Unconventional T cells in colon adenocarcinomas

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Cover illustration: Cells in a colon tumour. 4',6-diamidino-2-phenylindole (DAPI) staining of the nuclei of all cells present in a frozen section of a colon tumour. The image was kindly provided by Louis Szeponik

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"Good judgement comes from experience, and a lot of that comes from bad judgement".

- Will Rogers

In loving memory of **Nils Harold Rodin**

ABSTRACT

Many factors influence the initiation and growth of tumours. The infiltration and activity of immune cells in the tumour microenvironment are widely recognised as key factors affecting the clinical outcome of cancer. In this context, conventional T cells have been studied thoroughly but much less is known about the enigmatic populations of unconventional T cells. Of particular interest are the γδ and mucosal-associated invariant T (MAIT) cells as they, among other things, are (relatively) abundant in humans, possess the ability to recognise transformed host cells, and secrete cytokines, as well as cytotoxic effector proteins. The aim of this thesis was to investigate the potential role of unconventional T cells in the immune response to tumours and specifically if they possess the ability to kill tumour cells. We used tissue from colon tumours, unaffected colon mucosa, and blood from patients undergoing curative resection surgery at the Sahlgrenska University Hospital, along with blood samples from healthy blood donors, to investigate the phenotype and effector functions of MAIT and γδ T cells. We demonstrated that MAIT cells accumulate in colon tumours while γδ T cell infiltration is reduced. The γδ T cells present in colon tumours contained a subset of $V\delta1^{-}V\delta2^{-}$ cells with potential tumour-promoting properties. Furthermore, we showed that a portion of the tumour-infiltrating MAIT cells were functionally impaired and characterised by expression of PD-1 and Tim3. The $PD\text{-}1^{+}Tim3^{high}$ MAIT cells had a reduced capacity to produce cytokines and effector proteins, compared to their $PD\text{-}1^{-}Tim3^{-}$ counterparts. We also showed that these functionally impaired, or exhausted, MAIT cells could be partially re-activated in the presence of monoclonal antibodies towards PD-1 (Pembrolizumab). In a third study, we demonstrated that circulating and tumour-infiltrating MAIT cells from patients and circulating MAIT cells from healthy donors can be readily expanded in vitro. Expanded MAIT cells regardless of tissue origin were highly cytotoxic and effectively killed both epithelial cancer cell lines as well as primary tumour cells derived from colon cancer patients. Lastly, we also showed that the cytotoxicity of expanded MAIT cells was partly dependent on the activity of one or more serine proteases, as blocking the activity of all serine protease activity reduced the cytotoxicity of expanded MAIT cells.

In conclusion, this thesis highlights some of the complexities of γδ and MAIT cell responses in tumour immunity, but also that specific subsets,

such as MAIT cells, can be expanded and potentially used as future treatments against colon cancer.

Keywords: Unconventional T cells, MAIT cells, γδ T cells, colorectal cancer, tumour immunity

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SAMMANFATTNING PÅ SVENSKA

Tjocktarmscancer är den näst dödligaste cancerformen i världen, med omkring 900 000 dödsfall varje år och tydliga kopplingar mellan den västerländska livsstilen och risken för att utveckla tjocktarmscancer har kunnat påvisas. Dessutom ser utvecklingen vad gäller tjocktarmscancer ut att gå i fel riktning och en ökning av antalet fall är sannolikt att vänta inom de närmsta 10-20 åren. Till de vanligaste riskfaktorerna för att utveckla tjocktarmscancer hör alkohol, rökning, rött kött, fiberfattig kost och övervikt/fetma. Trots att vi idag har blivit bättre på att tidigt upptäcka cancer och att flera olika behandlingsalternativ finns så är prognosen för vissa typer av tjocktarmscancer fortfarande dålig. Den vanligaste behandlingsformen är partiell resektion av tjocktarmen för att avlägsna tumören, detta kan ibland kombineras med cellgifter för att minska tumören inför operation och/eller för att minska risken för återfall efter operation. I de fall då operation inte är möjligt eller då palliativ vård är det enda alternativet så kan cellgifter användas för att minska symptom och bromsa sjukdomsförloppet. Idag kan även behandlingar som riktar sig specifikt mot att förbättra aktiviteten hos de immunceller som finns i tumören nyttjas (s.k. immunterapier).

T celler är en celltyp inom immunförsvaret som i huvudsak skyddar kroppen mot virusangrepp och tumörer, oftast genom att direkt döda de celler som infekterats eller börjat utvecklas till tumörer. Vissa typer av T celler kan även påverka andra immunceller genom produktionen och frisättningen av olika molekyler eller via direkt kontakt mellan receptorer på cellernas ytor. Bland T cellerna finns en särskild grupp som kallas okonventionella T celler och som kännetecknas av att de delar egenskaper med celler som tillhör kroppens medfödda immunförsvar. Dessa okonventionella T celler finns ofta i de organ eller vävnader som fungerar som barriärer mellan kroppen och yttre hot, till exempel i kroppens slemhinnor där de skyddar oss från både infektioner och neoplastiska förändringar. Likt övriga T celler finns de också i blodet hos både friska och sjuka individer. Okonventionella T cellers förmåga att kunna känna igen även små förändringar hos kroppens egna celler och snabbt kunna aktiveras gör dem till en effektiv del av immunsvaret mot både infektioner och cancer.

Även om det forskats mycket på T cellers roll inom tumörimmunitet så är det fortfarande mycket som vi inte vet, särskilt om de okonventionella T

cellerna och deras roll i kroppens försvar mot cancer. I denna avhandling har vi undersökt γδ och MAIT (de två vanligaste typerna av okonventionella T celler) cellers funktioner hos patienter med tjocktarmscancer. I prover från tumör, normal tarmvävnad och blod från patienter som opererats för tjocktarmscancer vid Sahlgrenska universitetssjukhuset, tillsammans med blodprover från friska individer, har vi studerat egenskaper och funktioner hos γδ och MAIT celler. Vi har visat att MAIT celler anrikas i tumörerna hos cancerpatienterna medan andelen γδ T celler minskar. Bland de γδ T celler som finns i tumörerna finns också en särskild grupp celler som troligtvis bidrar till att förbättra tumörcellernas chans att överleva. Utöver det har vi också visat att en del av de MAIT celler som finns i tumörerna kan vara inaktiverade och sakna förmågan att effektivt bidra till ett immunsvar, då de saknar förmågan att producera flertalet för immunsvaret viktiga ämnen. Däremot har vi kunnat visa att dessa inaktiverade MAIT celler kan återaktiveras i närvaro av monoklonala antikroppar mot receptorn PD-1 (Pembrolizumab). Vi har också visat att MAIT celler från både tumörer och blodet i såväl friska som sjuka individer kan expanderas och att de har en stor förmåga att döda tumörceller, från både etablerade cellinjer och sådana som odlats direkt från tumörer i tjocktarmen.

Sammanfattningsvis så understryker denna avhandling komplexiteten i okonventionella T cellers roll i immunsvaret mot tjocktarmscancer men den visar också hur specifika grupper, så som MAIT celler, i framtiden skulle kunna användas som en del av en behandlingsstrategi.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Rodin, W. Szeponik, L. Rangelova, T. Sundström, P. Hogg, S. Wettergren, Y. Cosma, A. Bexe Lindskog, E. Quiding Järbrink, M. **Tumour-infiltrating non-Vδ1Vδ2 γδ T cells have tumour-promoting functions in humans.** *Manuscript*

II. Rodin, W. Sundström, P. Ahlmanner, F. Szeponik, L. Kajetan Zajt, K. Wettergren, Y. Bexe Lindskog, E. Quiding Järbrink, M. **Exhaustion in tumour-infiltrating Mucosal-Associated Invariant T (MAIT) cells from colon cancer patients.** Cancer Immunology and Immunotherapy 2021; 70(12): 1-15.

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III. Rodin, W. Verveda, A. Sundström, P. Hogg, S. Kajetan Zajt, K. Rangelova, T. Trajanoski, Z. Kristenson, L. Bergh Thóren, F. Wettergren, Y. Bexe Lindskog, E. Quiding Järbrink, M. **Tumour-infiltrating MAIT cells kill target cells by a granzyme B dependent mechanism.** *Manuscript*

CONTENT

ABBREVIATIONS

5-A-RU	5-Amino-6-(D-ribitylamino)uracil
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
ADAM17	A disintegrin and metalloprotease 17
ADCC	Antibody-dependent cellular cytotoxicity
APD	Avalanche photodiode
APC	Antigen-presenting cell
BCMA	B cell maturation antigen
BM	Basement membrane
Breg	Regulatory B cell
CAR	Chimeric antigen receptor
CCSCs	Colon cancer stem cells
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtype
CRC	Colorectal cancer
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DFS	Disease-free survival
e-MDSC	Early-onset myeloid-derived suppressor cell
EMT	Endothelial-mesenchymal transition
EPCR	Endothelial cell protein C receptor
FACS	Fluorescence-activated cell sorting
FAP	Familial adenomatous polyposis
FC	Flow cytometry
FMO	Fluorescence minus one
FPR1	Formyl peptide receptor 1
GVHD	Graft-versus host disease

HCC	Hepatocellular carcinoma
HDI	Human development index
HLA-DR	Human leukocyte antigen isotype DR
HMB-PP	(E)-4-hydroxi-3-metyl-but-2-enylpyrofosfat
HMGB1	High-mobility group box 1-protein
HNPCC	Hereditary nonpolyposis colorectal cancer
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
IHC	Immunohistochemistry
ILC	Innate lymphoid cell
ILF	Isolated lymfoid follicle
IPP	Isopentenyl pyrophosphate
ISC	Intestinal stem cell
LP	Lamina propria
M-MDSC	Monocytic-myeloid-derived suppressor cell
MACS	Magnetic cell sorting
MHC I	Major histocompatibility complex class I
MDSC	Myeloid-derived suppressor cell
MICA/B	MHC class I polypeptide-related sequence A/B
MLN	Mesenteric lymph node
MMR	Mismatch repair
MR1	Major histocompatibility complex, class I-related protein
MSI	Microsatellite instability
MSS	Microsatellite stable
mTNF	Membrane-bound TNF
NKT cell	Natural killer T cell
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
NSCLC	Non-small cell lung cancer
PMA	Phorbol 12-myristate 13-acetate
PMN-MDSC	Polymorphonuclear-myeloid-derived suppressor cell

PMT	Photomultiplier tube
PNT	Peroxynitrite
PP	Peyer´s patches
PPDTCs	Primary patient-derived tumour cells
sTNF	Soluble TNF
TA	Tumour antigen
TAA	Tumour-associated antigen
TAM	Tumour-associated macrophage
TAN	Tumour-associated neutrophil
TCR	T cell receptor
TGF	Transforming growth factor
Th cell	Helper T cell
TIL	Tumour-infiltrating lymphocyte
TME	Tumour microenvironment
TOF	Time-of-flight
Treg	Regulatory T cell
TSA	Tumour-specific antigen
UMAP	Uniform manifold proximation and projection

RECEPTORS, CYTOKINES AND EFFECTOR MOLECULES

CD272/BTLA	B- and T-lymphocyte attenuator. Co-inhibitory receptor belonging to the CD28 superfamily. Expression on tumour-infiltrating lymphocytes has been linked to tumour progression and poor prognosis in glioblastoma and melanoma patients[1].
CD1	Nonpolymorphic MHC-related molecules that present small molecule, lipid, and glycolipid antigens. Classified into the four isoforms a, b, c, and d[2].
CD16	Fc receptor (FcγRIII) expressed by several different immune cells, including NK cells and some T cells. CD16 mediates antibody-dependent cellular cytotoxicity upon binding to antigen loaded IgG antibodies[3, 4].
CD25	Interleukin-2 receptor α-chain. Together with the β (CD122) and γ (CD132) chains, CD25 makes up the IL-2 receptor. IL-2 signalling via the IL-2 receptor is crucial for the regulation, proliferation, and differentiation of T and NK cells[5, 6].
CD28	Co-stimulatory receptor, for the ligands CD80 and CD86, expressed mainly by naïve T cells. TCR stimulation without co-stimulatory signalling from CD28 results in anergy in the T cell[7].
CD38	Cyclic ADP ribose hydrolase. Expressed as part of cellular membranes, either cell surface or in intracellular compartments. CD38 has both enzymatic properties, linked to intracellular signalling, as well as adhesive receptor functions. Inflammation, cell migration, cytokine release, phagocytosis, and antigen-presentation have all been associated with CD38 expression[8].
CD39	Ectonucleosidase triphosphate diphosphohydrolase-1 (ENTPD1). Expressed on the surface of some immune cells, such as Tregs, and converts extracellular ATP into adenosine (together with CD73), which exerts immunosuppressive functions[9].
CD45RA	CD45 isoform expressed by naïve T cells[10].
CD45RO	CD45 isoform expressed by activated and memory T cells[10].

CD56	Neural cell adhesion molecule (NCAM). Hallmark NK cell marker but also expressed by other immune cells, such as T cells, DCs, and monocytes. CD56 expression seems to be intrinsically linked to degree of activation and indicate potent immunostimulatory functions[11].
CD62L	L-selectin. Cell adhesion molecule expressed by circulating immune cells involved in the adhesion of immune cells during extravasation into the tissue[12].
CD66a	Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Cell surface signalling receptor with pleiotropic functions on different immune cells. In T cells, CD66a is almost exclusively expressed by activated cells and involved in regulating T cell activity and induction of tolerance[13].
CD69	Early activation marker expressed on T and NK cells as well as a marker of tissue residency in T cells[14].
CD84	Surface receptor in the signalling lymphocyte activation molecule (SLAM) family. CD84 expression in the TME has been shown to mediate immunosuppression in T cells in myeloma and leukaemia[15, 16].
CD86	Ligand for CD28 expressed by antigen presenting cells[17].
CD94/KLRD1	Heterodimerizes with one of the seven isoforms in the NKG2 family of C-type lectin-like receptors to form inhibitory or activating surface bound receptors expressed by NK and T cells.[18].
CD95	Fas receptor (apoptosis antigen 1 or TNFRSF6), forms the death-inducing signalling complex (DISC) when interacting with its ligand (FasL/CD178) leading to apoptosis of the Fas expressing cell[19].
CD103	Integrin alpha E (ITGAE). Expressed by intraepithelial lymphocytes and involved in the homing and retention of tissue resident T cells[20].
CD127	Interleukin-7 receptor a-chain. Together with the common cytokine receptor γ-chain, CD127 forms the IL-7 receptor[21].
CD134/OX40	Tumour necrosis factor receptor superfamily, member 4 (TNFRSF4). Binds its ligand (OX40L), mainly expressed by APCs. OX40 is mainly expressed by activated T cells and has immunomodulatory properties[22].
CD137/4-1BB	Tumour necrosis factor receptor superfamily member 9 (TNFRSF9). Co-stimulatory molecule expressed by antigen-activated T cells capable of enhancing the effector functions of the activated T cell[23].

CD158	Killer cell immunoglobulin-like receptor (KIR) 2DL1/S1/S3/S5. HLA-binding receptors with activating or inhibitory properties, depending on the length of the cytoplasmic tail (long (L) = inhibitory and short (S) = activating[24].
CD158b	KIR2DL2/L3. Inhibitory KIR[24].
CD158e1	KIR3DL1. Inhibitory KIR[24].
CD159a/NKG2A	Inhibitory C-type lectin-like receptor in the NKG2 family of receptors[25, 26].
CD161	Killer cell lectin-like receptor subfamily B, member 1 (KLRB1). CD161 is constitutively expressed by all MAIT cells and some NK cells[27, 28].
CD178/FasL	Fas ligand is expressed by cytotoxic immune cells. FasL expressing cells are capable of inducing apoptosis in Fas (CD95) expressing cells[19].
CD226/DNAM1	DNAX accessory molecule-1. Activating receptor expressed by both NK and T cells[29].
CD244/2B4	Natural killer cell receptor 244. CD244 is a member of the signalling lymphocyte activation molecule (SLAM) family of receptors and expressed by NK cells and some T cells. Binds to its ligand CD48. The expression level, number of binding events between CD244 and its ligand, and level of intracellular SLAM-associated protein determines if the signal is activating or inhibitory[30].
CD352	SLAM family member 6 (SLAMF6). Co-receptor expressed by activated T cells[31].
CTLA4	Cytotoxic T-lymphocyte-associated protein 4. Inhibitory co-receptor in the immunoglobulin superfamily, homologous to CD28. Mainly expressed by activated T cells and capable of binding the same ligands (CD80 and CD86) as CD28[32].
FoxP3	Forkhead box P3. Transcription factor that functions as the master regulator for the development of regulatory T cells. Also expressed by activated (non-regulatory) T cells[33].
ICOS	Inducible T-cell co-stimulator. Expression is up-regulated on activated T cells and binding of the ICOS-ligand enhances and/or modulates the T cell response[34].
Ki-67	Cellular proliferation marker. A nuclear protein expressed by proliferating cells[35].

Lag3	Lymphocyte-activation gene 3. Inhibitory co-receptor expressed by activated T cells. Expression levels are correlated to the level of inhibition, and increased and persistent expression of Lag3 has been observed on exhausted T cells[36].
NKG2D	Also known as killer cell lectin like receptor K1 (KLRK1). NKG2D is an activating C-type lectin-like receptor in the NKG2 family of receptors. Typically expressed by NK cells and some T cell subsets[37].
NKp30	Natural cytotoxicity receptor 3. Expressed by NK cells and some T cells. Three isoforms of NKp30 have been identified with both activating (NKp30a and NKp30b) and inhibitory (NKp30c) functions[38].
NKp80	Killer cell lectin like receptor F1 (KLRF1). Activating receptor expressed by NK cells and some T cells.[39]
PD-1 (CD279)	Programmed cell death protein 1. Inhibitory co-receptor mainly expressed by activated T cells. PD-1 binds the programmed death ligand 1 and 2, expressed by many different immune and non-immune cells[40, 41].
PD-L1 (CD274) and PD-L2 (CD273)	Programmed death ligand 1/2. See PD-1.
RoRγt	Retinoic acid-related orphan receptor gamma. RoRγt is a transcription factor, crucial for the development of several subsets of T cells, type 3 innate lymfoid cells, and some IL-17 producing neutrophils[42].
T-bet	T-box transcription factor 21. T-bet is a transcription factor involved in the development and function of both innate and adaptive immune cells, such as T cells, B cells, innate lymphoid cells, NK cells and DCs[43].
TIGIT	T cell immunoreceptor with Ig and ITIM domains. TIGIT is an inhibitory co-receptor expressed by activated T and NK cells. Several known ligands, such as CD155 and CD112, have been identified which are shared by the activating co-receptor DNAM1[44].
Tim3	T-cell immunoglobulin and mucin-domain containing-3, also known as Hepatitis A virus cellular receptor 2 (HAVCR2). Tim3 is an inhibitory co-receptor expressed by activated T cells, DCs, macrophages, MDSCs, and NK cells. Several known ligands, such as galectin 9, HMGB1, and CEACAM1 have been identified[45].
TNF	Tumour necrosis factor. A soluble or membrane bound protein with pleiotropic functions in both health and disease. One of the main functions of TNF is induction and promotion of the inflammatory response. TNF is expressed by several different immune cells, including T cells[46].

TRAIL	TNF-related apoptosis-inducing ligand. A soluble or membrane bound protein capable of inducing apoptosis in target cells expressing the TRAIL receptors 1 (death receptor 4) or 2 (death receptor 5). Two additional receptors, called decoy receptors, also binds TRAIL but to not induce apoptosis in the cells expressing the decoy receptors. Several different types of cells express TRAIL but the most common ones are NK and T cells[47].
ULBP	UL16 binding protein. Ligand for the activating receptor NKG2D. ULPB is often upregulated on cells as a result of cellular stress[29].
VISTA	V-domain immunoglobulin suppressor of T cell activation. VISTA is an inhibiting receptor mainly expressed by myeloid cells or precursor cells in the haematopoietic niche. There is some VISTA expression by T cells, but it is mainly by naïve T cells[48].

INTRODUCTION

MUCOSAL SURFACES, THE LARGE INTESTINE AND BARRIER FUNCTIONS

Mucosal surfaces are present in organs and tissues responsible for many essential functions, such as digestion, nutrient uptake, gas exchange, and reproduction. These mucosal surfaces create barriers, consisting of thin layers of highly specialised cells, between the inside of the human body and the outside environment[49]. Consequently, the mucosal surfaces are in constant contact with foreign substances, viruses, and bacteria (both commensal and pathogenic). At the mucosal surfaces, the immune system plays a crucial role in protection from disease or damage whilst maintaining tolerance to unharmful substances and commensal bacteria[49].

The large intestine (**figure 1**) consists of the caecum (which is joined with the small intestine), the ascending (left) colon, the transverse colon, the descending (right) colon, the sigmoid colon and end with the rectum[50]. The large intestine is mainly responsible for water uptake and for maintaining a reservoir of commensal bacteria that contribute to the production of vitamins and digestion of nutrients essential for our health[49]. It has also been shown (in germ-free animals) that the presence of commensal bacteria is crucial for the development of a functioning immune system[51]. Additionally, later in life, the commensal bacteria are believed to have even more far-reaching effects on human health by affecting our psychological well-being through the gut-brain axis[52].

The epithelial layer of the large intestine is organised into a multitude of invaginations and unlike the small intestine, it lacks the villi that protrude into the lumen, making it relatively smooth[53]. These invaginations, or crypts of Lieberkühn, harbour the replicative intestinal stem cells (ISC) that give rise to the many specialized cells of the large intestine[54]. Exactly how the ISCs are distributed throughout the crypts is still debated, as the lack of well-defined lineage markers for different types of ISCs has hampered the efforts to define their exact postions[55].

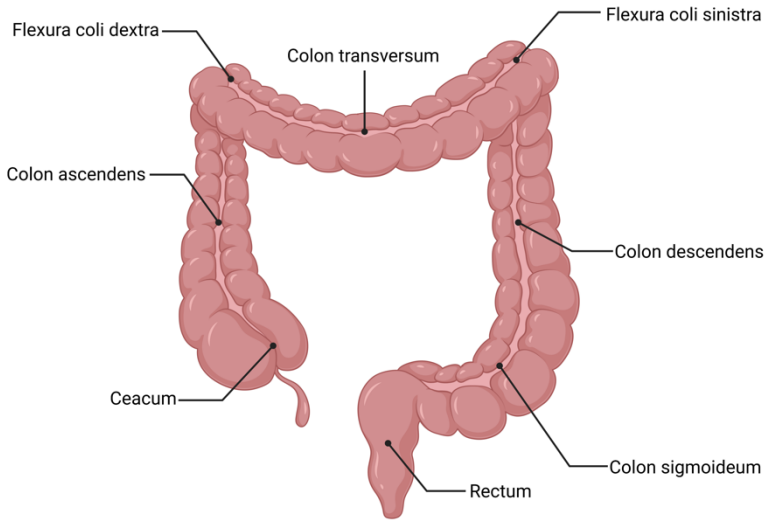

Figure 1. Anatomy of the large intestine. The large intestine consists of the caecum, ascending colon (colon ascendens), transverse colon (colon transversum), descending colon (colon descendens), sigmoid colon (colon sigmoideum), and rectum. Created using BioRender.com.

However, it is known that some ISCs are located at the very bottom of the crypts, interspaced between deep crypt secretory cells, while other ISCs are located at (or near) the +4 position (further from the bottom) of the crypts[56, 57]. The ISCs differentiate into proliferative progenitor cells, called transient amplifying cells, with the capacity to further differentiate into enterocytes, goblet cells, and enteroendocrine cells[58].

The intestinal epithelium is mostly composed of absorptive enterocytes, mucin-secreting goblet cells, and a small number of hormone-producing enteroendocrine cells[59]. As ISCs differentiate into the different cell types present in the epithelial layer the cells are slowly pushed upwards and out of the crypts while old and damaged cells are shed into the lumen, constantly renewing the intestinal epithelial layer[60]. Every 3-7 days most of the cells in the epithelial layer of the large intestine are replaced[61]. This rapid renewal of the intestinal epithelial layer helps to ensure that the structural integrity of the intestinal barrier is maintained and it is one of the many mechanisms that protect us from infection[60].

The mucin produced by goblet cells is the main component of the intestinal mucous layer that protects the intestinal epithelium and it can be divided into an inner and outer layer[62]. The outer mucus layer (closest to the lumen) is loose and facilitates the transport of foreign substances, viruses, and bacteria away from the intestinal epithelium[62]. The inner mucus layer (closest to the epithelial cells) is much denser and also harbours the protective IgA antibodies, produced by B-cells in the lamina propria (LP), and antimicrobial peptides produced by cells in the intestinal epithelium[62, 63, 64].

Although not technically a part of the intestinal epithelium, the intraepithelial lymphocytes (IELs) are interspersed between the cells of the epithelium, enabling a rapid response towards pathogens and transformed cells[49]. The IEL compartment is composed of both γδ and αβ T cells, as well as some innate lymphoid cells (ILCs)[65]. While most of the T cells are αβ T cells, as much as 60% of the IELs can be γδ T cells in certain parts of the intestine[66]. Most of the γδ T cells and ILCs are tissue-resident from early life, while tissue-residency in the intraepithelial αβ T cells is likely induced as a result of infection or inflammation[65]. A striking difference between the intraepithelial γδ T cells and circulating αβ T cells is the constitutive up-regulated expression of genes associated with effector functions in the γδ T cells[67]. While the IELs are a heterogeneous group of cells, asymmetrically distributed throughout the gut, they share the expression of the adhesion molecule integrin αE (CD103) and the activation and tissue-residency marker CD69[66]. Another feature of IELs is the expression of markers associated with NK cells, making IELs proficient in recognising both infected and transformed cells[66, 68]. However, while IELs express several markers associated with effector-type immune responses, they have also been linked to the initiation and progression of diseases, such as inflammatory bowel disease (IBD)[65, 66]. Beneath the epithelium, the basement membrane (BM), composed of collagen type IV, proteoglycans, and glycoproteins, separates the epithelial cells and underlying lamina propria[69, 70]. While allowing gas exchange and the passage of nutrients, the BM prevents cells and larger molecules from crossing between the epithelial surface and underlying LP[70]. The lamina propria is a layer of connective tissue rich in blood vessels, fibroblasts, and various immune cells. Additionally, both nerve cells and afferent lymphatic vessels, that drain to the superior mesenteric lymph nodes (MLNs), can be found in the LP[71]. While the large intestine lacks the Peyer's patches (PPs) of the small intestine, similar structures referred to as isolated lymphoid follicles (ILFs) have been described in the colonic lamina propria[72]. The main difference between Peyer's

patches and ILFs appears to be that ILFs are less organised and sometimes lack the specialised cells of Peyer's patches, making them a mix of PPs and simpler lymphoid cell aggregates[72].

The immune cells of the lamina propria include both innate and adaptive immune cells, that together with the IELs, form a specialised mucosal immune system capable of protecting the intestinal epithelium from microbial attack, finding, and destroying transformed or infected cells while maintaining tolerance to commensal bacteria and food antigens[49]. Like the epithelium, the LP harbour cytotoxic T and NK cells that facilitate the destruction of infected or transformed cells[49]. However, unlike the epithelium (that only contain IELs and specialised macrophages called Langerhans cells), the LP also contain additional cells, such as antigen-presenting dendritic cells (DCs), granulocytic cells, and IgA producing plasma cells[49]. The fibroblasts make up the mesenchymal niche of the lamina propria and supply growth factors that support proliferation and differentiation of the ISCs and transient amplifying cells of the intestinal epithelium[73]. Together, the epithelium, BM, lamina propria, and muscularis mucosa (a layer of smooth muscle cells) form the mucosa of the large intestine (**figure 2**). Located outside of the mucosa (further from the lumen), the submucosa, muscularis propria, and serosa (or adventitia, depending on anatomical location) form the lining of the intestine[69].

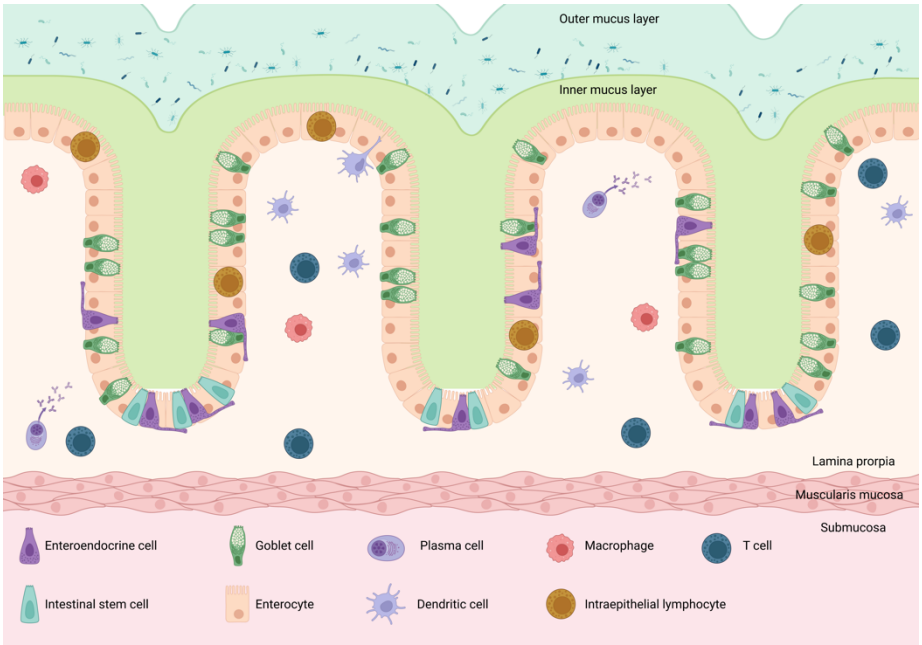

Figure 2. Cells of the large intestinal mucosa. Enterocytes, goblet cells, enteroendocrine cells, and intestinal stem cells make up the epithelial layer. The cytotoxic IELs reside between the cells of the epithelium, surveying the epithelium for signs of infection or malignancy. In the LP additional types of immune cells reside, such as antigen-presenting cells, T cells, NK cells, phagocytic cells, and plasma cells. Below the epithelium and LP, the muscularis mucosa and submucosa are found. Created using BioRender.com.

COLORECTAL CANCER

Colorectal cancer (CRC) is a malignancy found in the caecum, proximal colon (ascending and transverse), distal colon (descending and sigmoid), and rectum. While colorectal tumours (**figure 3**) are far from homogenous, they share several essential features and are therefore usually grouped together[74].

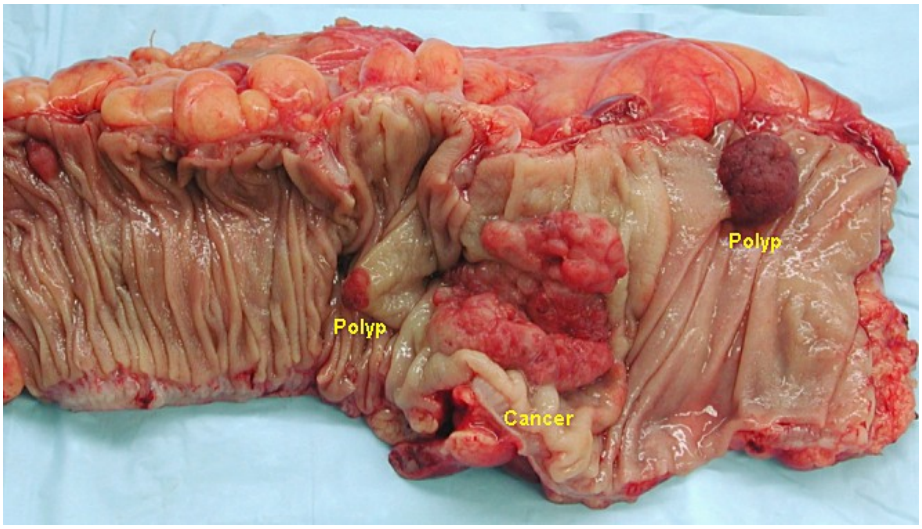

Figure 3. Colon cancer. Resected section of the large intestine with two polyps and a colon tumour. Adapted from "Colon Cancer" by Emmanuelm at English Wikipedia (Original text: Emmanuelm (talk)) is licensed under CC BY 3.0.

EPIDEMIOLOGY & RISK FACTORS

Globally, colorectal cancer is the 2nd most prevalent type of cancer in both men and women; in total, CRC accounts for more than 10% of all reported cases of cancer[75]. With over 1.9 million new diagnoses and more than 900 000 deaths annually, CRC is also the 2nd most lethal type of cancer[75]. The highest incidence and mortality of CRC is found in countries ranking from high (0.700 – 0.799) to very high (≥0.800) on the human development index (HDI, ranges from 0.0 – 1.0)[75, 76]. Strikingly, the CRC incidence is approximately 4 times higher in countries with a high HDI compared to those with a low HDI[75]. Much of the difference in CRC incidence is attributed to the diet and lifestyle common in countries with a higher HDI (**figure 4**). A CRC-promoting diet and lifestyle is generally characterized by low levels of physical activity combined with a high intake of red meat, alcohol, and sugar[77, 78, 79]. Additionally, tobacco use has also been linked to an increased risk of developing CRC[77]. Another CRC-associated lifestyle factor is being overweight or obese, which also increases the risk of developing CRC[78]. Furthermore, in individuals suffering from diabetes mellitus (increased blood sugar levels), the overall risk of developing CRC is increased[80]. The age of CRC onset is also lower in diabetes patients compared to CRC patients not suffering from diabetes[81].

It is estimated that the global CRC incidence will increase by as much as 50% in the coming 10 to 20 years, as the diet and lifestyle of developing countries become more similar to that of western countries[82]. As age is also related to CRC incidence, the historical and continued increase in life expectancy is also a contributing factor to the increase in CRC incidence (as well as other types of cancer)[74, 83]. Interestingly, while the incidence of CRC is highest in countries with the highest HDI, the mortality is equivalent to countries with a medium HDI[74]. This discrepancy between incidence and mortality in countries with a high HDI is likely due to a combination of preventative measures and access to better healthcare compared to countries with a lower HDI[74]. An example of this is how the 5-year survival in CRC (avg. 60-70%) is affected by the tumour stage at the time of diagnosis, where earlier diagnosis (diagnosing the CRC at an earlier stage) correlates to better survival[84, 85].

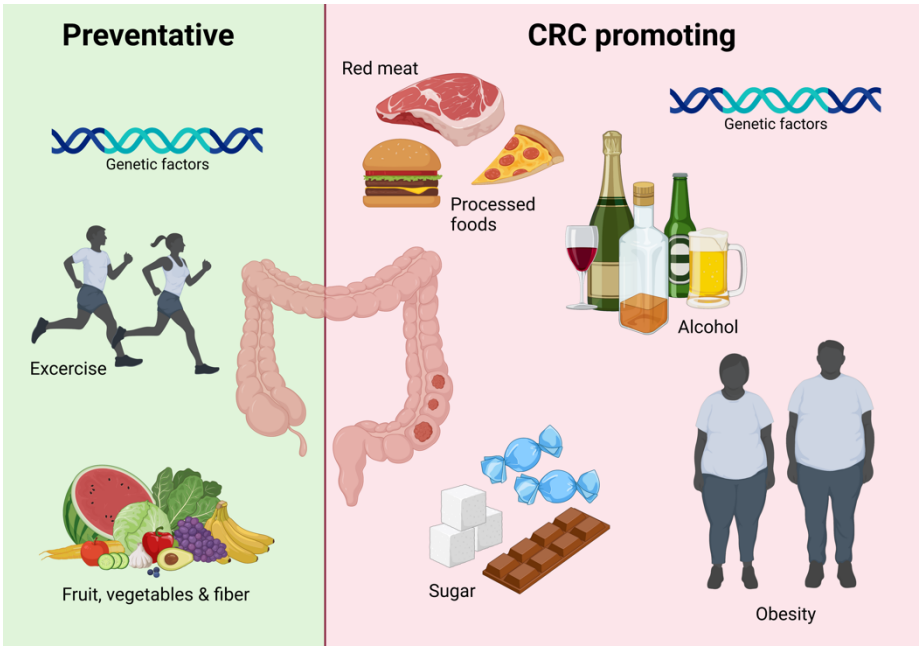

Figure 4. Lifestyle factors influencing the risk of developing CRC. Lack of exercise and a diet consisting of red meat, processed foods, alcohol, and sugar have been shown to increase the risk of developing CRC. In contrast, exercise, and a diet rich in fibres, fruits, and vegetables have been shown to protect from CRC. Genetic factors are known to both increase and decrease the risk if CRC. Created using BioRender.com.

Approximately two-thirds of all CRC cases are sporadic and the result of spontaneous somatic mutations, while the remaining cases are caused by some kind of hereditary component[86]. The well-defined hereditary conditions contributing to CRC development include Lynch syndrome, also called hereditary nonpolyposis colorectal cancer (HNPCC), *MUTYH*-associated polyposis, Peutz-Jeghers syndrome, Juvenile polyposis syndrome, hereditary mixed polyposis syndrome, serrated polyposis syndrome, and Familial adenomatous polyposis (FAP)[86]. However, these well-defined hereditary conditions include only 5% of all cases of hereditary CRC[86]. FAP is the result of a defect in the tumour suppressive adenomatous polyposis coli gene, while HNPCC is the result of defects in the mismatch repair genes, and *MUTYH*-associated polyposis is the result of defects in a DNA glycosylase (affecting the base excision repair mechanism)[86].

The remaining cases of hereditary CRC are grouped based on family history, rather than on defined genetic conditions. Having at least one first-degree relative presenting with CRC, at any point in life, correlates with a higher risk of developing CRC[87]. Additionally, there is a strong correlation between having one or more relatives presenting with CRC at a young age (<40 years old) and the likelihood of developing CRC, suggesting other, currently unknown, hereditary components[87].

Another risk factor associated with CRC is gender, as CRC incidence and mortality are approximately 30% higher in males compared to females[75]. While it is not clear what this difference between males and females can be attributed to, it has been shown that the levels of testosterone and oestradiol/oestrogen may have an effect[88, 89]. Interestingly, hormonal replacement therapy has been shown to reduce CRC-related mortality in female CRC patients[90]. Furthermore, inflammatory bowel disease (Chron´s disease and ulcerative colitis) is also a risk factor associated with CRC development and progression as tumour development is promoted by the state of chronic inflammation present in the intestines during IBD[74]. It has been shown that regular (at least bi-weekly) ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) decrease the risk of tumour development by as much as 20%, indicating that mild inflammation in the intestines is sufficient to increase the risk of tumour development[74]. However, as long-term use of NSAIDs increase the risk of intestinal bleeding and heart attack, it is not recommended as a preventative measure in the general population[74].

As mentioned above, gender, socioeconomic status, hereditary conditions, other diseases, and age are all factors that have been associated with an increased risk of developing CRC. However, it is difficult to say which of these factors is the most important as many of them are affected by, or associated with, each other.

ONCOGENESIS & SUBTYPES

Most CRC cases (>90%) begin as precancerous neoplastic lesions, or polyps, in the colon[74]. The polyps originate from intestinal stem cells in the crypts of the colon, that acquire mutations over time, driving the progression from healthy mucosa, to adenomatous polyps, adenocarcinomas, and eventually to invasive carcinomas[91] (**figure 5**). Metastatic disease and survival of the primary tumour are believed to be furthered by colon cancer stem cells (CCSCs) which are largely undifferentiated cells that constitute only a small fraction of the total cell mass of the tumour[91]. CCSCs are heterogenous and capable of initiating the growth of histologically similar tumours in different recipients and locations[91].

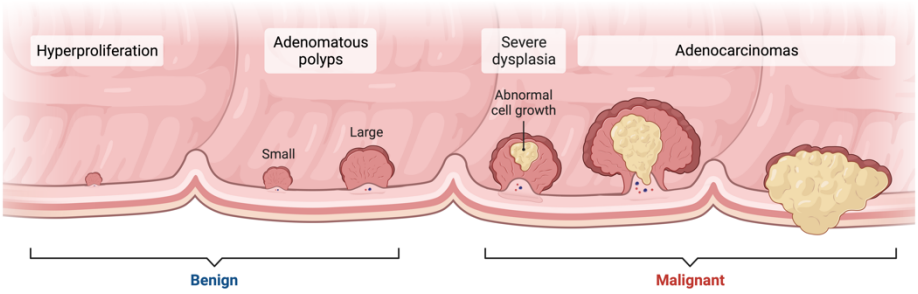

Figure 5. Formation of primary colon tumours. Polyps form as cells of the epithelium acquire enough mutations to enter a hyperproliferative state. The polyps continue to grow and can eventually form adenocarcinomas. Adapted from "Benign and Malignant Colon Cancer" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

Historically CRC has been divided into three groups based on the tumour being located in the proximal colon, distal colon, or rectum. This broad division is not without merit. However, current research better shows the heterogeneity of colorectal tumours by dividing them into molecular subtypes. Today, CRC is often classified based on mutations in the mismatch repair (MMR) genes. Tumours without mutations in the MMR genes are called microsatellite stable (MSS) and constitute ~85% of all colorectal tumours[92]. MSS tumours often have mutations in the chromosomal instability (CIN) pathway, characterised by an increased mutational burden in driver genes, such as *APC*, *Kras*, and *TP53*[93]. The remaining 15% of CRC cases often have deficient MMR machinery and are classified as part of the microsatellite instability (MSI) category[93]. MSI tumours can be further divided into two categories based on high (MSI-H) or low (MSI-low) mutational burden[93]. Furthermore, a small number of CRC tumours are classified as part of the CpG island methylator phenotype (CIMP), characterized by hypermethylation of promotor regions in some tumour suppressor genes[93].

Several other systems for classifying CRC tumours, based on different gene expression data and clinical parameters, have been developed in recent years[93]. To resolve the inconsistencies between several of these classification systems, Guinney et. al. created the consensus molecular subtypes (CMS) (**figure 6**). The CMS classification system consists of four categories based on the mutational burden, immune cell infiltration and immune response, mesenchymal alterations, and metabolic features of the tumours[94]. CMS categories function not only as prognostic markers for the efficacy of chemo-, radio-, or immunotherapy but also as a predictor of the clinical outcome as CRC progression varies between the different CMS categories[94]. CMS1 tumours are characterized by high immune cell infiltration and a strong MSI signature ($CIMP^{high}$). CMS2 are canonical tumours, described as immune deserts with a CIN phenotype. CMS3 tumours also have a low immune cell infiltration but are instead characterized by metabolic dysregulation and mutations in the *Kras* and *APC* genes. CMS4 tumours are characterized by mesenchymal alterations, an inflamed immune cell profile, and high somatic copy number alterations[94]. Another aspect of CRC is that of developing metastatic disease, where the cancerous cells undergo epithelial-mesenchymal transition (EMT) and leave the site of the primary tumour[95]. Using the circulatory system these cells can then travel to other tissues or organs and establish secondary tumours (metastases)[96]. Metastatic spread can also occur locally through bordering tissues[96]. In CRC the main cause of mortality is metastatic disease and roughly 20% of all new cases present

with metastases at diagnosis and an additional 25-50% of primary CRC tumours will later develop metastases[96, 97]. While metastatic growth is possible in any tissue or organ, the most common metastatic areas in CRC are lymph nodes, liver, peritoneum, and lungs[96]. Additionally, around 20% of all patients with metastatic disease will present with synchronous metastases in different organs[98].

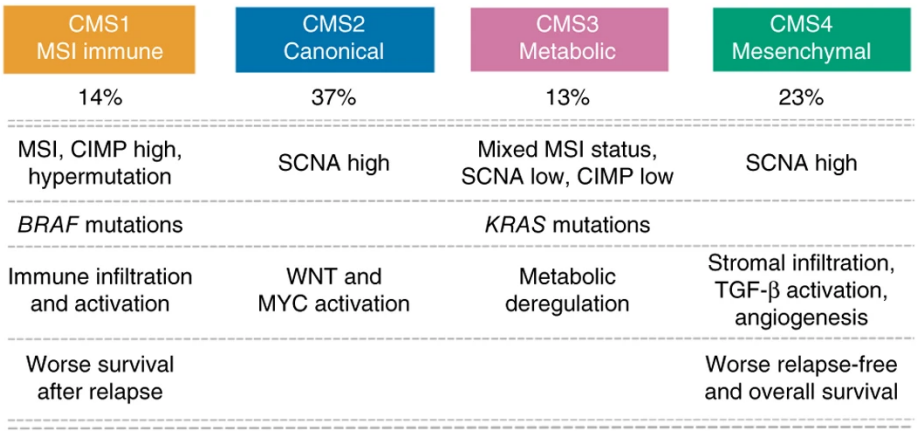

Figure 6. Overview of CMS categories. CMS1 tumours are highly infiltrated by activated immune cells and are highly mutated. CMS2 are immune deserts with high somatic copy number alterations. CMS3 generally have a lower mutational burden, compared to all other CMS categories, low immune cell infiltration, and metabolic dysregulation. CMS4 tumours have mesenchymal alterations, an inflamed immune cells profile along with high somatic copy number alterations. ©2015 Guinney, J., Dienstmann, R., Wang, X. et al. (Licensed under CC BY 4.0)[94]

In a clinical setting, the treatment course is mainly based on the TNM classification system (**table 1**)[99]. The first letter (T) of the TNM classification system describes the spread of the primary tumour into, and trough, the different tissue layers of the colon, while the second letter (N) describes the metastatic spread to regional lymph nodes[99]. The last letter (M) describes the type and number of distant metastases found[99]. Together, the information from the three parameters of the TNM classification system are used to assign a stage (**table 2**) to the tumour[99]. It is often the stage that is referred to (as this is the combined "score" of severity) when disease outcome is discussed e.g., in relation to a new type of treatment.

Although not part of the tumour stage or TNM classification system, the MMR status of the tumour(s) can also provide additional information when devising a treatment plan[99].

Table 1. TNM classification of CRC.

Primary tumour (T)	
TX	Primary tumour spread not known
T0	No indication of primary tumour
Tis	Carcinoma *in situ* – invasion of the epithelium or lamina propria
T1	Invasion of the submucosa Subclassified according to how far into the submucosa the tumour has spread: Sm1 = 1/3 of the submucosa Sm2 = 2/3 of the submucosa Sm3 = spans the entire submucosa
T2	Invasion into m. propria
T3	Invasion through m. propria and subsequent spread into the peri colorectal tissue
T4a	Spread to the surface of the visceral peritoneum
T4b	Invasion of other organs and/or tissues
Regional lymph node metastasis (N)	
NX	Spread to regional lymph nodes not assessed
N0	No metastasis to regional lymph nodes
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumour deposits
N2	Metastasis in 4, or more, regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7, or more, regional lymph nodes
Distant metastatic spread (M)	
M0	No distant metastatic spread
M1	Known distant metastatic spread
M1a	Metastatic spread into one organ or tissue but without peritoneal invasion
M1b	Metastatic spread into more than one organ/tissue
M1c	Metastatic spread into the peritoneum (with or without additional metastatic spread into other organs/tissues)

Note. Nationellt vårdprogram tjock- och ändtarmscancer[99].

Table 2. Tumour stage.

Stage	T	N	M
0	Tis	N0	M0
I	T1/T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3-T4a	N1/N1c	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

Note. Nationellt vårdprogram tjock- och ändtarmscancer [99].

TREATMENT

CRC screening (though colonoscopy or stool tests) is becoming more common, especially in the older population, and facilitates early detection of adenomas and tumours. However, at least 25% of all CRC cases are diagnosed at more advanced stages and present with a dramatically reduced 5-year survival compared to those diagnosed at an early stage. The 5-year survival for patients with stage IV tumours, at the time of diagnosis, is 11% and for those with stage I tumours it is more than 90%[74, 100]. Regardless of the stage at diagnosis, the preferred treatment for CRC is the surgical removal of all primary and secondary tumours through partial or complete resection of the affected tissue[74]. However, CRC diagnosed at more advanced stages, especially those that present with metastatic disease, cannot always be surgically treated. For instance, only 20% of patients with hepatic metastasis can be treated surgically[101]. In cases where the tumour is unresectable, radio- (rectal tumours and metastatic disease), chemo-, or immunotherapy can be used as treatment options, either as standalone treatments or as combination therapies[74]. In some cases, radio- or chemotherapy is also utilised to shrink the tumour and enable surgical resection[74].

While it is possible to effectively treat colorectal cancer, especially early-stage tumours where resection is possible, there is a lack of treatments that are effective against all types of tumours. This calls for further research into both detection and prevention, but perhaps more urgent is the need for treatments and therapies effective against different subtypes of colorectal tumours. This is further highlighted by the expected increase in CRC incidence over the coming decade.

UNCONVENTIONAL T CELLS

Unconventional T cells are characterised by a semi-invariable T cell receptor (TCR) along with expression of surface markers associated with both conventional αβ T cells and innate immune cells, making unconventional T cells a bridge between the adaptive and innate immune system[102, 103]. Unlike most conventional T cells, the majority of the unconventional T cells home directly to peripheral tissues, rather than to secondary lymphoid organs, and are ready to perform their effector functions immediately upon activation[104, 105]. Unconventional T cells encompass a wide variety of different cells including γδ T cells, MAIT cells, and several different subsets of Cd1 reactive αβ T cells[106] (**table 3**).

Table 3. Comparison between MAIT cells, γδ T cells, and iNKT cells.

	MAIT cells	γδ T cells	iNKT cells
Tissue	Circulation (1-10%), mucosal sites (variable), liver (up to 50%)	Circulation (up to 5%), mucosal sites, skin	Circulation, mucosal sites, liver
Reactivity	MR1	CD1 or MHC independent	CD1d
TCR	Vα7.2-Jα33/12/20	γδ TCR	Vα24-Jα18:Vβ11
Subsets	$CD8^+$, $CD4^+$, DN	γ and δ chains define subsets	Based on function (like Th-cells)
Hallmark antigen(s)	5-OP-RU	Phosphoantigens, surface molecules	α-GalCer
Transcription factor(s)	RoRγt, PLZF, Eomes, Tbet	RoRγt, Tbet	RoRγt, PLZF, GATA3, Tbet
Effector molecules	Granzyme B, perforin	Granzyme B, perforin	Granzyme B, perforin

Cytokines	IFNy, TNF, IL-2, IL-17, IL-22	IFNy, TNF, IL-17	IFNy, IL-2, IL-4, IL-10, IL-13, IL-17, IL-22

γδ T CELLS

γδ T cells make up 0.5-5% of all T cells and are characterized by a semi-invariant TCR consisting of one γ chain and one δ chain, like the conventional T cells, the γδ TCR also undergoes somatic recombination and selection in the thymus[106, 107]. However, the number of Variable (V), Diversity (D), and Joining (J) segments available for the γδ TCR is much more limited than for the αβ TCR[106]. Additionally, the γ and δ chains pair selectively with certain segments, which further limits the diversity of the γδ TCR[106]. Although γδ T cells recognize a limited number of antigens, most of the cells present outside of the thymus are fully differentiated effector cells, capable of immediate response upon activation[106].

In humans, γδ T cells are divided into subsets based on which variable segment is incorporated in the δ chain and can be further delineated based on the variable segment incorporated in the γ chain[108]. In total, 8 different δ chains and 6 different γ chains have been identified in humans, based on sequencing data[109]. The most common, and best characterized, subsets of γδ T cells are the Vδ2 and Vδ1 cells[109].

Vδ2 cells are mainly found in the peripheral circulation where they can make up as much as 90% of the total γδ T cell population[106]. However, while they are most abundant in blood, Vδ2 cells also home to the intestinal mucosa as well as infiltrate solid tumours (*paper I*, **fig. 1G**)[108]. The Vδ2 cells are generally divided into 2 subsets based on the expression of the Vγ9 chain, with $V\gamma9^+V\delta2^+$ cells being the most common[106]. The $V\gamma9^+V\delta2^+$ cells are described as more innate-like cells, with a less diverse TCR repertoire compared to other γδ T cells[110]. $V\gamma9^+V\delta2^+$ cells are capable of recognizing prenyl pyrophosphate metabolites (phosphoantigens), such as (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) derived from the non-mevalonate pathway of isoprenoid synthesis in microbes and isopentenyl pyrophosphate (IPP) derived from the mevalonate dependent pathway of isoprenoid synthesis in mammalian cells[110]. IPP expression is up-regulated in some tumour cells, enabling $V\gamma9^+V\delta2^+$ cells to recognize and destroy them through the release of perforin and granzyme or indirectly via IFNγ and TNF[111]. While phosphoantigens are known to activate $V\gamma9^+V\delta2^+$ cells it is so far poorly understood how they are presented to the γδ T

cells[112]. Much less is known about the small population of $V\gamma9^{-}V\delta2^{+}$ cells, but they have been described as more adaptive-like compared to the more abundant Vγ9 expressing Vδ2 cells[110]. Unlike other Vδ2 cells, the $V\gamma9^{-}V\delta2^{+}$ cells preferentially home to tissues and are unable to respond to phosphoantigens[110, 113].

Compared to the Vδ2 cells, Vδ1 cells constitute a much smaller population of the γδ T cells and are mainly found in the intestinal mucosa, skin, and hepatic tissue[111]. The Vδ1 cells have a diverse TCR repertoire and have also been shown to possess a stronger cytotoxic activity towards tumour cells, compared to most Vδ2 cells[111]. Generally, Vδ1 cell populations are polyclonal as clonal expansion is uncommon[111]. Using the TCR and other cell surface receptors, such as NK cell associated surface receptors, Vδ1 cells can recognise stress-induced self-ligands, such as the MHC class I polypeptide-related sequence A and B (MICA/B), endothelial cell protein C receptor (EPCR) and UL16 binding protein 1 (ULBP) that are up-regulated in some tumour cells[111].

Activated γδ T cells (**figure 7**) respond through rapid proliferation and production of cytokines and cytotoxic mediators, although the response varies between the different subsets of γδ T cells, how they were activated, and also based on the composition of the local environment where the cell is activated[107]. Tumour-infiltrating γδ T cells can produce both pro-inflammatory cytokines, such as IFNγ and TNF, as well as tumour-promoting cytokines, such as IL-17[107]. γδ T cell cytotoxicity is known to be mediated through the binding of TNF-related apoptosis-inducing ligand (TRAIL) or Fas-ligand (FasL) with their respective ligands, as well as through the release of granzymes, perforin, and granulysin[107]. Some γδ T cell subsets also express FcγR III (CD16) and can destroy tumour cells through antibody-dependent cellular cytotoxicity (ADCC)[107].

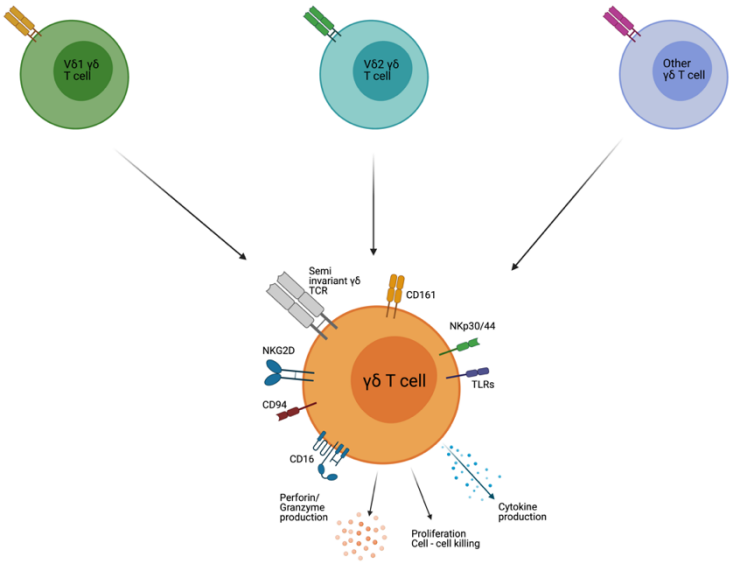

Figure 7. Subsets and functions of γδ T cells. γδ T cells are commonly grouped into Vδ1, and Vδ2 cells, depending on the tissue or context other subsets can also be identified. Different subsets of γδ T cells respond in different ways, but the most common responses involve the release of cytotoxic effector proteins, cytokine production, and direct killing via surface receptors. Furthermore, different subsets can express different surface markers and some of the commonly expressed surface markers among γδ T cells are shown in this figure. Created using BioRender.com.

To date, the availability of commercial antibodies towards the different δ chains in the TCR is limited[†], likely impeding much of the research effort into γδ T cell biology and narrowing our understanding of these cells in both health and disease.

MUCOSAL-ASSOCIATED INVARIANT T CELLS

Mucosal-associated invariant T (MAIT) cells express a semi-invariant αβ TCR with the Vα7.2-Jα33/12/20 chain paired with one of only a few different β chains (mainly Vβ2/13), resulting in a TCR with low diversity[114, 115]. The MAIT cell TCR recognizes small metabolically associated antigens from microbes presented by the evolutionary

[†]commercial antibodies towards the TCR Vδ1 and Vδ2 chains are currently available.

conserved MHC class 1 related molecule (MR1)[114]. Two of the most common antigens associated with MAIT cells are the short-lived and unstable molecules 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)[114]. 5-OE-RU and 5-OP-RU are formed when the vitamin B2 precursor 5-amino-6-D-ribitylaminouracil (5-A-RU), found in both fungi and bacteria, reacts with one of the two aldehydes, glyoxal or methylglyoxal[114]. Upon binding the MR1 molecule, 5-OE-RU and 5-OP-RU are stabilized and can react with the MAIT cell TCR[114]. In addition to vitamin B2-derived antigens, other bacterial antigens, viral antigens, synthetic antigens, and metabolically associated antigens derived from human cells have been shown to activate MAIT cells, although at much higher concentrations compared to 5-OE-RU and 5-OP-RU[116, 117, 118, 119]. Studies have shown that MAIT cell responses are hampered towards bacteria where the riboflavin pathway (vitB2) is absent or deficient[114]. Furthermore, recent efforts in characterising new antigens capable of interacting with MR1 and MAIT cells show that there are both activating and inhibitory antigens that modulate the response of MAIT cells interacting with antigen-loaded MR1 molecules[120].

MAIT cells are present in the circulation and mucosal tissues of the gut, intestine, lungs, and genital tracts (**figure 8**)[121]. In the blood they can make up 10% of all circulating T cells and in some tissues, such as the liver, they can make up as many as 50% of all T cells[121, 122]. Mature MAIT cells constitutively express CD161 and often also express CD26 and CD218. It is generally possible to identify MAIT cells based on the co-expression of Vα7.2 and CD161. However, in some tissues and conditions it is possible that some Vα7.2$^+$ non-MAIT cells also express the hallmark "MAIT cell markers" and therefore using an antigen loaded MR1 tetramer can be necessary to successfully identify MAIT cells[122].

MAIT cells develop in the thymus and are selected based on TCR recognition of MR1 expressed by CD4$^+$CD8$^+$ cortical thymocytes, unlike conventional T cells that are selected based on their ability to recognise MHC molecules expressed by thymic epithelial cells[28, 123, 124]. Interestingly, MAIT cells and type I NKT cells show remarkable similarity in early development as well as in transcriptional profile and function[125]. In humans, MAIT and type I NKT cell frequencies vary greatly from person to person, but they have also been shown to be correlated to one and other[122].

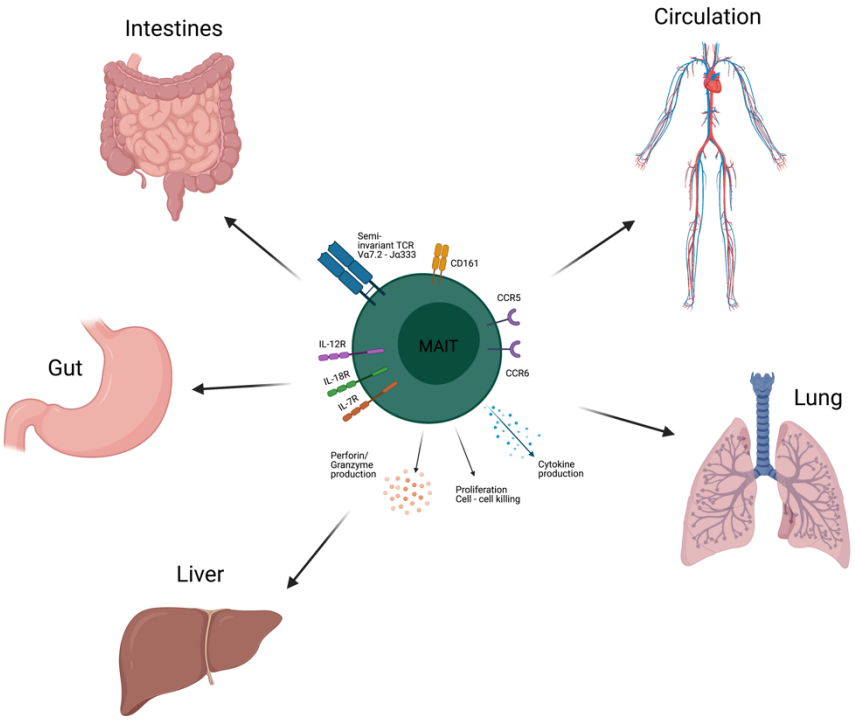

Figure 8. MAIT cell distribution. MAIT cells are found in the circulation, lungs, liver, gut, intestines, and genital tracts (not shown). Created using BioRender.com

As effector T cells, MAIT cells are capable of rapid production and release of both cytokines and other effector molecules. In response to both TCR mediated and TCR independent activation they can produce pro-inflammatory cytokines, such as IFNy, TNF, and IL-17[115]. MAIT cells also produce cytotoxic mediators, such as granzymes, granulysin, and perforin[115].

OTHER TYPES OF UNCONVENTIONAL T CELLS

CD1-restricted αβ T cells recognize antigens bound to the non-classical MHC protein CD1 and are classified based on the isoform of CD1 the T cell interacts with[126]. Similar to MAIT cells, the CD1-restricted T cells can be identified using antigen-loading tetramers[126]. Group I CD1-restricted T cells include cells reactive towards the isoforms CD1a, CD1b, and

CD1c. While group II CD1-restricted T cells encompass αβ T cells reactive towards CD1d, commonly referred to as Natural Killer T (NKT) cells as they share several features of both T and NK cells[126]. The CD1d molecule presents glycolipid antigens while group I CD1-molecules present lipid antigens, both classes of antigens can be derived from either microbial activity or from host cells[126]. The group II CD1-restricted NKT cells can be further divided into three separate subsets referred to as classical or invariant NKT (iNKT) cells, non-classical or diverse NKT cells, and NKT-like cells[127, 128]. The iNKT cells are characterised by their expression of the Vα24-Jα18:Vβ11 TCR and their ability to recognise α-GalCer[126]. All CD1-restricted T cells develop in the thymus, and they are selected based on their ability to recognise the various isoforms of CD1 on $CD4^+CD8^+$ cortical thymocytes[128].

The ratios between MAIT cells and CD1-restricted T cells differ between humans and rodents, with MAIT cells being relatively abundant in humans and CD1-restricted T cells virtually absent, while the ratio is inverted in mice[128]. Thus, most of what we know about CD1-restricted T cells stems from research done in mice. Despite the differences in antigen recognition, CD1-restricted T cells seem to have similar functions as MAIT cells in both infections and tumour immunity.

TUMOUR IMMUNITY

The hallmarks of cancer (originally proposed by Hanahan and Weinberg in 2000) include "avoiding immune destruction" and "tumour-promoting inflammation" as two of the now 8 hallmarks and 2 enabling characteristics of cancer progression (as of 2022 an additional two hallmarks and 2 enabling characteristics have been proposed) (**figure 9**)[129, 130, 131]. Together, the hallmarks "avoiding immune destruction" and "tumour-promoting inflammation" are cornerstones of the concept of tumour immunity. Crucial to the concept of tumour immunity and the subsequent outcome of tumour-immune reactions are the expression of cytokines, chemokines, effector proteins, immune checkpoint molecules, and neoantigens by immune and tumour cells.

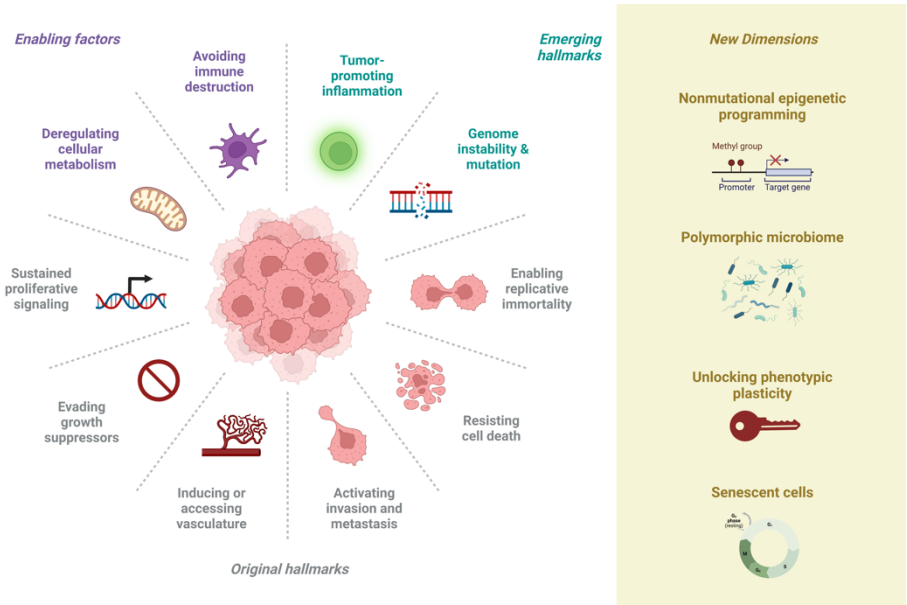

Figure 9. Hallmarks of cancer. The original hallmarks, enabling factors, emerging hallmarks, and new dimensions as proposed by Hanahan and Weinberg. Adapted from "Hallmarks of Cancer: Circle" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

Cytokines are small immunomodulatory proteins that are mainly associated with immune cells[132]. However, unlike hormones, almost all nucleated cells can produce and secrete one or several different kinds of cytokines[132]. Cytokines include interleukins, interferons, proteins belonging to the tumour necrosis factor (TNF) superfamily, chemokines, colony-stimulating factors (CSFs), monokines, transforming growth factors (TGFs) , and lymphokines[132]. Depending on the context cytokines can be either pro- or anti-inflammatory[132].

Interleukins are small soluble proteins that are responsible for much of the signalling between immune cells, both during homeostatic conditions and disease[133]. The interleukins can be divided into superfamilies or families based on structural similarities and shared features[133]. The structural similarities also mean that interleukins in the same family or superfamily to some extent can share receptors and signalling pathways[133]. As a group, the interleukins have pleiotropic effects and can have activating, proliferative, inhibiting, and stimulating effects depending on the context and cell types involved[133]. Furthermore, certain cell types or

subsets of cells, like T cells, can be skewed towards the expression of groups of cytokines with similar functions, that promote for instance pro- or anti-inflammatory responses. The specific functions of some cytokines, in a cancer setting, are covered in the chapter – "inhibiting and promoting immune responses".

There are 21 different interferons divided across 3 major subtypes (type-I, -II, and -III) that exert their function through signalling via cell surface receptor complexes composed of low- and high-affinity heterodimers[134]. Initially, interferons were named after their ability to interfere with viral replication in host cells, but research has shown that they have far-reaching effects in almost all kinds of diseases. The 16 type-I and 4 type-III interferons are mainly the product of innate immune cells and trigger similar anti-viral programmes, mediated by ISGF3 in host cells[135]. In contrast, the single type-II interferon (IFNγ) is mainly produced by NK cells and activated T cells (although some APCs have also been shown to produce IFNγ)[135]. Like its cousins, IFNγ also stimulates anti-viral activity but unlike the other two types of interferons, it primarily functions as a potent activator of macrophages, stimulating phagocytosis, upregulating MHC II, and increasing the intracellular killing of microbial pathogens[136]. IFNγ production and secretion in T cells is triggered by IL-12, IL-15, IL-18, and type-I interferon-signalling[137]. Furthermore, IFNγ functions as a differentiation signal for cytotoxic T cells as well as stimulates the pro-inflammatory and tumoricidal phenotypes of macrophages[137].

Proteins belonging to the tumour necrosis factor superfamily are not necessarily cytokines as some are membrane-bound, while others are soluble, and a few, such as TNF, exist either as a membrane-bound ligand (mTNF) or as a soluble cytokine (sTNF)[138]. TNF is initially membrane-bound and the soluble form is created via enzymatic cleavage of mTNF[138]. Soluble TNF primarily interacts with the transmembrane receptor TNFR1 that induces apoptosis, necrosis, or cell survival depending on the ubiquitination state of the receptor[138]. In contrast, mTNF interacts with both TNFR1 and TNFR2, where interaction with the latter induces cell survival through proliferation[138]. While the main source of TNF is monocytes or macrophages, almost all other immune cells, including T cells, can produce TNF[138]. Like many other cytokines, TNF has a dual role, known to both induce cell death in cancer cells and mediate a pro-inflammatory immune response while also promoting the chronic inflammatory conditions in autoimmune diseases, such as rheumatoid arthritis, Chron's disease, ankylosing spondylitis, and psoriasis[138]. As we will later discuss the importance of the tumour

microenvironment (TME) and how its composition of cells and soluble factors shape the immune response and tumour progression it is worth noting that TNF in a cancer setting is likely to both drive tumour progression but also play a role in the antitumour immune response, depending on the other aspects of the TME. In the TNF superfamily, there are, apart from TNF, 18 other known members, including lymphotoxin-alpha (LT-α/TNF-β), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) all of which can mediate cytotoxicity in target cells expressing the cognate receptors[139].

Chemokines are a large family of chemoattracting cytokines that interact with specialised G-protein coupled chemokine receptors, expressed by both immune and non-immune cells[140]. The most well-studied function of chemokines is their ability to induce cellular movement, thereby influencing the recruitment of immune cells[140]. However, chemokines are now known to share many of the signalling capabilities of other cytokines[140]. Based on structural differences in primary amino acid sequence, chemokines can be divided into five subfamilies: CC, CXC, CX3C, and XC[140].

Immune checkpoint molecules are membrane-bound receptors that are upregulated on activated immune cells[141]. They are crucial for regulating the activity of activated immune cells during homeostatic conditions and prevent them from causing unspecific or excessive damage to the host[141]. Through interaction with their ligands, the checkpoint molecules can prevent activation, reduce activity, or induce anergy in immune cells[141]. Efforts in modulating immune cell activity by blocking immune checkpoint molecules, or their ligands, have resulted in several different treatment options for both solid and haematological malignancies[141]. Furthermore, many more immune therapies using blocking antibodies towards checkpoint molecules are in development and their use is becoming increasingly common[142]. Checkpoint molecules that are known to impair immune cell responses through inactivation include, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), programmed cell death protein 1 (PD-1), B- and T-cell lymphocyte attenuator (BTLA), T-cell immunoglobulin and mucin domain-containing 3 (Tim-3), V-domain Ig suppressor of T-cell activation (VISTA), and lymphocyte-activation gene (LAG3)[141].

Some tumours can exploit immune checkpoint molecules by expressing their ligands (such as PD-L1 and L2) and can thereby induce a state of inactivity in the infiltrating immune cells, facilitating immune evasion.

This state of inactivity in immune cells is called exhaustion which is now a well-described state in effector T cells in both cancer and chronic infections[143]. Exhaustion in T cells can occur as a result of failed memory cell development due to continuous antigen stimulation[143]. Exhausted T cells become anergic, lose the ability to produce cytokines and effector proteins, and upregulate the expression of checkpoint molecules[143]. In conventional $CD8^+$ T cells, the state of exhaustion can sometimes be reversed using monoclonal antibodies targeting PD-1 or PD-L1[143]. However, continuous antigen exposure will eventually make the state of anergy irreversible[143]. Exhaustion is not exclusively found in effector T cells but has also been described in both B and NK cells[143].

Neoantigens, or more specifically, tumour antigens (TAs) arise as part of protein synthesis and can be divided into tumour-associated antigens (TAAs) and tumour-specific antigens (TSAs)[144]. TSAs are exclusively present in tumour cells, while TAAs can be presented by both tumour cells and non-transformed host cells[144]. Overexpression of unmodified proteins is the most common type of TAA; however, TAAs also include proteins and molecules with an altered expression and overexpression of non-protein antigens[144]. In contrast, TSAs are mutated antigens exclusive to the tumour or a specific clone of tumour cells[144].

TUMOUR MICROENVIRONMENT

The immediate environment surrounding the tumour, referred to as the tumour microenvironment, is crucial to the local enhancement or suppression of the immune response[145]. The TME is the sum of all cells (immune cells, tumour cells and surrounding non-transformed host cells), soluble factors, ECM, and biomechanical features of the tumour and its immediate surrounding (border)[145]. The composition of the TME is heavily influenced by the tissue of origin and the molecular characteristics of the specific tumour[145]. The TME is a crucial factor in promoting or inhibiting metastatic spread in most cancers, as well as the efficacy of anti-cancer-related drugs and therapies[145]. A crucial feature of the TME is the infiltration and phenotype of immune cells, and tumours are generally classified as hot (immune cell infiltration) or cold (without immune cell infiltration, can be further divided into excluded or ignored tumours)[145]. Additional features of the TME are the production of cytokines and chemokines, the expression of immune checkpoint molecules, and the expression of tumour antigens and ligands[145]. Biomechanical features of

the TME, such as the availability of oxygen inside the tumour, also affect the immune response[145].

INHIBITING AND PROMOTING IMMUNE RESPONSES

NK CELLS

NK cells are cytotoxic lymphocytes belonging to the innate arm of the immune system and generally contribute to the anti-tumour immune response[146]. NK cells express activating and inhibitory surface markers that enable them to recognize transformed or infected cells, e.g., through the absence of major histocompatibility complex I (MHC-I) on certain cancer cells[146]. Tumour-infiltrating NK cells are one of the major sources of intratumoral IFNγ; they can also kill cancer cells using soluble factors such as perforin, granzyme, and tumour necrosis factor[146]. Furthermore, NK cells also kill target cells through cell-to-cell contact via surface markers such as TRAIL and Fas-ligand[146]. IL-15 in the TME is known to recruit NK cells, and infiltration of NK cells into CRC and gastric tumours has been associated with improved outcome[146]. Additionally, NK cells expressing NKp46 have been shown to convey a protective effect in several metastatic cancers[146].

T CELLS

T cells are adaptive immune cells with the capacity to recognize a wide array of antigens and respond to cancer in many different ways[146]. Broadly, T cells are divided into cytotoxic T cells (CTLs), helper T cells (Th cells), and unconventional T cells[146]. Cytotoxic T cells express the co-receptor CD8, and they are potent effector cells capable of killing infected and transformed cells through the release of perforin and granzyme[146]. Additionally, CTLs can eliminate target cells via the expression of surface receptors such as FasL and TRAIL[146]. In contrast to the CTLs, helper T cells are identified by the expression of the co-stimulatory receptor CD4 and encompass a much more heterogenous cell population[146]. Subsets found within the population of $CD4^+$ helper T cells are regulatory T cells (Tregs), Th1-, and Th17 skewed cells[147]. Tregs are immunosuppressive T cells characterised by expression of the transcription factor forkhead box P3 (FoxP3) and interleukin-2 receptor α-chain (IL2Rα/CD25)[147]. Under normal conditions, Tregs are essential

for the induction of tolerance and prevention of autoimmune reactions by suppressing T cell activity[148]. However, in a cancer setting, they have been implicated in the promotion of tumour progression[149].

Tregs suppress the activity of other immune cells through several different mechanisms, one of which is the sequestering of IL-2 from the microenvironment, which inhibits both T and NK cell activity[148]. Tregs can also inhibit T cell activity through enzymatically cleaving extracellular ATP, using the ectoenzymes CD39 and CD73, into adenosine which binds to the receptor 2A on T cells and suppresses activity[148]. Additionally, Some Tregs can directly kill activated effector T and NK cells through the expression, and release, of perforin and granzyme B[148].

Regulatory T cells also express checkpoint inhibitors and ligands, such as CTLA4, Lag3, and PD-L1, that can induce anergy in T cells and modulate the responses of antigen-presenting cells (APCs)[148]. Antigen-presenting cells (APCs), such as DCs, can also be directly inhibited by Tregs through cell-cell contact[148]. Additionally, Tregs produce the immunosuppressive cytokines IL-35, TGF-β, and IL-10[148]. Most Tregs are programmed as regulatory cells already in the thymus, apart from a small number that can be induced at the site of activity (inducible Tregs)[147]. Induction of Tregs outside the thymus is driven by IL-2 and TGF-β[148].

The non-regulatory $CD4^+$ T cells that exit the thymus as naïve cells differentiate into cells with various effector profiles, upon TCR ligation and through signals from various cytokines[147]. As the $CD4^+$ cells are far more heterogenous than their $CD8^+$ counterparts, the infiltration of specific subsets of helper T cells has been associated with tumour-promoting immune responses. In contrast, other subsets of helper T cells have been shown to facilitate tumour rejection[147]. For instance, Th1 polarized cells are characterized by expression of interferon-γ, IL-2, and TNF, and have been shown to improve the anti-tumour immune response by recruitment of macrophages and NK cells as well as being crucial for an effective CTL response[150]. Th1 polarized cells can also exert direct cytolytic activity on tumour cells via IFNγ and TNF as well as through surface receptors, such as TRAIL and FasL[150]. The polarization of $CD4^+$ T cells into Th1 cells is driven by IL-12[151].

Another subset of helper T cells that highlights the duality of the helper T cell responses in tumour immunity is the population of Th17 cells. Th17 cells have been implicated in both the rejection and progression of

tumours and are mainly characterized by the production of IL-17[152]. IL-17 expression in tumours drives a tumour-promoting response by inducing the production of other pro-inflammatory mediators, recruitment of myeloid cells, activation of cancer-associated fibroblasts, and increasing tumour-specific angiogenesis[153]. Surprisingly, IL-17 production by Th17 cells has also been implicated in the elimination of cancer cells through enhanced recruitment and activation of CTLs[154]. Polarisation of naïve $CD4^+$ T cells to become Th17 cells is largely driven by IL-6 and TGF-β[152].

In general, T cell infiltration in tumours has been associated with an improved outcome in several types of solid cancers such as melanoma, colorectal, breast, lung, ovarian, gastric, prostate, and renal tumours[155, 156, 157, 158, 159, 160, 161, 162, 163]. Especially favourable is the infiltration of $CD8^+$ cytotoxic T cells and $CD4^+$ T cells with a Th1 polarized profile, while Th17 cells and Tregs mainly correlate with a worse patient outcome[146, 152, 161].

The role of unconventional T cells in tumour immunity is addressed in the last section of this chapter – "unconventional T cells in tumour immunity".

B CELLS

While some evidence suggests B cells can have anti-tumour properties, the overwhelming majority of research shows that B cells contribute to tumour growth and inhibition of the anti-tumour immune response[164]. For instance, in mice depleted of B cells, it has been shown that tumour growth and spread are reduced[146]. Furthermore, a subset of regulatory B cells (Bregs) has been described as B cells producing cytokines and other pro-tumorigenic factors that, similarly to Tregs, drive the tumour-promoting immune response[146]. The Bregs are a heterogenous population of cells that lack common markers or transcription factors, such as CD25 and FoxP3 in Tregs[146]. Instead, Bregs are identified solely based on their capacity to promote tumour growth and suppress the immune response, often through the production of IL-10, IL-35, and TGF-β or via surface markers such as FasL, CD1d, and PD-1/PD-L1[165].

MYELOID-DERIVED SUPPRESSOR CELLS

Myeloid-derived suppressor cells (MDSCs) are derived from the myeloid lineage and encompass cells with diverse markers and origins[166]. Human MDSCs are divided into two lineages, polymorphonuclear (PMN-MDSCs, $CD11b^+CD14^-CD15^+/CD66b^+$) and monocytic (M-MDSCs, $CD11b^+CD14^+HLA\text{-}DR^{+/low}CD15^-$)[166]. Additionally, a subset of remarkably heterogenous MDSCs, described as immature or "early-onset" (e-MDSCs) have been identified that lack expression of any lineage markers along with human leukocyte antigen-DR (HLA-DR) but that are positive for CD33[166]. MDSCs contribute to tumour progression by promoting angiogenesis and EMT while facilitating an immunosuppressive microenvironment and directly inhibiting immune cells with an anti-tumour function[167]. Through the production of nitric oxide (NO), reactive oxygen species, and peroxynitrite (PNT) MDSCs have been shown to inhibit T cell activity by interfering with the TCR-associated signalling cascade, directly affecting their ability to respond to antigens, or through induction of apoptosis[167]. Also, NO and PNT, along with disintegrin, high mobility group box protein 1 (HMGB1), and disintegrin and metalloprotease 17 (ADAM17), expressed by some MDSCs prevent T cell homing to the tumour by decreasing the surface expression of L-selectin and E-selectin on T and endothelial cells respectively[167]. Inactivation of CCL2 has also been reported as a consequence of peroxynitrite in tumours[167]. Furthermore, PNT expression in mice has been reported to prevent antigen presentation by tumour cells through structural alterations of the MHC class I molecules, thus preventing recognition by CTLs[168]. MDSCs can also inhibit T cell activity by consuming essential amino acids such as cysteine and L-arginine from the TME[166]. Similar to Tregs, MDSCs also express the ectoenzymes CD39 and CD73 that can hydrolyse and cleave soluble ATP into adenosine[167]. It is well known that T cell activity can be regulated through an ATP/adenosine axis where extracellular ATP exerts a pro-inflammatory effect while adenosine exerts a suppressive effect[167]. Tumour-infiltrating MDSCs also contribute to T cell suppression through secretion of IL-10 and TGF-β as well as through expression of the checkpoint inhibitor ligand PD-L1[167]. Furthermore, it has also been reported that TGF-β can inhibit the activity of NK cells and that MDSCs in hepatocellular carcinomas specifically inhibit the activity of $NKp30^+$ NK cells[169, 170].

MACROPHAGES

Macrophages differentiate from circulating monocytes after leaving the circulatory system and entering the tissue[146]. Activated macrophages are generally divided into M1 and M2, where M1 macrophages are considered pro-inflammatory and M2 macrophages are considered to be anti-inflammatory[146]. Macrophages in the tumours or TME are often referred to as tumour-associated macrophages (TAMs) and are generally associated with a tumour-promoting activity[146]. Through secretion of IL-10 and TGF-β TAMs contribute to immunosuppression[146]. Tumour-associated macrophages have also been reported to promote tumour growth and spread, by stimulating angiogenesis, tumour cell proliferation, and EMT[146].

NEUTROPHILS

Neutrophils, the most common immune cell in the circulation, are early responders and key drivers of inflammation in many different settings[146]. In recent years, studies on neutrophils in the tumours and TME have reported that high levels of tumour-associated neutrophils (TANs) are generally associated with tumour progression and metastasis[146]. However, they have also been described as a very heterogenous population with proposed N1 and N2 phenotypes (similar to macrophages), so it is fair to say that their role is not yet fully understood. It is likely that, like other immune cells, the neutrophil response is dependent on the interactions between many different cell types and that they can act in different ways depending on the context.

DENDRITIC CELLS

Dendritic cells can be found in almost all tissues of the human body, the only exception being the parenchyma of the brain[171]. The primary function of DCs is to present antigens to T cells. Depending on the setting and co-stimulatory signals, this can induce either tolerance to the antigen or elicit an effector-type response[146]. This makes the dendritic cells crucial for initiating T cell responses towards tumours. For instance, it has been shown that DCs that lack the expression of formyl peptide receptor 1 (FPR1) are associated with poor survival in CRC patients undergoing adjuvant chemotherapy[172]. Another example of the

importance of dendritic cells is that depletion of CD141^{+} DCs in stage I lung tumours correlate with impaired NK and T cell function[173].

An example of some of the complex interactions of a few immune cells in the TME can be seen in **figure 10**.

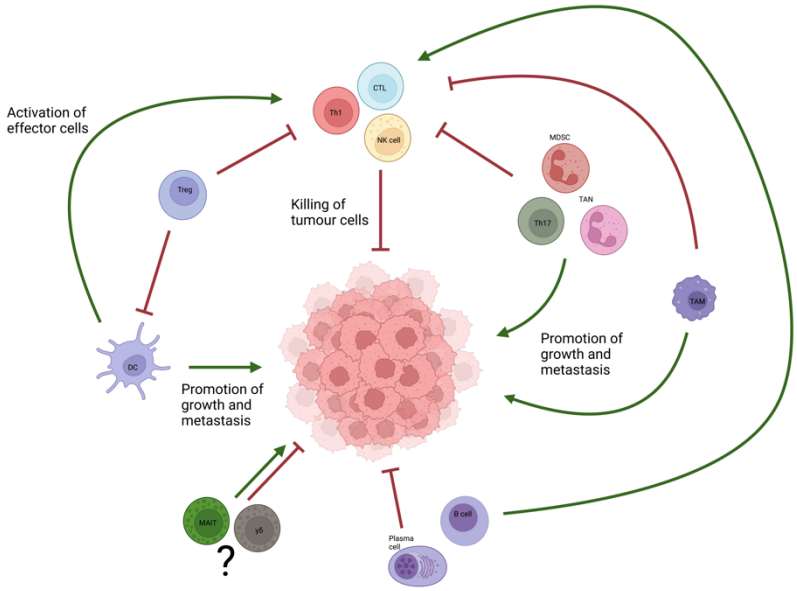

Figure 10. The complex interactions of the TME. The interactions between immune cells in the TME is complex and highly dependent on the cellular composition and presence of various soluble factors. Created using BioRender.com

IMMUNE EVASION

A key feature of tumour cells that heavily affects disease progression is the ability of some tumour cells to avoid detection by the immune system. This process is called immune evasion and can be achieved in several different ways. **Figure 11** shows the possible outcomes of the tumour-immune cell reaction.

Some tumour cells lose the ability to present antigens, through mutations affecting the antigen-processing machinery, which prevents them from being recognized by cytotoxic T and NK cells. These 'invisible cells' will

be favoured by selection pressure and become the dominant clone in the tumour, enabling further growth or metastases formation without being detected by the immune system[174]. Similarly, mutations that lead to alterations or loss of tumour-associated antigens that are recognized by the immune system can also favour clonal expansion of those tumour cells with an altered antigen expression[174]. This change or loss in antigen expression is common in cell therapies where transplanted cells often only target a single tumour antigen, such as CD19 in leukaemia[175].

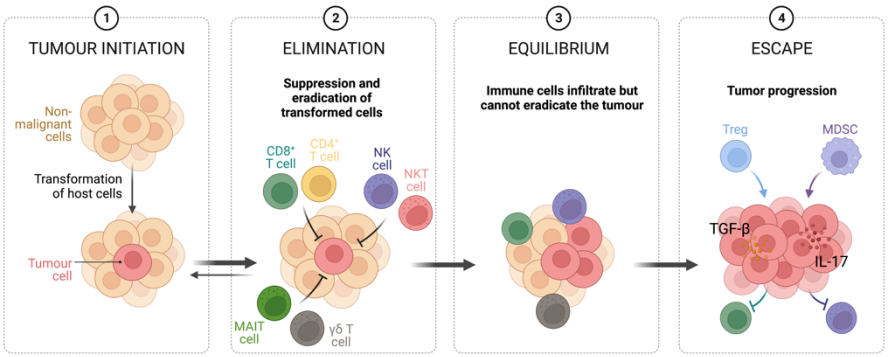

Figure 11. Different outcomes of the tumour immune interaction. After initial tumour formation, the immune system can either recognize and eliminate tumour cells, the tumour cells and immune cells can exist in equilibrium, or the tumour cells can escape the immune system and continue to grow. Adapted from "Cancer Immunoediting" by BioRender (2023). Retrieved from https://app.biorender.com/biorender-templates.

Another way tumour cells can evade the immune system is by inducing tolerance in infiltrating cytotoxic immune cells, this can happen when tumour cells still capable of antigen presentation lose the expression of co-stimulatory molecules[174]. Some tumour cells also up-regulate the expression of inhibitory ligands that enable the suppression of infiltrating immune cells[174]. Furthermore, tumour cells can also down-regulate surface markers normally used as death receptors, evading detection and recognition by NK and T cells[174]. It has also been shown that tumour cells can actively delete tumour-infiltrating cytotoxic T and NK cells through the induction of apoptosis[174].

As previously mentioned, cytokines and other soluble factors present in the TME are crucial in shaping the immune response. Tumour cells and other cells associated with the tumours can produce cytokines and

soluble factors that directly and indirectly suppress immune cells[174]. Also, tumour-mediated chemokine production can also recruit immune cells, such as Tregs and MDSCs that can inhibit the activity of cytotoxic T cells[174]. Additionally, alternatively-activated macrophages can be recruited to the tumour and contribute to the tumour-promoting inflammation and angiogenesis, or even facilitate metastasis[174].

UNCONVENTIONAL T CELLS IN TUMOUR IMMUNITY

While unconventional T cells are generally less numerous compared to their conventional counterparts, they can still constitute much of the immune cell niche in certain tissues[128]. Furthermore, as terminally differentiated effector cells, the unconventional T cells are also likely to be a part of the early immune response during tumour formation and shape much of the subsequent immune response.

As previously mentioned, MAIT cells are mainly activated by metabolically associated antigens derived from microbial pathways, but also from host cells and a few synthetic antigens[114, 116, 117, 118, 119]. Furthermore, MAIT cells can also be activated by cytokine signals alone, for example by IL-12 and IL-18[115]. In mice, both TCR and cytokine mediated activation trigger different transcriptional profiles, that are clearly defined by IL-17 and IFNγ production respectively, however, in humans this dichotomy is less clear[176]. Activated MAIT cells also have the capacity to contribute to wound healing when activated by TCR stimulation and cytokine signals[177]. In mucosal-associated cancers, most studies report that MAIT cells accumulate in the tumours compared to the surrounding healthy mucosa[178, 179]. Furthermore, the levels of circulating MAIT cells are also decreased, in for instance gastric cancer[180]. Exactly by what mechanism tumour-infiltrating MAIT cells are activated is not fully understood but as the intestinal barrier is impaired in most mucosal-associated cancer it is possible that bacterial antigens reach the immune cells[181]. Furthermore, as IL-12 and 18 are both present in tumours, it is likely that cytokines in the TME are also contributing to MAIT cells activation[182].

Exhaustion has been shown to affect MAIT cells in a similar fashion as conventional T cells, resulting in a reduced capacity to perform effector functions and an increased expression of checkpoint molecules, such as PD-1 and Tim-3[183]. In CRC, CD39 expression on tumour-infiltrating

MAIT cells has also been linked to TCR-mediated exhaustion[184, 185, 186]. Furthermore, tumour-infiltrating MAIT cells generally have a reduced capacity to produce cytokines, such as IFNγ, TNF, and IL-17 compared to in the adjacent mucosa[178, 183]. Both TCR dependent and independent activation of MAIT cells can trigger release of the granzymes A, B, and K, along with perforin that have the capacity to induce apoptosis in tumour cells[187, 188]. Of all the serine proteases produced by MAIT cells, granzyme K is of particular interest as it can induce apoptosis in target cells in the absence of the caspase pathway. The caspase pathway is commonly mutated in tumour cells which protects them from the cytolytic activity of caspase dependent granzymes[189, 190].

Like MAIT cells, the effector functions of γδ T cells include production and release of cytokines and cytotoxic effector molecules, ADCC, and effector functions mediated via surface receptors[191]. These effector functions can be triggered by recognition of both non-peptide antigens and surface molecules, which are upregulated by tumour cells[111, 191]. One example of γδ T cell activity in cancer is in response to increased IPP expression by tumour cells, that enables $V\gamma9^{+}V\delta2^{+}$ cells to recognize and destroy them, through the release of cytolytic effector molecules or indirectly via cytokines, such as TNF and IFNγ[111]. Other subsets of γδ T cells, such as Vδ1 cells, utilise surface receptors that binds to ligands up-regulated (due to increased metabolism or stress) on the tumour cells to recognize and mediate effector functions[111]. One example of receptor mediated cytotoxicity towards tumour cells is how $TRAIL^{+}$ γδ T cells have a reduced capacity to induce cell death in tumour cells that downregulate TRAIL receptor 4[192]. In solid cancers γδ T cells are generally associated with a good prognosis but in some cancers, there are conflicting reports or a direct association with a poor prognosis. In ovarian[193, 194], colon[195, 196, 197], and breast cancer[198, 199, 200, 201, 202] γδ T cell infiltration has been associated with both good and poor prognosis, while in HCC[203], gastric cancer[204, 205], renal cancer[206, 207], and GBM[208] infiltration has been associated with a good prognosis. While, in oral cancer the infiltration of γδ T cells is associated with a poor prognosis[209]. In many of these tumour types, infiltration of Th1-like γδ T cells, often of the Vδ1 subtype, producing IFNγ seem to be the main correlate of protection. However, other subsets or specific markers expressed by γδ T cells have also been shown to be associated with a good prognosis. In CRC, the infiltration of $V\gamma9^{+}V\delta2^{+}$ cells is linked to an increase in disease-free survival; also, the presence of Vδ1 cells, expressing the NK cell receptor

NKp46, in the mucosal tissue surrounding the tumours correlated with a reduced risk of developing metastases[210].

As already touched upon, murine unconventional T cells seem to be more clearly defined into subsets, based on the expression of certain transcription factors. An example of this is how IFNγ and IL-17 producing MAIT cells are defined by expression of T-bet and RoRγt[211]. The same clear division of unconventional T cells into functional subsets is generally not seen in humans. This likely means that the results from mice are difficult to extrapolate to humans or that what is seen in one tumour type is not necessarily true for others, as the subset or phenotype of unconventional T cells can vary greatly among different types of tumours and patients. From much of the research in the field of tumour immunity, in humans, it is evident that while a few cells (such as cytotoxic T and NK cells) can undoubtedly participate in the eradication of tumours, the role of most immune cells is far more complex and context-dependent. For this reason, continued efforts to understand how the microenvironment and immune landscape of tumours shape disease progression and treatment outcomes are needed.

IMMUNOTHERAPIES

It has been more than 100 years since Paul Ehrlich hypothesized that the immune system could recognize and protect us from cancer, and over 50 years since Thomas and Burnet laid the foundation for the theory of immunosurveillance. However, the use of treatments that focus on employing the immune system has only come into clinical use during the last deacade[212, 213, 214, 215]. According to the cancer research institute, more than 4000 (2020) different immunotherapies are being researched, and over 2500 (2022) cell therapies are in development. However, most of these potential treatments are still in pre-clinical research, and only a handful are currently approved for patient use (93 drug-based and eight cell-based therapies)[216, 217].

CHECKPOINT INHIBITORS

In 2011 and 2014, the first immune checkpoint inhibitors were approved for clinical use. These drugs target CTLA-4 (Ipilimumab) and PD-1

(Nivolumab and Pembrolizumab), respectively. Since then, research into these checkpoint inhibitors has continued, and currently, ten different treatments focusing on CTLA-4, PD-1, or PD-L1 are approved for use[218]. Additionally, several other targets, such as LAG-3, TIGIT, VISTA, and TIM-3, are being investigated as potential targets for new immunotherapies[219].

While the targeted receptor or cell type varies, the underlying principles for all checkpoint inhibitors are the same (**figure 12**). The aim is to enhance the effect of immune cells by blocking the interaction between an inhibiting receptor (expressed by the immune cell) and its ligand (expressed by tumour cells or other immune cells)[219]. One example of this is how monoclonal antibodies towards either the PD-1 molecule (expressed by T cells) or the PD-L1 ligand (expressed by tumour cells and some immune cells) can be used to prevent tumour cell-mediated T cell inactivation[220]. The presence of immune cells, expressing checkpoint molecules, cells expressing the cognate ligands, and TAAs in the TME are all necessary to enhance the anti-tumour immune response through immune checkpoint therapy[219, 220]. Furthermore, the degree of exhaustion in the infiltrating immune cells will affect the efficacy of immune therapy as terminally exhausted cells become anergic and lose their capacity to perform their effector functions[221]. Since immune cell infiltration and their degree of exhaustion vary dramatically (both between and within different forms of cancer) the efficacy of checkpoint inhibitors also varies greatly[221]. Furthermore, has it been shown that therapies targeting checkpoint inhibitors or co-receptors increase the anti-tumour activity of effector T cells, partly by inhibiting the activity of Tregs[222]. Therapies that specifically deplete Tregs, often via some form of modified CD25 antibody, also enhance the anti-tumour immune response[223].

Checkpoint inhibitors targeting PD-1 and PD-L1 have been shown to be effective against CRC tumours where there is infiltration of immune cells and are becoming increasingly common in a clinical setting[218, 220].

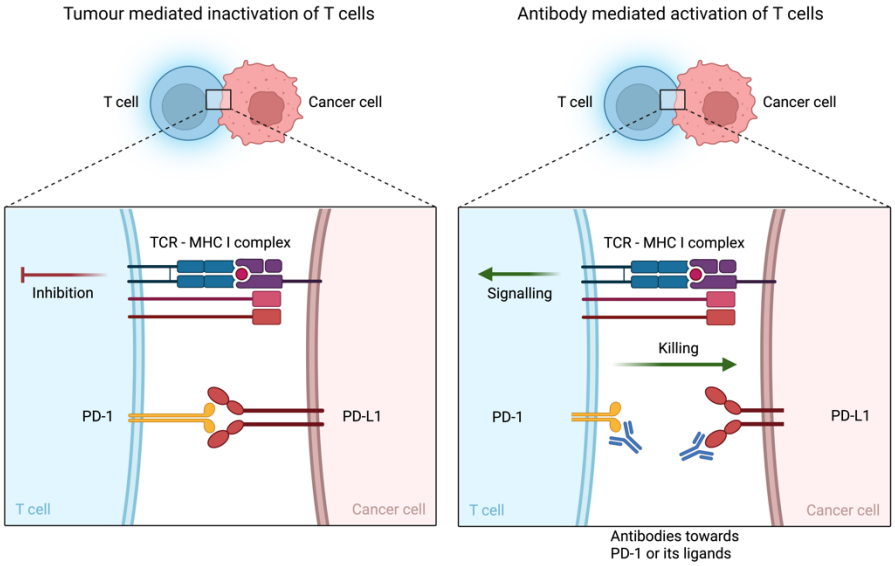

Figure 12. Principles of PD-1 and PD-L1 immunotherapy. When PD-1 binds one of its cognate ligands PD-L1 or L2 (L1 shown here) it leads to the inactivation of the T cell. In the presence of antibodies towards either PD-1 or PD-L1, the inhibitory signal is blocked and the TCR-MHC I interaction leads to an effector response in the T cell. Created using BioRender.com.

CELL THERAPIES

The few cell therapies used in a clinical setting today are based on re-transplanting *in vitro* modified host cells to improve the anti-tumour response. Currently, these treatments are only used for blood cancers where the modified cells are easily transfused to the patient and do not need to home to a specific tissue site[217]. There are two main approaches to cell-based treatment; tumour-infiltrating lymphocytes (TILs) and lymphocytes with modified antigen receptors, called chimeric antigen receptors (CARs)[224]. While there are TIL therapies in clinical trials, the only approved therapies for clinical use are therapies utilising the CAR approach[224, 225].

In TIL therapy, T cells recognizing tumour antigens are isolated, expanded, and transplanted back to the patient[224]. Immunomodulatory cytokines or checkpoint inhibitors can also be administered concurrently with the transplanted cells to increase the efficacy of the TIL therapy[224]. TIL therapy is dependent on tumour-reactive host cells already present in

the patient and aims to increase the numbers of already existing tumour-reactive TILs, the main issue with this approach is expanding sufficient numbers of cells[224].

In CAR-cell therapy, the isolated cells are genetically modified, using either retroviral vectors or CRISPR/Cas9, to express a chimeric antigen receptor (**figure 13**)[224]. Several generations of CAR-cell therapies, with different compositions of the antigen receptors, have been developed. For instance, second and third-generation CARs have signalling domains from co-stimulatory molecules associated with the intracellular motifs of the antigen receptor that will boost the activation signals and enable them to persist longer, compared to normal and first generation CAR cells[175]. Fourth-generation CARs (often referred to as TRUCKs or armoured CARs) also include signalling domains that trigger the expression of cytokines and co-stimulatory molecules, making the cells even more potent by becoming self-sufficient for specific cytokines or activation signals[175]. Cells intended for CAR therapies are usually harvested through leukapheresis but can also be isolated from whole blood or cord blood[224].

All six approved CAR T cell therapies are engineered to target B cells in blood cancer: four target CD19, and two target the B cell maturation antigen (BCMA)[224]. As already mentioned, no cell-based therapies have been approved for clinical use in solid tumours; however, there are currently both TIL and CAR cell therapies in various phases of clinical testing[219, 224].

Research into cell-based therapies is continuing to expand, and attempts at using other immune cells, such as NK cells, have been made. In general, the efficacy of cell-based therapies is good, and the main issues these potential treatments face are off-target cytotoxicity and homing to the tumour. Additionally, unconventional T cells have been suggested as potential candidates for cell-based therapies. As unconventional T cells are terminally differentiated effector cells, they possess the capacity to elicit a strong and rapid response. Furthermore, unconventional T cells, such as MAIT cells, also have the advantage of being HLA-unrestricted, making it (theoretically) possible to transplant them between different donors without the risk of graft-versus-host disease (GVHD). However, before using them in a therapeutic setting, further insights into the biology of unconventional T cells are needed.

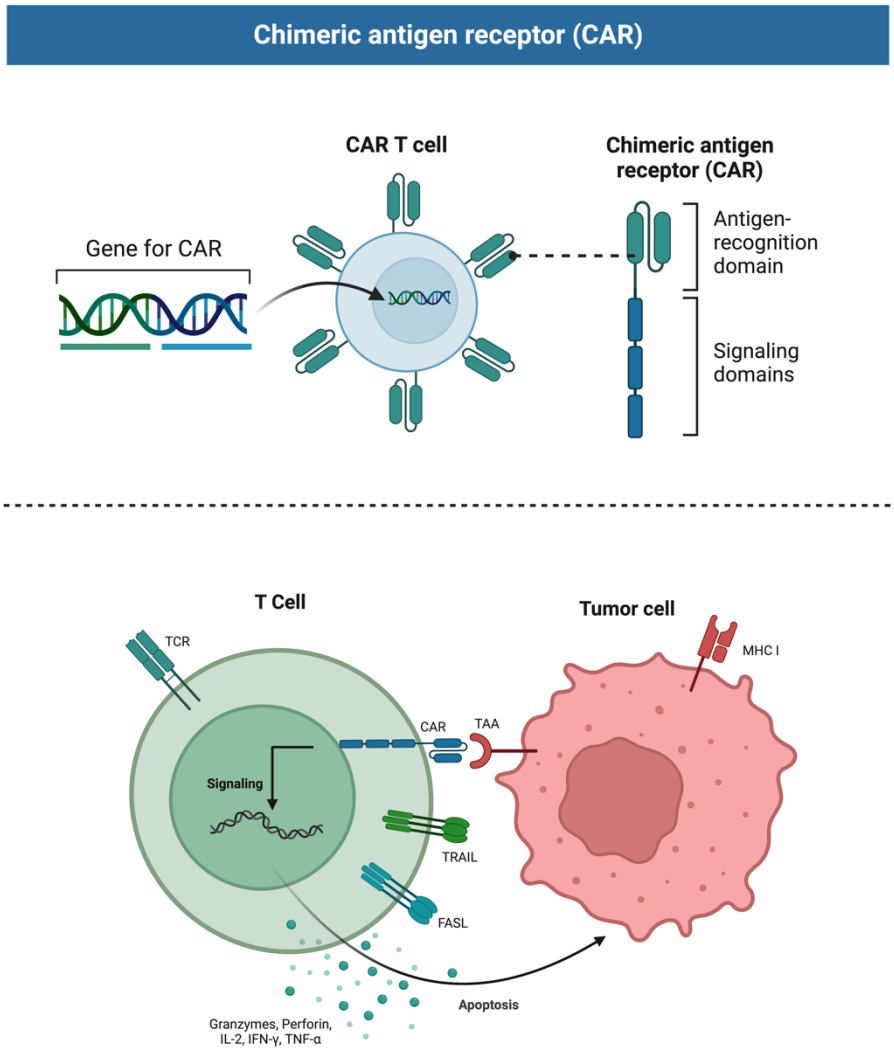

Figure 13. Principles of CAR T cell therapies. CAR T cells are engineered by inserting genes encoding for the modified T cell receptor. The CAR is specific for an antigen expressed by the tumour cell and post-generation one CARs have intracellular signalling domains associated with co-stimulatory molecules. Tumour cell recognition via the CAR triggers a potent cytotoxic immune response. Created using BioRender.com.

AIMS

This thesis investigates the phenotype and effector functions of tumour-infiltrating unconventional T cells from colon cancer patients. Additionally, we studied the potential of using *in vitro* expanded MAIT cells in adoptive cell therapies. This is important to be able to understand how different unconventional T cells contribute to tumour immunity and if they are potential options in future immunotherapies.

Specific aims:

I Determine if tumour-infiltrating MAIT cells have reduced effector functions and if anti-PD1 treatment affects the activation state of potentially exhausted MAIT cells.

II Decide what subsets of γδ T cells infiltrate colon tumours and how their effector functions can contribute to the immune response.

III Investigate if MAIT cells can be expanded *in vitro*, their cytotoxic capacity towards tumour cells, and by what means the cytotoxicity is mediated.

METHODS

PATIENTS & BLOOD DONORS

104 colon cancer patients (46 females, 58 males, aged 37 – 92 years, median age 74 years), undergoing curative resection surgery of colon tumour(s) (n = 100) or metastases (n = 4) at the Sahlgrenska university hospital (Östra Sjukhuset) were recruited by research nurses from the surgical oncology laboratory. The patients gave informed written consent before participating in the study, and the Regional Research Ethics Committee in Västra Götaland provided ethical approval to perform the study. Patients suffering from autoimmune disease or who had undergone chemo-, radio, or immunotherapy in the 2 years prior to the surgery were also excluded from the study. Tumour tissue, macroscopically unaffected colon mucosa (taken a minimum of 10 cm away from the tumour border), and blood samples were collected from each enrolled patient. In some cases, unaffected colon mucosa or blood samples could not be collected.

Additionally, 18 stage III or IV non-small cell lung cancer (NSCLC) patients treated with monoclonal antibodies towards PD-1 (Pembrolizumab, Nivolumab) or PD-L1 (Durvalumab, Atezolizumab) were included in the thesis. The patients were treated during 4 treatment cycles, according to the approved drug intervals and health conditions of the patients. Venous blood was collected in EDTA-coated tubes at baseline (before treatment) and after each treatment cycle. Whole blood was diluted in 10% DMSO and stored in liquid nitrogen until analysis.

We also used blood samples from 26 healthy volunteers (11 females, 7 males, aged 23-67, median age 34 years and 8 donors from the blood bank of unknown gender and age) to set up methods, and as controls in some experiments. The blood samples were collected as buffy coats (component laboratory, Sahlgrenska University hospital) or as venous blood samples at our department (in heparin tubes by a trained staff member according to standard practices). All blood donors consented to the blood collection and subsequent use of their samples.

CELL ISOLATION

Cells from tissue samples were isolated through enzymatic digestion (**figure 14**), commonly used to isolate cells from various tissues. The advantage of enzymatic digestion is that relatively large cell yields can be achieved from small samples. As our samples generally weighed less than 2 grams, the use of digestive enzymes was necessary. The main downside of using digestive enzymes is the destruction of some cell surface markers; this makes proper titration and optimization of the protocol crucial in order to avoid unnecessary impact on the cells. Also, proper controls (in our case, peripheral blood treated with digestive enzymes) are important to ensure that the absence of markers in the subsequent analysis was not due to the digestive enzymes. Most heavily affected by enzymatic digestion were the chemokine and cytokine receptors. However, it is possible to restore the expression of most of the affected receptors through incubation of the cells under physiological conditions for ~24 hours. As a substitute for enzymatic digestion, mechanical disruption of the tissue can be used to isolate cells. However, this often leads to a lower yield due to low efficiency and unnecessary cell death caused by mechanical stress.

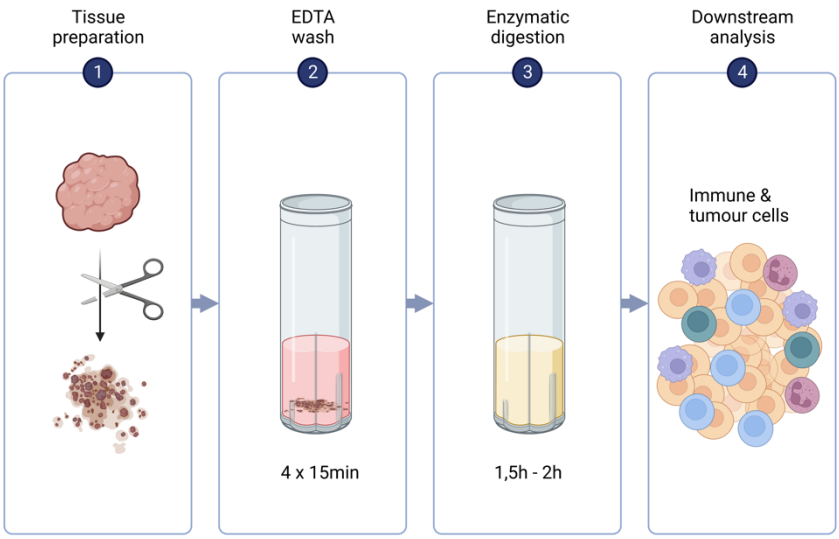

Figure 14. Overview of the isolation process. Tissue samples were manually processed into smaller pieces (~0.5cm), treated with 4 rounds of EDTA (in order to remove most of the epithelial cells), and digested using liberase TM and DNAse I for up to 2 hours. The resulting cell suspension was filtered and then used in various analyses. Created using BioRender.com.

PBMCs from CRC patients and healthy donors were isolated using density gradient centrifugation, which is common practice when isolating immune cells from blood samples. Density gradient centrifugation is an isolation method with a high yield that causes few or no changes to the cells.

Blood samples from NSCLC patients were thawed and centrifuged twice to remove the DMSO and then used immediately for flow cytometry analysis or stimulation assays.

FLOW CYTOMETRY

Being one of the most commonly used methods in the field of immunology, flow cytometry (FC) enables multiparameter analysis of single-cell suspension in a high throughput system. In short, cells in single-cell suspensions are labelled with fluorescently labelled antibodies towards surface, intracellular, and intranuclear targets (generally proteins) and passed through a system of lasers and detectors. Single cell data is generated as the fluidics system allows fluorescent data from each cell to be detected individually. As the cells pass the lasers (different instruments use different laser/detector configurations), the fluorochromes on the labelled antibodies are excited, and photons are emitted. The photons are filtered, amplified, and the signal is recovered via detectors that ensure that photons from the different fluorochromes cause minimum interference with one another.

The fluorochromes are excited by a specific wavelength of light, and the emitted photons, or light, are of a higher wavelength. As each laser often excites more than a single fluorochrome, the emitted light is passed through long-pass filters (light above a specific wavelength can pass) and band-pass filters (light between two wavelengths can pass) that separate light of various wavelengths from each other. The filtered light is converted to a digital signal through a photomultiplier tube (PMT) or avalanche photodiode (APD) that amplifies the signal. Although the intricate system of spatially separated lasers and filters ensures that most of the emitted light is recovered by the correct detector, there is still some spill over of unspecific signals due to the spectral overlap of certain fluorochromes. To avoid unspecific signal detection, a compensation matrix is generated, where unspecific signal spill over is digitally removed

from the processed data. However, a downside of this electronic compensation is that signal resolution tends to decrease.

In order to generate high-quality flow cytometric data, designing the antibody panel (the mix of antibodies used to stain and analyse the sample) to avoid unnecessary spectral overlap between the selected fluorochromes, as well as the use of proper controls, are crucial steps. Several different controls are used in flow cytometry, each with unique advantages and limitations. The most commonly used control is the fluorescence minus one (FMO) control, where the antibody of interest is omitted from one sample. Thus, a negative baseline for that marker can be established. The biggest drawback of FMO controls is that the antibodies in a sample generate a certain amount of background signal. The background signal can be affected when one or more antibodies are omitted. Another type of control that, in theory, does not suffer from the same issues as FMOs, is isotype control. In isotype controls, the antibody of interest is replaced with an antibody of the same isotype and conjugated to the same fluorochrome, but specific towards an epitope not present in the sample. The difference in specificity will prevent binding to the intended target, but the presence of the fluorochrome-conjugated antibody will contribute to the background fluorescence. However, fluorochrome-conjugated antibodies of different isotypes are not identical in every other aspect. Even minor differences in the molecular structure can affect the binding of the antibody, and by extension, the fluorescence signal generated. Another way of validating the detected signal is to use an "in-sample control", where the signal from the antibody can be compared to another cell type with a known expression of the marker of interest. For example, if the expression of a receptor on a rare subset of T cells is investigated and conventional T cells are known to express this receptor the staining can be compared between the two cell subsets.

As instruments become more advanced and able to detect more markers, the experimental design and subsequent analysis will become increasingly difficult. In flow cytometers capable of detecting up to ~20 markers, the use of conventional analysis software is both practical and an accepted practice. However, as the number of markers increases the need for computer-based algorithms and bioinformatic tools is becoming increasingly important.

CELL SORTING

FLUORESCENCE-ACTIVATED CELL SORTING

Fluorescence-activated cell sorting (FACS) is a technique based on flow cytometry, where antibody-labelled cells can be sorted based on the fluorescence signal. In FACS, cells are not analysed and discarded but instead transported to a sorting chamber, where the cells are organised into a droplet stream. The cell-containing droplets will then be given a positive or negative charge and sorted using two electrically charged deflector plates. Depending on the type of FACS instrument, more than one population can be sorted at a time. As droplets, rather than cells, are sorted, some care should be taken when setting up the experiment. If the speed at which the cells are processed is too high compared to the rate at which the droplets are formed, one droplet may end up containing more than one cell. Depending on the instrument settings, these droplets may end up being either discarded or included in the sort, which may affect the final yield or purity of the sorted cells. Another factor to consider is the mechanical stress the cells are subjected to, which may affect their viability or function in downstream applications. It is possible to reduce the stress that the cells suffer by reducing the pressure (and rate) at which the cells are sorted, using media that resist turning alkaline at atmospheric CO_2 levels, and keeping both the sample and sorted cells cool.

MAGNETIC-ACTIVATED CELL SORTING

Magnetic-activated cell sorting (MACS) employ antibodies conjugated to magnetic particles that enable the recovery or depletion of specific cell subsets. Compared to FACS, magnetic separation is generally less time-consuming and often less harmful to the cells. However, depending on the type of reagent used, the magnetic particles may interfere with downstream applications. Magnetic separation also has higher throughput compared to FACS and is usually of a comparable yield and purity. The main disadvantage of MACS is that it can be difficult to purify small cell populations efficiently without negatively impacting the yield.

MASS CYTOMETRY

The underlying principle of detecting various protein targets with labelled antibodies is similar in mass and flow cytometry. However, in mass cytometry, the antibodies are labelled with stable metal isotopes, rather than fluorescent molecules (**figure 15**). Like FC, mass cytometry can generate data on the expression of surface, intracellular, and intranuclear markers. However, mass cytometry has a much lower throughput compared to FC but with the advantage of the metal isotopes not being affected by spectral overlap to the same extent as in FC. Previously the number of markers detectable with mass cytometry (>50) has been higher compared to FC, but with the introduction of spectral flow cytometers, this is changing. As with spectral flow cytometry, the data from mass cytometry is often not suited for conventional analysis and instead require that clustering or dimensional reduction algorithm are used to make biological interpretations of the data. Although mass cytometers possess a fluidics system reminiscent of the one seen in flow cytometers, the data acquisition method differs dramatically. In mass cytometry, the cells are transferred from the liquid stream into a droplet spray, and the liquid is evaporated in a heat chamber. The cells are then ionized by an argon torch, at a temperature of around 5000 kelvins. This ionization process converts the labelled cells into an ion particle cloud that are passed through a high-pass ion filter (quadrupole) which removes low-mass ions, non-ionic particles, and photons, leaving only the ions derived from the metal isotope tags. The filtered ion particles are launched at short interval (13μs in the Helios™ system), by an electric current, into the time-of-flight (TOF) chamber. The mass of the ions can then be determined based on the time it takes for the particle to travel the (known) distance through the TOF chamber. The smaller the ion the faster it will travel through the TOF chamber. Inside the TOF chamber, a constant vacuum is maintained so that no particles will interfere with the trajectory of the ions. Finally, the detector then converts every event into a digital signal and can display the data in a dot plot. Unlike FC, no data on forward or side scatter be analysed as the cells are disintegrated, and only the resulting metal ions are detected. To separate the signals from cells and any unbound metal isotope, specific Cell-ID and barcode labels are used. In mass cytometry, cell viability is often detected using cisplatin, which enters the cytosol of dying and dead cells and binds covalently to intracellular proteins, enabling detection in the 194Pt channel.

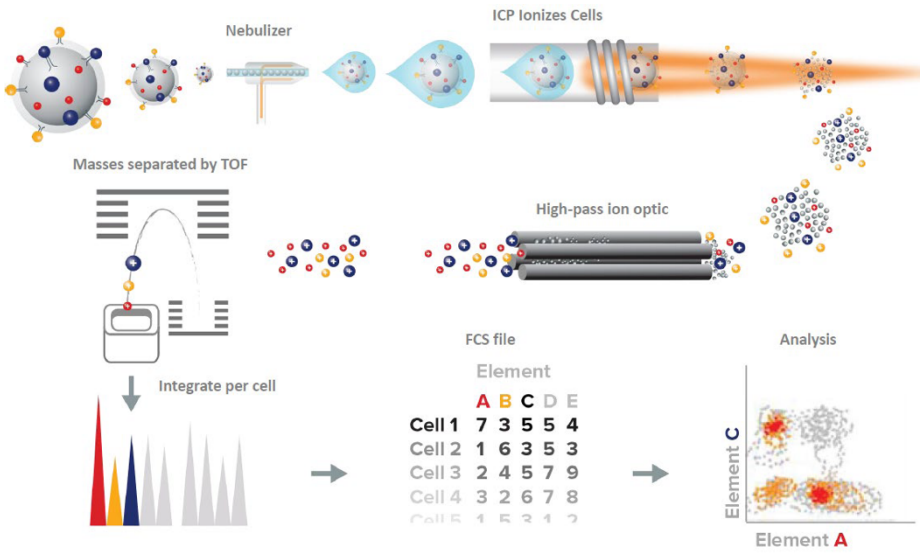

Figure 15. Principles of CyTOF. Cells labelled with antibodies tagged with stable metal isotopes are transferred to a droplet spray. The water is vaporised in a heat chamber and the cells are ionized by an argon torch. The resulting ion cloud is filtered to remove any residual low-mass ions, not from the antibody tags (such ions, that are naturally present in the cells). The ions are then fired through the time-of-flight chamber and the signal is interpreted. Courtesy of Fluidigm.

HIGH-DIMENSIONAL DATA ANALYSIS

Techniques such as mass cytometry, spectral flow cytometry, and RNA sequencing generate comparatively large datasets. These large datasets are advantageous, especially when formulating new hypotheses or investigating broad differences between cell subsets in various settings. However, large datasets pose a few unique issues. The first issue is that analysing the data without using a computer-based algorithm or other bioinformatic tools is almost impossible. Luckily, recent developments in analysis software enable many researchers to analyse their data without needing external help from bioinformaticians or computer programmers. Another issue is that much of the data will have to be validated through further experiments as it is difficult to include proper controls for all the features investigated. As such, one should be careful in making biological interpretations based solely on results from big-data experiments. Experiment design and proper quality control of the samples, reagents, and procedures become extra important when generating high-dimensional data, as downstream analyses rely on consistency and often

cannot detect batch effects or technical variations. A common way of analysing high-dimensional flow or mass cytometry data is by employing a combination of dimensional reduction algorithms (such as UMAP or t-SNE) and clustering algorithms (such as Phenograph or FlowSome). These algorithms aim to visualize three-dimensional data in a two-dimensional space, often by creating coloured overlays of the clustered data on a "map" of the cells. A more targeted analysis is generally required after this point to identify the properties of the relevant clusters or cells.

IMMUNOFLUORESCENCE MICROSCOPY

Immunofluorescence microscopy, or immunohistochemistry (IHC), is a relatively low throughput method, but in contrast to flow and mass cytometry it preserves the localisation of the cells in the tissue. IHC relies on the same basic principle as flow cytometry, the labelling of protein targets with fluorescently tagged antibodies. However, IHC analyses frozen or embedded tissue sections using a fluorescence microscope instead of analysing single-cell suspensions. Signal detection in the tissue sections relies on the fluorochromes on the antibodies being excited by one or more lasers, and through different filters and a camera, an image can be recorded. Conventional fluorescence microscopes are usually limited to around 6-8 markers, but recent advances in the field are now increasing the limit to around 40-50 markers (mainly through consecutive rounds of labelling of different protein targets with the same fluorescence molecules and recording an image between each step). Images of the tissue can be captured both as single images and through the digital stitching of consecutively captured images (scanning), generating a digital image with a very high resolution. These high-resolution images are suitable for more advanced and detailed analysis, often employing some kind of computer-based algorithm or AI image recognition software. As the number of markers increases and more easy-to-use analysis programs become commercially available, this technique may become increasingly important as it can generate data similar to that of flow cytometry whilst preserving the localisation of the investigated cells.

TARGETED RNA SEQUENCING

Non-targeted RNA sequencing, the conventional form of sequencing, investigates all RNA in the sample (often explicitly focused on mRNA) and aligns the sequenced genes with an existing genome. In contrast, targeted RNA sequences only investigate a set of pre-determined RNA sequences through hybridising probes. The probes are complementary RNA strands tagged with fluorescent molecules that can be detected using an imaging system following hybridisation with RNA present in the sample. An advantage of the targeted over the conventional approach is that it is both faster and cheaper. However, other potentially important findings detected in non-targeted sequencing may be overlooked when looking for specific sequences.

Targeted RNA sequencing is generally more easily analysed as pre-existing algorithms and software are tailored to the panel of probes used. The RNA sequences analysed are quantified relative to the expression level of all other analytes in the sample. Furthermore, absolute quantification of the expression of specific genes can be done through mixing in specific RNA sequences at known concentrations.

An unfortunate practice is how RNA expression is sometimes used instead of proteomic analysis; due to post-translational modifications of proteins, the mRNA and protein levels rarely correlate.

CELL CULTURE

The culture of various cell types was done throughout this thesis. This included both commercially available cell lines as well as primary cells derived from patients. In paper II, we used monocytic THP-1 cells, stimulated with fixed *E. coli*, in our studies of how anti-PD-1 therapy affects the expression of cytokines and effector proteins by tumour-infiltrating MAIT cells. In paper III, we used HeLa, HT29, HCT116 cells, and primary patient-derived tumour cells (PPDTCs) as target cells when investigating the cytotoxicity of expanded MAIT cells.

MAIT CELL EXPANSION

While the number of MAIT cells possible to isolate from most human tissues is generally sufficient for analysis, it is rarely possible to perform robust cytotoxicity assays with ex-vivo isolated MAIT cells. For this reason, we devised a method of expanding both tumour-infiltrating and circulating MAIT cells from CRC patients (**figure 16**). MAIT cell expansion was achieved through short-term culture (7 days) of single cell suspensions of PBMCs, in the presence of recombinant human IL-2 and IL-7, along with the MAIT cell antigen 5-OP-RU. After seven days of culture, MAIT cells expanded and became the dominant cell subset present in the culture (≥80%) and could be purified using MACS. As with all functional experiments, it is imperative to know the limitations of the experimental setup. In our case, the MAIT cells used are subjected to antigen stimulation and subsequently might have an altered activation pattern compared to those present *in situ*. However, it could be argued that intra-tumoral MAIT cells, to some degree, are activated by antigens or surface markers present in the tumour and are therefore comparable with expanded MAIT cells.

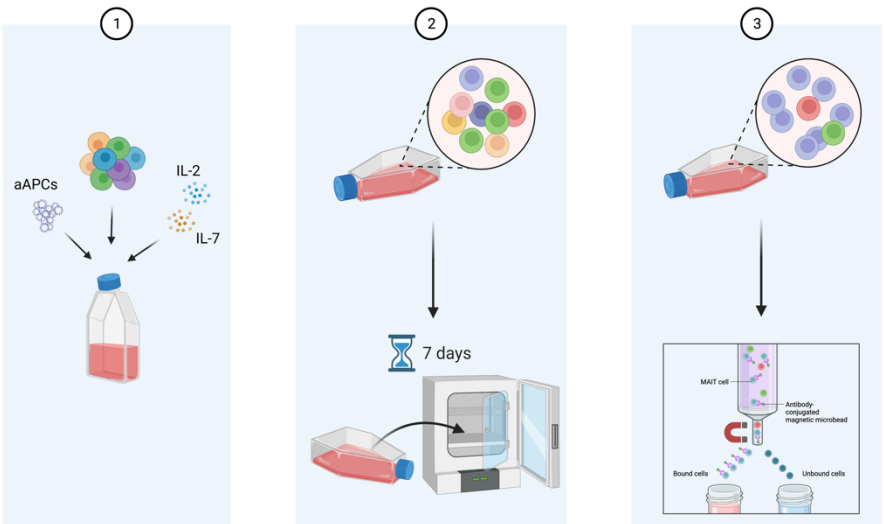

Figure 16. MAIT cell expansion. Lamina propria lymphocytes or PBMCs are cultured for 7 days in the presence of aAPCs, IL-2, and IL-7. Expanded MAIT cells can then be isolated using antibodies to the T cell receptor and MACS. Created using BioRender.com.

PRIMARY PATIENT-DERIVED TUMOUR CELLS

To better mimic the heterogeneity of CRC tumours we used primary patient-derived tumour cells (**figure 17**), cultured as spheroids, in our cytotoxicity and blocking assays. The PPDTCs were grown essentially as described[226]. Briefly, Rspondin- and Noggin-producing cells were cultured for 7 days to create conditioned media. Media for the primary patient-derived tumour cells was prepared by supplementing Advanced DMEM/F12 media with Glutamax, HEPES, Penicillin/Streptomycin, Rspondin- and Noggin-conditoned media, B27, nAc, A83_01, SB202190, Mouse EGF, Y27632, and Primocin. PPDTCs were then grown in an extracellular matrix substitute (Geltrex®) and the cell media was renewed every third day until the cells were either split and transferred into fresh matrix gel or used in the cytotoxicity assay. While cell lines are an excellent way of testing various hypothesis in research the use of PPDTCs in cytotoxicity assays is advantageous, as these cells have the many different unique mutations and characteristics of the primary tumour. However, much of the advantage is lost if only one or two different PPDTC lines are used, instead a large number of PPDTCs needs to be screened in order to determine the effectiveness of a specific function.

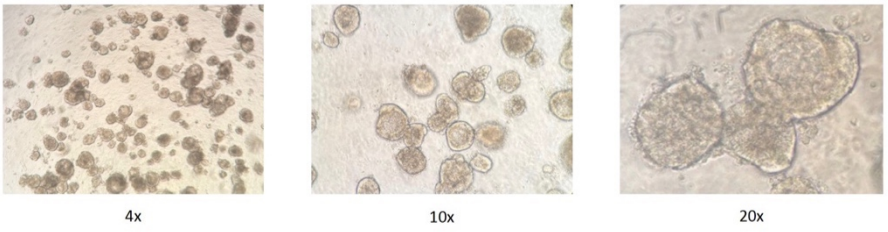

Figure 17. Colon tumour cells. Primary patient-derived tumour cells grown as spheroids in Geltrex®, photographed at 4x (left), 10x (middle), and 20x (right) magnification.

FUNCTIONAL ASSAYS

Human *in vivo* experiments are not possible to perform outside of clinical trials; instead, animal models or *in vitro* assays are used to study complex interactions. The use of animal models is necessary at different stages in a research project, for instance, when testing the vaccine response of a novel compound or investigating the homing of a cell type. However, much of the research in animal models proves to be entirely obsolete as it

cannot be translated to a human setting, and some of the research done in mice could have been done in more advanced (human) *in vitro* assays. Also, for ethical reasons, the use of animals in research should be limited. In the future, we will likely need to become better at using substitutes for animal models.

In vitro assays provide relatively easily manipulated experimental systems that are often cheaper and have higher throughput compared to animal models. Also, with the continued development of organoids, tissue printing and more advanced analysis methods, functional assays can be made more and more like the tissue or physiological condition studied. Regardless of the experimental system used, the key is to know the limitations of the selected system and, thus, what research questions it answers.

STIMULATION ASSAYS

In all studies, we investigated the expression of cytokines and effector proteins by unconventional T cells in colon and lung cancer patients, as well as in healthy donors.

We mainly employed polyclonal stimulation assays where the cell is activated in the absence of its antigen, for instance, through stimulation with phorbol 12-myristate 13-acetate (PMA) and Ionomycin or antibodies towards CD3 and CD28. Since polyclonal stimulation does not rely on antigen as a means of activation, it is usually a good method for investigating the functional capacity of what a cell can do, rather than what response is induced by a specific antigen. This is an important distinction, as some antigens elicit a stronger or weaker response while others may elicit a polarised response. One example of this is how different cytokines, along with stimulation of the CD3 receptor, lead to the activation of distinctly different transcriptional profiles in helper T cells[150, 155]. The downside of polyclonal stimulation is that some aspects of the response elicited by a specific antigen or surface receptor can be overlooked. However, it is rarely feasible to test all the available antigens or surface receptors on a cell or in the TME.

In MAIT cells, we studied the effect of antigen-specific activation using the 5-OP-RU antigen and the effect of polyclonal stimulation. In γδ T cells we studied the effect of polyclonal stimulation.

IMMUNE CHECKPOINT BLOCKING

In paper II we investigated the *in vitro* effects of anti-PD1 treatment on tumour-infiltrating MAIT cells.

Cell suspensions isolated from colon tumours and unaffected mucosa were stimulated using plate-bound αCD3 antibodies, soluble αCD28 antibodies, and recombinant-human IL-2, in the presence or absence of antibodies towards PD-1 (Pembrolizumab). The expression of CD25 was evaluated on the MAIT cells. Additionally, PD-L2$^+$ THP-1 cells were incubated with fixed *E. Coli* and used to stimulate MAIT cells from colon tumours and unaffected mucosa in the presence or absence of αPD-1 antibodies (Pembrolizumab) and the expression of HLA-DR and CD38 was evaluated on the MAIT cells.

CYTOTOXICITY ASSAY

As part of the final study (paper III), we evaluated the cytotoxic capacity of expanded MAIT cells towards both cell lines and PPDTCs (**figure 18**).

Target cells, between 70-90% confluency, were stimulated for 24h using IFNγ, IL-1β, and TNF to mimic the state of inflammation found in tumours. Following stimulation, the target cells were harvested. In parallel, expanded MAIT cells were purified using MACS towards TCR Vα7.2. Target and effector cells were then cocultured for 16h in the presence or absence of blocking antibodies towards CD161, TRAIL, and FasL. We also employed the small inhibitor molecule 3,4-dichloroisocoumarine to block serine protease activity in some assays. The frequency of live target cells was evaluated using a live/dead exclusion dye and the poly-caspase dye fam-FLICA. We used a cytotoxicity index[‡] to compare the effect of different treatments and effector-to-target cell ratios on MAIT cell cytotoxicity.

‡ $Cytotoxicity\ index = \frac{\%\ \text{dead cells in the sample} - \%\ \text{dead cells in the control}}{100 - \%\ \text{dead cells in the control}}$

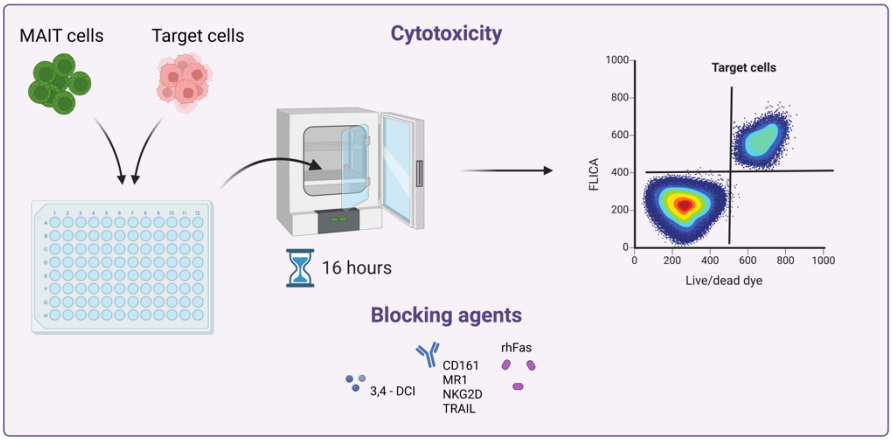

Figure 18. Cytotoxicity assay. Expanded MAIT cells were co-cultured with target cells at increasing (1:1, 2:1, 4:1, 6:1, and 10:1) MAIT:target cell ratios, in the presence or absence of various blocking agents, for 16 hours. The viability of the target cells was evaluated using flow cytometry. Created using BioRender.com.

STATISTICS

The Wilcoxon matched-pairs signed rank test and the Mann-Whitney U test were used to evaluate the statistical significance of paired and unpaired samples, respectively. p values < 0.05 were considered to be statistically significant. In graphs and figures, dots represent individual samples, and the horizontal lines (or bars) the median. GraphPad PRISM was used for all statistical analyses.

RESULTS AND DISCUSSION

As previously mentioned, one of the key factors of tumour progression is the composition of immune cells infiltrating the tumour and its immediate surroundings[145, 152, 161]. In contrast to how well conventional tumour-infiltrating T cells have been studied, much less is known about the infiltration and function of unconventional T cells. Still, in colon cancer (along with several other types of cancer), evidence of both pro- and anti-tumour activity of unconventional T cells has been observed[111, 178, 179, 192, 210]. Therefore, we were interested in what type of unconventional T cells infiltrate colon tumours, and if they are likely to contribute to tumour growth or the anti-tumour immune response. Specifically, we investigated the infiltration and composition of the MAIT and γδ T cell populations along with their potential effector functions in a cancer setting.

INCREASED INFILTRATION OF MAIT CELLS AND REDUCED INFILTRATION OF γδ T CELLS IN COLON TUMOURS

First, we analysed the infiltration of MAIT and γδ T cells, using flow cytometry, in the tumours of colon cancer patients, and found that the frequency and number of γδ T cells infiltrating colon tumours were reduced compared to blood and unaffected colon mucosa (*paper I*, **fig. 1B-C**). In contrast, the frequencies of MAIT cells in colon tumours were increased compared to the surrounding unaffected mucosa (*paper II*, **supplementary fig. 1C**). To further characterise the tumour-infiltrating MAIT and γδ T cell populations, we also used flow cytometry to investigate the expression of some common lineage, phenotype, and exhaustion markers. We also analysed the general composition of γδ T cell subsets based on TCR composition.

As expected, we found that circulating γδ T cells were dominated by the Vδ2 subset with almost no $V\delta1^+$ or $V\delta1^-V\delta2^-$ γδ T cells (*paper I*, **fig. 1F**). In contrast to the circulation, tumour infiltrating γδ T cells were mainly $V\delta2^+$ or $V\delta1^-V\delta2^-$ and only a few where $V\delta1^+$ (*paper I*, **fig. 1G**). In the surrounding unaffected mucosa, the dominating subset was the $V\delta1^-V\delta2^-$ γδ T cells while $V\delta2^+$ γδ T cells were less common and $V\delta1^+$ cells were scarce (*paper I*, **fig. 1E**). To further characterise the TCRs, we analysed

the expression of the Vγ9 chain among Vδ1$^+$, Vδ2$^+$and Vδ1$^-$Vδ2$^-$ γδ T cells and found that while it is expressed by both Vδ1$^+$ and Vδ1$^-$Vδ2$^-$ γδ T cells, it is most commonly paired with the Vδ2 chain (*paper I*, **supplementary fig. 1** and **fig. 2B**).

MAIT cells, on the other hand, are classified as Vα7.2$^+$CD161^{high} (in humans they are generally not divided into subgroups based on the β-chain they express) or classified based on their reactivity towards MR1 tetramers loaded with the 5-OP-RU antigen. In our studies, we used MR1 tetramers when possible. However, as this is not always possible, we focused on the CD8$^+$ and double negative (CD4$^-$CD8$^-$; DN) MAIT cells as these can be unambiguously identified using antibodies towards Vα7.2 and CD161. In general, MAIT cells from the circulation of both healthy blood donors, colon cancer patients, and those present in the unaffected colon mucosa express CD8 (*paper II*, **supplementary fig. 1C**). Interestingly, the frequencies of CD8$^+$ MAIT cells were reduced in colon tumours compared to both the circulation and surrounding unaffected colon mucosa (*paper II*, **supplementary fig. 1C**).

We also investigated the CD8 expression in tumour-infiltrating γδ T cells and found that while only some of the γδ T cells were positive for CD8, expression was detected in all tissues (blood, unaffected colon mucosa, and tumours) and in all subsets (Vδ1$^+$, Vδ2$^+$, and Vδ1$^-$Vδ2$^-$) of γδ T cells (*paper I*, **supplementary fig. 2**). Furthermore, the frequency of Vδ1$^+$CD8$^+$ γδ T cells were significantly higher compared to the frequency of Vδ2$^+$CD8$^+$ γδ T cells in the colon tumours (*paper I*, **supplementary fig. 2C**).

In Gentles' study[227], γδ T cells along with the surface marker CD161 (*KLRB1*) were identified as the strongest predictors of beneficial outcomes across a meta-study covering 39 different types of malignancies. This indicates that a reduced infiltration of γδ T cells would likely be associated with tumour progression, while increased infiltration of CD161 expressing cells, such as some conventional αβ T cells, some γδ T cells, and all MAIT cells would be associated with an improved outcome. However, a more recent meta-analysis, covering 50 different malignancies, focused on the Vγ9$^+$Vδ2$^+$ γδ T cells and showed that their abundance was unrelated to clinicopathological features and associated with both disease progression and resolution[228]. As for the MAIT cells, studies have shown that their role is ambiguous and that while they constitutively express CD161 it is not certain that they contribute to a beneficial outcome[229].

In CRC, CD39 expressing Vδ1 cells have been associated with immunosuppression through the adenosine pathway, and the recruitment of immune-suppressing MDSCs has been associated with IL-17 production in γδ T cells[230, 231]. Also, in breast cancer, high infiltration of $V\delta1^+$ γδ T cells was also associated with suppression of T cell proliferation and DC maturation, as well as being the most significant prognostic marker of reduced disease-free survival (DFS)[232, 233]. In contrast, another study identified infiltration of γδ T cells in CRC tumours to be associated with an improved 5-year DFS[234]. Another study showed that $NKp46^+V\delta1^+$ cells did not only show a strong Th1-phenotype but also that their accumulation in the unaffected colon mucosa was associated with a decreased risk of developing late-stage metastatic CRC[210]. Furthermore, in CRC liver metastases, it was shown that infiltration of $CD69^+V\delta1^+$ cells correlated with a reduced metastatic tumour burden as well as improved overall survival[235].

In our study, we found very few $V\delta1^+$ γδ T cells in the colon tissue, while other studies have reported that they are the dominating subset. A possible explanation for this could be differences between the tumours of the patients in our cohort compared to those in other studies. Another possible reason is the use of different antibody clones when identifying the cells or the use of less-than-optimal gating strategies. We have observed that αβ T cells will occasionally stain positively for the pan-γδ TCR or more rarely for antibodies towards individual δ chains. In our data we have gated out the αβ T cells to remove the risk of having cells that are falsely perceived as γδ T cells but if this is not done the composition of the γδ T cell population might be misinterpreted.

MAIT cell infiltration is observed in colon, liver, brain, cervical, and oesophageal tumours, and their presence has been linked to both beneficial and poor patient prognosis depending on the tumour type and observed MAIT cell functions[178, 179, 183, 186, 236, 237, 238, 239, 240, 241]. For instance, accumulation of MAIT cells in colon, hepatic, and oesophageal tumours have been linked to a poor prognosis[179, 227, 238]. In contrast to these findings, some studies have reported that tumour-infiltrating MAIT cells produce tumour-promoting cytokines, and others have demonstrated cytotoxic capacity and a Th1-like response[229].

Taken together this highlights the complex phenotypes of both MAIT and γδ T cells and shows how they are far more dependent on context than previously thought. The population of unconventional T cells likely require deeper analysis of the phenotypes and functions in order to

elucidate their potential contribution to the tumour immune response in different types of cancers.

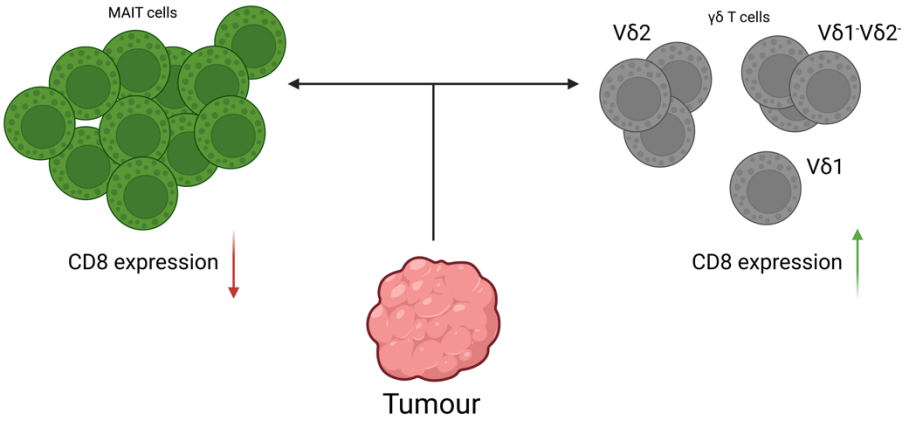

Figure 19. First graphical result summary. MAIT cells accumulate in colon tumours, but the frequencies of CD8$^+$ MAIT cells are reduced. The numbers and frequencies of γδ T cells are reduced in colon tumours. The γδ T cells are mainly Vδ2$^+$ or Vδ1$^-$Vδ2$^-$, the few Vδ1$^+$ cells have an increased expression of CD8, compared to the other γδ T cell subsets in the tumours. Created using BioRender.com.

EXHAUSTION IN TUMOUR-INFILTRATING MAIT CELLS

To further characterise the tumour-infiltrating MAIT cells we analysed the expression of the surface markers PD-1, Tim3, CD39, TIGIT, BTLA, and LAG3, as they have all been used to identify exhausted conventional T cells[143, 242] .

The frequency of PD-1$^+$ MAIT cells was similar in both the unaffected colon mucosa and the tumours, although it should be noted that the mean fluorescence intensity for PD-1 in the tumour-infiltrating PD-1$^+$ MAIT cells was significantly higher compared to those in the unaffected colon mucosa (*paper II*, **fig. 1A** and **supplementary fig. 2**). However, compared to blood, the expression of PD-1 was significantly higher in MAIT cells present in tissue (*paper II*, **fig. 1A**). As the surface marker Tim3, together with PD-1, can be used to identify terminally exhausted T cells in both chronic viral infections and tumours[243, 244] we also included it in our analysis. In tumour-infiltrating MAIT cells, the expression of Tim3 was significantly higher compared to in the surrounding unaffected colon mucosa, and for tumour-infiltrating CD8$^+$ MAIT cells it was also higher compared to in the circulation (*paper II*, **fig. 1B**). Furthermore, most of

the tumour-infiltrating MAIT cells that were PD-1 positive also expressed Tim3 (*paper II*, **fig. 1C**).

When we extended our analysis to also encompass CD39, we found the expression to be significantly higher in $CD8^+$ MAIT cells (but not DN MAIT cells) present in the tissue compared to in the circulation (*paper II*, **fig. 2A**). Further analysis also showed that BTLA and LAG3 expression was almost undetectable in most MAIT cells from the tissue while the frequency of $BTLA^+CD8^+$ MAIT cells was significantly higher in the circulation compared to in the tumour (*paper II*, **fig. 2C and D**). As exhausted T cells tend to co-express multiple inhibitory receptors[242] we also investigated the expression of CD39, TIGIT, and BTLA among the $PD\text{-}1^+Tim3^+$ and $PD\text{-}1^+Tim3^-$ MAIT cells. This analysis was done in a subset of the patients, where we had sufficient numbers of cells (>100 $PD\text{-}1^+Tim3^+$ and $PD\text{-}1^+Tim3^-$ MAIT cells respectively) to get reliable data. While expression of both TIGIT and BTLA was elevated among the $PD\text{-}1^+Tim3^+$ MAIT cells compared to the $PD\text{-}1^+Tim3^-$ MAIT cells (in most patients), the most striking difference was among the CD39 positive cells. We found a dramatic increase in CD39 expression among the $PD\text{-}1^+Tim3^+$ $CD8^+$ MAIT cells ($p<0{,}005$) compared to the $PD\text{-}1^+Tim3^-$ $CD8^+$ MAIT cells (*paper II*, **fig. 3A-C**). Interestingly, a recent study showed that $CD8^+PD\text{-}1^{high}CD39^+$ conventional T cells were at least as suppressive as Tregs[245]. Like Tregs, this suppression was mediated through extracellular adenosine generated via the CD39[245].

We also investigated the proliferative capacity of exhausted MAIT cells in colon tumours, based on their expression of Ki-67. In $PD\text{-}1^+Tim3^+$ $CD8^+$ MAIT cells the expression of Ki-67 was significantly up-regulated ($p<0.05$), compared to their non-exhausted counterparts ($PD\text{-}1^-Tim3^-$) (*paper II*, **fig. 4b**). We also found the same tendencies in double negative MAIT cells (*paper II*, **fig. 4b**). In viral infections, the functional effect of exhaustion in conventional T cells is loss of effector functions[143, 242]. Also, we have previously shown that the frequency of IFNγ producing MAIT cells are reduced in colon tumours[143, 242], indicating some form of functional exhaustion in tumour-infiltrating MAIT cells. Therefore, we also investigated the expression of other effector molecules involved in T cell mediated tumour immunity (IFNγ, Granzyme B, IL-2, and TNF), following short-term polyclonal stimulation (as this does not affect the expression of PD-1 or Tim3) on tumour-infiltrating $PD\text{-}1^+Tim3^+$ and $PD\text{-}1^-Tim3^-$ MAIT cells. We specifically investigated the polyfunctionality i.e., the capacity to produce several of the investigated effector molecules simultaneously. In tumour-infiltrating $PD\text{-}1^+Tim3^+$ MAIT cells the

polyfunctionality index was significantly lower compared to in the PD-1$^-$ Tim3$^-$ MAIT cells (*paper II*, **fig. 4C**). Specifically, there was a reduction in cells capable of producing all four, or three, of the investigated effector molecules, and an enrichment of cells capable of only producing a single type of effector molecule in the tumour-infiltrating PD-1$^+$Tim3$^+$ MAIT cells (*Paper II*, **fig. 4b**).

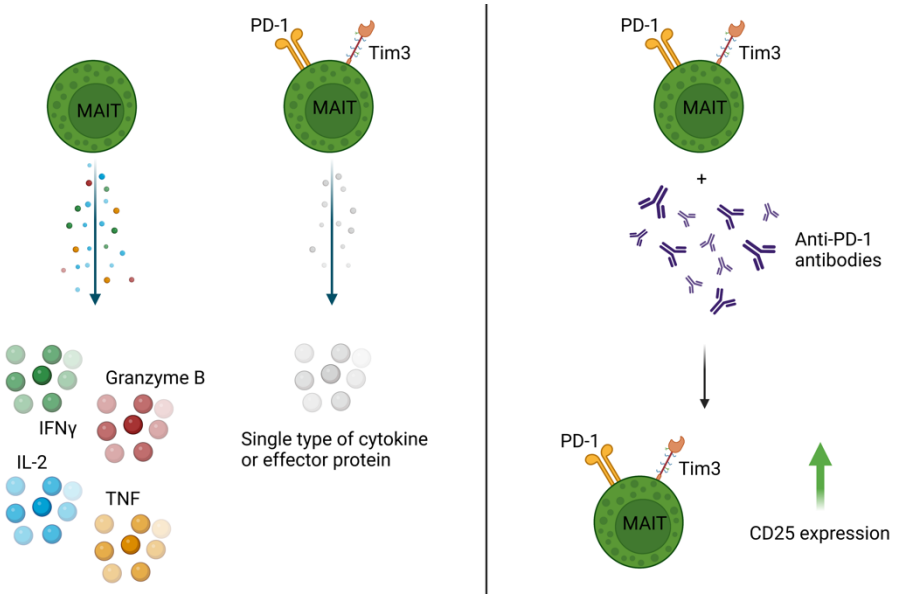

Figure 20. Second graphical result summary. MAIT cells positive for PD-1 and Tim3 have a reduced polyfunctionality index, or reduced capacity to produce different cytokines and effector proteins. PD-1$^+$Tim3$^+$ cells treated with monoclonal antibodies towards PD-1 increases their CD25 expression following stimulation. Created using BioRender.com.

PD-1 ligands (PD-L1 and PD-L2) are often widely expressed in the tumour microenvironment, and interactions between PD-1 and its ligands have been shown to reduce TCR-mediated signalling[246, 247]. However, the effector functions of PD-1-expressing T cells can be restored by blocking antibodies towards either PD-1 or its ligands[246, 247]. We investigated the effect of blocking PD-1, using a monoclonal antibody towards PD-1 (Pembrolizumab), on tumour-infiltrating MAIT cells following stimulation using anti-CD3 and anti-CD28. To better mimic the signals MAIT cells are exposed to in the tumour microenvironment, we used the bulk lamina propria cell fraction.

Following anti-PD-1 treatment, the expression of CD25 was significantly ($p<0.05$) increased in tumour-infiltrating MAIT cells compared to cells that did not receive anti-PD-1 treatment (*paper II*, **fig. 6A**). The effects of anti-PD-1 treatment varied between patients, but a reasonable response (> 15% increased expression of CD25) was detected in half of the patients analysed. In contrast, this effect was not observed in MAIT cells from unaffected colon mucosa or circulation (*paper II*, **fig. 6A**).

In a separate set of lung cancer patients (NSCLC) that were treated with blocking antibodies towards PD-1 (Pembrolizumab, Nivolumab) or PD-L1 (Durvalumab, Atezolizumab) we evaluated the MAIT cell response using flow cytometry. Expression of the activation markers CD25, CD69, HLA-DR, and CD38, as well as Ki-67, was analysed on circulating *ex vivo* isolated MAIT cells before and after treatment. Previous studies have indicated that invigoration of circulating T cells in response to PD-1 blockade can mainly be detected early during treatment[248, 249, 250, 251], therefore we thus focused on the potential response after the first treatment cycle. In patients that would later respond to treatment (partial responders, PD) we observed a significantly increased expression of CD69 and HLA-DR before and after the first treatment cycle (data not shown). In patients with stable disease (SD) or progressive disease (PD) a similar increased expression was not observed, this was also true for Ki-67 and CD25 expression in all patients regardless of response (data not shown).

We also analysed the expression of PD-1 and CD39, as co-expression of these markers has been linked to exhaustion in conventional T cells[252]. While PD-1 expression on circulating MAIT cells was detected both before and during treatment, CD39 expression was virtually absent from all PD-1$^+$ MAIT cells (data not shown). The lack of CD39 expression on the PD-1 positive MAIT cells most likely indicates that they are activated rather than exhausted. Interestingly, while PD-1 expression after the first treatment cycle (only analysed in patients treated with PD-L1 blocking antibodies) was upregulated in all patients regardless of the treatment outcome (data not shown), the PD-1 expression before the start of treatment was significantly higher in patients with a partial response compared to patients with both stable and progressive disease (**fig. 21A**). Furthermore, we also analysed the expression of TNF, IL-2, IFNγ, and granzyme B on circulating MAIT cells following polyclonal stimulation with PMA and Ionomycin. There was substantial upregulation of all

molecules, but there was no difference in expression between the different treatment responses (data not shown). However, there was a significant increase in the polyfunctionality index of circulating MAIT cells following the first treatment cycle, an increase not observed in the corresponding $CD8^+$ non-MAIT T cells (**fig. 21B-C**). The increase in polyfunctionality of circulating MAIT cells indicates a (re)activation of the MAIT cells, in response to treatment, consistent with what we observed in tumour-infiltrating MAIT cells in colon cancer patients (paper II).

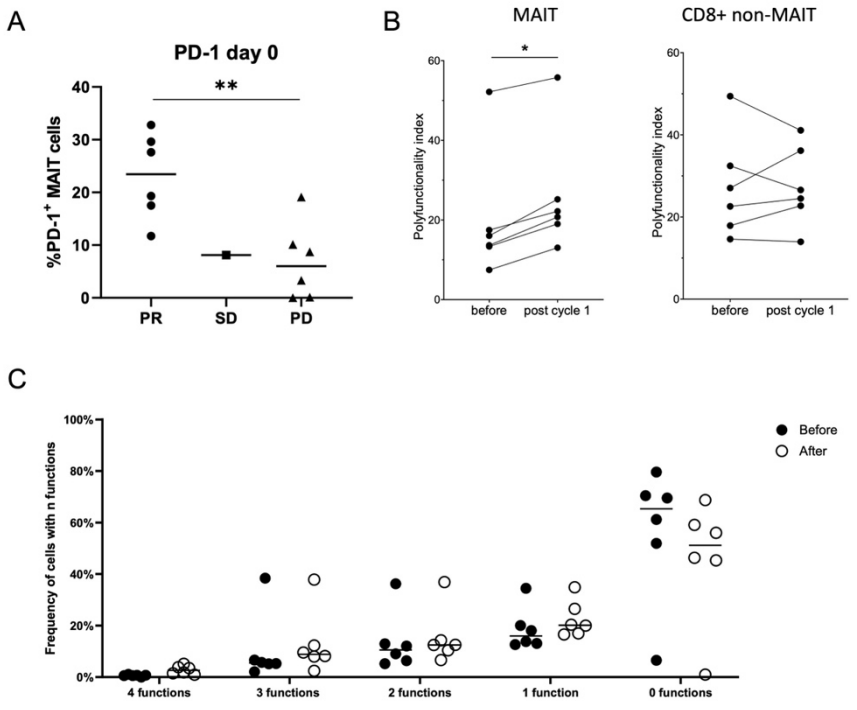

Figure 21. Polyfunctionality in circulating MAIT cells from NSCLC patients. The frequency of MAIT cells expressing PD-1 was analysed, using flow cytometry, in whole blood of patients with partial response (PR), stable disease (SD), and progressive disease (PD) before treatment with anti-PD-1 or anti-PD-L1 antibodies (**A**). The polyfunctionality index was evaluated using flow cytometry in PMA/ionomycin stimulated circulating MAIT and $CD8^+$ non-MAIT cells (**B**). The distribution of MAIT cells, before or after treatment, among groups with different number of effector functions (expression of TNF, IL-2, IFNγ, and granzyme B) is shown in **C**. n=13.

To a large extent, these results reflect what is already known about conventional T cells, where PD-1 expression also tends to be increased among the tumour-infiltrating cells and be indicative of functional impairment[143]. This has been observed in Hodgkin's lymphoma, melanoma, hepatocellular carcinoma (HCC), and gastric tumours where increased expression of PD-1 on $CD8^+$ T cells has been shown to result in functional impairment[253, 254, 255, 256, 257]. Furthermore, exhaustion functional impairment, with respect to IFNy, TNF, and IL-2 production, has also been shown in tumour-infiltrating $PD\text{-}1^+Tim3^+CD8^+$ T cells (in both mouse models and melanoma patients)[258, 259].

In MAIT cells, exhaustion based on PD-1 expression was originally suggested in chronic viral infections and later observed in both HCC and CRC[183, 260, 261, 262, 263]. In HCC an enrichment of $PD\text{-}1^+Tim3^+$ MAIT cells was also observed[183].

In a meta-analysis covering more than 16 000 patients, and several cancer types, across 91 different clinical trials, the overall response rate in treatments targeting PD-1 and PD-L1 was between 15 and 24 percent[264]. Tim-3 has also been investigated in the context of immune checkpoint therapy and animal studies have shown that targeting both Tim-3 and PD-1 restores the function of tumour-infiltrating T cells in both solid and haematological cancers[259, 265, 266]. Furthermore, early clinical trials using anti-Tim3 antibodies in combination with chemotherapy (Decitabine) have shown to be both safe and with promising effects in myelodysplastic syndromes and acute myeloid leukaemia patients[267]. Taken together, our results show that MAIT cells are likely to benefit from immunotherapies targeting both PD-1 and PD-1 ligands, as well as Tim3, in a fashion similar to how immune therapies affect conventional T cells. This indicates that MAIT cells could be reinvigorated and contribute to the anti-tumour immune response.

MEMORY POPULATIONS IN TUMOUR-INFILTRATING γδ T CELLS

Based on the expression of CD45RA and CD27, γδ T cells can be divided into naïve ($CD45RA^+CD27^+$), central memory (T_{CM}, $CD45RA^-CD27^+$), effector memory (T_{EM}, $CD45RA^-CD27^-$), and terminally-differentiated effector memory (T_{EMRA}, $CD45RA^+CD27^-$) cells[268].

We found that tumour-infiltrating Vδ1$^+$ and Vδ1$^-$Vδ2$^-$ γδ T cells were relatively evenly distributed among the four populations, with naïve cells being slightly more common among the Vδ1$^+$ γδ T cells and central memory cells being slightly more common among the Vδ1$^-$Vδ2$^-$ γδ T cells (*paper I*, **fig. 2C and E**). In contrast, the tumour-infiltrating Vδ2$^+$ γδ T cells were dominated by a central memory phenotype and the remaining cells were either naïve or effector memory cells (*paper I*, **fig. 2D**). While the γδ T cells in both the tumour and surrounding unaffected mucosa were relatively similarly distributed among the different memory populations, the distribution in the circulation was quite different. Circulating Vδ1$^+$ cells were mainly T_{EMRA}, while Vδ2$^+$ cells were evenly distributed among all four populations, and Vδ1$^-$Vδ2$^-$ cells were either naïve or of a T_{EMRA} phenotype (*paper I*, **fig. 2C-E**).

In NSCLC, the infiltration of CD45RA$^-$CD27$^-$ (T_{EM}) Vδ1$^+$ γδ T cells was found to correlate with improved relapse-free survival following excision of the tumours[269]. Another study found that T_{EMRA} γδ T cells (CD45RA$^+$CD27$^-$) could be stratified into 2 separate populations, those with high or intermediary CD45RA expression[268]. The CD45RA^{high} γδ T_{EMRA} cells showed early signs of exhaustion, through reduced proliferative capacity, while CD45^{int} γδ T_{EMRA} cells could be readily expanded *in vitro*[268]. Furthermore, they showed that γδ T_{EMRA} cells were enriched in the blood of cancer patients (NSCLC, oesophageal cancer, and HCC)[268]. In our study, we observed a high proportion of T_{EMRA} cells among the circulating Vδ1$^+$ γδ T cells and a moderate proportion among the Vδ1$^-$Vδ2$^-$ γδ T cells. However, as we did not stratify the T_{EMRA} cells into high and intermediary CD45RA expression it is not clear whether these were potentially exhausted cells or not.

MAIT AND γδ T CELLS FORM DISTINCT SUBSETS IN COLON TUMOURS

To get a comprehensive, and unbiased, view of the immune landscape of tumour-infiltrating γδ T cells we used both mass cytometry and targeted RNA sequencing to analyse *ex-vivo* isolated cells.

Analysis of the mass cytometry data (using the UMAP algorithm) encompassed 43 different markers and 301 946 γδ T cells (from blood, unaffected colon mucosa, and tumours). The initial clustering analysis revealed that blood and tissue cells form spatially separated clusters,

indicating little or no overlap in expression profile (*paper II*, **fig. 3A-B**). Further analysis into the 97 385 cells from the unaffected colon mucosa and tumours revealed a total of 32 clusters, of which 8 were exclusively present in tumour tissue and 2 in the unaffected colon mucosa (data not shown). When clustering the 59 110 γδ T cells isolated from the tumours separately we identified 24 clusters, and based on the expression of Vδ1 and Vδ2 we could identify 3 meta-clusters ($V\delta1^+$, $V\delta2^+$, and $V\delta1^-V\delta2^-$) (*paper II*, **fig. 3C-D**).

Several markers, such as CD69, CD45RO, FasL (CD178), NKG2D, CD84, and CD39 were expressed by most tumour-infiltrating γδ T cells some were however only expressed by a few (*paper II*, **fig. 3E)**. For instance, expression of CD103, TIGIT, PD-1, and CD38 were generally expressed by $V\delta1^+$ γδ T cells (*paper II*, **fig. 3E**) while expression of ICOS, CD45RO, Fas (CD95), and FoxP3 was associated with the single cluster of $V\delta1^-V\delta2^-$ γδ T cells (*paper II*, **fig. 3E**). In contrast, there were no clear patterns in expression among the $V\delta2^+$ γδ T cells, instead individual clusters were denoted by the expression of one or two markers, such as NKp30 and 4-1BB (cluster 22) or CD56 (cluster 10) (*paper II*, **fig. 3E**).

In a separate set of patients, we investigated the expression of 594 immune cell-associated genes in sorted $V\delta1^+$, $V\delta2^+$, and $V\delta1^-V\delta2^-$ γδ T cells, of which 76 genes were found to be differentially expressed among the investigated cell subsets (*paper II*, **supplemental table 2**). In general, expression of genes associated with cytotoxic effector functions was higher in both $V\delta1^+$ and $V\delta2^+$ cells compared to $V\delta1^-V\delta2^-$ γδ T cells (**table 4**) (*paper II*, **fig. 4A-C**). In contrast, the $V\delta1^-V\delta2^-$ γδ T cells had a higher expression of genes associated with an inflammatory or tumour-promoting immune response and antigen presentation (**table 5**) (*paper II*, **fig. 4A-B**). It should be noted that both mass cytometry and targeted RNA sequencing were performed on *ex vivo* isolated cells, hence few effector molecules such as cytokines could be detected. Taken together, these analyses show that while $V\delta1^+$ and $V\delta2^+$ cells seem to possess different phenotypes, they both express markers that are associated with cytotoxic effector functions. In contrast, the $V\delta1^-V\delta2^-$ cells appear to have a more tumour-promoting function, as they express both markers associated with an innate inflammatory immune response, as well as genes with a more direct tumour-promoting function.

Table 4. Genes upregulated in Vδ1$^+$ and Vδ2$^+$ γδ T cells compared to Vδ1$^-$Vδ2$^-$ γδ T cells.

Cell subset	Genes
Vδ1$^+$ γδ T cells	Granulysin, perforin, CD244, NCR1, and KIR subgroups 1 and 2
Vδ2$^+$ γδ T cells	Perforin, granzyme B, granzyme K, ZBTB16, and the killer cell lectin-like receptors (KLR) G1, C1, and B1

Table 5. Genes upregulated in Vδ1$^-$Vδ2$^-$ γδ T cells compared to Vδ1$^+$ and Vδ2$^+$ γδ T cells.

Cell subset	Genes
Vδ1$^-$Vδ2$^-$ γδ T cells	TGFBI, IL-8, CXCL1, CXCL2, galectin 3, PCDHLA-DQA1, HLA-DPA1, and HLA-DRB3

TGFBI and Galectin 3 expression have both been linked to potential tumour-promoting activities. Expression of TGFBI in tumours has been linked to a poor prognosis, with evidence of TAM-derived TGFBI being responsible for facilitating tumour growth and immunosuppression in both glioblastoma and ovarian cancer[270, 271, 272, 273]. Galectin 3 expression in Vδ1$^-$Vδ2$^-$ γδ T cells, in both healthy and tumour tissue, has been shown to be associated with the expression of IL-17 and a Th17 phenotype[274, 275]. To understand what type of immune response the tumour-infiltrating γδ T cells can contribute to, we analysed the expression of Th1 and Th17 associated cytokines and cytolytic effector molecules, following stimulation with PMA and Ionomycin. Vδ1$^+$ γδ T cells had a moderate expression of IFNγ, granzyme B, and TNF while Vδ2$^+$ γδ T cells had a higher expression of the same markers (*paper II*, **fig. 5A-C**). While Vδ1$^-$Vδ2$^-$ γδ T cells had a similar expression of IFNγ, granzyme B, and TNF as the Vδ1$^+$ cells they were also the only cell subset capable of substantial IL-17 production, at least in a few patients (*paper II*, **fig. 5A-C**).

As previously mentioned, the balance between Th1- and Th17-type responses is key in determining tumour progression[150, 152]. In our data it is evident that there is a difference in what type of immune response the various subsets of tumour-infiltrating γδ T cells are likely to contribute to. Vδ1$^-$Vδ2$^-$ γδ T cells appear to be more skewed towards a Th17 type response, or at least have an impaired Th1 response compared to the other tumour-infiltrating γδ T cells. Deficient Th1 responses has been linked to both direct immunosuppression, through recruitment of PMN-MDSCs, promotion of tumour growth and metastases, as well as correlate with worse clinicopathological features of the tumours[231, 276, 277, 278].

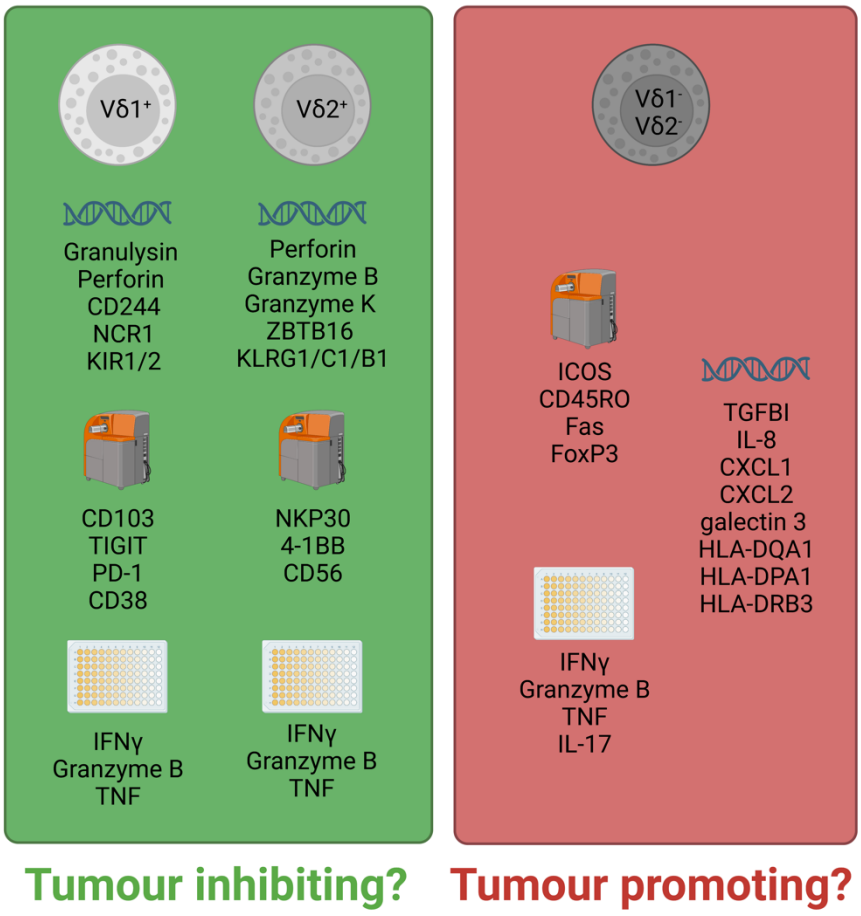

Figure 22. Third graphical result summary. Targeted sequencing, proteomic analysis, and functional assays indicate that Vδ1+ and Vδ2+ γδ T cells have tumour inhibiting capacity while Vδ1-Vδ2- γδ T cells are likely to contribute to tumour progression. Created using BioRender.com.

We also performed UMAP and Phenograph analysis on mass cytometry data of 31 057 MAIT cells isolated from the circulation, tumours, and unaffected colon mucosa from the same 15 CRC patients used in paper I (**fig. 23A**). In total 21 clusters were identified, of which 3 (cluster 4, 16, and 18) were unique for circulating MAIT cells (**fig. 23B**). Of the remaining 18 clusters all were found in both unaffected colon mucosa and tumour tissue (**fig. 23C-D**). However, local enrichment of specific clusters in the tumour and unaffected mucosa was observed. In addition,

we also performed in-depth analysis of circulating (**supplementary fig. 1**) and tissue infiltrating (**supplementary fig. 2**) MAIT cells separately.

Cluster 4 was composed of $CD8^+$ cells with a moderate expression of CD127, CD84, CD352, CD28, and NKG2D (**fig. 23E**). Interestingly, cluster 4, together with cluster 18, were the only expressing CD56 (**fig. 23E**). Cluster 18 was remarkably similar to cluster 4, and mainly differed in the lack of CD8 expression (**fig. 23E**). Cluster 16 was composed of naïve CD8 expressing MAIT cells with a co-expression of CD38, CD84, CD352, and TIGIT (**fig. 23E**). The in-depth analysis of the 4 146 MAIT cells from the circulation resulted in 12 separate clusters (**supplementary fig. 1A**). Interestingly, no expression of CD158b, CD159a, NKp30, Tim3, Lag3, CD158e1, or CD86 was detected among any of the circulating MAIT cells (**supplementary fig. 1B**).

The majority of tissue-infiltrating MAIT cells were either of a memory phenotype or activated cells ($CD45RO^+$), with a moderate to high expression of CD38, CD127, CD28, CD95, and TIGIT (**fig. 23E**). In the tumours there were two particularly interesting clusters that were enriched, clusters 12 and 21. Cluster 12 had a high expression of PD-1 and CD39, indicating potentially exhausted MAIT cells[252] (**fig. 23E**). In addition to also expressing PD-1 and CD39, the cells encompassing cluster 21 had a high expression of Tim3, Lag3, and 4-1BB (**fig. 23E**). Furthermore, the same cells (cluster 21) expressed CD158b, NKp30, CD86, and CD28 (**fig. 22E**). In the unaffected tissue the clusters 3 and 13 encompassed $CD8^+$ cells that were more enriched compared to the tumour tissue. These two clusters were both characterised by an increased expression of NKG2D and mainly differed in that the cells forming cluster 3 had a higher expression of CD127 and CD103 than those in cluster 13 (**fig. 23E**).

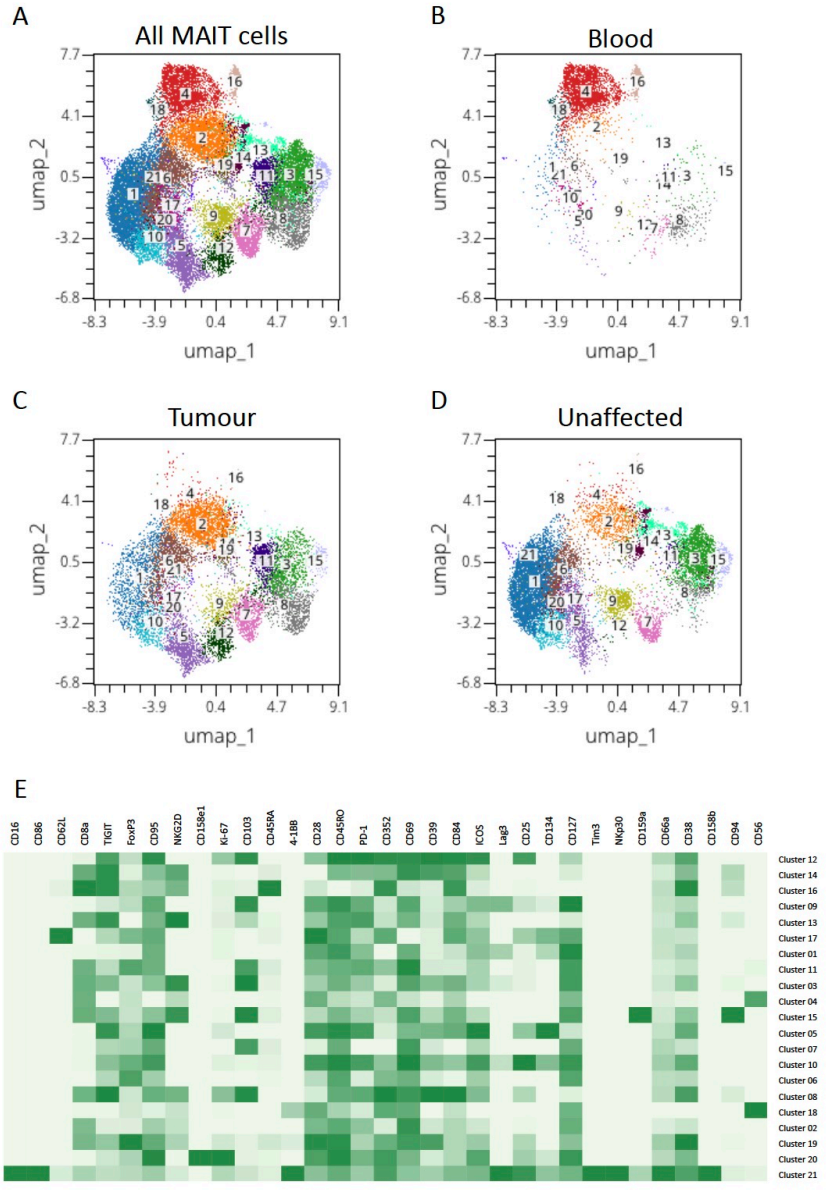

Figure 23. Clustering analysis of CRC MAIT cells. Single cell suspensions were isolated from tumours, corresponding unaffected colon mucosa, and blood, and analysed using mass cytometry. MAIT cells were analysed using the UMAP dimensional reduction algorithm together with the Phenograph clustering algorithm in concatenated data from blood, tumour, and unaffected tissue (**A**). Spatial distribution of circulating (**B**), tumour-infiltrating (**C**), and MAIT cells from the unaffected colon mucosa (**D**) within the UMAP-Phenograph plot. Heatmap of clusters identified in circulating MAIT cells using the UMAP dimensional reduction and phenograph clustering algorithm (**E**). n=15.

To investigate the expression of markers that could modulate the cytolytic activity in MAIT cells, we used flow cytometry and analysed the *ex vivo* expression of some NK cell markers investigated in the mass cytometry panel (CD56, CD94, CD158, NKG2D, and CD95) as well as a few additional markers (NKp80, 2B4, DNAM1, and NKG2A) on circulating MAIT cells from healthy donors, patients, and colon tumours.

We found that consistent with the mass cytometry data (where CD56 was heavily expressed in the blood), the CD56 expression was significantly increased in circulating MAIT cells, from both healthy donors ($p<0.05$) and patients ($p<0.005$), compared to tumour-infiltrating MAIT cells (**fig. 24**). Also, CD94 was expressed to a larger extent by tumour-infiltrating MAIT cells compared to circulating MAIT cells ($p<0.05$) (**fig. 24**). Furthermore, there seemed to be tendencies towards a high expression of CD158, 2B4, and NKG2D among tumour-infiltrating MAIT cells in some of the patients (**fig. 24**).

Interestingly, expression of the same markers (CD158, 2B4, and NKG2D) was lower in circulating MAIT cells from both the same patient and healthy donors compared to in the tumour-infiltrating MAIT cells (**fig. 24**). Also, expression of NKp80 was significantly higher among circulating MAIT cells compared to patient-derived MAIT cells (**fig. 24**). In circulating MAIT cells from healthy donors we also observed a trend towards a slightly increased expression of DNAM1 and NKG2A, compared to patient-derived MAIT cells (**fig. 24**). However, this increase in expression was not found to be significant and was likely caused by an increased expression in just a few of the patient samples (**fig. 24**).

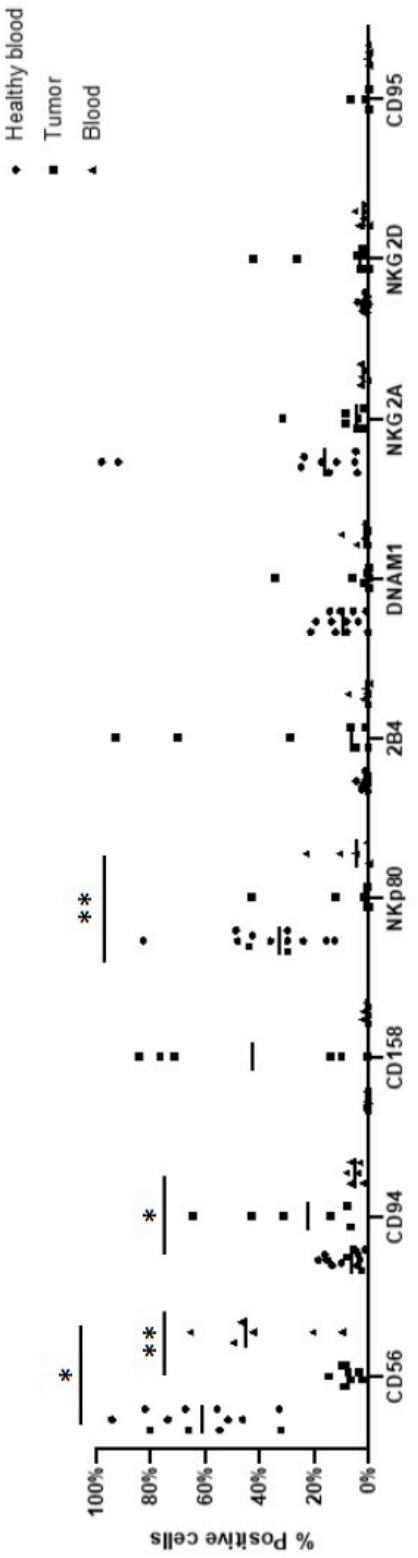

Figure 24. NK cell markers on MAIT cells. Single cell suspensions were isolated from tumours and blood samples and analysed, for the expression of CD56, CD94, CD158, NKp80, 2B4, DNAM1, NKG2A, NKg2D, and CD95, using flow cytometry. n = 12 healthy donors (blood) and 6 CRC patients (tumour and blood).

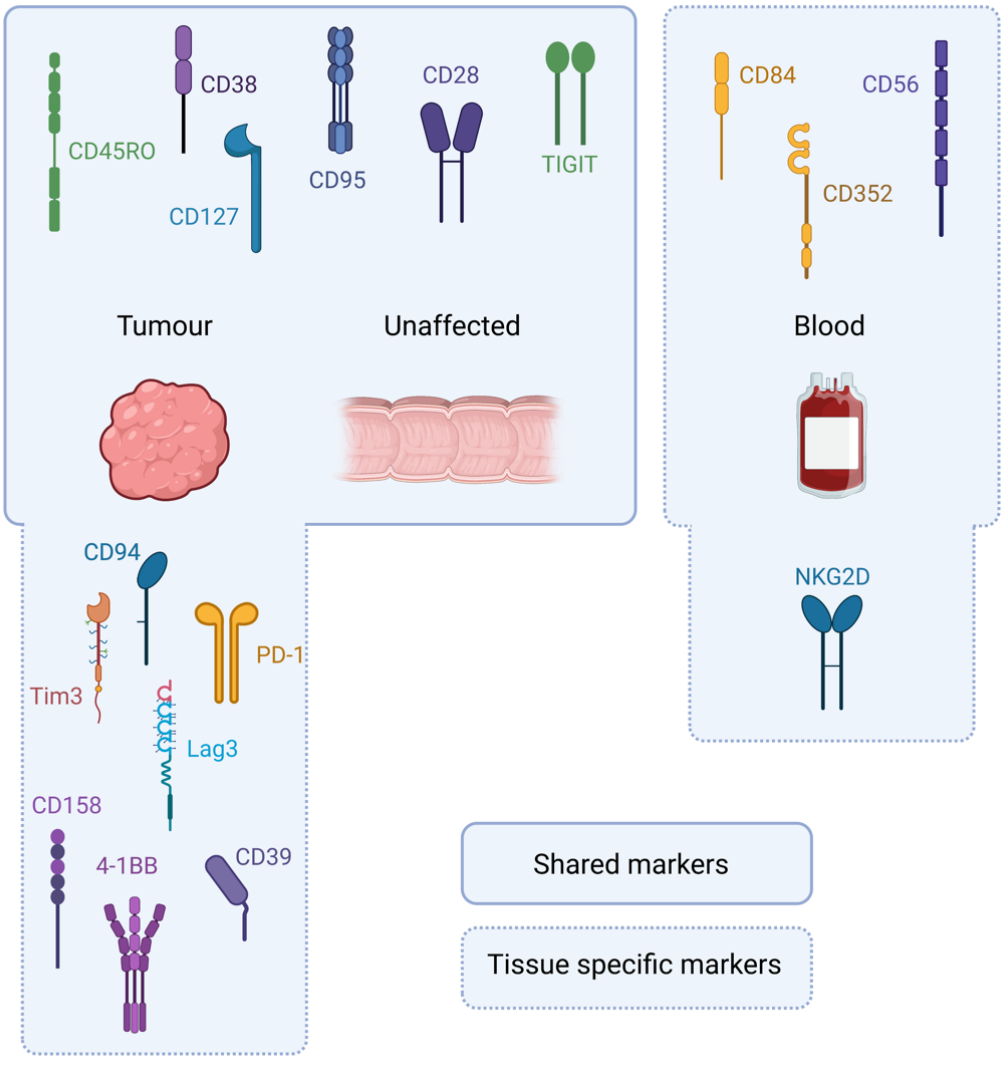

Figure 25. Fourth graphical result summary. Markers expressed by MAIT cells in tumours, unaffected colon mucosa, and blood from CRC patients. Some markers are shared among most cells in a tissue, and some are shared between cells of different tissues (filled line). Some markers were unique to, or more highly expressed in, clusters enriched in specific tissues (dotted lines). Created using BioRender.com.

UNCONVENTIONAL T CELLS AND CLINICOPATHOLOGICAL FEATURES OF COLON TUMOURS

As we have identified several different subsets of tumour-infiltrating MAIT and γδ T cells we were interested in if the infiltration of these cells were related to the clinicopathological features of the tumours. As we observed a reduced polyfunctionality in PD-1^{high}Tim3^{+} MAIT cells and potentially tumour-promoting functions in Vδ1^{-}Vδ2^{-} γδ T cells we focused our analysis on these subsets.

Infiltration of PD-1^{high}Tim3^{+} MAIT cells into colon tumours was not related to T-stage, stage, age, or MSI-status (*paper II*, **supplemental fig. 4**). Similarly, γδ T cell infiltration into colon tumours was not affected by differentiation grade, tumour stage, MSI-status, or location (*paper I*, **fig. 6B**). However, while no difference was significant, there were tendencies towards an increased infiltration of Vδ1^{-}Vδ2^{-} γδ T cells in highly differentiated and MSI-high tumours, but also a decreased infiltration of Vδ1^{-}Vδ2^{-} γδ T cells with advancing stage (*paper I*, **fig. 6A**). These results are quite possibly affected by the fact that our dataset is largely made up of MSS tumours that are late stage and poorly differentiated.

We also performed a survival analysis in 93, surgically treated, CRC patients. This dataset mainly encompassed not included in the other studies as sufficient time needed to elapse prior to evaluating the patient outcome. In our analysis we excluded stage IV tumours as these are rarely cured through surgery, we also excluded stage I tumours as these are relatively rare in our patient cohorts and these patients seldom have relapses.

Using flow cytometry, we evaluated the frequencies of MAIT cells in the tumours and unaffected colon mucosa and correlated this with survival data from the patients. We compared the relapse-free survival (RFS) of patients with a MAIT cell frequency that was higher or lower than the median frequency of tumour-infiltrating MAIT cells (**fig. 26A**). We also compared the RFS of patients in the highest or lowest quartile of tumour-infiltrating MAIT cells (**fig. 26B**). In addition to this we compared the RFS of patients with more or less than three times as many MAIT cells in the tumours as the unaffected colon mucosa, as previously described by Zabijak et. al.[179] (**fig. 26C**). Zabijak et. al. found that an accumulation of MAIT cells in the tumour compared to the surrounding tissue correlated to a poor prognosis[179]. In contrast to their results, we could find no

indication of a correlation between increased MAIT cell infiltration in the tumours and a decreased RFS.

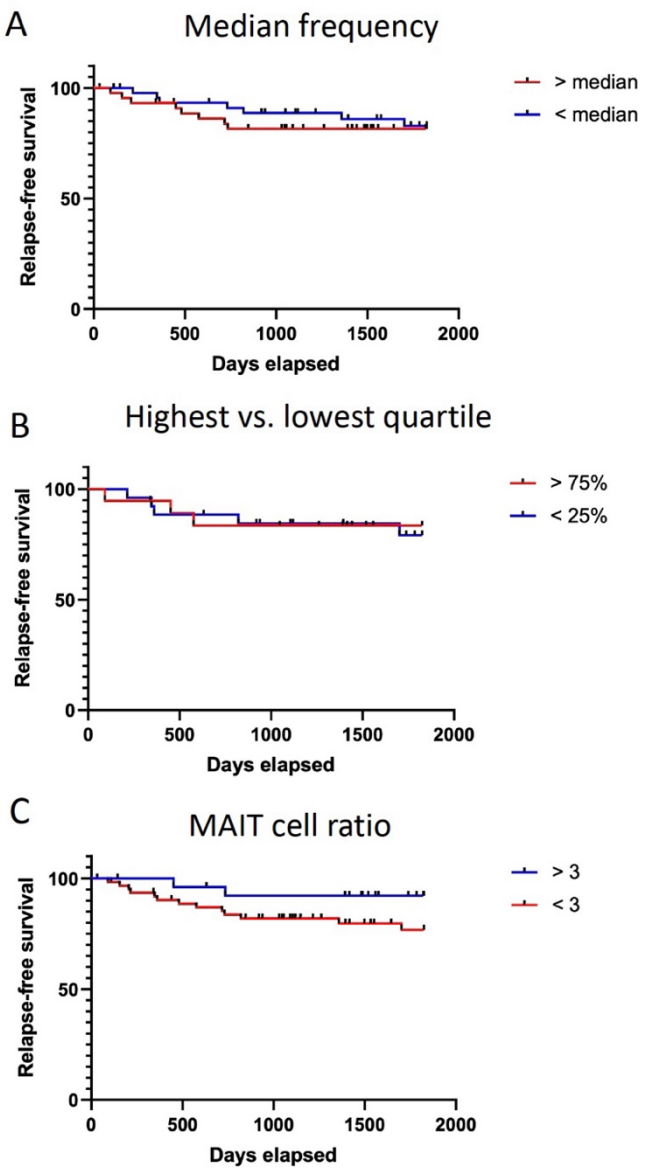

Figure 26. Patient survival in relation to frequencies of tumour infiltrating MAIT cells in stage II and III colon tumours. Relapse-free survival (RFS) in patients with a MAIT cell infiltration above or below the median, n=93 (p=0,64) (**A**). RFS in patients with a MAIT cell infiltration in the highest or lowest quartile, n=45 (p=0,88) (**B**). RFS in patients with a MAIT cell infiltration more than or less than three times higher in the tumours compared the unaffected colon mucosa, n=93 (p=0,13) (**C**).

THE THERAPEUTIC POTENTIAL OF MAIT CELLS

In order to investigate the potential use of MAIT cells as a treatment for colon cancer, we established a method of short-term expansion using 5-OP-RU conjugated latex microparticles in the presence of IL-2 and IL-7.

Over the course of 7 days of culture, a median 25-fold expansion was achieved in circulating MAIT cells from healthy donors, and similar levels of expansion was observed in both colon cancer patients and patients with metastatic colon cancer (peritoneal metastases) (*paper III*, **fig. 1A**). Tumour-infiltrating MAIT cells, and those from unaffected colon mucosa, also expanded readily, however, they did not expand as efficient as those from the circulation (*paper III*, **fig. 1A**). While there was no correlation between expansion and gender or age of the donor or patient (data not shown) there seemed to be a tendency towards greater expansion in individuals with a low frequency of circulating MAIT cells. In individuals with less than 3% circulating MAIT cells (among the T cells) the median fold increase was 36, and in individuals with more than 3% circulating MAIT cells (among the T cells) the median fold increase was only 10 (*paper III*, **supplementary fig. 1**).

As many expansion techniques utilise irradiated allogeneic feeder cells we investigated, in a separate set of experiments, if this led to a greater MAIT cell expansion. MAIT cells were cultured as previously described (in paper III) and after 7 days of culture, irradiated allogeneic PBMCs were added at a ratio of 1:200 between MAIT and feeder cells. Following the addition of feeder cells, the MAIT cells expanded for another 7 days (14 days in total), which led to a significantly ($p<0.005$) higher expansion compared to expansion without feeder cells **(fig. 27**). In this set of healthy donors, the median fold increase during 7 days of culture was 11 and the median fold increase during 14 days of culture was 142 (**fig. 27**). Furthermore, MAIT expansion after 7 days of culture, without the addition of feeder cells, was not possible. However, while they did not expand further the expanded MAIT cells persisted in culture for 5 – 7 days without additional stimulation (data not shown). When using feeder cells, the MAIT cells stopped expanding at around day 14 of culture but persisted with undiminished numbers for another 7 days, after which we observed a rather rapid decline in numbers and viability (data not shown).

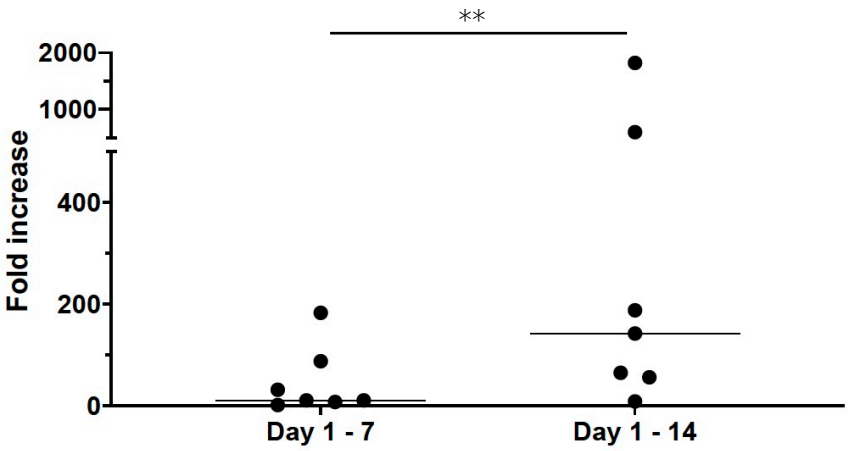

Figure 27. MAIT cell expansions with and without irradiated feeder cells. MAIT cells were expanded for one week using aAPCs, IL-2, and IL-7, after which feeder cells were added and the cultures were continued for an additional 7 days. Day 1-7 shows the MAIT cell expansion during the first week without feeder cells and Day 1-14 shows the total fold increase of the same cultures after feeder cells were added. n=7.

As we had no direct need for the increase in MAIT cell numbers that expansion using irradiated feeder cells brought and as the feeder cells caused some hurdles when isolating expanded MAIT cells, we proceeded to use short-term expansion of MAIT cells over the course of 7 days. However, it is promising that larger expansions of MAIT cells can be achieved, as the adoptive cell therapies currently investigated require a large number of cells to be effective[279].

To assess the effects of expansion on the MAIT cells, we analysed the expression of several NK and T cell-associated cytotoxicity markers and cytokines, using flow cytometry. To get an insight into the kinetics of the expression we performed this analysis before, during (day 3), and after expansion.

In circulating MAIT cells, the expression of CD56, KLRG1, KIR2DL/S2/S3/S5 was upregulated after 7 days of expansion (*paper III*, **fig. 1B**). Furthermore, the expression of the cytotoxic effector molecules GrA, GrB, and Perforin was also upregulated in a similar fashion as the NK cell-associated markers (*paper III*, **fig. 1E**). However, the cytotoxicity-associated surface receptors FasL and TRAIL were not dramatically affected by the expansion but showed tendencies towards a slight

increase in expression after 7 days (*paper III*, **fig. 1G**). Consistent with both NK-associated markers and cytotoxic effector molecules the cytokines IFNγ, GM-CSF, and TNF were also upregulated first after 7 days of expansion (*paper III*, **fig. 1D**). In contrast, NKG2A expression was downregulated and the expression of NKp46 and GrK was unchanged (*paper III*, **fig. 1C** and **1F**). Unsurprisingly, the expression of the cell-cycle protein Ki-67 increased steadily over the course of the expansion (day 3 and day 7) (*paper III*, **supplementary fig. 2**).

To investigate the cytotoxic capacity of expanded MAIT cells, we co-cultured target cells with expanded MAIT cells from the circulation of both healthy donors and patients (colon cancer and peritoneal metastasis), and from the tumours. In these assays we measured the specific killing of target cells. As target cells we used both epithelial cancer cell lines (HeLa, HCT116, and HT29) and primary patient-derived tumour cells from colon cancer patients.

Expanded MAIT cells from all tissues readily killed tumour cells in a dose-dependent manner (*paper III*, **fig. 2A-D**). Interestingly, MAIT cells could induce substantial cytotoxicity in the target cells already at MAIT to target cell ratios as low as 1:1 and 4:1. Furthermore, tumour-derived expanded MAIT cells seem to possess a stronger cytotoxic activity compared to blood-derived MAIT cells (*paper III*, **fig. 2B**). We also measured the cytotoxic activity of MAIT cells expanded from the circulation of healthy donors towards primary patient-derived tumour cells and found that MAIT cells also exhibited a similarly robust cytotoxic response towards these cells (*paper III*, **fig. 4**).

Having shown that both circulating and tumour-infiltrating MAIT cells are cytotoxic we were interested in how this cytotoxicity is mediated. To investigate this, we blocked various surface receptors known to be associated with cytotoxicity in T and NK cells, along with the activity of the cytotoxic serine proteases[280, 281]. When blocking the surface receptors TRAIL and FasL we saw that the cytotoxicity towards HCT116 cells was reduced slightly in the higher MAIT to target cell ratios (4:1) (*paper III*, **fig. 3D** and **F**). In contrast, blocking the activity of CD161 seemed to have less of an effect (*paper III*, **fig. 3B**). However, blocking the serine protease activity of expanded circulating MAIT cells resulted in a more pronounced decrease in cytotoxic activity towards both HeLa and HCT116 cells (*paper III*, **fig. 3G** and **H**).

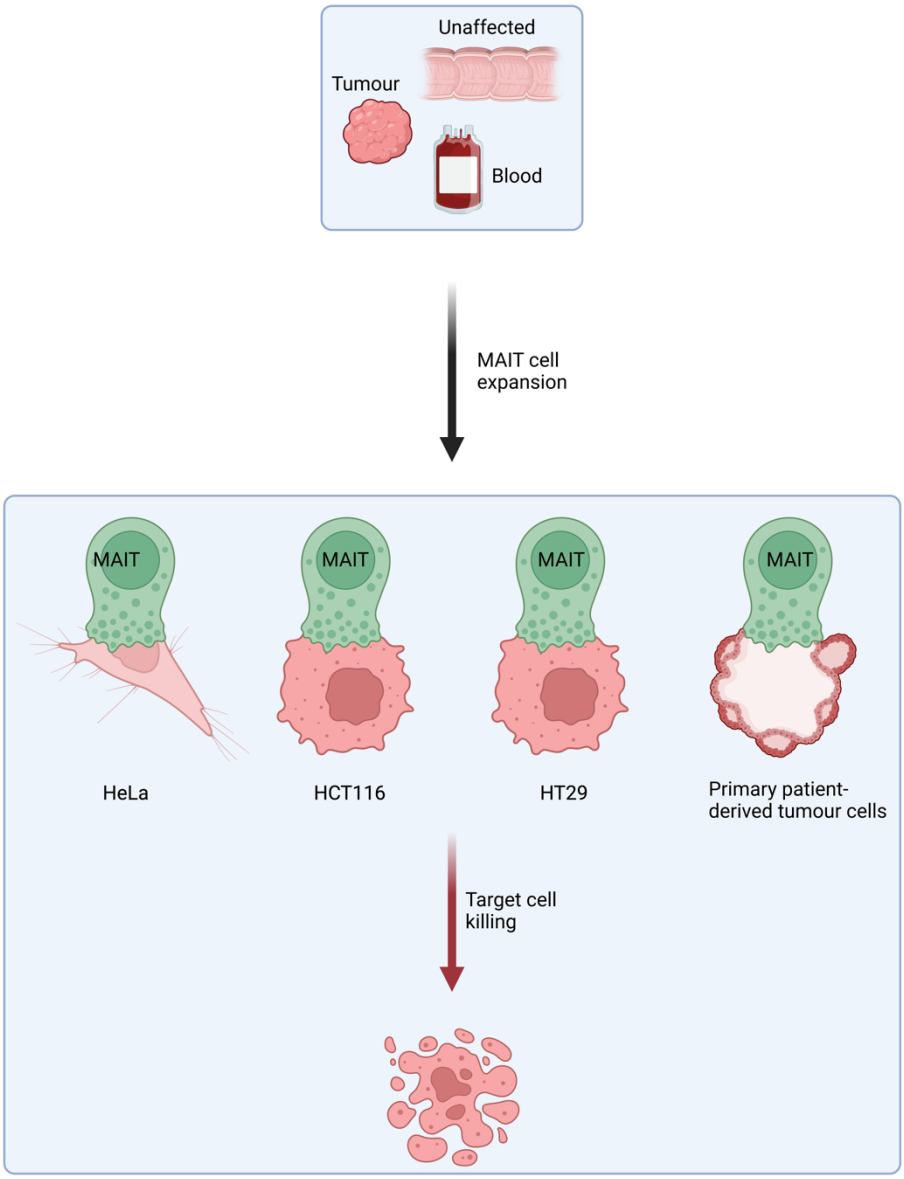

Figure 28. Fifth graphical result summary. MAIT cells expanded from tumours, unaffected colon mucosa, and blood readily kill tumour cells from epithelial cancer cell lines (HeLa, HCT116, and HCT29) and primary patient-derived tumour cells. Created using BioRender.com.

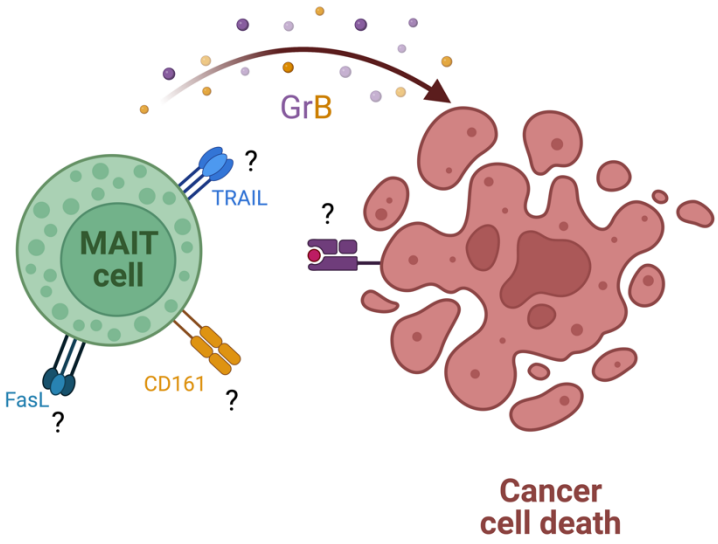

Figure 29. Sixth graphical result summary. MAIT cell cytotoxicity is partially dependent on serine proteases and as GrB is highly expressed by expanded MAIT cells it is a likely candidate. Other receptors, cytokines, or effector molecules may also be involved as blocking TRAIL, FasL, and CD161 can either increase or reduce the cytotoxic activity of MAIT cells depending on the type of target cell. Furthermore, it is also not known which receptor or marker on the cancer cell is responsible for the recognition event as blocking MR1 has no effect on the MAIT cell cytotoxicity. This highlights the complexity of MAIT cell cytotoxicity as it seems to be dependent on multiple modes of action and utilises different mechanisms depending on the targeted cell type. Created using BioRender.com.

Consistent with other studies, we show that MAIT cells can be readily expanded in vitro and possess a potent cytolytic activity following expansion[282, 283]. As the MAIT cells become activated following expansion, due to the TCR-mediated signals from the aAPCs and cytokines, it is fair to say that this study focuses on the potential use of MAIT cells in functional therapies rather than the actual state of MAIT cells in colon tumours. However, as the epithelium in CRC tumours is more permeable, compared to in the unaffected colon mucosa, and tumour-infiltrating MAIT cells have an activated or exhausted phenotype this could indicate that MAIT cell antigens reach the tumours and induces local activation and recruitment of MAIT cells[181, 229]. Furthermore, a recent study also suggested that MAIT cell antigens are

present in serum and would thus also be able to reach the colon tumours[284].

While expanded MAIT cells from all tissues effectively killed target cells derived from both ovarian cancer (HeLa) and colon cancer (HT29 and HCT116) those from the tumour exhibited a slightly increased cytotoxic capacity, this may indicate that they are already antigen-experienced or primed by factors in the TME[115]. Similar results were observed in MAIT cells from acute hepatitis A where MAIT cells from hepatic tissue were more cytotoxic compared to circulating MAIT cells[285]. Also, the expanded MAIT cells from the circulation of both healthy donors and CRC patients could kill primary patient-derived tumour cells, established from at least one CRC patient, which is promising for their use in potential treatments. Furthermore, we observed the cytotoxicity of expanded MAIT cells to be partly dependent on the activity of one or more serine proteases (as Granzyme B is highly expressed in expanded MAIT cells it is a likely candidate). The dependency on serine protease activity is interesting as the over-expression of serine protease inhibitors by tumour cells, to circumvent cytotoxic T and NK cells have been shown to be an effective way of immune evasion[286, 287]. We also observed varied effects of blocking FasL, TRAIL, and CD161 on expanded MAIT cells depending on which type of target cell was used. This may partly depend on the relatively small dataset we have so far or indicate that the activity of these surface markers is related to factors expressed by the tumour cells. It is possible that the MAIT cell-to-target cell ratio could also affect which receptors are used by MAIT cells, for both target cell recognition and cytolytic activity.

CONCLUSIONS & FUTURE PERSPECTIVES

In this thesis, we show how the numbers and frequencies of γδ T cells are reduced in colon tumours and that the remaining tumour-infiltrating γδ T cells contain a subset with an unknown TCR composition and potentially tumour-promoting functions. We also show how MAIT cells accumulate in colon tumours, but that a substantial number of them are exhausted and therefore functionally impaired. We also demonstrated that exhausted MAIT cells respond to immune checkpoint therapy, indicating that they could participate in the immune response of patients treated with PD-1 or PD-1-ligand-blocking antibodies. This further highlights the need to understand the properties of tumour-infiltrating unconventional T cells to better harness their capacity in cancer therapies.

As fully differentiated effector T cells the unconventional T cells are capable of rapid response upon activation. The composition of cells infiltrating the tumours, or of those present in the tissue prior to tumour formation, could be crucial in determining the type of immune response mounted. Therefore, further improving our knowledge of the composition and function of unconventional T cells in cancer is essential. It is especially important as understanding how unconventional T cells influence tumour immunity might function as a future prognostic marker for tumour progression or treatment outcome. However, for unconventional T cells to effectively function as a prognostic marker more knowledge about their phenotype and function is needed. Also, it would be preferable to develop future prognostic assays so they can be carried out quickly, cheaply, and without causing further distress to the patients. While investigating the tumour-infiltrating cells will always be the most relevant in solid tumours, getting a comparative analysis from unaffected control tissue, blood samples, and levels of relevant serum proteins increases the likelihood of identifying potential biomarkers.

Lastly, we showed that expanded MAIT cells possess cytotoxic activity towards several different epithelial cancer cell lines as well as towards tumour cells derived directly from colon cancer patients, indicating that MAIT cells could be a promising new candidate in the field of immunotherapy. As these are preliminary results an extended investigation into what type of colon tumour cells the expanded MAIT cells can effectively kill, exactly how their cytotoxicity is mediated, and if

they exhibit unspecific cytotoxicity towards other cell types is needed. Furthermore, potential treatments using MAIT cells would likely require larger numbers of cells, than what we have generated in the lab. A larger number of MAIT cells could be generated using irradiated feeder cells or possibly through expansion in a bioreactor. As we have not compared the properties of MAIT cells expanded using feeder cells with those expanded without, this would need investigation. Another issue that still needs to be investigated is whether factors in the tumours are inhibiting MAIT cell proliferation or cytotoxicity, as suggested by the fact that some tumour-infiltrating MAIT cells are exhausted. Also, if this is the case, investigations into how, or if, these factors can be modulated to improve the effect of expanded MAIT cells would be needed. Furthermore, the question of tissue homing is yet to be investigated in expanded MAIT cells, which would obviously be of great importance if attempting to transplant expanded cells.

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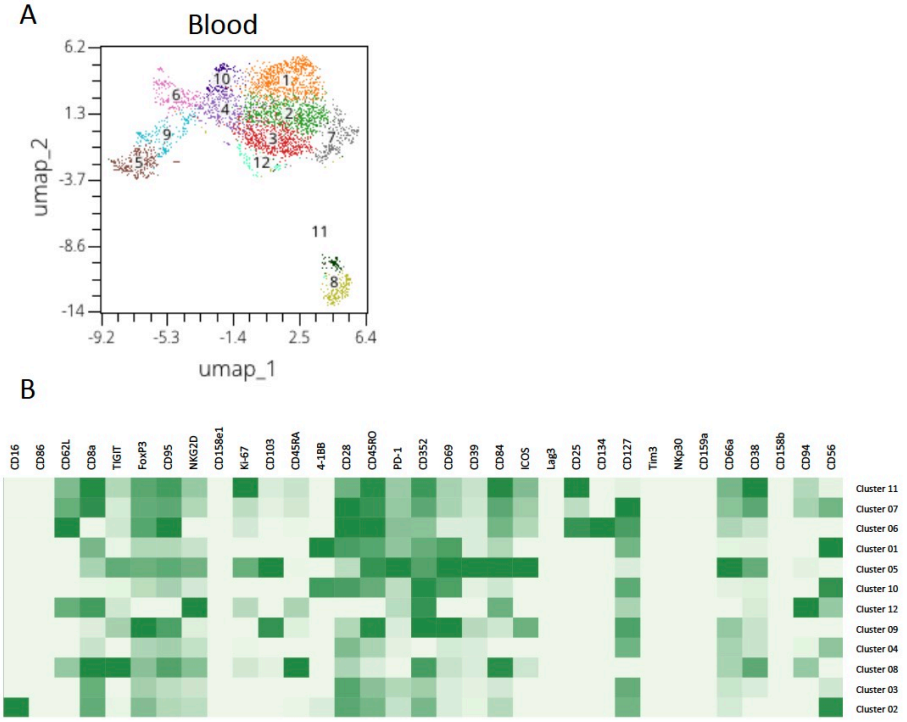
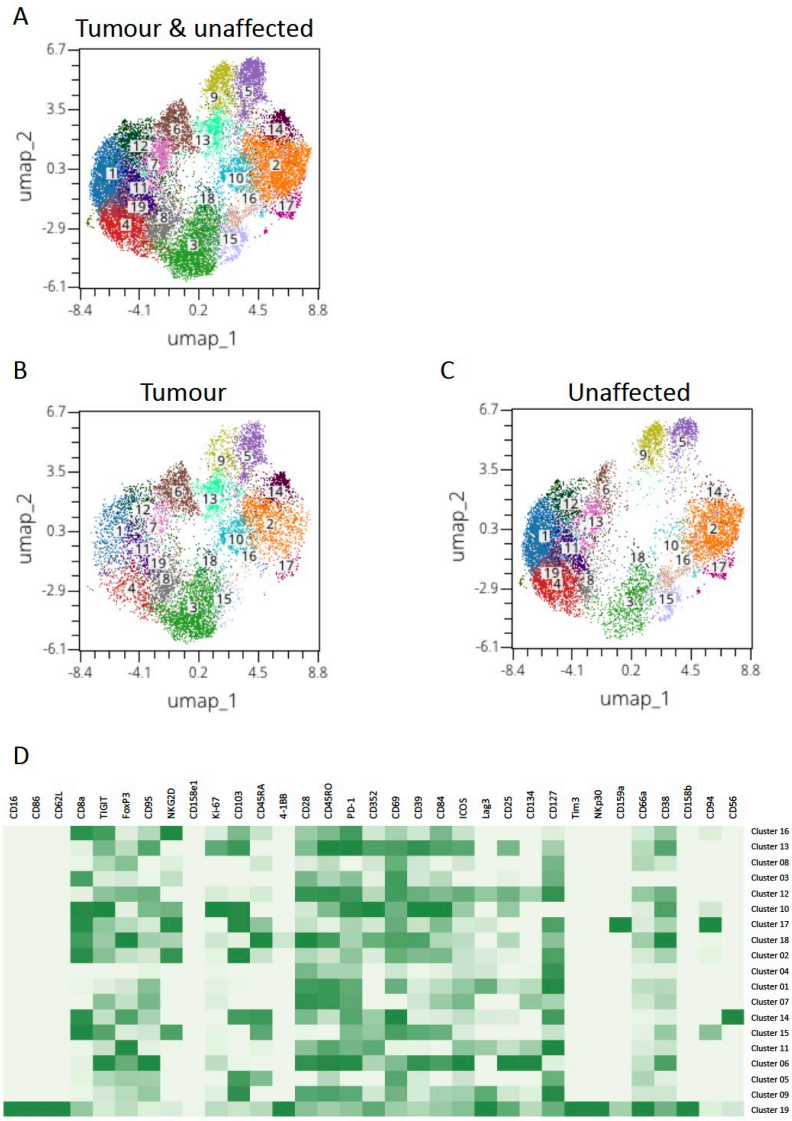
SUPPLEMENTARY INFORMATION



Supplementary figure 1. Clustering analysis of circulating MAIT cells in colon cancer. Single cell suspensions were isolated from blood and analysed using mass cytometry. MAIT cells were analysed using the UMAP dimensional reduction algorithm together with the Phenograph clustering algorithm in concatenated data from blood (A). Heatmap of clusters identified in circulating MAITT cells using the UMAP dimensional reduction and phenograph clustering algorithm (B). n=15.



Supplementary figure 2. Clustering analysis of tissue MAIT cells in colon cancer patients. Single cell suspensions were isolated from tumours and corresponding unaffected colon mucosa and analysed using mass cytometry. MAIT cells were analysed using the UMAP dimensional reduction algorithm together with the Phenograph clustering algorithm in concatenated data from tumour, and unaffected tissue (**A**). Spatial distribution of tumour-infiltrating (**B**) and MAIT cells from the unaffected colon mucosa (**C**) within the UMAP-Phenograph plot. Heatmap of clusters identified in circulating MAITT cells using the UMAP dimensional reduction and phenograph clustering algorithm (**D**). n=15.