, which runs counter to this hypothesis (123).

Redundancy Between NR4A Family Members

An important area of consideration is potential redundancy between NR4A family members. As noted previously, there is significant homology between the DNA binding domain of all three members and they all target the same NBRE and NuRE motifs in mice (107). Both murine and human transcriptomic data similarly suggest that exhausted CD8+ T cells upregulate multiple members of the NR4A family simultaneously, however the individual transcripts that are co-expressed are quite variable between studies, with some demonstrating increases in *Nr4a2* and

Nr4a3 expression, while others demonstrated a simultaneous rise in *Nr4a1* and *Nr4a3* (99, 120, 122).

There is also significant evidence of functional redundancy. Notably, Chen *et al* found that NR4A TKO most significantly improved depth and durability of tumour response, although NR4A2 and NR4A3 single KO had a smaller but statistically significant improvement in survival and tumour control. That said, this group did not evaluate double KO of NR4A family members, so it is unknown whether one of these may be dispensable (121). Conversely, Liu *et al* did find that NR4A1 single KO reduced exhaustion in chronic LCMV (clone 13) infection in mice, however this model used $CD4^+$ OT-II cells rather than $CD8^+$ T cells (116). In human CAR-T cells, Jung *et al* intriguingly showed that both *Nr4a1* and *Nr4a3* transcripts were upregulated in the context of BLIMP-1 KO, however they chose to target NR4A3 as it was more significantly upregulated. They demonstrated that NR4A3 single KO had no effect on CAR-T exhaustion or efficacy, however they did demonstrate a synergy in a multiplex KO of BLIMP-1 and NR4A3 (123). This once again raises the question of synergy or redundancy not just of members of the NR4A family with each other, but also with other pro-exhaustion and pro-effector TFs, which can be overcome by multiplex editing. However, the fact that *Nr4a1* and *Nr4a3* transcripts increased simultaneously suggests that they may be functionally redundant, which makes both interesting targets for multiplex editing.

If TKO is known to result in significant functional improvements in CD8+ CAR-T cells, why should we care if an individual member of the NR4A family is dispensable? Beyond the obvious scientific value of better understanding NR4A family function in human T cells, there are also significant translational advantages to defining a more limited number of TFs to be edited. One is the possibility of off-target effects during CRISPR/Cas9 editing, which are of course increased when more sgRNAs are introduced. On top of the standard off-target risks, simultaneous editing using double-stranded breaks can result in chromosomal translocations. The more simultaneous double-stranded breaks, the more opportunities for chromosomal translocations, which would introduce serious safety risks for an eventual clinical grade product. While this risk can be

mitigated through editing techniques such as sequential editing with amphiphilic peptides, use of nickases, or use of catalytically dead Cas enzymes with base- or prime-editing, fewer targets nonetheless means less off-target risk.

Another potential advantage concerns the function of CD4+ T cells. NR4A2 KO is known in murine models to have a tolerogenic effect by reducing CD4+ T cell effector functions. Notably, NR4A2 KO decreases severity of EAE, an experimental model of CD4+ T cell-mediated autoimmune disease (124-126). Thus, it is possible that NR4A TKO will have a deleterious effect on CD4+ T cell function in an eventual cellular therapy through NR4A2 KO, and it would be advantageous to perform only a NR4A1/3 double KO if NR4A2 is dispensable in CD8+ T cells. While much attention is often paid to CD8+ effector cells in the cellular therapy world, CD4+ T cells are known to be required for cellular persistence and long-term efficacy. In fact, a recent publication demonstrated CD4+ effector-like CAR-T cell persistence ten years after adoptive transfer in two patients with durable disease control, which further demonstrates the importance of CD4+ CAR-T cells in long-term outcomes (209). This could also be critical to the success of expanded products in individual donors who respond more to MHC-II restricted epitopes, who would be more dependent on CD4+ T cell function for the product to be effective.

Possible Technical Explanations for Observed Results

Sub-Optimal CRISPR/Cas9 Editing

One significant disadvantage in CRISPR/Cas9 gene editing is that no edit will ever be 100% efficient, leading to the inevitable presence of unedited cells in the experimental population. In circumstances where a reliable antibody exists for the target, flow cytometry studies can be gated for the KO population, or sorting can be used if it is a surface marker to ensure a homogenous population. However, in our case, since NR4A3 is intracellular and no reliable antibody could be found, these sorting and gating strategies could not be used in our experimental design. As a result, it is impossible to say whether an individual cell being analyzed is edited or unedited. Furthermore, since T7E1 is a largely qualitative technique, a precise editing efficiency cannot be

determined. One strategy to mitigate this would be to use a Sanger sequencing-based technique such as TIDE or ICE to calculate on-target editing efficiency more precisely.

Another concern related to editing efficiency is that NR4A3 KO could introduce an initial proliferative disadvantage compared to WT, and the NR4A3 unedited population, however small, could initially outcompete NR4A3 KO. That said, Odagiu *et* al found that NR4A3 KO OT-I cells outcompeted wild type cells in a murine tumour model after competitive transfer (208). One way to mitigate this concern would be to perform KO validation using a quantitative method at baseline and at the end of the culture to determine whether population dynamics lead to a loss of NR4A3 KO cells over time. Another way to overcome the lack of an antibody would be to use a knock-in of a fluorescent marker such as GFP as an HDR template rather than NHEJ to introduce the KO. While efficiency would be lower than with a NHEJ-based KO, this would allow gating or sorting to ensure that the analyzed population is homogenously KO. A simpler technique could also be to introduce a shRNA with a fluorescent marker, however as discussed previously this would require pre-activation of the T cell to allow viral transduction and could adversely affect the results.

Additionally, electroporation is quite traumatic to T cells and may more severely affect more delicate subpopulations, which could skew our results by destroying the subpopulations we wish to expand. This is particularly true in unstimulated T cells as in our experimental design. In a 2018 pre-print, which was never subsequently published in a peer-reviewed journal, differential effects on transfection efficiency and cellular survival were observed among naïve and memory CD4+ T cell subsets, with T_{CM} having higher transfection efficiency but lower survival (210). While this data has not been peer-reviewed, it does raise the possibility that the use of electroporation to transduce RNP may indeed have adversely affected our results. Unfortunately, a clear alternative to electroporation to deliver the gene edit is elusive, as viral transduction would require T cell activation (negating the advantage of editing unstimulated T cells), nanoparticles are toxic to T cells, and microinjection would be grossly inefficient. An amphiphilic peptide could potentially be

an option in the future given recent data supporting their use in primary human T cell editing (138).

Antigen-Independent T Cell Activation

The use of antigen-independent T cell activation for serial stimulation itself is not physiologic and may lead to stronger post-TCR and co-stimulatory signaling than antigen-specific activation by target cells or APCs. This in turn may lead to faster differentiation into T_{eff} or T_{EM} or faster induction of exhaustion due to stronger TCR signalling, thus skewing our data. Another technical factor to consider is that cytokine production was measured after TCR-independent pharmacological stimulation with PMA/ionomycin. Odagiu *et al* found an increase in cytokine production when they used ovalbumin peptide stimulation to measure cytokines in NR4A3 KO OT-I cells, but observed no difference when PMA/ionomycin pharmacological stimulation was used (117). As a result, it is possible that NR4As do not interact with an unidentified partner in the post-TCR cascade, thus negating the effect of NR4A3 KO on cytokine production. However, it should be noted that PMA/ionomycin stimulation strongly induces NR4A family expression, which would not support this hypothesis. Finally, because of antigen-independent activation, we were unable to assess specific cytotoxicity which would require antigen-specific T cells.

Inability to Produce EBV-Specific NR4A3 KO T Cells

Integration of a same-day electroporation step into the rapid expansion workflow using unenriched PBMCs failed to produce antigen-specific T cell expansion. This may have occurred due to the toxicity of electroporation itself differentially affecting APCs present within the PBMC pellet, thus abrogating peptide presentation, or memory T cells which are selectively expanded using the rapid expansion protocol. Also, electroporation and TCR stimulation in short succession may have been too traumatic and thus resulted in excess mortality. Finally, electroporation of unselected PBMCs would introduce NR4A3 KO into all the cells within the mix, including APCs, and may have been deleterious for their antigen presenting function, thus resulting in a failure to expand antigen-specific cells. In fact, Boulet *et al* showed that NR4A3 is essential for differentiation of monocyte-derived dendritic cells (MoDCs) after microbial stimulation, which suggests that it may play an important role in other APCs (211).

Genetic heterogeneity of human donors may also explain why variable responses to the same EBNA1 and LMP2 peptide libraries are observed, as in our case where the donor had a good EliSpot response to EBNA1 but not to LMP2. Since pooled cells containing both CD4+ and CD8+ cells are used to perform EliSpots, the fact that peptide libraries are used rather than APCs so it is impossible to determine whether the given antigens are MHC-I or MHC-II restricted, and since we could only differentiate CD4+ and CD8+ cytokine-producing cells using a flow cytometry-based method, we cannot determine which cells (CD4+ vs CD8+) are producing IFN-γ in this assay. A way to answer this question in the future could be to perform peptide library stimulation followed by intracellular flow cytometry to evaluate cytokine production, which would allow differentiation of the cell type in antigen-specific T cells, would allow determination of the effect on production of other effector cytokines such as TNF-α or IL-2, and would allow evaluation of polyfunctionality in antigen-specific T cells.

Perspectives and Subsequent Experiments

Multiplex Editing of Multiple NR4A Family TFs

Given the significant biological plausibility and experimental evidence of functional redundancy, multiplex editing of NR4A family TFs is a high priority. This will be done in a combinatorial fashion to properly evaluate potential dispensability of one of the NR4A family TFs. Thus, the previously described non-specific serial stimulation workflow will be performed with NR4A1, NR4A2, and NR4A3 single KO, NR4A1/2, NR4A1/3 and NR4A2/3 double KO, and TKO enriched human T cells. This will establish which combinations produce the desired phenotypic and functional effects to select the appropriate multiplex KO for further development. Cytokine production will be measured after non-specific TCR stimulation with anti-CD3 antibody rather than PMA/ionomycin in case of interaction with an unidentified partner within the post-TCR cascade. The NR4A1/2 antibody and KO validations that have already been performed will greatly accelerate this experiment.

This multiplex editing faces the risks discussed in chapter 2, particularly when considering translational applications. Simultaneously introducing more than one sgRNA increases the risk of off target editing, particularly whole chromosome translocations due to the introduction of more than one DSB on different chromosomes (chromosomes 2, 9, and 11 for the NR4A family, for example). This could have unforeseen consequences, even oncogenesis, that need to be carefully considered if this product is to be one day administered to humans. Deep sequencing techniques (such as GUIDE-Seq) will need to be applied to any resulting product before releasing it for administration. Ways to mitigate this risk would be sequential editing using a less toxic delivery method such as an amphiphilic peptide, or by using an editing method that does not rely on DSBs, such as base editing.

Bulk RNA-seq and ATAC-seq will also be performed on promising combinations to further understand how these multiplex KOs affect cellular and molecular processes within the T cell. These may also identify compensatory upregulation of other TFs as potential future targets for multiplex editing. As mentioned previously these experiments will also include SLAMF6 as a marker to evaluate T_{ex} precursors and sequencing-based techniques to quantify CRISPR editing efficiency after the first activation and at the end of the culture. Of course, it will also be critical to assess off-target editing given the notable risk of chromosomal translocation.

NR4A3 KO in Selected Naïve and Memory T Cells

Naïve and memory T cells will either be magnetically enriched or sorted using flow cytometry followed by NR4A3 KO in the selected populations to further evaluate the differences between NR4A3 function in naïve and memory cells. These experiments will also incorporate RNA-seq and ATAC-seq to further understand how NR4A3 acts differently in these T cell subsets. While these experiments will be biologically important, its use will be limited from a translational perspective as current clinical cellular production processes target activated or memory T cells for expansion, particularly rapid expansion protocols for VST production.

Antigen-Specific T Cell Production and Serial Stimulation

Further experiments will be performed to optimize introducing an electroporation step into the VST expansion protocol. Electroporation will be performed on unstimulated T cells, and these will be allowed to rest for three days in a G-Rex supplemented with IL-7 at 10ng/mL, analogous to the current workflow for non-specific stimulation. We hypothesize that allowing T cells to rest and recover after electroporation may be less traumatic than immediately stimulating them. Furthermore, peptide pulsed, irradiated DCs will be used as an APC as they are well-established in VST production as the most efficient APCs and will not be subjected to electroporation or NR4A KO, thus eliminating the possibility that electroporation or NR4A3 KO of the APCs affected their viability or function (194). Peptide pulsed, irradiated PHA blasts will be used for serial antigen-specific stimulation as they more closely resemble target cells than DCs and they resulted in successful expansion of VSTs in our experiments.

Should the above measures not be successful to produce KO antigen-specific T cells, another option would be to generate CAR-T cells. A viral transduction step could be introduced to the workflow three days after non-specific stimulation, followed by serial stimulation with antigen-expressing cell lines. While this would not be as physiologic as TCR-mediated serial stimulation, it would still represent a potentially translatable workflow for clinical studies and would be both antigen-specific and have a pre-defined target cell to measure cytotoxicity. Another advantage of generating CARs is that they could be further evaluated by adoptive transfer into mice inoculated with tumours expressing the target antigen, allowing for pre-clinical *in vivo* evaluation of the KO (121). From a translational perspective, using a CAR-T cell for pre-clinical studies would also be advantageous as they are already in extensive clinical use.

Conclusion

While NR4A3 KO did not result in preferential differentiation into T_{CM} or an increase in cytokine production as hypothesized, it did produce a consistent decrease in Tim-3 expression which may represent a decrease in terminal T_{ex}. These data, and other recent works that have examined the interaction between NR4A3 and other TFs, indicate that redundancy likely explains these results, and supports further work to identify and target its redundant partners through multiplex editing.

While we have not yet been able to produce KO VSTs, future experiments will optimize this workflow so that antigen-specific cells can be tested in the same manner. In the long term, we expect this work to result in clinical-grade anti-infectious and anti-cancer cellular immunotherapies that will provide patients with long term protection against infection and cancer relapse. This will allow transplant patients to live longer and better lives without the constant threat of viral reactivation and viral-associated cancers.

References

1. Fischer A, Notarangelo LD, Neven B, Cavazzana M, Puck JM. Severe combined immunodeficiencies and related disorders (Primer). Nature Reviews: Disease Primers. 2015;1(1).

2. Proksch E, Brandner JM, Jensen JM. The skin: an indispensable barrier. Exp Dermatol. 2008;17(12):1063-72.

3. Family L, Li Y, Chen LH, Page JH, Klippel ZK, Chao C. A Study of Novel Febrile Neutropenia Risk Factors Related to Bone Marrow or Immune Suppression, Barrier Function, and Bacterial Flora. J Natl Compr Canc Netw. 2018;16(10):1201-8.

4. Shishido SN, Varahan S, Yuan K, Li X, Fleming SD. Humoral innate immune response and disease. Clin Immunol. 2012;144(2):142-58.

5. Gao X, Ding J, Liao C, Xu J, Liu X, Lu W. Defensins: The natural peptide antibiotic. Adv Drug Deliv Rev. 2021;179:114008.

6. Murphy KW, C. Janeway's Immunobiology: 9th Edition. New York: Garland Science, Taylor & Francis Group, LLC; 2017.

7. Björkström NK, Strunz B, Ljunggren H-G. Natural killer cells in antiviral immunity. Nature Reviews Immunology. 2022;22(2):112-23.

8. Michelson DA, Hase K, Kaisho T, Benoist C, Mathis D. Thymic epithelial cells co-opt lineage-defining transcription factors to eliminate autoreactive T cells. Cell. 2022;185(14):2542-58.e18.

9. Lee H-G, Cho M-J, Choi J-M. Bystander CD4+ T cells: crossroads between innate and adaptive immunity. Experimental & Molecular Medicine. 2020;52(8):1255-63.

10. Masopust D, Sivula CP, Jameson SC. Of Mice, Dirty Mice, and Men: Using Mice To Understand Human Immunology. J Immunol. 2017;199(2):383-8.

11. Kim H, Kim M, Im SK, Fang S. Mouse Cre-LoxP system: general principles to determine tissue-specific roles of target genes. Lab Anim Res. 2018;34(4):147-59.

12. Kumar BV, Connors TJ, Farber DL. Human T Cell Development, Localization, and Function throughout Life. Immunity. 2018;48(2):202-13.

13. Moon JJ, Chu HH, Hataye J, Pagán AJ, Pepper M, McLachlan JB, et al. Tracking epitope-specific T cells. Nat Protoc. 2009;4(4):565-81.

14. Sullivan BM, Emonet SF, Welch MJ, Lee AM, Campbell KP, de la Torre JC, Oldstone MB. Point mutation in the glycoprotein of lymphocytic choriomeningitis virus is necessary for receptor binding, dendritic cell infection, and long-term persistence. Proc Natl Acad Sci U S A. 2011;108(7):2969-74.

15. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity. 2007;27(2):281-95.

16. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. Journal of Experimental Medicine. 2008;205(3):625-40.

17. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nature Immunology. 2003;4(12):1191-8.

18. Hand TW, Morre M, Kaech SM. Expression of IL-7 receptor α is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. Proceedings of the National Academy of Sciences. 2007;104(28):11730-5.

19. Chen Y, Zander R, Khatun A, Schauder DM, Cui W. Transcriptional and Epigenetic Regulation of Effector and Memory CD8 T Cell Differentiation. Front Immunol. 2018;9:2826.

20. Teixeiro E, Daniels MA, Hamilton SE, Schrum AG, Bragado R, Jameson SC, Palmer E. Different T Cell Receptor Signals Determine CD8+ Memory Versus Effector Development. Science. 2009;323(5913):502-5.

21. Sabins NC, Chornoguz O, Leander K, Kaplan F, Carter R, Kinder M, et al. TIM-3 Engagement Promotes Effector Memory T Cell Differentiation of Human Antigen-Specific CD8 T Cells by Activating mTORC1. The Journal of Immunology. 2017;199(12):4091-102.

22. Bally APR, Tang Y, Lee JT, Barwick BG, Martinez R, Evavold BD, Boss JM. Conserved Region C Functions To Regulate PD-1 Expression and Subsequent CD8 T Cell Memory. The Journal of Immunology. 2017;198(1):205-17.

23. Kallies A, Xin A, Belz GT, Nutt SL. Blimp-1 Transcription Factor Is Required for the Differentiation of Effector CD8⁺ T Cells and Memory Responses. Immunity. 2009;31(2):283-95.

24. Roychoudhuri R, Clever D, Li P, Wakabayashi Y, Quinn KM, Klebanoff CA, et al. BACH2 regulates CD8+ T cell differentiation by controlling access of AP-1 factors to enhancers. Nature Immunology. 2016;17(7):851-60.

25. Yao S, Buzo Bruno F, Pham D, Jiang L, Taparowsky Elizabeth J, Kaplan Mark H, Sun J. Interferon Regulatory Factor 4 Sustains CD8⁺ T Cell Expansion and Effector Differentiation. Immunity. 2013;39(5):833-45.

26. Milner JJ, Toma C, Yu B, Zhang K, Omilusik K, Phan AT, et al. Runx3 programs CD8(+) T cell residency in non-lymphoid tissues and tumours. Nature. 2017;552(7684):253-7.

27. Wang D, Diao H, Getzler AJ, Rogal W, Frederick MA, Milner J, et al. The Transcription Factor Runx3 Establishes Chromatin Accessibility of cis-Regulatory Landscapes that Drive Memory Cytotoxic T Lymphocyte Formation. Immunity. 2018;48(4):659-74.e6.

28. Takemoto N, Intlekofer AM, Northrup JT, Wherry EJ, Reiner SL. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. J Immunol. 2006;177(11):7515-9.

29. Danilo M, Chennupati V, Silva JG, Siegert S, Held W. Suppression of Tcf1 by Inflammatory Cytokines Facilitates Effector CD8 T Cell Differentiation. Cell Reports. 2018;22(8):2107-17.

30. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. Journal of Experimental Medicine. 2005;202(5):637-50.

31. Cui W, Joshi NS, Jiang A, Kaech SM. Effects of Signal 3 during CD8 T cell priming: Bystander production of IL-12 enhances effector T cell expansion but promotes terminal differentiation. Vaccine. 2009;27(15):2177-87.

32. Richer MJ, Pewe LL, Hancox LS, Hartwig SM, Varga SM, Harty JT. Inflammatory IL-15 is required for optimal memory T cell responses. The Journal of Clinical Investigation. 2015;125(9):3477-90.

33. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs that Promote the Differentiation of Effector Cytolytic T Cells. Immunity. 2010;32(1):79-90.

34. Blattman JN, Grayson JM, Wherry EJ, Kaech SM, Smith KA, Ahmed R. Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo. Nature Medicine. 2003;9(5):540-7.

35. Dominguez CX, Amezquita RA, Guan T, Marshall HD, Joshi NS, Kleinstein SH, Kaech SM. The transcription factors ZEB2 and T-bet cooperate to program cytotoxic T cell terminal differentiation in response to LCMV viral infection. Journal of Experimental Medicine. 2015;212(12):2041-56.

36. Guan T, Dominguez CX, Amezquita RA, Laidlaw BJ, Cheng J, Henao-Mejia J, et al. ZEB1, ZEB2, and the miR-200 family form a counterregulatory network to regulate CD8+ T cell fates. Journal of Experimental Medicine. 2018;215(4):1153-68.

37. Zhou X, Yu S, Zhao DM, Harty JT, Badovinac VP, Xue HH. Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1. Immunity. 2010;33(2):229-40.

38. Yu B, Zhang K, Milner JJ, Toma C, Chen R, Scott-Browne JP, et al. Epigenetic landscapes reveal transcription factors that regulate CD8+ T cell differentiation. Nature Immunology. 2017;18(5):573-82.

39. Rao Rajesh R, Li Q, Bupp Melanie RG, Shrikant Protul A. Transcription Factor Foxo1 Represses T-bet-Mediated Effector Functions and Promotes Memory CD8⁺ T Cell Differentiation. Immunity. 2012;36(3):374-87.

40. Scott-Browne JP, López-Moyado IF, Trifari S, Wong V, Chavez L, Rao A, Pereira RM. Dynamic Changes in Chromatin Accessibility Occur in CD8⁺ T Cells Responding to Viral Infection. Immunity. 2016;45(6):1327-40.

41. Chang JT, Palanivel VR, Kinjyo I, Schambach F, Intlekofer AM, Banerjee A, et al. Asymmetric T Lymphocyte Division in the Initiation of Adaptive Immune Responses. Science. 2007;315(5819):1687-91.

42. Akondy RS, Fitch M, Edupuganti S, Yang S, Kissick HT, Li KW, et al. Origin and differentiation of human memory CD8 T cells after vaccination. Nature. 2017;552(7685):362-7.

43. Smith NL, Patel RK, Reynaldi A, Grenier JK, Wang J, Watson NB, et al. Developmental Origin Governs CD8⁺ T Cell Fate Decisions during Infection. Cell. 2018;174(1):117-30.e14.
44. Martin MD, Badovinac VP. Defining Memory CD8 T Cell. Front Immunol. 2018;9:2692.
45. Gonzalez NM, Zou D, Gu A, Chen W. Schrödinger's T Cells: Molecular Insights Into Stemness and Exhaustion. Front Immunol. 2021;12:725618.
46. Graef P, Buchholz Veit R, Stemberger C, Flossdorf M, Henkel L, Schiemann M, et al. Serial Transfer of Single-Cell-Derived Immunocompetence Reveals Stemness of CD8⁺ Central Memory T Cells. Immunity. 2014;41(1):116-26.
47. Bachmann MF, Barner M, Viola A, Kopf M. Distinct kinetics of cytokine production and cytolysis in effector and memory T cells after viral infection. Eur J Immunol. 1999;29(1):291-9.
48. Veiga-Fernandes H, Walter U, Bourgeois C, McLean A, Rocha B. Response of naïve and memory CD8+ T cells to antigen stimulation in vivo. Nature Immunology. 2000;1(1):47-53.
49. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest. 2008;118(1):294-305.
50. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. Nat Med. 2011;17(10):1290-7.
51. Li Y, Wu D, Yang X, Zhou S. Immunotherapeutic Potential of T Memory Stem Cells. Frontiers in Oncology. 2021;11.
52. Di Benedetto S, Derhovanessian E, Steinhagen-Thiessen E, Goldeck D, Müller L, Pawelec G. Impact of age, sex and CMV-infection on peripheral T cell phenotypes: results from the Berlin BASE-II Study. Biogerontology. 2015;16(5):631-43.
53. Gordon CL, Miron M, Thome JJ, Matsuoka N, Weiner J, Rak MA, et al. Tissue reservoirs of antiviral T cell immunity in persistent human CMV infection. J Exp Med. 2017;214(3):651-67.
54. Abdelsamed HA, Moustaki A, Fan Y, Dogra P, Ghoneim HE, Zebley CC, et al. Human memory CD8 T cell effector potential is epigenetically preserved during in vivo homeostasis. J Exp Med. 2017;214(6):1593-606.

55. Durek P, Nordström K, Gasparoni G, Salhab A, Kressler C, de Almeida M, et al. Epigenomic Profiling of Human CD4(+) T Cells Supports a Linear Differentiation Model and Highlights Molecular Regulators of Memory Development. Immunity. 2016;45(5):1148-61.

56. Pulko V, Davies JS, Martinez C, Lanteri MC, Busch MP, Diamond MS, et al. Human memory T cells with a naive phenotype accumulate with aging and respond to persistent viruses. Nature Immunology. 2016;17(8):966-75.

57. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. Journal of Experimental Medicine. 1990;171(3):801-17.

58. Wherry EJ, Teichgräber V, Becker TC, Masopust D, Kaech SM, Antia R, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol. 2003;4(3):225-34.

59. Jabbari A, Harty JT. Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype. J Exp Med. 2006;203(4):919-32.

60. Kim J, Kim AR, Shin EC. Cytomegalovirus Infection and Memory T Cell Inflation. Immune Netw. 2015;15(4):186-90.

61. Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrançois L, Farber DL. Cutting Edge: Tissue-Retentive Lung Memory CD4 T Cells Mediate Optimal Protection to Respiratory Virus Infection. The Journal of Immunology. 2011;187(11):5510-4.

62. Zhu J, Koelle DM, Cao J, Vazquez J, Huang ML, Hladik F, et al. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. J Exp Med. 2007;204(3):595-603.

63. Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8αα+ skin-resident T cells in human herpes virus infection. Nature. 2013;497(7450):494-7.

64. Purwar R, Campbell J, Murphy G, Richards WG, Clark RA, Kupper TS. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. PLoS One. 2011;6(1):e16245.

65. Turner DL, Bickham KL, Thome JJ, Kim CY, D'Ovidio F, Wherry EJ, Farber DL. Lung niches for the generation and maintenance of tissue-resident memory T cells. Mucosal Immunol. 2014;7(3):501-10.

66. Pallett LJ, Davies J, Colbeck EJ, Robertson F, Hansi N, Easom NJW, et al. IL-2(high) tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. J Exp Med. 2017;214(6):1567-80.

67. Woon HG, Braun A, Li J, Smith C, Edwards J, Sierro F, et al. Compartmentalization of Total and Virus-Specific Tissue-Resident Memory CD8+ T Cells in Human Lymphoid Organs. PLoS Pathog. 2016;12(8):e1005799.

68. Mackay LK, Braun A, Macleod BL, Collins N, Tebartz C, Bedoui S, et al. Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. J Immunol. 2015;194(5):2059-63.

69. Shiow LR, Rosen DB, Brdičková N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature. 2006;440(7083):540-4.

70. Mackay LK, Minnich M, Kragten NA, Liao Y, Nota B, Seillet C, et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. Science. 2016;352(6284):459-63.

71. Kumar BV, Ma W, Miron M, Granot T, Guyer RS, Carpenter DJ, et al. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. Cell Rep. 2017;20(12):2921-34.

72. Vieira Braga FA, Hertoghs KM, Kragten NA, Doody GM, Barnes NA, Remmerswaal EB, et al. Blimp-1 homolog Hobit identifies effector-type lymphocytes in humans. Eur J Immunol. 2015;45(10):2945-58.

73. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon ML, et al. The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. Nat Immunol. 2013;14(12):1294-301.

74. Mackay LK, Wynne-Jones E, Freestone D, Pellicci DG, Mielke LA, Newman DM, et al. T-box Transcription Factors Combine with the Cytokines TGF-β and IL-15 to Control Tissue-Resident Memory T Cell Fate. Immunity. 2015;43(6):1101-11.

75. Hombrink P, Helbig C, Backer RA, Piet B, Oja AE, Stark R, et al. Programs for the persistence, vigilance and control of human CD8(+) lung-resident memory T cells. Nat Immunol. 2016;17(12):1467-78.

76. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature. 1993;362(6422):758-61.

77. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJD, Suresh M, Altman JD, Ahmed R. Viral Immune Evasion Due to Persistence of Activated T Cells Without Effector Function. Journal of Experimental Medicine. 1998;188(12):2205-13.

78. McLane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. Annual Review of Immunology. 2015;37(1):457-95.

79. Blank CU, Haining WN, Held W, Hogan PG, Kallies A, Lugli E, et al. Defining 'T cell exhaustion'. Nature Reviews Immunology. 2019;19(11):665-74.

80. Yamamoto R, Nishikori M, Kitawaki T, Sakai T, Hishizawa M, Tashima M, et al. PD-1–PD-1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. Blood. 2008;111(6):3220-4.

81. Fourcade J, Kudela P, Sun Z, Shen H, Land SR, Lenzner D, et al. PD-1 Is a Regulator of NY-ESO-1-Specific CD8+ T Cell Expansion in Melanoma Patients1. The Journal of Immunology. 2009;182(9):5240-9.

82. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, Rosenberg SA. Tumor antigen–specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood. 2009;114(8):1537-44.

83. Gehring AJ, Ho ZZ, Tan AT, Aung MO, Lee KH, Tan KC, et al. Profile of Tumor Antigen-Specific CD8 T Cells in Patients With Hepatitis B Virus-Related Hepatocellular Carcinoma. Gastroenterology. 2009;137(2):682-90.

84. Saito H, Kuroda H, Matsunaga T, Osaki T, Ikeguchi M. Increased PD-1 expression on CD4+ and CD8+ T cells is involved in immune evasion in gastric cancer. J Surg Oncol. 2013;107(5):517-22.

85. Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in immunotherapy. Blood. 2018;131(1):58-67.

86. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3(5):541-7.

87. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science. 1995;270(5238):985-8.

88. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 2006;439(7077):682-7.

89. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1–mediated inhibition. Science. 2017;355(6332):1428-33.

90. Rota G, Niogret C, Dang AT, Barros CR, Fonta NP, Alfei F, et al. Shp-2 Is Dispensable for Establishing T Cell Exhaustion and for PD-1 Signaling In Vivo. Cell Reports. 2018;23(1):39-49.

91. Frebel H, Nindl V, Schuepbach RA, Braunschweiler T, Richter K, Vogel J, et al. Programmed death 1 protects from fatal circulatory failure during systemic virus infection of mice. Journal of Experimental Medicine. 2012;209(13):2485-99.

92. Rangachari M, Zhu C, Sakuishi K, Xiao S, Karman J, Chen A, et al. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3–mediated cell death and exhaustion. Nat Med. 2012;18(9):1394-400.

93. Wolf Y, Anderson AC, Kuchroo VK. TIM3 comes of age as an inhibitory receptor. Nature Reviews Immunology. 2020;20(3):173-85.

94. Takumi M, Daisuke S, Il-mi O, Taku O. LAG-3: from molecular functions to clinical applications. Journal for ImmunoTherapy of Cancer. 2020;8(2):e001014.

95. Joe-Marc C, Hassane MZ. TIGIT in cancer immunotherapy. Journal for ImmunoTherapy of Cancer. 2020;8(2):e000957.

96. Mueller SN, Ahmed R. High antigen levels are the cause of T cell exhaustion during chronic viral infection. Proceedings of the National Academy of Sciences. 2009;106(21):8623-8.

97. Wherry EJ, Ha S-J, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular Signature of CD8⁺ T Cell Exhaustion during Chronic Viral Infection. Immunity. 2007;27(4):670-84.
98. Quigley M, Pereyra F, Nilsson B, Porichis F, Fonseca C, Eichbaum Q, et al. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. Nature Medicine. 2010;16(10):1147-51.
99. Martinez GJ, Pereira RM, Äijö T, Kim EY, Marangoni F, Pipkin ME, et al. The Transcription Factor NFAT Promotes Exhaustion of Activated CD8⁺ T Cells. Immunity. 2015;42(2):265-78.
100. Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MBA. Interleukin-10 determines viral clearance or persistence in vivo. Nature Medicine. 2006;12(11):1301-9.
101. Brooks DG, Ha S-J, Elsaesser H, Sharpe AH, Freeman GJ, Oldstone MBA. IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. Proceedings of the National Academy of Sciences. 2008;105(51):20428-33.
102. Tinoco R, Alcalde V, Yang Y, Sauer K, Zuniga EI. Cell-Intrinsic Transforming Growth Factor-β Signaling Mediates Virus-Specific CD8⁺ T Cell Deletion and Viral Persistence In Vivo. Immunity. 2009;31(1):145-57.
103. Lukhele S, Rabbo DA, Guo M, Shen J, Elsaesser HJ, Quevedo R, et al. The transcription factor IRF2 drives interferon-mediated CD8(+) T cell exhaustion to restrict anti-tumor immunity. Immunity. 2022;55(12):2369-85.e10.
104. Hudson WH, Gensheimer J, Hashimoto M, Wieland A, Valanparambil RM, Li P, et al. Proliferating Transitory T Cells with an Effector-like Transcriptional Signature Emerge from PD-1(+) Stem-like CD8(+) T Cells during Chronic Infection. Immunity. 2019;51(6):1043-58.e4.
105. Beltra JC, Manne S, Abdel-Hakeem MS, Kurachi M, Giles JR, Chen Z, et al. Developmental Relationships of Four Exhausted CD8(+) T Cell Subsets Reveals Underlying Transcriptional and Epigenetic Landscape Control Mechanisms. Immunity. 2020;52(5):825-41.e8.
106. Zander R, Schauder D, Xin G, Nguyen C, Wu X, Zajac A, Cui W. CD4(+) T Cell Help Is Required for the Formation of a Cytolytic CD8(+) T Cell Subset that Protects against Chronic Infection and Cancer. Immunity. 2019;51(6):1028-42.e4.

107. Safe S, Jin UH, Morpurgo B, Abudayyeh A, Singh M, Tjalkens RB. Nuclear receptor 4A (NR4A) family - orphans no more. J Steroid Biochem Mol Biol. 2016;157:48-60.

108. Odagiu L, May J, Boulet S, Baldwin TA, Labrecque N. Role of the Orphan Nuclear Receptor NR4A Family in T-Cell Biology. Front Endocrinol (Lausanne). 2020;11:624122.

109. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, Hogquist KA. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J Exp Med. 2011;208(6):1279-89.

110. Richard AC, Lun ATL, Lau WWY, Göttgens B, Marioni JC, Griffiths GM. T cell cytolytic capacity is independent of initial stimulation strength. Nature Immunology. 2018;19(8):849-58.

111. Cheng LE, Chan FK, Cado D, Winoto A. Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. Embo j. 1997;16(8):1865-75.

112. Jennings E, Elliot TAE, Thawait N, Kanabar S, Yam-Puc JC, Ono M, et al. Nr4a1 and Nr4a3 Reporter Mice Are Differentially Sensitive to T Cell Receptor Signal Strength and Duration. Cell Rep. 2020;33(5):108328.

113. Fassett MS, Jiang W, D'Alise AM, Mathis D, Benoist C. Nuclear receptor Nr4a1 modulates both regulatory T-cell (Treg) differentiation and clonal deletion. Proc Natl Acad Sci U S A. 2012;109(10):3891-6.

114. Hu QN, Suen AYW, Henao Caviedes LM, Baldwin TA. Nur77 Regulates Nondeletional Mechanisms of Tolerance in T Cells. J Immunol. 2017;199(9):3147-57.

115. Nowyhed HN, Huynh TR, Thomas GD, Blatchley A, Hedrick CC. Cutting Edge: The Orphan Nuclear Receptor Nr4a1 Regulates CD8+ T Cell Expansion and Effector Function through Direct Repression of Irf4. J Immunol. 2015;195(8):3515-9.

116. Liu X, Wang Y, Lu H, Li J, Yan X, Xiao M, et al. Genome-wide analysis identifies NR4A1 as a key mediator of T cell dysfunction. Nature. 2019;567(7749):525-9.

117. Odagiu L, Boulet S, Maurice De Sousa D, Daudelin J-F, Nicolas S, Labrecque N. Early programming of CD8+ T cell response by the orphan nuclear receptor NR4A3. Proceedings of the National Academy of Sciences. 2020;117(39):24392-402.

118. Boddupalli CS, Nair S, Gray SM, Nowyhed HN, Verma R, Gibson JA, et al. ABC transporters and NR4A1 identify a quiescent subset of tissue-resident memory T cells. J Clin Invest. 2016;126(10):3905-16.

119. Kurd NS, He Z, Louis TL, Milner JJ, Omilusik KD, Jin W, et al. Early precursors and molecular determinants of tissue-resident memory CD8(+) T lymphocytes revealed by single-cell RNA sequencing. Sci Immunol. 2020;5(47).

120. Giordano M, Henin C, Maurizio J, Imbratta C, Bourdely P, Buferne M, et al. Molecular profiling of CD8 T cells in autochthonous melanoma identifies Maf as driver of exhaustion. Embo j. 2015;34(15):2042-58.

121. Chen J, López-Moyado IF, Seo H, Lio CJ, Hempleman LJ, Sekiya T, et al. NR4A transcription factors limit CAR T cell function in solid tumours. Nature. 2019;567(7749):530-4.

122. Yang R, Cheng S, Luo N, Gao R, Yu K, Kang B, et al. Distinct epigenetic features of tumor-reactive CD8+ T cells in colorectal cancer patients revealed by genome-wide DNA methylation analysis. Genome Biology. 2019;21(1):2.

123. Jung I-Y, Narayan V, McDonald S, Rech AJ, Bartoszek R, Hong G, et al. BLIMP1 and NR4A3 transcription factors reciprocally regulate antitumor CAR T cell stemness and exhaustion. Science Translational Medicine.14(670):eabn7336.

124. Doi Y, Oki S, Ozawa T, Hohjoh H, Miyake S, Yamamura T. Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. Proceedings of the National Academy of Sciences. 2008;105(24):8381-6.

125. Raveney BJ, Oki S, Yamamura T. Nuclear receptor NR4A2 orchestrates Th17 cell-mediated autoimmune inflammation via IL-21 signalling. PLoS One. 2013;8(2):e56595.

126. Liebmann M, Hucke S, Koch K, Eschborn M, Ghelman J, Chasan AI, et al. Nur77 serves as a molecular brake of the metabolic switch during T cell activation to restrict autoimmunity. Proceedings of the National Academy of Sciences. 2018;115(34):E8017-E26.

127. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. Immunity. 2003;19(5):739-48.

128. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346(6213):1258096.

129. Westermann L, Neubauer B, Köttgen M. Nobel Prize 2020 in Chemistry honors CRISPR: a tool for rewriting the code of life. Pflugers Arch. 2021;473(1):1-2.

130. Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal Transduction and Targeted Therapy. 2020;5(1):1.

131. Han H. RNA Interference to Knock Down Gene Expression. Methods Mol Biol. 2018;1706:293-302.

132. Okuma A. Generation of CAR-T Cells by Lentiviral Transduction. Methods Mol Biol. 2021;2312:3-14.

133. Tao J, Bauer DE, Chiarle R. Assessing and advancing the safety of CRISPR-Cas tools: from DNA to RNA editing. Nature Communications. 2023;14(1):212.

134. Micklethwaite KP, Gowrishankar K, Gloss BS, Li Z, Street JA, Moezzi L, et al. Investigation of product-derived lymphoma following infusion of piggyBac-modified CD19 chimeric antigen receptor T cells. Blood. 2021;138(16):1391-405.

135. Hirano S, Abudayyeh OO, Gootenberg JS, Horii T, Ishitani R, Hatada I, et al. Structural basis for the promiscuous PAM recognition by Corynebacterium diphtheriae Cas9. Nature Communications. 2019;10(1):1968.

136. Nakagawa R, Ishiguro S, Okazaki S, Mori H, Tanaka M, Aburatani H, et al. Engineered Campylobacter jejuni Cas9 variant with enhanced activity and broader targeting range. Communications Biology. 2022;5(1):211.

137. Rezalotfi A, Fritz L, Förster R, Bošnjak B. Challenges of CRISPR-Based Gene Editing in Primary T Cells. Int J Mol Sci. 2022;23(3).

138. Foss DV, Muldoon JJ, Nguyen DN, Carr D, Sahu SU, Hunsinger JM, et al. Peptide-mediated delivery of CRISPR enzymes for the efficient editing of primary human lymphocytes. Nature Biomedical Engineering. 2023;7(5):647-60.

139. Albanese M, Ruhle A, Mittermaier J, Mejías-Pérez E, Gapp M, Linder A, et al. Rapid, efficient and activation-neutral gene editing of polyclonal primary human resting CD4+ T cells allows complex functional analyses. Nature Methods. 2022;19(1):81-9.

140. Antoniou P, Miccio A, Brusson M. Base and Prime Editing Technologies for Blood Disorders. Front Genome Ed. 2021;3:618406.

141. Schober K, Müller TR, Gökmen F, Grassmann S, Effenberger M, Poltorak M, et al. Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. Nature Biomedical Engineering. 2019;3(12):974-84.

142. Vouillot L, Thélie A, Pollet N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3 (Bethesda). 2015;5(3):407-15.

143. Sentmanat MF, Peters ST, Florian CP, Connelly JP, Pruett-Miller SM. A Survey of Validation Strategies for CRISPR-Cas9 Editing. Scientific Reports. 2018;8(1):888.

144. Brinkman EK, van Steensel B. Rapid quantitative evaluation of CRISPR genome editing by TIDE and TIDER. CRISPR Gene Editing: Methods and Protocols. 2019:29-44.

145. Conant D, Hsiau T, Rossi N, Oki J, Maures T, Waite K, et al. Inference of CRISPR Edits from Sanger Trace Data. Crispr j. 2022;5(1):123-30.

146. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nat Biotechnol. 2018;36(8):765-71.

147. Wienert B, Cromer MK. CRISPR nuclease off-target activity and mitigation strategies. Front Genome Ed. 2022;4:1050507.

148. Atkins A, Chung C-H, Allen AG, Dampier W, Gurrola TE, Sariyer IK, et al. Off-Target Analysis in Gene Editing and Applications for Clinical Translation of CRISPR/Cas9 in HIV-1 Therapy. Frontiers in Genome Editing. 2021;3.

149. Dötsch S, Svec M, Schober K, Hammel M, Wanisch A, Gökmen F, et al. Long-term persistence and functionality of adoptively transferred antigen-specific T cells with genetically ablated PD-1 expression. Proc Natl Acad Sci U S A. 2023;120(10):e2200626120.

150. Zhao Z, Shi L, Zhang W, Han J, Zhang S, Fu Z, Cai J. CRISPR knock out of programmed cell death protein 1 enhances anti-tumor activity of cytotoxic T lymphocytes. Oncotarget. 2018;9(4):5208-15.

151. Trefny MP, Kirchhammer N, Auf der Maur P, Natoli M, Schmid D, Germann M, et al. Deletion of SNX9 alleviates CD8 T cell exhaustion for effective cellular cancer immunotherapy. Nat Commun. 2023;14(1):86.

152. Oh SA, Senger K, Madireddi S, Akhmetzyanova I, Ishizuka IE, Tarighat S, et al. High-efficiency nonviral CRISPR/Cas9-mediated gene editing of human T cells using plasmid donor DNA. J Exp Med. 2022;219(5).

153. Sahin U, Toprak SK, Atilla PA, Atilla E, Demirer T. An overview of infectious complications after allogeneic hematopoietic stem cell transplantation. J Infect Chemother. 2016;22(8):505-14.

154. Fishman JA. Infection in solid-organ transplant recipients. N Engl J Med. 2007;357(25):2601-14.

155. Cornelissen JJ, Blaise D. Hematopoietic stem cell transplantation for patients with AML in first complete remission. Blood. 2016;127(1):62-70.

156. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. J Clin Invest. 2019;129(6):2357-73.

157. Purine Analogues. LiverTox: Clinical and Research Information on Drug-Induced Liver Injury. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012.

158. Muranski P, Boni A, Wrzesinski C, Citrin DE, Rosenberg SA, Childs R, Restifo NP. Increased intensity lymphodepletion and adoptive immunotherapy--how far can we go? Nat Clin Pract Oncol. 2006;3(12):668-81.

159. Scheinberg P, Nunez O, Weinstein B, Scheinberg P, Biancotto A, Wu CO, Young NS. Horse versus rabbit antithymocyte globulin in acquired aplastic anemia. N Engl J Med. 2011;365(5):430-8.

160. Mikulska M, Lanini S, Gudiol C, Drgona L, Ippolito G, Fernández-Ruiz M, Salzberger B. ESCMID Study Group for Infections in Compromised Hosts (ESGICH) Consensus Document on the safety of targeted and biological therapies: an infectious diseases perspective (Agents targeting lymphoid cells surface antigens [I]: CD19, CD20 and CD52). Clin Microbiol Infect. 2018;24 Suppl 2:S71-s82.

161. Aktas S, Colak T, Baskin E, Sevmis S, Ozdemir H, Moray G, et al. Comparison of basiliximab and daclizumab with triple immunosuppression in renal transplantation. Transplant Proc. 2011;43(2):453-7.

162. Jorge S, Guerra J, Silva S, Santana A, Mil-Homens C, Prata MM. Induction immunosuppressive therapy in renal transplantation: does basiliximab make the difference? Transplant Proc. 2008;40(3):693-6.

163. Wang K, Xu X, Fan M. Induction therapy of basiliximab versus antithymocyte globulin in renal allograft: a systematic review and meta-analysis. Clin Exp Nephrol. 2018;22(3):684-93.

164. Barbarino JM, Staatz CE, Venkataramanan R, Klein TE, Altman RB. PharmGKB summary: cyclosporine and tacrolimus pathways. Pharmacogenet Genomics. 2013;23(10):563-85.

165. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology. 2000;47(2-3):85-118.

166. Fox RI, Herrmann ML, Frangou CG, Wahl GM, Morris RE, Strand V, Kirschbaum BJ. Mechanism of action for leflunomide in rheumatoid arthritis. Clin Immunol. 1999;93(3):198-208.

167. Sehgal SN. Sirolimus: its discovery, biological properties, and mechanism of action. Transplant Proc. 2003;35(3 Suppl):7s-14s.

168. Czock D, Keller F, Rasche FM, Häussler U. Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids. Clin Pharmacokinet. 2005;44(1):61-98.

169. Cohen JI. Herpesvirus latency. J Clin Invest. 2020;130(7):3361-9.

170. Green M, Michaels MG. Infections in Pediatric Solid Organ Transplant Recipients. Journal of the Pediatric Infectious Diseases Society. 2012;1(2):144-51.

171. Ramanan P, Razonable RR. Cytomegalovirus infections in solid organ transplantation: a review. Infect Chemother. 2013;45(3):260-71.

172. Chemaly RF, Chou S, Einsele H, Griffiths P, Avery R, Razonable RR, et al. Definitions of Resistant and Refractory Cytomegalovirus Infection and Disease in Transplant Recipients for Use in Clinical Trials. Clin Infect Dis. 2019;68(8):1420-6.

173. Marty FM, Ljungman P, Chemaly RF, Maertens J, Dadwal SS, Duarte RF, et al. Letermovir Prophylaxis for Cytomegalovirus in Hematopoietic-Cell Transplantation. N Engl J Med. 2017;377(25):2433-44.

174. Limaye AP, Budde K, Humar A, Vincenti F, Kuypers DRJ, Carroll RP, et al. Letermovir vs Valganciclovir for Prophylaxis of Cytomegalovirus in High-Risk Kidney Transplant Recipients: A Randomized Clinical Trial. Jama. 2023.

175. Avery RK, Alain S, Alexander BD, Blumberg EA, Chemaly RF, Cordonnier C, et al. Maribavir for Refractory Cytomegalovirus Infections With or Without Resistance Post-Transplant: Results From a Phase 3 Randomized Clinical Trial. Clin Infect Dis. 2022;75(4):690-701.

176. Seeley WW, Marty FM, Holmes TM, Upchurch K, Soiffer RJ, Antin JH, et al. Post-transplant acute limbic encephalitis: clinical features and relationship to HHV6. Neurology. 2007;69(2):156-65.

177. Tzannou I, Papadopoulou A, Naik S, Leung K, Martinez CA, Ramos CA, et al. Off-the-Shelf Virus-Specific T Cells to Treat BK Virus, Human Herpesvirus 6, Cytomegalovirus, Epstein-Barr Virus, and Adenovirus Infections After Allogeneic Hematopoietic Stem-Cell Transplantation. Journal of Clinical Oncology. 2017;35(31):3547-57.

178. Kempkes B, Robertson ES. Epstein-Barr virus latency: current and future perspectives. Curr Opin Virol. 2015;14:138-44.

179. Münz C. Latency and lytic replication in Epstein–Barr virus-associated oncogenesis. Nature Reviews Microbiology. 2019;17(11):691-700.

180. Price AM, Luftig MA. To be or not IIb: a multi-step process for Epstein-Barr virus latency establishment and consequences for B cell tumorigenesis. PLoS Pathog. 2015;11(3):e1004656.

181. Young LS, Dawson CW, Eliopoulos AG. The expression and function of Epstein-Barr virus encoded latent genes. Molecular Pathology. 2000;53(5):238-47.

182. Allen UD, Preiksaitis JK. Post-transplant lymphoproliferative disorders, Epstein-Barr virus infection, and disease in solid organ transplantation: Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. Clin Transplant. 2019;33(9):e13652.

183. Malonga GA, Jary A, Leducq V, Moudiongui Mboungou Malanda D, Boumba ALM, Chicaud E, et al. Seroprevalence and molecular diversity of Human Herpesvirus 8 among people living with HIV in Brazzaville, Congo. Scientific Reports. 2021;11(1):17442.

184. Pinto M, Dobson S. BK and JC virus: a review. J Infect. 2014;68 Suppl 1:S2-8.

185. Szymonowicz KA, Chen J. Biological and clinical aspects of HPV-related cancers. Cancer Biol Med. 2020;17(4):864-78.

186. Cortese I, Beck ES, Al-Louzi O, Ohayon J, Andrada F, Osuorah I, et al. BK virus-specific T cells for immunotherapy of progressive multifocal leukoencephalopathy: an open-label, single-cohort pilot study. Lancet Neurol. 2021;20(8):639-52.

187. Balduzzi A, Lucchini G, Hirsch HH, Basso S, Cioni M, Rovelli A, et al. Polyomavirus JC-targeted T-cell therapy for progressive multiple leukoencephalopathy in a hematopoietic cell transplantation recipient. Bone Marrow Transplant. 2011;46(7):987-92.

188. Garrote H, de la Fuente A, Oña R, Rodríguez I, Echevarría JE, Sepúlveda JM, García JF. Long-term survival in a patient with progressive multifocal leukoencephalopathy after therapy with rituximab, fludarabine and cyclophosphamide for chronic lymphocytic leukemia. Experimental Hematology & Oncology. 2015;4(1):8.

189. Olson A, Lin R, Marin D, Rafei H, Bdaiwi MH, Thall PF, et al. Third-Party BK Virus-Specific Cytotoxic T Lymphocyte Therapy for Hemorrhagic Cystitis Following Allotransplantation. J Clin Oncol. 2021;39(24):2710-9.

190. Stevanović S, Helman SR, Wunderlich JR, Langhan MM, Doran SL, Kwong MLM, et al. A Phase II Study of Tumor-infiltrating Lymphocyte Therapy for Human Papillomavirus-associated Epithelial Cancers. Clin Cancer Res. 2019;25(5):1486-93.

191. Cesaro S, Berger M, Tridello G, Mikulska M, Ward KN, Ljungman P, et al. A survey on incidence and management of adenovirus infection after allogeneic HSCT. Bone Marrow Transplant. 2019;54(8):1275-80.

192. Gerdemann U, Keirnan JM, Katari UL, Yanagisawa R, Christin AS, Huye LE, et al. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. Mol Ther. 2012;20(8):1622-32.

193. O'Reilly RJ. T-cells: Third Party Parity for Viral Infections. Transplant Cell Ther. 2023;29(5):285-6.

194. Boudreau G, Carli C, Lamarche C, Rulleau C, Bonnaure G, Néron S, Delisle JS. Leukoreduction system chambers are a reliable cellular source for the manufacturing of T-cell therapeutics. Transfusion. 2019;59(4):1300-11.

195. Feuchtinger T, Richard C, Joachim S, Scheible MH, Schumm M, Hamprecht K, et al. Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. J Immunother. 2008;31(2):199-206.

196. Neuenhahn M, Albrecht J, Odendahl M, Schlott F, Dössinger G, Schiemann M, et al. Transfer of minimally manipulated CMV-specific T cells from stem cell or third-party donors to treat CMV infection after allo-HSCT. Leukemia. 2017;31(10):2161-71.

197. Haque T, Amlot PL, Helling N, Thomas JA, Sweny P, Rolles K, et al. Reconstitution of EBV-Specific T Cell Immunity in Solid Organ Transplant Recipients1. The Journal of Immunology. 1998;160(12):6204-9.

198. Cameron JE, Fewell C, Yin Q, McBride J, Wang X, Lin Z, Flemington EK. Epstein-Barr virus growth/latency III program alters cellular microRNA expression. Virology. 2008;382(2):257-66.

199. Chapuis AG, Afanasiev OK, Iyer JG, Paulson KG, Parvathaneni U, Hwang JH, et al. Regression of metastatic Merkel cell carcinoma following transfer of polyomavirus-specific T cells and therapies capable of re-inducing HLA class-I. Cancer Immunol Res. 2014;2(1):27-36.

200. Peghin M, Castaldo N, Tascini C, Bassetti M, Graziano E, Givone F, et al. Successful JC virus-targeted T-cell therapy for progressive multifocal leukoencephalopathy in a lung transplant recipient. J Heart Lung Transplant. 2022;41(7):991-6.

201. Muftuoglu M, Olson A, Marin D, Ahmed S, Mulanovich V, Tummala S, et al. Allogeneic BK Virus–Specific T Cells for Progressive Multifocal Leukoencephalopathy. New England Journal of Medicine. 2018;379(15):1443-51.

202. Feucht J, Opherk K, Lang P, Kayser S, Hartl L, Bethge W, et al. Adoptive T-cell therapy with hexon-specific Th1 cells as a treatment of refractory adenovirus infection after HSCT. Blood. 2015;125(12):1986-94.

203. Patel S, Lang H, Sani G, Freeman AF, Leiding J, Hanley PJ, et al. Mycobacteria-Specific T Cells May Be Expanded From Healthy Donors and Are Near Absent in Primary Immunodeficiency Disorders. Front Immunol. 2019;10:621.

204. Castillo P, Wright KE, Kontoyiannis DP, Walsh T, Patel S, Chorvinsky E, et al. A New Method for Reactivating and Expanding T Cells Specific for Rhizopus oryzae. Mol Ther Methods Clin Dev. 2018;9:305-12.

205. Menger L, Gouble A, Marzolini MA, Pachnio A, Bergerhoff K, Henry JY, et al. TALEN-mediated genetic inactivation of the glucocorticoid receptor in cytomegalovirus-specific T cells. Blood. 2015;126(26):2781-9.

206. Amini L, Wagner DL, Rössler U, Zarrinrad G, Wagner LF, Vollmer T, et al. CRISPR-Cas9-Edited Tacrolimus-Resistant Antiviral T Cells for Advanced Adoptive Immunotherapy in Transplant Recipients. Mol Ther. 2021;29(1):32-46.

207. Ottaviano G, Georgiadis C, Gkazi SA, Syed F, Zhan H, Etuk A, et al. Phase 1 clinical trial of CRISPR-engineered CAR19 universal T cells for treatment of children with refractory B cell leukemia. Sci Transl Med. 2022;14(668):eabq3010.

208. Odagiu L, Sousa DMD, Boulet S, Labrecque N. NR4A3 deficiency in CD8⁺ T cells improves adoptive T cell therapy of cancer. bioRxiv. 2023:2023.04.21.537841.

209. Melenhorst JJ, Chen GM, Wang M, Porter DL, Chen C, Collins MA, et al. Decade-long leukaemia remissions with persistence of CD4(+) CAR T cells. Nature. 2022;602(7897):503-9.

210. Pinar A, Bülent Arman A, Eric C, Jeff H. Viable and efficient electroporation-based genetic manipulation of unstimulated human T cells. bioRxiv. 2019:466243.

211. Boulet S, Daudelin JF, Odagiu L, Pelletier AN, Yun TJ, Lesage S, et al. The orphan nuclear receptor NR4A3 controls the differentiation of monocyte-derived dendritic cells following microbial stimulation. Proc Natl Acad Sci U S A. 2019;116(30):15150-9.