

Investigating the role of Th17 cells in colorectal cancer



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Abstract

In the two decades since their discovery, T helper type 17 (Th17) cells have become widely accepted as key immune sentinels at mucosal barriers such as the gut. During homeostasis, they play an important role in protecting against microbial translocation by strengthening the epithelial cell layer present within these niches and by enhancing the production of antimicrobial peptides. These environments can be highly dynamic and Th17 cells have evolved to display functional plasticity, rapidly upregulating a number of inflammatory programmes in response to physiological or immunological stimuli.

However, aberrant activation of these inflammatory programmes has the capacity to drive a number of pathological processes, including the initiation and development of colorectal cancer (CRC). Despite an abundance of literature suggesting that Th17 cells are deleterious in CRC, accumulating evidence has implied that some Th17 populations may serve a more protective role. One reason for these seemingly antithetical functions may be due to the transcriptional heterogeneity which Th17 cells display.

In this thesis, we first explore the extent of this heterogeneity in human CRC using a variety of computational techniques before validating our findings *ex vivo* using patient resections. We describe a population of CD69⁺ CD103⁺ Th17 cells that are enriched in the tumours of some patients. We speculate that this population, which we term Th17 resident memory (Th17RM) cells due to their enrichment with several tissue resident memory T cell markers, develop from a pool of homeostatic Th17 cells in the presence of inflammatory cytokines such as IFN- γ , TNF- α , TGF- β 1 and IL-18 derived from tumour associated macrophages. Th17RM cells are clonally expanded and exhibit gene programmes that suggest they proliferate in

response to antigen within the tumour microenvironment. Tumours that are enriched with Th17RM-associated genes correlate with improved prognosis in patients with colon adenocarcinoma and this protection is similarly observed in those with melanoma. However, such protection is not afforded to patients with rectal adenocarcinoma, lung or breast carcinomas. Th17RM cells are enriched with cytotoxic molecules such as granzyme A, granzyme B, IFN- γ and TNF- α and can be found embedded within the tumour epithelium, suggesting that this protection might at least partially be mediated by direct cytotoxicity.

Declaration of authorship

We utilised single cell RNA-sequencing datasets uploaded to the Gene Expression Omnibus repository under the accession codes GSE108989 and GSE146771 that were sequenced by the laboratories of Zemin Zhang and Xin Yu, respectively^{1,2}. To investigate intercellular communication networks, we used a publicly available spatial transcriptomics dataset curated by the Gao laboratory³.

The writing of this thesis, including analysis of the above single cell RNA-sequencing and spatial transcriptomics datasets and generation of data presented hereon is solely my own.

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List of abbreviations

ACK	Ammonium-chloride-potassium
AID	Activation-induced cytidine deaminase
<i>AIRE</i>	Autoimmune regulator gene
APC	Antigen presenting cell
<i>APC</i>	Adenomatous polyposis coli gene
ATAC-seq	Assay for transposase-accessible chromatin with sequencing
β_2 M	Beta ₂ microglobulin
BCR	B cell receptor
BCL-2	B cell lymphoma-2
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
CDR3	Complementarity determining region 3
CMS	Consensus molecular subtype
CO ₂	Carbon dioxide
CRC	Colorectal cancer
CTLA-4	Cytotoxic T lymphocyte associated protein-4
CXCL	Chemokine ligand
CXCR	Chemokine receptor

DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	1,4-Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FcR	Fragment crystallisable receptor
FGF-2	Fibroblast growth factor 2
FLT3L	Feline McDouglas sarcoma (FMS)-like tyrosine kinase ligand
FMO	Fluorescence minus one
FoxP3	Forkhead box protein P3
<i>g</i>	Gravity
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GO	Gene ontology
GPR183	G protein-coupled receptor 183
GSEA	Gene set enrichment analysis
H&E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNSCC	Head and neck squamous cell carcinoma
IBD	Inflammatory bowel disease

ICOS	Inducer of co-stimulation
ICOSL	Inducer of co-stimulation ligand
IFN- α	Interferon-alpha
IFN- β	Interferon-beta
IFN- γ	Interferon-gamma
IL	Interleukin
ILC3	Type 3 innate lymphoid cell
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
IR	Infrared
IRF4	Interferon regulatory factor 4
JAML	Junctional adhesion molecule-like protein
LAG-3	Lymphocyte activation gene-3
MAIT	Mucosal associated invariant T
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
MOG ₃₃₋₅₅	Myelin oligodendrocyte glycoprotein ₃₃₋₅₅
NHS	National Health Service
NK	Natural killer
NKT	Natural killer T
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PD-1	Programmed cell death-1
PFA	Paraformaldehyde

PGE ₂	Prostaglandin E ₂
PHA-L	Phytohaemagglutinin-L
PMA	Phorbol 12-myristate 13-acetate
RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROR _γ t	Retinoic acid receptor-related orphan receptor gamma t
RPMI	Roswell Park Memorial Institute
S1P	Sphingosine-1-phosphate
S1PR1	Sphingosine-1-phosphate receptor 1
SCA-1	Stem cell antigen-1
SCID	Severe combined immunodeficiency
scRNA-seq	Single cell RNA-sequencing
TCF-1	T cell factor-1
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
Tfh	T follicular helper
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TIM-3	T cell immunoglobulin and mucin domain-3
TNF	Tumour necrosis factor
TNF- α	Tumour necrosis factor alpha

Treg	T regulatory
UK	United Kingdom
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier
V(D)J	Variable-diversity-joining
VEGF	Vascular endothelial growth factor
° C	Degrees Celsius

Chapter 1: Introduction

1.1 The aetiology of colorectal cancer

1.1.1 Epidemiology and risk factors

CRC, cancer of the colon or rectum, is the third most common cancer and cause of cancer-related death in both men and women worldwide. Over 2 million new cases are reported annually and almost 1 million deaths can be attributed to CRC per year⁴.

In those over 50 years of age from high-income countries, CRC incidences have remained relatively stable which can partially be explained by the success of routine screening methods that target this age group, namely faecal immunochemical testing in the UK. This trend has not been seen in low- to middle-income countries and the number of new CRC cases has instead been steadily rising. Additionally, the incidence of CRC in young, European individuals (clinically defined as < 50 years of age) has risen rapidly in the past decade, with the strongest increase of 7.9% per year observed in those aged between 20 and 29 years⁵.

Although the exact reasons for this rise remain unknown, it is plausible that a combination of poor dietary habits, alcohol intake, low physical activity, obesity and antibiotic use drive this phenomenon⁶. Exposure to these risk factors can have broad consequences; not only can they cause genetic and epigenetic alterations in the epithelial cells that line the gut but they can also impact the microbiota and host immune cells which reside at this interface. The risk of developing CRC is further increased in individuals who have a family history of disease and, accordingly, a positive family history is documented in approximately 15-20% of CRC patients⁷.

A specific subgroup of CRC patients is made up of those affected by hereditary CRC syndromes. Whilst this population is relatively small, accounting for 5-15% of CRC patients⁸, it is important that these individuals are identified early to allow for diligent surveillance as a significant risk of CRC-associated mortality exists.

The most common syndrome in this category is Lynch syndrome, caused by mutations in DNA mismatch repair genes such as *MLH1* or *MSH2*⁸. The impairment of these machineries increases the likelihood of DNA mutations during cell division, particularly in microsatellite DNA fragments with repetitive nucleotide sequences. Individuals with Lynch syndrome are also predisposed to cancers of at least eight other organs: the endometrium, stomach, small intestine, hepatobiliary system, upper urologic tract, ovary, pancreas, breast and prostate⁹.

The second most common syndrome is familial adenomatous polyposis (FAP), caused by mutations in *APC*, a tumour suppressor gene that functions to sequester the Wnt signalling target β -catenin¹⁰. A considerable number of patients with FAP develop large adenomas that progress to CRC at a young age. Other far rarer hereditary syndromes include those belonging to the hamartomatous polyposis cluster of syndromes, such as Peutz-Jeghers syndrome (mutations in *LKB1*) and juvenile polyposis (mutations in *SMAD4*), as well as the hereditary syndrome characterised by recessively inherited mutations in *MYH*¹¹.

Finally, chronic colitis due to IBD is another major risk factor for CRC. Encouragingly, the number of CRC cases that arise secondary to IBD (currently about 1% of CRC cases) appears to be falling, with the success of anti-inflammatory strategies and improved surveillance thought to be driving this trend¹².

1.1.2 Tumourigenesis and cancer development

The environmental and genetic factors that cause CRC do so by promoting the acquisition and accumulation of driver mutations in the epithelial cells that line the colon or rectum. The majority of these tumours become progressively genomically unstable in a well-documented, stepwise fashion commonly referred to as the 'adenoma-carcinoma sequence' that culminates in the formation of invasive carcinoma⁷.

Premalignant epithelial cells usually initiate this dysplastic trajectory with inactivating mutations in *APC*. The accumulation of β -catenin signals the triggering of pathological epithelial proliferation and the development of early polyps. Next, the acquisition of mutations in *KRAS* or *BRAF* drive pathological activation of MAPK signalling pathways, which further enhance cellular proliferation and survival mechanisms. Inactivation of canonical TGF- β /SMAD tumour suppressor signalling exacerbates this failure to regulate proliferation and, finally, loss of the critical tumour suppressor *TP53* (p53) permits outgrowth into the adenocarcinoma stage. This sequence describes that taken by tubular and tubulovillous adenomatous polyp lesions as they progress towards CRC and encompasses around 90% of CRC tumours.

However, some tumours can evolve from a subset of polyps known as sessile serrated polyps. These emerge by distinct molecular and histological events whereby dysregulated Wnt signalling is induced via mutations in *CTNNB1* (β -catenin) and loss of MAPK signalling is usually associated with mutated *BRAF*, *PIK3CA* and *TGFBR2*. Tumours of this origin arise more frequently on the right side of the colon (caecum, ascending and transverse colon) and commonly display microsatellite instability with heightened aberrant CpG island DNA

methylation, known as a CpG island methylator phenotype (CIMP). Conversely, tumours on the left side (descending and sigmoid colon plus rectum) are more commonly microsatellite stable and a larger proportion carry mutant *KRAS*.

1.1.3 Classification systems in colorectal cancer

These compounded genetic instabilities can result in somatic gene copy number alterations (SCNA) and the acquisition of additional mutations that contribute to the remarkable tumour heterogeneity observed between CRC patients¹³. This variation has translational implications and one of the most prominent is observed in those with either proficient (pMMR) or deficient (dMMR) mismatch repair systems. In patients that lose this regulatory machinery, the high rate of somatic mutations can lead to an abundance of neoantigens that are capable of eliciting tumour-specific T cell responses¹⁴. These patients have a high (~50%) response to immune checkpoint blockade therapy, whereas those which have intact machinery have low mutational burden and are largely unresponsive to immunotherapy¹⁵⁻¹⁷.

Recent work has set out to delineate commonalities within this variation, which has led to the generation of the consensus molecular subtype (CMS) classification system^{18,19} that is now routinely used as part of the clinical categorisation of tumours. This classification system encompasses intrinsic tumour characteristics as well as categorising immune hubs present within each tumour niche (**Table 1.1**).

CMS subtype	CMS1	CMS2	CMS3	CMS4
Pathotype	MSI immune	Canonical	Metabolic	Mesenchymal
Incidence	14%	37%	13%	23%
Molecular features	MSI CIMP high Hypermutation	SCNA high	Mixed MSI status CIMP low SCNA low	SCNA high
Key mutation	<i>BRAF</i>		<i>KRAS</i>	
Immune landscape	High infiltration High activation		Metabolic dysregulation	Stromal rich TGF- β activation Angiogenesis
Prognostic information	Worse survival after relapse			Worse relapse-free and overall survival
Histological description²⁰	Serrated	Tubular	Tubular	Mesenchymal serrated
Anatomical location^{21,22}	Proximal colon	Distal colon & rectum	No specific enrichment	Distal colon & rectum

Table 1.1 | Consensus molecular subtype (CMS) classification system in CRC. CIMP = CpG island methylator phenotype, MSI = microsatellite instability, SCNA = somatic gene copy number alteration. Table adapted from Guinney *et al.*¹⁸.

1.1.4 Patient management and current treatment strategies

For patients with early stage disease, surgery remains the mainstay treatment. In those with more progressive or metastatic disease, combined surgery with neoadjuvant chemotherapy

is more commonly used and fluoropyrimidines, irinotecan, oxaliplatin and trifluridine chemotherapeutic agents are most frequently used²³. Tumour heterogeneity can play a big part in determining which treatments patients receive and this is especially highlighted by the use of immunotherapies. As mentioned previously, many patients with dMMR tumours exhibit robust responses to anti-PD-1 immunotherapy¹⁵⁻¹⁷. However, these individuals make up only a fraction of CRC patients and anti-PD-1 immunotherapy may actually heighten patient insensitivity to conventional chemo- and radiotherapies in some individuals by directly suppressing intrinsic PD-1 signalling in tumour cells²⁴.

CAR T cell therapies have also proved to be a revolutionary tool in the arsenal against cancer. Whilst their use has proved enormously successful in the treatment of haematological malignancies, comparable translational success in CRC (like many other solid tumours) remains to be seen. The longevity of CAR T cells *in vivo* remains a major limitation to their use, as is their inability to penetrate impermeable tumours²⁵. Improving our understanding about which phenotypes that the most successful CAR T cells adopt *in vivo* to eradicate solid tumours will be necessary to augment their efficacy.

1.2 The human innate immune system

Maintaining homeostasis is necessary for the survival of any organism. Infection, abnormal or damaged cellular material pose considerable threat to this balance and organisms have evolved intricate and elegant ways of dealing with such challenges. The human immune system broadly comprises two major mechanisms that defend against these threats: firstly, germline-encoded receptors activate innate immune components; secondly, antigen-specific

receptors initiate adaptive immune responses²⁶. These systems are not distinct entities and successful immune responses often rely on a complex interplay between the two.

The innate immune system acts as a first line of defence and utilises pattern recognition receptors (PRRs) to detect potential threats. These receptors have broad specificities for common structural motifs or patterns that can be derived from microorganisms (pathogen-associated molecular patterns, PAMPs) or from host cell damage (damage-associated molecular patterns, DAMPs). PAMPs are often essential for the survival of microorganisms and thus generally have molecular structures that cannot be found in host cells. These include lipopolysaccharide or lipoteichoic acid found in bacterial cell walls, bacterial DNA or the fungal cell wall component β -glucan. DAMPs, such as heat shock proteins, ATP, uric acid or calreticulin, are released by dying cells and initiate sterile inflammation²⁷.

Key classes of innate immune cells that express these PRRs include DCs, macrophages, monocytes, granulocytes (neutrophils, eosinophils, basophils and mast cells) and NK cells. Despite stemming from non-haematopoietic origins, fibroblasts, epithelial and endothelial cells can also express PRRs and are thus considered by some to be part of the innate immune system²⁸.

Toll-like receptors (TLRs) are perhaps the best known PRR, with NOD-like, RIG-I-like, AIM-2-like and C-type lectin receptors comprising the other major receptor types²⁹. Upon ligation, each aforementioned receptor initiates extensive signal transduction cascades that activate $\text{NK-}\kappa\text{B}$, AP-1, STAT3 and NFAT transcription factors which upregulate pro-inflammatory cytokines and type I interferons²⁶. Several signalling pathways also promote the assembly of

NLRP3 or AIM2 inflammasomes which induce the secretion of IL-1 β and IL-18 to initiate pyroptosis, a form of pro-inflammatory cell death³⁰.

The induction of inflammation by innate immune cells represents an important feature in many favourable anti-tumour immune responses. Pro-inflammatory macrophages boast robust phagocytic activity and antigen presentation capabilities which function to both directly eradicate tumour cells and prime components of the adaptive immune system. Specific cell type functionalities can also be particularly advantageous. An enriched presence of CD1c⁺ DCs (cDC2s) within the tumour has been mechanistically linked with greater CD4⁺ T cell mediated tumour rejection by enhanced cross-presentation of tumour antigens³¹. As such, many studies are focussing on manipulating the innate immune system for therapeutic benefit³².

However, the tumour microenvironment hosts a variety of complex signalling networks and administering exogenously generated innate immune cells, such as DCs, is not always sufficient to recapitulate the anti-tumour immune responses seen in some studies. Some tumours are adapted to secrete large amounts of VEGF, for example, which inhibits the activity of FLT3L that is essential for the development and persistence of cDC2s, and indeed other DCs^{33,34}. Thus, it is necessary to consider the broader immune landscape to most efficiently manipulate host immunity.

1.3 The human adaptive immune system

The second branch of the human immune system belongs to that of adaptive immunity. In contrast with the broad recognition specificity demonstrated by PRRs, receptors in the adaptive system recognise precise antigens. This is owed to each adaptive receptor being

assembled from variable and constant fragments through an imprecise, stochastic process termed V(D)J recombination, achieved via the activity of RAG1 and RAG2 endonucleases^{35,36}. This process yields an astonishingly vast number of possible receptor combinations – in the range of 10^{20} – and patients with defective *RAG1* or *RAG2* can exhibit a remarkable level of immunodeficiency³⁷.

There are two main cell types which express these adaptive receptors: B cells and T cells.

1.3.1 B cells

The initiation of B cell responses requires interactions between native antigens and a BCR, which usually takes place in secondary lymphoid organs such as lymph nodes, Peyer's patches or the spleen. The cells types that present antigens vary depending on the tissue, although specialised stromal cells called follicular DCs most commonly facilitate this process³⁸.

Upon encounter with antigen, BCR-antigen complexes activate intracellular signalling cascades that drive proliferation before becoming internalised and undergoing a series of processing steps which allow B cells to function as APCs by presenting processed antigen on MHC class II. Activated B cells then move to the interface of the B cell follicle and T cell zone where they interact with Tfh cells via CD40/CD40L and receive activatory signals via IL-21. In this scenario, B cells differentiate into germinal centre B cells and undergo class switching. B cells that do not receive these signals from Tfh cells instead become plasma cells that produce low-affinity antibody^{39,40}.

Within germinal centres, B cells undergo rapid proliferation and somatic hypermutation. During this process, activation-induced deaminases induce mutations into the variable regions of immunoglobulin genes to increase diversity. At the same time, B cells must compete for antigen and growth factors and thus increasingly specific clones survive in an evolutionary process termed affinity selection. Finally, germinal centre B cells either differentiate into memory plasma cells, memory B cells or repeat the process to generate B cell clones with even greater antigen specificity⁴¹.

1.3.2 Bridging the innate and adaptive immune systems

As mentioned previously, the most favourable immune responses in cancer often occur when the innate and adaptive immune systems function harmoniously. One example of a beneficial collaboration is the immunological phenomenon termed epitope-spreading. Here, tumour antigens are first taken by professional APCs which prime neo-epitope-specific Tfh cells. These cells then trigger the expansion and differentiation of tumour-reactive plasma cells which further facilitates APC activation via FcR-mediated uptake of immune complexes⁴². This intricate chain of events is considered desirable in cancer owing to its ability to broaden antigen reactivity and foster multimodal tumour eradication.

As emphasised with the innate immune system, it is also important to consider the landscape in which tumour-associated B cells function in, to better appreciate their role they play in anti-tumour immune responses. Quantifying the absolute number of B cells present within a given tumour has mixed associations, with prognosis depending on the tumour type⁴³. Contrastingly, B cells found within mature tertiary lymphoid structures are largely associated

with favourable prognoses across a range of tumour types, including CRC, melanoma and breast cancer⁴⁴.

1.4 T cells

Research into the mechanisms that underlie T cell-mediated immunity in cancer has received substantial interest in the past few decades and the recent success of several cancer immunotherapies has only fuelled this further. Techniques such as scRNA-seq have led to an unprecedented ability to dissect T cell functionality in ever more specific factions which are beginning to become exploited by contemporary strategies such as CAR T immunotherapy. Comprehensively understanding the factors which elicit the most beneficial T cell adaptations will be key for the continued development of T cell based immunotherapies in cancer.

1.4.1 T cell development

All aspirational T cells begin as precursors arising from haematopoietic stem cells residing in the bone marrow in adults or within the foetal liver. These thymic seeding progenitors (TSPs) migrate to the thymus where they undergo a series of maturation and differentiation processes that ultimately dictate their fate as they transition into functional $\gamma\delta$, $\alpha\beta$ CD4⁺ or $\alpha\beta$ CD8⁺ T cells.

Upon entering the thymus, TSPs receive a wave of Notch signals from cortical epithelial cells which drives the upregulation of TCF-1 (*TCF7*), GATA-3 and Bcl11b⁴⁵⁻⁴⁷. Together, these transcription factors initiate thymopoiesis by suppressing differentiation programmes associated with B cells, myeloid cells or innate immune cells, enforcing the commitment of TSPs towards the T cell lineage⁴⁸⁻⁵¹. Next, these committed TSPs, which are double negative

for CD4 and CD8 (DN), make their second major fate decision by rearranging TCR β , TCR γ and TCR δ chains to continue developing as either $\gamma\delta$ or $\alpha\beta$ T cells. Although the exact mechanisms that underlie this bifurcation in fate are not completely understood, it is thought that strong and sustained Notch signalling drive TSPs to preferentially rearrange TCR γ and TCR δ chains and become $\gamma\delta$ T cells^{52,53}.

TSPs which upregulate TCR β must pass a series of additional checkpoints before they may become fully developed $\alpha\beta$ CD4⁺ or CD8⁺ single positive (SP) T cells. The first checkpoint is termed β -selection, whereby the rearranged TCR β chain associates with an invariant pre-TCR α chain and CD3 to create a pre-TCR signalling complex. During β -selection, these pre-TCRs must successfully interact with peptide-MHC on thymic stromal cells before a T cell can progress and become a double positive (DP, CD4⁺ CD8⁺) T cell. Cells which pass this checkpoint must then undergo allelic exclusion to restrict their TCR β chain clonotypes to one per cell and replace the pre-TCR α chain with a rearranged TCR α chain to form a mature $\alpha\beta$ TCR complex. T cells which complete this second process and possess functional TCR complexes are protected from apoptosis and undergo rapid proliferation as they develop into DP thymocytes⁵⁴.

In the final stages of $\alpha\beta$ T cell development, DP thymocytes that recognise peptide-MHC class I or II with a moderate affinity on cortical thymic epithelial cells (cTECs) are first spared from apoptosis in a process termed positive selection. This process ensures that TCRs can recognise antigens in the context of MHC molecules and therefore are 'useful' clones⁵⁵. DP thymocytes which do not interact with peptide-MHC on cTECs die in a process termed 'death by neglect'. Positive selection also functions to determine whether DP thymocytes progress towards the CD4⁺ or CD8⁺ T cell lineage as those which recognise MHC class I develop into

CD8⁺ thymocytes via loss of CD4 and *vice versa*⁵⁶. The importance of this step was cleverly demonstrated using 'flip-flop' mice, which had *Cd4* genes engineered into the *Cd8* locus; DP thymocytes with MHC class II-restricted TCRs were then misdirected to the CD8⁺ T cell lineage (and *vice versa*) and failed to mount effective immune responses following infection⁵⁷.

Finally, CCR4 and CCR7 on newly-generated SP thymocytes direct them into the thymic medulla where they interact with self-antigen presented on medullary thymic epithelial cells (mTECs), controlled by AIRE^{58,59}. SP thymocytes that strongly interact to peptide-MHC on mTECs, or any of the other self-antigen-presenting APCs present in the medulla such as DCs, B cells or thymic mimetic cells (cells that mimic diverse extrathymic cell types), undergo clonal deletion or become Treg cells. This process, termed negative selection, is crucial for the removal of self-reactive T cells and prevention of autoimmunity⁶⁰. The 'useful' and self-tolerant T cells which successfully complete this developmental process finally follow S1P gradients that direct them into the circulatory system.

Rarer MAIT and unconventional NKT cells are also produced in the thymus though are generated via slightly different developmental pathways. MAIT cells express a semi-invariant $\alpha\beta$ TCR that recognise microbial-derived vitamin B metabolites presented on MR1 and are thought to become positively selected by MR1-expressing DP cortical thymocytes⁶¹⁻⁶³. NKT cells, on the other hand, recognise glycolipids presented on the β_2 M-associated MHC class I-like molecule CD1d and developmentally branch off at the DP thymocyte stage^{64,65}.

1.4.2 T cell activation

Once released from the thymus, naïve T cells scour peripheral tissues in search for cognate antigen presented on MHC molecules. It is the interaction between peptide-MHC complexes

with the TCR in the periphery that initiates the process of T cell activation and allows naïve T cells to adopt more specialised functions. However, antigen recognition alone is not sufficient for complete activation and T cells must simultaneously receive additional co-stimulatory signals and cytokines to adopt a complete effector profile⁶⁶.

Co-signalling in T cells can be broadly partitioned into co-stimulatory and co-inhibitory pathways. These signalling molecules localise with the TCR-MHC-peptide complex within the immunological synapse during interactions with APCs and function to either promote or inhibit T cell activation and functionality. Co-stimulatory interactions between APCs and T cells such as CD80 or CD86/CD28, ICOSL/ICOS, CD70/CD27 or CD40/CD40L, induce T cell proliferation and survival. Conversely, co-inhibitory interactions such as CD80 or CD86/CTLA-4, PD-L1/PD-1, or between ligands that bind to LAG-3 or TIM-3 on T cells (exact ligands remain unknown), inhibit cell cycle machinery, promote T cell exhaustion and can trigger apoptosis⁶⁷. Preventing these co-inhibitory interactions has proved to be a revolutionary strategy that can re-ignite host immunity in cancer⁶⁸.

These co-signalling events can be mediated by several different cell types, although co-signalling molecules have been best described on professional APCs, which have the ability to capture and cross-present exogenous antigens to T cells on MHC class II. The best known examples of which include DCs, macrophages and B cells⁶⁹. The presentation of foreign antigen via MHC class II has been more recently been described on basophils, mast cells, eosinophils and tumour cells which have been recently termed 'amateur APCs', although the exact role they play in priming anti-tumour immune responses is less well-characterised^{69,70}.

1.4.3 An early dualistic paradigm for T cell immunity

In combination with TCR signalling and co-stimulatory activation, cytokines act as the third signal that imparts nuanced characteristics onto recipient T cells. The notion that T cells could express heterogeneous phenotypes was first described in the 1980s, with seminal experiments from Timothy Mossman and Robert Coffman revealing that some T cells made IFN- γ , IL-2 and GM-CSF (termed Th1 cells) whereas some produced IL-4 and IL-5 (termed Th2 cells), depending on their environment and stimulatory context⁷¹. Later experiments in the 1990s went on to show that IL-12 was essential for the induction of Th1 properties in naïve CD4⁺ T cell precursors^{72,73}, whilst IL-4 was alternatively required for the induction of Th2 properties^{74,75}.

Cytokines, rather than the type of antigen, are the crucial determinants of T cell effector phenotypes as naïve CD4⁺ T cells with the same TCR co-cultured with APCs can become polarised either way depending on the presence of IFN- γ or IL-4^{76,77}. Furthermore, each lineage can adopt the other phenotype by swapping the stimulation conditions early after differentiation⁷⁸. It was later demonstrated that IFN- γ and IL-4 initiate distinct intracellular signalling pathways in naïve CD4⁺ T cells, with STAT4 and the transcription factor T-bet responsible for Th1 cell development^{79,80} and STAT6 with GATA-3 responsible for Th2 cell development⁸¹⁻⁸³.

Importantly, these two lineages were thought to embody stable phenotypes that represented a clear division of labour to eliminate specific classes of immunogens; Th1- derived cytokines mediated the immune destruction of intracellular pathogens by potentiating CD8⁺ T cell and

macrophage responses, whilst Th2 cytokines promoted the activation of mast cells, eosinophils and basophils to clear extracellular parasites⁸⁴.

1.4.4 An expanding family of T cells

A third CD4⁺ population which preserved immune tolerance, Treg cells, was discovered in the late 1980s. Controlled by the master transcription factor FoxP3⁸⁵⁻⁸⁷ and characterised by rich expression of the high-affinity IL-2 receptor α chain CD25⁸⁸, Treg cells actively restrained inflammation via an array of suppressive mechanisms including secretion of IL-10 and TGF- β 1 in conjunction with the expression of CTLA-4⁸⁹⁻⁹², production of immunosuppressive adenosine⁹³ as well as by directly killing other effector immune cells⁹⁴.

The functional importance of these regulatory cells was elegantly demonstrated in early studies utilising the T cell transfer model of colitis, whereby the transfer of Treg cells into SCID immunodeficient hosts abrogated the inflammation driven by co-transferred CD4⁺ CD45RB^{high} cells^{95,96}. It is now well-established that both thymic-derived natural Treg (nTreg) cells, which arise during thymic positive selection, and peripherally-induced Treg (iTreg) cells, which develop from naïve T cells in the presence of TGF- β 1, have important regulatory functions that extend beyond protecting against colitis. In mice, a systemic defect in Treg cells driven by deleterious mutations in Foxp3 results in lethal multi-organ autoimmunity⁹⁷. Human males with comparably defective mutations (FOXP3 resides on the X chromosome) develop IPEX syndrome, which comprises a similar multi-system autoimmune phenotype^{98,99}.

1.4.5 T cells can exhibit polyfunctionality

The discovery of IL-17⁺ Th17 (discussed in detail later) and IL-21⁺ Tfh cells in the early 2000s not only invalidated the dualistic principle of T cell immunity but also scrutinised the idea that each T cell lineage could be responsible for only one, distinct cytokine profile. Tfh cells (marked by CXCR5) performed a functionally unique role by helping with the rearrangement, class switching and affinity maturation of immunoglobulin in B cells, as well as aiding in the formation of follicular structures¹⁰⁰⁻¹⁰². However, they were found to mediate these effects via secretion of canonical T helper cytokines including IFN- γ ^{103,104}, IL-4¹⁰⁵⁻¹⁰⁷, IL-10¹⁰⁸ and IL-17¹⁰⁹. Furthermore, the Tfh master transcription factor Bcl-6 functions unlike other master regulators of T cell transcription, instead operating as a transcriptional repressor that binds to the promoters of T-bet, GATA-3 and ROR γ t (Th17 master transcription factor)^{110,111}. Whether Tfh cells truly represent a stable, terminal developmental fate remains contentious and some argue that the adoption of Tfh-like effector programmes are a consequence of compartmentalised adaptations given their elevated propensity to localise within B cell follicles¹¹².

Th17 cells can express a similarly multifunctional cytokine profile depending on the cytokine environment. In response to homeostatic IL-27 in the gut, Th17 cells can co-express IL-10 (under the control of c-Maf) along with their characteristic cytokine IL-17, and function like Treg cells to suppress effector T cell activity and restrain inflammation¹¹³. Alternatively, upon exposure to IL-1 β or IL-23, Th17 cells can upregulate T-bet alongside ROR γ t which allows them to secrete large amounts of IFN- γ like Th1 cells¹¹⁴⁻¹¹⁶.

More contemporary facets of the T helper lineage include Th9, Th22 and Th25 cells based on their expression of IL-9, IL-22 and IL-25, respectively¹¹⁷⁻¹²⁰. However, the polyfunctional profiles displayed by Tfh and Th17 cells suggests that such a nomenclature is too simplistic. Furthermore, it does not account for T cell plasticity which can occur in response to dynamic cytokine fluxes that are commonly encountered *in vivo*. Our understanding of EAE, the mouse model of multiple sclerosis, represents an exemplar which highlights the pitfall for such a nomenclature where, for so long, Th1 cells were thought to drive disease progression due to the causative role for IFN- γ ¹²¹. This assumption held true for many years until it became appreciated that Th17 cells that had evolved to co-express IFN- γ were the pivotal drivers of pathology¹²².

Modern technologies such as scRNA-seq have enabled researchers to simultaneously explore multiple transcription factors, cytokines and signalling receptors in individual cells. As a direct consequence of their utilisation, it has now become appreciated that most T cell effector programmes can exhibit marked complexity, especially within dynamic tissue environments such as the gut¹²³. Several new models have been proposed that aim to accommodate these ever expanding T cell profiles and generally recommend that future classifications should consider the broader effector programmes elicited, rather than focussing on single cytokines, and consider the cell types affected by each profile¹²⁴⁻¹²⁶.

1.5 Th17 cells

As mentioned previously, EAE was thought to be driven by Th1 cells. IFN- γ played a causative role in disease progression and the loss of T-bet was sufficient to prevent pathology¹²⁷. However, a series of findings in the early 2000s challenged this notion. Firstly, the loss of

STAT1, which lies upstream of T-bet, could not elicit a similar phenotype¹²⁷. The discovery of IL-23 as a novel cytokine added further doubt to the idea that Th1 cells were the causative pathogenic population, as loss of IL-23 signalling but not Th1-inducing IL-12 protected mice from disease^{128,129}. Adoptively transferring IL-17⁺ CD4⁺ T cells expanded with IL-23 was sufficient for the induction of EAE in susceptible mice and, in 2005, Th17 cells were proposed to represent an independent T cell subset¹²². In 2006, ROR γ t was identified as the master regulator of Th17 cells and has since been used alongside IL-17 and the surface marker CCR6 to study the role of Th17 cells in both homeostasis and disease^{130,131}.

1.5.1 The role of Th17 cells during homeostasis

During steady state conditions, Th17 cells are predominantly found at mucosal sites where they function to maintain barrier integrity and protect against microbial translocation (**Fig. 1.1**). These protective functions are largely due to the well-characterised activities of Th17-derived IL-17A, IL-17F and IL-22. Together, and often in synergy, these cytokines act on epithelial cells to strengthen the structural and physical barriers present within these niches, including supporting tight junction formation¹³², promoting the production of mucins¹³³ and stimulating the secretion of antimicrobial peptides¹³⁴⁻¹³⁶.

IL-17 and IL-22 also function to protect the health of epithelial cells themselves. IL-22 protects intestinal stem cells from genotoxic stress¹³⁷ and IL-17 is a key regulator of their proliferation and differentiation¹³⁸. The epithelial lining of the gut is one of the most rapidly self-renewing tissues in the body, turning over a new gut lining approximately every three days¹³⁹, and so a constant presence of IL-17 is vital for its homeostatic maintenance.

Tissue homeostasis is not a static event, however, and tissue resident cells must rapidly respond to infection or injury by evoking inflammatory responses in order to return to steady state. As such, IL-17 signals via non-haematopoietic cells to upregulate the production of potent neutrophil chemoattractants, such as G-CSF, CXCL12 and CXCL5¹⁴⁰⁻¹⁴², which act as first responders to inflammatory insults. In order to limit the tissue damage elicited by inflammatory processes, Th17-derived IL-17 also cooperates with FGF-2, secreted by Treg cells, to expedite epithelial repair programmes¹⁴³.

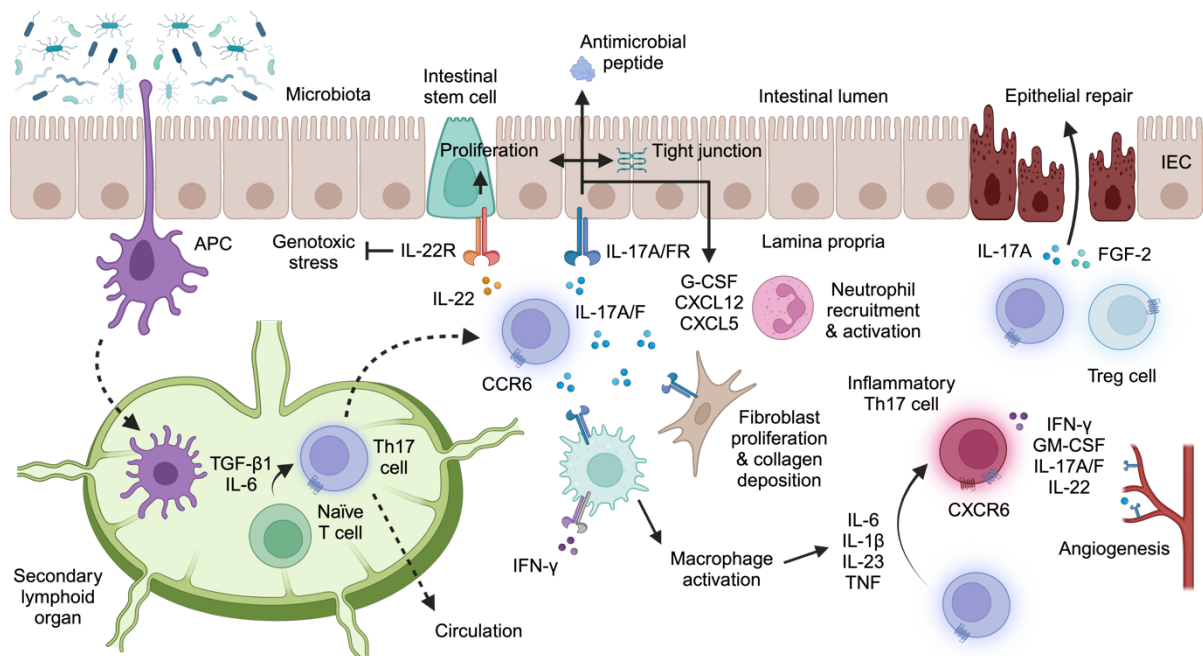


Figure 1.1 | Th17 cells during homeostasis and inflammation. Schematic depicting the role of Th17 cells during intestinal homeostasis, inflammation and upon resolution of inflammation. APCs migrate from tissue to prime naïve CD4⁺ T cells in secondary lymphoid organs, which differentiate into Th17 cells. Chemokine receptors such as CCR6 recruit Th17 cells back into the tissue where they secrete IL-17A, IL-17F and IL-22. Upon the induction of inflammation, cytokines such as IL-23 and IL-1β can induce the production of IFN-γ and GM-CSF in Th17 cells, alongside IL-17 and IL-22. GM-CSF and IL-17 can promote angiogenesis by stimulating vascular endothelial cells to produce VEGF^{144,145}. IEC = intestinal epithelial cell.

1.5.2 Induction and maintenance of Th17 cells

The earliest investigations into the key drivers of Th17 cell functionality focused on cytokine combinations which were able to induce ROR γ t expression and elicit IL-17 production in naïve CD4⁺ T cells. IL-6 and TGF- β 1 were early candidates based on these parameters^{130,146,147}. However, this model of polarisation was complicated by ensuing studies which showed that naïve CD4⁺ T cells polarised with other cytokines, such as IL-1 β alone or in combination with IL-6 and IL-23, could also induce ROR γ t and IL-17. In contrast to the cells induced by IL-6 and TGF- β 1, these Th17 cells expressed a far more inflammatory phenotype, characterised by the secretion of IFN- γ alongside IL-17^{114,115}. Furthermore, after adopting this population into MOG₃₅₋₅₅ immunised mice, these IFN- γ ⁺ Th17 cells could rapidly instigate EAE, unlike their IFN- γ ⁻ counterparts¹⁴⁸⁻¹⁵⁰. The development of more sophisticated techniques to explore how each of these Th17 profiles is linked *in vivo*, such as fate mapping using *Il17a*^{Cre} fate reporter mice has since revealed that IL-17⁺ Th17 cells constitute a progenitor pool for IFN- γ ⁺ IL-17⁺ Th17 cells during inflammation¹⁵¹. Furthermore, the integration of ATAC-seq with scRNA-seq has led to the prediction of several regulatory factors which regulate each of these Th17 phenotypes. *EST1*, *BACH2*, *FOSL2* and *RBPJ* are predicted to control IL-17⁺ Th17 cells whilst *ATF3*, *BHLHE40*, *FOS*, *NR4A1*, *EOMES* and *TBX21* (T-bet) are predicted to control IFN- γ ⁺ Th17 cells¹⁵².

More recently, a number of additional cytokines have been implicated in the development of Th17 cells which highlights their exquisite sensitivity to local environmental cues. Th17 cells appear to be particularly sensitive to members of the TGF- β superfamily of cytokines, with activin A and TGF- β 3 driving homeostatic and pathogenic phenotypes, respectively. IL-27 can drive IL-10 production and a homeostatic profile in Th17 cells, which may be derived from

resident Treg cells *in vivo*^{153,154}. Additionally, IL-21 appears to act as an 'intermediary' cytokine by preparing Th17 cells to respond to IL-23 signals via upregulation of IL-23R^{155,156}. This would agree with suggestions that IL-23 functions to maintain inflammatory characteristics in Th17 cells, rather than induce them directly¹⁵⁷.

More physiologically, hypoxia, high levels of sodium chloride, growth hormone-related hormone or serum amyloid A have all been reported to upregulate of inflammatory characteristics in Th17 cells whilst melatonin alternatively suppresses their development¹⁵⁸⁻¹⁶². The complexity and diversity of local niches within the human body would suggest that an assortment of Th17 cell phenotypes can be identified *in vivo*. Recent scRNA-seq profiling of Th17 cells during homeostasis confirmed that Th17 cells exhibit tissue-specific gene signatures¹⁶³, although how and indeed if this translational variation engenders functional variation remains unknown.

In vivo, the microbiota act as key upstream modulators of a variety of tissue cytokine environments and play a crucial role in the induction of Th17 cells via APCs. Seminal work revealed that the presentation of SFB-derived antigens on MHC class II by CD11c⁺ DCs in mice, within either the lamina propria or secondary lymphoid organs, was sufficient for the induction of Th17 cells with a phenotype analogous to those primed with IL-6 and TGF- β ^{130,164-168}. Contrastingly, *Candida albicans* - and *Citrobacter rodentium* -pulsed monocytes primed Th17 cells with rich IFN- γ expression and *Staphylococcus aureus* -specific Th17 cells produced IL-17 and IL-10 upon restimulation¹⁶⁸⁻¹⁷⁰.

1.6 Th17 cell heterogeneity in cancer

1.6.1 Microbial perturbations and Th17 cell phenotype

The potential implications that arise as a consequence of microbial and Th17 diversity are particularly relevant in cancer, in which dysbiosis constitutes an important hallmark of disease¹⁷¹. Furthermore, as the composition of such altered microbiota communities can vary so considerably between cancer patients¹⁷², it is pressing that the functional implications of tumour-associated Th17 heterogeneity are established.

It has been established that dysbiosis in cancer patients can impact the number of Th17 cells present within the tumour. This principle can be demonstrated by reconstitution studies, whereby germ-free mice orally gavaged with microbiota from donors with CRC have a higher Th17 count than those from healthy donors¹⁷³. Although there is no obvious consensus about whether certain microbes elicit superior or more deleterious immune protection than others, some bacterial strains enriched in the microbiota of CRC patients have been more associated with promoting the infiltration of Th17 cells, including *Peptostreptococcus anaerobius*, *Bacteroides fragilis*, *Fusobacterium nucleatum* and *Parvimonas micra*¹⁷⁴⁻¹⁷⁹. Whether these Th17 cells are recruited from the circulation or become differentiated from a naïve pool remains to be established, as is the effect of these bacteria on Th17 functionality.

The microbiota may also regulate the spatial enrichment of Th17 cells within the tumour microenvironment. In CRC, tumour progression and any resulting breakdown in barrier integrity likely facilitates the close proximity between host immunity and the microbiota, permitting microbiota to inhabit regions previously devoid of their presence¹⁸⁰. This tumour-associated translocation could generate pockets of tissue more conducive to Th17 cell

polarisation and increased levels of IL-6 and IL-23 have been described in colorectal tumours colonised with bacteria¹⁸¹.

1.6.2 Tumour microenvironment-induced heterogeneity

Numerous cell types, particularly myeloid and stromal cells, play a crucial role in shaping the profile of tumour Th17 cells¹⁸². The vast majority of cytokines mentioned previously which influence the recruitment, differentiation and expansion of Th17 cells have strong associations with macrophages, DCs and fibroblasts. IL-1 β secretion from tumour associated macrophages, for example, can strongly induce IL-17 production in naïve CD4⁺ T cells¹⁸³, whilst CCL20 from cancer-associated fibroblasts can recruit them into the tumour microenvironment via CCR6^{184,185}. Furthermore, IL-23 derived from macrophages and DCs within the tumour can fuel strong IFN- γ production in Th17 cells^{183,186-188}.

Not all tumour milieus function to strengthen Th17 profiles, however. High levels of TGF- β and PGE₂ reportedly synergise to skew Th17 cells towards a regulatory phenotype. These 'ex-Th17 Treg cells', which can maintain IL-17 expression alongside upregulation of FoxP3, facilitate tumour growth in a mouse model of ovarian cancer and similar populations of IL-17⁺ FoxP3⁺ cells have been identified in ovarian cancer patients¹⁸⁹. As highlighted by the CMS classification of CRC, tumours can exhibit remarkable heterogeneity and it is likely that Th17 cells similarly display different effector functionalities in different clusters of CRC patients. The extent of this inter-Th17 cell heterogeneity in CRC, or indeed other cancers, remains to be explored.

1.7 The role of Th17 cells in cancer immunity

1.7.1 Th17 cells can shape the tumour cell landscape

The relationship between Th17 cells and the tumour landscape is reciprocal and Th17 cells can also influence the cells present in these niches. IL-17 functions as a potent chemoattractant and can recruit several cell types into the tumour, including macrophages, myeloid-derived suppressor cells, eosinophils and neutrophils^{182,190-193}. Once recruited, these immune cells can elicit further changes to this landscape by promoting angiogenesis and driving the recruitment of additional immune cells¹⁹⁴. More directly, CCR6⁺ DCs, Treg cells, CD8⁺ T cells and other Th17 cells can all follow CCL20 chemotactic gradients generated by Th17 cells within the tumour^{193,195-197}.

Th17 cells can also shape the transcriptional programme of fibroblast by activating collagen deposition genes which can function to exclude T cells from the tumour^{198,199}. Mechanistically, this process may aid the formation of 'cold' tumours, whereby immune cells fail to infiltrate the tumour. Endothelial cells are another major responder to Th17 cell cytokines and IL-17 can stimulate the production of VEGF by vascular endothelial cells which can fuel tumour neovascularisation^{144,200,201}.

1.7.2 Th17 cells can directly promote cancer progression

Whilst the functional plasticity exhibited by Th17 cells undoubtedly impacts their role in anti-tumour immunity, it is likely that intrinsic tumour properties and contextual factors within the local milieu also influence their effect on disease progression. The proliferative and stem-inducing activities of IL-17 and IL-22 that are so beneficial during epithelial homeostasis also define two important hallmarks of cancer¹⁷¹ and tumour cells have adapted to exploit this

homeostatic signalling. IL-17R is overexpressed in pancreatic epithelial cells after oncogenic activation of *Kras*, for example, which renders newly neoplastic cell hypersensitive to IL-17-driven proliferation²⁰². A similar phenomenon is observed in colonic epithelial cells which have lost normal function of *Apc* and homeostatic levels of IL-17 can instead drive pathogenic, uncontrolled cell-cycle progression whilst protecting cells from apoptosis²⁰³.

In conjunction with mutant *KRAS*, IL-22 can similarly drive hyperproliferation in colonic epithelial cells via aberrant activation of the STAT3 signalling pathway²⁰⁴⁻²⁰⁷. Importantly though, IL-22 also drives stemness properties in epithelial cells, upregulating genes including *NANOG*, *SOX2* and *POU5F1*²⁰⁸. Cancer stem-like cells are less differentiated than normal malignant cells and can rapidly give rise to new tumour cells, rendering their presence highly advantageous for tumour progression²⁰⁹. Th17 cells may also play a more direct role in tumour extravasation as both IL-17 and IL-22 have been implicated in the initiation of epithelial-mesenchymal-transition. Both cytokines can repress both the epithelial marker E-cadherin and the mesenchymal marker Vimentin whilst activating the key EMT-associated transcription factors Snail, Slug and ZEB1^{207,210}.

Inflammatory processes are known to exacerbate the acquisition of these somatic mutations and it is now widely appreciated that several environmental factors, infection and chronic inflammation and autoimmune conditions can predispose tissue to oncogenic mutations; the most notable being smoking, *Helicobacter pylori*-induced gastritis and IBD, respectively²¹¹. Subclinical systemic inflammation, such as that seen in obesity, can also promote or increase the risk of several cancers²¹². Whilst Th17 cells have been implicated in the development and sustenance of all the aforementioned pathologies²¹³⁻²¹⁸, the acquisition of such tissue

mutations as a consequence of Th17 presence is unlikely. Rather, the support that Th17 cells provide in maintaining chronic inflammation is plausibly a more important mechanism²¹⁹.

1.7.3 Th17 cells can directly protect against cancer

Not all interactions between Th17 cells and the tumour are deleterious, however. Producing IFN- γ appears to be a particularly beneficial adaption in tumour Th17 cells and adoptively transferring tumour antigen-specific IFN- γ ⁺ Th17 cells can efficiently eradicate established melanoma in mice^{187,220}. Properties specific to Th17 cells were important facilitators of this anti-tumour ability as adoptively transferring tumour antigen-specific Th1 cells could not confer the same level of protection¹⁸⁷.

One hypothesis is that Th17 cells may display enhanced persistence within the tumour microenvironment. Like tumour cells, it appears that the expression of stem-associated genes, such as *TCF7* and *BCL2*, might provide a survival advantage for tumour Th17 cells, making them refractory to senescence and permitting long-term immunity^{188,221}. Whether the Th17 cells associated with worsened prognosis express comparable levels of these stem-associated genes and which environmental drivers encourage this phenotype remains unclear. IL-6 may somewhat play a role²²², however comprehensively understanding the complete cytokine milieu which fuels this profile, as well as defining persistence-associated surface markers and transcription factors, will be important to determine.

The tissue tropism of Th17 cells for mucosal sites might also rationalise these observations and it would be interesting to explore whether IFN- γ ⁺ Th17 cells offer similarly heightened protection compared with Th1 cells against tumours of non-mucosal origin. It also remains unclear whether a reservoir of stem-like Th17 cells exists in which IFN- γ ⁺ Th17 cells are

differentiated from. Such a regenerative pool has been recently identified in mice^{113,163,223}, however further work is required to characterise a comparable population in humans. Finally, whether the production of IFN- γ is the only direct mean by which Th17 cells can elicit anti-tumour protection (other than cell recruitment) remains to be determined. Studies in multiple sclerosis have shown that Th17 cells can directly kill human neurons and oligodendrocytes^{224,225} via granzyme-mediated cytotoxicity and CD26^{high} Th17 cells, which mediate efficient protection against mesothelioma, pancreatic cancer and melanoma in mice, also express granzyme B²²⁶. However, the exact modality of anti-tumour function in this CD26^{high} population was undetermined and it remains unclear if Th17 cells can directly kill tumour cells.

1.8 T cell memory

1.8.1 The family of memory T cells

After receiving the three signals from APCs required for full activation and polarisation, naïve CD4⁺ T cells undergo a proliferative burst before they begin to extensively produce their respective effector molecules. This process is highly energy intensive and the majority of these activated T cells will only mediate short-lived immune protection before undergoing apoptosis. However, a small portion of these antigen-experienced T cells will form long-lived memory cells that can persist long beyond the clearance of pathogen. Such memory populations elicit protective, anamnestic immunity by rapidly reconstituting antigen-specific T cell pools upon re-encounter with the same antigen. Additionally, memory T cells can produce effector cytokines without the same dependence on co-stimulatory signals that naïve T cells have²²⁷⁻²²⁹.

Technologies such as flow cytometry and scRNA-seq have enabled the dissection of T cell memory based on defined surface markers and by varying degrees of multipotency. This diversity is advantageous and allows for variable differentiation pathways, localisation, recall ability and effector functionality to maximise the surveillance and protection offered by memory cells.

The least differentiated memory population, termed stem-like memory T cells (TSCM), was initially identified after observations that a population of T cells with a naïve-like phenotype (CD45RA⁺ CCR7⁺ CD27⁺) could sustain GVHD upon serial transplantation into allogeneic hosts. Furthermore, this novel population could reconstitute a complete effector pool and other memory subsets whilst maintaining its own pool through self-renewal^{230,231}. The characterisation of CD95 (Fas receptor), IL-2R β , CXCR3 and SCA-1 as surface markers which could be used to differentiate TSCM cells from naïve T cells^{232,233} permitted the identification of an analogous population in the circulatory and lymphatic systems of humans only a few years later^{234,235}. *Ex vivo*, the IL-2 family members IL-7 and IL-15 are necessary for the differentiation of TSCM from naïve precursors as well as for their homeostatic turnover²³¹.

Acting as a bridge between TSCM cells and more functional memory profiles are central memory T (TCM) cells. TCM cells can be defined by the expression of lymph node homing markers such as CCR7 and CD62L (SELL, L-selectin), as well as expression of CD45RO²²⁷ - the CD45 isoform associated with T cell activation²³⁶. Effector memory T (TEM) cells, on the other hand, have a weaker capacity for self-renewal, lack the expression of CD62L and CCR7 but have a greater propensity for induction of effector functions upon restimulation. Furthermore, TEM cells upregulate the marker CD69 which serves to promote peripheral tissue retention by binding to and sequestering S1PR1 which mediates T cell egress²³⁷⁻²⁴¹.

Resident memory T (TRM) cells are a recent addition to the family of memory phenotypes and can be identified by the co-expression of CD69 with integrin α E (CD103), which binds to E-cadherin on epithelial cells. As such, TRM cells have been identified in a number of mucosal sites, including the skin, lungs, gut and vaginal mucosae²⁴². Early parabiosis experiments hinted that TRM cells are restricted to these peripheral tissues²⁴³, although more recent studies have suggested that TRM cells can migrate to regional lymph nodes upon induction of anamnestic immunity²⁴⁴. In CD8⁺ TRM cells, TGF- β 1 acts as the master regulator of their differentiation and key driver of CD103 expression, with IL-15 sustaining their presence in non-lymphoid tissues²⁴⁵. In CD4⁺ TRM cells, which are relatively understudied, IL-15 and IL-7 are thought to be implicated in their development^{246,247}. The role of TGF- β 1 is more controversial in CD4⁺ TRM cells as few studies have reported the expression of CD103 in this population^{246,248,249}.

Finally, at the most differentiated end of the spectrum lies terminally differentiated effector T (TEMRA) cells which usually have the most cytotoxic profile²⁵⁰. However, these functionalities co-exist alongside exhaustion, which broadly describes a state of late stage differentiation where T cells become hypofunctional and upregulate a number of inhibitory receptors, such as PD-1, CTLA-4 and LAG-3²⁵¹.

1.8.2 Memory T cells in tumour immunity

Recent work has identified memory T cells as key determinants of cancer immunity through their role in replenishing effector populations²⁵²⁻²⁵⁴. Memory populations that express tumour-specific TCRs have also been found to persist long after tumour eradication which

suggests that cancer survivors can maintain durable memory that protect against future recurrence²⁵⁵.

Despite their primary function as an effector reservoir, memory T cells can be active cytokine secretors and it is thought that this functionality acts as a second mechanism by which memory T cells can suppress tumour development. TRM cells appear to be a key memory population that acts in this manner and a number of studies have reported rich expression of granzymes, perforin, IFN- γ and TNF in TRM cells²⁵⁶⁻²⁵⁸. Granzymes and perforin directly lyse and kill tumour cells whilst IFN- γ and TNF have been shown to promote a phenomenon known as tumour dormancy, whereby tumour cells become quiescent and tumour growth is arrested^{259,260}.

The production of these cytokines can also trigger DC maturation and epitope spreading which recruits and activates additional effector cells into the tumour microenvironment²⁶¹. Furthermore, the expression of several immune checkpoints on memory T cells renders them highly sensitive to immune checkpoint blockade and numerous studies have correlated heightened tumour infiltration of memory T cells before the administration of therapy with improved patient response²⁶².

One factor that complicates the study of memory T cells in cancer, as opposed to studying those which develop secondary to most infections, is the presence of persistent antigen within the tumour microenvironment. Whilst *bona fide* memory T cells adopt a relatively quiescent state, the chronic stimulatory conditions that tumour-reactive memory T cells exist within can quickly drive them along an exhausted trajectory. How, and indeed if, memory T cells can persist in these environments whilst retaining effective functionality remains to be

established. It is possible that memory T cells may rely on migratory circuits into tumour lymphoid tissue to avoid chronic stimulation, as TRM cells have been tracked moving into local draining lymph nodes after stimulation with antigen and transient rest is known to restore their T cell functionality^{244,263}.

Additionally, whether the type of effector programme that a given memory T cell adopts can impact longevity requires further study. The elevated expression levels of *TCF7* and *BCL2* in Th17 cells makes them more refractory to senescence than other canonical T helper programmes and this stemness is attributed with their superior tumour clearance over Th1 cells due to augmented persistence within the tumour microenvironment^{188,264}. A recent report of protective Tc17-like CD8⁺ TRM cells in melanoma could suggest that the acquisition of Th17-like features by memory T cells may be a mechanism by which exhaustion is avoided and persistence is achieved in TRM cells²⁶⁵ and whether analogous populations of Th17-like CD4⁺ TRM cells exist in cancer is unclear.

1.8.3 Thesis aims

The range of adaptations that Th17 cells can acquire within the tumour microenvironment remains unclear. Furthermore, how Th17 heterogeneity might impact anti-tumour immune responses is also poorly understood. Therefore, this thesis seeks to achieve the following:

1. Establish whether Th17 cell heterogeneity exists in CRC.
2. Determine whether distinct Th17 profiles associate with certain tumour landscapes.
3. Explore the functional role for each Th17 profile and determine how their presence within the tumour impacts patient prognosis.

Chapter 2: Exploring Th17 cell heterogeneity in human colorectal cancer

2.1 Introduction

The role of Th17 cells in cancer remains contentious. Some reports suggest that the chemokines secreted by Th17 cells can recruit populations more typically associated with tumour eradication, such as CD8⁺ T cells and NK cells^{193,266,267}, and some suggest that Th17 cells can directly suppress tumour growth^{187,188,222,264}. However, accumulating evidence implies that some Th17 populations may serve a more deleterious role. This multitude of conflicting reports might be a product of undocumented heterogeneity within populations of intratumoural Th17 cells. As tumours progress, extraordinary levels of heterogeneity can arise, both between and within tumour types. This heterogeneity is often accompanied with vastly different cytokine, chemokine and metabolite milieus, the key manipulators of local immune cell phenotypes including Th17 cells which strongly suggests that Th17 cells can differentially adapt as tumours evolve over time.

Despite the many potential avenues in which Th17 cells might become polarised within the tumour, a considerable number of studies still simply correlate the number of IL-17 expressors or overall levels of IL-17 with patient outcome^{268,269}, disregarding the broader tumour landscape or phenotypic adaptations these Th17 cells may exhibit. The studies which do explore Th17 functionality often choose to focus on cells with phenotypes analogous to those described in EAE. This biased approach has proved somewhat fruitful, revealing a protective role for IFN- γ ⁺ Th17 cells in melanoma, which can eradicate established tumours more efficiently than Th1 cells despite secreting comparable levels of IFN- γ ¹⁸⁷. These

observations have also laid the foundation for more comprehensive profiling investigations of these intratumoural IFN- γ ⁺ Th17 cells, revealing the expression of genes associated with stemness and self-renewal that aid with long-term Th17 tumour protection^{188,222,264,270}.

However, this bias to seek out and investigate IFN- γ ⁺ Th17 cells has also stifled the exploration of prospective, novel effector programmes which Th17 cells may adopt in the many complex milieus and niches found in tumours. Some groups have attempted to utilise reports of novel surface markers found on Th17 cells in non-tumour settings, such as CD26²⁷¹, and translate these findings to explore whether these same markers can demarcate intratumoural Th17 cells with distinct effector profiles. Using this process, researchers have found that Th17 cells expressing high levels of CD26 also express higher levels of granzyme B, IFN- γ , TNF- α and IL-17A in mouse mesothelioma than those with intermediate levels of CD26²²⁶. Although this is one example of successful exploration, this strategy is limited by the publishing rate of contemporary Th17 markers and the laborious process of investigating Th17 populations one marker at a time.

There is therefore a necessity to broadly explore the existence of novel Th17 populations in cancer. Understanding the signatures which demarcate specific functions or niches could be used to stratify patients based on a histological cancer pathotype and it might also allow for the selective use of treatments to optimise their efficacy.

2.2 Aims

Here we focus on CRC due to the abundance of Th17 cells in the gut and the important role they play during homeostasis. In this first experimental chapter, we aim to utilise scRNA-seq and establish a novel workflow to explore Th17 heterogeneity in an unbiased manner. Using this strategy, we aim to define undescribed Th17 adaptations and seek to establish how each population temporally relates to one another.

2.3 Results

2.3.1 Utilising scRNA-seq to identify Th17 cells in human CRC

Most studies that utilise mouse models of cancer rely on techniques such as fate mapping or *Il17a* reporters to track Th17 cells *in vivo* and these technologies have underpinned huge advancements in our understanding of Th17 biology. However, to investigate Th17 cells in human cancer, such tools cannot be utilised. To overcome these limitations, we opted to explore Th17 heterogeneity using data collected from a recently published, publicly available scRNA-seq dataset (GSE108989)¹. In this dataset, single cells from venous blood, resected healthy adjacent and tumour tissue of 12 treatment-naïve CRC patients were antibody stained before CD4⁺ and CD8⁺ T cells were collected by FACS. Libraries were constructed, amplified and purified before each cell and its TCR was sequenced using Smart-seq2 scRNA-seq technology (**Fig. 2.1**), a highly sensitive and accurate method of profiling²⁷². In total, transcriptomic data from 11,138 T cells and 7,274 TCR sequences were acquired.

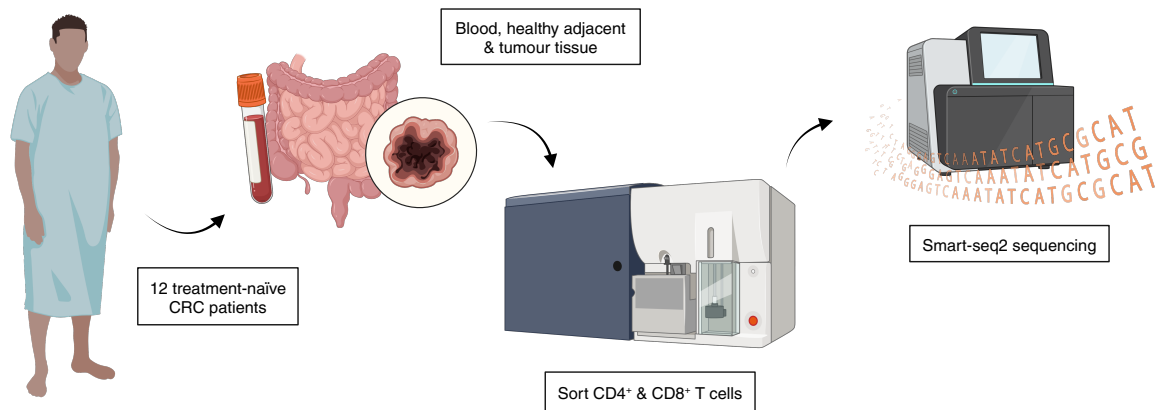


Figure 2.1 | Generating a T cell enriched scRNA-seq dataset from CRC patients. A schematic of the methodology used to generate the T cell enriched, publicly available scRNA-seq dataset (adapted from Zhang *et al.*¹) which we utilised to transcriptomically explore Th17 cell heterogeneity in CRC. Venous blood, healthy normal adjacent and freshly resected tumour tissue was obtained from 12 treatment naïve CRC patients. CD4⁺ and CD8⁺ T cells were sorted using FACS and sequenced along with their TCR using Smart-seq2 technology.

K nearest neighbours were then calculated using the *FindNeighbours* function in the R package Seurat before each cell was plotted on a UMAP plot for general visualisation purposes (**Fig. 2.2A**). T cells broadly grouped together based on sample location with distinct regions of the UMAP exclusively composed of cells from the blood and tumour. T cells derived from healthy normal adjacent tissue appeared more dispersed.

To explore putative Th17-like populations within this dataset, the expression of *RORC* (ROR γ t) was examined in each T cell. We chose to explore *RORC* expression, rather than a more classical marker of Th17 function such as *IL17A* (IL-17A), due to appreciable functional heterogeneity described in the Th17 literature. In Th17 cells exposed to high levels of IL-23 and IL-1 β , for example, IFN- γ secretion is upregulated alongside a simultaneous reduction in IL-17A secretion. Yet, despite suppressed levels of IL-17A in these cells, ROR γ t remains an

essential transcription factor that functions to both stabilise and propagate this phenotype²⁷³. In GSE108989, *RORC* was identified in cells originating from all three tissue types (**Fig. 2.2B**, **Fig. 2.2C**). Overall, *RORC*⁺ cells made up 5.9% (199/3,350) of the total blood T cells, 6.3% (165/2,637) of the total T cells in normal healthy tissue and 8.23% (424/5,149) of the total T cells in tumour tissue.

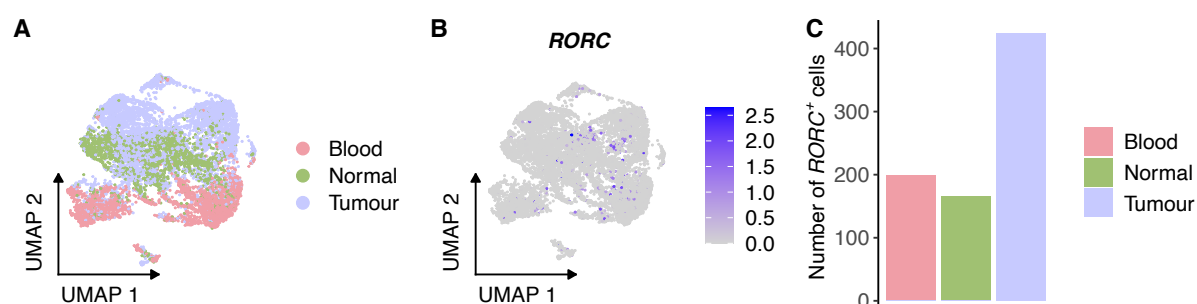


Figure 2.2 | *RORC* transcripts are detected in T cells located within the blood, healthy normal adjacent and tumour tissue of CRC patients. (A) UMAP of T cells sequenced in GSE108989, coloured by origin tissue type. (B) UMAP of *RORC* transcript detection, where colour represents expression level. Grey represents low frequency of transcripts, whilst blue represents a high frequency of transcripts. (C) Bar plot representing the number of *RORC*⁺ cells per tissue sample.

To characterise these putative Th17-like cells with increased granularity, positive *RORC* expressors from GSE108989 were subset out and new neighbours were identified using k means clustering and a shared nearest neighbours based clustering algorithm. This process generated seven transcriptionally distinct clusters (**Fig. 2.3A**) and the most differentially expressed genes were determined using Wilcoxon rank sum test with Bonferroni correction (**Fig. 2.3B**) and used to help annotate each cluster.

Clusters 0, 1 and 2 expressed the highest overall level of *RORC* and expressed high levels of *CCR6*, a chemokine receptor that recognises CCL20 and is often used as a putative surface marker for Th17 cells¹³¹. All seven clusters also expressed *RORA* (*RORα*), a transcription factor which binds to and stabilises *RORγt* to sustain the expression of type 17 effector functions²⁷⁴, suggesting that the transcriptomic expression of *RORC* also indicates functional transcription factor activity.

Focussing on the more individual signatures belonging to each cluster, cluster 0 appeared to represent a highly activated community. Cells in cluster 0 strongly expressed *CD69*, a marker of T cell activation and tissue residency^{237,275}, alongside the transcription factors *NR4A2* and *JUNB*, both of which are reported to augment typically pro-inflammatory characteristics in Th17 cells, including IL-23R expression with IFN- γ and GM-CSF secretion²⁷⁶⁻²⁷⁹.

Cluster 1 exhibited more stem-like properties and was enriched with a number of receptors involved in T cell tissue egress and lymph node homing, including *S1PR1*, *SELL* (CD62L) and *CCR7*, as well as transcription factors known to antagonise the acquisition of T cell effector properties (*LEF1*, *TCF7* (TCF-1) and *KLF2*)²⁸⁰.

The transcriptomic signature of cells in cluster 2 overlapped somewhat with those from cluster 0, with both communities expressing *CCL20*. More distinctly though, cluster 2 cells expressed the activation marker *CD38* and the cytokine *IL26*, which is reportedly produced by a population of circulating *CCR6*⁺ *CCR4*⁺ memory T cells that do not express IL-17A but rapidly upregulate IL-17A upon exposure to TGF- β ¹²⁸¹. Conflictingly, *IL17A* transcripts were also detected in cluster 2 cells (**Fig. 2.3C**), suggesting that they may not completely overlap with this recently described Th17 precursor population.

The most differentially expressed genes in cells belonging to clusters 3-6 aligned towards non-Th17 populations and were annotated accordingly, with signatures associated with CD8⁺ or NKT cells (*CD8A*, *NKG7*), Tfh cells (*CXCR5*, *PDCD1* (PD-1), *BCL6*), Treg (*FOXP3*, *CCR8*) and cycling T cells (*MKI67*(Ki67)), respectively.

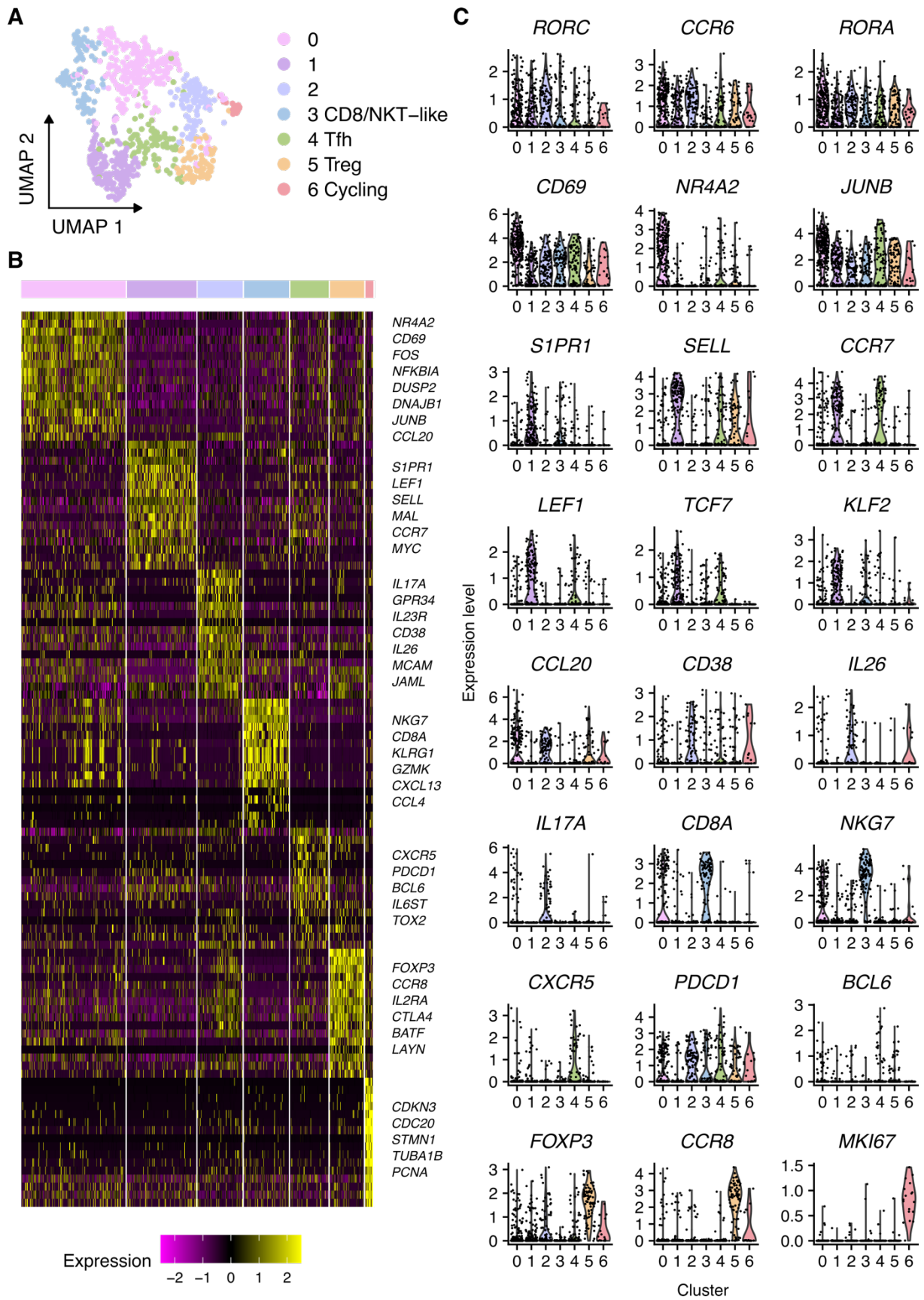


Figure 2.3 | Seven distinct clusters of *RORC*⁺ *CD3D*⁺ T cells can be identified in scRNA-seq data from GSE10989. Cells simultaneously expressing *RORC* and *CD3D* transcripts were subset and grouped by first calculating k nearest neighbours then by identifying clusters using a shared nearest neighbours based clustering algorithm from the Seurat package in R. **(A)** Annotated UMAP of *RORC*⁺ *CD3D*⁺ clusters. **(B)** To inform the naming of each cluster annotation, differential gene expression analysis was performed to determine the most enriched genes within each cluster. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed genes, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. **(C)** Violin plots of a selection of genes used to inform cluster annotations.

Given the ubiquitously high expression levels of *RORC* in cluster 0, 1 and 2, as well as the lack of distinctive signature annotation found in the literature, the cells belonging to these clusters were subset out and new neighbours were identified. Clusters were then formed and visualised on a UMAP plot (**Fig. 2.4A**), with differential gene expression analysis used to inform the labelling of each cluster (**Fig. 2.4B**).

This time, four transcriptomically distinct communities were observed. The newly formed clusters 0, 1 and 2 transcriptomically aligned with the previously described clusters 0, 1 and 2 (**Fig. 2.4C**) and were renamed Th17-like_0, Th17-like_1 and Th17-like_2, respectively (**Fig. 2.4A**).

However, despite the previous elimination of a CD8⁺ T cell cluster, a new group of what appeared to be highly cytotoxic *SLAMF7*⁺ CD8⁺ T cells was formed²⁸², indicating that contaminant cells had filtered through the Th17-like subsetting. We postulated that this CD8⁺ T cell cluster might represent a group of cytotoxic Tc17 cells, however they did not express *IL17A* nor *IL23R*, two key transcripts associated with a Tc17 phenotype²⁸³ (**Fig. 2.4C**).

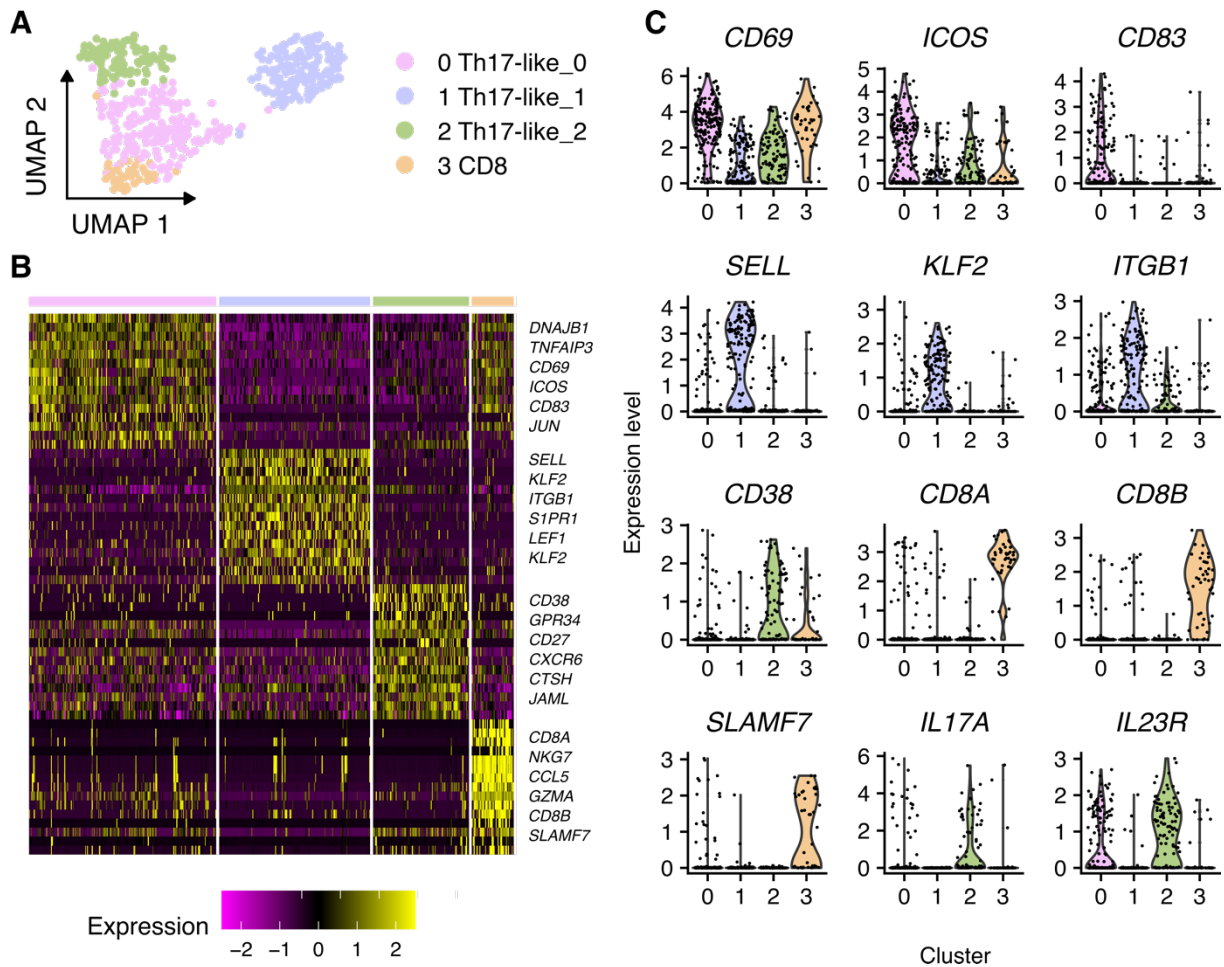


Figure 2.4 | A contaminant CD8⁺ T cell cluster is identified after re-clustering Th17-like groups. Clusters 0, 1 and 2 from the previous clustering of *RORC*⁺ *CD3D*⁺ T cells were re-clustered to identify any contaminant cell populations. **(A)** Annotated output UMAP after re-clustering. **(B)** To inform the naming of each cluster annotation, differential gene expression analysis was performed to determine the most enriched genes within each cluster. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed genes, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. **(C)** Violin plots of a selection of genes used to inform cluster annotations.

To further purify the Th17-like clusters, each Th17-like population was subset out, with new neighbours calculated and the updated clusters visualised on a UMAP plot (**Fig. 2.5A**). To validate the complete removal of CD8⁺ T cell contaminants, *CD8A* and *CD8B* expression levels

were superimposed onto separate UMAP plots (**Fig. 2.5B**). Rather surprisingly, very small communities of CD8⁺ T cells that were not defined as individual clusters clearly remained.

In response to these findings, we gradually raised the clustering resolution used, a process which permits the identification of more communities within a given dataset. At the resolution level that captured each CD8⁺ T cell group as a distinct cluster, a plethora of other clusters had also been formed (**Fig. 2.3C**). Differential gene expression analysis revealed that a number of these, previously annotated as Th17-like, were enriched with the Treg master transcription factor *FOXP3* (**Fig. 2.5D**). Curiously, some of these *FOXP3*⁺ cells also expressed *IL17A*, especially those in cluster 7 which appeared to demarcate highly activated, pro-tumourigenic Treg cells expressing the IL-1 decoy receptor *IL1R2*²⁸⁴⁻²⁸⁶ (**Fig. 2.5E, 2.5F**).

Our findings that some T cells co-express ROR γ t with FoxP3 does not describe a novel population. In the intestinal and colonic lamina propria of both mice and humans, microbiota induced Treg cells frequently co-express FoxP3 with ROR γ t and these double positive Tregs play a crucial role in restraining local inflammatory processes²⁸⁷⁻²⁹⁰. The ontology of our double positive T cells, however, was harder to determine. ROR γ t⁺ Th17 cells can upregulate FoxP3 and acquire immunosuppressive qualities in the presence of TGF- β 1^{189,291,292} and, conversely, FoxP3⁺ Treg cells can upregulate ROR γ t and IL-17A in the presence of the inflammatory cytokines IL-6 and IL-1 β ²⁹³⁻²⁹⁵. Whether our *RORC*⁺ *FOXP3*⁺ T cells were Treg cells with Th17-like properties or Th17 cells with Treg like properties was difficult to establish.

Although we had little doubt that these *RORC*⁺ *FOXP3*⁺ *IL1R2*⁺ cells, and indeed other double positive T cells would provide interesting insights into the general diversity of T cell profiles within CRC, we rationalised that the strict expulsion of all potential contaminant Treg (and

CD8⁺ T) cells would allow us to focus on populations which we could confidently describe as Th17 cells in our downstream analysis. Therefore, all cells with a *CD8A* expression level of > 0.1 (**Fig. 2.5G**) or *FOXP3* expression level of > 0.01 were excluded from our analysis (**Fig. 2.5H**). The resulting Treg/CD8⁺ T cell deplete clusters were renamed Th17_A, Th17_B and Th17_C (**Fig. 2.5I**) and to validate that all three Th17 populations did not express *CD8A* or *FOXP3* transcripts, the expression of each respective gene was superimposed onto a UMAP plot (**Fig. 2.5J**).

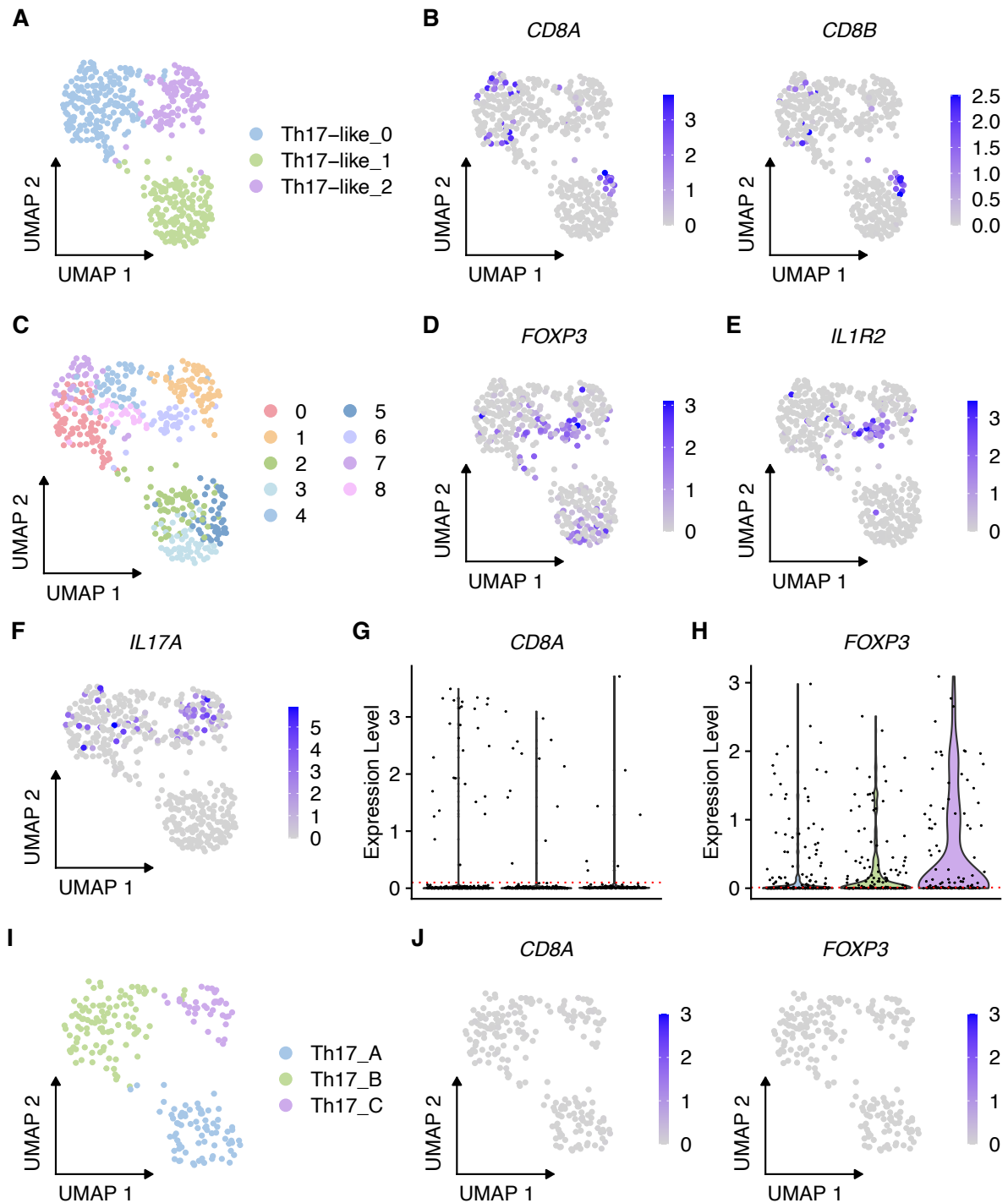


Figure 2.5 | Removal of persistent CD8⁺ T cell and Treg contaminants. Despite previous removal of a contaminant CD8⁺ T cell population, clustering the remaining Th17-like populations revealed persistent contamination with CD8⁺ T cells and a number of *FOXP3*⁺ cells. **(A)** UMAP plot after removal of the contaminating CD8⁺ T cell cluster. **(B)** Expression of *CD8A* and *CD8B* transcripts superimposed onto a UMAP plot. **(C)** To investigate any further

contamination, the clustering resolution was increased to identify a larger number of communities. Output UMAP is displayed. Expression of (D) *FOXP3*, (E) *IL1R2* and (F) *IL17A* transcripts superimposed onto the UMAP plot. Violin plots showing the expression level of (G) *CD8A* and (H) *FOXP3*, split by Th17-like cluster. The red dashed line on (G) and (H) marks the expression level threshold for exclusion, where cells above the line were removed. (I) Output UMAP after expression level exclusion. (J) The expression levels of *CD8A* and *FOXP3* superimposed onto the UMAP in (I) to confirm the complete elimination of *CD8A* and *FOXP3* expressing cells.

2.3.2 Transcriptionally distinct Th17 cell populations exist in human colorectal cancer

To comprehensively establish the gene signatures that discriminate between each Th17 cluster, differential gene expression analysis was performed, comparing each Th17 population with one another.

In both pairwise comparisons (**Fig. 2.6A**, **Fig. 2.6B**), Th17_A cells displayed a gene signature which strongly aligned with that of a stem-like memory or central memory T cell; devoid of functional effector molecules and rich in markers associated with secondary lymphoid organ homing (*SELL* (CD62L), *CCR7*) and T cell persistence (*LEF1*, *IL7R* (IL-7R α), *TCF7* (TCF-1)). *CD29* (integrin $\beta 1$) was also enriched, which demarcates highly cytotoxic CD4⁺ and CD8⁺ T cells^{296,297}, although such effector functions in Th17_A cells were not evident. A large number of genes encoding ribosomal proteins (*RPL3*, *RPL4*, *RPL5*, *RPL13*, *RPS6*) were also notably enriched in Th17_A cells, the accumulation of which might mechanistically facilitate the repaid engagement of translational machinery in response to an activating stimulus^{298,299}. Enrichment of *TXNIP* (thioredoxin-interacting protein) would also lend to the notion that Th17_A cells represent a functionally quiescent Th17 population, as Txnip is concomitantly

upregulated during the contraction phase of Th2 immunity and generation of long-lived memory Th2 cells in mice³⁰⁰. Mechanistically, TXNIP can also regulate T cell metabolism by simultaneously promoting oxidative phosphorylation and suppressing the uptake of glucose, a key energy source required for the production of inflammatory cytokines in Th17 cells^{168,301,302}.

Whether the signature of Th17_A cells aligned more towards a stem-like memory phenotype, characterised by antigen inexperience, or a central memory phenotype, characterised by quiescence and longevity following activation, was difficult to determine given the considerable overlap between the two profiles³⁰³. As the expression level of *RORC* and *CCR6* in Th17_A cells was comparable to that observed in Th17_B and Th17_C cells, we reasoned that Th17_A cells were more likely to represent a polarised central memory state given the activatory requirements that are required to adopt a type 17 phenotype³⁰⁴⁻³⁰⁶. We later characterise Th17_A cells as CD45RO⁺ by flow cytometry, validating this assumption (**Section 2.3.5**).

Comparatively, Th17_B cells were enriched with a substantial number of AP-1 transcription factor motifs (*JUN*, *JUNB*, *JUND*, *FOS*), members of the NR4A orphan nuclear receptor superfamily (*NR4A1*, *NR4A2*) and STAT3-modulating phosphatases (*DUSP1*, *DUSP2*), the majority of which have been associated with the induction of a range of cytokines in Th17 cells, including IL-17A, IL-21, GM-CSF and IFN- γ ^{276-279,307,308}. *SOC3*, a negative feedback regulator which lies downstream of IL-6 and IL-21 induced STAT3 signalling³⁰⁹, was also heavily upregulated, and in combination suggest that Th17_B cells are located within a niche rich in Th17 polarising cytokines. Fewer differential cell surface markers were detected in Th17_B cells with *CD83*, *CXCR4*, *CD69* and *ICOS* making up the most notable candidates.

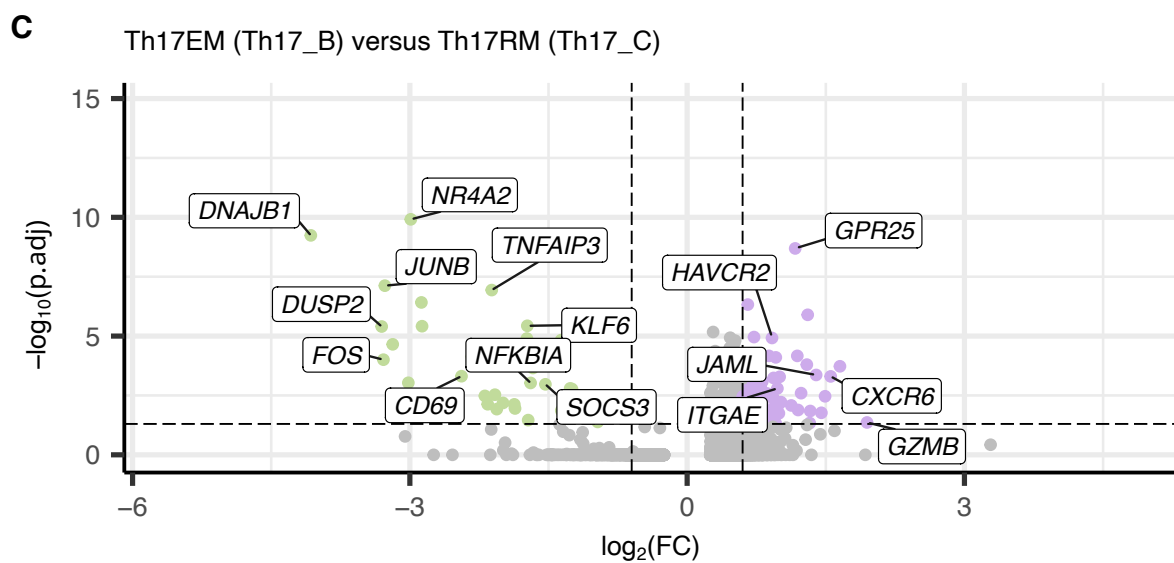
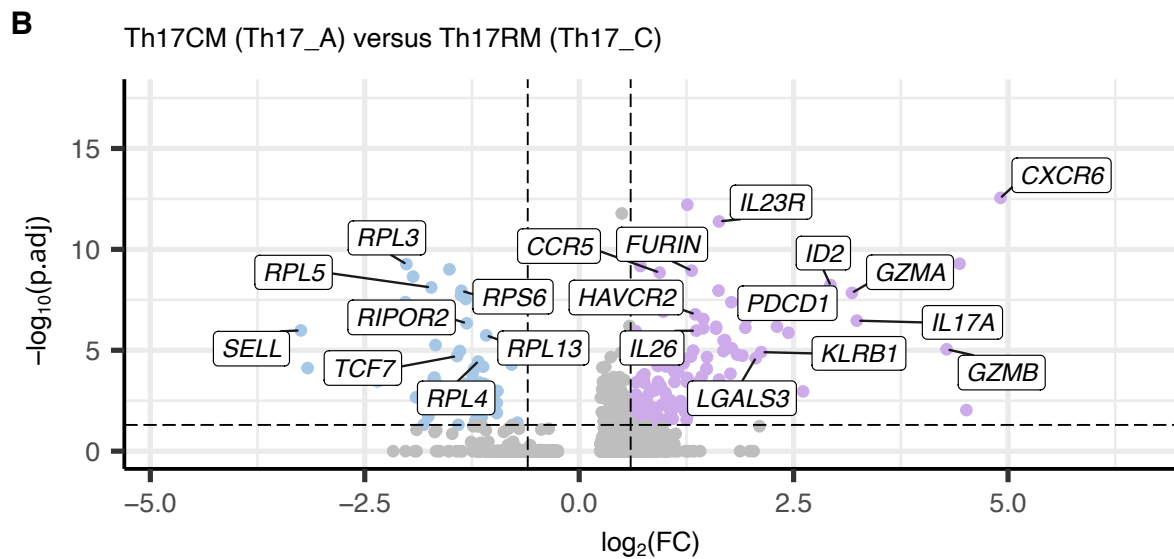
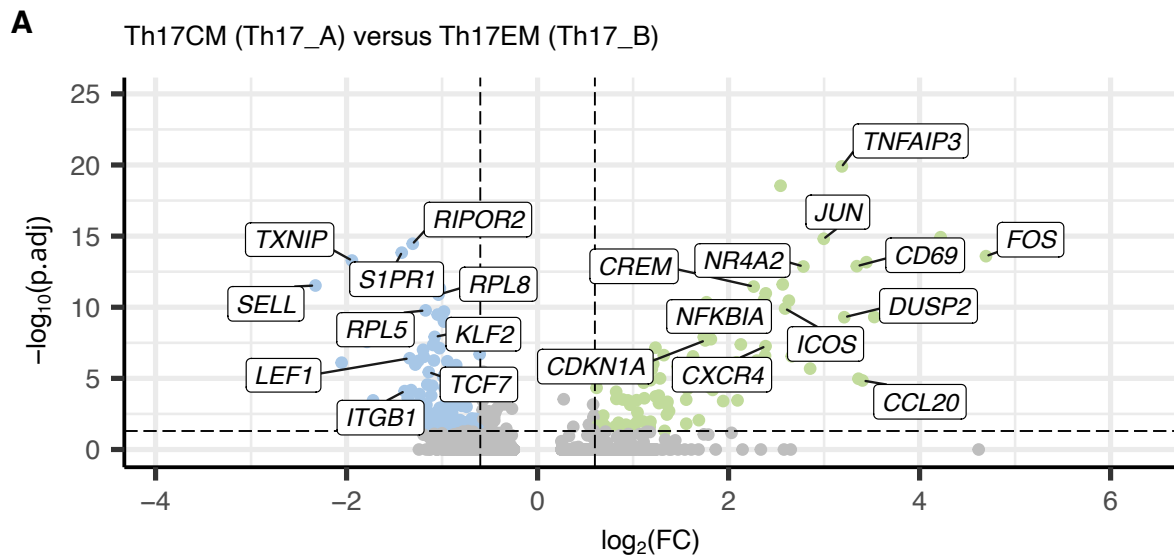
Antigen-specific Th17 cells simulated with ICOS signalling, rather than more traditional CD28 co-stimulatory pathways, augment their production of IFN- γ and their anti-tumour capacity is superior to that of their CD28-stimulated counterparts in mice bearing human mesothelioma tumour xenografts^{310,311}. In skin CD8⁺ T cells, ICOS signalling cooperates with c-Maf and IL-7 as part of a circuit that preferentially induces the production of IL-17, highlighting its importance in the propagation of type 17 immunity³¹².

More uniquely, the number of differentially expressed genes on Th17_C cells varied considerably depending on the comparator population. Against Th17_A cells, Th17_C cells appeared far more functionally active, strongly upregulating a number of effector cytokines including *IL17A* and *IL26* as well as the cytolytic serine proteases *GZMA* (granzyme A) and *GZMB* (granzyme B) (**Fig. 2.6B**). However, when compared against Th17_B cells, many of these functional disparities were lost (**Fig. 2.6C**). The most differentially expressed genes here, of which there were far fewer, were dominated by markers of resident memory T cells²⁷⁵ (*ITGAE*, *CXCR6*) and a number of adhesion molecules (*LGALS3*), hinting that the major differences between Th17_C and Th17_B cells may arise as a consequence of different tropisms.

CD103 (encoded by *ITGAE*, integrin α E) strongly binds to E-cadherin, a cell adhesion molecule found abundantly on the surface of epithelial cells³¹³, and the chemokine receptor CXCR6 regulates T cell trafficking to and retention within inflamed mucosal sites³¹⁴⁻³¹⁶, suggesting that Th17_C cells may differentially reside at an inflammatory epithelial interface. More specifically to Th17 cells, CXCR6 expression is often associated with secretion of IFN- γ , GM-CSF and TNF- α , with CXCR6⁺ Th17 cells readily inducing EAE upon adoptive transfer into MOG₃₅₋₅₅ immunised mice^{163,317} (though these cytokines were not upregulated in Th17_C cells, data not shown).

The consistent upregulation of the co-stimulatory molecule *JAML* in Th17_B cells during both comparisons was another interesting observation, as JAML has recently been found to define a population of memory CD8⁺ T cells whose tumour infiltration is positively associated with survival in non-small cell lung carcinoma patients³¹⁸. *ENTPD1*, which encodes the ATP-cleaving ectonucleotidase CD39, has been suggested to protect T cells from ATP-induced apoptosis within ATP rich tumour microenvironments³¹⁹ and was also consistently upregulated in Th17_C cells. Co-expression of CD39 with CD103 has also been recently shown to define tumour antigen-specific CD8⁺ T cells in both head and neck squamous cell carcinoma and CRC patients³²⁰⁻³²³, suggesting that Th17_C cells might form an important population of tumour Th17 cells.

The transcriptional alignment of each Th17 population towards different T cell differentiation states³²⁴ prompted us to reconsider our nomenclature. As Th17_A cells were enriched with lymph node migration markers, like central memory T cells, we reclassified them as Th17CM cells, the nomenclature we use henceforth. Highly activated Th17_B cells were renamed Th17EM, in line with an effector memory T cell phenotype. Finally, the collective expression of *ITGAE*, *CXCR6* and *JAML* in Th17_C cells led to their recategorisation as Th17RM cells due to their transcriptional overlap with the resident memory phenotype.



● Enriched in Th17CM ● Enriched in Th17EM ● Enriched in Th17RM ● NS

Figure 2.6 | Differential gene expression comparing each Th17 cell population with one another. Differential gene expression analysis was performed, comparing each Th17 population with one another. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed genes, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. Volcano plots displaying the differentially expressed genes, where **(A)** displays those differentially upregulated in Th17CM (left) and Th17EM (right) cells, **(B)** displays those differentially upregulated in Th17CM (left) and Th17RM (right) cells and **(C)** displays those differentially upregulated in Th17EM (left) and Th17RM (right) cells. A selection of genes have been annotated. Dashed lines are drawn at $\log_2$ fold change = 0.5 and $p_{\text{adj}} = 0.05$. Blue data points are genes upregulated in Th17CM cells, green data points are those upregulated in Th17EM and cells and purple data points are those upregulated in Th17RM cells. Grey data points represent genes which did not meet the threshold for significance. NS = not significant, FC = fold change.

To validate the alignment of our Th17 populations with these three T cell states of differentiation, we used GSEA³²⁵ to respectively explore the enrichment of central memory, effector memory and resident memory gene sets, as curated by two reference publications^{275,326}, within our newly termed Th17CM (**Fig. 2.7A**), Th17EM (**Fig. 2.7B**) and Th17RM cells (**Fig. 2.7C**). As predicted, each gene set was significantly enriched within in each respective Th17 population. To confirm the specificity of each enrichment, we next compared mismatched pairs e.g. the TCM gene set with Th17EM and Th17RM cells. Negative enrichment was observed for most mismatched pairs (**Fig. S2.1A-E**) although the enrichment score for the TRM signature in Th17EM cells was not significant. We could not contrast the TEM gene set with Th17RM cells as there were no significant gene overlaps and this was perhaps due to the few genes specifically enriched in Th17RM cells.

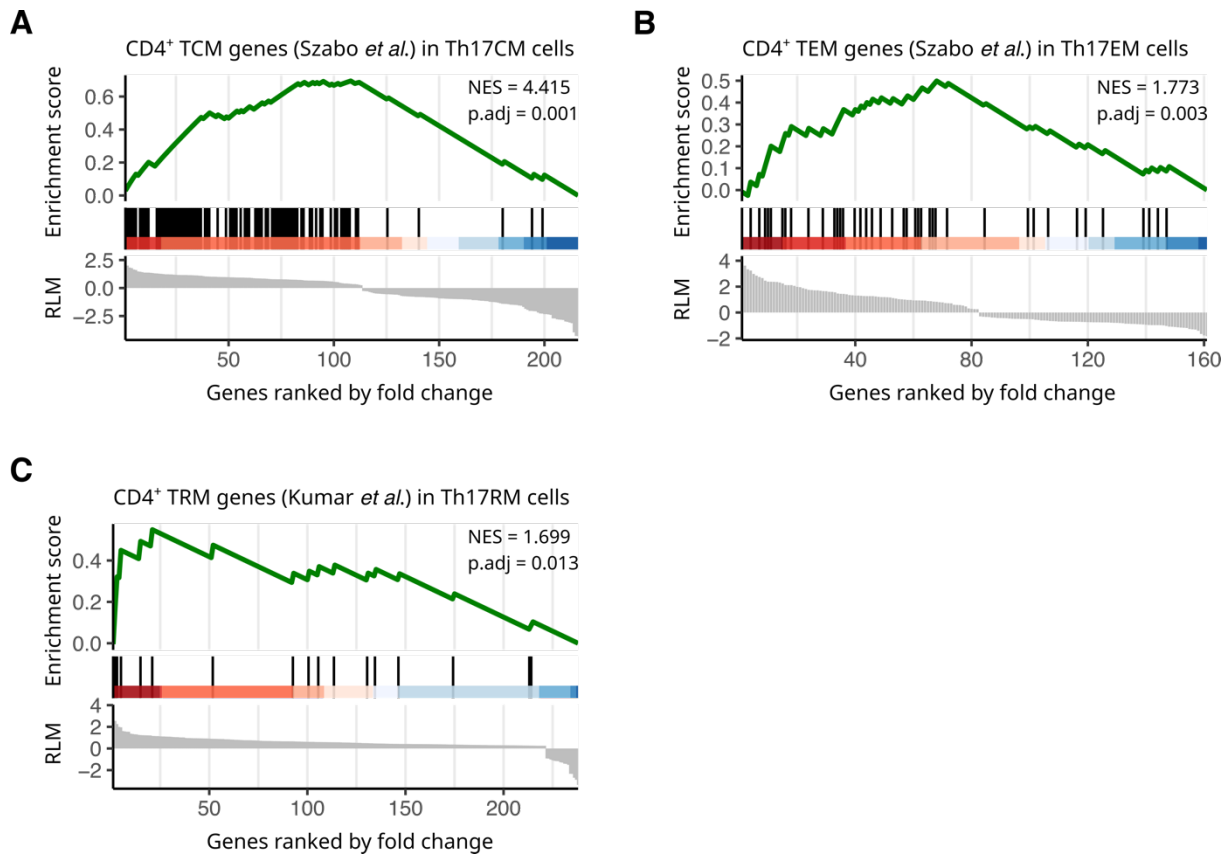


Figure 2.7 | Enrichment analysis for each Th17 population demonstrates alignment with unique T cell activation states. GSEA was used to score the enrichment analysis of central memory (TCM), effector memory (TEM) and resident memory (TRM) T cell gene signatures^{275,326}, in Th17CM, Th17EM and Th17RM cells, respectively. NES = normalised enrichment score, p.adj = adjusted p value, RLM = ranked list metric.

2.3.3 A pan-Th17 surface marker remains elusive

A persistent challenge when working with Th17 cells is the lack of unifying, identifiable surface marker. Unlike immunosuppressive Treg cells, for example, where FoxP3 activity is well correlated with high surface expression of CD25 and diminished presence of the IL-7R α subunit (CD127)^{88,92,327,328}, reliable, analogous markers are yet to be defined for ROR γ t activity.

For many years, CCR6 has been used as a putative surface marker for Th17 cells, despite its expression on a number of other cell types, including Tregs³²⁹, B cells³³⁰ and dendritic cells¹⁹⁵. IL-23R has more recently been used as another surrogate surface marker for Th17 cells, although reliable antibody clones against this receptor are yet to be established and both Th1 and Treg cells are also able to respond to IL-23 via IL-23R signalling³³¹⁻³³³. Markers for more nuanced Th17 phenotypes, such as CXCR6 for IFN- γ ⁺ Th17 cells^{163,317} or gasdermin E for IL-1 α ⁺ Th17 cells¹⁷⁰ are gradually becoming elucidated however the vast majority of reliable, pan-Th17 cell markers remain intracellular, such as STAT3³³⁴, IRF4³³⁵, BATF³³⁶ and ROR γ t³³⁷.

We speculated that the depth of sequencing and range of T cell phenotypes available in GSE108989 would enable us to uniquely explore putative pan-Th17 surface markers by contrasting our Th17 populations with other T cell clusters in the dataset. Differential gene expression analysis was performed, as described previously, comparing each Th17 population with all the other T cells subsets, collectively termed 'other'. Any overlapping genes which were significantly differentially upregulated in Th17 cells were then collated and assessed with the aim of identifying novel surface markers for Th17 identification (**Fig. 2.8A**).

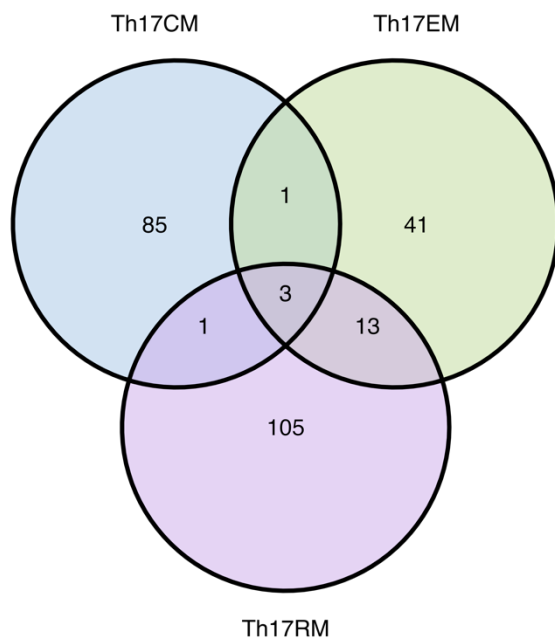
Rather surprisingly, only three genes were commonly upregulated in our Th17 cells, compared with the 'other' T cells: *RORC*, as expected due to the clustering hierarchy used; CD40L (CD40 ligand, CD154), a co-stimulatory molecule usually associated with Tfh cells although its expression is reported on Th17 cells that have been induced by strong antigenic signalling³³⁸; and TNFRSF25 (DR3, death receptor 3), whose interaction with its cognate ligand TL1A drives Th17 differentiation and proliferation³³⁹ (**Fig. 2.8B**).

To explore commonality between Th17 populations, we repeated the process but grouped each Th17 population in pairs with one another. Only *IL7R*, the receptor for a key anti-apoptotic and lymphocyte survival cytokine IL-7^{340,341}, was similarly upregulated in both Th17CM and Th17EM cells. Th17CM and Th17RM cells shared the upregulation of *S100A4*, a gene with undefined functions in T cells.

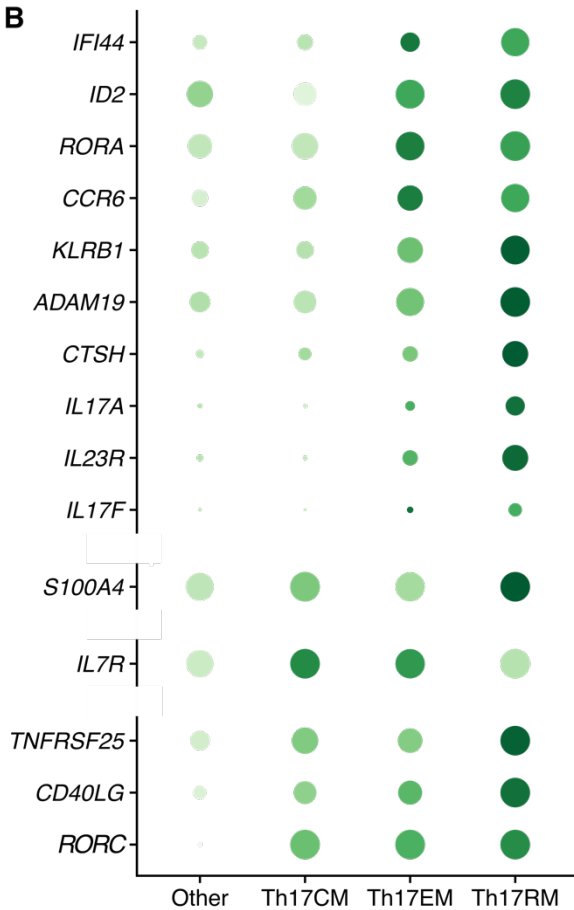
The most overlapping phenotype belonged to Th17EM and Th17RM cells, with 13 significantly upregulated genes shared between them. The majority of these genes constituted established Th17 markers (*IL17A*, *IL17F*, *IL23R*, *CCR6*, *KLRB1*, *RORA*) and some, such as *ID2*, have been referenced as part of broader analyses of T cell differentiation^{342,343}. *CSTH* (cathepsin H) has also been previously identified in Th17 cells as part of RNA-seq screening although no investigations into its function have been carried out^{344,345}.

As part of a final exploration into the differentially expressed genes that define each Th17 population, we characterised the genes enriched in each individual Th17 cohort, as compared with all other T cells within GSE108989 (**Fig. 2.8C**). Most hits had been identified by our previous intra-Th17 cell analysis (**Fig. 2.7**). Of note though from this analysis, both *BHLHE40* and *MCAM* have established associations with inflammatory cytokine induction in Th17 cells^{150,274,346} and were significantly upregulated in Th17EM and Th17RM cells, respectively. Additionally, *RUNX2* is a key transcription factor that potentiates a cytotoxic resident memory phenotype in CD8⁺ T cells, with its expression in this population associated with improved prognostic outcome in patients with metastatic melanoma³⁴⁷.

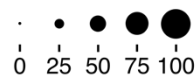
A



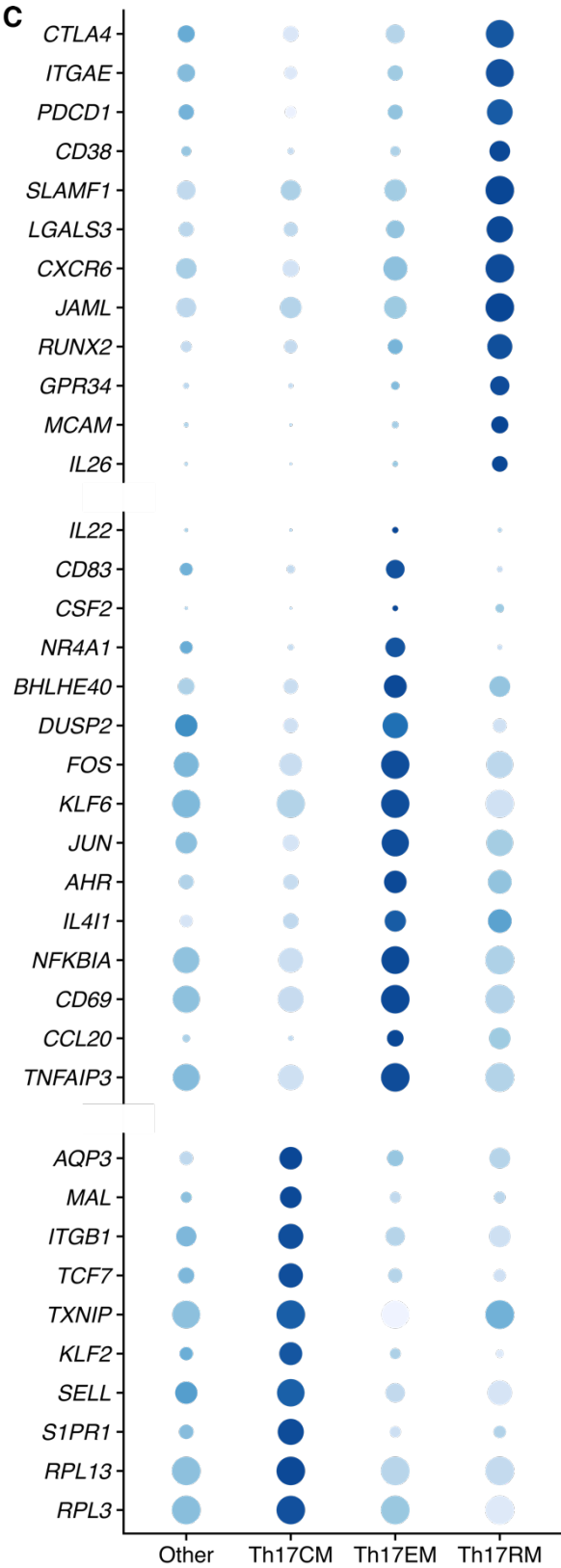
B



Percentage of cells in group



C



Average expression across subset

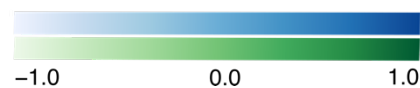


Figure 2.8 | Differential gene expression comparing Th17 cell populations with other T cells in GSE108989. Differential gene expression analysis was performed comparing each Th17 population with other T cell communities in GSE108989, collectively termed 'other'. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed genes, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. **(A)** Venn diagram showing the number of significantly regulated genes shared by > 1 Th17 population. Dot plots showing a selection of significantly upregulated genes either **(B)** shared by > 1 Th17 population or **(C)** significantly upregulated in only one Th17 population. Dot size represents the percentage of cells in each population expressing the gene and colour saturation indicates the relative expression level, scaled across each population.

2.3.4 Each Th17 population is spatially enriched

Th17 functionality is highly influenced by the tissue milieu. As each Th17 populations we have identified thus far is transcriptionally distinct, we hypothesised that each might display a unique tissue tropism. To investigate this, we first plotted each Th17 population on a UMAP then overlaid the tissue type from which each Th17 cell was identified. We observed a distinctive segregation of Th17 population by tissue type, with Th17CM cells almost exclusively restricted to the circulation, Th17EM cells broadly observed within tissue and Th17RM cells highly enriched within tumour tissue (**Fig. 2.9A**). Overall, Th17CM cells made up 2.2% (74/3,350) of the circulating compartment, Th17EM cells made up 3.6% (99/2,637) of the healthy T cell compartment and Th17RM cells made up 0.7% (36/5,149) of the tumour T cell compartment in GSE108989.

Whilst these enrichment patterns were quite striking, they could have been skewed by individual patients with particularly high counts for one specific Th17 population. To test this, we first stratified the Th17 cells from each patient by sample location (**Fig. 2.9B**) and next by

Th17 annotation (**Fig. 2.9C**). Th17 cells were found in 92% (11/12) of the patients (data not shown for the patient in which Th17 cells were not identified), with reasonable representation from all three tissue types per patient; Th17 cells were identified in blood samples from 83% (10/12) of the patients, in 75% (9/12) from normal healthy adjacent tissue samples and 92% (11/12) of the patients had Th17 cells identified within the tumour (**Fig. 2.9B**).

Looking at the distribution of Th17 cell annotations by patient, 93% of patients had circulating Th17CM cells and Th17EM cells were found in 83% of patients. Rather conflictingly however, Th17RM cells were only found in 25% (3/12) of patients, with the large majority of these identified in a single patient, P0701 (**Fig. 2.9C**).

Given the small patient sample size of this dataset, we were underpowered to determine whether or not this inverse Gaussian Th17RM distribution correlated with any clinical metadata. Differential niche selection due to sample heterogeneity may have also influenced the Th17 populations isolated from each patient, as some Th17 phenotypes may be more enriched within the submucosa or epithelium, for example, than others. Correlating the presence of Th17RM cells with other niche specific cell types, such as *RSPO3*⁺ *GREM1*⁺ fibroblasts localised nearby intestinal crypt bases³⁴⁸, might have allowed for further exploration. However, as GSE108989 was a T cell enriched dataset, this option was not available to us.

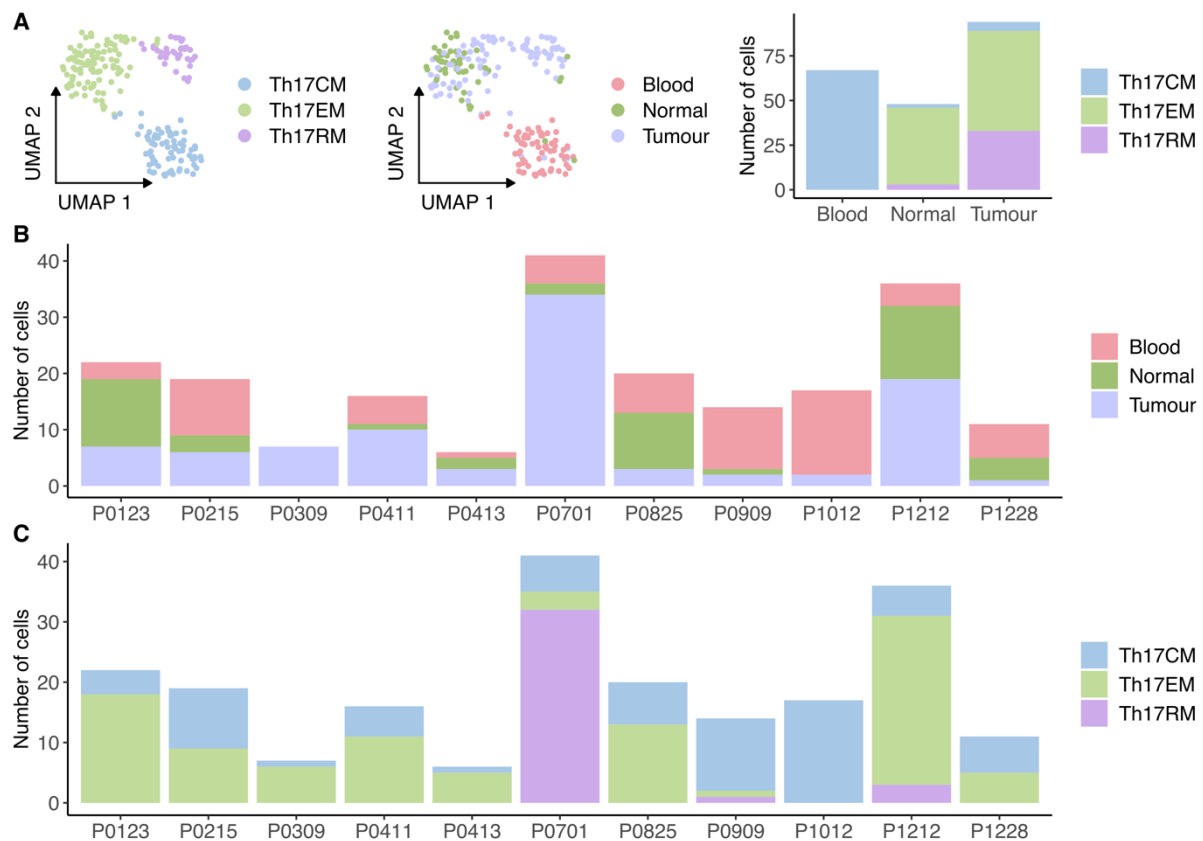


Figure 2.9 | Each sampled tissue location is enriched with a different Th17 population.

(A) UMAP of each Th17 population and the tissue location in which they were identified in and quantitative representation of Th17 population distribution across sampled tissue locations. Stratification of the number of cells from each (B) tissue location and (C) Th17 population across patient samples.

2.3.5 Each Th17 population can be identified *ex vivo* in CRC patients

To validate whether these computational findings were representative of that seen in CRC patients, we explored the expression of a selection of surface markers by flow cytometry that characterised each Th17 population on Th17 cells from CRC patients. Fresh single cells were obtained from venous blood, healthy normal adjacent and tumour tissue from CRC patients undergoing surgery before extracellular staining for the relevant markers, permeabilisation

and intranuclear staining for transcription factor analysis (gating strategy with ROR γ t FMO and isotype control shown in **Fig. 2.10**).

As predicted computationally, the expression of SELL (CD62L) and CD29 (integrin β 1) were both significantly enriched on circulating Th17 cells, compared with those isolated from normal and tumour tissue (**Fig. 2.11A, Fig. 2.11D**). The relative lack of surface markers that were predicted to differentially define Th17EM cells was also reflected at the protein level. Although significantly higher levels of CD69 were detected on Th17 cells from normal healthy adjacent tissue, the majority of tumour derived Th17 cells also expressed CD69 (**Fig. 2.11B, Fig. 2.11D**). No other Th17EM markers computationally predicted (CD83, CXCR4, ICOS) were enriched in Th17 cells from healthy tissue either (data not shown).

In the tumour tissue, Th17 cells expressed significantly more CD103 and CD39 compared with blood and normal Th17 cells. Although there was a trend towards Th17 cells expressing CD38 in the tumour, there were not significantly more CD38⁺ Th17 cells in the tumour compared with in normal tissue (**Fig. 2.11C, Fig. 2.11D**).

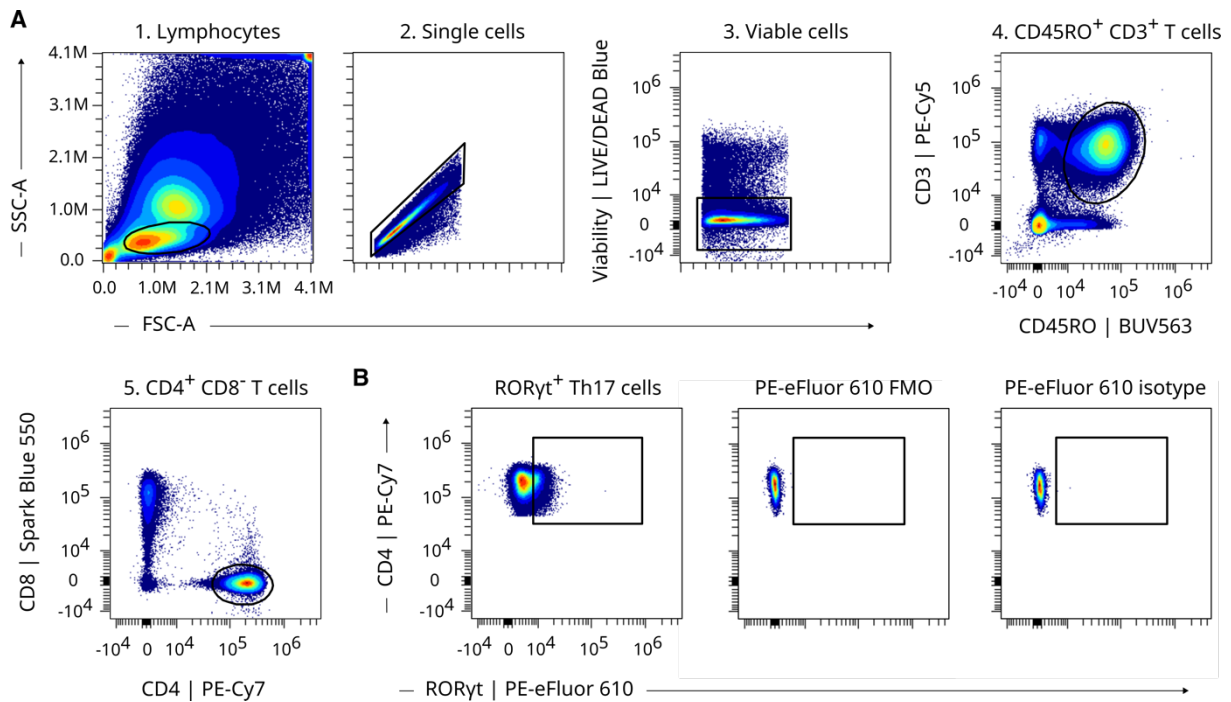


Figure 2.10 | Gating strategy used to identify Th17 cells by flow cytometry. To validate the existence of Th17 populations identified by scRNA-seq in CRC patients, Th17 cells from fresh venous blood, healthy normal adjacent and resected tumour tissue patients undergoing CRC surgery were first identified by flow cytometry. **(A)** Gating strategy used to characterise CD4⁺ T cells (single, viable CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ lymphocytes). **(B)** Identification of Th17 cells (RORγt⁺ CD4⁺ T cells) (left), with RORγt FMO (middle) and rat IgG2a isotype control (right) staining shown.

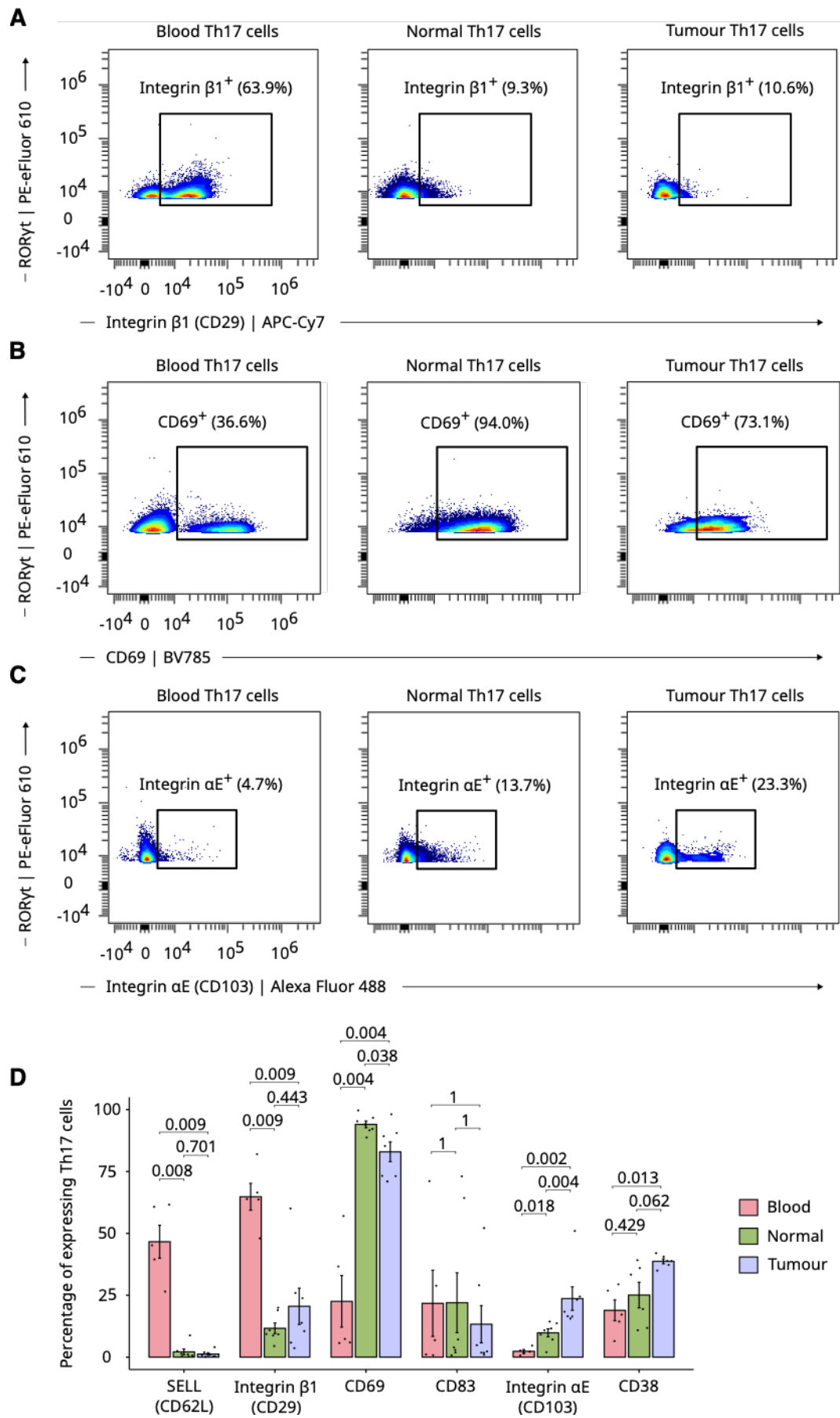


Figure 2.11 | Th17 populations identified by scRNA-seq can be found *ex vivo* in CRC patients. Freshly isolated single cells from venous blood, healthy normal adjacent (normal) and resected tumour tissue collected from patients undergoing CRC surgery were used to analyse Th17 cell marker expression by flow cytometry. **(A)** The gating strategy used to identify single, viable CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ ROR γ t⁺ (Th17) cells [DP165; GI 9214] alongside **(B)** a ROR γ t FMO [DP165; GI 9214] and a rat IgG2a, κ isotype control [DP165; GI 9214]. The expression profiles of **(C)** integrin β 1 (CD29), **(D)** CD69 and **(E)** integrin α E (CD103) on blood, normal tumour tissue-derived Th17 cells are shown. The percentage of Th17 cells per tissue sample expressing each respective marker are shown above the gate. **(F)** The frequency of Th17 populations expressing different markers stratified by tissue location (blood, $n = 5$; normal and tumour, $n = 7$). Wilcoxon tests with Benjamini-Hochberg correction was used to calculate the statistical comparisons. Adjusted p values (p.adj) are numerically shown above each comparison and $p.\text{adj} \leq 0.05$ is considered statistically significant.

2.3.6 Each Th17 cell may highlight a single moment in a continuum of adaptations

The development of fate reporter mice to track cells has greatly improved our understanding of Th17 cell plasticity, allowing researchers to follow the natural history of individual Th17 cells from the earliest stages of pathogenesis through to the resolution of inflammation. Since the first reported use of an *IL17a* fate reporter model¹⁵¹, it has been established that Th17 cells can not only co-express cytokines associate with other T cell subsets, such as IFN- γ ¹⁶³ or IL-10³³⁷, but are plastic enough to become entirely reprogrammed into distinctive T cell populations such as Tfh³⁴⁹ or Tr1²⁹² cells.

Our identification of three Th17 populations in human tissue meant that we could only observe each Th17 state in the ‘snapshot’ of time when the tissues were processed. However, techniques such as pseudotime analysis are designed to computationally predict the

developmental trajectories that cells may take using these ‘snapshot’ data points. Using the pseudotime functions from the Monocle 3 package in R, we predicted that Th17CM cells represent the earliest population along the developmental trajectory, with Th17EM cells constituting an intermediate population and Th17RM cells embodying the most terminally differentiated community (**Fig. 2.12A**). These predictions aligned with comparable chronological analyses performed in CD8⁺ T cells, where transcriptional downregulation of *S1pr1* in the most naïve populations is inversely correlated with surface CD69 expression, which itself precedes the expression of CD103 in resident memory CD8⁺ T cells^{350,351}. A comparable sequence of surface marker changes was observed as cells transitioned from Th17CM to Th17EM and finally Th17RM functional states (**Fig. 2.12B-G**).

We found the strict confinement of *CXCR4* to Th17EM cells in comparison with the gradual upregulation of *CXCR6* in late Th17EM/Th17RM cells intriguing. CXCL12, the ligand for CXCR4, is classified as a homeostatic chemokine due to its constitutive expression in lymphoid, circulatory and peripheral tissues, functioning to facilitate broad immunosurveillance³⁵². On the other hand, CXCL16, the ligand for CXCR6, is far more niche specific. The secretion of CXCL16 is restricted to inflammatory environments, with cancer associated fibroblasts³⁵³, dendritic cells³¹⁵, inflammatory macrophages³⁵⁴ and tumour cells³⁵⁵ known to be the main producers. The distribution of these chemokine receptors by pseudotime further implies that Th17EM and Th17RM have different tropisms. It suggests that Th17EM cells are recruited into inflammatory tumours where they rapidly lose the ability to leave, reducing their ability to respond to follow homeostatic CXCL12 gradients and upregulating tissue retention integrins such as CD103 (**Fig. 2.12D-G**).

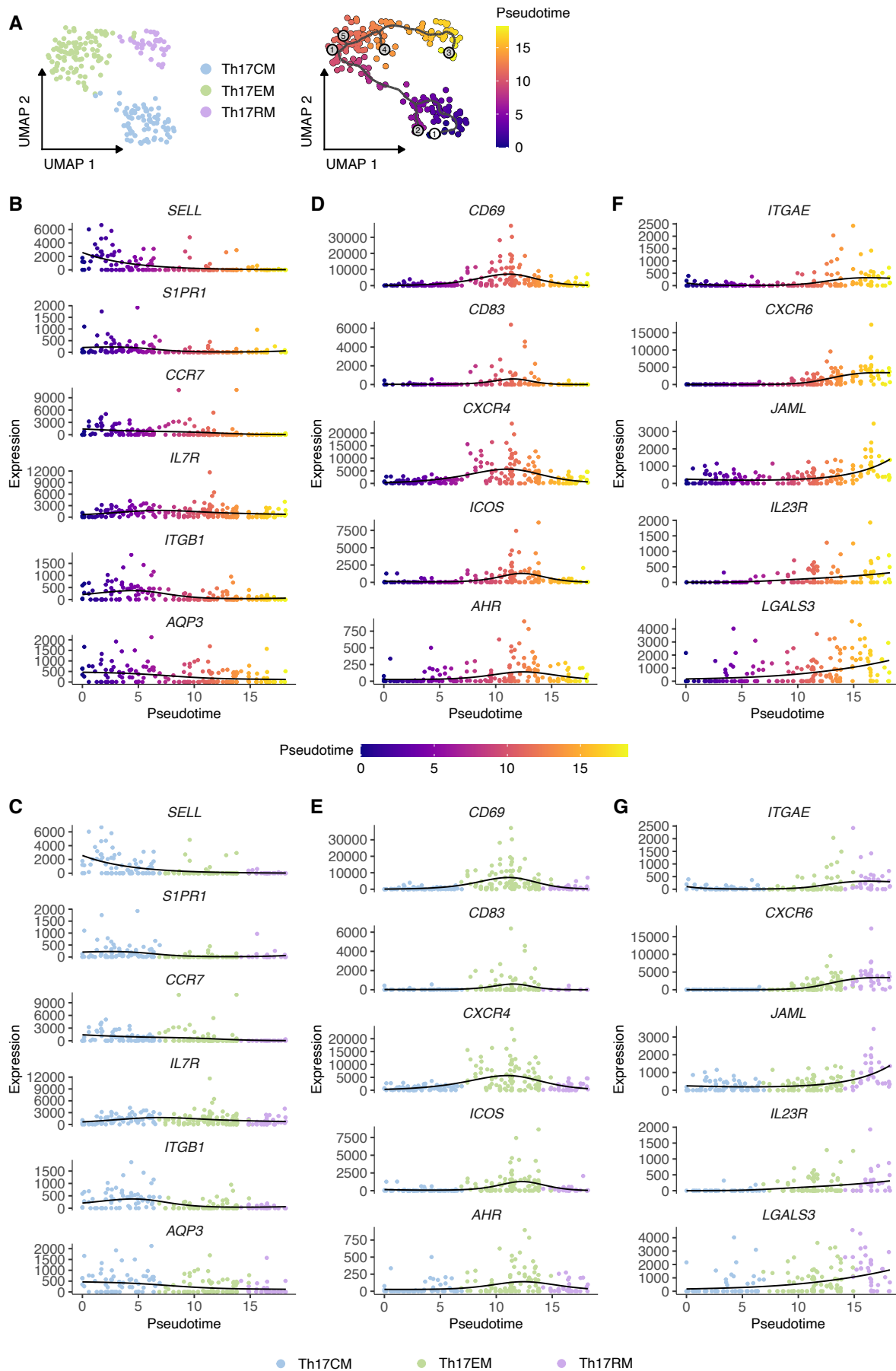


Figure 2.12 | Pseudotime analysis of surface marker genes expressed by Th17 cells. (A) UMAP of Th17 cells with pseudotime trajectory. Selection of surface markers projected by pseudotime, coloured by pseudotime and cluster for (B) (C) Th17CM, (D) (E) Th17EM or (F) (G) Th17RM cells.

Transcription factors are the key drivers of these dynamic cellular changes over time. As such, we explored how a selection of key transcription factors identified during prior differential gene expression analysis fluctuated across pseudotime.

Neither the transcription factors that control Th17CM nor Th17RM cells were normally distributed, with a clear skewing towards their respective cell populations. *TCF7*, *LEF1*, *KLF2* and *ID3* were all restricted to the earliest events in pseudotime (**Fig. 2.13A**). *ID2* and *RUNX2*, Th17RM transcription factors, displayed the reverse trend (**Fig. 2.13B**). We also explored how *PRDM1* (BLIMP-1) and *ZNF683* (Hobit), two additional transcription factors that act as key regulators of resident memory properties in CD4⁺ T cells³⁵⁶, vary with pseudotime, expecting them to follow a similar trend to *ID2* and *RUNX2*. It was instead surprising how ubiquitously low the expression of *PRDM1* and *ZNF683* was across pseudotime (**Fig. 2.13B**) and it would be informative to explore the expression of BLIMP-1 and Hobit by flow cytometry to see if these expression levels translate into functional transcription factor activity.

Prior differential gene expression analysis of Th17EM cells was dominated by transcription factors and so we were particularly intrigued to understand how their expression ranged with pseudotime. Quite unlike the patterns seen in Th17CM and Th17RM cells, transcription factor expression in Th17EM cells appeared to exist in three temporal waves; with the AP-1 transcription factor motifs *JUN*, *JUNB* and *FOS*, as well as *DUSP1* collaboratively enriched in early pseudotime Th17EM cells, *DUSP2*, *NR4A2*, *NR4A1* and *KLF6* enriched at an intermediate

timepoint, and *BHLHE40* and *CREM* upregulated together in latest moments in pseudotime (**Fig. 2.13C**). Coincidentally, three branch points within the Th17EM cluster were also identified by pseudotime (**Fig. 2.13A**), perhaps highlighting the presence of three distinct fate points experienced by Th17EM cells.

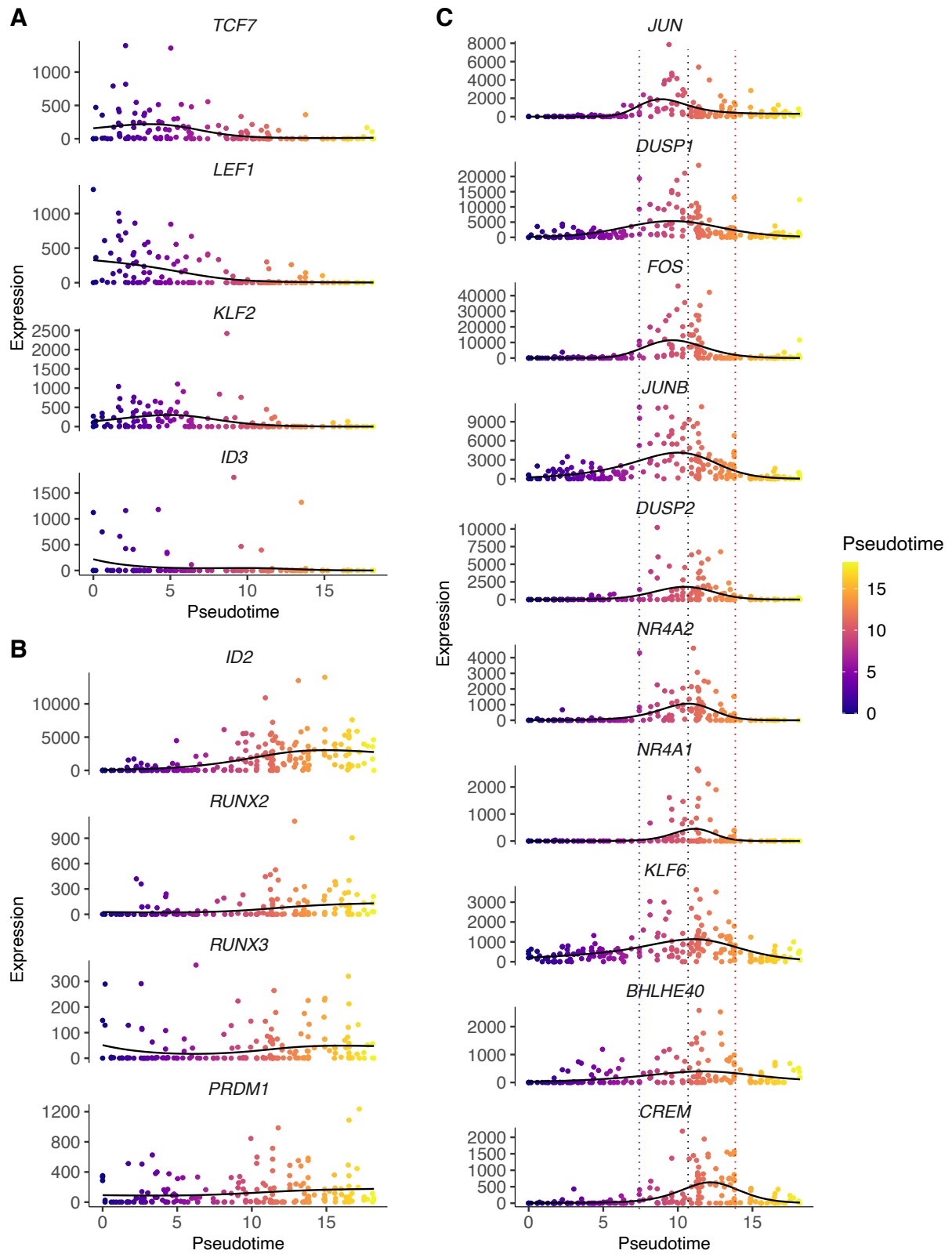


Figure 2.13 | Each Th17 population is temporally regulated by transcription factors. (A) Th17CM, **(B)** Th17RM and **(C)** Th17EM transcription factor expression plotted by pseudotime.

Blue, black and red vertical dashed lines in (C) represent the early, intermediate and late timepoints of transcription factor expression.

2.4 Discussion

Using GSE108989, a publicly available, T cell enriched scRNA-seq dataset from patients with CRC, we identify three transcriptionally distinct Th17 populations. Each population is enriched within a unique tissue environment and expresses a gene profile that aligns with a defined T cell adaptive state. Th17CM cells, rich in lymph node homing marker, are found in the circulation and exhibit stem-like features. Th17EM cells are found in normal, healthy tissue and display a highly activated profile. Th17RM cells are associated with the tumour microenvironment and may develop from Th17EM cells in response to a tumour-related stimulus.

2.4.1 Why are unifying Th17 markers so elusive?

Identifying a reliable marker of Th17 cells has proven intriguingly difficult since their initial identification two decades ago. ROR γ t was identified early on as the key transcription factor that dictates IL-17 expression¹³⁰ and, since then, a host of other intracellular regulators of Th17 cells have been elucidated, including STAT3, IRF4 and BATF.

Convincing surface markers have remained more elusive. For many years, CCR6 has been used as a putative Th17-specific chemokine receptor due to its expression on both mouse and human Th17 cells, as well as its reasonably uniform expression level in Th17 cells found in the small and large intestine, Peyer's patches and spleen³⁵⁷. We performed differential gene expression analysis comparing our three Th17 populations with all other T cells from GSE108989 with the aim of identifying novel markers for Th17 identification. Only three

significantly enriched genes were detected; *RORC*, *CD40L* and *TNFRSF25*(DR1). *CCR6* was not significant, aligning with reports of its expression in other T cell subsets, primarily Treg cells³⁵⁸.

One potential reason as to why so few markers exclusively associate with Th17 cells is their tropic enrichment at mucosal barriers. Especially in the gut, rapid epithelial cell turnover combined with close proximity to a staggering number of microbes means these niches are highly dynamic environments. The T cells which patrol these borders, such as Th17 cells, Treg cells, ILC3s and MAIT cells, must possess a capacity to rapidly induce inflammation in response to aberrant epithelial proliferation or loss of barrier integrity, as well as a capacity to halt inflammatory responses as quickly to prevent excess tissue damage. The necessity for such multifaceted effector functions could therefore explain why the majority of Th17 associated markers, such as CCR6, CXCR6 and CD161, have been described in all three of these mucosal T cell subsets as well as a number of other mucosal-resident cells such as DCs and NK cells^{315,359,360}. Furthermore, memory signatures have been extensively described in CD8⁺ T cells. The considerable transcriptional overlap between each Th17 population and TCM, TEM and TRM CD8⁺ T cells would also lend to the idea that Th17 cell effector functions are tightly linked with signals from local niches.

Due to the paucity of surface markers revealed by our scRNA-seq analysis, it may be better to define Th17 populations using combinatorial signatures that define more specific effector functions, such as the Th17CM, Th17EM and Th17RM cells we describe. This nomenclature follows a principle to that akin to describing Th17 cells as 'non-pathogenic' or 'pathogenic', a popular nomenclature now widely used in the literature based on the ability of Th17 cells to

produce IFN- γ . However, these terms describe the function of Th17 cells in EAE and it can be confusing, or even misleading, when used to name Th17 cells in the context of cancer.

2.4.2 The importance of ROR γ t⁺ FoxP3⁺ Th17 cells in the tumour

To obtain a pure population of Th17 cells for downstream analysis, *RORC*⁺ T cells expressing *FOXP3* transcripts were removed from our clustering output. Under homeostatic conditions, ROR γ t⁺ FoxP3⁺ T cells are commonly found in gut lamina propria and are induced by symbiotic members of the microbiota, including multiple members belonging to the genera *Firmicutes*, *Bacteroides* and *Fusobacteria*. Functionally, these double positive (DP) T cells play an important role in restraining local inflammation²⁸⁷. Despite a necessity for these DP T cells in maintaining gut homeostasis, we chose to exclude double expressors from our downstream analysis due to difficulty in identifying their ontogeny. Whilst some Th17 cells can acquire and co-express FoxP3, they do not define a stable population but mark a transitional state as Th17 cells transdifferentiate into Treg cells¹⁸⁹. In the reverse direction, the acquisition and co-expression of ROR γ t in Treg cells appears more stable, permitting the sustained presence of polarising cytokines. Whilst there are no reports of Th17 cells co-expressing FoxP3 then returning back to a Th17 profile, ROR γ t⁺ Treg cells that rest from IL-6 and IL-1 β *in vitro* fail to produce IL-17 upon restimulation and display equivalent levels of suppression as compared with Treg cells isolated *ex vivo*²⁹³. Together, these data suggest that the cells expressing both *RORC* and *FOXP3* in our clusters have either lost or are set to lose their Th17 functionality.

With more time, it would have been interesting to explore the functional impact of these DP clusters on tumour immunity. Given the presence of DP Treg cells in the gut lamina propria during homeostasis²⁸⁷, it is perhaps not surprising that other studies have concluded they

play a role in polyposis, one of the earliest events as tissue transitions from healthy to neoplastic. In this context, DP T cells have been shown to exhibit reduced suppressive ability which permits mast cell degranulation, an essential process in the development of intestinal polyps³⁶¹, and enhances the infiltration of cytotoxic cells into hyperplastic tissue regions³⁶². Despite these convincing observations, contradictory functions for DP T cells have been reported in other pathologies. In EAE, DP T cells exhibit potent immunosuppression and their induction is sufficient to completely abolish Th17-mediated CNS inflammation³⁶³. The extent of overlap between the transcriptional profiles of these two contrasting T cell populations has not been established. Heterogeneity within DP T cells, likely a result of distinct tissue milieus, might underpin one reason as to why these two DP populations display seemingly antithetical functions.

2.4.3 What phenotype, or phenotypes, best capture the earliest timepoint in the evolution of Th17 cells?

It is now well established that Th17 cells, as a population, can exhibit remarkable plasticity. However, whether all homeostatic Th17 cells display the same capacity to differentiate along distinct trajectories is not known. Furthermore, a thorough phenotypic characterisation of the intermediary facets in which Th17 cells transition through as they evolve has also not been performed.

Recent scRNA-seq analysis of IL-17⁺ T cells from multiple tissues in *Il17a* reporter mice has provided evidence to suggest that homeostatic Th17 cells exhibit strong tissue-specific signatures¹⁶³. These observations might suggest that distinct tissue-specific Th17 populations are poised to respond differently to identical environmental cues. In support of

this hypothesis, polarising Th17 cells *in vitro* with TGF- β 1, IL-23 and IL-6 largely generates IFN- γ ⁺ Th17 cells, yet a small population of IL-10⁺ Th17 cells is also produced³⁶⁴.

Despite our pseudotime analysis being correlative, the numerous branch points detected in such few Th17EM cells, in combination with the seemingly trimodal temporal expression of immunomodulatory genes, makes the idea that Th17EM cells capture a functionally homogenous population with diverging trajectories intriguing.

One major limitation with pseudotime analysis as a technique is the necessity to manually annotate the 'root' community before trajectories are calculated. Normally the root, the least differentiated population, is determined by prior biological knowledge. Across the three Th17 populations, Th17CM cells exhibit the most commonality with naïve T cells, with both subsets expressing an overlapping signature composed of *CCR7* and *SELL*, amongst a host of other stem-like markers³⁰³. However, how naïve Th17CM cells really are remains unclear. The first study to describe TCM cells suggested they develop directly from CD45RA⁺ naïve T cells²²⁷ and differentiate in response to low affinity ligand³⁶⁵. However, we demonstrate that Th17CM cells express CD45RO which would suggest that they have been previously activated. Thus, whether Th17CM cells instead comprise a population of contracted, long lived effector cells and potentially deriving from cells kindred to those in the Th17EM community, is perhaps not unreasonable.

Examining the three distinct temporal transcription factor clusters within the Th17EM community might hint to the notion that Th17EM cells represent the biological 'root' community and plastic gateway population that gives rise to a spectrum of phenotypic adaptations. There is evidence to suggest that AP-1 transcription factor motifs found within

the first temporal cluster, such as *JUN*, *JUNB* and *FOS*, represent key drivers of stem-like persistence and protection against exhaustion in T cells³⁶⁶ and therefore may demarcate the gateway Th17 phenotype that gives rise to Th17CM cells. The expression of *BHLHE40* and *CREM*, activated downstream of IL-1 β signalling and found in Th17EM cells at the latest timepoint, has been heavily associated with the induction of an inflammatory façade in Th17 cells, with both transcription factors regulating GM-CSF production. Furthermore, the suppression of either transcription factor is sufficient to protect mice against EAE^{346,367-369}. This could indicate that the Th17EM cells that branch at the latest point in pseudotime follow a trajectory towards Th17RM cells, or a similarly pro-inflammatory equivalent.

The presence of proteins belonging to the NR4A orphan nuclear receptor superfamily in T cells is tightly linked with the induction of a regulatory phenotype, forming a positive feedback cycle with FoxP3 to promote the development of Treg precursor cells³⁷⁰. Furthermore, NR4A factors can mechanistically suppress AP-1 activity³⁷¹ and therefore this intermediate timepoint captured by pseudotime might embody early precursor cells in the well-documented pathway of Th17 to Treg transdifferentiation.

Calculating pseudotime positions on a broader range of cell types might be one way to explore this hypothetical model. Utilising RNA velocity comprises another computational technique that may offer alternative insights into the interconnected relationships between each Th17 population and other T cell states. We did attempt to perform this RNA velocity analysis however logistical difficulties obtaining the raw FASTQ files from the laboratory that published GSE108989 prevented us from taking this analysis further. It would also be interesting to profile the gene signatures Th17 cells within homeostatic tissue based on relative expression of AP-1 transcription factors, NR4A family member or CREM and explore

whether each putative subpopulation spatially co-localises with one another. Selectively blocking AP-1 transcription factors, NR4A members or CREM signalling in tissue-derived Th17 cells in the presence of polarising cytokine combinations could offer additional insights into the relevance of progenitor heterogeneity.

2.4.4 Do Th17CM cells represent a Th17 reservoir?

One early study exploring the natural history of Th17 cells during intranasal infection with *Listeria monocytogenes* in mice concluded that they comprise short-lived effector cells. Although robust type 17 immunity was elicited after initial infection, few antigen-specific IL-17A⁺ cells could be detected three months after the resolution of infection³⁷² and this fleeting Th17 presence was attributed to low expression of CD27, a marker of T cell longevity³⁷³. A separate report which concluded that IL-2, a cytokine that provides essential survival signals for all other T cell subsets, antagonises Th17 differentiation³⁷⁴ further eluded to the notion that type 17 immunity merely represents a fugacious cellular state.

It is likely that antigen specific Th17 cells transdifferentiated during the resolution of infection and adopted regulatory features²⁹², giving the illusion of a short-lived population if only tallying IL-17⁺ effector cells. As such, a contradictory study published only a year later reported that Th17 cells in fact express high levels *Tcf7*, self-renew and demonstrate superior persistence and anti-tumour activity in a mouse model of melanoma, as compared with Th1 cells¹⁸⁸. Since then, numerous reports of durable Th17 memory responses have been described, both in response to microbial infection^{248,375,376} and in cancer^{222,264,377}.

However, true memory reservoirs required for anamnestic immune responses not only display features of multipotency, such as *Tcf7* expression, but usually lack robust effector

functions³⁷⁸, unlike the memory Th17 cells reported in the previous studies. The more recent identification of metabolically and transcriptomically distinct CD27⁺ and CD27⁻ Th17 populations alluded to the existence of such a reservoir in Th17 cells. CD27⁺ Th17 cells were enriched in the circulation of mice, perhaps explaining why CD27⁻ cells were found in the inflamed mucosa of mice challenged with *L. monocytogenes*³⁷². CD27⁺ Th17 cells also displayed low metabolic activity and gave rise to CD27⁻ IL-17A⁺ Th17 cells, which themselves gave rise to an EAE-inducing IFN- γ ⁺ Th17 cells²²³.

To our knowledge, a true Th17 reservoir has not been described in humans. The Th17CM cells we describe are similarly enriched in the circulation, do not display prominent effector cytokine transcripts and also express high levels of *CD27* (**Fig. S2.2**). They do not express *SLAMF6*, another recently described marker in 'stem-like' Th17 cells, though this Slamf6⁺ population, whilst expressing high levels of *Tcf7*, was found in mouse intestinal tissue and secreted IL-17A¹⁶³. We therefore speculate that Th17CM cells function as a reservoir in humans, sustaining a homeostatic effector pool that itself gives rise to Th17 populations with more nuanced functions that develop in response to non-homeostatic milieu. Isolating Th17CM cells from the circulation, stimulating them with anti-CD3 antibody and overlapping the resulting cytokine signature with Th17EM cells could be one way to establish this putative relationship.

2.5 Conclusions

To our knowledge, the identification of Th17 cells by *RORC* expression in a human CRC dataset represents a novel and unbiased method to explore Th17 heterogeneity. The advent of deep sequencing methodologies has aided the discovery of numerous clinically relevant T cell

populations with remarkable granularity. Within the tumour, some of these distinct T cell adaptations mark subsets of cells particularly responsive to novel immunotherapy strategies. Therefore, understanding the functional importance of our Th17 signatures, as well as which cells they co-exist with in the niches in which they can be found, will be key to leveraging any translational benefits that may arise from their presence. These are key questions which we aim to address in the forthcoming chapters.

Chapter 3: Investigating the tumour niches in which Th17RM cells reside in

3.1 Introduction

For a given T cell to functionally contribute to an anti-tumour immune response, it is usually a prerequisite that they reside within the tumour microenvironment. However, it is becoming increasingly appreciated that simply identifying immune cells within the tumour provides limited information regarding their functional impact. Considering additional features, such as their association with certain immune populations or residence within a specific niche, can substantially impact their prognostic value. Intratumoural tertiary lymphoid structures are one good example of this; these organised, ectopic lymphoid organs found in non-lymphoid tumour tissue such as the colonic submucosa in CRC, provide a specialised niche whereby antigen-laden APCs can educate tumour infiltrating lymphocytes and foster robust, adaptive immune responses⁴⁴. Accordingly, the prognostic impact of intratumoural lymphocytes is heightened when their presence is also affiliated with the presence of tertiary lymphoid structures in a number of cancers³⁷⁹⁻³⁸⁴.

There is evidence to suggest that distinctive tumour niches can regulate the anti-tumour capacity of Th17 cells. By histologically screening a large CRC patient cohort, a recent investigation concluded that the enrichment of Th17 cells in different tumoural regions provided differing prognostic information; on one hand, positive *in situ* staining for intraepithelial IL-17⁺ CD4⁺ (Th17) cells was associated with improved prognosis by virtue of Th17-mediated recruitment of CD8⁺ T cells and CD16⁺ myeloperoxidase-secreting neutrophils¹⁹³. Contrastingly, the presence of Th17 cells embedded within the peritumoral

stroma provided no prognostic benefit, perhaps indicating that either stromal Th17 cells fail to acquire the functionalities necessary to traffic CD8⁺ T cells and neutrophils, or their residence within the stromal niche physically inhibits their recruitment towards adjacent tumour cells. Regardless, this exemplar highlights the importance of considering both the composition and organisation of the tumour microenvironment in tandem and might provide one explanation as to why such contrasting evidence exists for the role of Th17 cells in cancer.

Alterations to the cytokine milieu drive nuanced Th17 profiles. In the previous chapter, we identify three distinct Th17 profiles in human CRC, with Th17CM cells expressing CD62L, Th17EM cells expressing CD69 and Th17RM cells additionally expressing CD103 alongside CD69. Utilising pseudotime trajectory analysis, we predicted that Th17RM cells arise from a population of Th17EM cells, adopting phenotypic adaptations that permit their specialised habitation at epithelial interfaces rich with E-cadherin, the ligand for CD103. As Th17EM cells can be identified in both healthy and neoplastic tissue but Th17RM cells are specifically enriched within the tumour, we place a particular focus on what may differentially regulate the differentiation of Th17RM cells in this chapter.

In vivo, Th17 polarisation is largely attributed to cells belonging to the myeloid lineage. Both DCs and macrophages are key producers of the classic polarising combinations of IL-6 and TGF- β 1 or IL-1 β and IL-23. Like Th17 cells though, both DCs and macrophages exhibit a diverse range of phenotypes in cancer, with each phenotype associated with a similarly broad amalgamation of cytokines, co-stimulatory and immunosuppressive molecules that have the potential to differentially shape and refine Th17 tumour immunity³⁸⁵⁻³⁸⁷.

A clear understanding of the cell types that Th17EM and Th17RM cells interact with and establishing whether their transcriptomic differences are a product of regional enrichment will be key to modulating these populations therapeutically.

3.2 Aims

In this chapter, we aim to characterise the cell types that co-localise with the non-circulating Th17 phenotypes described in Chapter 3, placing a particular focus on tumour Th17RM cells due to their apparent absence within homeostatic tissue. Using ligand-receptor analysis algorithms, we aim to define the ligands responsible for the acquisition of each phenotype and intend to use these ligand directories to predict which patients may exhibit Th17RM enrichment. Finally, we aim to validate the intercellular interactions predicted by ligand-receptor analysis using techniques such as spatial transcriptomics and multiplex fluorescence imaging.

3.3 Results

3.3.1 Using cellular deconvolution to explore the immune landscape of colon adenocarcinoma patients

The T cell enriched scRNA-seq dataset curated by Zhang *et al.* (GSE108989)¹ was informative due to the depth of sequencing and large number of cells used. However, to explore the cellular composition of the tumours in which Th17EM and Th17RM cells can be found, a dataset containing a broader range of cell types is required.

To initially explore the broad immune landscape that associate with Th17EM or Th17RM cells in the tumour, we exploited the substantial amount of publicly available data stored in The

Cancer Genome Atlas colon adenocarcinoma (TCGA-COAD) repository³⁸⁸. The TCGA-COAD repository contains bulk RNA-seq information from the resected tumours of 522 colon adenocarcinoma patients, providing us with information about the broad RNA signatures expressed in each tumour in conjunction with a considerable number of patients for reliable statistical analysis.

We first deconvoluted the bulk RNA-seq information from each patient using CIBERSORTx, a computational tool which can infer cell type abundance from bulk RNA-seq profiles³⁸⁹, and predicted the tumour immune landscape per patient. We used the LM22 immune signature matrix to deconvolute each sample, a specially developed gene expression matrix for use with CIBERSORTx which contains the transcriptional signatures of 22 immune cells from both myeloid and lymphoid cell lineages³⁹⁰ (**Fig. S3.1**).

We next planned to curate uniquely identifying gene signatures for Th17EM and Th17RM cells which would be used to score each patient using GSVA, a method which estimates the variation of gene sets over a sample population in an unsupervised manner³⁹¹. TCGA-COAD patients would then be divided into equal tertiles based on their Th17EM or Th17RM signature score, with the associated immune landscapes from those in the lowest and highest tertiles compared against each other (**Fig. 3.1**)

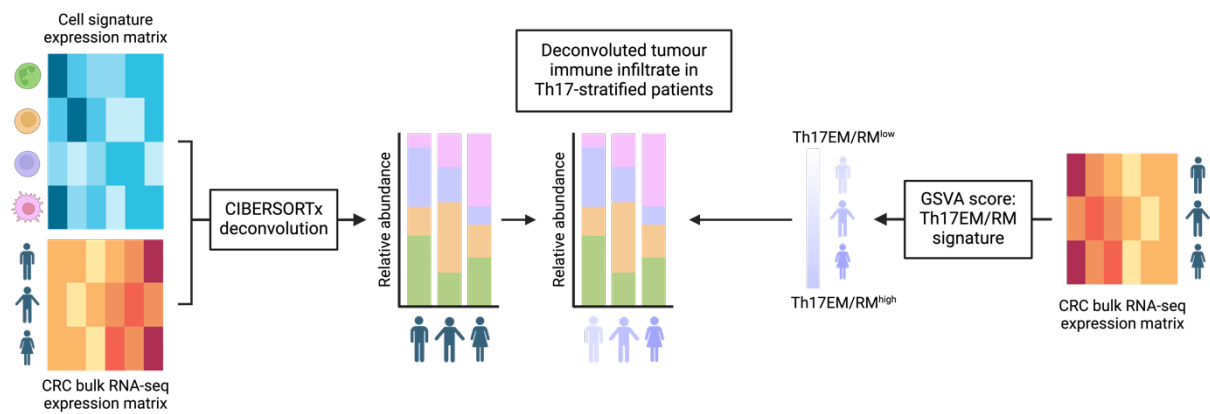


Figure 3.1 | Deconvoluting tumour immune cell infiltrate in colon adenocarcinoma patients. A schematic of the workflow used to determine the broad immune cell landscape associated with the presence of Th17 cells in colon adenocarcinoma patients. Patient scoring using a Th17RM gene signature is used as an example in the schematic. CIBERSORTx is first used to deconvolute bulk RNA-seq data from The Cancer Genome Atlas colon adenocarcinoma (TCGA-COAD) cohort using a cell signature expression matrix specifically designed for utilisation with CIBERSORTx (LM22). CIBERSORTx then outputs the relative abundance of each cell type per patient, who is then scored based on their expression of a curated Th17RM signature. The immune landscape of those expressing the lowest Th17RM signature score (Th17RM^{low}) is finally compared with that seen in the highest scoring patients (Th17RM^{high}).

3.3.2 Curating Th17EM and Th17RM cell gene signatures

To curate the Th17 signatures that we would use to score each TCGA-COAD patient with, differential gene expression analysis was first performed using the original GSE108989 scRNA-seq dataset, comparing Th17EM or Th17RM cells with all non-Th17EM/RM cells, grouped together and collectively termed “other T cells”. For each Th17 population, we combined the markers validated at protein level (ROR γ t with CD69 or CD103) with the top five most differentially expressed genes at the transcriptomic level to curate each respective Th17 signature.

For Th17EM cells, the five most differentially upregulated genes consisted of *IL22*, *CCL20*, *IL17F*, *IL17A* and *CD69*, and we combined these transcriptional outputs with ROR γ t (*RORC*) to make a six gene Th17EM cell signature (**Fig. 3.2A**). To validate the specificity of this signature, we scored each cell in GSE108989 using the *AddModuleScore* function in the Seurat package from R and contrasted the scores for each cell type annotation. Our six gene Th17EM signature was able to significantly differentiate Th17EM cells from all other T cell subsets (**Fig. 3.2B**), validating its reliability.

We next repeated this process for Th17RM cells and found that the five most differentially upregulated genes were *IL17A*, *KLRB1* (CD161), *CTSH* (cathepsin H), *CXCR6* and *LGALS3* (galectin-3) (**Fig. 3.3A**). In combination with the ROR γ t and CD103 (*ITGAE*) validated at the protein level, this seven gene Th17RM signature was similarly able to differentiate Th17RM cells from all other T cells in GSE108989 (**Fig. 3.3B**).

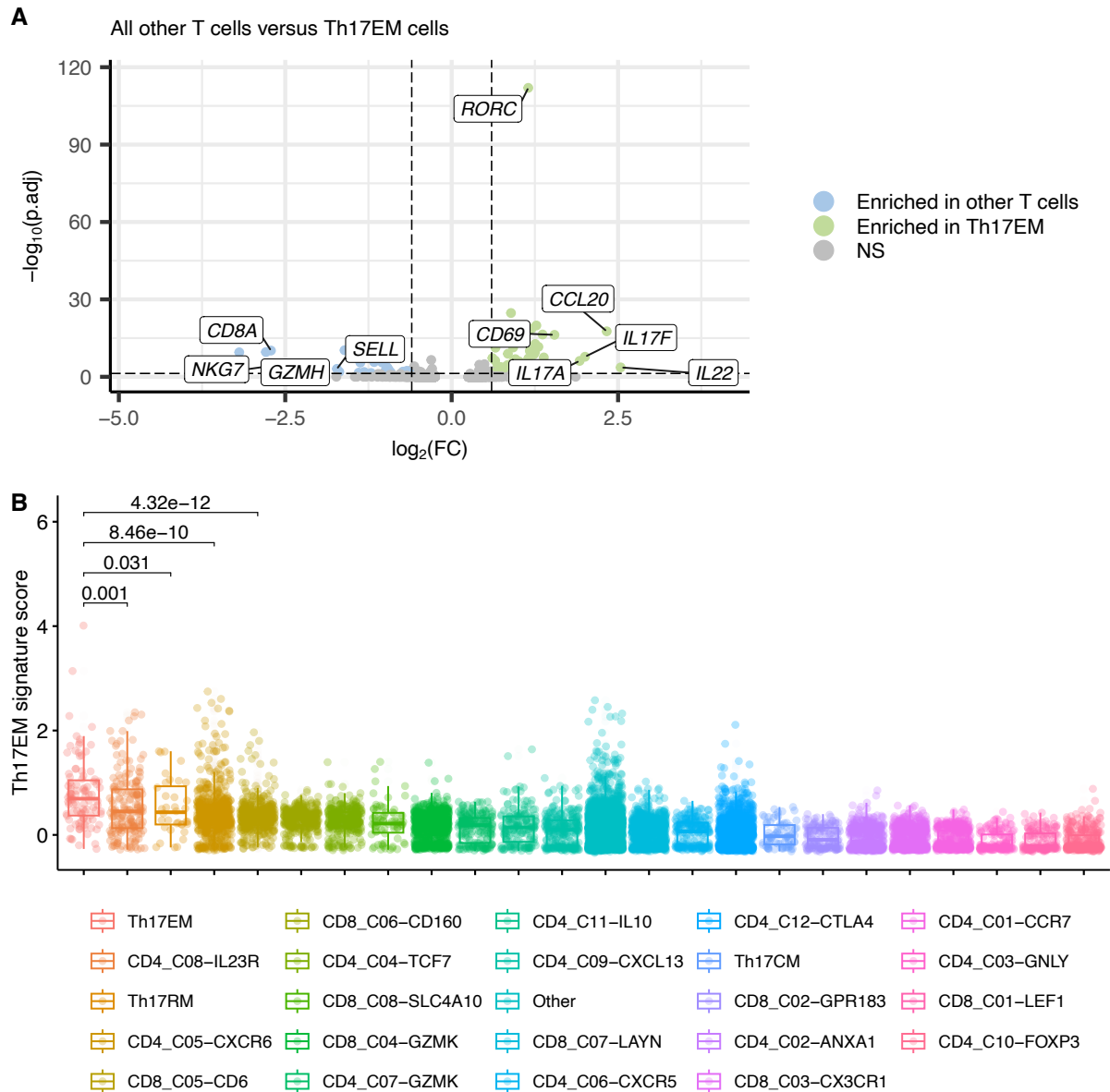


Figure 3.2 | Curating a Th17EM cell signature. Differential gene expression analysis was performed by comparing Th17EM cells with all other T cell clusters in GSE108989. **(A)** Volcano plot displaying the differentially expressed genes. Green dots represent genes differentially enriched in Th17EM cells and blue dots represent genes differentially enriched in non-Th17EM (other) T cells. Wilcoxon rank sum test with Bonferroni correction was used to identify differentially upregulated genes in Th17EM cells, with $\log_2$ fold change (FC) ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. Dashed lines are drawn where $\log_2$ fold change = 0.5 and $p_{\text{adj}} = 0.05$. **(B)** Each cluster was scored using the six gene Th17EM signature (*RORC*, *CD69*, *IL22*, *CCL20*, *IL17F*, *IL17A*). Signature scores are displayed as box and

whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cluster. Each data point represents an individual cell. Pairwise Wilcoxon tests with Benjamini-Hochberg correction were used to calculate the statistical comparisons, with Th17EM cells set as the reference population. The four least statistically significant adjusted p values are displayed. $p_{\text{adj}} \leq 0.05$ is considered statistically significant.

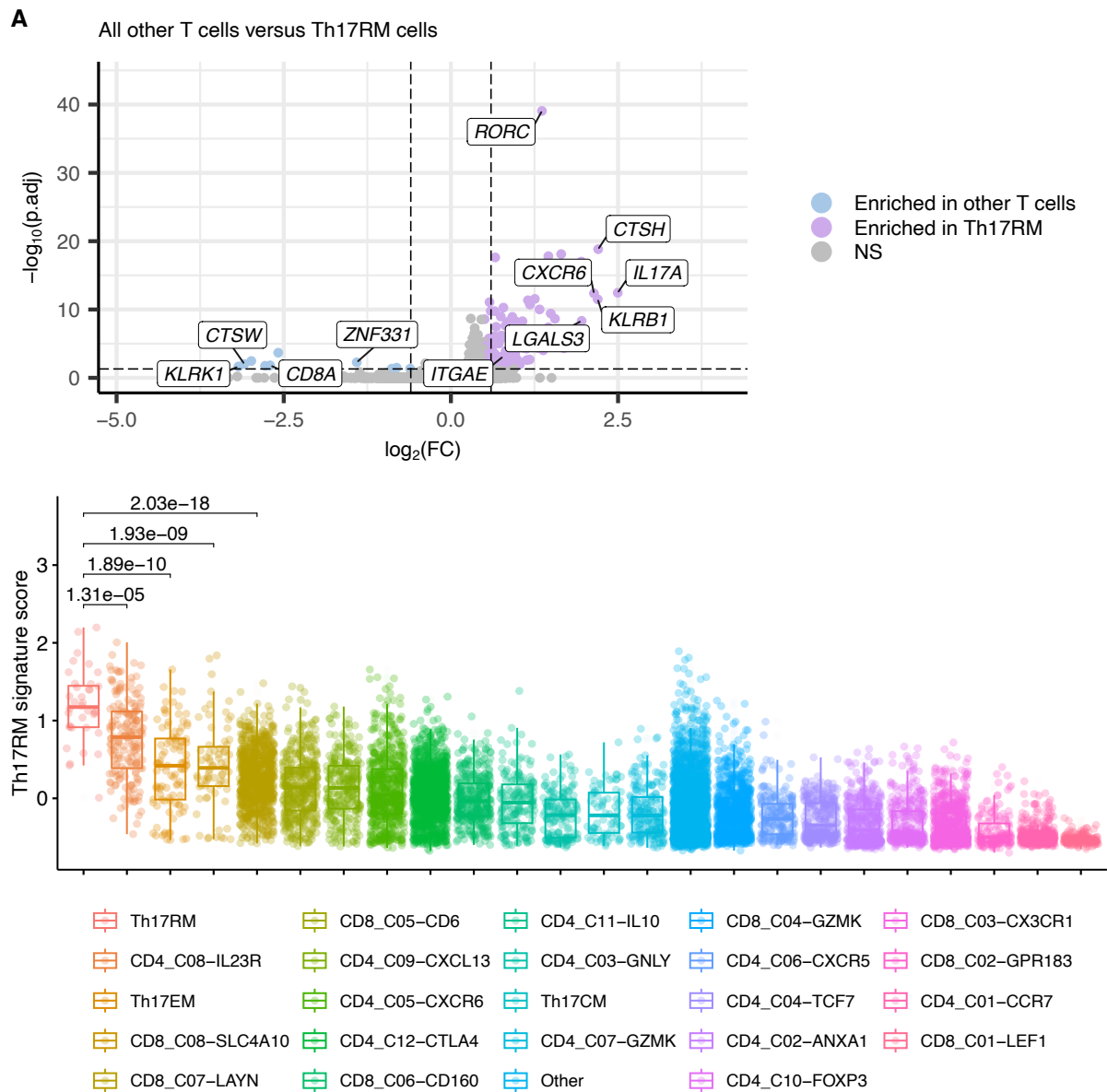


Figure 3.3 | Curating a Th17RM cell signature. Differential gene expression analysis was performed by comparing Th17RM cells with all other T cell clusters in GSE108989. **(A)** Volcano plot displaying the differentially expressed genes. Purple dots represent genes differentially

enriched in Th17RM cells and blue dots represent genes differentially enriched in non-Th17RM (other) T cells. Wilcoxon rank sum test with Bonferroni correction was used to identify differentially upregulated genes in Th17RM cells, with $\log_2$ fold change (FC) ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. Dashed lines are drawn where $\log_2$ fold change = 0.5 and $p_{\text{adj}} = 0.05$. **(B)** Each cluster was scored using the seven gene Th17RM signature (*RORC*, *ITGAE*, *IL17A*, *CTSH*, *KLRB1*, *CXCR6*, *LGALS3*). Signature scores are displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cluster. Each data point represents an individual cell. Pairwise Wilcoxon tests with Benjamini-Hochberg correction were used to calculate the statistical comparisons, with Th17RM cells set as the reference population. The four least statistically significant adjusted p values are displayed. $p_{\text{adj}} \leq 0.05$ is considered statistically significant.

3.3.3 Th17EM and Th17RM cells might feature as part of different tumour immune responses

To explore the immune landscape of patients with high or low levels of Th17EM or Th17RM cells based on signature expression in bulk RNA-seq data from the tumour, we chose to compare patients in the lowest scoring tertile with those in the highest. This would maximise the differences observed and give a more reliable indication as to which populations were alternatively enriched.

The immune landscape in patients with high Th17EM signature score (Th17EM^{high}) seemed to capture a humoral tumour response, with activated CD4⁺ T cells, dendritic cells, plasma cells and Tfh cells displaying the most significant levels of enrichment (**Fig. 3.4A**). Together, these cells constitute major cell type components found within mature tertiary lymphoid structures, which are usually associated with more favourable prognosis in a variety of cancer types³⁹². Conversely, unpolarised (M0) macrophages and resting mast cells were more enriched in

patients with a lower Th17EM signature score (Th17EM^{low}), indicative of a homeostatic epithelial barrier.

In patients with a high Th17RM signature score (Th17RM^{high}), the immune landscape was considerably altered. Most prominently in the myeloid compartment, patients had far more resting dendritic cells than those with a low Th17RM signature score (Th17RM^{low}). These cells were perhaps replaced with those from the macrophage lineage as a significant increase in the proportion of activated macrophages, displaying both inflammatory (M1) and anti-inflammatory (M2) phenotypes, was seen (**Fig. 3.4B**). A higher proportion of resting mast cells was also observed in Th17RM^{high} patients, as well as infiltration of CD4⁺, CD8⁺ and $\gamma\delta$ T cells subsets, which may indicate more robust activation of the cellular arm of adaptive tumour immunity.

Consistent across both Th17EM^{high} and Th17RM^{high} cohorts was the upregulation activated CD4⁺ T cells, Tfh cells and Treg cells. Generally, the presence of Treg cells in the tumour microenvironment is indicative of hindered immunosurveillance, largely due to their ability to reduce APC-mediated effector T cell activation, production of large quantities of immunosuppressive adenosine, ability to starve effector T cells of the stimulatory cytokine IL-2 and by direct killing of anti-tumour immune populations via secreted granzymes and perforin³⁹³. Together, these effector functions can promote development and progression of tumours. However, whilst deconvolution techniques such as CIBERSORTx are able to hint at the presence of immune populations within the tumour, one major limitation is their inability to allude to cellular tissue functions. In the presence of high levels of type I (IFN- α , IFN- β) or type II (IFN- γ) interferons, for example, Treg cells exhibit heightened functional fragility which can instead result in augmented anti-tumour immunity^{394,395}. Therefore, the enhanced

presence of Treg cells in Th17EM^{high} and Th17RM^{high} patients does not necessarily stipulate immunosuppression and establishing which cell types that Treg cells in Th17EM^{high} and Th17RM^{high} patients co-localise with would be more informative, as the type I interferons from the M1 macrophages or type II interferons from the CD8⁺ T cells similarly enriched in Th17RM^{high} patients could negate any suppressive Treg activity.

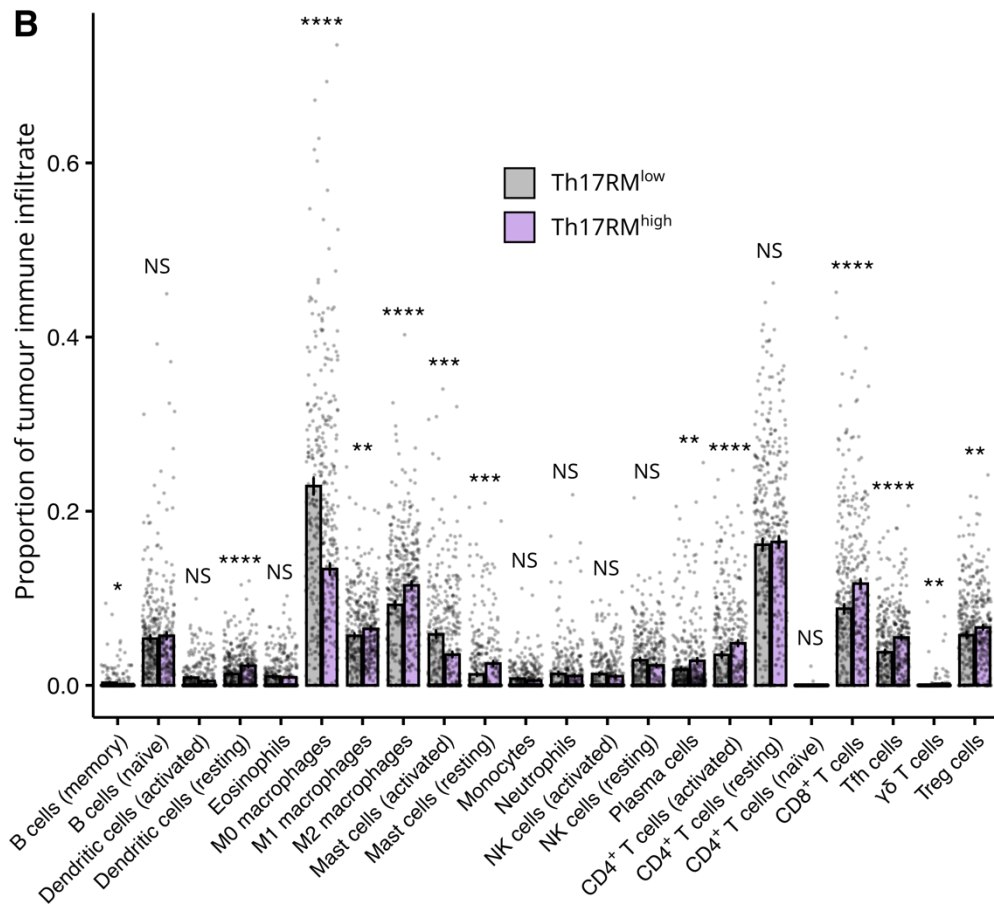
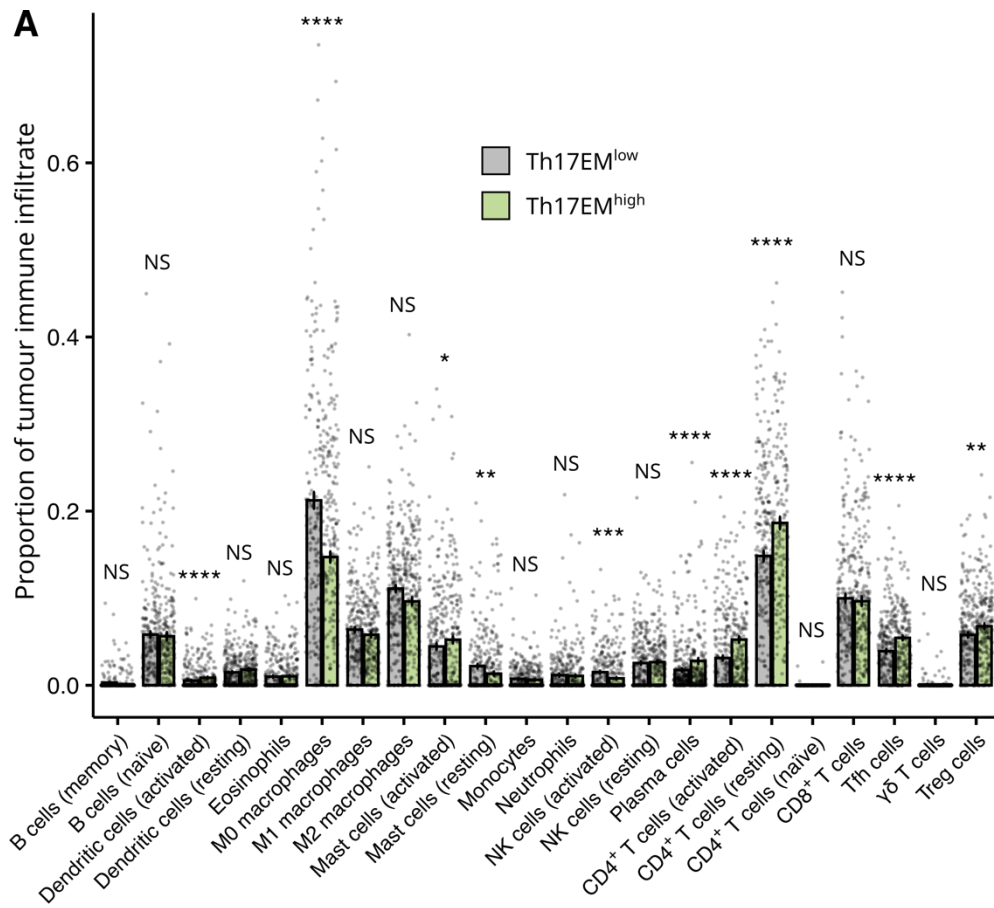


Figure 3.4 | Th17EM and Th17RM cells are associated with different immune landscapes in TCGA-COAD patients. GSVA was used to score patients from The Cancer Genome Atlas colon adenocarcinoma (TCGA-COAD) cohort based on the expression of our curated six gene Th17EM cell signature or seven gene Th17RM cell signature. This signature score was then used to divide the cohort into equal tertiles, with patients in the lowest tertile termed Th17EM/RM^{low} and those in the highest scoring tertile named Th17EM/RM^{high}. Direct comparison of cell type proportions in TCGA-COAD patients based on (A) Th17EM signature or (B) Th17RM signature. Wilcoxon test was performed for statistical analysis. Mean and standard error are displayed. NS (not significant), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

3.3.4 Identification of Th17 cells in a cell type replete scRNA-seq dataset

To unbiasedly investigate intercellular communication networks that exist between Th17 cells and their interacting counterparts by scRNA-seq, we were required to identify each Th17 population in a dataset containing all cell types. Our original scRNA-seq, GSE108989, was not sufficient as it was enriched for T cells.

We therefore selected another publicly available dataset, GSE146771, for this analysis. GSE146771 was curated by sequencing both the CD45⁺ and CD45⁻ components of blood, healthy normal adjacent and tumour tissue from 18 CRC patients using Smart-seq2, totalling 10,468 cells². Epithelial cells (hE), malignant cells (hC), fibroblasts (hF), myeloid cells (hM), B cells (hB), ILCs (hI), CD4⁺ and CD8⁺ T cells (hT) were captured in the sequencing (**Fig. S3.2**).

To identify putative Th17 cells, the same clustering strategy was employed as described in Chapter 3. *RORC*⁺*CD3D*⁺ cells were firstly subset and clustered before differential gene expression analysis was performed (**Fig. 3.5A**), which identified communities of Th17CM-, Th17EM- and Th17RM-like cells. Th17RM-like cells high expressed high levels of *IL23R* with

low levels of *FOXP3*, whilst Th17EM-like cells conformed to a profile intermediate to that of *LEF1*⁺ Th17CM-like and Th17RM-like cells (**Fig. 3.5B**). To confirm the alignment of these Th17-like populations to our previous Th17 annotations, we projected the newly curated signatures onto each of the *RORC*⁺*CD3D*⁺ cells. The Th17RM signature was significantly enriched in Th17RM-like cells in this new dataset (**Fig. 3.5C, 3.5D**). However, the Th17EM signature failed to differentiate between Th17EM-like and Th17RM-like cells, indicating a stronger transcriptional overlap between these two clusters in this dataset (**Fig. 3.5E**).

After the clustering of *RORC*⁺*CD3D*⁺ cells in our original scRNA-seq, we encountered some contamination from extraneous cell types which required further subsetting and re-clustering to remove. Given the considerable number of cells present in our new, putative Th17-like clusters, we speculated that a similar contamination had occurred and therefore repeated the process of subsetting and re-clustering each Th17-like cluster. Differential gene expression was then re-performed to aid with annotating the new output clusters.

As predicted, a number of contaminants were identified, including Tfh cells, another CD8⁺ population and two communities of Treg cells (**Fig. 3.6A, 3.6B**). To again confirm the robustness of our Th17 annotations, we projected each signature onto the cells. The Th17RM signature was again most associated with Th17RM-like cells (**Fig. 3.6C, 3.6D**) and the Th17EM signature similarly failed to distinguish between Th17EM and Th17RM cells (**Fig. 3.6E**).

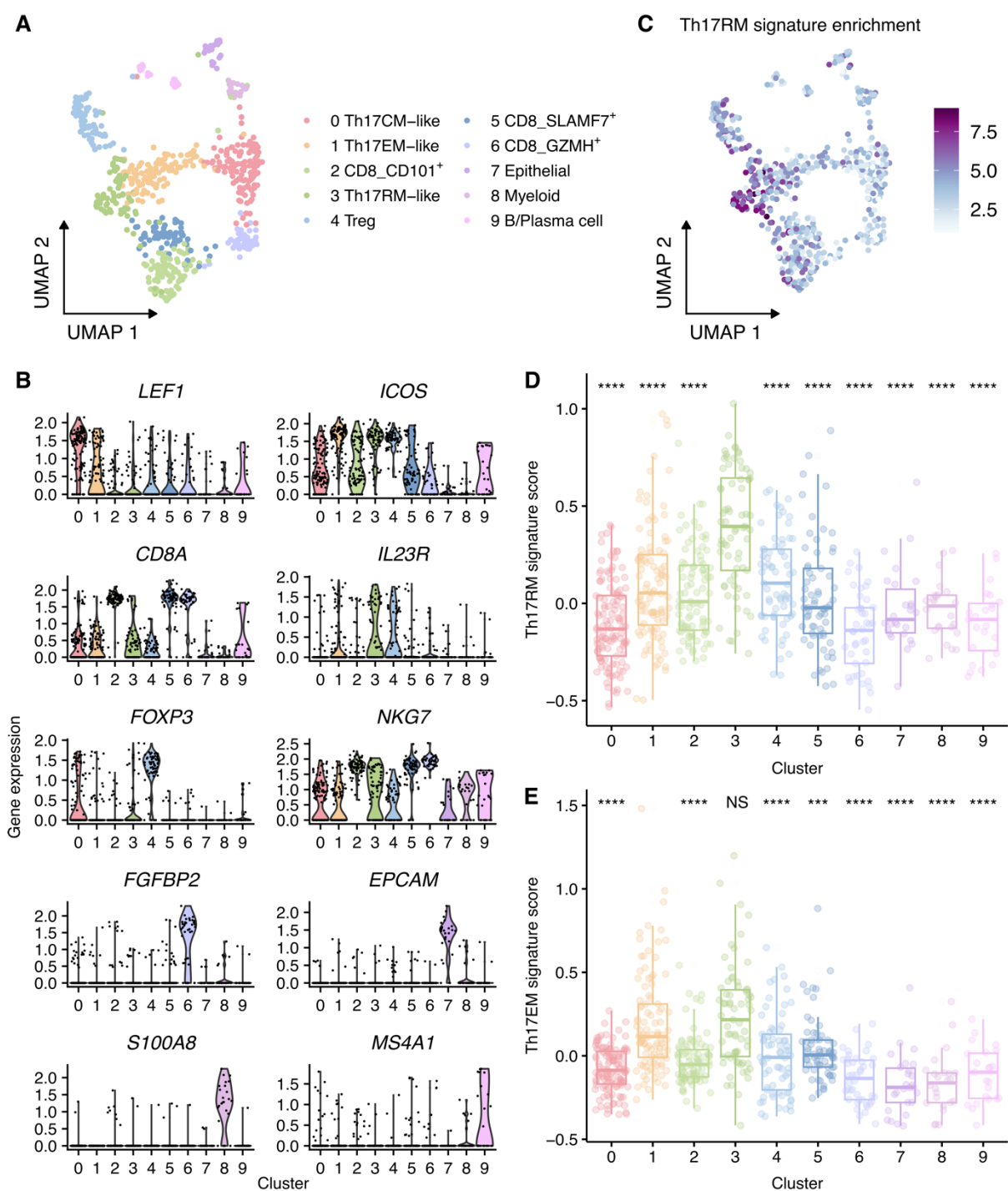


Figure 3.5 | Identification of Th17RM-like cells in a validation cohort. To validate the existence of Th17RM cells in a second cohort, cells expressing both *RORC* and *CD3D* were subset out of the scRNA-seq dataset GSE146771. **(A)** UMAP of *RORC*⁺ *CD3D*⁺ cells with cluster annotations based on the output of differential gene expression analysis. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed

genes, with $\log_2$ fold change ≥ 0.5 and $p.\text{adj}$ (adjusted p value) ≤ 0.05 considered statistically significant. **(B)** Violin plots of differentially expressed genes. **(C)** Cells were scored using the seven gene Th17RM signature as curated previously with the *AddModuleScore* function in the Seurat package. Relative score enrichment is denoted by the colour gradient and displayed by UMAP. **(D)** Th17RM and **(E)** Th17EM signature scores displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cluster. Each data point represents an individual cell. Pairwise Wilcoxon tests with Benjamini-Hochberg correction were used to calculate the statistical comparisons, with (D) Th17RM and (E) Th17EM cells set as the reference population. The four least statistically significant adjusted p values ($p.\text{adj}$) are displayed. $p.\text{adj} \leq 0.05$ is considered statistically significant. The colours used in (B), (D) and (E) match the colours assigned to each cluster in (A).

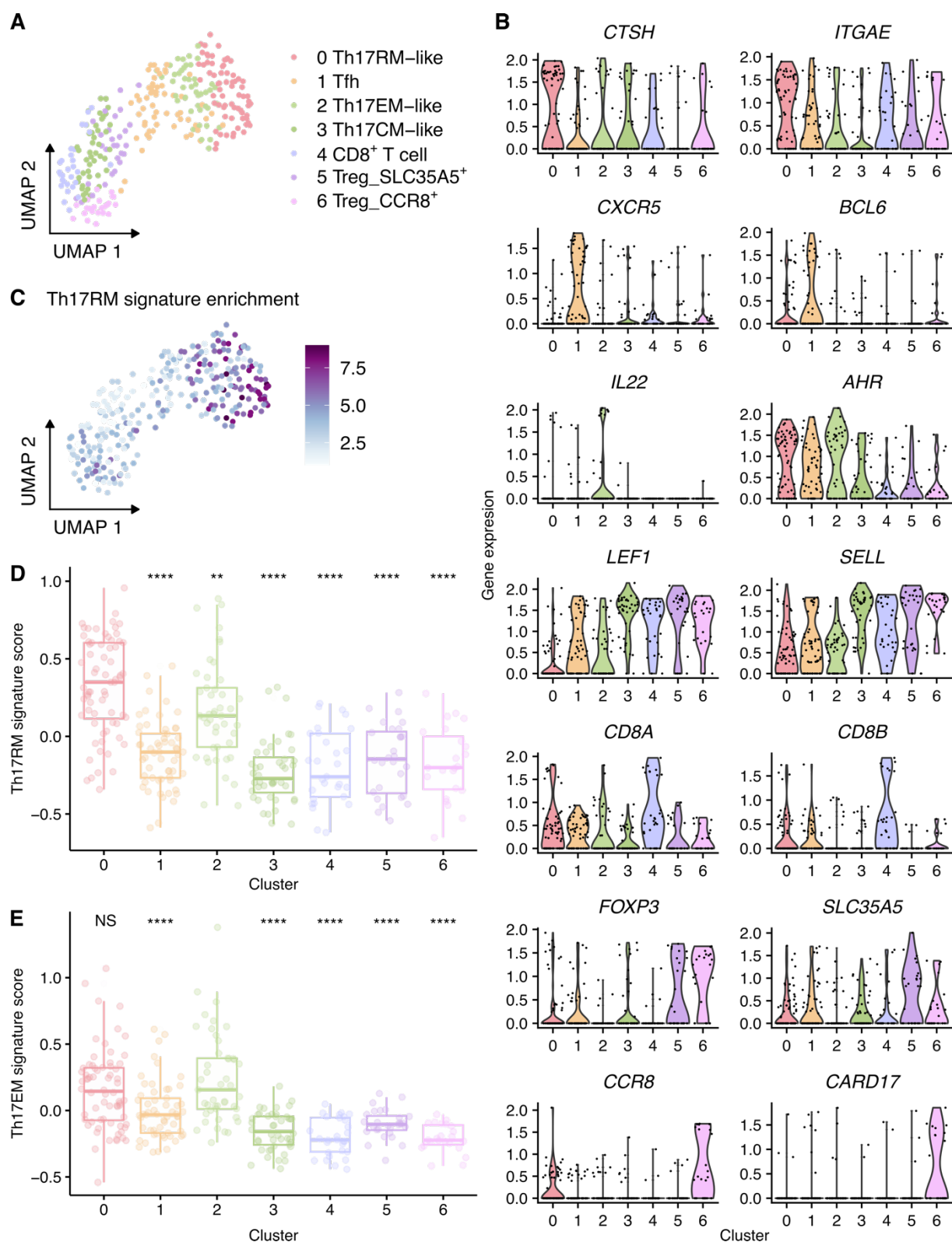


Figure 3.6 | Refining the selection of Th17RM cells in GSE146771. To remove contaminant cells from the previously characterised Th17RM-like population, a second round of clustering was performed. **(A)** UMAP of Th17RM-like cells re-clustered and re-annotated based on the

output of differential gene expression analysis. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed genes, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. **(B)** Violin plots of differentially expressed genes. **(C)** Cells were scored using the seven gene Th17RM signature as curated previously with the *AddModuleScore* function in the Seurat package. Relative score enrichment is denoted by the colour gradient and displayed by UMAP. **(D)** Th17RM and **(E)** Th17EM signature scores displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cluster. Each data point represents an individual cell. Pairwise Wilcoxon tests with Benjamini-Hochberg correction were used to calculate the statistical comparisons, with (D) Th17RM and (E) Th17EM cells set as the reference population. The four least statistically significant adjusted p values (p_{adj}) are displayed. $p_{\text{adj}} \leq 0.05$ is considered statistically significant. The colours used in (B), (D) and (E) match the colours assigned to each cluster in (A).

Finally, to validate that the tissue enrichment of each Th17 phenotype held true in this new scRNA-seq dataset, each cell was plotted on a UMAP (**Fig. 3.7A**) and the sample location of origin was overlaid (**Fig. 3.7B**). In line with that seen in the original GSE108989 dataset, Th17CM cells in GSE146771 were restricted to the blood, whilst Th17EM cells were the most dominant Th17 population in healthy normal adjacent tissue. Despite finding a few Th17RM cells in healthy tissue, the vast majority were found within in the tumour (**Fig. 3.7B**), in agreement with our *ex vivo* findings (**Fig. 3.11D**). One reason for the detection of these Th17RM cells in normal tissue could be a sampling issue, as the healthy adjacent tissue captured may have not been peripheral enough from the tumour site.

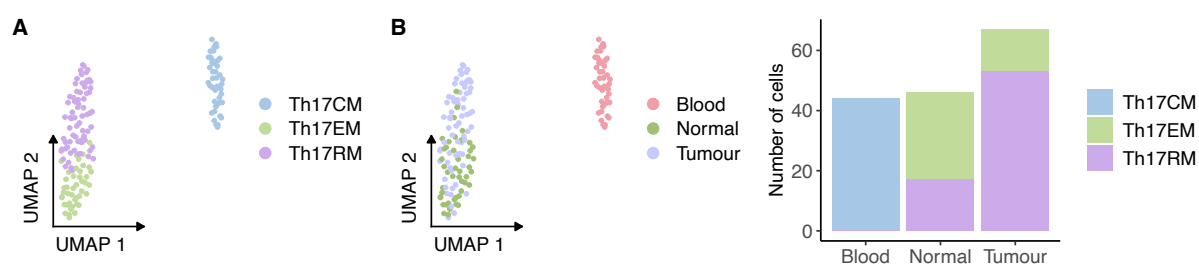


Figure 3.7 | Th17 cells in GSE146771 share the same tissue enrichments. (A) Th17CM, Th17EM and Th17RM clusters displayed by UMAP. **(B)** Qualitative and quantitative tissue distributions for each Th17 population.

As a final check to validate the reliability of our Th17 annotations, each cell in our new scRNA-seq dataset was scored with the two Th17 signatures. As shown previously, many Th17RM cells also scored highly for the Th17EM signature, although the signature was able to distinguish Th17EM cells from all the other cell types (**Fig. 3.8**).

Despite our Th17RM signature reliably distinguishing Th17-like cells in this new dataset, when projected against all the existing cellular annotations we noticed that it was unable to distinguish Th17RM cells from a population of CD4⁺ T cells expressing *IL23R* (hT08_CD4-IL23R) (**Fig. 3.9**). One explanation could be that some cells annotated as hT08_CD4-IL23R also express ROR γ t but *RORC* transcripts were not detected by the sequencing given the established difficulty detecting transcription factors by scRNA-seq.

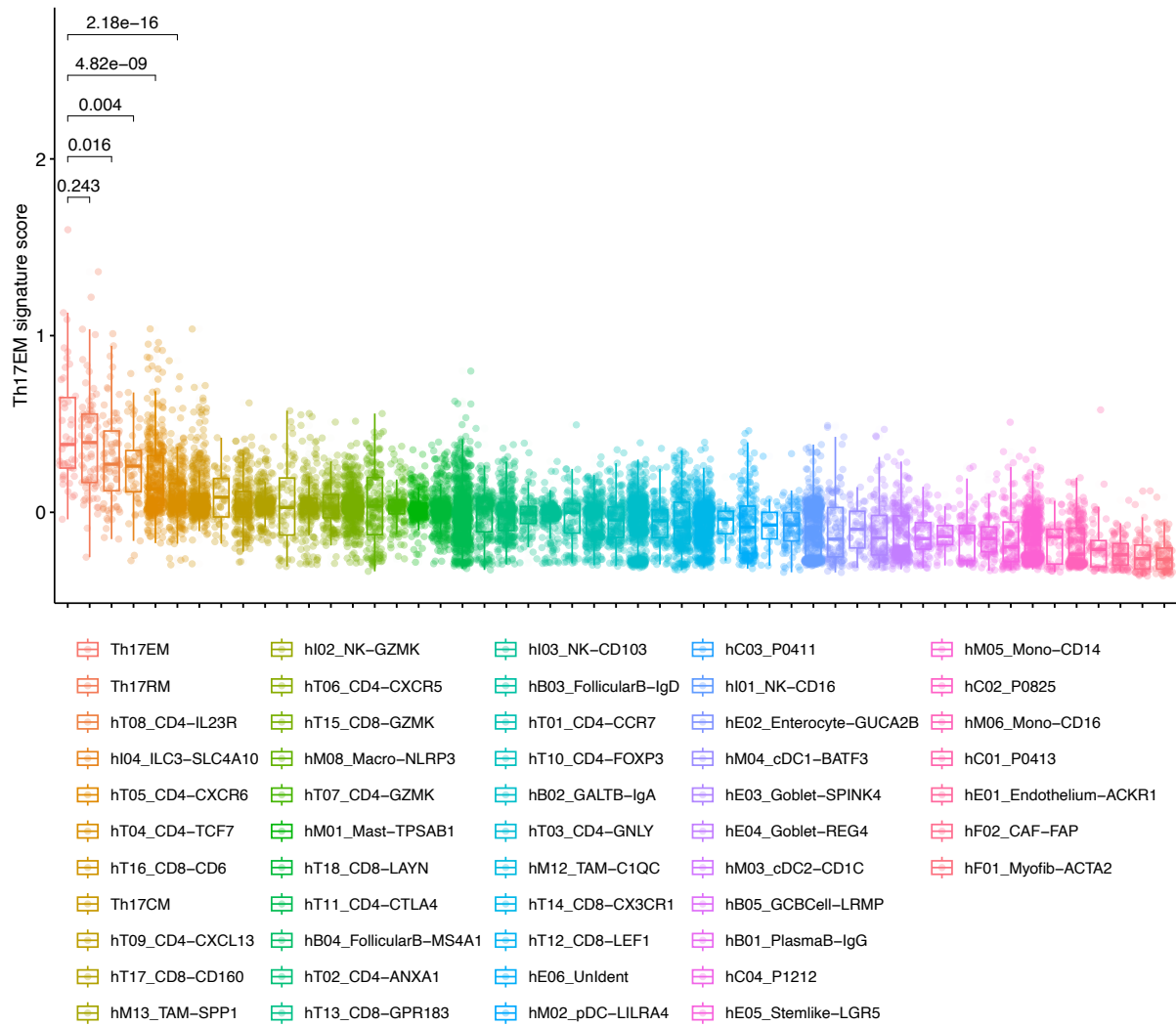


Figure 3.8 | Projecting the six gene Th17EM signature into GSE146771. Clusters in GSE146771 were scored using the six gene Th17EM signature as curated previously with the *AddModuleScore* function in the Seurat package. Th17EM signature score displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cluster. Each data point represents an individual cell. Pairwise Wilcoxon tests with Benjamini-Hochberg correction were used to calculate the statistical comparisons, with Th17EM cells set as the reference population and an adjusted p value ≤ 0.05 is considered statistically significant. The five least statistically significant pairwise comparisons are displayed.

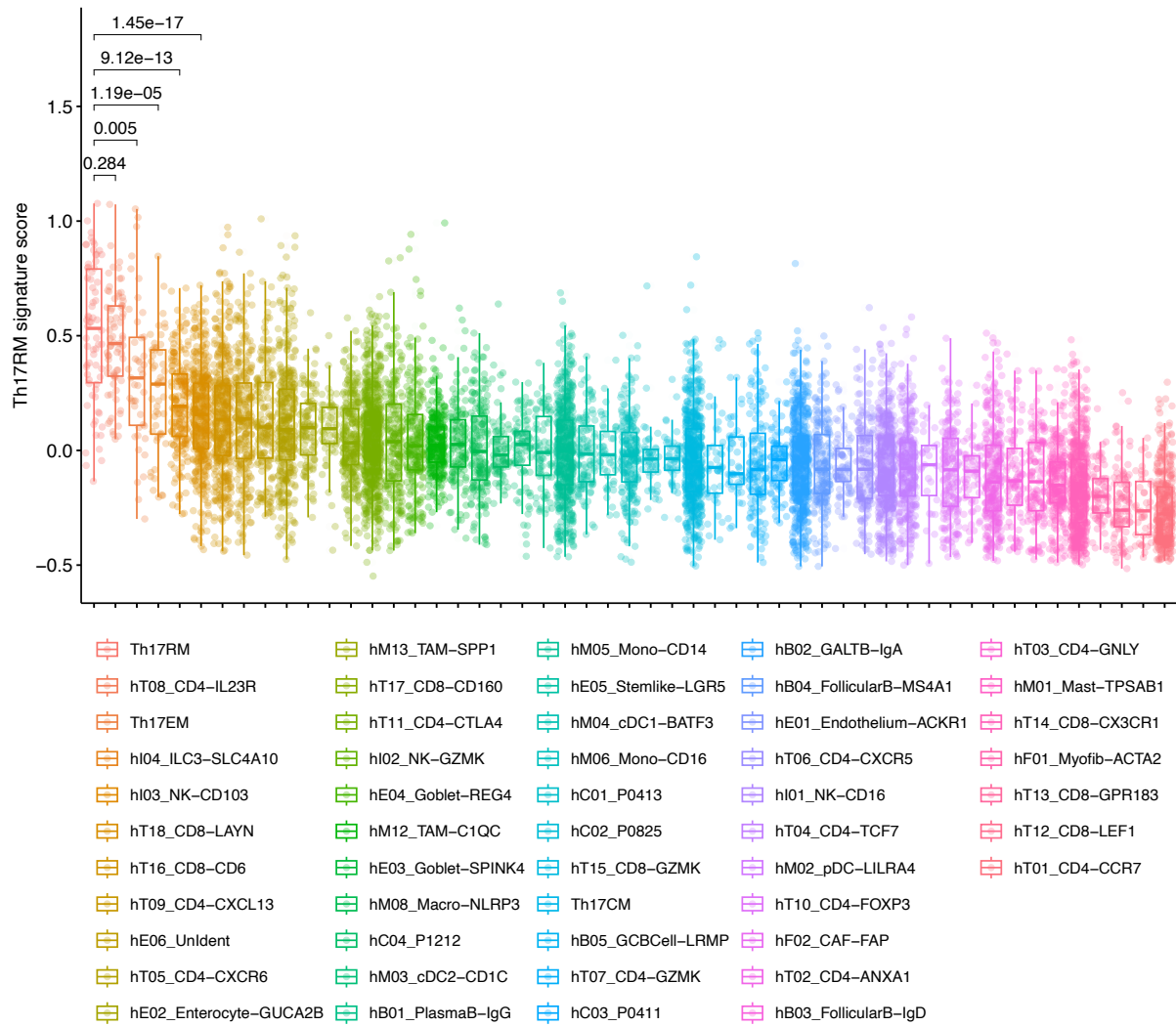


Figure 3.9 | Projecting the seven gene Th17RM signature into GSE146771. Clusters in GSE146771 were scored using the seven gene Th17RM signature as curated previously with the *AddModuleScore* function in the Seurat package. Th17RM signature score displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cluster. Each data point represents an individual cell. Pairwise Wilcoxon tests with Benjamini-Hochberg correction were used to calculate the statistical comparisons, with Th17RM cells set as the reference population and an adjusted p value ≤ 0.05 is considered statistically significant. The five least statistically significant pairwise comparisons are displayed.

3.3.5 Th17RM cells are driven by a highly inflammatory tumour milieu

The consequence of exposure to variable ligand combinations during Th17 polarisation is well established. It is therefore likely that the unique transcriptional signatures attributed with Th17EM and Th17RM cells arise from different ligand interactions within distinct cellular niches. To better understand these phenotypic drivers, as well as garnering information regarding the chemokine gradients which may recruit these Th17 cells and the cell types from which they originate, we employed NicheNet, a ligand-receptor network interaction package. Similar to other ligand-receptor network analysis tools, such as CellPhoneDB³⁹⁶ or CellChat³⁹⁷, NicheNet utilises a public repository of known ligands, receptors and their documented interactions to quantitatively infer intercellular communication networks from scRNA-seq data.

In principle, the receptors expressed by each Th17 cell type are matched with sender cells expressing their cognate ligands, enabling the computational elucidation of communication networks. Unlike CellPhoneDB or CellChat, NicheNet has the additional benefit of calculating and accounting for active signal transduction networks within the recipient cell, incorporating this information to generate a continuous score for the most likely ligand-receptor interactions³⁹⁸. For example, if *IL23R* and *IFNGR1* are equally expressed in Th17RM cells and *IL23A* and *IFNG* transcripts are detected at equal levels in macrophages and CD8⁺ T cells, respectively, CellPhoneDB and CellChat would conclude that both ligands equally contribute to the Th17RM expression profile. However, if target genes downstream of IL-23 signalling are more upregulated than those downstream of IFN- γ signalling, as informed by the literature, NicheNet would infer that IL-23 plays a stronger role than IFN- γ in dictating the Th17RM transcriptional programme.

To capture as many reliable intercellular interactions as possible, every cell type in GSE146771 was set as a potential sender population with Th17EM or Th17RM cells set as the recipient population. Ligand activity was defined as the Pearson correlation coefficient between the vector of the recipient cell gene signature and the target genes score for that ligand, with a correlation coefficient over 0.075 used as a threshold to define active ligands based on recent utilisation of this technique³⁹⁹.

Initially focussing on Th17RM cells due to their enrichment within the tumour, we found that a highly inflammatory tumour milieu was associated with their phenotype, with the highest ranking correlation coefficients belonging to IL-23, IFN- γ , IL-1 β , TNF, CCL2, IL-6, TGF- β 1, IL-18, ICAM-1 ADAM17 and CD40 (**Fig. 3.10A**), as determined by the combined scoring of ligand-Th17RM receptor interactions (**Fig. 3.10B**) and downstream target gene expression (**Fig. 3.10C**).

A number of these ligands have previously been associated with influencing nuanced Th17 programmes. Together, IL-6 and TGF- β 1 instruct the secretion of IL-17 in CD4⁺ T cells and the addition of IL-23 and IL-1 β stimulates additional pro-inflammatory characteristics *in vitro* including the secretion of IFN- γ , GM-CSF and TNF- α ^{115,146}. The CCR2/CCL2 axis drives Th17 recruitment into the CNS during late, but not early, EAE and it is thought that persistent TCR stimulation plus IL-23 exposure are prerequisite conditions for CCR2 upregulation on the surface of Th17 cells⁴⁰⁰.

Meanwhile, the role of IL-18 in Th17 biology is less well understood. IL-18, an inflammasome-driven member of the IL-1 family of cytokines, initially binds to the IL-18R α subunit (encoded by *IL18R1*), which then recruits the IL-18R β subunit (*IL18RAP*) before the complete, high

affinity heterodimeric receptor can initiate downstream signalling cascades⁴⁰¹⁻⁴⁰⁴. Co-culturing naïve CD4⁺ T cells in Th17 polarising conditions in the presence of IL-18 somewhat reduces the number of IL-17⁺ cells produced as compared to those polarised in the absence of IL-18⁴⁰⁵, although a considerable number of Th17 cells can still be differentiated and colonic inflammation is not reduced in *Il18r1*^{-/-} mice after the induction of experimental colitis, suggesting that IL-18 might not functionally impact the acquisition of inflammatory properties⁴⁰⁵. It has been speculated that IL-18 might partially antagonise IL-1 β driven Th17 polarisation, as Th17 count remains unchanged in *IL1r1*^{-/-} or *Myd88*^{-/-} CD4⁺ T cells⁴⁰⁵, however Th17RM cells appear to respond to IL-1 β (**Fig. 3.10A**), suggesting that IL-18 may function differently in human Th17RM cells.

IL-18 is predicted to control the transcription of *CCL5*, *CCL20* and *IFNG* in Th17RM cells (**Fig. 3.10C**), which could collaboratively promote the recruitment and activation of CCR5⁺ (receptor for CCL5) CCR6⁺ (receptor for CCL20) CD8⁺ T cells into the tumour, a population which has been shown to be highly anti-tumourigenic in CRC¹⁹³. Furthermore, how IL-18 ligation can modulate the phenotype of already differentiated Th17 cells, rather than that of naïve T cells⁴⁰⁵, also remains undetermined and will be a crucial to test the activity of these ligands in future *in vitro* experiments. In CD8⁺ T cells, IL-18 functions to amplify the production of IFN- γ ⁴⁰⁶⁻⁴⁰⁸ and works synergistically with other cytokines such as IL-12 to elicit the strongest production of IFN- γ ^{402,409}. In our NicheNet output, *IFNG* (IFN- γ) is both predicted to be upregulated in response to IL-18 (**Fig. 3.10C**) and predicted to influence the Th17RM transcriptional programme (**Fig. 3.10B**), hinting that IL-18 may function to initiate an autocrine positive feedback cycle that strengthens the Th17RM phenotype.

The high correlation coefficient of ADAM17 (a disintegrin and metalloproteinase 17) was also surprising. Although ADAM17 has been previously implicated in Th17 biology, it does not influence their phenotype by direct interaction. ADAM17 functions to cleave membrane IL-6R into its soluble form which facilitate IL-6 trans-signalling, a form of IL-6 signalling that can exacerbate inflammatory processes by expanding the range of cells capable of responding to IL-6 beyond those expressing IL-6R⁴¹⁰. Blocking IL-6 trans-signalling does inhibit the expansion of Th17 cells *in vitro*⁴¹¹ although it is thought that classical IL-6 signalling is primarily required for the maintenance of inflammatory Th17 characteristics⁴¹². Which form of IL-6 signalling Th17RM cells respond to within the tumour is difficult to interpret from our results. ADAM17 also cleaves pro-TNF- α into its active, soluble form⁴¹³.

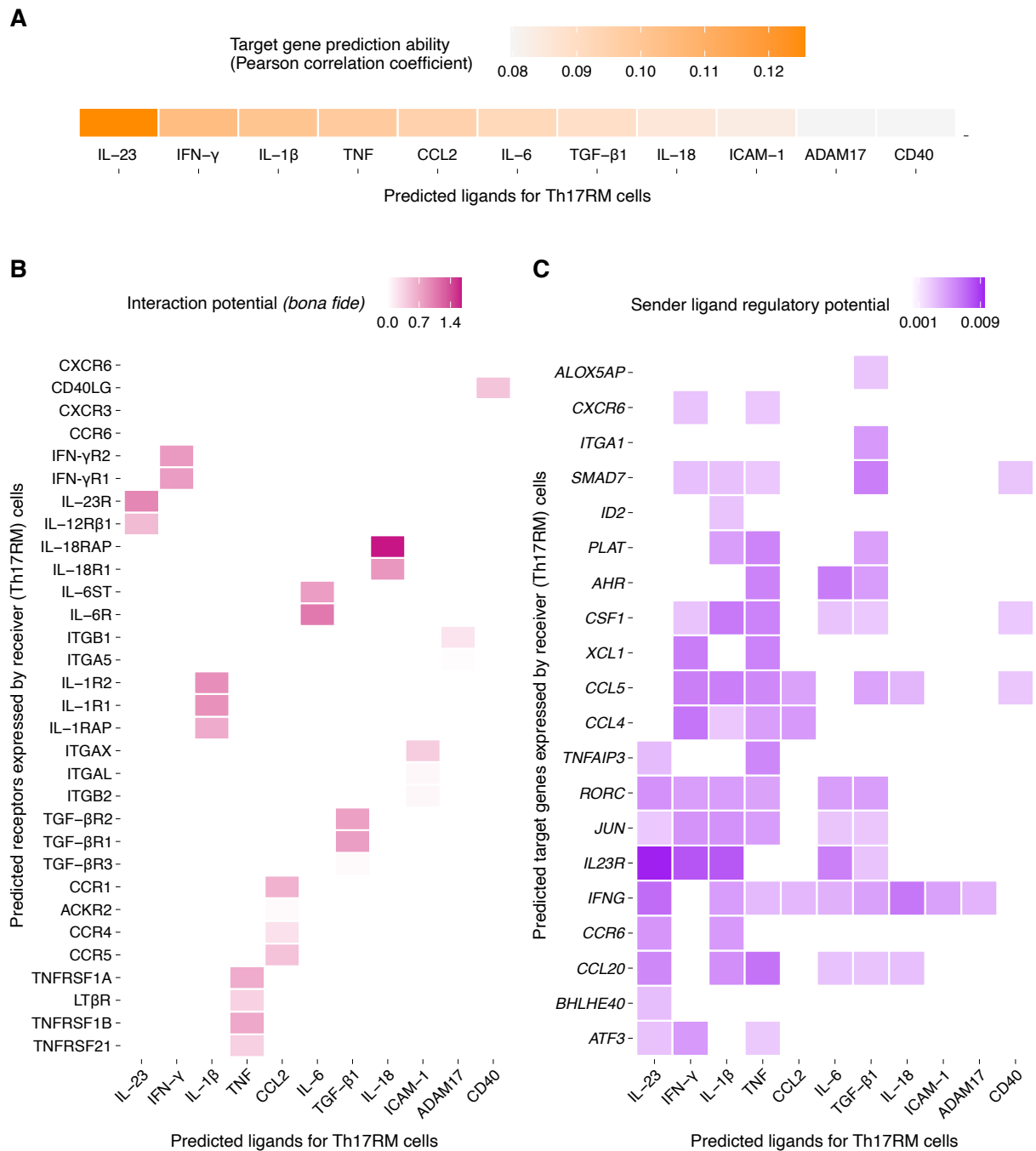


Figure 3.10 | Ligands, such as IL-23, IFN- γ , IL-1 β , TNF, IL-16, TGF- β 1 and IL-18 are predicted to drive a Th17RM transcriptional programme. NicheNet ligand-receptor network analysis was used to predict the ligands that functionally interact with Th17RM cells. (A) The predicted ligands which are most likely to interact with Th17RM cells based on (B) the level of cognate receptor expression and (C) the expression of downstream target genes for each respective signalling pathway.

Comparatively, the cytokines NicheNet predicted to most strongly interact with Th17EM cells allude to the notion that Th17EM cells may self-regulate a number of their transcriptional programmes. We were initially surprised to see that IL-17A had the highest correlation coefficient (**Fig. 3.11A**), binding to the heterodimeric IL-17R (**Fig. 3.11B**), composed of IL17RA and IL-17RC chains⁴¹⁴, and upregulating a broad array of genes in Th17EM cells (**Fig. 3.11C**). However, an IL-17 mediated regulatory autocrine loop has been recently described in Th17 cells that functions to restrain the secretion of GM-CSF, IL-17F and IL-22 to restrict their pathogenicity in EAE⁴¹⁵, making the prospect of a similar autocrine loop in Th17EM cells a plausible conclusion. IL-1 α can also function in an autocrine manner in Th17 cells, although the resulting phenotype contradicts that seen with IL-17A regulation. Autocrine IL-1 α signalling represses the production of IL-10 whilst concomitantly upregulates secretion of IL-1 α , IFN- γ and IL-17A released by Th17 cells to instead enforce their pathogenicity¹⁷⁰.

The correlation coefficient of leukaemia inhibitory factor (LIF) was also surprisingly high, a cytokine which functions to antagonise IL-6 driven Th17 differentiation by upregulating the level of the STAT3 inhibitor SOCS3⁴¹⁶. Although this corroborates with the increased *SOCS3* identified in our previous Th17EM transcriptional analysis (**Fig. 2.6A, 2.6C**), the functional impact of LIF signalling contradicts the strong Th17 signature associated with Th17EM cells. Furthermore, IL-6 exhibits a correlation coefficient of 0.09 in Th17EM cells (data not shown), similar to that calculated in Th17RM cells (**Fig. 3.10A**) and instructs a phenotype more similar to the one we have described in Th17EM cells⁴¹⁷, challenging the biological relevance of LIF as an output cytokine.

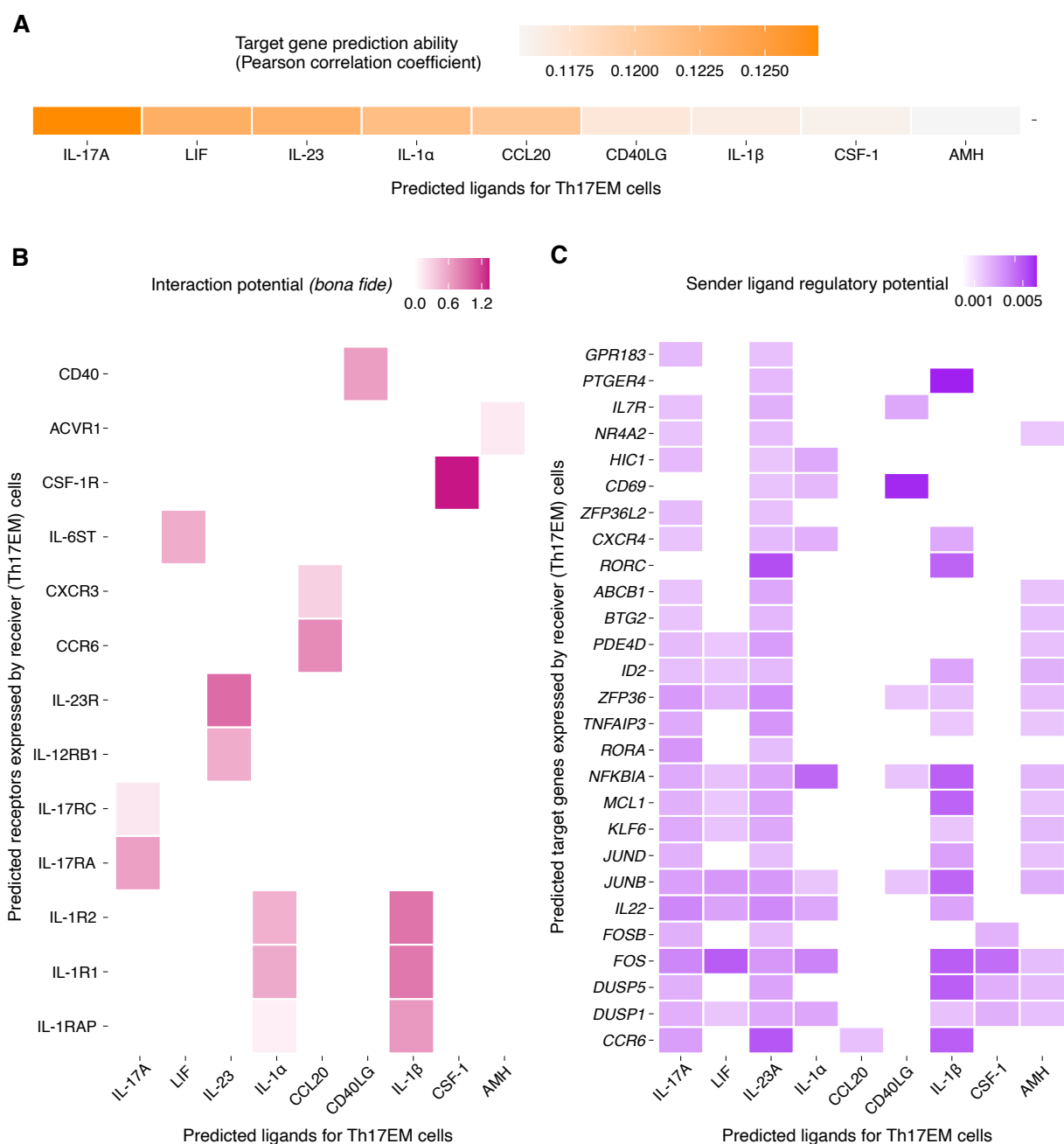


Figure 3.11 | Th17EM cells might self-regulate their phenotype more than Th17RM cells.

NicheNet ligand-receptor network analysis was used to predict the ligands that functionally interact with Th17EM cells. **(A)** The predicted ligands which are most likely to interact with Th17EM cells based on **(B)** the level of cognate receptor expression **(C)** and the expression of downstream target genes of each respective signalling pathway.

3.3.6 Inflammatory, tumour-residing macrophages produce the majority of Th17RM predicted ligands

We next sought to describe the tumour niche that drives Th17EM cells to acquire resident memory characteristics by establishing the major sender cell types for Th17RM ligands. Firstly, we broadly visualised the cell type distribution for the top sender ligand-receptor pairs using a Circos plot, where each connecting arrow was colour coded depending on the specificity of the ligand to a distinct sender cell type. Ligands that had transcripts detected in numerous cell types were coloured green and those with transcripts found almost exclusively, if not exclusively, in one cell type were coloured a non-green colour depending on the cell type in question.

The majority of sender ligands were expressed by multiple different cell types, with only *CCL2* and *IL1B* uniquely expressed by fibroblasts and myeloid cells, respectively (**Fig. 3.12A, 3.12B**). The expression of the more extensively shared ligands agreed with observations from within the literature. CCL20, for example, was found to be more enriched in the Th17 cells, of which they are known to be a dominant producer¹¹⁵, with lower expression levels also being detected in malignant cells. What was more surprising was that Th17EM and Th17RM cells were relatively enriched for *IL23A* (IL-23p19), as IL-23 secretion is typically associated with cells that derive from the myeloid lineage⁴¹⁸. One explanation could be the detection of non-functional IL-23p19 transcripts in these Th17 populations, having been recently shown that following TCR ligation, CD4⁺ T cells can secrete IL-23p19 but not the IL-23p40 additionally required to form functional IL-23⁴¹⁹.

To explore the sender cell types with increased granularity, we next plotted the minor cell type annotations against each predicted ligand (**Fig. 3.12C**). A cluster of three inflammatory macrophages, hM08_Macro_NLRP3, hM12_TAM_C1QC and hM13_TAM_SPP1, were enriched with nearly all the Th17RM sender ligands, strongly suggesting the existence of Th17RM cell/inflammatory macrophage niche within the tumour. It has been previously established that osteopontin (*SPP1*) from APCs in the inflamed lungs of smokers is known to drive IL-17 and IFN- γ secretion in CD4⁺ T cells²¹³, as are ligands downstream of NLRP3 inflammasome activation such as IL-1 β ¹¹⁵.

Other notable sender populations included hM03_cDC2-CD1C and hM04_cDC1_BATF3 cells, which have been reported to elicit strong Th17 responses *in vivo*⁴²⁰⁻⁴²². Within the tumour, these dendritic cell populations have also been associated with the generation of robust anti-tumour responses in a number of mouse models of cancer, including against fibrosarcoma and melanoma^{423,424} by mediating the capture of tumour antigens and cross-priming T cells to generate immunotherapy responsive, resident memory CD8⁺ T cells⁴²⁵⁻⁴²⁷. Additionally, BATF3⁺ DCs also characteristically express CD103⁴²⁸, further suggesting that these cells could plausibly co-localise with CD103⁺ Th17RM cells at an E-cadherin⁺ epithelial interface.

Exploring ligand expression across all cell types within GSE146771 also revealed potential heterogeneity between patient tumours which could impact the Th17 cell façades found within the microenvironment. The malignant cell clusters hC01_P0413 and hC02_P0825 appeared to be rich in *IL18* and low in *CCL20*, whilst the malignant clusters hC03_P0411 and hC04_P1212 displayed the reverse profile (**Fig. 3.12C**). It is intriguing to speculate that CCL20⁺ tumours might be more likely to recruit Th17 cells from non-malignant tissue, which could possibly display more Th17EM-like properties, whilst tumours that secrete IL-18 more actively

induce Th17RM-like features in local Th17 cells already present within the tumour niche. Using immunohistochemistry to stain a varied CRC cohort for IL-18, CCL20, Th17EM and Th17RM markers could be an informative way of validating this conjecture.

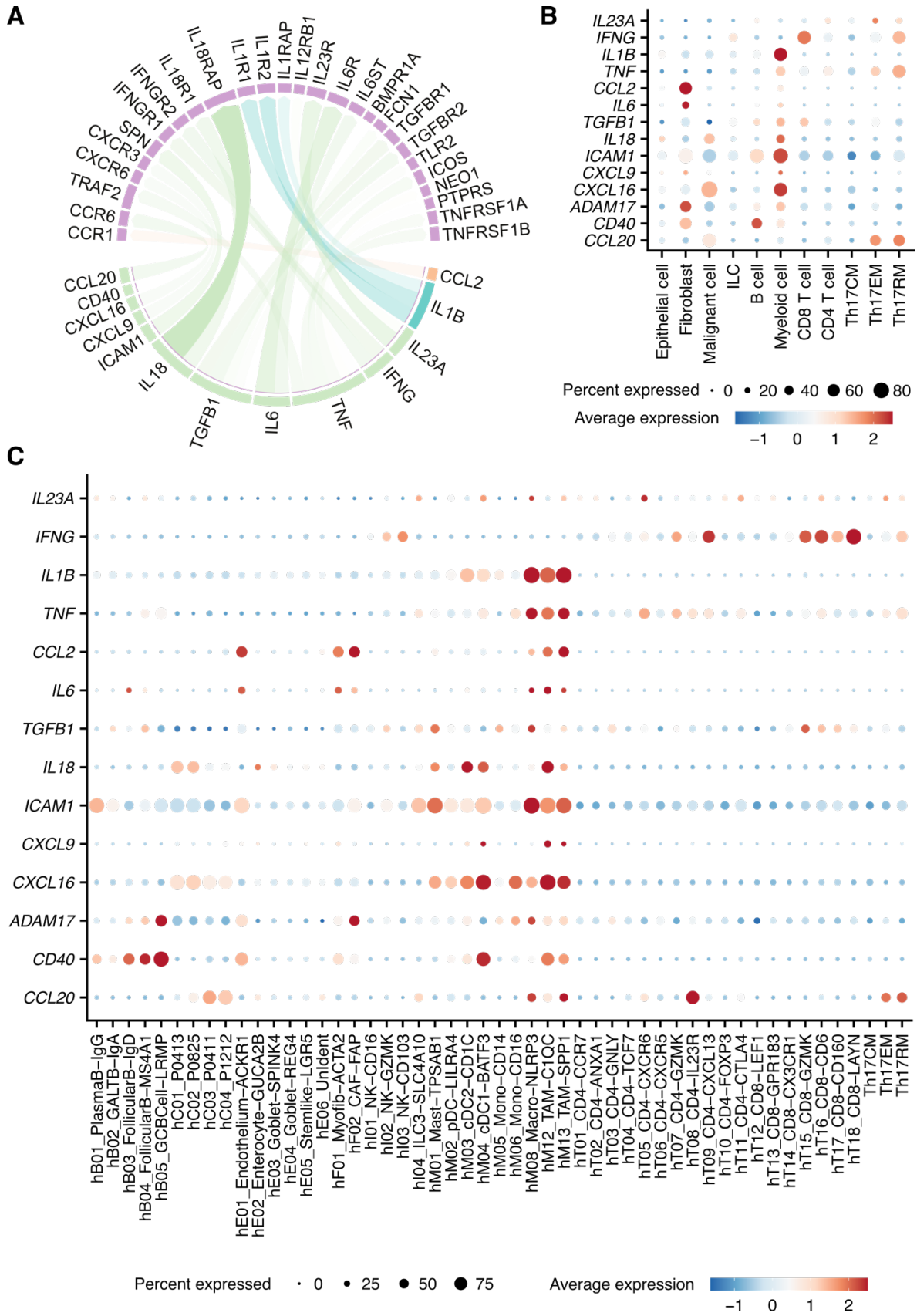


Figure 3.12 | Macrophages are the dominant producers of Th17RM cell ligands. The expression of predicted ligands for Th17RM cells was transcriptomically explored in B cell, malignant cell, epithelial cell, fibroblast, innate immune cell, myeloid cell and T cell clusters in GSE146771. **(A)** Circos plot displaying predicted Th17RM ligands on the bottom semicircle and putative cognate Th17RM receptors on the top semicircle. Connections are coloured by cell type, where peach represents ligands exclusively expressed by fibroblasts, blue representing ligands exclusively expressed by myeloid cells and green representing ligands expressed by > 1 cell type. **(B)** Dot plot displaying the expression and distribution of predicted ligands across broad cell type annotations. **(C)** Dot plot displaying the expression and distribution of predicted ligands across specific cell type annotations. For (B) and (C), dot size represents the percentage of cells in each cell type annotation expressing the ligand. Dot colour plus colour saturation indicates the relative expression level, scaled across the total number of populations.

3.3.7 Some putative Th17RM ligands are enriched in the tumours of Th17RM^{high} patients

We postulated that the expression of these putative Th17RM ligands, if they functionally associate with the recruitment or development of Th17RM cells *in vivo*, should be enriched in the tumours of TCGA-COAD patients displaying the highest GSVA Th17RM signature score. To explore this hypothesis, we contrasted the expression level of ligands with a correlation coefficient ≥ 0.075 in Th17RM^{low} and Th17RM^{high} patients (**Fig. 3.13**), as stratified previously. 10 of the 14 ligands were significantly upregulated in Th17RM^{high} patients, including *CXCL9*, *IL18* and *IFNG* which displayed the strongest positive shift. Rather surprisingly, some of the top hit cytokines from our NicheNet analysis, and indeed from the literature – *IL23A*, *IL1B* and *IL6* – displayed no significant difference in the bulk RNA-seq data of Th17RM^{high} and Th17RM^{low} colon adenocarcinoma patients. We considered the possibility that the lack of upregulation in these cytokines could be a consequence of the location in which the tumour

samples were taken, as the scRNA-seq suggested that dense epithelial regions could be particularly depleted of all three cytokines (**Fig. 3.12B**). However, the large sample size of the TCGA-COAD cohort should compensate for such a confounder. Another plausible explanation is that IL-23, IL-1 β and IL-6 are implicated in the development of both Th17EM and Th17RM cells.

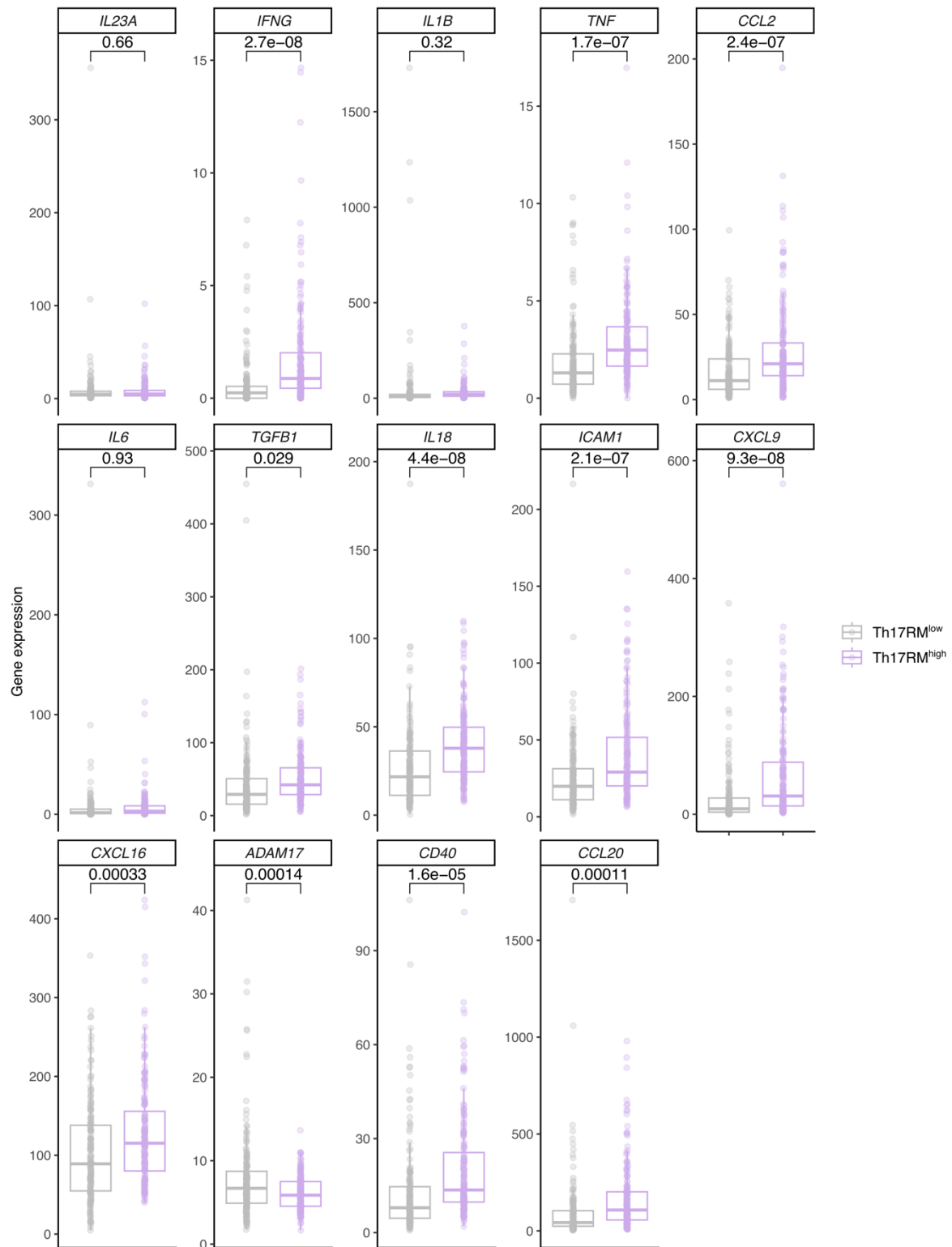


Figure 3.13 | The expression of putative Th17RM ligands positively correlates with Th17RM signature score in bulk RNA-seq data from colon adenocarcinoma patients. GSVA was used to score patients from The Cancer Genome Atlas colon adenocarcinoma

(TCGA-COAD) cohort based on the expression of our curated seven gene Th17RM cell signature. Signature score was used to divide the cohort into equal tertiles, with patients in the lowest tertile termed Th17RM^{low} and those in the highest scoring tertile named Th17RM^{high}. The expression level of each putative Th17RM ligand in bulk RNA-seq data from Th17RM^{low} and Th17RM^{high} patients was then compared against one another. Data are displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cluster. Each data point represents an individual patient. Two way unpaired t-test was performed for statistical analysis and p values are numerically displayed above each comparison. $p \leq 0.05$ is considered statistically significant.

3.3.8 Th17RM intercellular interactions are difficult to observe by spatial transcriptomics

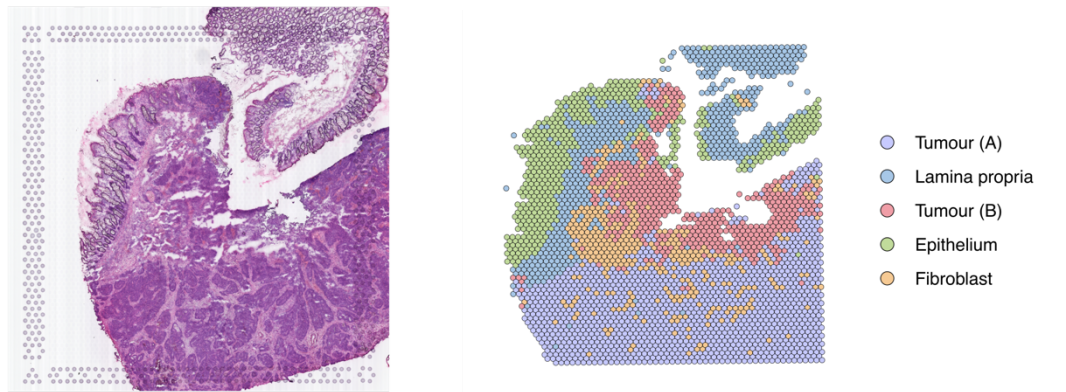
Ligand-receptor network analysis revealed a predictive association between inflammatory tumour associated macrophages and the acquisition of resident memory characteristics in Th17 cells. We next sought to confirm these intercellular interactions and observe more direct co-localisation between the two populations within the same tumour niche. We approached this aim twofold; firstly, by utilising spatial transcriptomics (**Section 3.3.9**) and secondly, by using multiplex fluorescence imaging (**Section 3.3.10**).

For our initial exploration using spatial transcriptomics, we made use of a dataset composed of tissue obtained from four CRC patients. Patients 1 and 2 were treatment naïve, whilst 3 and 4 were partial responders to neoadjuvant chemotherapy. Each patient also had metastatic dissemination to the liver³. To firstly demarcate the histological regions of interest for each tissue sample, unsupervised spot clustering was performed alongside differential

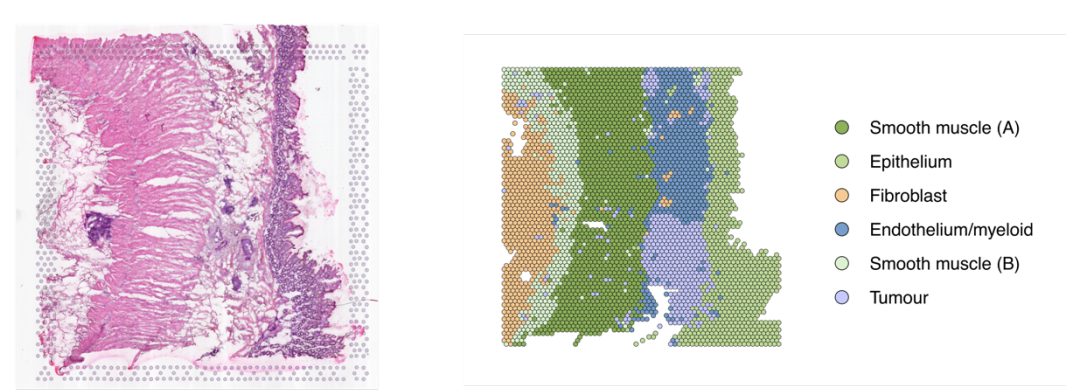
gene expression analysis, with the output of each used to facilitate the manual annotation of each tissue region. Considerable tissue heterogeneity was observed between each patient.

The tumour region of patient 1 could be dissected into two clusters, with tumour region A expressing a number of ribosomal stress proteins (*RPS27A*, *RPS15A*) and tumour region B more enriched with genes associated with cellular proliferation (*REG3A*, *REG1A*, *EIF2S2*). Region A also appeared to be punctuated by fibroblast (*COL3A1*, *COL12A1*, *COL4A1*) clusters. A considerable portion of lamina propria (*IGHG1*, *PIGR*, *SERPINA1*) and healthy epithelium (*MUC1*, *MUC2*, *SPINK4*) was also defined in patient 1 (**Fig. 3.15A**). Large portions of the tissue from patients 2 and 3 were made up of smooth muscle cells (*MYH11*, *TAGLN*, *MYL9*, *SMTN*, *FLNC*), with smaller interspersed endothelial clusters (*MCAM*, *VIM*, *PLN*) identified (**Fig. 3.15B**, **Fig. 3.15C**). The tumour region of patient 4 appeared to be encapsulated and was separated from a fibroblast zone by healthy epithelium and lamina propria (**Fig. 3.15D**).

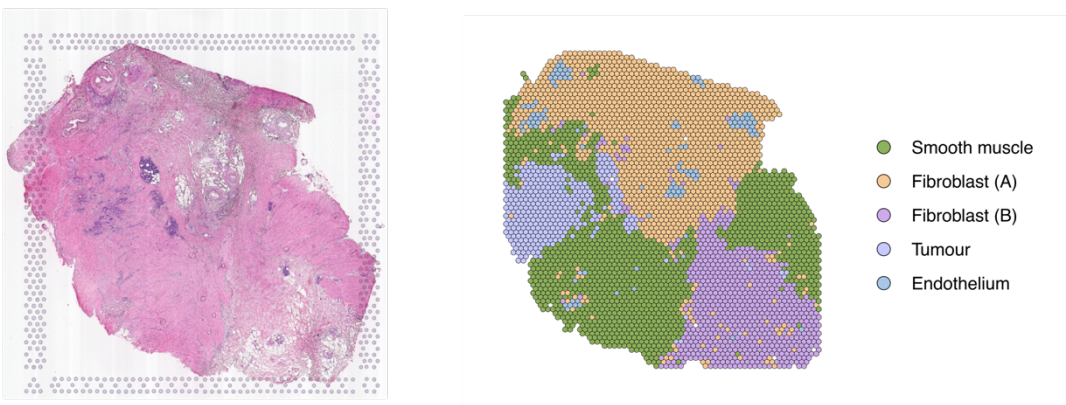
A



B



C



D

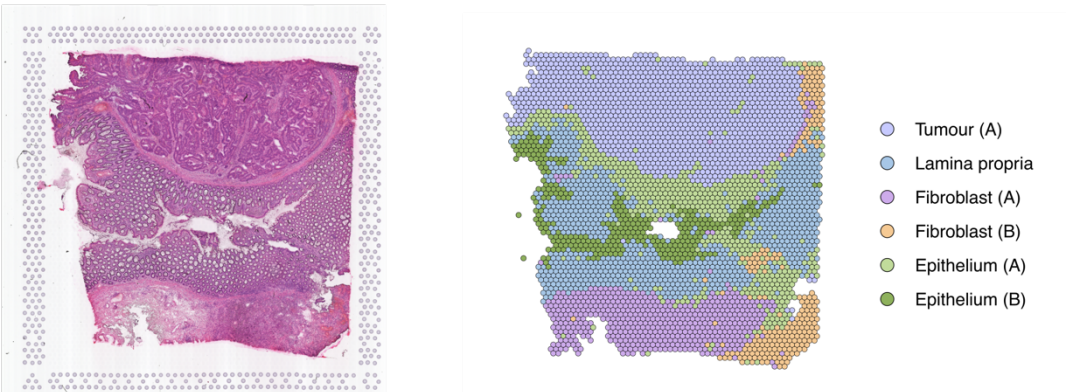


Figure 3.15 | Identifying tissue niches in CRC patients using spatial transcriptomics.

Histological sections from four CRC patients were processed by Wu *et al.*³ for analysis by spatial transcriptomics using the 10X Visium platform. Each processed slide was downloaded and data spots were clustered using *FindClusters* function in the Seurat package in R. Differential gene expression analysis was used to inform the tissue niche cluster annotations. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed genes, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. (A), (B), (C) and (D) represent CRC patients 1-4. H&E staining of each tissue section are displayed on the left and annotated tissue niche clusters are displayed on the right.

In order to visualise the distribution of Th17RM cells and inflammatory tumour associated macrophages, the most differentially expressed genes by each GSE146771 cluster annotation were overlapped onto the tissue spots sequenced by spatial transcriptomics to determine the probabilistic correlation that a given cell was present per spot using an anchor-based integration method, as initially described by the Satija laboratory⁴²⁹. Once the anchors were established, each spot was scored per each cell type using the *TransferData* function in Seurat and scaled by colour for visualisation.

To test how reliably the cell type annotations were projected onto each tissue section, we first explored the distribution of enterocyte and goblet cell clusters as control populations, which we rationalised should be enriched within the adjacent epithelial regions of each tumour slide. As expected, both cell types were observed to be strongly enriched in regions manually labelled as epithelium in patients 1 (**Fig. 3.16**), 2, 3 and 4 (**Fig. S3.3A-C**), suggesting that the cell type annotations had been reliably transferred onto the tissues.

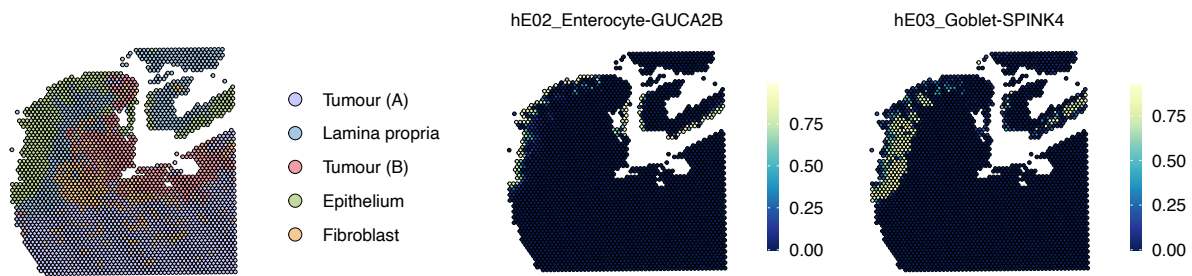


Figure 3.16 | Validating the transfer of cell type annotations from GSE146771 onto each spatial transcriptomics tissue section. To explore the distribution of different cell types within the tissue of each CRC patient, data spots were individually scored based on the expression of cell type signatures using the *TransferData* function in Seurat. Differential gene expression analysis was used to generate a gene signature for each cell type. Wilcoxon rank sum test with Bonferroni correction was used to identify significantly differentially expressed genes, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. To validate the reliability of signature scoring per spot, the distribution of two well-characterised epithelial subtypes was visually inspected. Spot colour represents the probability that a given cell type can be found within that region, with blue representing low probability and yellow representing high probability. Annotated tissue niches are displayed on the left and distribution of hE02_Enterocyte_GUCA2B plus hE03_Goblet-SPINK4 cells are displayed on the right.

Next, we explored the distribution of tissue Th17 cells and the selection of myeloid cells most strongly predicted to interact with Th17RM cells (**Fig. 3.17A**). As predicted, Th17EM cells were largely restricted to healthy lamina propria and some regions annotated as epithelium. On the other hand, Th17RM cells were the dominant Th17 population within the tumour niche (**Fig. S3.4**).

The five selected myeloid populations all seemed to be enriched in a similar zone within the tumour. Although large myeloid dominated regions are not uncommon in CRC⁴³⁰, we wondered whether these results could also arise from a sequencing bias if spots became

dominated by a single cell population as each spot (~50 μm) contains multiple cells. For example, if 10 hM03_cDC2-CD1C cells were present in a spot along with 1 Th17RM cell, the Th17 transcripts might become diluted to the point where they are minimally detected. To support this idea, Th17RM cells were spatially associated most other T cell phenotypes and no T cell we investigated was strongly enriched in the healthy epithelial region (**Fig. S3.5**), presumably due to the abundance of epithelial transcripts despite intraepithelial lymphocytes comprising an important cell population within this niche⁴³¹. It was therefore less surprising when we contrasted the localisation scores of Th17RM cells and each myeloid population and found that there was no co-habitation observed, with the R values essentially 0 for all five cell types due to the majority of Th17RM scoring spots devoid of myeloid cells and vice versa (**Fig. 3.17B**). The frequency of positive co-localisation spots was also low so we ultimately concluded that our spatial transcriptomics analysis was insufficient to reliably confirm interactions within the tumour.

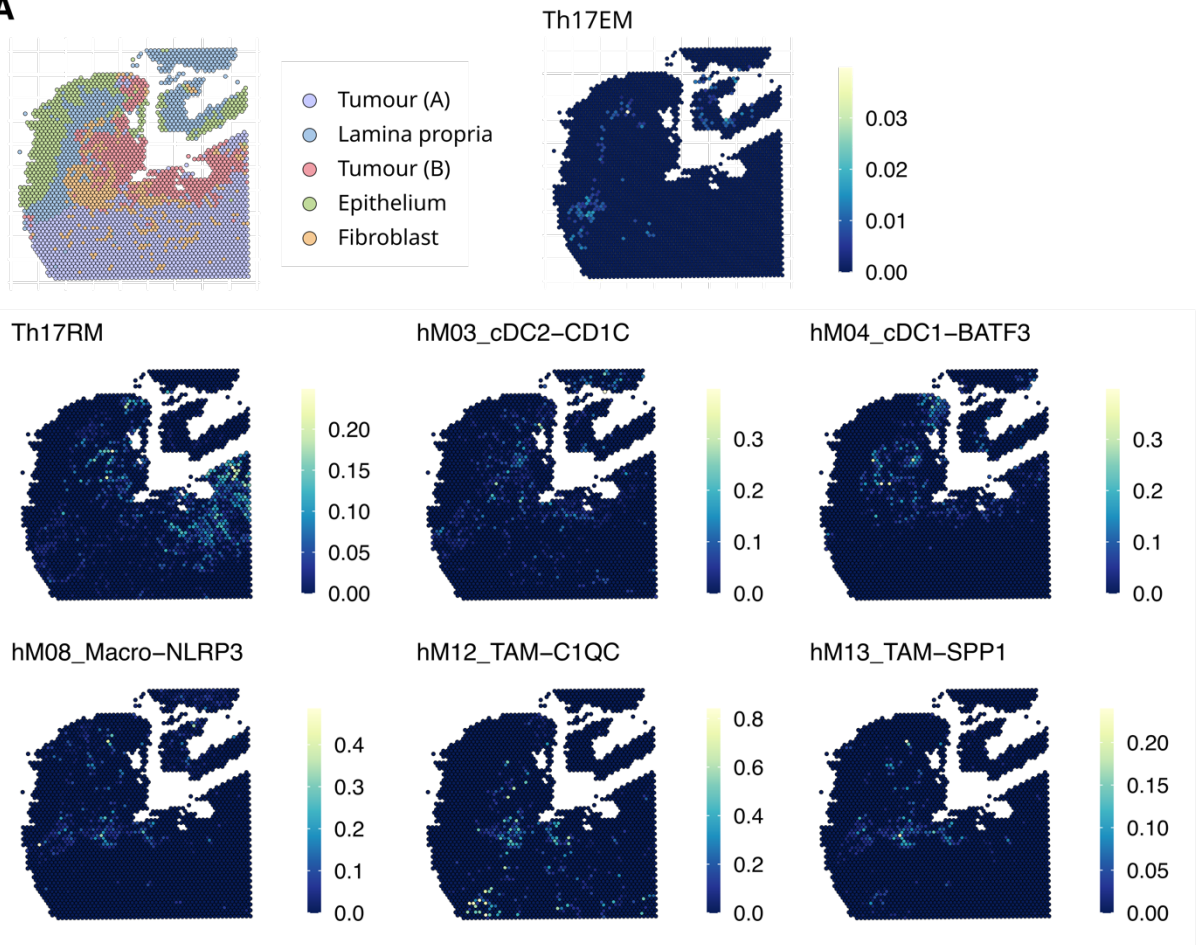
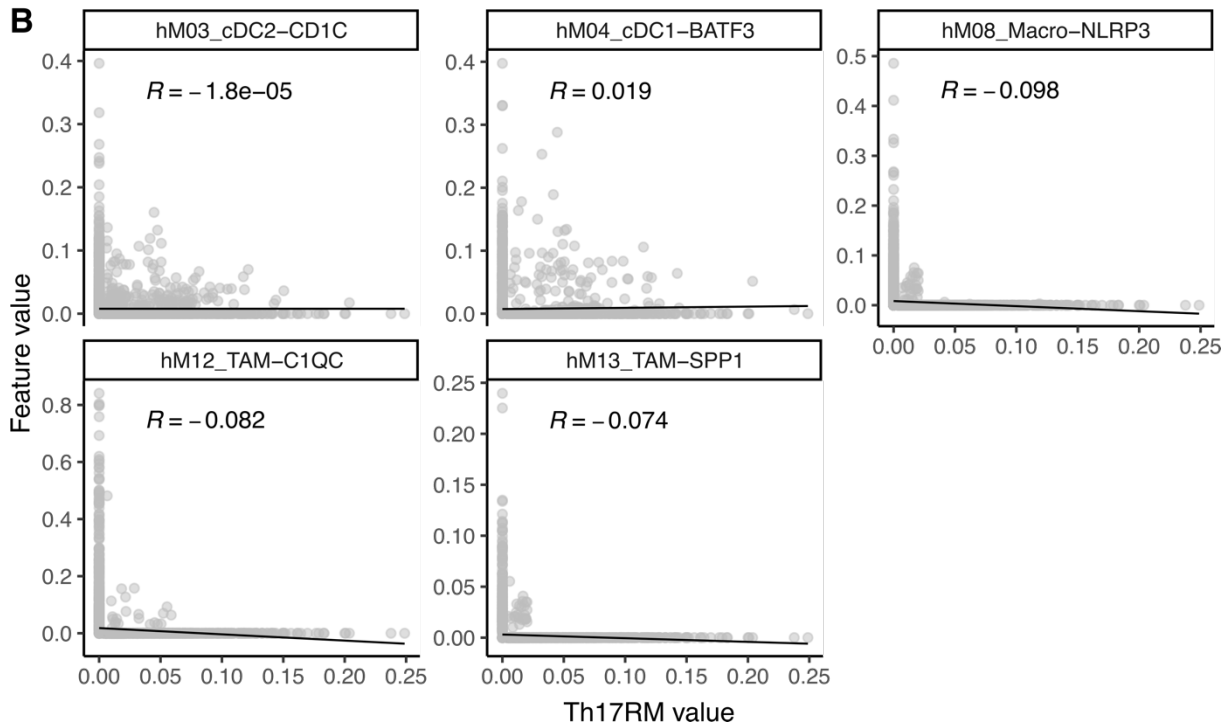
A**B**

Figure 3.17 | There is no correlation between the tissue distribution of Th17RM cells and myeloid cells by spatial transcriptomics. To explore whether correlations between the distribution of tissue Th17 and myeloid cells exist in CRC, signatures for each cell type were projected onto individual spots on a tissue from patient 1 processed by 10X Visium for spatial transcriptomics. (A) Histological annotations with probability scores for Th17EM cells, Th17RM cells and a selection of myeloid cell types per tissue spot. Spot colour represents the probability that a given cell type can be found within that region, with blue representing low probability and yellow representing high probability. (B) Correlation between Th17RM spot probabilities and myeloid spot probabilities in CRC patient 1. Each data point represents a spot on the tissue. Pearson correlation is displayed with corresponding R value.

3.3.9 Multiplex fluorescence imaging reveals that Th17RM cells co-localise CD68 CD11c⁺ inflammatory macrophages

In light of the inconclusive results from our spatial transcriptomics, we next probed the spatial enrichment and intercellular interactions of Th17RM cells within the tumour at higher resolution using multiplex fluorescence imaging. This technique permits qualitative analysis at the protein level whilst incorporating spatial information within the tissue. Each tissue sample can be stained with up to three fluorescent antibodies at a time before images are acquired, fluorescence is quenched and the process is repeated. In theory, this process can be repeated an unlimited number of times and thus an unlimited number of proteins probed, with tissue degradation being the limiting factor.

Studying human Th17 cells *in situ* is challenging due to a lack of reliable ROR γ t antibody for use with FFPE tissue, precluding direct histological identification by transcription factor expression. We therefore first had to test and validate an anti-human ROR γ t antibody to permit the demarcation of Th17RM cells within the tissue. To identify Th17 cells by flow

cytometry we used the rat IgG2a anti-human ROR γ t clone AFKJS-9 (**Fig. 2.10**) which, to our knowledge, has only been validated for use by flow cytometry. We speculated that this clone might yield similar staining success in human FFPE tissue sections, which are similarly fixed the cells prepared for intranuclear staining by flow cytometry with 4% PFA.

To test this hypothesis, sequential FFPE sections from a resected CRC tumour were selected for preliminary validation of the clone AFKJS-9 and we chose to focus on a region of cells adjacent to an epithelial crypt (**Fig. 3.18A**). A rat IgG2a isotype with Alexa Fluor 647 secondary detection antibody (**Fig. 3.18B**) or secondary detection antibody only (**Fig. 3.18C**) were used as negative controls to confirm that AFKJS-9 was not binding non-specifically via FcR-mediated interactions. For the full stain, whilst some non-specific binding was observed, particularly in spaces demarcating the epithelial border, convincing intranuclear staining was observed in a number of lymphocyte sized cells (**Fig. 3.18D**). Furthermore, the virtual H&E section confirmed that a considerable quantity of background staining could be attributed to erythrocyte autofluorescence, which we were unable to remove (**Fig. 3.18A**). Therefore, we chose to focus on ROR γ t⁺ DAPI⁺ cells only for our downstream analysis.

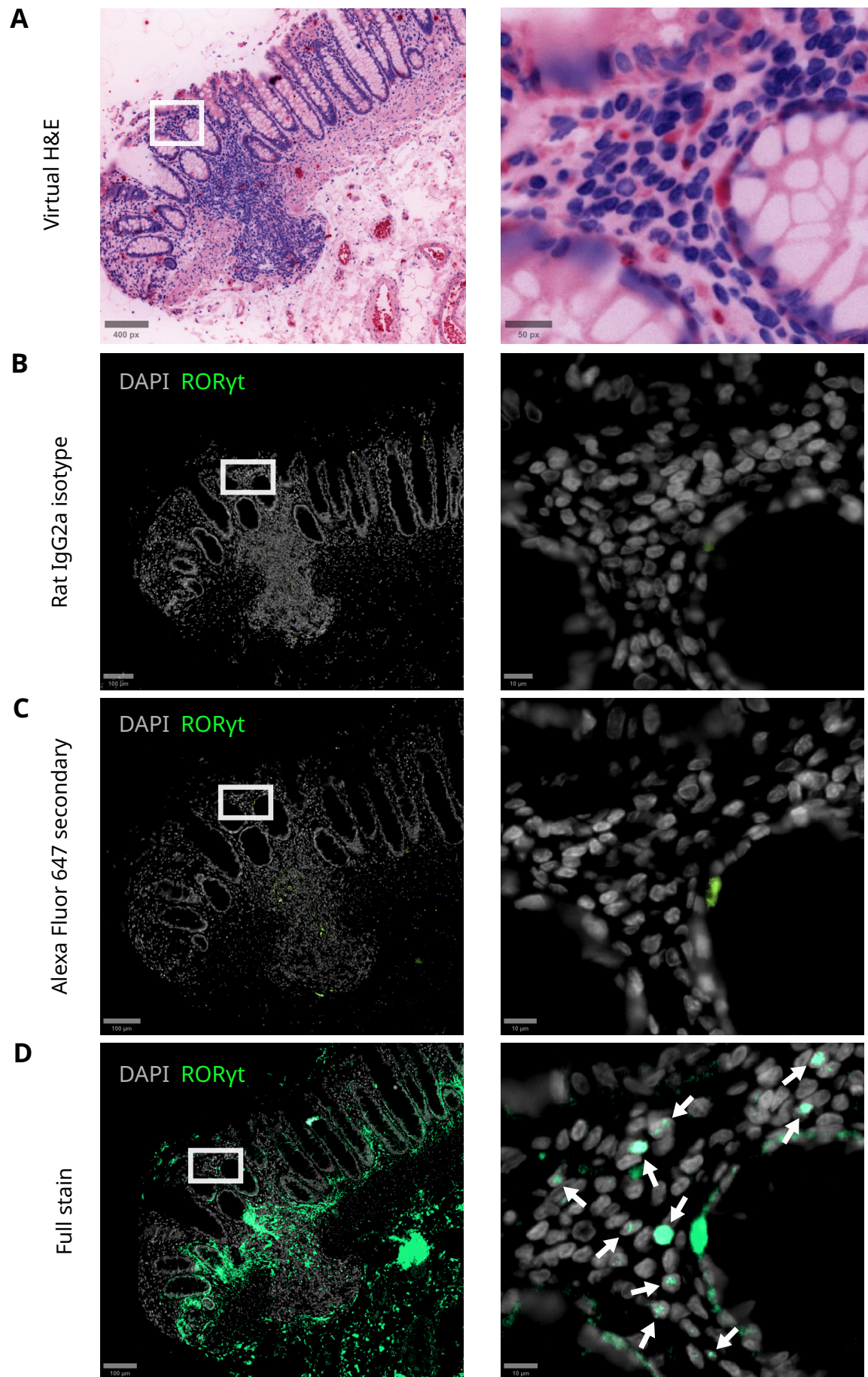


Figure 3.18 | AFKJS-9, a monoclonal antibody against mouse and human ROR γ t, exhibits intranuclear staining on FFPE slides from CRC patients. Validation of AFKJS-9, a rat IgG2a monoclonal antibody against mouse and human ROR γ t, using sequential FFPE sections from CRC patient GI 8416. Images were acquired with an IN CELL 2500HS widefield imaging system and multiplexed using the Cell DIVE platform. (A) Virtual H&E staining where the left image scale bar represents 400 pixels and the right represents 50 pixels. Immunofluorescent images taken with (B) rat IgG2a isotype control plus goat anti-rat Alexa Fluor 647, (C) goat anti-rat Alexa Fluor 647 or (D) AFKJS-9 plus goat anti-rat Alexa Fluor 647. Scale bar on the left image represents 100 μ m and on the right image represents 10 μ m for (A), (B), (C) and (D). White arrows represent positive intranuclear staining of ROR γ t in (D).

We next validated a number of surface antibodies which we planned on using to identify Th17RM cells, including CD45RO, CD3, CD4, CD103, CXCR6 and CD161. A number of additional antibodies were also validated, such as CD8, FoxP3, T-bet (did not work), IL-23p19, CD40, E-cadherin, Ki67 and CD20, which would be used to identify additional cell types within the tissues and negatively demarcate Th17RM cells (such as CD8 and FoxP3). Multiplexed rounds of imaging revealed that Th17RM cells, which we defined as CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ ROR γ t⁺ CD103⁺ CXCR6⁺ CD161⁺ cells, could be identified in a number of our patients (**Fig. 3.19A**).

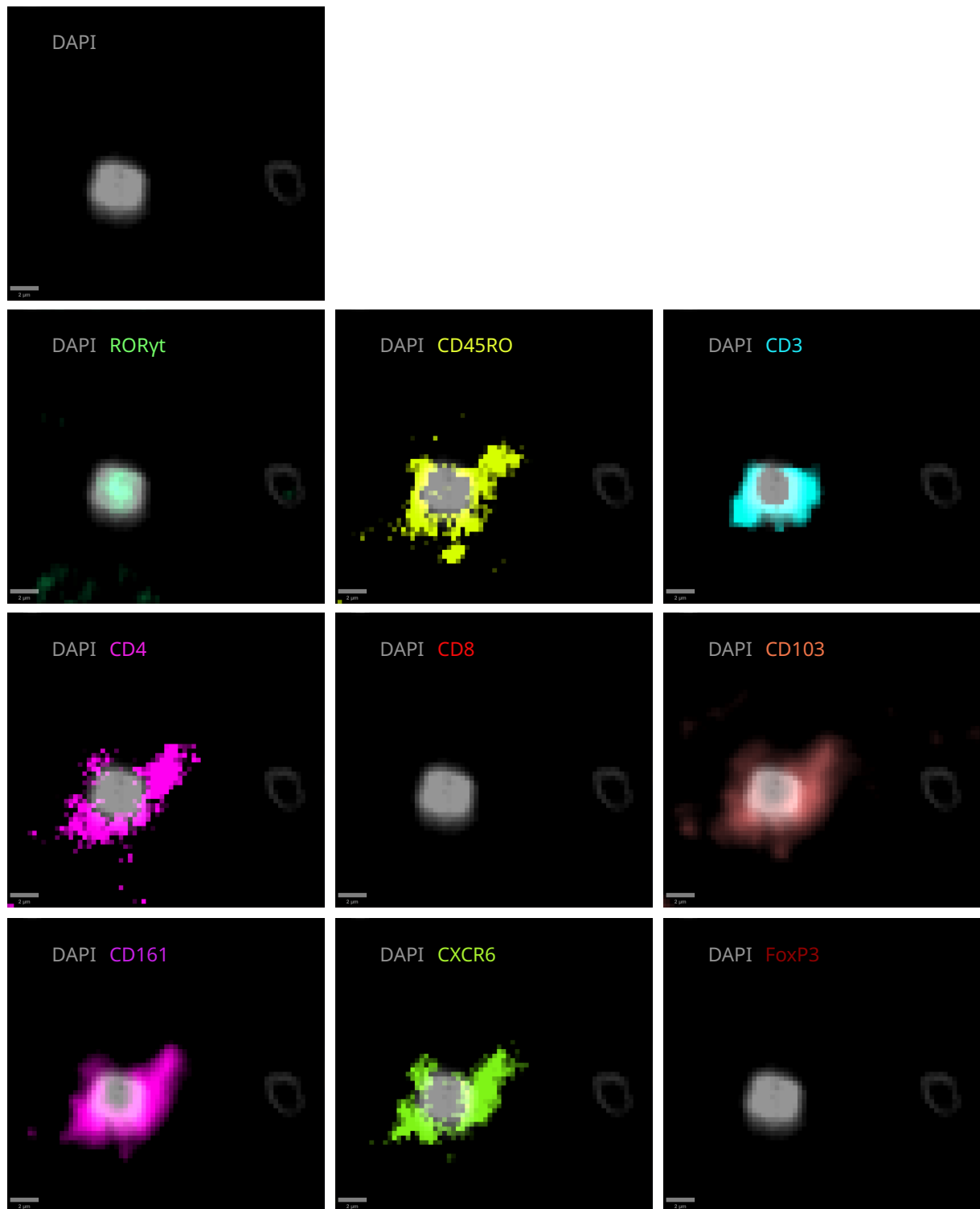
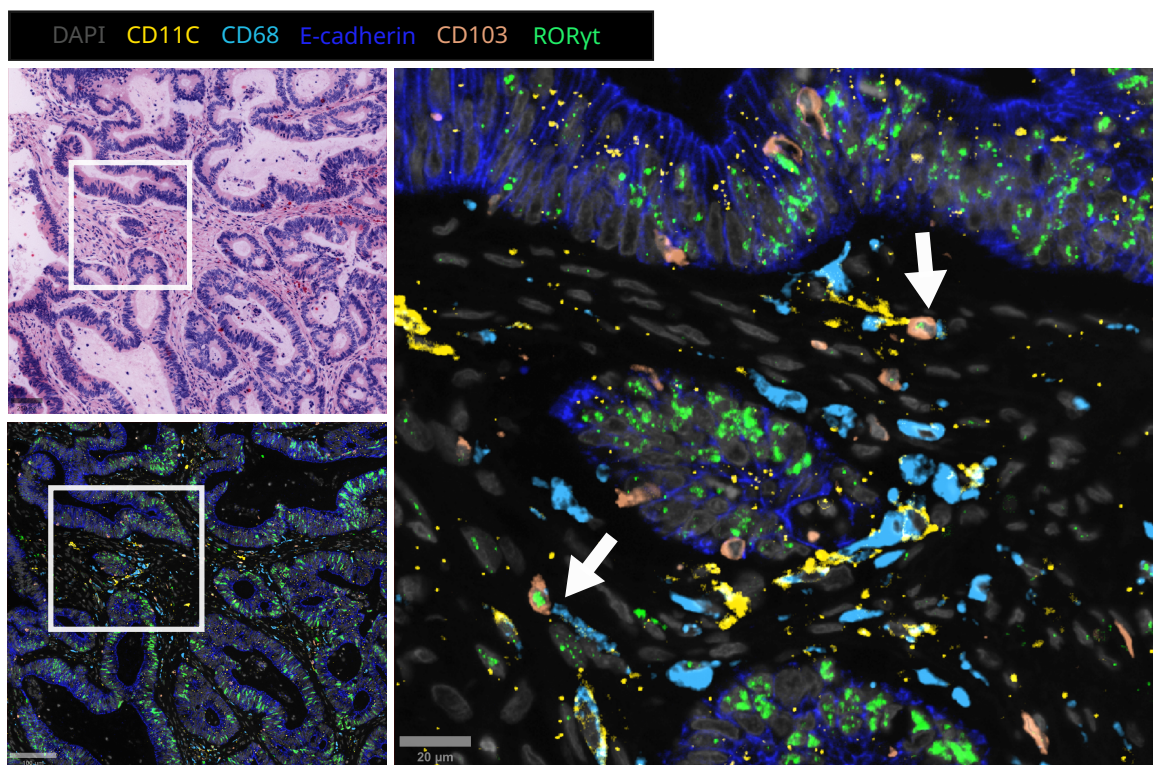
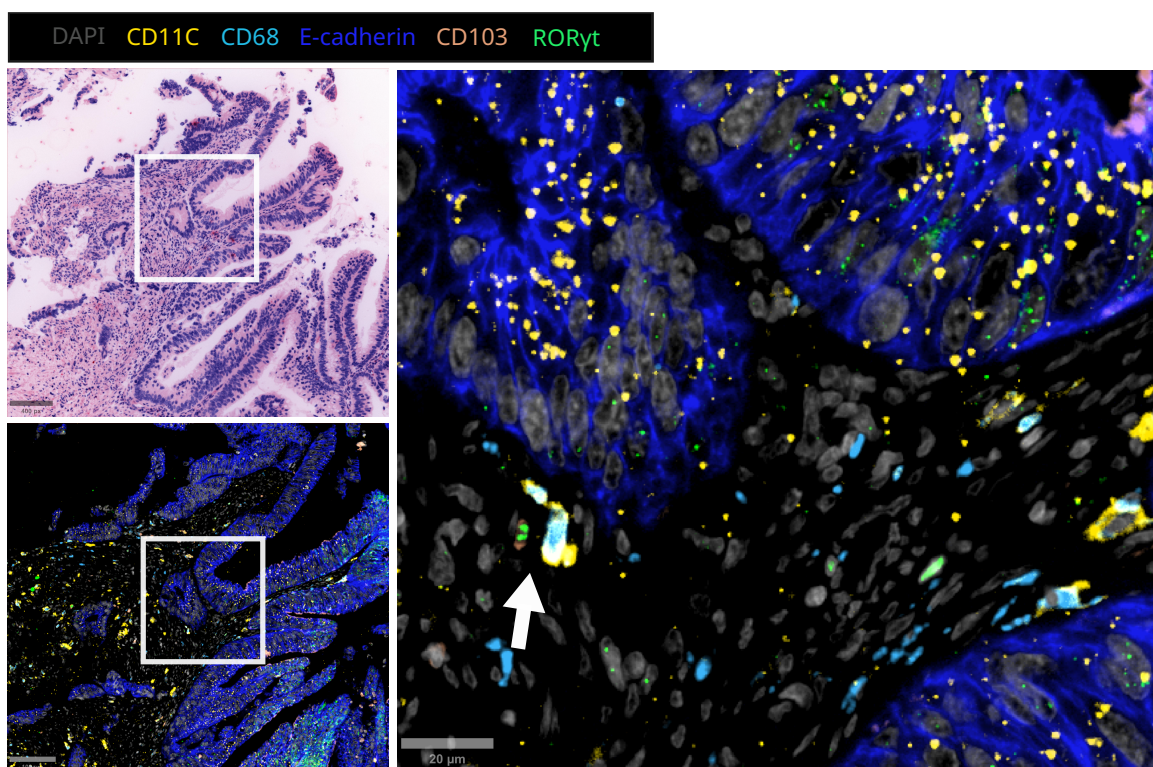


Figure 3.19 | Th17RM cells can be identified in CRC patients *in situ*. Multiplexed staining of FFPE tissue sections from CRC patients was used to identify Th17RM cells *in situ*. Images were acquired with an IN CELL 2500HS widefield imaging system and multiplexed using the Cell DIVE platform. Each image shows representative single colour images of the marker

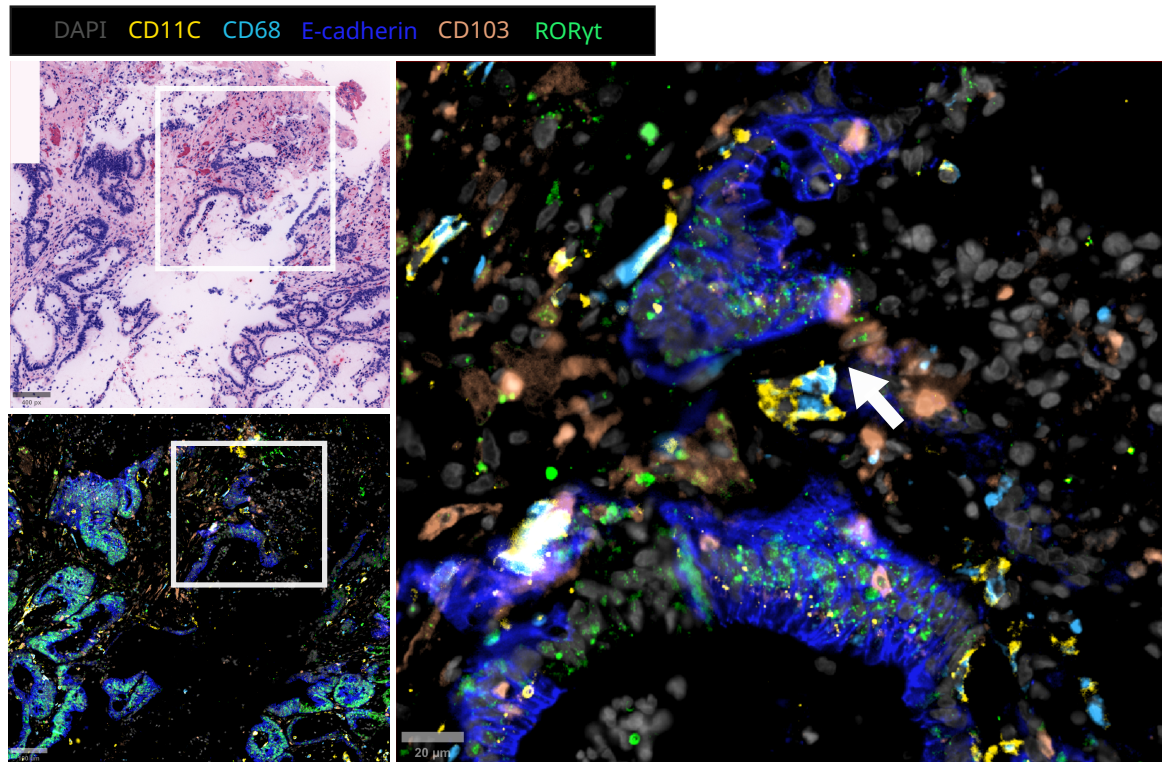
staining used to identify Th17RM cells, classed as CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ ROR γ t⁺ CD103⁺ CXCR6⁺ CD161⁺ cells. Scale bar represents 2 μ m.

One drawback of using immunohistochemistry over a computational strategy, such as spatial transcriptomics, is the relative inflexibility to explore nuanced cellular heterogeneity without directly staining tissue sections with an extensive panel of markers. The number of myeloid populations identified by scRNA-seq capable of producing the broad inflammatory cocktail speculated to drive Th17RM cells would result in validating an unrealistic quantity of markers to identify a precise sender phenotype *in situ*. Furthermore, we speculated that Th17RM cells are not a product of an individual intercellular interaction but are rather compartmentalised within a broader inflammatory tumour niche and the range of potential sender cell types for cytokines such as IL-18, which have epithelial and myeloid origins due to the ubiquity of the inflammasome⁴³², reinforcing this notion.

As such, to streamline the prerequisite antibody validation and optimisation phase, we sought to determine whether Th17RM cells are found in proximity to CD68⁺ macrophages that express CD11c. This combination of markers identifies gut macrophages which are known to exhibit specialised pro-inflammatory functions in IBD, by secreting IL-23, IL-1 β and TNF- α ^{433,434}, as well as mediating the cross-presentation of antigens from apoptotic tumour cells to augment anti-tumour CD8⁺ T cell immunity⁴³⁵. We observed a number of Th17RM cells in very close proximity with CD68⁺ CD11c⁺ macrophages (**Fig. 3.20A-C**) and, across three select patients with a high Th17RM count, quantitatively determined that Th17RM cells tended to be in closer proximity with CD68⁺ CD11c⁺ macrophages than their CD68⁺ CD11c⁻ counterparts, although this was not statistically significant (**Fig. 3.20D**).

A**B**

C



D

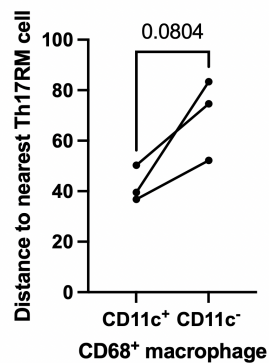


Figure 3.20 | Th17RM cells are found adjacent to CD68⁺ CD11c⁺ tumour associated macrophages. Multiplexed staining of FFPE tissue sections from 3 CRC patients was used to qualitatively and quantitatively explore the relationship between Th17RM (CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ RORγt⁺ CD103⁺ CXCR6⁺ CD161⁺) cells and CD68⁺ CD11c⁺ tumour associated macrophages *in situ*. Cellular co-localisation in patient (A) GI 8790, (B) GI 8870 and (C) GI 8898. Images were acquired with an IN CELL 2500HS widefield imaging system and were multiplexed using the Cell DIVE platform. Scale bars represent 100 µm (top left, virtual H&E), 100 µm (bottom left) and 20 µm (right) per patient and staining in (A), (B) and (C) is representative of all 3 patients. (D) Bar graph representing the spatial proximity of Th17RM

cells with CD68⁺ CD11c⁻ or CD68⁺ CD11c⁺ macrophages. Paired T test used for statistical comparison.

Although a considerable number of Th17RM cells were found adjacent to inflammatory macrophages, a striking number of Th17RM cells were also found embedded within the tumour epithelium (**Fig. S3.6**). As tumour epithelial cells strongly express E-cadherin, the ligand for CD103, we were not surprised to observe Th17RM cells positioned at this epithelial interface and we explore potential functional implications of this localisation in Chapter 5. Additionally, tumour cells are important producers of IL-18, TGF- β 1 and the chemokines CCL20 and CXCL16, all of which Th17RM cells are predicted to respond to (**Fig. 3.11C**). Whether Th17RM cells become differentiated by inflammatory macrophages and then migrate towards the malignant epithelium, or can be polarised by either cell type, is difficult to determine using immunohistochemistry but these observations permit the postulation of both mechanisms which are not mutually exclusive. Our observations that tumour cells exhibit heterogeneity in the level of IL-18 produced between patients (**Fig. 3.11C**) might support the idea that each mechanism can shape Th17 immunity depending on the tumour type, with CCL20 rich tumour cells favouring Th17 recruitment and IL-18 rich tumours favouring the differentiation of local Th17 cells.

3.3.10 Later tumour stages have a higher Th17RM score in TCGA-COAD

Finally, to probe the intrinsic properties of the tumours in which Th17RM cells develop, we ran multivariate Cox regression analysis on the TCGA-COAD cohort based on GSVA Th17RM signature score and ran regression analysis on variety of tumour features, including location, patient gender and tumour stage (**Fig. 3.21**). We tried to assess tumour mutation status and CMS classification although had difficulty accessing these metadata.

Th17RM score was not significantly affected by tumour location and nor by patient gender. Tumour stage, on the other hand, was influenced by Th17RM score in almost directly proportional manner, with stage T4 (HR = 8.79, 1.79-43.20, $p = 0.007$) and T4a (HR = 11.08, 2.03-60.32, $p = 0.005$) displaying the largest hazard ratios. This progressive association could be a consequence of sample preparation, with later stage tumour samples spanning more tissue niches and perhaps resulting in a heightened likelihood of Th17RM cells being captured for sequencing.

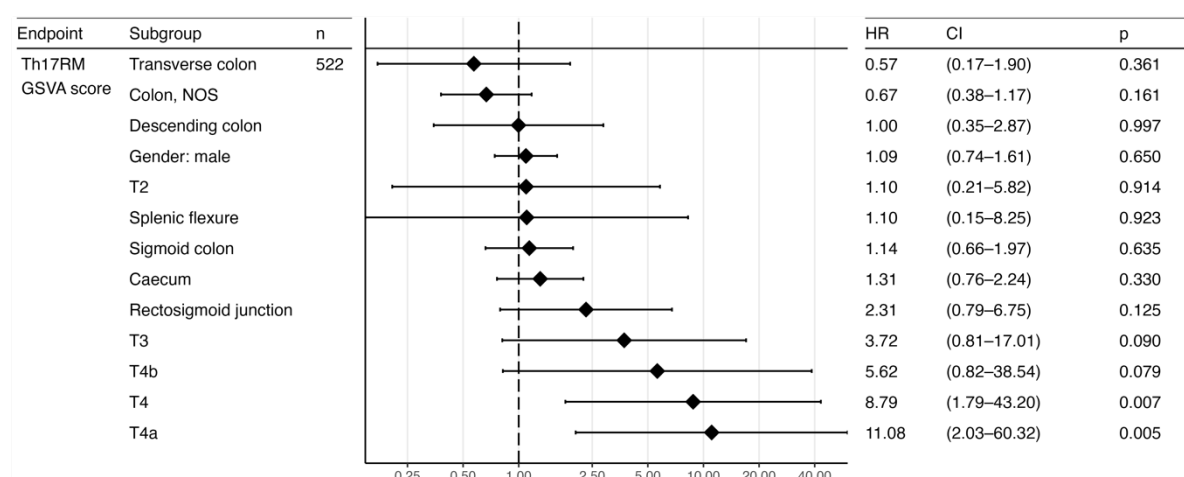


Figure 3.21 | Higher Th17RM GSV score associates with more developed tumours.

Multivariate regression analysis comparing Th17RM GSV score with clinical variables within The Cancer Genome Atlas colon adenocarcinoma (TCGA-COAD) cohort. Cox proportional hazards regression was used to calculate hazard ratios and statistical significance. HR = hazard ratio, CI = confidence interval. $p \leq 0.05$ is considered statistically significant.

3.4 Discussion

The functional profile of Th17 cells is highly susceptible to the local cytokine milieu. As we have previously described three transcriptionally distinct Th17 populations: Th17CM, Th17EM

and Th17RM cells, we deduced it was highly likely that these differences were a consequence of presiding within unique tissue microenvironments.

3.4.1 Deconvoluting the tumour immune landscape

We first assessed the relative tumour compositions of patients with high or low levels of Th17EM or Th17RM cells using a large, bulk RNA-seq cohort of colon adenocarcinoma patients. Several *in silico* deconvolution techniques have been developed in the past decade, with each capable of estimating the cellular composition of a given tissue sample from its bulk gene expression profile. These deconvolution techniques can be divided into partial or complete deconvolution methods, with partial methods relying on an input file of markers which are known to be expressed in a cell-specific manner and complete methods revealing cellular tissue compositions without any prior knowledge about the underlying cell types⁴³⁶.

In complex tissue types, such as that seen in dissected tumours, partial deconvolution techniques are considered more robust⁴³⁷ and so we chose to use the partial methodology utilised by the CIBERSORTx algorithm, which has been validated in tissue from HNSCC patients and optimised for immune cell deconvolution³⁸⁹. Moreover, a recent comparison of several deconvolution methodologies in bulk RNA-seq tissue from human brain tissue ranked CIBERSORTx as the most reliable algorithm to impute cellular fractions with⁴³⁷.

We used the TCGA-COAD cohort patient cohort to first explore the composition of the immune environment associated with Th17EM or Th17RM cells, using deconvoluted results with the LM22 cell signature matrix specially curated for use with CIBERSORTx. In patients with a higher Th17EM signature score, we found more prominent infiltration of activated CD4⁺ T cells, DCs, plasma cells and Tfh cells. This was an associative analysis and did not allow

us to determine which cell types modulated one another. However, it is known that CCR6⁺ DCs and CCR6⁺ B cells can migrate into the tumour tissue following CCL20 chemotactic gradients^{266,438}, a key cytokine produced by Th17EM cells (**Fig. 2.8C**) that might explain these associations. The collective upregulation of DCs, plasma cells and Tfh cells also alludes to the presence of tertiary lymphoid structures in some of these patients with a high Th17EM signature score. Th17 cytokines such as IL-17 and IL-22 have been implicated in the formation of these structures during chronic inflammatory processes⁴³⁹⁻⁴⁴² although a direct mechanistic link is yet to be described. Histologically associating the presence of TLSs in CRC patients with the presence of Th17EM cells would provide further insights into this speculative association.

Th17 cells have also been linked with the recruitment of CD8⁺ T cells into the tumour site^{183,193,266} and such an enrichment was observed in patients with a high Th17RM score, meaning that Th17RM cells may have particular adaptations that exacerbate this process. One mechanism of recruitment is mediated by the synergistic actions of IFN- γ and IL-17, which together act on tissue resident macrophages to upregulate the CXCL9 and CXCL10 that CXCR3⁺ CD8⁺ T cells migrate towards¹⁸³. As the number of polarised macrophages was also increased in patients with a high Th17RM signature score, it is plausible that escalated CXCL9 and CXCL10 gradients are generated in response to the presence of Th17RM cells. These two chemokines can also be abundantly expressed in both the stromal and epithelial compartment⁴⁴³ and so repeating our deconvolution with a broader input signature matrix, which could be generated from the scRNA-seq dataset, would facilitate more comprehensive cellular deconvolution of the tumour microenvironment.

3.4.2 Elucidating the signalling networks that polarise Th17RM cells

Next, to probe which ligands and cellular interactions drive the acquisition of tissue resident memory characteristics in Th17RM cells, we performed NicheNet ligand receptor analysis to compare putative ligand drivers with those predicted to drive Th17EM cells. Th17RM cells were responsive to an assortment of cytokines, including IL-23, IFN- γ , IL-1 β , TNF, TGF- β 1 and IL-18. However, without the additional *in vitro* validation step for each ligand, or ligand combinations, it remains unclear as to what contribution each speculative cytokine adds to the overall profile of Th17RM cells.

Comparing our results to the known ligand drivers that skew CD4⁺ TRM cells would intuitively confirm ligands of interest for further validation. However, our present knowledge regarding the factors required for the tissue establishment and maintenance of CD4⁺ TRM cells is poor. In CD8⁺ TRM cells, which have been far more extensively studied, TGF- β 1 constitutes the master regulator of their differentiation and is the key driver for CD103 expression, with IL-15 sustaining their presence in non-lymphoid tissues²⁴⁵. A key reason as to why TGF- β 1 has not been similarly implicated in the development of CD4⁺ TRM cells is a reported lack of CD103 surface expression in these cells^{246,248,249}. However, this is in stark contrast to our findings – we have described the expression of CD103 on Th17RM cells at the transcriptomic level with scRNA-seq and twice at the protein level using flow cytometry and immunohistochemistry. Thus, directly comparing our NicheNet output with the existing CD4⁺ TRM literature may not be the most reliable method to establish ligands of interest.

To our knowledge, only one other report has described CD103 on a population of Th17 cells. These cells were located within the skin, required IL-23 for their maintenance and mediated

anamnestic immunity against infection with *C. albicans*. CD301b⁺ myeloid cells were an important producer of the IL-23 necessary for the persistence of these Th17 TRM cells⁴⁴⁴. However, other than the initial infection with *C. albicans*, the exact ligands or driver cell population which initially permitted the generation of these CD69⁺ CD103⁺ Th17 cells, remains to be determined.

NicheNet did indicate that Th17RM cells respond to TGF- β 1, which could mean that they upregulate CD103 by a mechanism comparable to that seen in CD8⁺ TRM cells. However, TGF- β 1 signalling has been implicated in the development of Th17 cells for two decades without surface CD103 being reported. One possibility is that the presence of additional cytokines can alter the biological impact of TGF- β 1 signalling. This phenomenon has been well-characterised in the context of Treg or Th17 skewing where the additional presence of IL-6 is enough to oppose the regulatory effects of TGF- β 1 signalling alone⁴⁴⁵. Similar amendments to the activity of TGF- β 1 have been reported in CD8⁺ TRM cells, with IFN- β or IL-12 supplementation sufficient to effectively abrogate the stimulatory effect that TGF- β 1 has on CD103 upregulation⁴⁴⁶.

Not all cytokines have this suppressive compounded effect, however. When IL-18 signals in tandem with TGF- β 1 in naïve CD8⁺ T cells, no observable changes to surface CD103 levels can be seen, meaning that the important polarising effects of TGF- β 1 can indeed be maintained with additional cytokine stimulation⁴⁴⁶. The augmented anti-tumour properties that CAR T cells exhibit in response to IL-18 signalling⁴⁴⁷ additionally make IL-18 a particularly interesting speculative Th17RM ligand and investigating the effect of TGF- β 1 plus IL-18 exposure on Th17 cells *in vitro* could be an interesting next step.

It is also feasible that Th17 cells might express alternative signalling networks not observed in other CD4⁺ T cells. TGF- β 1 mediates the upregulation of CD103 via the Runx2 and Runx3 transcription factors in CD8⁺ TRM cells, which are additionally responsible for controlling CD8⁺ TRM cytotoxicity^{347,448}. Runx3 has not been detailed in CD4⁺ T cells and this deficiency has been shown to directly underpin their reported lack of responsivity towards TGF- β 1⁴⁴⁹. We did explore the expression of both Runx2 and Runx3 in our Th17 populations and found there was a positive trend in expression as cells progressed from Th17EM to Th17RM phenotypic states (**Fig. 2.13B**). However, we did not directly compare these expression levels with that seen in CD8⁺ T cells to give an indication as to whether this expression level was of that required for biologically active signalling. Exploring the activity of Smad3, which signals downstream of TGF- β R ligation⁴⁵⁰, could be further used to insinuate TGF- β 1 responsivity in Th17RM cells.

Finally, whilst NicheNet was able to provide a list of putative ligand drivers for Th17RM cells, it will be necessary to assess contribution of each to the expression profile of Th17 cells *in vitro*. We have worked under the assumption that all Th17EM cells transition into Th17RM cells under the appropriate polarising conditions but we should not overlook the possibility that transitional Th17 cells may exhibit disparate propensities for these driver signals. Within lymphoid tissues, persistent exposure to TGF- β 1 in naïve CD8⁺ T cells can actually favour their differentiation along the TRM trajectory upon immune challenge⁴⁵¹ and this environmentally determined proclivity for TRM differentiation could mean that Th17 cells in pre-defined niches experienced an enhanced reaction to putative Th17RM ligands.

3.4.3 Heterogeneity in Th17RM polarising myeloid populations

We initially assessed the expression of the Th17RM ligands by each broad class of cell within the scRNA-seq dataset GSE146771. Myeloid cells were the major sender population, enriched with ligands such as IL-1 β , IFN- γ , TNF, TGF- β 1 and IL-18. To explore these phenotypes of these myeloid sender cells with more granularity, we assessed ligand expression across each of the minor cell type annotations. Here, it was quite clear that a cluster of three macrophages (*NLRP3*⁺, *C1QC*⁺, *SPP1*⁺) and two DC populations (*CD1C*⁺, *BATF3*⁺) were poised to secrete Th17RM ligands.

Heterogeneity in the cell types that are capable of polarising TRM cells is similarly reported in the literature. In mice, IL-15, IL-12 and the co-stimulatory molecule CD24 have been shown to be essential for the differentiation of mouse skin and lung TRM cells, with all three being provided by *BATF3*⁺ DCs during cross-priming of dead cell antigen⁴⁵². Such cross-priming events by *BATF3*⁺ DCs are also necessary for the development of TRM cells in the tumour setting, driving the acquisition of TRM properties in circulating central memory T cells which enter the tissue and mediate effective tumour control⁴²⁷. The exploitation of scRNA-seq technology in recent years has also led to further dissection of the DC community and unique states are being continuously described⁴⁵³. In the wake of such advances, novel *CD11c*⁺ DCs marked by *CD1c*, *CD163* and *CD14* have been implicated in the preferential induction of a TRM-like transcriptome during priming, explained by their ability to provide active TGF- β 1 during these early T cell interactions^{454,455}. DCs with this combination of surface markers have also been reported to abundantly produce the cytokines IL-23 and TNF- α and exhibit superior ability to induce Th17 cells⁴⁵⁶.

We predicted interactions between Th17RM and DCs expressing both CD1c and BATF3 from NicheNet. However, exploring functional heterogeneity within each DC cluster could give additional clues as to which is more associated with Th17RM cells, if not a combination of all three. To further pinpoint the exact interactions that occur during Th17RM polarisation, recently developed techniques such as CosMx could aid with more precise characterisation regarding the myeloid cells that Th17RM cells interact with whilst retaining important spatial information concerning these interactions.

The identity of the macrophage phenotypes that best induce TRM cells in tumour tissue is less well understood, making it difficult to draw comparisons between our three identified clusters and what has been established. ‘Hot’ tumours with rich M1 macrophage infiltration have been associated with elevated TRM presence and better prognosis in lung cancer patients⁴⁵⁷, which aligns well with our conclusion that Th17RM cells reside in close proximity to CD11c⁺ macrophages and observations in viral infections where macrophages play a key role in sustaining protective CD4⁺ TRM cells⁴⁵⁸. Furthermore, although macrophage-derived IL-18 has not been directly implicated in TRM formation or sustenance, the role of TRM cells as rapid responders to secondary immune insults would strongly suggest some form of interplay between these two cell types.

3.4.4 Expanding the search for Th17RM interactions beyond the tumour

We chose to validate putative Th17RM cell interactions *in situ* using tumour tissue from CRC patients by spatial transcriptomics and immunohistochemistry. The selective enrichment of Th17RM cells by scRNA-seq within the tumour constituted the major rationale behind this decision, whilst tissue availability from our CRC patient cohort formed a second. We expected

to observe convincing interactions between Th17RM cells and their corresponding sender cells in these tissue sections and, whilst we did observe Th17RM cells in closer proximity to CD11c⁺ macrophages than their CD11c⁻ counterparts, some Th17RM cells were found in niches where macrophages were sparse. Therefore, it remains unclear whether the proximity of Th17RM cells with these CD11c⁺ macrophages is indicative of polarisation.

Within the literature, exactly where TRM cells become polarised remains inconclusive. Some reports describe the priming of CD8⁺ TRM cells adjacent to the epithelial border during both homeostasis and in the tumour setting due to epithelial expression of α_v integrins^{459,460}. Others have suggested that the induction of TRM cells takes place within lymphoid tissue by specialised APCs that similarly express these TGF- β 1-activating α_v integrins⁴⁵¹. The tissue distribution of different subpopulations of TRM cells is also reported to vary^{461,462} and so the location in which priming occurs could also impact TRM phenotype.

It has also been posited that TRM cells are far less static within non-lymphoid tissues than previously thought, despite the expression of markers that promote their retention such as CD69, CD103 and CXCR6³¹⁵. Circulating CD4⁺ TRM cells have been found in the circulation⁴⁶³ and, upon the induction of anamnestic immune responses, some TRM cells leave the tissue and migrate into local draining lymph nodes²⁴⁴. Additionally, pathogen specific CD8⁺ TRM cells can be found in all peripheral tissues regardless of whether these sites were directly infected and established TRM can further redistribute themselves based on the level of tissue inflammation, preferentially relocating into the most inflammatory milieu, regardless of the inflammatory stimulus^{243,464}. This phenomenon is likely intended to provide global protection in the wake of local immune insults, though it does raise the possibility that Th17RM cells,

despite being found within the tumour, are not polarised here and the key intercellular interactions that drive their functional profile occur within more distal sites.

To broaden the scope of our exploration into these intercellular interactions, it could be highly informative to stain tissue samples from the local draining lymph nodes of CRC patients, such as those within the mesenteric lymphatic network. Such tissue samples were not available to us although future collaborative work may facilitate the acquisition of these tissues. Another resourceful option might be to computationally explore Th17RM interactions in scRNA-seq datasets that sequence draining lymph nodes¹⁹.

3.4.5 Heterogeneity within Th17RM cells

Whilst exploring the distribution of Th17RM cells by immunohistochemistry, we noticed that a number of T cells displayed a 'partial' Th17RM phenotype, characterised by the expression of CD103 with either CD161 or CXCR6 (on top of a baseline expression combination of CD45O⁺ CD3⁺ CD4⁺ CD8⁻ ROR γ t⁺), but not both (**Fig. S3.7**). We were unsure how to classify these cells and did not investigate their proximity to either Th17RM or CD11c⁺ macrophages, however they may be indicative of heterogeneity within tissue Th17RM cells.

Functional variability within CD8⁺ TRM cells has been described. The expression of CD49a, for example, can clearly dissect CD8⁺ TRM cells on a compartmental and functional level within the skin. CD49a⁺ TRM preferentially secrete IFN- γ and granzyme B and accumulate within the epidermis, eradicating local melanocytes where they propagate a vitiligo-like pathology in response to IL-15. Contrastingly, CD49a⁻ TRM are characterised by the secretion of IL-17, are compartmentalised within the dermis and epidermis and are expanded in skin biopsies from patients with psoriasis⁴⁶².

Whether the co-expression of CD161 or CXCR6 alongside CD103 discriminates between functionally diverse Th17RM cells remains unclear. Their differential expression may also describe Th17RM cells moving into niches with a different ligand makeup, as chemokine receptors become internalised after receptor ligation or TCR stimulation⁴⁶⁵.

3.5 Conclusions

Th17 cells are highly adaptive within their local environments and can display nuanced phenotypes in response to fluctuations in ligand availability. Because of this, we rationalised that the tumours niches that house Th17EM and Th17RM are composed of different cellular landscapes. In this chapter, we find that patients who have tumours enriched with Th17RM-associated genes cells also display greater infiltration of activated macrophages, CD8⁺ and $\gamma\delta$ T cells compared with tumours enriched with Th17EM-associated genes. Ligand-receptor network analysis revealed that these macrophages are likely to promote the skewing of Th17EM cells into Th17RM cells by secreting a myriad of inflammatory cytokines, including IFN- γ , TNF, TGF- β 1 and IL-18, which are also found at higher levels in patients with higher Th17RM-associated genes. This proposed mechanism requires further validation *in vitro* to confirm the unique contribution of each ligand towards the complete Th17RM profile. Finally, we explore these interactions *in situ* and confirm that macrophages expressing the inflammatory marker CD11c are in closer proximity to Th17RM cells than those devoid of this marker.

Chapter 4: Exploring the function of Th17RM cells within the tumour microenvironment

4.1 Introduction

The contribution of T cells during anti-tumour immune responses is complex. The enormous spectrum of phenotypes, tissue adaptations and tumour contexts in which they function can make deciphering their effect on protective immune responses a challenge. However, certain T cell profiles have been more associated with better patient outcomes than others. The acquisition of a TRM phenotype is one of these more beneficial profiles and the accumulation of TRM cells within the tumour is more associated with favourable outcome in a number of cancers, including CRC^{258,320,466-468}.

One mechanism by which TRM cells achieve these robust anti-tumour responses is by initiating epitope spreading cascades via local dendritic cells²⁶¹, a process whereby primary anti-tumour responses facilitate additional secondary responses against novel antigens that arise from tumour cell death. Increasing evidence from experimental work in mouse tumour models and from observations in patient samples has also supported a role for TRM cells in the direct eradication of tumour cells, as well the propagation and maintenance of broader protective immune responses. Not only do both CD4⁺ and CD8⁺ T cells expressing CD103 display heightened cytotoxic functionalities than their CD103-negative counterparts^{466,469} but it is now postulated that these effector functions can be selectively executed towards tumour cells in a cancer setting. Here, CD103⁺ TRM cells are thought to encompass highly expanded tumour-reactive T cell clones that can physically interact with tumour cells in an MHC-dependent manner³²⁰.

Appreciating the importance of TRM cells has precipitated translational benefits too. A heightened frequency of intratumoural TRM cells is not only indicative of more favourable patient outcome but their presence within the tumour is now becoming appreciated as a useful biomarker that can stratify patient response to some immunotherapies^{265,460,470,471} and cancer vaccination strategies⁴⁷²⁻⁴⁷⁴.

We have previously described the existence of Th17RM cells, a population of CD103⁺ Th17 cells which reside within inflammatory tumour niches alongside specialised antigen-presenting macrophages, demarcated by the expression of CD11c. The functional implications of this cell type on tumour development and patient outcome, however, remain undetermined. Understanding the immunological consequence of accommodating intratumoural Th17RM cells could not only dictate whether resources are utilised to promote or inhibit their development *in vivo* but might also describe a beneficial phenotype that can be manipulated to inform the next advances in CAR T cell immunotherapies.

4.2 Aims

In this final experimental chapter, we aim to establish the functional programmes that are enriched in Th17 cells which acquire TRM characteristics (Th17RM) and establish how their cytokine profile differs from tumour-resident Th17 cells that fail to upregulate these properties. By exploring intercellular interactions using multiplexed immunohistochemistry, we probe how these effector functions might impact tumour development. Finally, we explore whether Th17RM cells feature as part of desirable anti-tumour immune responses in CRC patients, as well exploring whether these associations are conserved in a number of other cancer types.

4.3 Results

4.3.1 Th17RM cells might clonally expand in response to tumour antigen

Whilst the exploration of differentially expressed genes is necessary to elucidate identifiable population markers by scRNA-seq, studying them in isolation does not allow for their contextualisation within the broader signalling pathways or communication networks in which they may function. To translate the assortment of differentially expressed genes enriched in Th17RM cells into biologically relevant functionalities, overrepresentation analysis was employed using terms from the Gene Ontology biological processes category. This analysis enabled the catalogue of differentially expressed genes to be clustered and annotated based on the role they play as part of established biological processes.

Consistent with the activated effector profile of Th17RM cells, their tissue tropism and the comparative abundance of stem-like genes enriched in Th17CM cells (**Fig. 2.6B**), the most overrepresented pathways in Th17RM cells compared with Th17CM cells unsurprisingly constituted a number of T cell activation programmes and cellular adhesion pathways (**Fig. 4.1A**). This was also seen when compared with Th17EM cells (**Fig. S4.1**). Previous differential gene expression analysis had revealed a more similar transcriptional profile in Th17RM and Th17EM cells, with fewer upregulated genes defining Th17RM cells (**Fig. 2.6C**). Thus, the number of significantly enriched pathways in Th17RM cells after overrepresentation analysis was more surprising. TCR signalling, cellular proliferation and metabolic adaptations comprised the major differential pathways (**Fig. 4.1B**) which, in combination, describe the earliest events downstream of antigen recognition. In response to TCR ligation with cognate peptide-MHC and co-stimulatory signals, T cells switch their metabolic preferences towards

aerobic glycolysis, a state which rapidly, yet inefficiently, produces ATP to support the rapid production of cytokines and fuel intrinsic proliferative machineries^{475,476}.

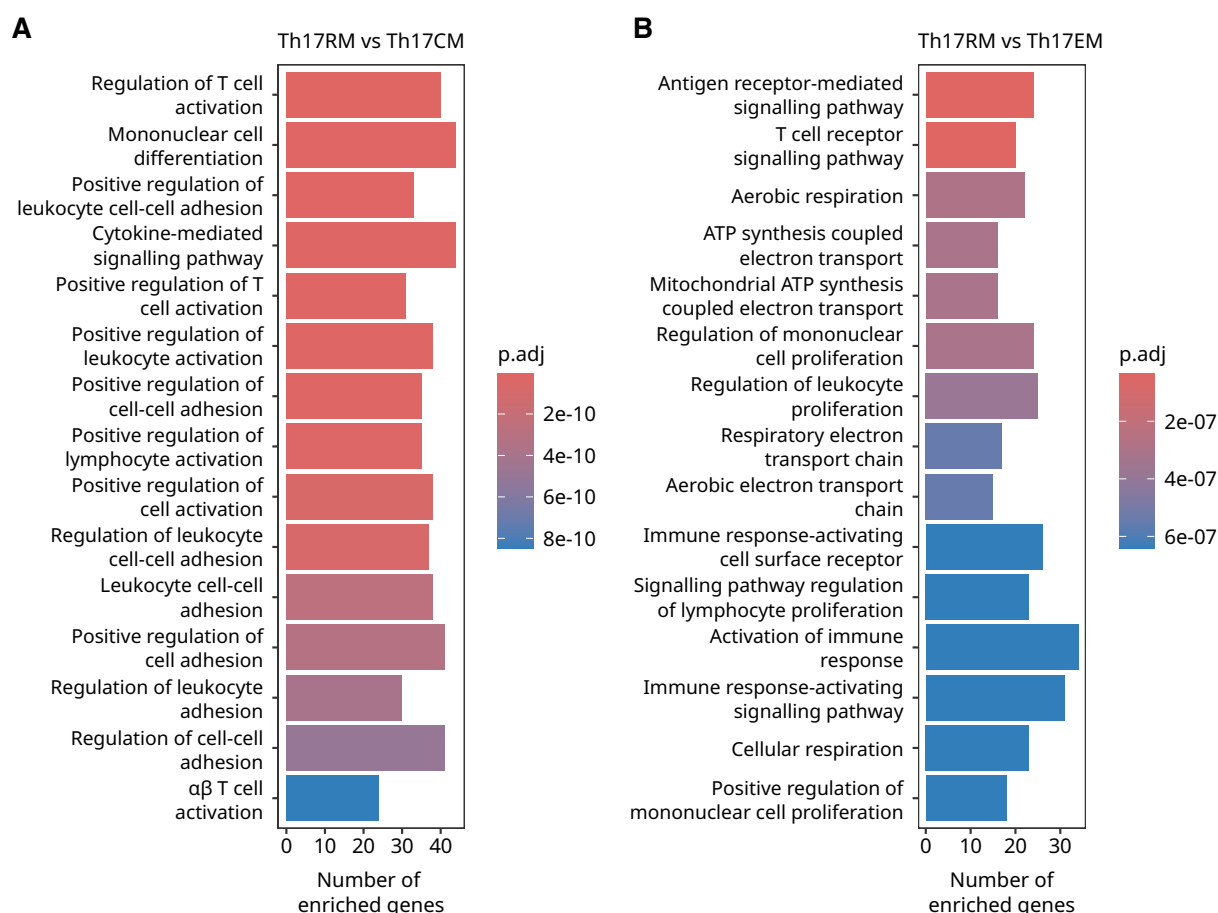


Figure 4.1 | Gene Ontology indicates that Th17RM cells are responding to antigen. Gene Ontology (GO) biological process overrepresentation analysis was used to explore the relationship between differentially expressed genes in Th17RM cells, as compared with (A) Th17CM and (B) Th17EM cells, and enriched functional pathways. Wilcoxon rank sum test with Bonferroni correction was used to identify differentially upregulated genes in Th17RM cells, with $\log_2$ fold change ≥ 0.5 and p.adj (adjusted p value) ≤ 0.05 considered significant. Significantly upregulated genes were then mapped onto terms within the GO biological processes ontology. One-sided hypergeometric test with Benjamini-Hochberg correction was used to determine the functional significance of each biological process. The top 15 most significantly upregulated biological processes are illustrated.

It is tempting to speculate that Th17RM cells may develop and proliferate in response to tumour antigen presented on APCs such as dendritic cells, tumour associated macrophages or epithelial cells. CD103, the key differentially expressed surface integrin that we have used to demarcate Th17RM cells, is selectively expressed by tumour reactive CD8⁺ T cells in a number of different malignancies, including CRC, and forms part of a signalling complex with CD61 that enhances the TCR signalling and downstream cytotoxicity of tumour infiltrating T cells^{320,477,478}. Moreover, the enhanced proliferation and cytotoxicity of CD103⁺ CD8⁺ T cells in non-small cell lung cancer patients is associated with IL-17 expression, with the accumulation of these CD103⁺ Tc17-like cells in treatment-naïve patients also functioning as a positive predictor of response to anti-PD-1 immunotherapy²⁶⁵. Furthermore, IL-18, a key cytokine upregulated alongside the presence of Th17RM cells (**Fig. 3.13**), can upregulate MHC class II expression on epithelial cells via an IFN- γ positive feedback loop⁴⁷⁹, potentially facilitating interactions between Th17RM and tumour cells.

However, despite major advances in machine learning and the development of high performance computational reconstruction models⁴⁸⁰⁻⁴⁸², imputing antigen specificity and reliably mapping cognate antigens to TCRs remains a challenging task⁴⁸³. Nevertheless, if the generation of Th17RM cells is a product of antigen recognition, we would expect to observe an expanded clonal repertoire within the Th17RM cluster. To investigate this idea, we explored the diversity of CDR3 TCR α chain sequences within each Th17 population, sequenced by Zhang *et al.* as part of the GSE108989 dataset¹, and compared this sequence diversity with that observed in each other T cell cluster.

Over 75% of the TCRs within Th17RM population ($n = 36$) contained an expanded clone, far more than the approximately 30% of Th17EM and 5% of Th17CM cells, suggesting they

constitute a greatly expanded cluster (**Fig. 4.2A**). Although cells in the *CX3CR1*⁺, *GZMK* (granzyme K)⁺ and *LAYN* (layilin)⁺ CD8⁺ T cells clusters contained a higher frequency of expanded clones, they also contained more overall cells ($n = 771$, $n = 840$, $n = 831$, respectively), making a direct comparison difficult to interpret. To account for this limitation, we utilised extrapolation by constructing rarefaction curves for each T cell cluster to enable a more reliable comparison of overall TCR diversity. Rarefaction analysis subsamples each population before calculating individual diversity indices using interpolation in order to extrapolate the results as if a complete population were sequenced, allowing comparisons between clusters of different starting sizes. After extrapolation, we found that the clonal diversity within Th17RM TCRs was similar to that observed in *CXCL13*⁺, *GNLY*(granulysin)⁺ and *GZMK*⁺ CD4⁺ as well as *CX3CR1*⁺ CD8⁺ T cell clusters (**Fig. 4.2B**). These populations have all been associated with efficacious anti-tumour responses by augmenting humoral responses⁴⁸⁴ and mediating tumour eradication by direct cytotoxicity^{485,486}, hinting that Th17RM cells may also exhibit similar functional benefits. Finally, we compared the extent of clonal overlap across all cells within the dataset (**Fig. 4.2C**). Potentially due to the small sample size within the Th17RM cell cluster, Th17RM cells showed relatively dissimilar clonal repertoire compared with other T cells. Most of their clonal overlap was shared with cells belonging to the *IL23R*⁺ CD4⁺ T cell cluster, perhaps unsurprisingly given the shared transcriptomic signature. Only one clone was shared with *CXCL13*⁺ CD4⁺ T cells and 2 were shared with those from the *CTLA4*⁺ CD4⁺ T cell cluster.

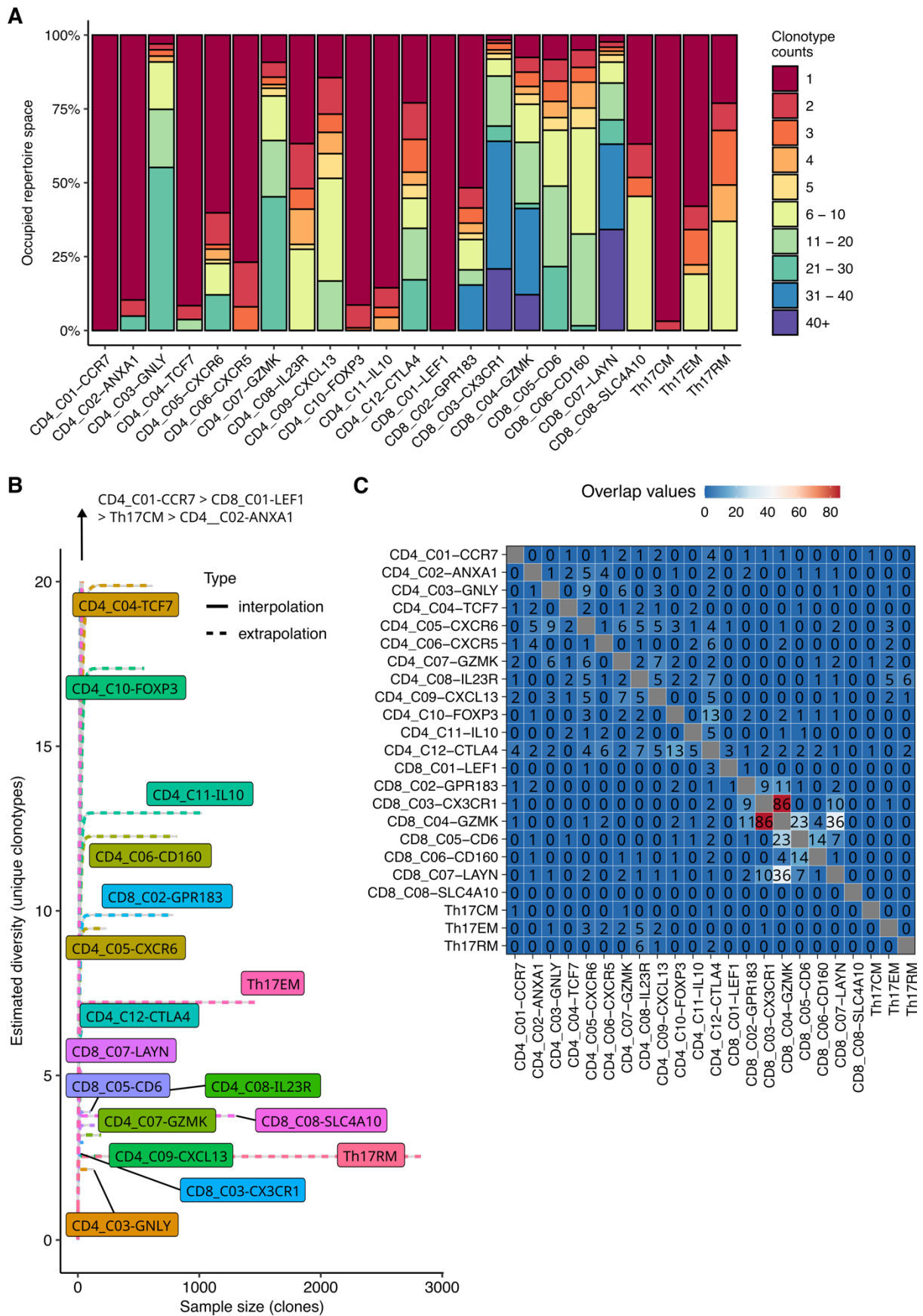


Figure 4.2 | Th17RM cells constitute a clonally expanded population. CDR3 nucleotide sequences for T cells in GSE108989 were determined by TCR α chain repertoire analysis, performed by Zhang *et al.*¹. Clonotype counts were calculated as the number of T cells sharing a common CDR3 nucleotide sequence. **(A)** The clonal repertoire profile of each T cell annotation where the frequency of CDR3 sequences is indicated by colour. **(B)** Rarefaction analysis to establish the relative clonal richness within each T cell population. Clonal diversity was first calculated using interpolation before being extrapolated to estimate the number of unique clonotypes within a complete T cell population. **(C)** The frequency of overlapping clonotypes between cell populations, indicated numerically and by colour.

4.3.2 Th17RM cells express cytotoxic effector molecules

For Th17RM cells to exhibit overlapping effector properties with anti-tumour CD8⁺ T cells, it is implied that they would be enriched with a similarly tumouricidal cytokine profile. Anti-tumour CD8⁺ T cells usually protect against tumour growth by releasing granules containing a cocktail of cytotoxic proteins, namely perforin, granulysin and granzymes. Mechanistically, perforin and granulysin create pores in the target cell membrane, allowing the entry of granzymes into the cytoplasm. Here these granzymes, particularly granzymes A and B, induce DNA breaks, trigger mitochondrial damage and activate caspases, initiating a number of apoptotic pathways that eventually lead to target cell death⁴⁸⁷⁻⁴⁹⁰.

To explore any commonalities that may exist between the cytokine profiles of CD8⁺ and Th17RM cells, we first explored the transcriptomic expression of these key tumouricidal cytokines, alongside a selection of more established Th17-related effector molecules. Nearly all Th17RM cells expressed transcripts for *GZMA* (granzyme A), *GZMB* (granzyme B), *PRF1* (perforin) and *GNLY* (granulysin). In fact, a higher percentage of Th17RM cells expressed more cytotoxic transcripts than transcripts encoding any other transcript tested, including *IL17A*

and *CCL20* (**Fig. 4.3A**). The cells expressing the highest levels of cytotoxic proteins appeared to localise in the bridge between Th17EM and Th17RM cells when plotted by UMAP (**Fig. 4.3B**), perhaps suggesting that these putatively cytotoxic effector functions are acquired early in the transition phase as Th17 cells accumulate resident memory adaptations.

We next sought to determine whether the expression of these genes was comparable to that measured in CD8⁺ T cells. The expression level of *GZMA*, *GZMB*, *PRF1* and *GNLY* in each Th17 population was contrasted with that detected in CD8⁺ T cell clusters from GSE108989. As anti-tumour properties have been described in CX3CR1⁺, granzyme K (*GZMK*)⁺ and layilin (*LAYN*)⁺ CD8⁺ T cells⁴⁹¹⁻⁴⁹⁵, we hypothesised that a comparable expression level in Th17RM cells might confer a similar degree of protective immunity.

No significant difference in the expression of *GZMA* was detected in Th17RM cells compared with the majority of high-expressing CD8⁺ T cells, with only *LAYN*⁺ CD8⁺ T cells expressing more (**Fig. 4.3C**). However, although Th17RM cells were the highest Th17 expressors of *GZMB* and *PRF1*, expression was significantly less than that measured in most CD8⁺ T cells (**Fig. 4.3D**, **Fig. 4.3E**). The median expression value of *GNLY* was almost 0 in all of the cell types investigated although a reasonable expression distribution in some of the CD8⁺ T cell clusters was observed (**Fig. 4.3F**).

Whether these differences in expression translate into functional differences remains unclear. An analogous comparison between cytotoxic CD4⁺ and CD8⁺ T cells in human melanoma showed comparable levels of *GZMA*, *GZMB*, *PRF1* and *GNLY* transcripts⁴⁹⁶, suggesting that cytolytic activity might be less robust than that seen in CD8⁺ T cells. However, whilst the release of cytotoxic granules is one mechanism by which CD4⁺ T cells can exhibit

cytotoxicity⁴⁸⁶, it is not the only mechanism. The production of IFN- γ is also sufficient for CD4⁺ T cells to elicit tumouricidal activity⁴⁹⁷, of which Th17RM cells additionally express (**Fig. 4.3A**).

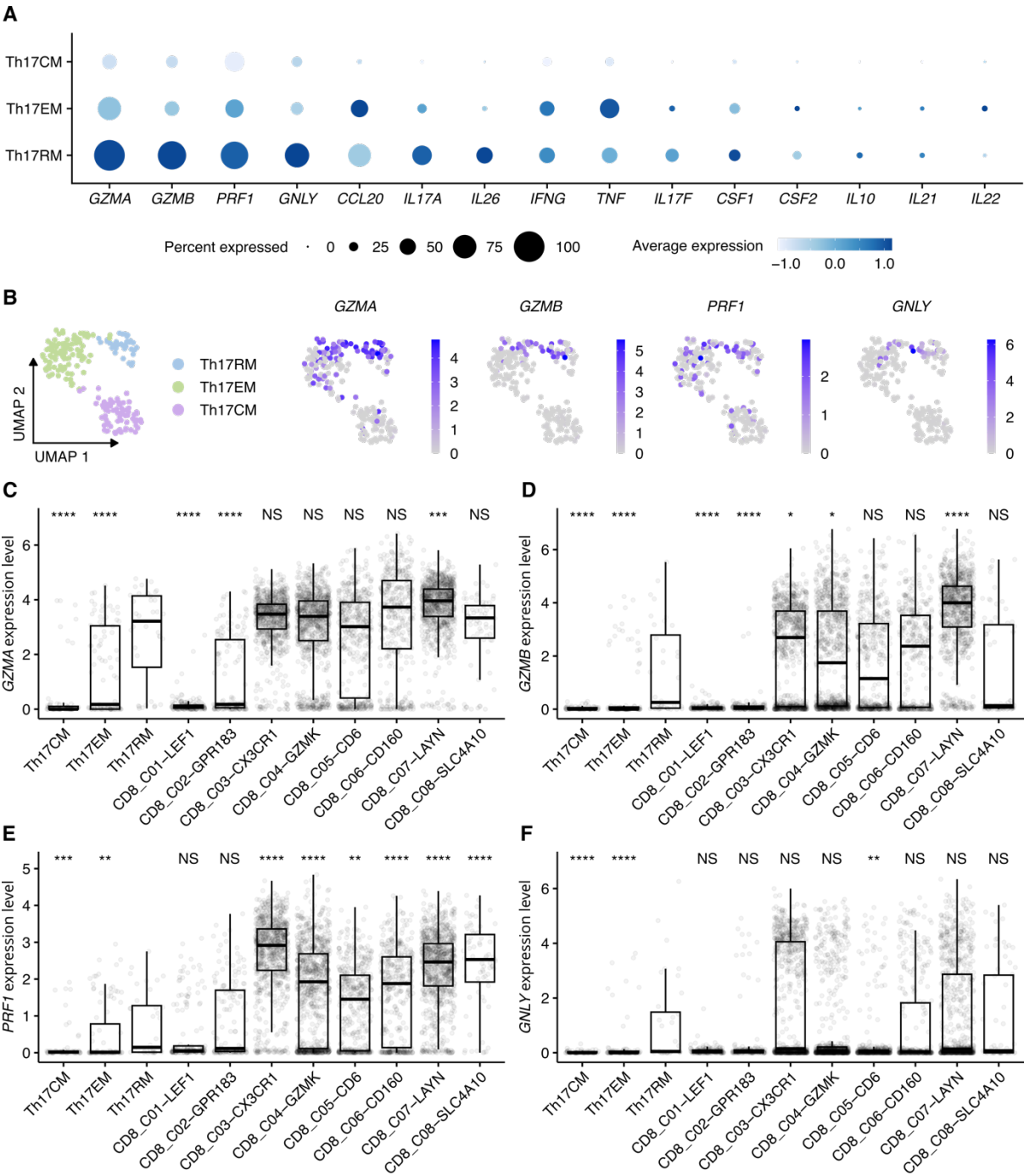


Figure 4.3 | Th17RM cells are transcriptomically enriched with cytotoxic effector molecules compared with other Th17 cell populations. (A) Quantitative representation of transcriptomic cytokine expression. Dot size represents the percentage of cells in each

population expressing the cytokine and colour saturation indicates the relative expression level, scaled across each population. (B) Normalised expression level and distribution of *GZMA* (granzyme A), *GZMB* (granzyme B), *PRF1* (perforin) and *GNLY* (granulysin) expression across Th17 UMAP clusters. Comparative normalised expression levels of (C) *GZMA*, (D) *GZMB*, (E) *PRF1* and (F) *GNLY* in Th17 and CD8⁺ T cell annotations. Data are displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range for each cytokine. Pairwise Wilcoxon tests with Benjamini-Hochberg correction were used to calculate the statistical comparisons, with Th17RM cells set as the reference population. Adjusted p values are shown, where NS (not significant), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

To establish whether the expression of cytokine transcripts translated into the secretion of functional cytokines, we liberated single cells from the primary tumour resections of four CRC patients then stimulated them with PMA and ionomycin in the presence of brefeldin A. In combination, these pharmacological agents mimic early TCR signal transduction pathways and block intracellular protein trafficking, trapping cytokines in the endoplasmic reticulum for downstream analysis.

To validate the reliability of our intracellular cytokine detection by flow cytometry, we first confirmed that positive staining for IL-17A was indicative of ROR γ t expression (**Fig. 4.4A**). Next, to address the hypothesis that a resident memory phenotype in Th17 cells is associated with heightened expression of cytotoxic molecules, the percentage of CD103⁺ Th17 (CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ ROR γ t⁺) cells positive for these proteins was compared with their CD103⁻ counterparts (**Fig. 4.4B**). Both granzyme A and granzyme B were significantly upregulated in CD103⁺ Th17 cells, as was IL-17A (**Fig. 4.4C**). Although there was a generally positive trend for both IFN- γ and TNF- α , neither reached statistical significance, alluding to the notion that CD103⁺ Th17 cells are not simply an analogous 'pathogenic' Th17 population found within the

tumour. Perforin expression was also measured however it was not detected in Th17 or CD8⁺ T cells, suggesting that the antibody staining did not work (data not shown).

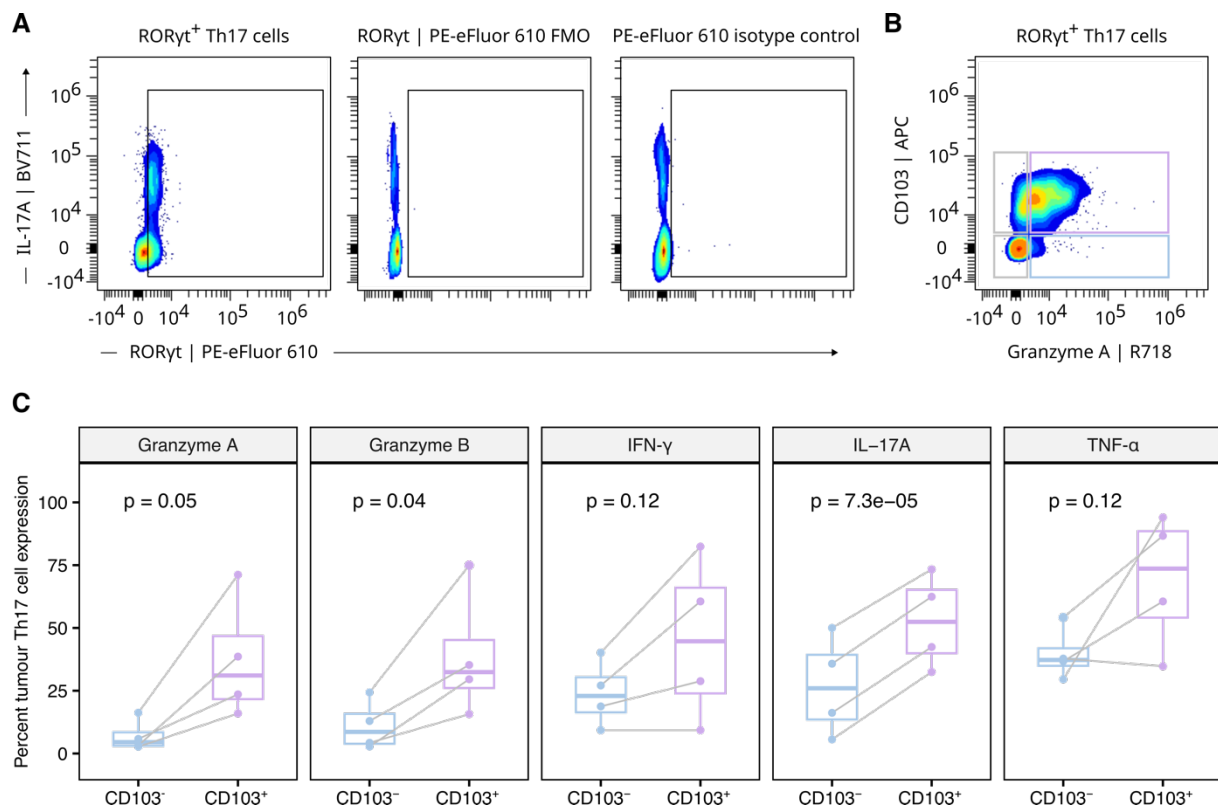


Figure 4.4 | Tumour CD103⁺ Th17 (Th17RM) cells produce more granzyme A, B and IL-17A than their CD103⁻ counterparts. Recently frozen (< 4 months) resected tumours from CRC patients was thawed and digested to liberate single cells, which were stimulated with PMA and ionomycin in the presence of brefeldin A for 6 hours. Stimulated cells were then extracellularly stained for Th17RM-identifiable markers before being fixed, permeabilised and intracellularly stained for cytokines. **(A)** Flow cytometry plots displaying the gating strategy to identify RORγt⁺ cells, alongside the same gates on RORγt PE-eFluor 610 FMO and rat IgG2a PE-eFluor 610 isotype control samples. **(B)** Gating strategy to identify cytokine⁺ non-Th17RM (CD45RO⁺ CD3⁺ CD8⁻ CD4⁺ RORγt⁺ CD103⁻) cells (blue box) and cytokine⁺ Th17RM (CD45RO⁺ CD3⁺ CD8⁻ CD4⁺ RORγt⁺ CD103⁺) cells (purple box). Percent cytokine expression in non-Th17RM and Th17RM populations was calculated as the number of cytokine⁺ cells divided by the total number of cells per population (non-Th17RM cell total = blue gate plus bottom left grey gate; Th17RM cell total = purple gate plus top left grey gate). Granzyme A flow

cytometry plot is representative of all the cytokines measured. **(C)** Percent cytokine expression in non-Th17RM (CD103⁻) and Th17RM (CD103⁺) cells. Data are displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range of the percent tumour Th17 population cytokine expression. $n = 4$ with paired t-test used to calculate statistical comparison. p values are shown in numerical form, where $p \leq 0.05$ is considered statistically significant.

4.3.3 Granzyme B⁺ Th17RM cells associate with tumour cells

The ability of cytotoxic anti-tumour T cells to eradicate tumour cells is not simply determined by volume of cytotoxic proteins released into the tumour microenvironment. The proximity of tumouricidal T populations to malignant cells represents another key feature that influences the efficacy of protective immune responses. Although anti-tumour T cells can often be physically identified within tumour masses, their prevention from directly interacting with tumour cells is frequently correlated with poor clinical outcome^{498,499}. Some of the best characterised mechanisms of immune exclusion include physical impediment by stromal cells enveloping tumour nests^{500,501} and the ineffectual utilisation of T cell engagement machinery by non-productive engagement with tumour associated macrophages⁵⁰².

We utilised multiplex immunofluorescence staining to characterise the precise tumour niche in which granzyme B⁺ Th17RM cells could be located. Whilst the absolute number of observable granzyme B⁺ Th17RM cells did vary considerably per patient within our staining cohort, perhaps due to tumour heterogeneity or the variable depth at which each tumour slide was cut, we did consistently find that the majority of these putatively cytotoxic Th17RM were embedded within the tumour epithelium (**Fig. 4.5A, 4.5B**). Exploring the expression level of apoptotic markers within tumour cells, such as cleaved caspase 3, would be a useful additional test for validating a functional cytotoxic relationship with Th17RM cells.

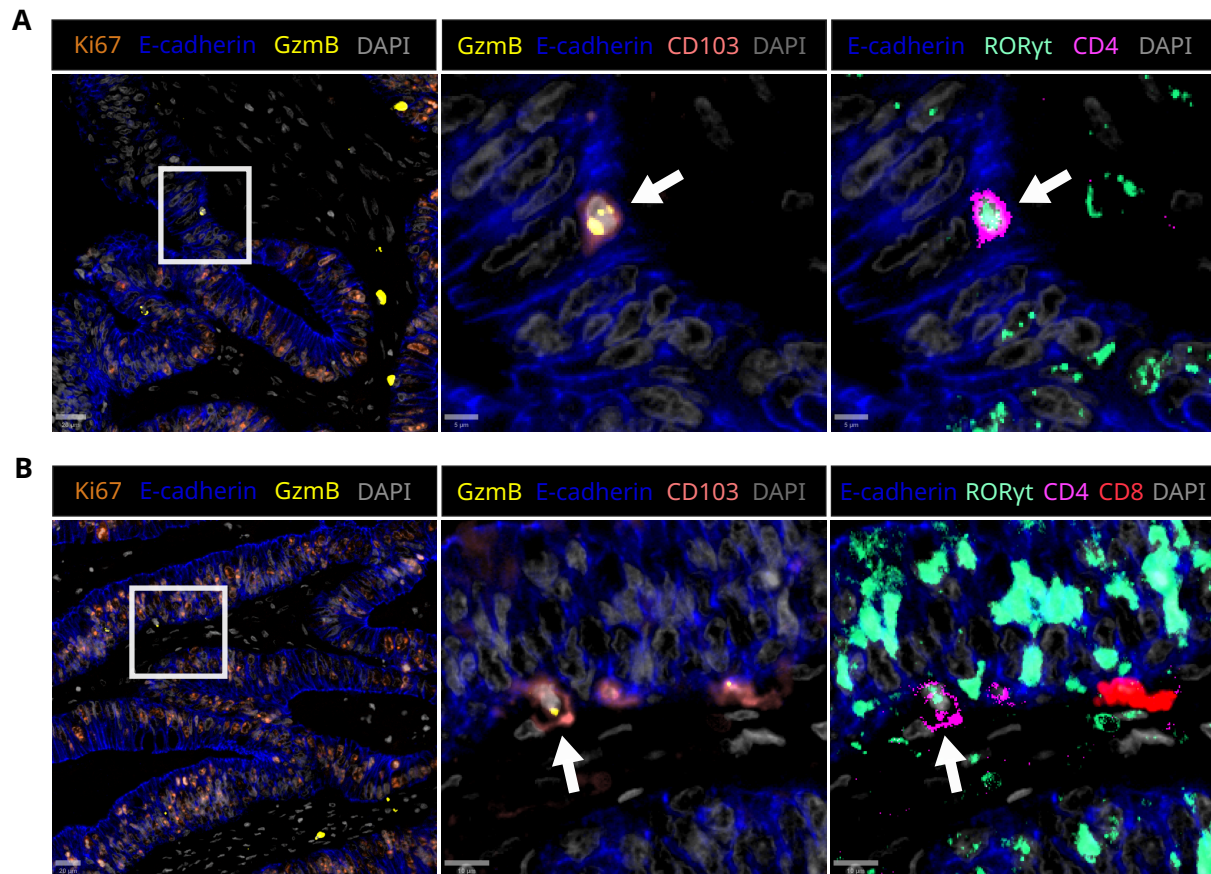


Figure 4.5 | Putatively cytotoxic Th17RM cells are proximal to the tumour epithelium.

Immunofluorescent staining of Ki67 (orange), E-cadherin (dark blue), granzyme B (GzmB, yellow), DAPI (grey), CD103 (peach), ROR γ t (aquamarine), CD4 (purple) and CD8 (red) in tumour sections from CRC patients to visualise the localisation of granzyme B⁺ Th17RM cells (white arrows) in **(A)** patient GI 8790 and **(B)** GI 8870. Th17RM cells are defined by positive staining for CD45RO, CD3, CD4, CD103, CD161 and CXCR6 and images are representative of staining in the whole patient cohort. Scale bars are 20 μ m in the left image and 5 μ m in the middle and right images.

4.3.4 High expression of a Th17RM signature is associated with favourable prognosis in colon adenocarcinoma

If the notion that Th17RM cells have tumouricidal properties is true, we would speculate that an increased number of Th17RM-tumour interactions would positively impact the clinical

outcome of CRC patients. Whilst we were able to quantify the frequency of observable *in situ* interactions in the CRC cohort used for immunofluorescence staining, we did not have enough patients to control for extraneous variables such as tumour stage and would not have the statistical power to reliably conclude whether the number of interactions had any impact on clinical outcome.

To mitigate these limitations, we utilised the large and clinically detailed colon and rectum adenocarcinoma TCGA cohorts to establish whether Th17RM cell infiltration impacts prognostic outcome. Like before, GSVA was used to score each patient based on the expression of our previously curated seven gene Th17RM signature within their bulk RNA-seq data. Each cohort was then divided into equal tertiles, with the highest scoring 33% of patients collectively termed Th17RM^{high}, those with medium score values termed Th17RM^{med} and those in the lowest scoring tertile termed Th17RM^{low}. Cox proportional hazards regression was then performed, incorporating the survival status of each patient before Kaplan-Meier curves were plotted to visualise the results.

In colon adenocarcinoma, a high Th17RM score was significantly associated with increased survival, compared with patients in both Th17RM^{med} and Th17RM^{low} groups (**Fig. 4.6A**). However, this immunological protection was not conserved in the rectal adenocarcinoma cohort, with no significant differences observed between any of the patient groups (**Fig. 4.6B**).

Although the medical and surgical interventions used for colon and rectal adenocarcinomas have considerable overlap, hence the two diseases are frequently amalgamated into the broad umbrella term of 'colorectal cancer', it is becoming increasingly appreciated that each

disease contains distinct histological and anatomical features that associate with variable clinical outcomes⁵⁰³. Some of these differences also extend to immunological heterogeneity. CMS1 tumours which are MSI and are more associated with the infiltration of anti-tumour T cells (**Table 1.1**), for example, are more frequently found in the colon^{504,505}. Conversely, CMS4 tumours are found more frequently in the rectum and are associated with stromal infiltration, rich TGF- β 1 signalling and worsened prognosis.

Observing the histology of the tumours analysed by multiplex fluorescence imaging which had high infiltration of Th17RM cells, they appeared to be serrated in nature with good immune infiltration (**Fig. 3.20**). This would suggest that these tumours belonged to CMS1 which might be why patients with rectal adenocarcinoma are not protected. Confirming the association between Th17RM cells and CMS1 tumours by extending our multivariate regression analysis to include CMS subtype (**Fig. 3.21**) will be a necessary step.

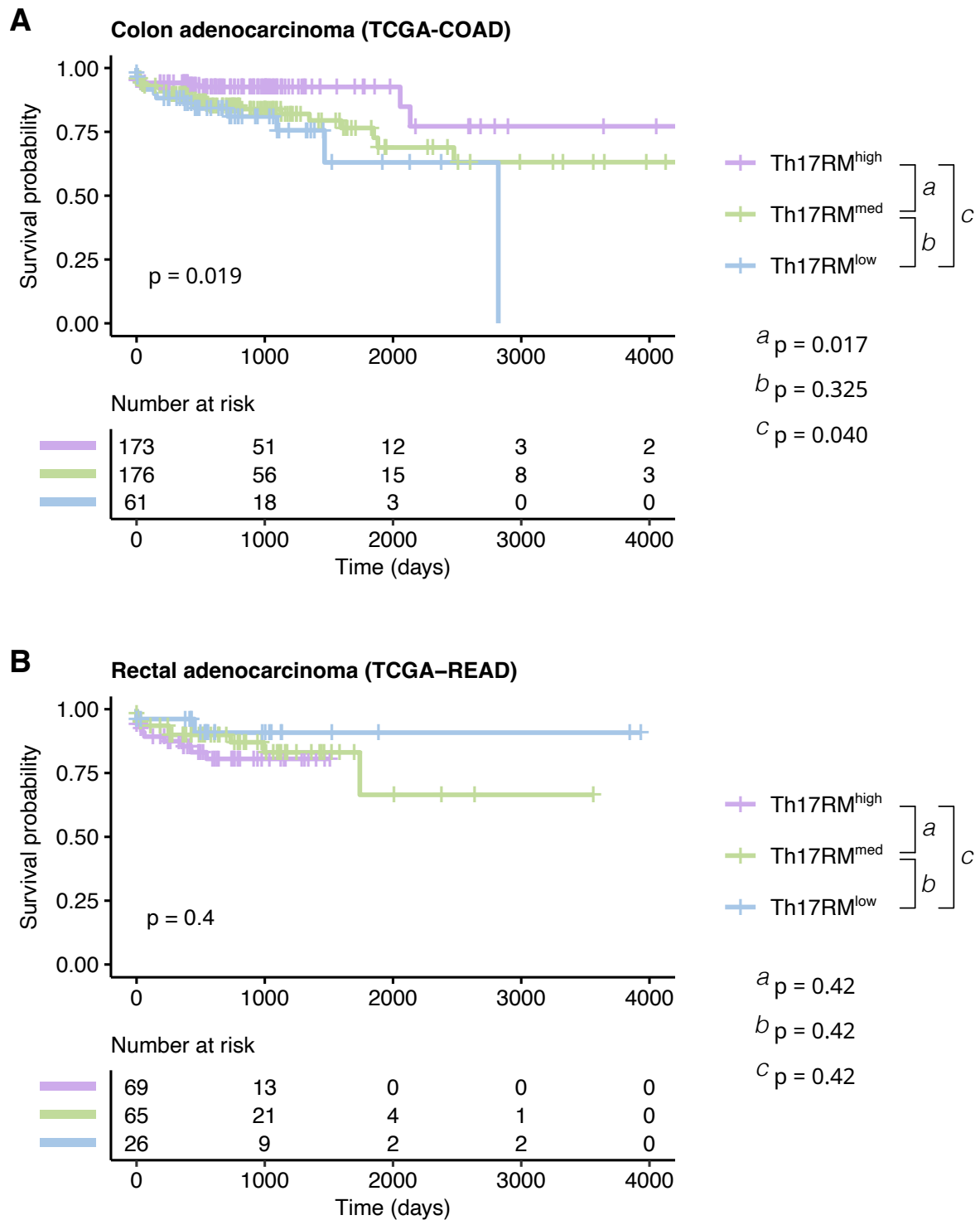


Figure 4.6 | The Th17RM cell signature is protective in colon adenocarcinoma but not in rectal adenocarcinoma. Patients from The Cancer Genome Atlas (**A**) colorectal adenocarcinoma (TCGA-COAD) and (**B**) rectal adenocarcinoma (TCGA-READ) cohorts were scored using GSVA with our previously curated seven gene Th17RM signature (**Fig. 3.2**). Each cohort was divided into equal tertiles, with patients in the lowest scoring tertile termed

Th17RM^{low}, those in the middle scoring tertile termed Th17RM^{med} and those in the highest scoring tertile termed Th17RM^{high}. Cox proportional hazards regression was then performed with the associated patient survival metadata before Kaplan-Meier survival curves were plotted, stratified by each GSVA tertile. p values calculated using Cox proportional hazard regression analysis are displayed on the Kaplan-Meier plots. Log-rank test with Benjamini-Hochberg correction was used to calculate pairwise statistical comparisons between each GSVA tertile. $p \leq 0.05$ is considered statistically significant.

4.3.5 The protective Th17RM signature extends to melanoma patients

As Th17 cells typically mediate tissue surveillance at mucosal barrier surfaces, they are implicated in a range of other cancers. Whether the presence of Th17 cells is prognostically beneficial or deleterious remains an open question and the extraordinary plasticity of Th17 cells, as well as the variety of cellular compositions that make up tumour milieus, likely contribute to the discordant conclusions reported and inconclusive stance taken by the scientific community. To explore whether the particular adaptations acquired by Th17RM cells correlate with immunological protection in non-CRC malignancies, we scored TCGA cohort patients from a number of cancer types in which Th17 cells have been implicated.

One highly studied cancer in the context of Th17 cells is melanoma and multiple reports that adoptively transferring *in vitro* polarised TCR transgenic Th17 cells into B16 melanoma expressing mice results in tumour eradication has led to the general consensus that Th17 cells may provide one form of therapeutic protection in melanoma^{187,188,266,270,506}. Like colon adenocarcinoma, cutaneous melanoma patients with the highest Th17RM score had a significantly improved survival probability (**Fig. 4.7A**), suggesting that putatively cytotoxic Th17RM cells may form an important mechanism of anti-tumour immunity. However, despite the striking outcome stratification seen in melanoma patients, Th17RM score had no impact

on survival probability in any of the other cancer types we tested, including lung adenocarcinoma, lung squamous cell carcinoma, invasive breast carcinoma and head and neck squamous cell carcinoma (**Fig. 4.7B**). Tumour heterogeneity might provide one explanation for these starkly contrasting observations, whilst the range of adaptations that TRM cells acquire in different tissues may form a second interpretation. Although a core cluster of markers are commonly used to demarcate TRM cells, such as CD103 and CD69, recent single cell profiling of lung, jejunum and skin-resident TRM cells revealed distinct tissue-specific gene signatures⁵⁰⁷. This may indicate that analogous, protective Th17RM cells in other cancer types might not share the same seven gene signature that defines those characterised in colon adenocarcinoma.

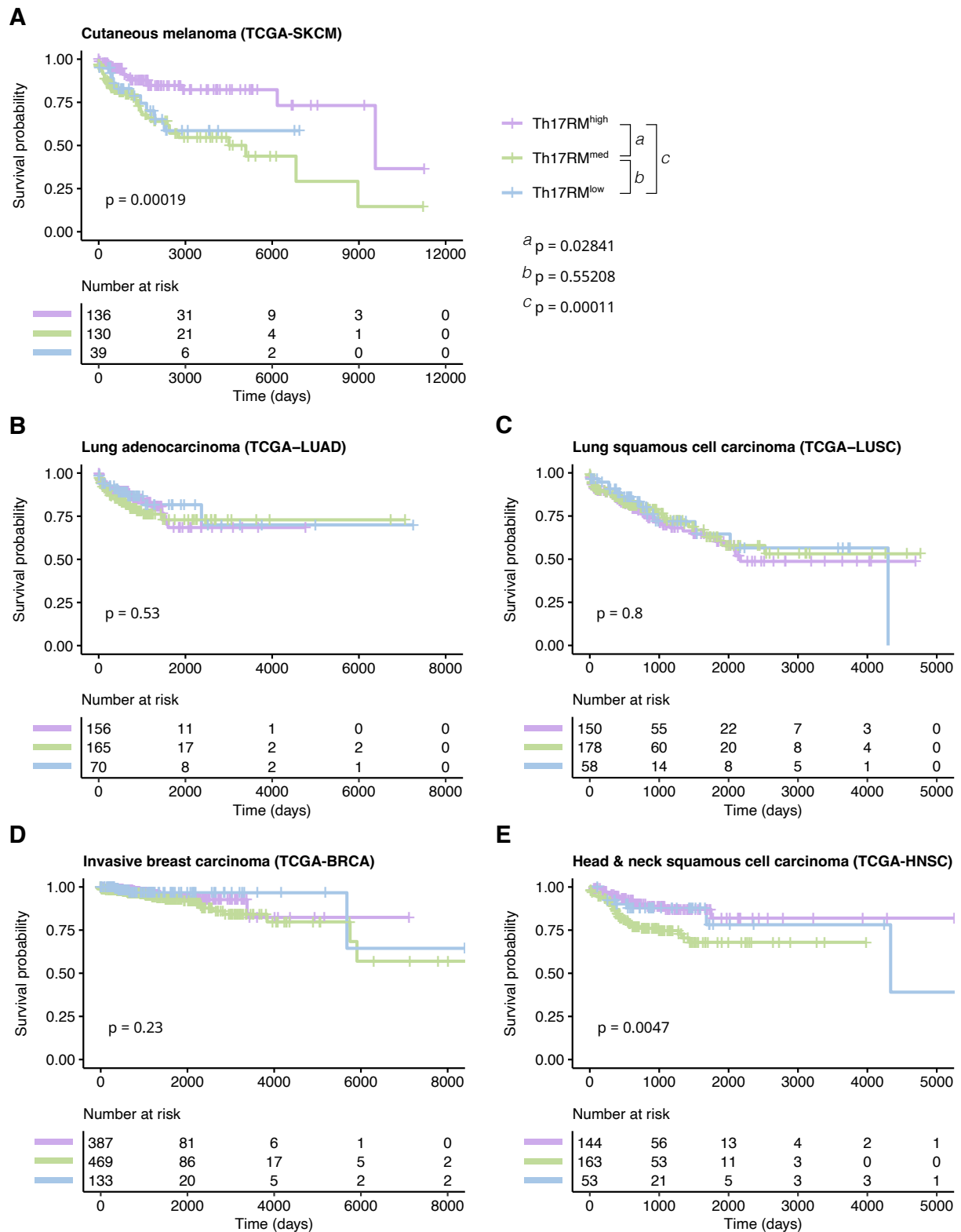


Figure 4.7 | A high Th17RM signature score is not universally protective in cancer. Patients from The Cancer Genome Atlas (**A**) cutaneous melanoma (TCGA-SKCM), (**B**) lung adenocarcinoma (TCGA-LUAD), (**C**) lung squamous cell carcinoma (TCGA-LUSC), (**D**) invasive

breast carcinoma (TCGA-BRCA) and (E) head and neck squamous cell carcinoma (TCGA-HNSC) cohorts were scored using GSVA with the seven gene Th17RM signature curated previously (Fig. 3.2). Each cohort was divided into tertiles, with patients in the lowest scoring tertile termed Th17RM^{low}, those in the middle scoring tertile termed Th17RM^{med} and those in the highest scoring tertile termed Th17RM^{high}. Cox proportional hazards regression was then performed on the associated patient survival metadata before Kaplan-Meier survival curves were plotted, stratified by each GSVA tertile. p values calculated using Cox proportional hazard regression analysis are displayed on the Kaplan-Meier plots. (A) Log-rank test with Benjamini-Hochberg correction was used to calculate pairwise statistical comparisons between GSVA tertiles. $p \leq 0.05$ is considered statistically significant.

4.3.6 Do Th17RM cells exhibit superior tumour control compared with Th17EM cells?

As the expression of CD103 on tumour Th17 cells is associated with augmented effector functionality and the Th17RM signature is protective in both colon adenocarcinoma and cutaneous melanoma patients, we speculated that Th17RM might have a direct role in restricting tumour cell viability. To test this hypothesis, we planned to isolate Th17 cells from tumour resections, dividing them into CD103⁺ and CD103⁻ fractions then co-culturing them with a CRC cell line to measure cytotoxicity.

In our previous *ex vivo* or *in situ* experiments, we have utilised the rat antibody clone AFKJS-9 to mark and identify ROR γ t⁺ Th17 cells. However, to establish heightened effector functionality in the CD103⁺ Th17 fraction translates into more efficient tumour control, we would be required to sort viable Th17RM cells from tissue using FACS. This means that the fixation and permeabilisation steps required for the intranuclear staining of ROR γ t would not be compatible with this experimental design as these processes are cytotoxic.

In mouse models, Th17 cells are frequently isolated from blood or tissue using *Rorc* or *Il17a* reporter mice. These reporters allow for the identification and subsequent sorting of cells expressing translated ROR γ t or Il17a without the need to fix or permeabilise the cells before downstream *in vitro* culture. However, these techniques are not compatible with human cells and so we were first required to validate a surface marker panel which could be used as a surrogate for ROR γ t expression and the subsequently the identification of Th17RM cells.

In situ, we defined Th17RM cells as CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ ROR γ t⁺ CD103⁺ CD161⁺ CXCR6⁺ cells. To firstly explore how effectively this surface marker combination encapsulates cells expressing ROR γ t, we stained three freshly resected tumours for analysis by flow cytometry and compared the expression of ROR γ t in the CD4⁺ T cell compartment with those additionally expressing CD103, CD161 and CXCR6. We saw a remarkably consistent increase in the percentage of cells expressing ROR γ t when co-expressing CD103⁺ CD161⁺ CXCR6⁺ alongside CD4. Approximately 72% of CD4⁺ CD103⁺ CD161⁺ CXCR6⁺ T cells expressed ROR γ t, as opposed to the approximately 35% of CD4⁺ T cells within the tumour expressing ROR γ t (**Fig. 4.8**). Although an arbitrary comparison, early studies using ROR γ t transgenic mice showed that only 65% of ROR γ t⁺ IL-17⁺ cells in the small intestine expressed the surface chemokine receptor CCR6, a commonly used surrogate marker for Th17 cells³⁵⁷, satisfying us that our surface panel of CD4⁺ CD103⁺ CD161⁺ CXCR6⁺ was sufficient specific to sort Th17RM cells without ROR γ t staining.

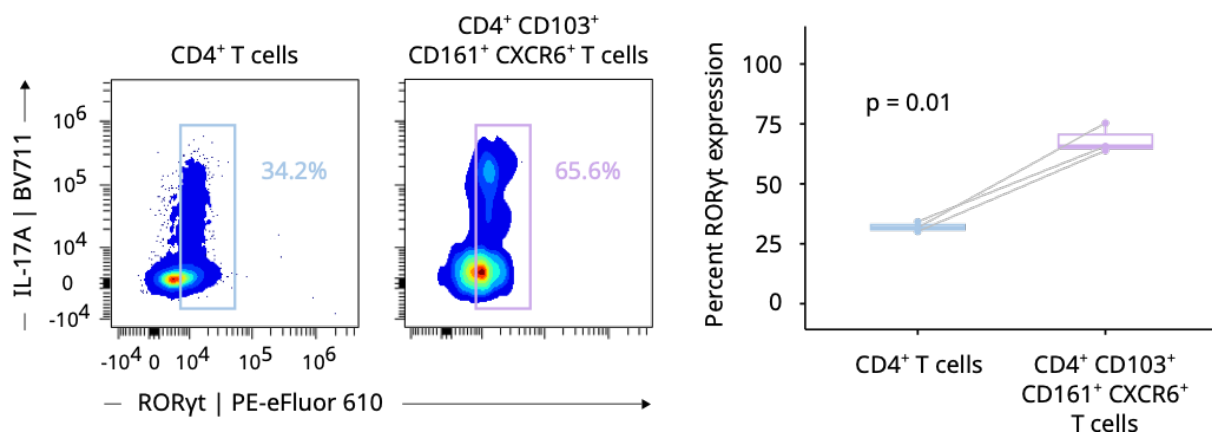


Figure 4.8 | CD103, CD161 and CXCR6 collectively define a population of CD4⁺ T cells enriched for RORγt. Freshly resected tumours from CRC patients were digested to liberate single cells, extracellularly stained for Th17RM-identifiable markers then fixed, permeabilised and intracellularly stained for RORγt. Representative flow cytometry plots (left) and quantification (right) of RORγt⁺ cells in CD4⁺ (CD45RO⁺ CD3⁺ CD4⁺ CD8⁻, gating as performed in **Fig. 3.10**) and CD4⁺ CD103⁺ CD161⁺ CXCR6⁺ T cell populations are shown. Quantitative data are displayed as box and whisker plots showing the median, 25th and 75th percentiles and two whiskers at the 1.5x interquartile range of the percent RORγt expression. $n = 3$ with paired t-test used to calculate statistical comparison. $p \leq 0.05$ is considered statistically significant.

As a first optimisation step for the co-culture assay, Colo205 cells (human CRC cell line) were cultured with magnetically enriched CD3⁺ T cells from PBMCs banked from healthy patients. The T cells were then stimulated with solitomab, PMA with ionomycin or PHA-L for 2 hours before killing efficacy was determined across a range of effector-target (ET) ratios, ranging from 0.125:1 to 1:1. Solitomab is a recombinant bispecific antibody that recognises human CD3 and EpCAM⁵⁰⁸ and we used this to activate and redirect T cells towards EpCAM⁺ Colo205 cells. PMA with ionomycin mimics early TCR signal transduction pathways and PHA-L is often used as an alternative for anti-CD3/28 stimulation in CAR T cells due to its ease of use and cost-effectiveness^{509,510}.

We observed clear a dose-dependent dose dependent killing of tumour cells in all three T cell stimulation conditions, as measured using a kit to detect the activity caspase-3, a reliable apoptotic marker⁵¹¹ (**Fig. 4.9A**). At the highest E:T ratio tested, PHA-L might slightly augment T cell mediated cytotoxicity. We next repeated the co-culture using CD3⁺ T cells isolated using FACS from the same bank of healthy patient PBMCs. This time, the number of apoptotic Colo205 cells was greatly reduced and the dose-dependent effect was lost (**Fig. 4.9B**). We are currently repeating these experiments to confirm these findings.

The underlying reason for this difference is unclear. The cell purity achieved with FACS is greater than that with magnetic enrichment and some contaminant populations from the latter process, such as APCs or NK cells, might therefore be enhancing the effect of T cell cytotoxicity by activating co-stimulatory networks or by directly mediating cytotoxicity, in the case of NK cells. FACS is also a longer and more abrasive process for cells than magnetic enrichment which could have an unwanted implications for T cell functionality. As of yet, these optimisation steps are ongoing with the ultimate aim of whether Th17RM cells possess adaptations which allow them to directly mediate tumour cytotoxicity.

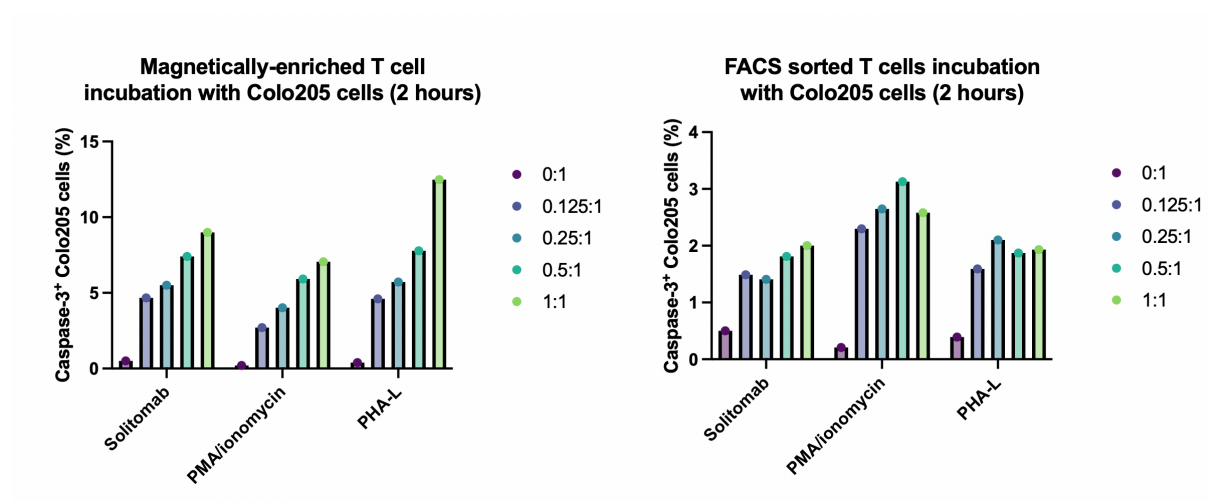


Figure 4.9 | The cytotoxicity achieved by T cells after magnetic enrichment from blood is not retained by FACS. CD3⁺ T cells were magnetically enriched or isolated by FACS from thawed, venous blood from healthy donors. T cells were stimulated with solitomab, PMA with ionomycin or PHA-L and co-incubated with Colo205 cells for 2 hours. T cell-mediated killing was assessed by activity of caspase-3. Each colour represents a different E:T ratio.

4.4 Discussion

Cytotoxicity against tumour cells is an important feature seen in the most robust anti-tumour T cell responses. For many years, the emphasis was placed on CD8⁺ T cells due to the longstanding notion that CD4⁺ T cell effector functions centre around cytokine production and CD8⁺ T cells mediate target cell killing. As research has progressed, studies in pre-clinical and clinical contexts have identified CD4⁺ T cells that not only express cytotoxic molecules but demonstrate functional cytotoxicity in a variety of disease contexts, including cancer⁵¹².

Multiple lines of evidence are also beginning to stress the influence that antigen recognition by CD4⁺ T cells has on dictating patient response to immunotherapy. In melanoma, the expression of tumour MHC class II correlates with both clinical response and overall survival following anti-PD-1 immunotherapy⁵¹³. Moreover, insights into how patients will respond to these treatments can be predicted before their administration, as individuals with the highest levels of intratumoural IFN- γ and tumour MHC class II expression experience the best overall survival following anti-PD-1 checkpoint blockade⁵¹⁴.

CD4⁺ T cells have also been directly implicated in the initiation of potent CD8⁺ immune responses in response to melanoma neoantigens⁵¹⁵ and CD4⁺ T cells are the primary population that becomes activated in response to some melanoma and glioblastoma neoantigen vaccination strategies⁵¹⁶⁻⁵¹⁸. In CRC, a recent large scale genetic screen has also

identified several neoantigens that elicit strong IFN- γ production from CD4⁺ tumour infiltrating lymphocytes⁵¹⁹.

The importance for MHC class II-mediated immunity in cancer has therefore made our observations that Th17RM cells are enriched with signalling pathways downstream of antigen-receptor signalling and comprise a clonally expanded population of particular interest. From these results, it is tempting to speculate the Th17RM cells proliferate in response to the ligation of tumour antigens presented on MHC class II. The inflammatory cytokine IL-18, implicated in the generation of Th17RM cells, may facilitate these interactions. IL-18 can promote MHC class II upregulation on epithelial cells by augmenting IFN- γ production in lymphocytes⁴⁷⁹, a cytokine also upregulated in CD103⁺ Th17 cells (**Fig. 4.4C**).

Tumour antigens are not the only peptides that Th17RM TCRs may react to within the tumour microenvironment. Melanoma tumour cells have been shown to present antigens from intracellular bacteria on their surface via MHC class I and II, namely those derived from *Staphylococcus caprae*, *Actinomyces odontolyticus*, *Fusobacterium nucleatum* and *Staphylococcus capitis*⁵²⁰. These findings have since been reiterated in glioblastoma⁵²¹ and, given the staggering density of microbes found in the human gut, it is almost impossible to think that microbial presentation does not also occur in CRC patients.

The possibility that Th17RM cells respond to microbially-derived antigens within the tumour does not necessarily indicate that they are unable to play a role in protective anti-tumour responses. The fact that microbially-derived antigens can look so 'foreign' to T cells, compared with many host tumour antigens that display relatively low immunogenicity, can actually elevate the strength of anti-tumour immune responses. To support this idea, injecting

tumours with attenuated bacterial spores can hasten the eradication of malignant tissue⁵²² and several studies have demonstrated improved response to immune checkpoint inhibitors in the presence of certain bacteria⁵²³⁻⁵²⁵. If tumour cells are destroyed by Th17RM cells, whether in response to tumour or microbial antigens, this could also initiate epitope spreading cascades via local DCs, a sequence of events which has also been shown to amplify adaptive anti-tumour immune responses²⁶¹.

Whether Th17RM cells mediate tumour protection by direct cytotoxicity or via the recruitment of other immune components remains an unresolved question which we are actively pursuing. We explored a variety of molecules expressed by Th17RM cells and found that they were heavily enriched for key genes that make up cytotoxic granules, including *PRF1*, *GZMA*, *GZMB* and *GNLY*. Whilst these transcripts were found at higher levels in Th17RM cells than Th17EM cells, all except for *GZMA* were found to be expressed at lower levels compared with a number of anti-tumour CD8⁺ T cell clusters in the same scRNA-seq dataset. This contrast might represent the more traditionally observed division of labour between CD4⁺ and CD8⁺ T cells, with CD8⁺ T cells being more predisposed to upregulate lytic killing machinery. We did not detect any *LAMP1* (CD107a) transcripts in Th17RM cells, a marker of cytotoxic granule release⁴⁸⁵, nor any transcripts of *KLRK1* (NKG2D), which could have suggested that Th17RM cells elicit TCR-independent cytotoxicity⁵²⁶ (**Fig. S4.2**).

However, redundancy is built into these killing mechanisms and lysis is not the only process by which T cells can eradicate tumours. Non-lytic mechanisms, such as the secretion of IFN- γ or TNF- α , can inhibit the proliferation of neoplastic cells⁵²⁷, as well as recruiting and activating CD8⁺ or NK cells within the tumour niche⁵²⁸. The production of these cytokines is a key way by which CD4⁺ T cells control tumour development⁴⁹⁷ and CD4⁺ T cells with these functions

can rescue T cell responses against MHC class I deficient tumours that escape direct targeting by CD8⁺ T cells⁵²⁹. Although neither reached statistical significance, there was a convincingly positive trend for both IFN- γ and TNF- α in CD103⁺ Th17 cells, suggesting that they might favour non-lytic cytotoxicity in CRC. Co-culturing Th17RM cells with a reporter CRC cell line in the presence of blocking antibodies against IFN- γ or TNF- α would be a preferable way to explore this hypothesis. Exploring the expression of *LAMP1* (LAMP-1, CD107a) or *FASLG* (Fas ligand, CD95L), two surface markers associated with degranulation in CD8⁺ or NK cells, could be another way to infer the reliance of Th17RM cells on lytic destruction.

By inference, the protective immunity offered by Th17RM cells is observed in colon adenocarcinoma and cutaneous melanoma patients, as tumours enriched for the Th17RM signature were significantly associated with a superior survival probability. We found it surprising that this signature offered no protection at all in a number of other cancers that we tested, including rectal adenocarcinoma, lung adenocarcinoma, lung squamous cell carcinoma, invasive breast carcinoma and head and neck squamous cell carcinoma. Tumour immunity is highly varied across tissue types, due to an abundance of tumour intrinsic and spatial factors, and the enrichment of Th17 cells in the skin and colon during homeostasis might favour the generation of protective Th17-mediated immunity the development of cancer.

4.5 Conclusions

In this final experimental chapter, we show that the gene expression profile enriched in Th17RM cells is one that reflects a cellular state procured in response to antigen recognition. We support the idea that Th17RM cells proliferate in response to tumour microenvironment-

associated antigens as TCR profiling shows the presence of highly expanded clones within this population. When questioning whether Th17RM cells function as part of broader efficacious anti-tumour immune responses, we find that patients enriched with the Th17RM signature in colon adenocarcinoma or cutaneous melanoma tumour tissue have significantly improved survival outcomes, although this protection is not universal among all cancer types. We postulate that the protective immunity conferred by Th17RM cells in these two cancers may be a result of heightened IFN- γ and TNF- α production. However, we also observe granzyme B⁺ Th17RM cells embedded within the neoplastic epithelium in CRC patients *in situ*, suggesting that Th17RM cells may also offer a degree of protection via tumour lysis. Validating this putative cytotoxicity will be a key final experiment to confirm that the presence of Th17RM cells may be advantageous in some cancer patients.

Chapter 5: Discussion

Since their discovery two decades ago, Th17 cells have been widely accepted as key members of the mucosal immune system. During homeostasis, they play a crucial role in protecting against microbial translocation by strengthening the structural barriers present within these niches. The highly dynamic nature of these microbial interfaces also means that Th17 cells are phenotypically plastic and are continually poised to rapidly upregulate a number of inflammatory characteristics in response to barrier breach or infection. However, like that seen in a variety of other immune cell types present within these environments, aberrant activation of their pro-inflammatory programmes has the capacity to drive a number of pathological processes, including the initiation and development of cancer.

Whilst targeting these inflammatory programmes might appear as an obvious strategy to limit tumour progression, some reports have also noted a role for Th17 cells in mediating anti-tumour immune responses by virtue of their ability to promote inflammation and recruit beneficial immune populations into the tumour niche²⁶⁶. Exactly why such contradictory findings have been reported for the role of Th17 cells in cancer is not fully understood. However, it is likely that a combination of intrinsic tumour-related properties and the remarkable plasticity demonstrated by Th17 cells is one underlying cause. We therefore aimed to probe the diverse range of Th17 cell phenotypes observed in patients with CRC, an anatomical region in which Th17 cells can usually be found in abundance.

Using deep scRNA-seq from a publicly available dataset, we have described the presence of three transcriptionally distinct populations of Th17 cells in CRC patients. These Th17 cells display adaptations which allow them to persist within different tissue types; with SELL

(CD62L)⁺ Th17CM cells enriched in the circulation, CD69⁺ Th17EM cells stationed in gut tissue during both homeostasis and cancer and CD69⁺ CD103⁺ Th17RM cells specifically enriched within the tumour microenvironment. The existence of each populations was validated in CRC patients using flow cytometry and we predicted that each transcriptional state is ontogenically related to one another using pseudotime trajectory analysis.

We found the expression of CD103 on Th17RM cells a particularly intriguing observation. CD103 functionally mediates cellular adhesion to E-cadherin⁺ epithelial cells and an increasing body of evidence is reporting its presence on a group of CD8⁺ T cells that functionally persist in tissue environments and is increasingly associating their presence with improved prognosis in a number of cancer types⁵³⁰. Less is known about the role of CD103 on CD4⁺ T cells and even less so about how its expression modulates Th17 cell functionality.

To explore what environmental drivers might signal the development of Th17RM cells, we first used a variety of computational techniques, such as cellular deconvolution and NicheNet ligand-receptor interaction analysis, to determine the cell types and ligands most likely to induce this specific Th17 transcriptional programme. Using a large cohort of patients with colon adenocarcinoma from The Cancer Genome Atlas repository (TCGA-COAD), we deduced that tumours rich in Th17RM-associated genes also had significantly higher infiltration of activated macrophages and CD8⁺ T cells. We hypothesised that these macrophages adopt an inflammatory profile, potentially in response to activation of the inflammasome, and secrete cytokines such as TGF- β 1, TNF- α and IL-18 that collaboratively drive the acquisition of Th17RM characteristics in proximal Th17EM cells. Additional cytokines enriched in Th17RM^{high} tumours, such as IFN- γ , may be derived from infiltrating CD8⁺ T cells or may signal in an autocrine manner via Th17RM cells themselves to further dictate nuanced transcriptional

programmes in this CD103⁺ population. The ligands that we have predicted to most likely influence Th17RM activity, especially that of TGF- β 1, are supported by robust evidence from the literature which has implicated their role in the upregulation of CD103 and strengthening of effector programmes in CD8⁺ tissue resident memory T cells. Confirming the optimal combinations and exact concentrations at which these cytokines most efficiently elicit a Th17RM phenotype *in vitro* constitutes an important ensuing experiment. Not only will understanding these parameters allow us to potentially control their differentiation *in vivo* for more translational studies but it opens the door to a readily-available pool of Th17RM cells for further functional testing *in vitro* – the lack of which embodies a considerable problem we have had to endure throughout this project.

Although investigating the properties of Th17RM cells *ex vivo* arguably manifests the most representative method to explore their functionality, obtaining resected tissue from CRC patients (either fresh or frozen) has proven to be a significant hurdle that has substantially bottlenecked our non-computational investigations. At best, resected tissue was collected from surgery once every two weeks. At worst, collections took place once every few months.

The fallout from the COVID-19 pandemic was partially responsible for this tissue shortage, with the Oxford Biobank team severely depleted of personnel. To add to this, the vast majority of the CRC surgeries overran into antisocial hours. Combined with a lack of available out-of-hours pathologists, this resulted in an unfortunate number of samples being stored in formalin for clinical examination the next morning, rendering them unusable for functional validation experiments.

We therefore attempted to elucidate as much information about Th17RM cells as we could via computational means or by using existing banks of tissue stored pre-pandemic by the lab. As such, we endeavoured to validate interactions between Th17RM cells and inflammatory macrophages firstly with a publicly available spatial transcriptomics dataset and secondly using multiplexed immunohistochemistry on a cohort of banked CRC patients. We found that none of the predicted myeloid populations correlated with Th17RM cells by spatial transcriptomics and considered the possibility that the limited RNA readouts per data spot became saturated by the presence of broad cell types. To support this speculation, we failed to detect the presence of any T cell subsets within the regions of healthy epithelium, despite intraepithelial lymphocytes representing an important cell type within this niche. Exploring the spatial relationship of Th17RM cells with inflammatory macrophages using more recently generated spatial transcriptomics datasets might allow us to work with higher quality RNA sequencing depths per spot. Correlating the presence of Th17RM cells with individual cytokines, such as TGF- β 1 or IL-18, could form another angle to validate our hypothesis.

Using multiplexed immunohistochemistry, we found that Th17RM cells were located in closer proximity to macrophages when they expressed the surface marker CD11c, a population with specialised antigen presentation functions and an inflammatory profile⁴³⁵. We used this marker because of the inflammatory cytokines thought to interact with Th17RM cells, although a more extensive phenotypic validation of the driver myeloid cell(s) in question will enhance our ability to generate Th17RM for future *in vivo* work. Recent advances in spatial techniques, such as the advent of the CosMx platform, which permits the subcellular localisation of transcripts on FFPE slides, could help to answer this question.

As mentioned previously, CD103 defines a population of CD8⁺ T cells which display enhanced persistence within tissues and possess enhanced effector functions than CD103⁻ counterparts. In the context of cancer, it is also becoming increasingly appreciated that the expression of CD103 in CD8⁺ T cells can also be used to identify tumour-reactive clones that exhibit potent tumouricidal effector functions and whose presence is associated with better survival outcomes various cancers. We wondered whether Th17RM cells had comparable functionalities and sought to explore whether their presence in the tumour niche similarly impacts patient prognosis.

Our initial probe into the functional pathways enriched in Th17RM cells using gene ontology was encouraging, as Th17RM cells displayed elevated levels of transcripts associated with antigen-induced signalling pathways compared with Th17EM cell controls. We also found that Th17RM cells constituted a highly expanded population by comparing clonal diversity with other tumour-associated T cell subsets. Whilst these results were convincing enough to conclude that Th17RM cells respond to antigen within the tumour microenvironment, formally comparing clonal diversity with CD103⁺ CD8⁺ T cells, which are known to react with tumour antigens, would further reinforce our conjecture that Th17RM cells are tumour-specific.

This exemplifies another limitation with some parts of our analysis – what we reflectively perceive as a lack of detailed cluster annotations. For both GSE108989 and GSE146771 scRNA-seq datasets, we utilised the existing cluster annotations made by the authors after the identification of Th17CM, Th17EM and Th17RM cells to conduct our analyses. A more stringent and personalised approach to cluster identification and labelling, such as that demonstrated when discerning each Th17 population, could have provided more insight for

downstream experiments. Our ligand-receptor interaction analysis could have particularly benefitted from these alterations and they might have helped pinpoint the exact myeloid population most likely to drive Th17RM cells. Pooling scRNA-seq datasets to increase the number of cells available to cluster, similar to that demonstrated in a recent analysis of CRC scRNA-seq data by Zhang *et al.*⁵³¹, could be one way to facilitate more detailed cluster annotations.

After observing that the presence of Th17RM cells was protective in patients with colon adenocarcinoma and cutaneous melanoma, we began to explore the mechanism by which these cells might mediate this protective immunity. At a transcriptional level, Th17RM cells were heavily enriched with cytotoxic markers when compared with the other Th17 profiles. Against CD8⁺ T cells, only *GZMA* was transcribed to a level that we assumed could be biologically functional. However, speculating on biological function by transcript expression is perhaps misguided as both granzyme A and B were significantly upregulated at the protein level in Th17RM cells. Protein expression of non-lytic tumouricidal cytokines, such as IFN- γ and TNF- α , was also raised (though not significant) in Th17RM cells, which perhaps indicates that protective immunity conferred by Th17RM cells is accomplished by multiple mechanisms. Observations that granzyme B⁺ Th17RM cells can be found within the tumour epithelium further alludes to a direct form of anti-tumour function, as well as any benefits that Th17RM cells may mediate via cellular recruitment.

Integrated redundancies that are built into the immune system are a feature that makes it such a powerful biological weapon. Cytotoxicity within the CD4⁺ compartment, for example, can effectively compensate for a loss of CD8⁺ driven immunity in tumours which downregulate the expression of MHC class I⁵²⁹. The array of effector mechanisms employed

by immune cells represents a second example, whereby malignant cells can be targeted by cellular, antibody or cytokine-mediated modalities to maximise successful eradication²⁸. A recent study describing the accumulation of highly beneficial IL-17⁺ CD103⁺ CD8⁺ TRM (Tc17RM) cells in anti-PD-1-responsive lung cancer patients²⁶⁵ therefore raises a question regarding the importance of the contribution made by Th17RM cells within a system built upon redundancy. It is injudicious to consider Tc17RM and Th17RM as mutually exclusive entities within efficacious anti-tumour immune responses. However, given the specialisation of CD8⁺ T cells for cytotoxicity, it is worth exploring their contribution. Mouse models of cancer represent the most complete system whereby this question can be functionally addressed and such observations could be made using *Cd4 & Rorc-split-ERT2^{Cre} x Itgae^{fl/fl}* mice, which would have a selective deletion of CD103 in CD4⁺ Rorγt⁺ T cells after the administration of tamoxifen.

A comprehensive understanding of Th17RM cell biology in CRC (as well as their functions in other cancers) has translational implications too that could inform future immunotherapeutic strategies. A considerable number of studies have begun to report the acquisition of Th17-like properties in adoptively transferred CAR T cells in the most complete responders, with these CAR T cells upregulating cytokines such as IL-17 and IL-22 *in vivo*^{222,532}. As a product of these observations, some of the most recent CAR T strategies have started experimenting with the polarising conditions used during the manufacturing process to expedite the acquisition of these properties. This strategy has so far proved fruitful and CAR T cells primed to exhibit Th17 functionalities by RORγ agonists or by ICOS stimulation can efficiently eradicate established tumours in mouse xenograft models of melanoma and mesothelioma by IFN-γ and IL-17A-dependent mechanisms^{222,264,306,506}. Furthermore, CAR T cells that are

conditioned to upregulate TRM characteristics by culturing them beforehand with TGF- β 1 similarly display the features of enhanced residency and stemness that align so well with efficacious responses⁵³³. It is therefore plausible that CAR T cells conditioned to adopt Th17RM cell characteristics during the production stage can amalgamate and further enhance these protective features.

It is therefore critical that we are able to conclusively establish the conditions or cell types most able to elicit these features in Th17 cells. The increasing pressure to develop novel cancer strategies that are able to take advantage of host immunity exemplifies this necessity. Th17 cells are key immune players at a number of barrier sites and we have shown that heterogeneity within this population can have functional consequences for anti-tumour immunity. In elderly patients whose immune system has become adversely impacted by immunosenescence, it is unclear whether these new immunomodulatory strategies will elicit maximal responses. However, with the rates of young-onset CRC on the rise, characterising the most beneficial immune phenotypes for exploitation should remain a priority. CD103⁺ Th17 cells might comprise one of these advantageous phenotypes and we hope that they may be exploited in the near future to provide therapeutic relief for some cancer patients.

Chapter 6: Materials and methods

6.1 Materials and reagents

6.1.1 Cell lines and culture

The Colo205 CRC cell line was obtained from The American Type Culture Collection and sustained in complete medium, composed of RPMI 1640 cell culture medium (Gibco, 11875093) with 10% FBS (Atlanta Biologicals, S12550H) and 100 U/mL penicillin-streptomycin (Sigma, P0781) at 37 °C with 5% CO₂.

6.1.2 Antibody library (flow cytometry)

Antigen	CD number	Fluorophore	Dilution	Clone	Cat #
CCR6	CD196	BUV737	1:100	11A9	612780
CCR7	CD197	BV510	1:200	2-L1-A	566760
CD3	CD3	PE-Cy5	1:200	UCHT1	300410
CD4	CD4	PE-Cy7	1:200	OKT4	317413
CD4	CD4	BV785	1:200	OKT4	317442
CD8	CD8	Spark Blue 550	1:200	SK1	344760
CD38	CD38	BUV805	1:200	HB7	742074
CD39	CD39	BV711	1:200	A1	328227
CD39	CD39	PerCP-Cy5.5	1:200	A1	328217
CD45RO	CD45RO	BUV563	1:200	UCHL1	748369
CD69	CD69	BV785	1:200	FN50	310932

CD83	CD83	BV421	1:200	HB15e	305323
CD161	CD161	BUV395	1:200	HP-3G10	748280
CXCR4	CD184	BV605	1:200	12G5	740418
CXCR6	CD186	BUV805	1:200	13B 1E	748448
FoxP3	-	PerCP-Cy5.5	1:100	PCH101	45-4776-41
FoxP3	-	Alexa Fluor 647	1:100	206D	320113
Granzyme A	-	R718	1:100	CB9	566976
Granzyme B	-	PE	1:100	QA16A02	372208
ICOS	CD278	Alexa Fluor 700	1:200	C398.4A	313528
IFN- γ	-	FITC	1:100	B27	506504
IFN- γ	-	APC-Cy7	1:100	B27	506523
IL-17A	-	BV605	1:100	BL168	512328
IL-22	-	PE-Cy7	1:100	22URTI	25-7229-42
Integrin α E	CD103	Alexa Fluor 488	1:200	Ber-ACT8	350208
Integrin α E	CD103	APC	1:200	B-Ly7	17-1038-42
Integrin β 1	CD29	APC-Cy7	1:200	TS2/16	303014
Perforin	-	Pacific Blue	1:100	dG9	308117
ROR γ t	-	PE-eFluor 610	1:100	AFKJS-9	61-6988-80
SELL	CD62L	BV650	1:200	DREG-56	304831
T-bet	-	Alexa Fluor 647	1:100	4B10	644804
TIM-3	CD366	BUV615	1:200	7D3	752363
TNF- α	-	BV510	1:100	MAB11	502950

Table 6.1 | Antibody library (flow cytometry).**6.1.3 Antibody library (multiplex fluorescence imaging)**

Antigen	CD #	Fluorophore	Dilution	Clone	Cat #
CCR7	CD197	Alexa Fluor 647	1:100	EPR23192-57	ab2755165
CD3	CD3	Alexa Fluor 555	1:100	SP162	ab245731
CD4	CD4	Alexa Fluor 488	1:100	EPR6855	ab196372
CD8 α	CD8	Alexa Fluor 647	1:100	C8/144B	372906
CD11c	CD11c	Alexa Fluor 555	1:50	EP1347Y	ab279329
CD20	CD20	Alexa Fluor 488	1:100	EP459Y	ab198941
CD45RA	CD45RA	Alexa Fluor 488	1:100	H100	304114
CD45RO	CD45RO	Alexa Fluor 488	1:100	UCHL1	304212
CD69	CD60	Alexa Fluor 488	1:100	8B6	NBP1-51607
Integrin α E	CD103	Alexa Fluor 647	1:100	EPR1466(2)	ab225153
CD161	CD161	Alexa Fluor 647	1:100	EPR26340-6	ab302565
CXCR5	CD185	Alexa Fluor 555	1:100	EPR23463-30	ab272936
CXCR6	CD186	Alexa Fluor 555	1:100	EPR24686-113	ab273116
Granzyme B	-	Alexa Fluor 555	1:100	D6E9W	79903SF
FoxP3	-	Alexa Fluor 555	1:50	D2W8E	74816SF
ROR γ t	-	Alexa Fluor 647	1:50	AFKJS-9	14-6988-82
T-bet	-	Alexa Fluor 647	1:50	D6N8B	27112SF
TCF-1	-	Alexa Fluor 555	1:50	C63D9	85942SF

Table 6.2 | Antibody library (multiplex fluorescence imaging).

6.2 *Ex vivo* methodology

6.2.1 Human subjects and ethics

CRC patients were recruited from the colorectal surgery department at the Churchill Hospital, Oxford University Hospitals NHS Foundation Trust, UK. All patients gave informed consent and collection was approved by the NHS National Research Ethics Service under the reference number 16/YH/0247.

6.2.2 Processing of human tissue

PBMCs from CRC patients were isolated from heparinised venous blood samples, whilst those from healthy donors were isolated from blood cones collected via the NHS Blood and Transplant service. PBMCs were enriched using Lymphoprep (STEMCELL) by density-gradient centrifugation from both sources, and cone-derived PBMCs were additionally incubated in ACK lysis buffer for 5 minutes at room temperature to maximise erythrocyte removal. Isolated PBMCs were then analysed downstream or resuspended in FBS with 10% DMSO (Sigma) in liquid nitrogen vapour phase for long-term storage.

Resected tissue samples were obtained after surgery and classified as either tumour or normal adjacent by a pathologist. Two 5 mm³ sections were initially cut, with the first suspended in RNAlater (Qiagen) at -20 °C for the preservation of RNA. The second section was stored in 4% (w/v) formaldehyde (Thermo Fisher) overnight at 4 °C before transferring to 70 % ethanol for long-term storage before histological analysis.

Banked tissue was suspended in cryopreservation medium (Merck), cooled slowly at a rate of -1 °C/minute until a temperature of -80 °C was reached, then stored in liquid nitrogen vapour phase for long-term storage. For freshly processed specimens, excess fat and submucosal tissue were manually removed using sterile forceps and scissors before being washed twice in PGA solution, containing PBS (Gibco), 100 U/mL penicillin-streptomycin, 50 µg/mL gentamycin (Sigma), 2 µg/mL amphotericin B (Sigma), 1 µg/mL ciprofloxacin (Sigma) and supplemented with 1 mM DTT (Roche), for 15 minutes at 37 °C on an orbital shaker to remove epithelial cells. Remaining tissue was then rinsed in RPMI 1640 with 5 mM HEPES (Sigma) before two incubations in digestion medium, composed of RPMI 1640, 5 mM HEPES, 0.3 U/mL collagenase A (Sigma) and 10 µg/mL DNase I (Roche), for 30 minutes each at 37 °C on an orbital shaker. Liberated cells were filtered through 70 µm cell strainer (Thermo Fisher) into FACS buffer, made up of PBS, 0.1% BSA (Sigma) and 5 mM EDTA (Invitrogen), before later downstream analysis.

6.2.3 Cytokine restimulation assay

Single cells obtained from tissue resections were incubated with a stimulation medium composed of complete medium supplemented with 50 ng/mL PMA (Sigma), 1 µg/mL ionomycin (Sigma) and 5 µg/mL brefeldin A (Biolegend), for 6 hours at 37 °C in 5% CO₂ before analysis by flow cytometry.

6.2.4 Flow cytometry, FACS and magnetic enrichment

Single cells were washed in FACS buffer, centrifuged at 300 x *g* for 5 minutes at room temperature, before resuspending in a blocking solution made up of human FcR blocking reagent (Miltenyi) and LIVE/DEAD fixable blue stain (Invitrogen) for 10 minutes at room

temperature. The cells were washed again in FACS buffer before staining for surface antibodies for 30 minutes at room temperature. For samples where only surface antigens were investigated, cells were fixed with fixation buffer (Biolegend) before being resuspended in FACS buffer. For the investigation of intracellular or intranuclear antigens, cells were incubated in fixation/permeabilisation solution (Thermo Fisher) for 30 minutes, washed and resuspended in permeabilisation buffer from the FoxP3 transcription factor staining kit (Thermo Fisher, 00-5521-00). Samples were then stained for 30 minutes at room temperature. Fluorescence was detected with an Aurora spectral cytometer (Cytek) and the data were processed with SpectroFlo (Cytek) and OMIQ (Dotmatics).

Samples prepared for FACS were processed similarly to those prepared for flow cytometry however non-viable cells were marked with LIVE/DEAD fixable near IR stain (Invitrogen, L34994) and were resuspended in FACS buffer supplemented with 200 µg/mL DNase I before sorting with a FACSAria III cell sorter (BD Biosciences). Data were processed using FACSDiva (BD Biosciences). For magnetic enrichment, T cells were positively selected with the EasySep human CD3⁺ positive selection kit (STEMCELL) as per the manufacturer's instructions.

6.2.5 Cytotoxicity assay

Isolated CD3⁺ T cells were cultured with either 1 µg/mL solitomab (Thermo Fisher), 50 ng/mL PMA (Sigma) with 1 µg/mL ionomycin (Sigma) or 2.5 µg/mL PHA-L (Sigma) for 2 hours in the presence of 4x10⁴ Colo205 cells/mL. Cells were then prepared for flow cytometry as usual with the addition of the NucView488 caspae-3 assay kit (Biotium) just before analysis, as per the manufacturer's instructions.

6.3 *In silico* methodology

6.3.1 R package library

R package	Version
Seurat	4.4.0
tidyverse	2.2.0
ggplot2	3.5.1
EnhancedVolcano	1.22.0
clusterProfiler	4.12.0
org.Hs.eg.db	3.19.1
enrichplot	1.24.0
fgsea	1.30.0
GSEABase	1.66.0
ggpubr	0.0.6
monocle3	1.3.7
viridis	0.6.5
ggh4x	0.2.8
tibble	3.2.1
MAST	1.30.0
tidyr	1.3.1
TCGAbiobanks	2.32.0
SummarizedExperiment	1.34.0
reshape	0.8.9

vctrs	0.6.5
GSVA	1.52.2
RColorBrewer	1.1.3
rstatix	0.7.2
nichenetr	2.1.5
circlize	0.4.16
patchwork	1.2.0
dplyr	1.1.4
png	0.1.8
gridExtra	2.3
stringr	1.5.1
grid	4.4.0
survival	3.5.8
survminer	0.4.9
survivalAnalysis	0.3.0

Table 6.3 | R package library.

6.3.2 scRNA-seq data quality control, pre-processing and clustering

Publicly available scRNA-seq datasets were downloaded from Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) with the accession numbers GSE108989, GSE146771 and processed using the Seurat package. Count matrices and corresponding metadata files were combined into a Seurat object using *CreateSeuratObject*. Cells with > 2% mitochondrial

reads were removed. The gene expression matrices were normalised and the total UMI counts per cell were transformed using the natural log scale. After selecting the 2,000 most variable genes using the *FindVariableFeatures* function, PCA was performed on the genes. *FindNeighbours* was then run on the top 30 principal components. *FindClusters* was set with a resolution ranging from 0.3 to 1.3 to explore cell clusters. Two-dimensional UMAP was used to represent cell clusters. To accommodate for multiple rounds of clustering, Seurat objects were subset to enrich for clusters of choice before repeating the process from the "*FindVariableFeatures*" stage. To cluster *RORC*⁺ populations, cells expressing a positive transcript count for *RORC* and *CD3D* were first subset from the scRNA-seq dataset before the process above was initiated. Differentially expressed genes were visualised using the *VlnPlot* function in Seurat, the *EnhancedVolcano* plot in EnhancedVolcano or the *DotPlot* function in Seurat. Figures were compiled using the *ggarrange* function in ggpubr throughout.

6.3.3 Gene set enrichment analysis

Enriched gene set expression profiles were determined using GSEA, a tool published by Subramanian *et al.*³²⁵. Significantly differentially expressed genes, as calculated in 6.3.2, were first ranked according to log₂ fold change. Custom gene lists for TCM, TEM and TRM cells (published by Szabo *et al.*³²⁶ and Kumar *et al.*²⁷⁵) were downloaded and normalised enrichment scores were calculated with 10,000 permutations and a p value cut off set to 0.05 using the *GSEA* function in the fgsea package. Normalised enrichment scores and ranked list metrics were visualised using the *gseaplot2* function in the GSEAbase package.

6.3.4 Pseudotime trajectory analysis

Pseudotime trajectory analysis was performed using the Monocle3 package, published by Cao *et al.*⁵³⁴. The Th17 Seurat object was first converted into a cell data set (CDS) before size factors were determined using the *estimate_size_factors* function. Cells were then re-clustered and the *learn_graph* function was employed to produce a trajectory through the CDS. The root was manually set using the *order_cells* function and pseudotime trajectories were visualised using *plot_cells*. Fluctuations in individual genes over pseudotime were plotted using the *plot_genes_in_pseudotime* function.

6.3.5 Curating Th17EM and Th17RM cell signatures

To calculate the transcriptional gene signatures for Th17EM and Th17RM cells, all T cell clusters other than Th17EM/RM in GSE108989 were grouped together in an 'other_clusters' variable before the *FindMarkers* function was employed with the MAST package. Genes with an adjusted p value of ≤ 0.05 and $\log_2$ fold change ≥ 0.5 were ranked by fold change and the top 5 were combined with the major flow cytometry identifiers (ROR γ t and CD69 or CD103, respectively) for downstream analysis. To determine how specific each signature was for the respective Th17 population, the *AddModuleScore* function from Seurat package was employed. Pairwise Wilcoxon tests with Benjamini-Hochberg correction was used to calculate the significance of each statistical pairwise comparison.

6.3.6 CIBERSORTx cellular deconvolution

CIBERSORTx, a tool published by the Alizadeh lab³⁸⁹, was used to deconvolute bulk RNA-seq data downloaded from the publicly-available TCGA-COAD repository. Cell fractions were imputed using the LM22 signature matrix, generated by the Alizadeh lab specifically for use

with the CIBERSORTx platform. Batch correction was disabled, as was quantile normalisation, as these settings were recommended for RNA-seq data. Deconvolution was run in absolute mode and 1,000 permutations were set for statistical analysis.

6.3.7 TCGA data processing and GSVA scoring

TCGA bulk RNA-seq expression matrices were downloaded using GDCquery from the TCGAbiolinks package. *Data.category* was set to 'transcriptome profiling', *data.type* set to 'gene expression quantification' and *workflow.type* set to 'STAR-Counts'. Data matrices were read and prepared using the *GDCprepare* function.

To score individuals in the TCGA-COAD cohort based on expression of the curated Th17EM/RM gene signatures, gene set variation analysis (GSVA) was employed, as described by Hänzelmann *et al.*³⁹¹. The *kcdf* was set to 'Gaussian' as the bulk RNA-seq matrix had already been normalised. Patients in the TCGA-COAD cohort were divided into equal tertiles based on the output GSVA score and each stratified group was matched back to their CIBERSORTx-deconvoluted profile to compare tumour immune landscapes. *Stat_compare_means* from the ggpubr package was used to calculate statistical significance between each group per cell type, using a Wilcoxon test.

6.3.8 Volcano plots

Differential gene expression analysis was performed using the *FindMarkers* function in the Seurat package (version 4.4.0). Top differentially expressed genes were selected based on their adjusted p value and annotated using the *EnhancedVolcano* function in the

EnhancedVolcano package (version 1.22.0). Each Volcano plot was combined into a single figure using the *ggarrange* function from the *ggpubr* package (version 0.6.0).

6.3.9 NicheNet ligand-receptor analysis

To explore ligand-receptor interactions, we utilised NicheNet, a tool created by the Saeys lab³⁹⁸. Th17EM/RM cells were set as the 'receiver' population and a list of sender and receiver genes were generated by comparing Th17 cells to all other cell types in GSE146771, classed as 'sender' cells. Differentially expressed genes in each Th17 population were determined by comparing them with CD4⁺ T cells, as the reference population. A minimum of 10% of the receiver cells were required to express the genes to be considered and 10% of the sender cells were required to express the considered sender ligands. Scoring of the predicted targets was based on a Pearson correlation coefficient, as described in the NicheNet vignette³⁹⁸.

6.3.10 Spatial transcriptomics

Gene-spot matrices generated by the Visium platform and paired histological images were downloaded from a publicly-available repository published by Wu *et al.*³. Spots were clustered using the same process utilised to clusters single cells with the Seurat package. To project cell signatures from GSE146771, anchors in the spatial transcriptomics dataset were generated using the *FindTransferAnchors* function and combined with the scRNA-seq gene signatures using *TransferData*. Differentially expressed genes with an adjusted p value of ≤ 0.05 and $\log_2$ fold change ≥ 0.5 were transferred. Spatial feature expression plots were generated with *SpatialFeaturePlot*.

6.3.11 Gene ontology

In order to compare differentially enriched pathways between each Th17 subpopulation, gene ontology was performed on genes where the $\log_2$ fold change ≥ 0.5 . Pathways enriched within the 'biological processes' ontology were selected after Benjamini-Hochberg correction.

6.3.12 T cell clonal analysis

For analysis of paired TCR sequencing from GSE108989, the R package immunarch was used. Clonality was explored using the *repClonality* function, with breaks defined as 1, 2-3, 4-10, 11-30, 31-100 and 100+. Overlap analysis was performed using the morisita method of the *repOverlap* function, which calculates the extent of sharing based on clonal abundance. Comparative clonal diversity was estimated using rarefaction, employed using the *repDiversity* function, due to the dissimilar cell numbers within each cluster.

6.3.13 Univariate patient survival and multivariate analysis

The clinical metadata columns 'days_to_last_follow_up', 'days_to_death' and 'vital_status' were incorporated into the GSVA data frame in preparation for patient survival analysis. *Surv* from the survival package was used to generate a survival object which was subjected to Cox proportional hazards regression. The Kaplan-Meier method was used to generate survival curves and the log-rank test was used to determine the overall statistical significance of differences in survival. Benjamini-Hochberg correction was used for multiple comparisons between tertiles. Survival curves were constructed using the survival and survminer packages. *Analyse_multivariate* from the survivalAnalysis package was used to generate multivariate hazard ratios and *forest_plot* was used to visualise the scores.

6.4 *In situ* methodology

6.4.1 Processing and multiplexed imaging of tissue

Resections were fixed in 4% paraformaldehyde for 24 hours before being transferred into 70% ethanol for long-term storage. Before processing, samples were placed into paraffin using a tissue processor and embedded in paraffin blocks in an anteroposterior orientation. Tissues were sectioned at 5 μm using a microtome.

Multiplex fluorescence imaging was performed to validate intercellular interactions and explore the tissue localisation of Th17 subpopulations. Sections of matched healthy control and tumour tissue were baked overnight at 60 °C. Slides were transferred into xylene for dewaxing followed by a series of rehydration steps in ethanol solutions (100%, 95%, 70% and 50%). Each step was repeated twice for 5 minutes each, followed by 2 washing steps in 1X PBS. The tissue was then permeabilised for 10 minutes in 0.3% Tris X-100 and washed for a further 5 minutes in 1X PBS. Heat-induced epitope retrieval was performed using a decloaking chamber with both citrate (pH 6.0) and Tris (pH 9.0) antigen retrieval solutions. The chamber was programmed to incubate the slides in citrate (Dako) solution for 20 minutes reaching 110 °C and 6.2 PSI, which was maintained for 4 minutes before cooldown was initiated. Slides were transferred to a Tris (Dako) solution for 20 minutes followed by an additional 10 minute incubation at room temperature (RT). Tissue slides were blocked with a 5% donkey serum (Sigma) and 5% BSA (Sigma) solution overnight at 4 °C. Slides were then washed in 1X PBS for 10 minutes and stained with DAPI (Sigma) for 15 minutes. Slides were washed again in 1X PBS for 5 minutes and coverslipped with 50 μL of a 50% glycerol (Sigma) and 4% propyl gallate (Sigma) mounting media.

6.4.2 Cell DIVE imaging of stained tissues

The GE Cell DIVE system was used to image tissues using the ImageApp imaging software. An initial image using a 10X objective lens of the entire tissue was used for ROI selection and the background autofluorescence of the tissue was captured using a 20X imaging round. FITC, Cy3 and Cy5 channels were used alongside the DAPI channel to create a virtual H&E image. This background imaging was used to subtract autofluorescence from all the subsequent rounds of staining.

Slide coverslips were removed in 1X PBS prior to staining. Each staining round consisted of a master mix of 3 antibodies prepared in antibody diluent (3% BSA in 1X PBS). The initial round used primary antibodies which were incubated in the dark for 1 hour at RT followed by 3 washes in 1X PBS. Secondary antibodies conjugated with either Alexa Fluor 488, 555 or 647 were then incubated for an additional hour at RT before being coverslipped and imaged.

Fluorophores were bleached between each round of staining using NaHCO_3 (0.1 M, pH 11.2 (Sigma)) and 3% H_2O_2 (Sigma). Slides were bleached 3 times for 15 minutes each with a 1 minute wash in 1X PBS in between bleaching rounds. Slides were then re-stained with DAPI for 2 minutes and washed in 1X PBS for 5 minutes in order to enable with image registration and alignment. Dye-inactivated slides were then imaged in order to calculate the new background subtraction (and for each subsequent background subtraction). Slides were then multiplexed with the next panel of 3 markers with iterative staining, bleaching and imaging. A negative control slide stained with isotype-matched antibodies was used at the same concentration as the corresponding primary antibodies and using the same exposure settings. QuPath software (v0.4.4) was used for image visualisation and isotype controls were

contrast-matched to samples stained with primary antibodies for comparison. The HALO (v3.0) nearest neighbour analysis was utilised to calculate intercellular distances.

Supplementary figures

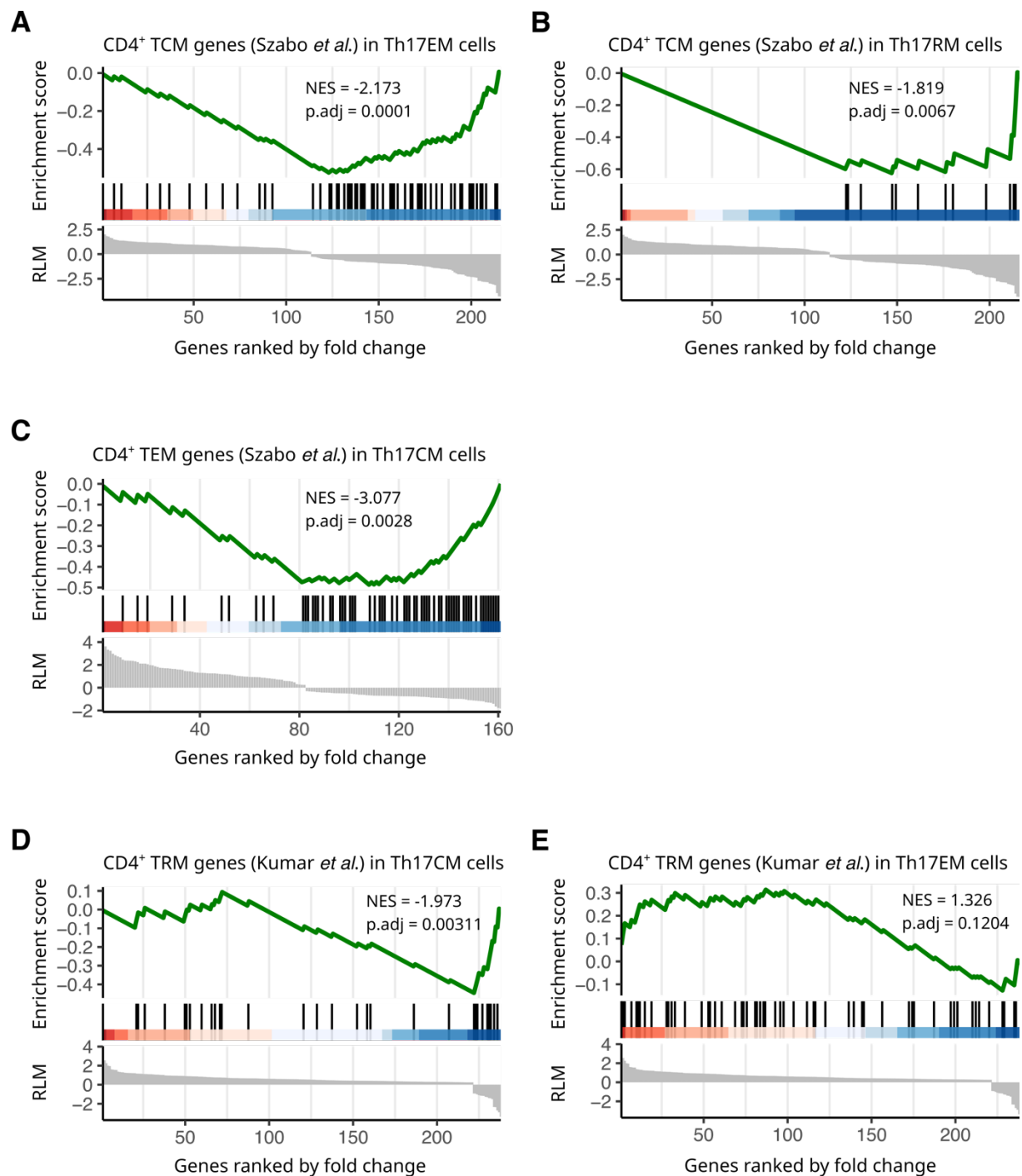


Figure S2.1 | Enrichment analysis for each Th17 population demonstrates specificity for each memory T cell state. GSEA was used to score the enrichment analysis of central memory (TCM), effector memory (TEM) and resident memory T (TRM) cell gene

signatures^{275,326}, in Th17CM, Th17EM and Th17RM cells, respectively. NES = normalised enrichment score, p.adj = adjusted p value, RLM = ranked list metric.

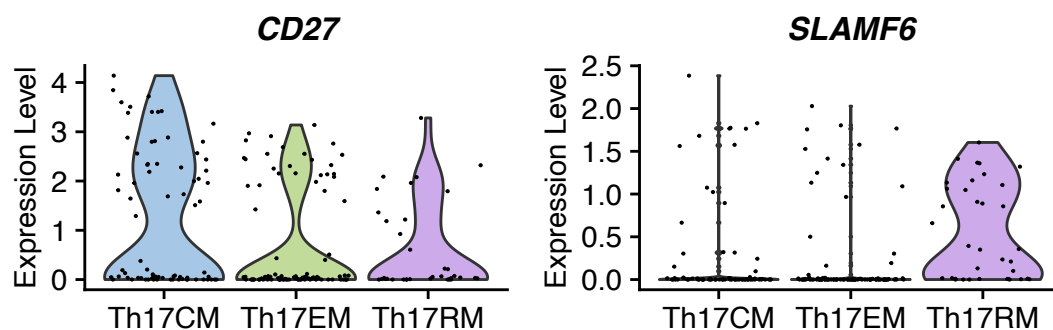


Figure S2.2 | Expression level of *CD27* and *SLAMF6* in each Th17 population in GSE108989.

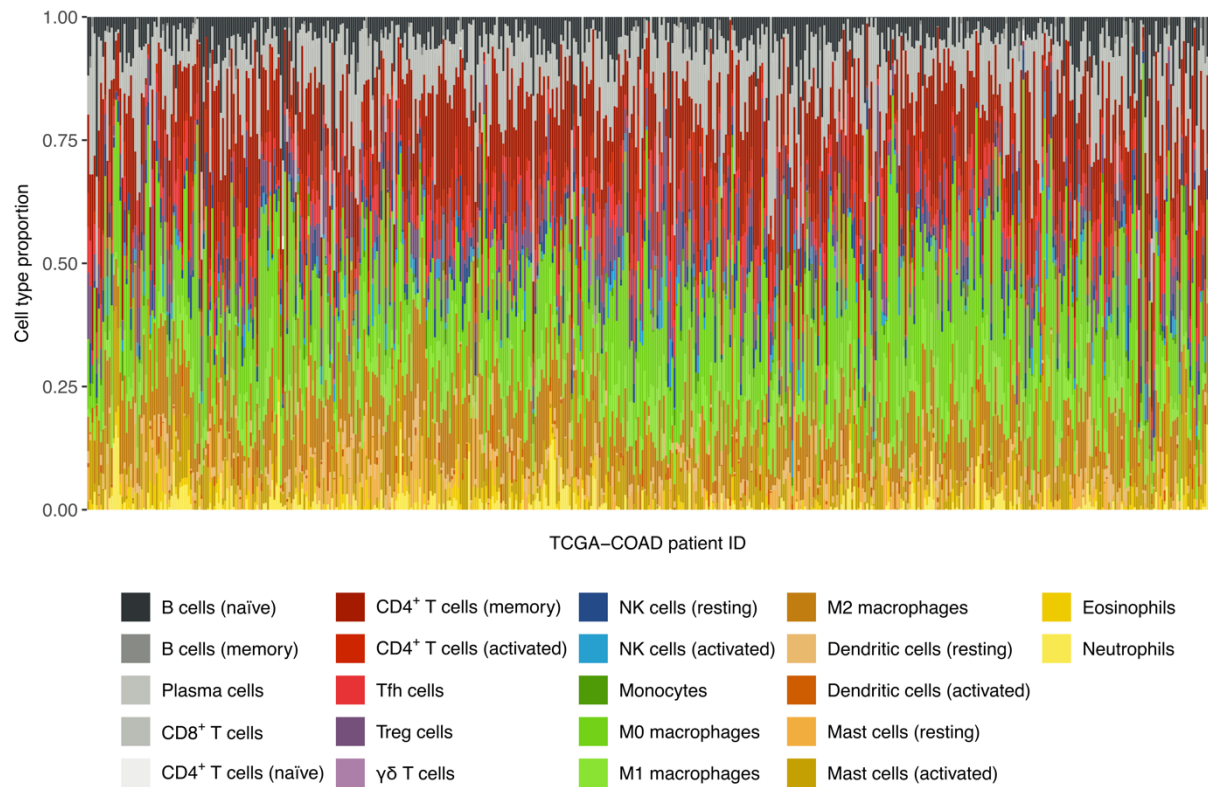


Figure S3.1 | Tumour immune landscapes across TCGA-COAD patients. Output cell type proportions from the CIBERSORTx deconvolution of TCGA-COAD patients using the LM22 signature matrix. Each column represents one patient.

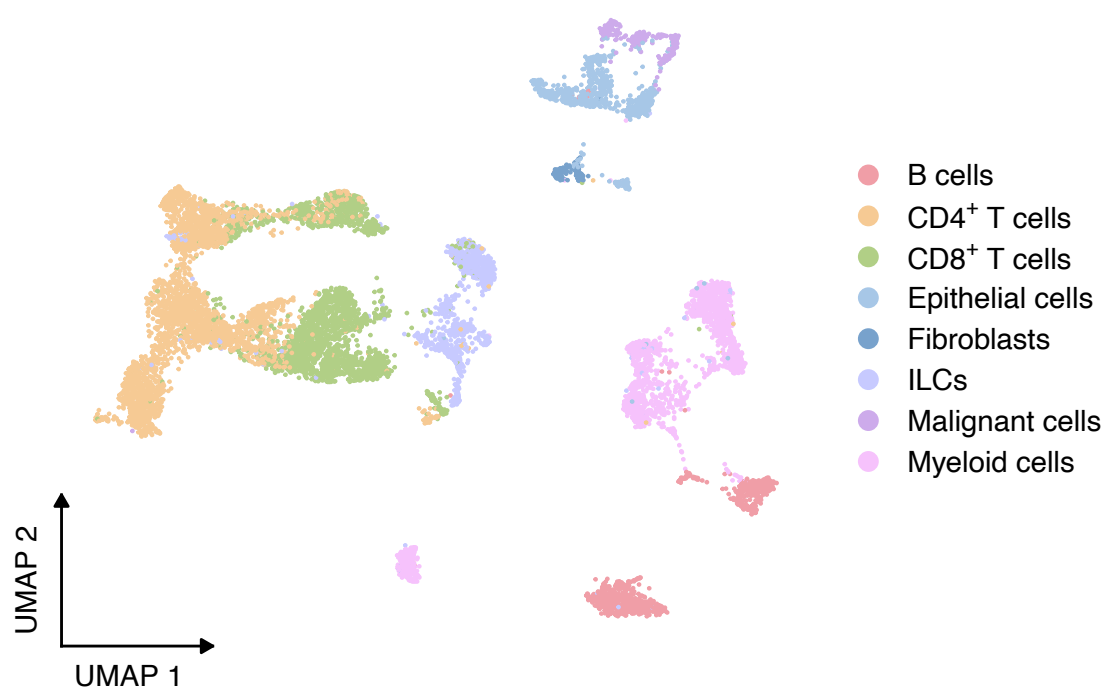


Figure S3.2 | UMAP clustering of GSE146771 scRNA-seq data.

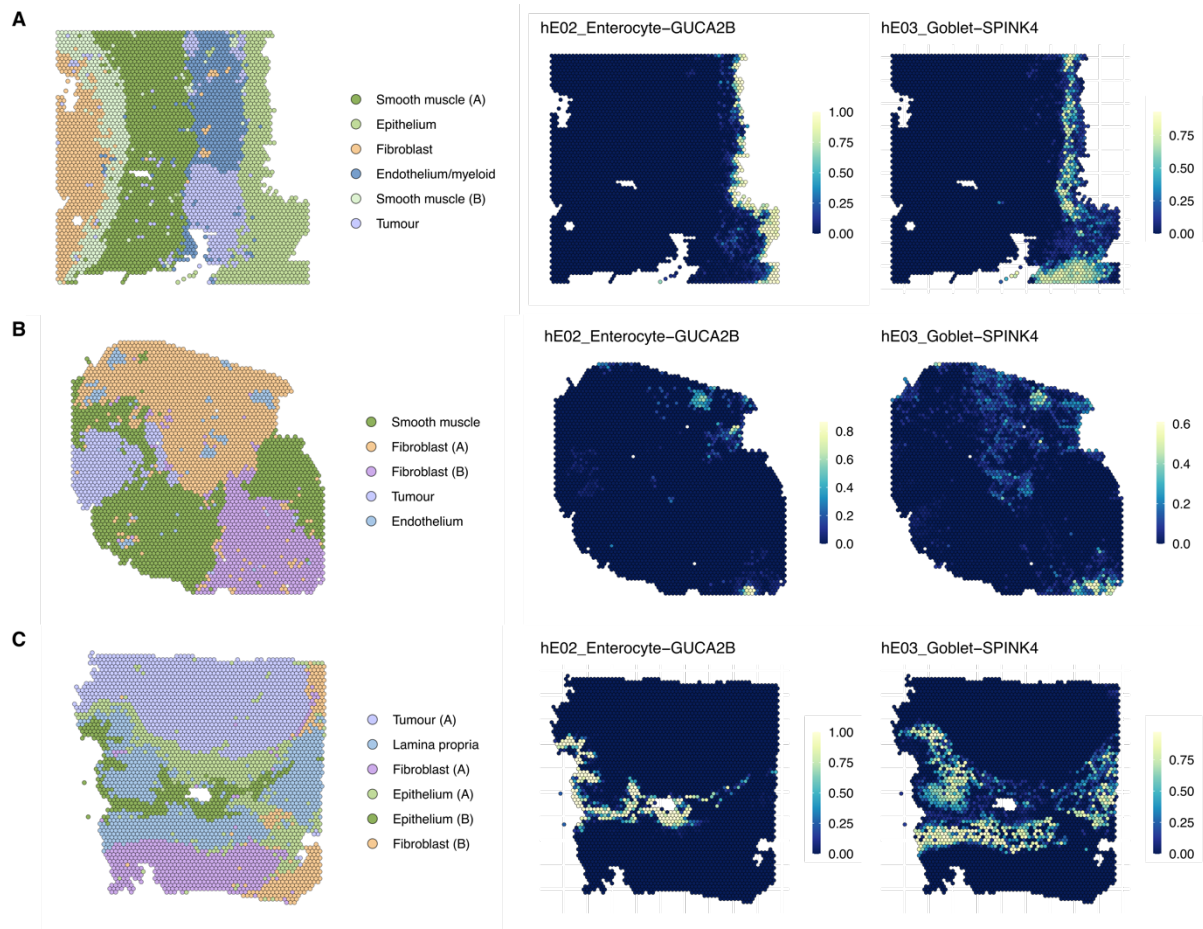


Figure S3.3 | Distribution of enterocytes and goblet cells by spatial transcriptomics.

Probability scores for hE02_Enterocyte-GUCA2B and hE03_Goblet-SPINK4 cells across tumour samples from **(A)** patient 2, **(B)** patient 3 and **(C)** patient 4. Histological annotations are in the left column. Spot colour in the middle and right column represent the probability that a given cell type can be found within that region, with blue representing low probability and yellow representing high probability.

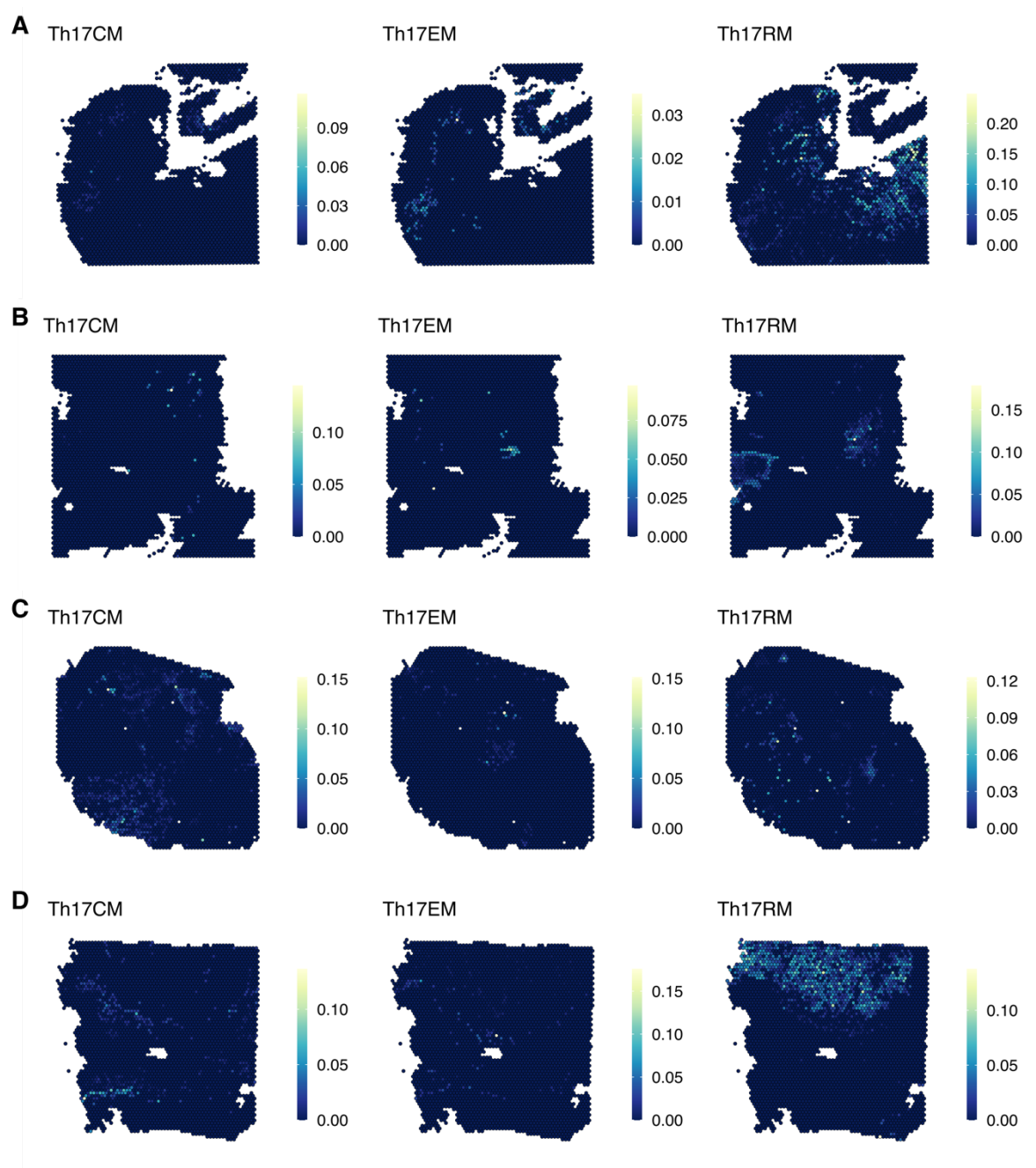


Figure S3.4 | Distribution of tumour Th17 cells. Probability scores for Th17CM, Th17EM and Th17RM cells across tumour samples from (A) patient 1, (B) patient 2, (C) patient 3 and (D) patient 4. Spot colour represents the probability that a given cell type can be found within that region, with blue representing low probability and yellow representing high probability.

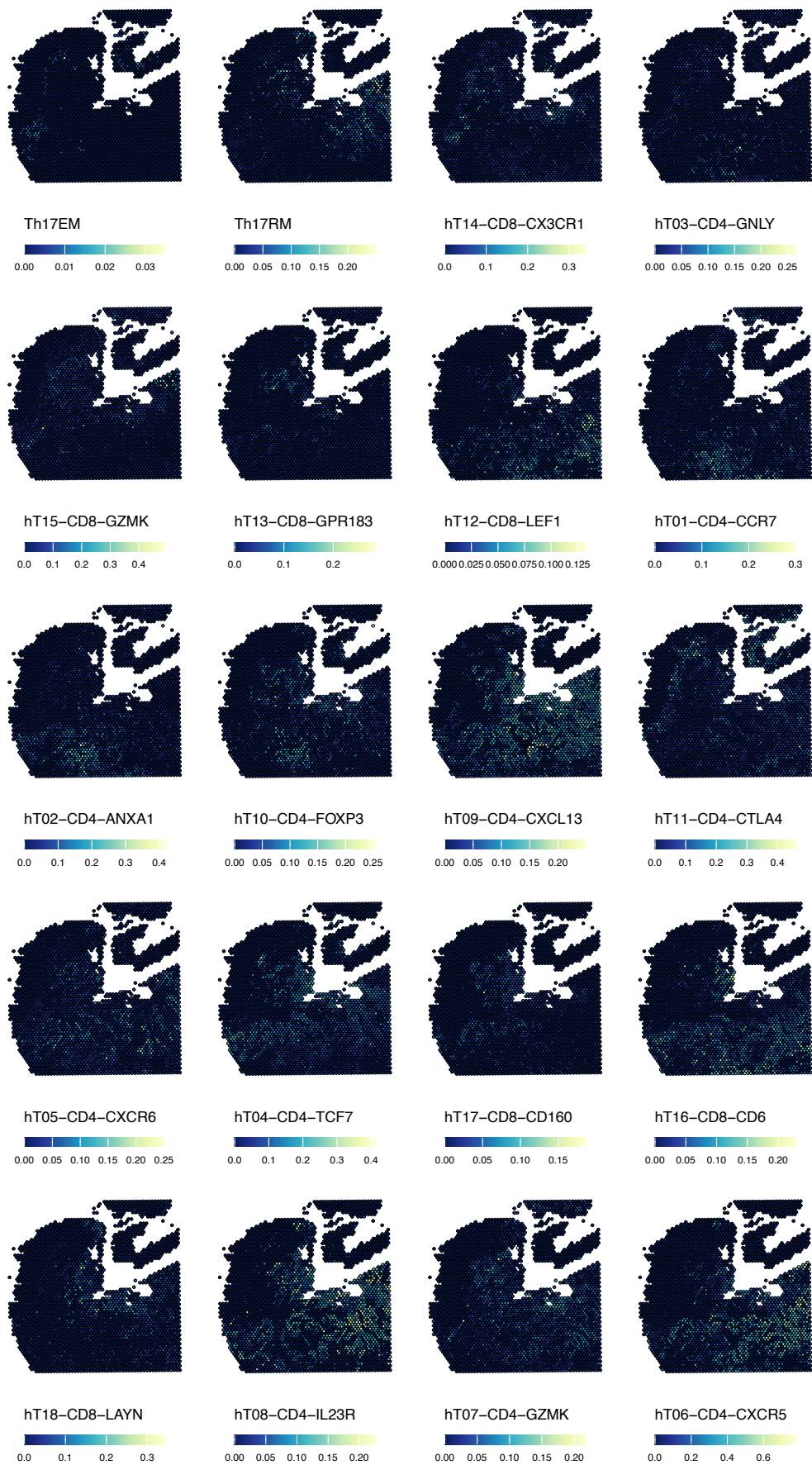


Figure S3.5 | Distribution of T cell subsets in patient 1. Probability scores for each T cell subset in the tumour sample from patient 1. Spot colour represents the probability that a given cell type can be found within that region, with blue representing low probability and yellow representing high probability.

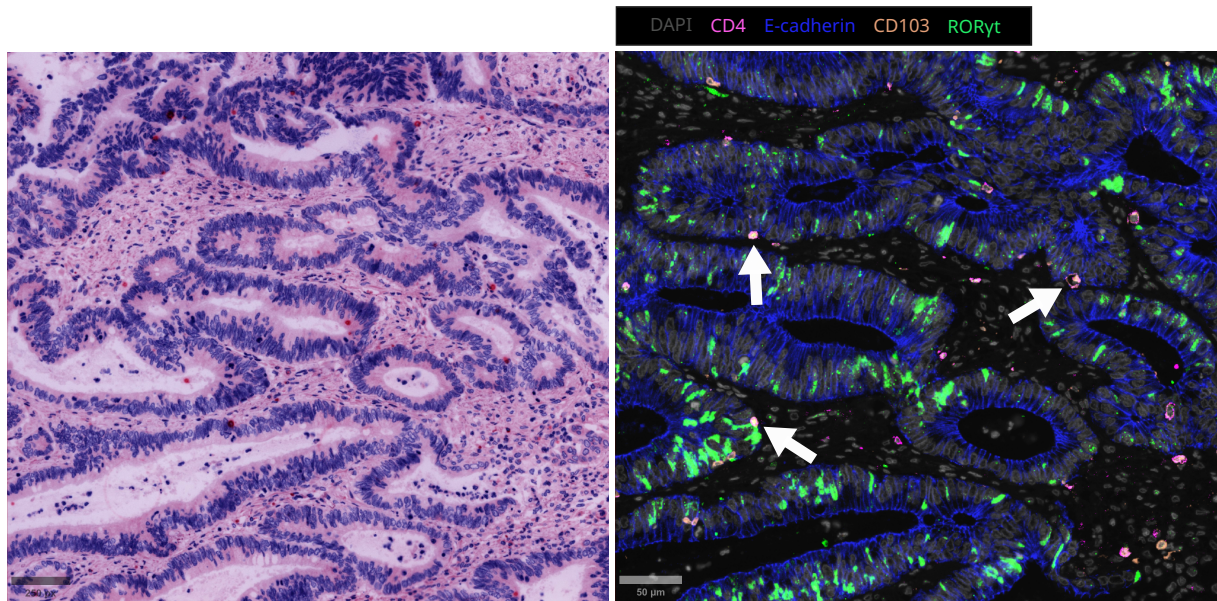


Figure S4.6 | Th17RM cells can be found within the tumour epithelium. Multiplexed staining of FFPE tissue with a Th17RM (CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ RORγt⁺ CD103⁺ CXCR6⁺ CD161⁺) cell embedded in the tumour epithelium (E-cadherin⁺), as indicated by a white arrow. Scale bar represents 50 μm.

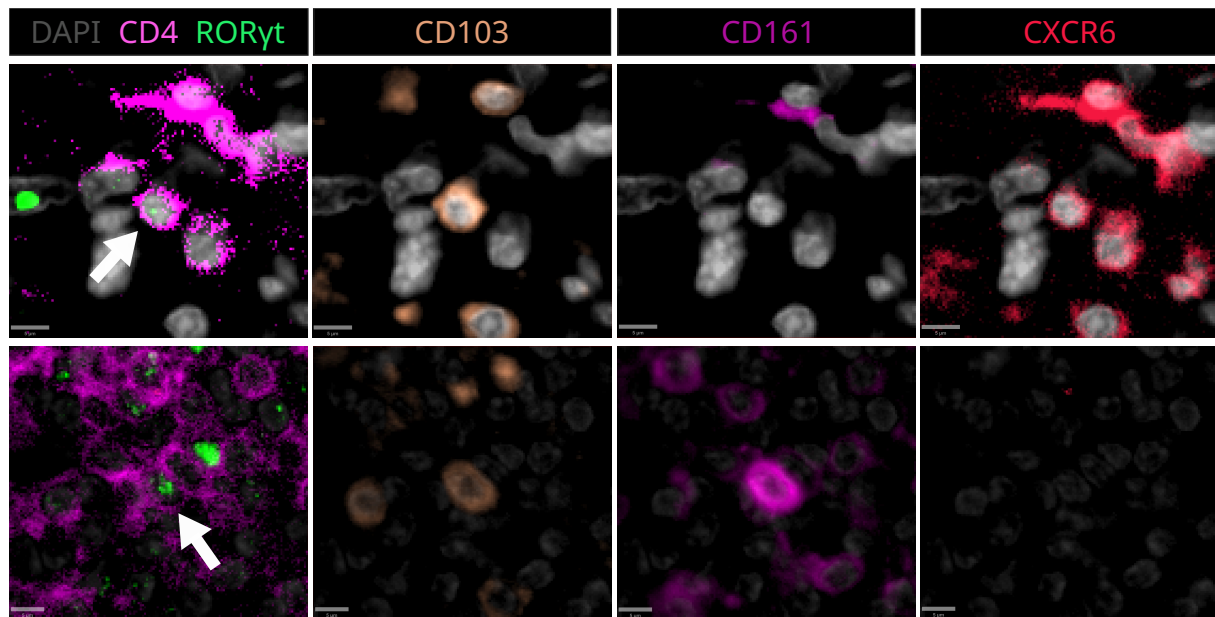


Figure S4.7 | CD103⁺ Th17 cells do not always co-express CD161 or CXCR6. Multiplexed staining of FFPE tissue with a CD103⁺ CD161⁺ CXCR6⁻ Th17 (CD45RO⁺ CD3⁺ CD4⁺ CD8⁻ RORγt⁺) cell from patient GI 8858 on the left and a CD103⁺ CD161⁻ CXCR6⁺ Th17 cell from patient GI 8841 on the right . Scale bar represents 5 μm.

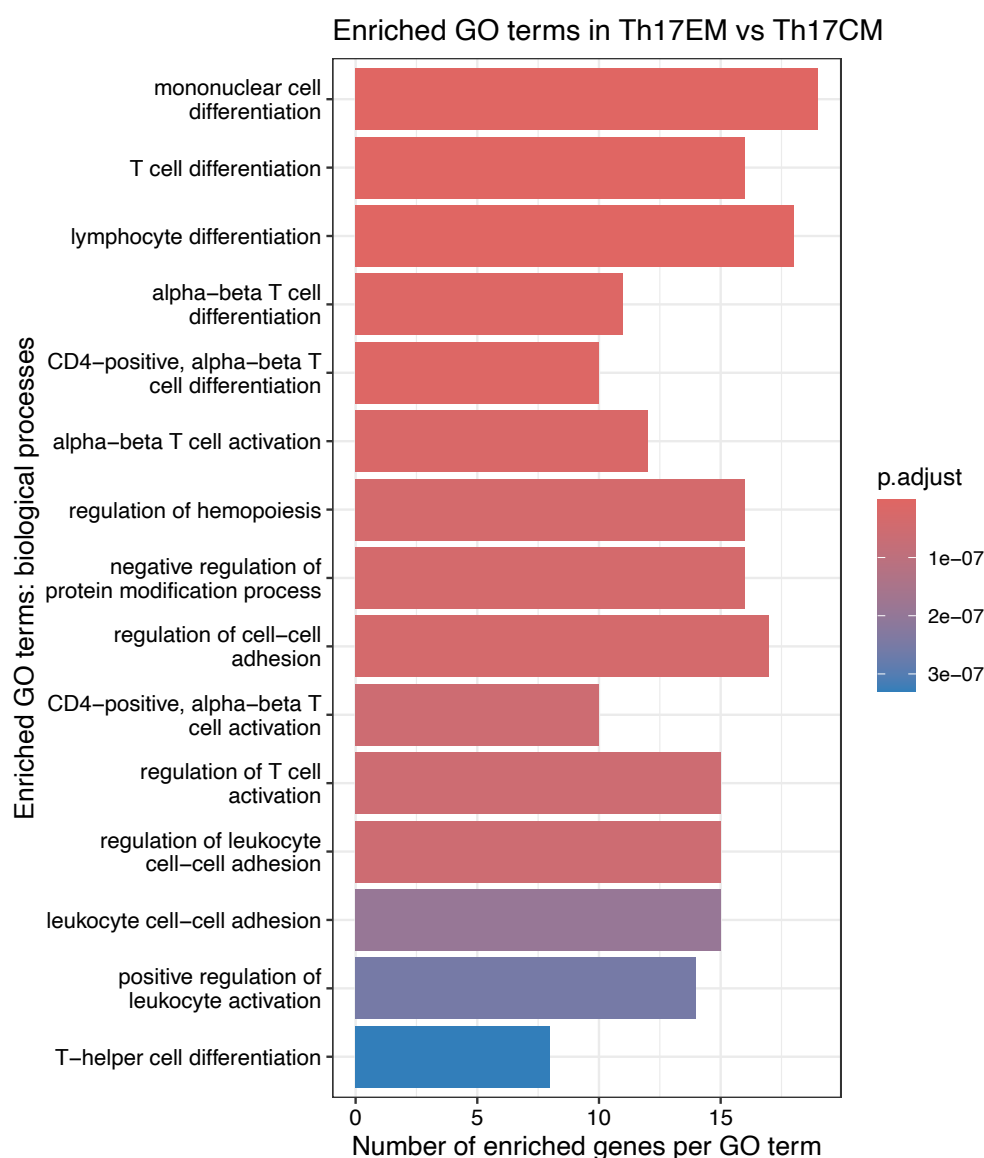


Figure S5.1 | Gene ontology indicates that Th17EM cells are more activated than Th17CM cells. Gene ontology (GO) biological process overrepresentation analysis was used to explore the relationship between differentially expressed genes in Th17EM cells, as compared with Th17CM cells. Wilcoxon rank sum test with Bonferroni correction was used to identify differentially upregulated genes in Th17RM cells, with $\log_2$ fold change ≥ 0.5 and p_{adj} (adjusted p value) ≤ 0.05 considered significant. Significantly upregulated genes were then mapped onto terms within the GO biological processes ontology. One-sided hypergeometric test with Benjamini-Hochberg correction was used to determine the functional significance of each biological process. The top 15 most significantly upregulated biological processes are illustrated.

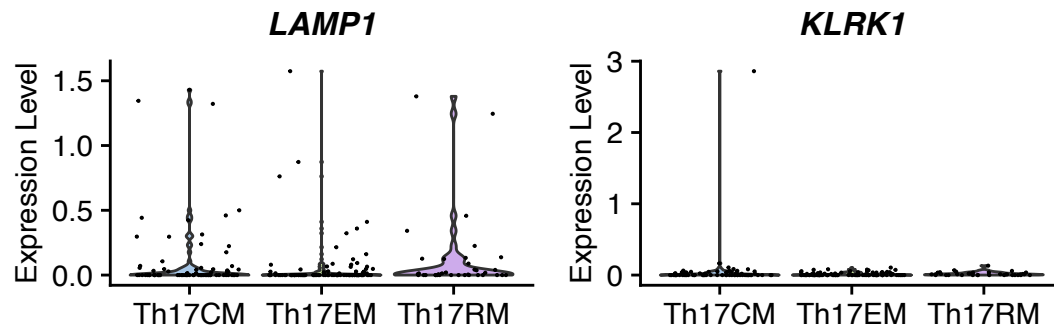


Figure S5.2 | Expression level of *LAMP1* (CD107a) and *KLRK1* (NKG2D) in each Th17 population in GSE108989.

References

- 1 Zhang, Y. *et al.* Deep single-cell RNA sequencing data of individual T cells from treatment-naïve colorectal cancer patients. *Scientific Data* **6** (2019). <https://doi.org:10.1038/s41597-019-0131-5>
- 2 Zhang, L. *et al.* Single-Cell Analyses Inform Mechanisms of Myeloid-Targeted Therapies in Colon Cancer. *Cell* **181**, 442-459.e429 (2020). <https://doi.org:10.1016/j.cell.2020.03.048>
- 3 Wu, Y. *et al.* Spatiotemporal Immune Landscape of Colorectal Cancer Liver Metastasis at Single-Cell Level. *Cancer Discovery* **12**, 134-153 (2022). <https://doi.org:10.1158/2159-8290.Cd-21-0316>
- 4 Siegel, R. L., Giaquinto, A. N. & Jemal, A. Cancer statistics, 2024. *CA: A Cancer Journal for Clinicians* **74**, 12-49 (2024). <https://doi.org:10.3322/caac.21820>
- 5 Vuik, F. E. R. *et al.* Increasing incidence of colorectal cancer in young adults in Europe over the last 25 years. *Gut* **68**, 1820 (2019). <https://doi.org:10.1136/gutjnl-2018-317592>
- 6 Spaander, M. C. W. *et al.* Young-onset colorectal cancer. *Nature Reviews Disease Primers* **9** (2023). <https://doi.org:10.1038/s41572-023-00432-7>
- 7 Kuipers, E. J. *et al.* Colorectal cancer. *Nature Reviews Disease Primers* **1**, 15065 (2015). <https://doi.org:10.1038/nrdp.2015.65>
- 8 Lynch, H. T. & De La Chapelle, A. Hereditary Colorectal Cancer. *New England Journal of Medicine* **348**, 919-932 (2003). <https://doi.org:10.1056/nejmra012242>
- 9 Peltomäki, P., Nyström, M., Mecklin, J.-P. & Seppälä, T. T. Lynch Syndrome Genetics and Clinical Implications. *Gastroenterology* **164**, 783-799 (2023). <https://doi.org:10.1053/j.gastro.2022.08.058>
- 10 Liu, J. *et al.* Wnt/ β -catenin signalling: function, biological mechanisms, and therapeutic opportunities. *Signal Transduction and Targeted Therapy* **7** (2022). <https://doi.org:10.1038/s41392-021-00762-6>

- 11 de la Chapelle, A. Genetic predisposition to colorectal cancer. *Nature Reviews Cancer* **4**, 769-780 (2004). <https://doi.org/10.1038/nrc1453>
- 12 Jess, T. *et al.* Decreasing Risk of Colorectal Cancer in Patients With Inflammatory Bowel Disease Over 30 Years. *Gastroenterology* **143**, 375-381.e371 (2012). <https://doi.org/10.1053/j.gastro.2012.04.016>
- 13 Valdeolivas, A. *et al.* Profiling the heterogeneity of colorectal cancer consensus molecular subtypes using spatial transcriptomics. *npj Precision Oncology* **8** (2024). <https://doi.org/10.1038/s41698-023-00488-4>
- 14 Pelka, K. *et al.* Spatially organized multicellular immune hubs in human colorectal cancer. *Cell* **184**, 4734-4752.e4720 (2021). <https://doi.org/10.1016/j.cell.2021.08.003>
- 15 Le, D. T. *et al.* Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* **357**, 409-413 (2017). <https://doi.org/doi:10.1126/science.aan6733>
- 16 André, T. *et al.* Pembrolizumab in Microsatellite-Instability–High Advanced Colorectal Cancer. *New England Journal of Medicine* **383**, 2207-2218 (2020). <https://doi.org/doi:10.1056/NEJMoa2017699>
- 17 Overman, M. J. *et al.* Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair–Deficient/Microsatellite Instability–High Metastatic Colorectal Cancer. *Journal of Clinical Oncology* **36**, 773-779 (2018). <https://doi.org/10.1200/jco.2017.76.9901>
- 18 Guinney, J. *et al.* The consensus molecular subtypes of colorectal cancer. *Nature Medicine* **21**, 1350-1356 (2015). <https://doi.org/10.1038/nm.3967>
- 19 Joanito, I. *et al.* Single-cell and bulk transcriptome sequencing identifies two epithelial tumor cell states and refines the consensus molecular classification of colorectal cancer. *Nature Genetics* **54**, 963-975 (2022). <https://doi.org/10.1038/s41588-022-01100-4>
- 20 Buikhuisen, J. Y., Torang, A. & Medema, J. P. Exploring and modelling colon cancer inter-tumour heterogeneity: opportunities and challenges. *Oncogenesis* **9** (2020). <https://doi.org/10.1038/s41389-020-00250-6>

- 21 Aiderus, A., Barker, N. & Tergaonkar, V. Serrated colorectal cancer: preclinical models and molecular pathways. *Trends in Cancer* **10**, 76-91 (2024).
<https://doi.org:10.1016/j.trecan.2023.09.011>
- 22 Valenzuela, G. *et al.* Consensus molecular subtypes of colorectal cancer in clinical practice: A translational approach. *World J Clin Oncol* **12**, 1000-1008 (2021).
<https://doi.org:10.5306/wjco.v12.i11.1000>
- 23 Meyerhardt Jeffrey, A. & Mayer Robert, J. Systemic Therapy for Colorectal Cancer. *New England Journal of Medicine* **352**, 476-487 (2005). <https://doi.org:10.1056/NEJMra040958>
- 24 Ieranò, C. *et al.* In PD-1+ human colon cancer cells NIVOLUMAB promotes survival and could protect tumor cells from conventional therapies. *Journal for ImmunoTherapy of Cancer* **10**, e004032 (2022). <https://doi.org:10.1136/jitc-2021-004032>
- 25 Albelda, S. M. CAR T cell therapy for patients with solid tumours: key lessons to learn and unlearn. *Nature Reviews Clinical Oncology* **21**, 47-66 (2024).
<https://doi.org:10.1038/s41571-023-00832-4>
- 26 Paludan, S. R., Pradeu, T., Masters, S. L. & Mogensen, T. H. Constitutive immune mechanisms: mediators of host defence and immune regulation. *Nature Reviews Immunology* **21**, 137-150 (2021). <https://doi.org:10.1038/s41577-020-0391-5>
- 27 Gong, T., Liu, L., Jiang, W. & Zhou, R. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. *Nature Reviews Immunology* **20**, 95-112 (2020).
<https://doi.org:10.1038/s41577-019-0215-7>
- 28 Gajewski, T. F., Schreiber, H. & Fu, Y.-X. Innate and adaptive immune cells in the tumor microenvironment. *Nature Immunology* **14**, 1014-1022 (2013).
<https://doi.org:10.1038/ni.2703>
- 29 Cao, X. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. *Nature Reviews Immunology* **16**, 35-50 (2016).
<https://doi.org:10.1038/nri.2015.8>

- 30 Sagulenko, V. *et al.* AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death & Differentiation* **20**, 1149-1160 (2013). <https://doi.org/10.1038/cdd.2013.37>
- 31 Binnewies, M. *et al.* Unleashing Type-2 Dendritic Cells to Drive Protective Antitumor CD4+ T Cell Immunity. *Cell* **177**, 556-571.e516 (2019). <https://doi.org/10.1016/j.cell.2019.02.005>
- 32 Wculek, S. K. *et al.* Dendritic cells in cancer immunology and immunotherapy. *Nature Reviews Immunology* **20**, 7-24 (2020). <https://doi.org/10.1038/s41577-019-0210-z>
- 33 Ohm, J. E. *et al.* Effect of Vascular Endothelial Growth Factor and FLT3 Ligand on Dendritic Cell Generation In Vivo. *The Journal of Immunology* **163**, 3260-3268 (1999). <https://doi.org/10.4049/jimmunol.163.6.3260>
- 34 Barry, K. C. *et al.* A natural killer–dendritic cell axis defines checkpoint therapy–responsive tumor microenvironments. *Nature Medicine* **24**, 1178-1191 (2018). <https://doi.org/10.1038/s41591-018-0085-8>
- 35 Mombaerts, P. *et al.* RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-877 (1992). [https://doi.org:https://doi.org/10.1016/0092-8674\(92\)90030-G](https://doi.org/https://doi.org/10.1016/0092-8674(92)90030-G)
- 36 Shinkai, Y. *et al.* RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**, 855-867 (1992). [https://doi.org:https://doi.org/10.1016/0092-8674\(92\)90029-C](https://doi.org/https://doi.org/10.1016/0092-8674(92)90029-C)
- 37 Schwarz, K. *et al.* RAG Mutations in Human B Cell-Negative SCID. *Science* **274**, 97-99 (1996). <https://doi.org/10.1126/science.274.5284.97>
- 38 Heesters, B. A., Myers, R. C. & Carroll, M. C. Follicular dendritic cells: dynamic antigen libraries. *Nature Reviews Immunology* **14**, 495-504 (2014). <https://doi.org/10.1038/nri3689>
- 39 Zotos, D. *et al.* IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. *Journal of Experimental Medicine* **207**, 365-378 (2010). <https://doi.org/10.1084/jem.20091777>

- 40 Ochiai, K. *et al.* Transcriptional Regulation of Germinal Center B and Plasma Cell Fates by Dynamical Control of IRF4. *Immunity* **38**, 918-929 (2013).
<https://doi.org:10.1016/j.immuni.2013.04.009>
- 41 Cyster, J. G. & Allen, C. D. C. B Cell Responses: Cell Interaction Dynamics and Decisions. *Cell* **177**, 524-540 (2019). <https://doi.org:10.1016/j.cell.2019.03.016>
- 42 Lo, J. A. *et al.* Epitope spreading toward wild-type melanocyte-lineage antigens rescues suboptimal immune checkpoint blockade responses. *Science Translational Medicine* **13**, eabd8636 (2021). <https://doi.org:10.1126/scitranslmed.abd8636>
- 43 Laumont, C. M., Banville, A. C., Gilardi, M., Hollern, D. P. & Nelson, B. H. Tumour-infiltrating B cells: immunological mechanisms, clinical impact and therapeutic opportunities. *Nature Reviews Cancer* **22**, 414-430 (2022).
<https://doi.org:10.1038/s41568-022-00466-1>
- 44 Schumacher, T. N. & Thommen, D. S. Tertiary lymphoid structures in cancer. *Science* **375**, eabf9419 (2022). <https://doi.org:10.1126/science.abf9419>
- 45 Radtke, F. *et al.* Deficient T Cell Fate Specification in Mice with an Induced Inactivation of Notch1. *Immunity* **10**, 547-558 (1999). [https://doi.org:10.1016/s1074-7613\(00\)80054-0](https://doi.org:10.1016/s1074-7613(00)80054-0)
- 46 Sambandam, A. *et al.* Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nature Immunology* **6**, 663-670 (2005).
<https://doi.org:10.1038/ni1216>
- 47 Hozumi, K. *et al.* Delta-like 4 is indispensable in thymic environment specific for T cell development. *The Journal of Experimental Medicine* **205**, 2507-2513 (2008).
<https://doi.org:10.1084/jem.20080134>
- 48 Garcia-Perez, L. *et al.* Functional definition of a transcription factor hierarchy regulating T cell lineage commitment. *Science Advances* **6**, eaaw7313 (2020).
<https://doi.org:10.1126/sciadv.aaw7313>
- 49 Ikawa, T. *et al.* An Essential Developmental Checkpoint for Production of the T Cell Lineage. *Science* **329**, 93-96 (2010). <https://doi.org:10.1126/science.1188995>

- 50 Li, P. *et al.* Reprogramming of T Cells to Natural Killer–Like Cells upon Bcl11b Deletion. *Science* **329**, 85-89 (2010). <https://doi.org:10.1126/science.1188063>
- 51 Van De Walle, I. *et al.* GATA3 induces human T-cell commitment by restraining Notch activity and repressing NK-cell fate. *Nature Communications* **7**, 11171 (2016). <https://doi.org:10.1038/ncomms11171>
- 52 García-Peydró, M., De Yébenes, V. G. & Toribio, M. A. L. Sustained Notch1 signaling instructs the earliest human intrathymic precursors to adopt a $\gamma \delta$ T-cell fate in fetal thymus organ culture. *Blood* **102**, 2444-2451 (2003). <https://doi.org:10.1182/blood-2002-10-3261>
- 53 Van De Walle, I. *et al.* Specific Notch receptor–ligand interactions control human TCR- $\alpha \beta$ / $\gamma \delta$ development by inducing differential Notch signal strength. *Journal of Experimental Medicine* **210**, 683-697 (2013). <https://doi.org:10.1084/jem.20121798>
- 54 Dutta, A., Zhao, B. & Love, P. E. New insights into TCR β -selection. *Trends in Immunology* **42**, 735-750 (2021). <https://doi.org:https://doi.org/10.1016/j.it.2021.06.005>
- 55 Zerrahn, J., Held, W. & Raulet, D. H. The MHC Reactivity of the T Cell Repertoire Prior to Positive and Negative Selection. *Cell* **88**, 627-636 (1997). [https://doi.org:10.1016/s0092-8674\(00\)81905-4](https://doi.org:10.1016/s0092-8674(00)81905-4)
- 56 Robey, E. A. *et al.* Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage. *Cell* **64**, 99-107 (1991). [https://doi.org:10.1016/0092-8674\(91\)90212-H](https://doi.org:10.1016/0092-8674(91)90212-H)
- 57 Shinzawa, M. *et al.* Reversal of the T cell immune system reveals the molecular basis for T cell lineage fate determination in the thymus. *Nature Immunology* **23**, 731-742 (2022). <https://doi.org:10.1038/s41590-022-01187-1>
- 58 Anderson, M. S. *et al.* Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein. *Science* **298**, 1395-1401 (2002). <https://doi.org:10.1126/science.1075958>
- 59 Anderson, M. S. *et al.* The Cellular Mechanism of Aire Control of T Cell Tolerance. *Immunity* **23**, 227-239 (2005). <https://doi.org:10.1016/j.immuni.2005.07.005>

- 60 Malchow, S. *et al.* Aire Enforces Immune Tolerance by Directing Autoreactive T Cells into the Regulatory T Cell Lineage. *Immunity* **44**, 1102-1113 (2016).
<https://doi.org:10.1016/j.immuni.2016.02.009>
- 61 Treiner, E. *et al.* Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* **422**, 164-169 (2003). <https://doi.org:10.1038/nature01433>
- 62 Legoux, F. *et al.* Microbial metabolites control the thymic development of mucosal-associated invariant T cells. *Science* **366**, 494-499 (2019).
<https://doi.org:10.1126/science.aaw2719>
- 63 Seach, N. *et al.* Double Positive Thymocytes Select Mucosal-Associated Invariant T Cells. *The Journal of Immunology* **191**, 6002-6009 (2013).
<https://doi.org:10.4049/jimmunol.1301212>
- 64 Gapin, L., Matsuda, J. L., Surh, C. D. & Kronenberg, M. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nature Immunology* **2**, 971-978 (2001). <https://doi.org:10.1038/ni710>
- 65 Egawa, T. *et al.* Genetic Evidence Supporting Selection of the V α 14i NKT Cell Lineage from Double-Positive Thymocyte Precursors. *Immunity* **22**, 705-716 (2005).
<https://doi.org:10.1016/j.immuni.2005.03.011>
- 66 June, C. H., Ledbetter, J. A., Gillespie, M. M., Lindsten, T. & Thompson, C. B. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Molecular and Cellular Biology* **7**, 4472-4481 (1987).
<https://doi.org:10.1128/mcb.7.12.4472>
- 67 Chen, L. & Flies, D. B. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature Reviews Immunology* **13**, 227-242 (2013). <https://doi.org:10.1038/nri3405>
- 68 Pardoll, D. M. The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews Cancer* **12**, 252-264 (2012). <https://doi.org:10.1038/nrc3239>
- 69 Schuijs, M. J., Hammad, H. & Lambrecht, B. N. Professional and 'Amateur' Antigen-Presenting Cells In Type 2 Immunity. *Trends in Immunology* **40**, 22-34 (2019).
<https://doi.org:10.1016/j.it.2018.11.001>

- 70 Darragh, L. B. & Karam, S. D. Amateur antigen-presenting cells in the tumor microenvironment. *Molecular Carcinogenesis* **61**, 153-164 (2022).
[https://doi.org:https://doi.org/10.1002/mc.23354](https://doi.org/https://doi.org/10.1002/mc.23354)
- 71 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-2357 (1986).
- 72 Hsieh, C.-S. *et al.* Development of TH1 CD4+ T Cells Through IL-12 Produced by Listeria-Induced Macrophages. *Science* **260**, 547-549 (1993).
[https://doi.org:10.1126/science.8097338](https://doi.org/10.1126/science.8097338)
- 73 Seder, R. A., Gazzinelli, R., Sher, A. & Paul, W. E. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proceedings of the National Academy of Sciences* **90**, 10188-10192 (1993). [https://doi.org:10.1073/pnas.90.21.10188](https://doi.org/10.1073/pnas.90.21.10188)
- 74 Swain, S. L., Weinberg, A. D., English, M. & Huston, G. IL-4 directs the development of Th2-like helper effectors. *The Journal of Immunology* **145**, 3796-3806 (1990).
[https://doi.org:10.4049/jimmunol.145.11.3796](https://doi.org/10.4049/jimmunol.145.11.3796)
- 75 Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D. & Paul, W. E. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *The Journal of experimental medicine* **172**, 921-929 (1990). [https://doi.org:10.1084/jem.172.3.921](https://doi.org/10.1084/jem.172.3.921)
- 76 Hsieh, C. S., Heimberger, A. B., Gold, J. S., O'Garra, A. & Murphy, K. M. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proceedings of the National Academy of Sciences* **89**, 6065-6069 (1992). [https://doi.org:10.1073/pnas.89.13.6065](https://doi.org/10.1073/pnas.89.13.6065)
- 77 Seder, R. A., Paul, W. E., Davis, M. M. & Fazekas De St Groth, B. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. *The Journal of experimental medicine* **176**, 1091-1098 (1992). [https://doi.org:10.1084/jem.176.4.1091](https://doi.org/10.1084/jem.176.4.1091)

- 78 Murphy, E. *et al.* Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *The Journal of experimental medicine* **183**, 901-913 (1996).
<https://doi.org:10.1084/jem.183.3.901>
- 79 Jacobson, N. G. *et al.* Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *The Journal of experimental medicine* **181**, 1755-1762 (1995).
<https://doi.org:10.1084/jem.181.5.1755>
- 80 Szabo, S. J. *et al.* A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. *Cell* **100**, 655-669 (2000). [https://doi.org:10.1016/s0092-8674\(00\)80702-3](https://doi.org:10.1016/s0092-8674(00)80702-3)
- 81 Kaplan, M. H., Schindler, U., Smiley, S. T. & Grusby, M. J. Stat6 Is Required for Mediating Responses to IL-4 and for the Development of Th2 Cells. *Immunity* **4**, 313-319 (1996).
[https://doi.org:10.1016/S1074-7613\(00\)80439-2](https://doi.org:10.1016/S1074-7613(00)80439-2)
- 82 Takeda, K. *et al.* Essential role of Stat6 in IL-4 signalling. *Nature* **380**, 627-630 (1996).
<https://doi.org:10.1038/380627a0>
- 83 Zheng, W.-P. & Flavell, R. A. The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. *Cell* **89**, 587-596 (1997).
[https://doi.org:10.1016/s0092-8674\(00\)80240-8](https://doi.org:10.1016/s0092-8674(00)80240-8)
- 84 Abbas, A. K., Murphy, K. M. & Sher, A. Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793 (1996). <https://doi.org:10.1038/383787a0>
- 85 Hori, S., Nomura, T. & Sakaguchi, S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* **299**, 1057-1061 (2003).
<https://doi.org:10.1126/science.1079490>
- 86 Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology* **4**, 330-336 (2003).
<https://doi.org:10.1038/ni904>
- 87 Khattri, R., Cox, T., Yasayko, S.-A. & Ramsdell, F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nature Immunology* **4**, 337-342 (2003).
<https://doi.org:10.1038/ni909>

- 88 Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *The Journal of Immunology* **155**, 1151-1164 (1995).
<https://doi.org/10.4049/jimmunol.155.3.1151>
- 89 Sakaguchi, S., Fukuma, K., Kuribayashi, K. & Masuda, T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *The Journal of experimental medicine* **161**, 72-87 (1985).
<https://doi.org/10.1084/jem.161.1.72>
- 90 Powrie, F. & Mason, D. OX-22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22low subset. *The Journal of experimental medicine* **172**, 1701-1708 (1990). <https://doi.org/10.1084/jem.172.6.1701>
- 91 Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. *The Journal of Experimental Medicine* **190**, 995-1004 (1999).
<https://doi.org/10.1084/jem.190.7.995>
- 92 Read, S., Malmström, V. & Powrie, F. Cytotoxic T Lymphocyte-Associated Antigen 4 Plays an Essential Role in the Function of Cd25+Cd4+ Regulatory Cells That Control Intestinal Inflammation. *The Journal of Experimental Medicine* **192**, 295-302 (2000).
<https://doi.org/10.1084/jem.192.2.295>
- 93 Deaglio, S. *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *The Journal of Experimental Medicine* **204**, 1257-1265 (2007). <https://doi.org/10.1084/jem.20062512>
- 94 Cao, X. *et al.* Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance. *Immunity* **27**, 635-646 (2007).
<https://doi.org/10.1016/j.immuni.2007.08.014>
- 95 Powrie, F., Leach, M. W., Mauze, S., Caddie, L. B. & Coffman, R. L. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17

- scid mice. *International Immunology* **5**, 1461-1471 (1993).
<https://doi.org:10.1093/intimm/5.11.1461>
- 96 Powrie, F. *et al.* Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553-562 (1994).
[https://doi.org:https://doi.org/10.1016/1074-7613\(94\)90045-0](https://doi.org:https://doi.org/10.1016/1074-7613(94)90045-0)
 - 97 Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* **27**, 68-73 (2001). <https://doi.org:10.1038/83784>
 - 98 Wildin, R. S. *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nature Genetics* **27**, 18-20 (2001).
<https://doi.org:10.1038/83707>
 - 99 Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics* **27**, 20-21 (2001). <https://doi.org:10.1038/83713>
 - 100 Breitfeld, D. *et al.* Follicular B Helper T Cells Express Cxc Chemokine Receptor 5, Localize to B Cell Follicles, and Support Immunoglobulin Production. *The Journal of Experimental Medicine* **192**, 1545-1552 (2000). <https://doi.org:10.1084/jem.192.11.1545>
 - 101 Schaerli, P. *et al.* Cxc Chemokine Receptor 5 Expression Defines Follicular Homing T Cells with B Cell Helper Function. *The Journal of Experimental Medicine* **192**, 1553-1562 (2000).
<https://doi.org:10.1084/jem.192.11.1553>
 - 102 Choi, Youn S. *et al.* ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. *Immunity* **34**, 932-946 (2011). <https://doi.org:10.1016/j.immuni.2011.03.023>
 - 103 Arroyo-Díaz, N. M. *et al.* Interferon- γ production by Tfh cells is required for CXCR3+ pre-memory B cell differentiation and subsequent lung-resident memory B cell responses. *Immunity* **56**, 2358-2372.e2355 (2023). <https://doi.org:10.1016/j.immuni.2023.08.015>

- 104 Powell, M. D., Read, K. A., Sreekumar, B. K., Jones, D. M. & Oestreich, K. J. IL-12 signaling drives the differentiation and function of a TH1-derived TFH1-like cell population. *Scientific Reports* **9** (2019). <https://doi.org/10.1038/s41598-019-50614-1>
- 105 Zaretsky, A. G. *et al.* T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *Journal of Experimental Medicine* **206**, 991-999 (2009). <https://doi.org/10.1084/jem.20090303>
- 106 King, I. L. & Mohrs, M. IL-4-producing CD4⁺ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *Journal of Experimental Medicine* **206**, 1001-1007 (2009). <https://doi.org/10.1084/jem.20090313>
- 107 Reinhardt, R. L., Liang, H.-E. & Locksley, R. M. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nature Immunology* **10**, 385-393 (2009). <https://doi.org/10.1038/ni.1715>
- 108 Xin, G. *et al.* Single-cell RNA sequencing unveils an IL-10-producing helper subset that sustains humoral immunity during persistent infection. *Nature Communications* **9** (2018). <https://doi.org/10.1038/s41467-018-07492-4>
- 109 Kim, V. *et al.* IL-17-producing follicular Th cells enhance plasma cell differentiation in lupus-prone mice. *JCI Insight* **7** (2022). <https://doi.org/10.1172/jci.insight.157332>
- 110 Yu, D. *et al.* The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment. *Immunity* **31**, 457-468 (2009). <https://doi.org/10.1016/j.immuni.2009.07.002>
- 111 Kusam, S., Toney, L. M., Sato, H. & Dent, A. L. Inhibition of Th2 Differentiation and GATA-3 Expression by BCL-6. *The Journal of Immunology* **170**, 2435-2441 (2003). <https://doi.org/10.4049/jimmunol.170.5.2435>
- 112 Olatunde, A. C., Hale, J. S. & Lamb, T. J. Cytokine-skewed Tfh cells: functional consequences for B cell help. *Trends in Immunology* **42**, 536-550 (2021). <https://doi.org/10.1016/j.it.2021.04.006>

- 113 Brockmann, L. *et al.* Intestinal microbiota-specific Th17 cells possess regulatory properties and suppress effector T cells via c-MAF and IL-10. *Immunity***56**, 2719-2735.e2717 (2023). <https://doi.org/10.1016/j.immuni.2023.11.003>
- 114 Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A. & Sallusto, F. Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nature Immunology***8**, 942-949 (2007). <https://doi.org/10.1038/ni1496>
- 115 Wilson, N. J. *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nature Immunology***8**, 950-957 (2007). <https://doi.org/10.1038/ni1497>
- 116 Yang, Y. *et al.* T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *Journal of Experimental Medicine***206**, 1549-1564 (2009). <https://doi.org/10.1084/jem.20082584>
- 117 Dardalhon, V. *et al.* IL-4 inhibits TGF- β -induced Foxp3+ T cells and, together with TGF- β , generates IL-9+ IL-10+ Foxp3– effector T cells. *Nature Immunology***9**, 1347-1355 (2008). <https://doi.org/10.1038/ni.1677>
- 118 Veldhoen, M. *et al.* Transforming growth factor- β 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature Immunology***9**, 1341-1346 (2008). <https://doi.org/10.1038/ni.1659>
- 119 Duhon, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. & Sallusto, F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nature Immunology***10**, 857-863 (2009). <https://doi.org/10.1038/ni.1767>
- 120 Fallon, P. G. *et al.* Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *The Journal of Experimental Medicine***203**, 1105-1116 (2006). <https://doi.org/10.1084/jem.20051615>
- 121 Constantinescu, C. S., Farooqi, N., O'Brien, K. & Gran, B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British Journal of Pharmacology***164**, 1079-1106 (2011). <https://doi.org/10.1111/j.1476-5381.2011.01302.x>

- 122 Langrish, C. L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *Journal of Experimental Medicine* **201**, 233-240 (2005).
<https://doi.org:10.1084/jem.20041257>
- 123 Tortola, L. *et al.* High-Dimensional T Helper Cell Profiling Reveals a Broad Diversity of Stably Committed Effector States and Uncovers Interlineage Relationships. *Immunity* **53**, 597-613.e596 (2020). <https://doi.org:10.1016/j.immuni.2020.07.001>
- 124 O'Shea, J. J. & Paul, W. E. Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4+ T Cells. *Science* **327**, 1098-1102 (2010).
<https://doi.org:doi:10.1126/science.1178334>
- 125 Eberl, G. & Pradeu, T. Towards a General Theory of Immunity? *Trends in Immunology* **39**, 261-263 (2018). <https://doi.org:https://doi.org/10.1016/j.it.2017.11.004>
- 126 Tuzlak, S. *et al.* Repositioning TH cell polarization from single cytokines to complex help. *Nature Immunology* **22**, 1210-1217 (2021). <https://doi.org:10.1038/s41590-021-01009-w>
- 127 Bettelli, E. *et al.* Loss of T-bet, But Not STAT1, Prevents the Development of Experimental Autoimmune Encephalomyelitis. *Journal of Experimental Medicine* **200**, 79-87 (2004).
<https://doi.org:10.1084/jem.20031819>
- 128 Cua, D. J. *et al.* Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**, 744-748 (2003).
<https://doi.org:10.1038/nature01355>
- 129 Chen, Y. *et al.* Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *The Journal of Clinical Investigation* **116**, 1317-1326 (2006). <https://doi.org:10.1172/JCI25308>
- 130 Ivanov, I. I. *et al.* The Orphan Nuclear Receptor ROR γ t Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell* **126**, 1121-1133 (2006).
<https://doi.org:10.1016/j.cell.2006.07.035>
- 131 Hirota, K. *et al.* Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *The Journal of Experimental Medicine* **204**, 2803-2812 (2007). <https://doi.org:10.1084/jem.20071397>

- 132 Tsai, P.-Y. *et al.* IL-22 Upregulates Epithelial Claudin-2 to Drive Diarrhea and Enteric Pathogen Clearance. *Cell Host & Microbe* **21**, 671-681.e674 (2017).
<https://doi.org:10.1016/j.chom.2017.05.009>
- 133 Sugimoto, K. *et al.* IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *Journal of Clinical Investigation* (2008). <https://doi.org:10.1172/jci33194>
- 134 Aujla, S. J. *et al.* IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nature Medicine* **14**, 275-281 (2008). <https://doi.org:10.1038/nm1710>
- 135 Zheng, Y. *et al.* Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine* **14**, 282-289 (2008).
<https://doi.org:10.1038/nm1720>
- 136 Kumar, P. *et al.* Intestinal Interleukin-17 Receptor Signaling Mediates Reciprocal Control of the Gut Microbiota and Autoimmune Inflammation. *Immunity* **44**, 659-671 (2016).
<https://doi.org:10.1016/j.immuni.2016.02.007>
- 137 Gronke, K. *et al.* Interleukin-22 protects intestinal stem cells against genotoxic stress. *Nature* **566**, 249-253 (2019). <https://doi.org:10.1038/s41586-019-0899-7>
- 138 Lin, X. *et al.* IL-17RA-signaling in Lgr5+ intestinal stem cells induces expression of transcription factor ATOH1 to promote secretory cell lineage commitment. *Immunity* **55**, 237-253.e238 (2022). <https://doi.org:https://doi.org/10.1016/j.immuni.2021.12.016>
- 139 Okada, T. *et al.* Microbiota-derived lactate accelerates colon epithelial cell turnover in starvation-refed mice. *Nature Communications* **4**, 1654 (2013).
<https://doi.org:10.1038/ncomms2668>
- 140 Ye, P. *et al.* Requirement of Interleukin 17 Receptor Signaling for Lung Cxc Chemokine and Granulocyte Colony-Stimulating Factor Expression, Neutrophil Recruitment, and Host Defense. *The Journal of Experimental Medicine* **194**, 519-528 (2001).
<https://doi.org:10.1084/jem.194.4.519>
- 141 Chen, K. *et al.* IL-17 Receptor Signaling in the Lung Epithelium Is Required for Mucosal Chemokine Gradients and Pulmonary Host Defense against K. pneumoniae. *Cell Host & Microbe* **20**, 596-605 (2016). <https://doi.org:10.1016/j.chom.2016.10.003>

- 142 McGinley, A. M. *et al.* Interleukin-17A Serves a Priming Role in Autoimmunity by Recruiting IL-1 β -Producing Myeloid Cells that Promote Pathogenic T Cells. *Immunity* **52**, 342-356.e346 (2020). <https://doi.org:10.1016/j.immuni.2020.01.002>
- 143 Song, X. *et al.* Growth Factor FGF2 Cooperates with Interleukin-17 to Repair Intestinal Epithelial Damage. *Immunity* **43**, 488-501 (2015). <https://doi.org:10.1016/j.immuni.2015.06.024>
- 144 Pan, B. *et al.* Interleukin-17 promotes angiogenesis by stimulating VEGF production of cancer cells via the STAT3/GIV signaling pathway in non-small-cell lung cancer. *Scientific Reports* **5**, 16053 (2015). <https://doi.org:10.1038/srep16053>
- 145 Zhao, J. *et al.* Granulocyte/Macrophage Colony-Stimulating Factor Influences Angiogenesis by Regulating the Coordinated Expression of VEGF and the Ang/Tie System. *PLoS ONE* **9**, e92691 (2014). <https://doi.org:10.1371/journal.pone.0092691>
- 146 Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235-238 (2006). <https://doi.org:10.1038/nature04753>
- 147 Mangan, P. R. *et al.* Transforming growth factor- β induces development of the TH17 lineage. *Nature* **441**, 231-234 (2006). <https://doi.org:10.1038/nature04754>
- 148 Chung, Y. *et al.* Critical Regulation of Early Th17 Cell Differentiation by Interleukin-1 Signaling. *Immunity* **30**, 576-587 (2009). <https://doi.org:10.1016/j.immuni.2009.02.007>
- 149 Ghoreschi, K. *et al.* Generation of pathogenic TH17 cells in the absence of TGF- β signalling. *Nature* **467**, 967-971 (2010). <https://doi.org:10.1038/nature09447>
- 150 Lee, Y. *et al.* Induction and molecular signature of pathogenic TH17 cells. *Nature Immunology* **13**, 991-999 (2012). <https://doi.org:10.1038/ni.2416>
- 151 Hirota, K. *et al.* Fate mapping of IL-17-producing T cells in inflammatory responses. *Nature Immunology* **12**, 255-263 (2011). <https://doi.org:10.1038/ni.1993>

- 152 Thakore, P. I. *et al.* The chromatin landscape of Th17 cells reveals mechanisms of diversification of regulatory and pro-inflammatory states. *bioRxiv*, 2022.2002.2026.482041 (2022). <https://doi.org:10.1101/2022.02.26.482041>
- 153 Chang, K.-K. *et al.* IL-27 triggers IL-10 production in Th17 cells via a c-Maf/ROR γ t/Blimp-1 signal to promote the progression of endometriosis. *Cell Death & Disease* **8**, e2666-e2666 (2017). <https://doi.org:10.1038/cddis.2017.95>
- 154 Lin, C.-H. *et al.* Selective IL-27 production by intestinal regulatory T cells permits gut-specific regulation of TH17 cell immunity. *Nature Immunology* **24**, 2108-2120 (2023). <https://doi.org:10.1038/s41590-023-01667-y>
- 155 Nurieva, R. *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* **448**, 480-483 (2007). <https://doi.org:10.1038/nature05969>
- 156 Zhou, L. *et al.* IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nature Immunology* **8**, 967-974 (2007). <https://doi.org:10.1038/ni1488>
- 157 Stritesky, G. L., Yeh, N. & Kaplan, M. H. IL-23 Promotes Maintenance but Not Commitment to the Th17 Lineage1. *The Journal of Immunology* **181**, 5948-5955 (2008). <https://doi.org:10.4049/jimmunol.181.9.5948>
- 158 Kleinewietfeld, M. *et al.* Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature* **496**, 518-522 (2013). <https://doi.org:10.1038/nature11868>
- 159 Du, L. *et al.* Growth hormone releasing hormone signaling promotes Th17 cell differentiation and autoimmune inflammation. *Nature Communications* **14** (2023). <https://doi.org:10.1038/s41467-023-39023-1>
- 160 Lee, J.-Y. *et al.* Serum Amyloid A Proteins Induce Pathogenic Th17 Cells and Promote Inflammatory Disease. *Cell* **180**, 79-91.e16 (2020). <https://doi.org:10.1016/j.cell.2019.11.026>
- 161 Huang, J. *et al.* Melatonin, an endogenous hormone, modulates Th17 cells via the reactive-oxygen species/TXNIP/HIF-1 α axis to alleviate autoimmune uveitis. *Journal of Neuroinflammation* **19** (2022). <https://doi.org:10.1186/s12974-022-02477-z>

- 162 Dang, E. V. *et al.* Control of TH17/Treg Balance by Hypoxia-Inducible Factor 1. *Cell* **146**, 772-784 (2011). <https://doi.org/10.1016/j.cell.2011.07.033>
- 163 Schnell, A. *et al.* Stem-like intestinal Th17 cells give rise to pathogenic effector T cells during autoimmunity. *Cell* (2021). <https://doi.org/10.1016/j.cell.2021.11.018>
- 164 Goto, Y. *et al.* Segmented Filamentous Bacteria Antigens Presented by Intestinal Dendritic Cells Drive Mucosal Th17 Cell Differentiation. *Immunity* **40**, 594-607 (2014). <https://doi.org/10.1016/j.immuni.2014.03.005>
- 165 Lécuyer, E. *et al.* Segmented Filamentous Bacterium Uses Secondary and Tertiary Lymphoid Tissues to Induce Gut IgA and Specific T Helper 17 Cell Responses. *Immunity* **40**, 608-620 (2014). <https://doi.org/10.1016/j.immuni.2014.03.009>
- 166 Sano, T. *et al.* An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. *Cell* **163**, 381-393 (2015). <https://doi.org/10.1016/j.cell.2015.08.061>
- 167 Atarashi, K. *et al.* Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell* **163**, 367-380 (2015). <https://doi.org/10.1016/j.cell.2015.08.058>
- 168 Omenetti, S. *et al.* The Intestine Harbors Functionally Distinct Homeostatic Tissue-Resident and Inflammatory Th17 Cells. *Immunity* **51**, 77-89.e76 (2019). <https://doi.org/10.1016/j.immuni.2019.05.004>
- 169 Zielinski, C. E. *et al.* Pathogen-induced human TH17 cells produce IFN- γ or IL-10 and are regulated by IL-1 β . *Nature* **484**, 514-518 (2012). <https://doi.org/10.1038/nature10957>
- 170 Chao, Y.-Y. *et al.* Human TH17 cells engage gasdermin E pores to release IL-1 α on NLRP3 inflammasome activation. *Nature Immunology* (2023). <https://doi.org/10.1038/s41590-022-01386-w>
- 171 Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discovery* **12**, 31-46 (2022). <https://doi.org/10.1158/2159-8290.cd-21-1059>

- 172 Liu, N.-N. *et al.* Multi-kingdom microbiota analyses identify bacterial–fungal interactions and biomarkers of colorectal cancer across cohorts. *Nature Microbiology* **7**, 238-250 (2022). <https://doi.org/10.1038/s41564-021-01030-7>
- 173 Wong, S. H. *et al.* Gavage of Fecal Samples From Patients With Colorectal Cancer Promotes Intestinal Carcinogenesis in Germ-Free and Conventional Mice. *Gastroenterology* **153**, 1621-1633.e1626 (2017). <https://doi.org/10.1053/j.gastro.2017.08.022>
- 174 Long, X. *et al.* Peptostreptococcus anaerobius promotes colorectal carcinogenesis and modulates tumour immunity. *Nature Microbiology* **4**, 2319-2330 (2019). <https://doi.org/10.1038/s41564-019-0541-3>
- 175 Wu, S. *et al.* A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nature Medicine* **15**, 1016-1022 (2009). <https://doi.org/10.1038/nm.2015>
- 176 Zhao, L. *et al.* Parvimonas micra promotes colorectal tumorigenesis and is associated with prognosis of colorectal cancer patients. *Oncogene* **41**, 4200-4210 (2022). <https://doi.org/10.1038/s41388-022-02395-7>
- 177 Ternes, D. *et al.* The gut microbial metabolite formate exacerbates colorectal cancer progression. *Nature Metabolism* **4**, 458-475 (2022). <https://doi.org/10.1038/s42255-022-00558-0>
- 178 Brennan, C. A. *et al.* Fusobacterium nucleatum drives a pro-inflammatory intestinal microenvironment through metabolite receptor-dependent modulation of IL-17 expression. *Gut Microbes* **13** (2021). <https://doi.org/10.1080/19490976.2021.1987780>
- 179 Kostic, Aleksandar D. *et al.* Fusobacterium nucleatum Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment. *Cell Host & Microbe* **14**, 207-215 (2013). [https://doi.org/https://doi.org/10.1016/j.chom.2013.07.007](https://doi.org/10.1016/j.chom.2013.07.007)
- 180 Janney, A., Powrie, F. & Mann, E. H. Host–microbiota maladaptation in colorectal cancer. *Nature* **585**, 509-517 (2020). <https://doi.org/10.1038/s41586-020-2729-3>

- 181 Grivennikov, S. I. *et al.* Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* **491**, 254-258 (2012).
<https://doi.org:10.1038/nature11465>
- 182 Yao, J. *et al.* Single-cell transcriptomic analysis in a mouse model deciphers cell transition states in the multistep development of esophageal cancer. *Nature Communications* **11** (2020). <https://doi.org:10.1038/s41467-020-17492-y>
- 183 Kryczek, I. *et al.* Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* **114**, 1141-1149 (2009).
<https://doi.org:10.1182/blood-2009-03-208249>
- 184 Walch-Rückheim, B. *et al.* Stromal Fibroblasts Induce CCL20 through IL6/C/EBP β to Support the Recruitment of Th17 Cells during Cervical Cancer Progression. *Cancer Research* **75**, 5248-5259 (2015). <https://doi.org:10.1158/0008-5472.CAN-15-0732>
- 185 Su, X. *et al.* Tumor Microenvironments Direct the Recruitment and Expansion of Human Th17 Cells. *The Journal of Immunology* **184**, 1630-1641 (2009).
<https://doi.org:10.4049/jimmunol.0902813>
- 186 Revu, S. *et al.* IL-23 and IL-1 β Drive Human Th17 Cell Differentiation and Metabolic Reprogramming in Absence of CD28 Costimulation. *Cell Reports* **22**, 2642-2653 (2018).
<https://doi.org:10.1016/j.celrep.2018.02.044>
- 187 Muranski, P. *et al.* Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* **112**, 362-373 (2008). <https://doi.org:10.1182/blood-2007-11-120998>
- 188 Muranski, P. *et al.* Th17 Cells Are Long Lived and Retain a Stem Cell-like Molecular Signature. *Immunity* **35**, 972-985 (2011). <https://doi.org:10.1016/j.immuni.2011.09.019>
- 189 Downs-Canner, S. *et al.* Suppressive IL-17A⁺Foxp3⁺ and ex-Th17 IL-17AnegFoxp3⁺ Treg cells are a source of tumour-associated Treg cells. *Nature Communications* **8**, 14649 (2017). <https://doi.org:10.1038/ncomms14649>
- 190 He, D. *et al.* IL-17 Promotes Tumor Development through the Induction of Tumor Promoting Microenvironments at Tumor Sites and Myeloid-Derived Suppressor Cells. *The*

- Journal of Immunology* **184**, 2281-2288 (2010).
<https://doi.org/10.4049/jimmunol.0902574>
- 191 Calcinotto, A. *et al.* Microbiota-driven interleukin-17-producing cells and eosinophils synergize to accelerate multiple myeloma progression. *Nature Communications* **9** (2018).
<https://doi.org/10.1038/s41467-018-07305-8>
 - 192 Benevides, L. *et al.* IL17 Promotes Mammary Tumor Progression by Changing the Behavior of Tumor Cells and Eliciting Tumorigenic Neutrophils Recruitment. *Cancer Research* **75**, 3788-3799 (2015). <https://doi.org/10.1158/0008-5472.CAN-15-0054>
 - 193 Amicarella, F. *et al.* Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. *Gut* **66**, 692-704 (2017). <https://doi.org/10.1136/gutjnl-2015-310016>
 - 194 Ponzetta, A. *et al.* Neutrophils Driving Unconventional T Cells Mediate Resistance against Murine Sarcomas and Selected Human Tumors. *Cell* **178**, 346-360.e324 (2019).
<https://doi.org/10.1016/j.cell.2019.05.047>
 - 195 Charles, J. *et al.* Characterization of Circulating Dendritic Cells in Melanoma: Role of CCR6 in Plasmacytoid Dendritic Cell Recruitment to the Tumor. *Journal of Investigative Dermatology* **130**, 1646-1656 (2010). <https://doi.org/10.1038/jid.2010.24>
 - 196 Chen, K.-J. *et al.* Selective Recruitment of Regulatory T Cell through CCR6-CCL20 in Hepatocellular Carcinoma Fosters Tumor Progression and Predicts Poor Prognosis. *PLoS ONE* **6**, e24671 (2011). <https://doi.org/10.1371/journal.pone.0024671>
 - 197 Yu, Q., Lou, X.-M. & He, Y. Preferential Recruitment of Th17 Cells to Cervical Cancer via CCR6-CCL20 Pathway. *PLOS ONE* **10**, e0120855 (2015).
<https://doi.org/10.1371/journal.pone.0120855>
 - 198 Mucciolo, G. *et al.* IL17A critically shapes the transcriptional program of fibroblasts in pancreatic cancer and switches on their protumorigenic functions. *Proceedings of the National Academy of Sciences* **118**, e2020395118 (2021).
<https://doi.org/10.1073/pnas.2020395118>

- 199 Chen, X. *et al.* IL-17-induced HIF1 α drives resistance to anti-PD-L1 via fibroblast-mediated immune exclusion. *Journal of Experimental Medicine* **219** (2022).
<https://doi.org:10.1084/jem.20210693>
- 200 Numasaki, M. *et al.* IL-17 Enhances the Net Angiogenic Activity and In Vivo Growth of Human Non-Small Cell Lung Cancer in SCID Mice through Promoting CXCR-2-Dependent Angiogenesis. *The Journal of Immunology* **175**, 6177-6189 (2005).
<https://doi.org:10.4049/jimmunol.175.9.6177>
- 201 Huang, Q. *et al.* IL-17 Promotes Angiogenic Factors IL-6, IL-8, and Vegf Production via Stat1 in Lung Adenocarcinoma. *Scientific Reports* **6**, 36551 (2016).
<https://doi.org:10.1038/srep36551>
- 202 McAllister, F. *et al.* Oncogenic Kras Activates a Hematopoietic-to-Epithelial IL-17 Signaling Axis in Preinvasive Pancreatic Neoplasia. *Cancer Cell* **25**, 621-637 (2014).
<https://doi.org:10.1016/j.ccr.2014.03.014>
- 203 Wang, K. *et al.* Interleukin-17 Receptor A Signaling in Transformed Enterocytes Promotes Early Colorectal Tumorigenesis. *Immunity* **41**, 1052-1063 (2014).
<https://doi.org:10.1016/j.immuni.2014.11.009>
- 204 McCuaig, S. *et al.* The Interleukin 22 Pathway Interacts with Mutant KRAS to Promote Poor Prognosis in Colon Cancer. *Clinical Cancer Research* **26**, 4313-4325 (2020).
<https://doi.org:10.1158/1078-0432.ccr-19-1086>
- 205 Xie, M.-H. *et al.* Interleukin (IL)-22, a Novel Human Cytokine That Signals through the Interferon Receptor-related Proteins CRF2-4 and IL-22R. *Journal of Biological Chemistry* **275**, 31335-31339 (2000). <https://doi.org:10.1074/jbc.m005304200>
- 206 Khosravi, N. *et al.* IL22 Promotes Kras-Mutant Lung Cancer by Induction of a Protumor Immune Response and Protection of Stemness Properties. *Cancer Immunology Research* **6**, 788-797 (2018). <https://doi.org:10.1158/2326-6066.cir-17-0655>
- 207 Perusina Lanfranca, M. *et al.* Interleukin 22 Signaling Regulates Acinar Cell Plasticity to Promote Pancreatic Tumor Development in Mice. *Gastroenterology* **158**, 1417-1432.e1411 (2020). <https://doi.org:10.1053/j.gastro.2019.12.010>

- 208 Kryczek, I. *et al.* IL-22+CD4+ T Cells Promote Colorectal Cancer Stemness via STAT3 Transcription Factor Activation and Induction of the Methyltransferase DOT1L. *Immunity* **40**, 772-784 (2014). <https://doi.org/10.1016/j.immuni.2014.03.010>
- 209 Clarke, M. F. & Fuller, M. Stem Cells and Cancer: Two Faces of Eve. *Cell* **124**, 1111-1115 (2006). <https://doi.org/10.1016/j.cell.2006.03.011>
- 210 Sun, Y., Pan, J., Mao, S. & Jin, J. IL-17/miR-192/IL-17Rs Regulatory Feedback Loop Facilitates Multiple Myeloma Progression. *PLoS ONE* **9**, e114647 (2014). <https://doi.org/10.1371/journal.pone.0114647>
- 211 Elinav, E. *et al.* Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nature Reviews Cancer* **13**, 759-771 (2013). <https://doi.org/10.1038/nrc3611>
- 212 Greten, F. R. & Grivnickov, S. I. Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity* **51**, 27-41 (2019). <https://doi.org/10.1016/j.immuni.2019.06.025>
- 213 Shan, M. *et al.* Cigarette Smoke Induction of Osteopontin (SPP1) Mediates TH17 Inflammation in Human and Experimental Emphysema. *Science Translational Medicine* **4**, 117ra119-117ra119 (2012). <https://doi.org/doi:10.1126/scitranslmed.3003041>
- 214 Baskara, I. *et al.* Cigarette smoking induces human CCR6+Th17 lymphocytes senescence and VEGF-A secretion. *Scientific Reports* **10** (2020). <https://doi.org/10.1038/s41598-020-63613-4>
- 215 Dixon, K. O. & Kuchroo, V. K. IL-18: throwing off the shackles to boost anti-tumor immunity. *Cell Research* **30**, 831-832 (2020). <https://doi.org/10.1038/s41422-020-00396-3>
- 216 Perez, L. G. *et al.* TGF- β signaling in Th17 cells promotes IL-22 production and colitis-associated colon cancer. *Nature Communications* **11** (2020). <https://doi.org/10.1038/s41467-020-16363-w>
- 217 Chen, L. *et al.* The role of Th17 cells in inflammatory bowel disease and the research progress. *Frontiers in Immunology* **13** (2023).

- 218 Endo, Y. *et al.* Obesity Drives Th17 Cell Differentiation by Inducing the Lipid Metabolic Kinase, ACC1. *Cell Reports* **12**, 1042-1055 (2015).
<https://doi.org:10.1016/j.celrep.2015.07.014>
- 219 Luo, Q. *et al.* An autonomous activation of interleukin-17 receptor signaling sustains inflammation and promotes disease progression. *Immunity*(2023).
<https://doi.org:https://doi.org/10.1016/j.immuni.2023.06.012>
- 220 Kryczek, I., Wei, S., Szeliga, W., Vatan, L. & Zou, W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* **114**, 357-359 (2009).
<https://doi.org:10.1182/blood-2008-09-177360>
- 221 Kryczek, I. *et al.* Human TH17 Cells Are Long-Lived Effector Memory Cells. *Science Translational Medicine* **3**, 104ra100-104ra100 (2011).
<https://doi.org:10.1126/scitranslmed.3002949>
- 222 Knochelmann, H. M. *et al.* IL6 Fuels Durable Memory for Th17 Cell-Mediated Responses to Tumors. *Cancer Research* **80**, 3920-3932 (2020). <https://doi.org:10.1158/0008-5472.CAN-19-3685>
- 223 Karmaus, P. W. F. *et al.* Metabolic heterogeneity underlies reciprocal fates of TH17 cell stemness and plasticity. *Nature* **565**, 101-105 (2019). <https://doi.org:10.1038/s41586-018-0806-7>
- 224 Kebir, H. *et al.* Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nature Medicine* **13**, 1173-1175 (2007).
<https://doi.org:10.1038/nm1651>
- 225 Jamann, H. *et al.* Contact-Dependent Granzyme B-Mediated Cytotoxicity of Th17-Polarized Cells Toward Human Oligodendrocytes. *Frontiers in Immunology* **13** (2022).
<https://doi.org:10.3389/fimmu.2022.850616>
- 226 Bailey, S. R. *et al.* Human CD26^{high} T cells elicit tumor immunity against multiple malignancies via enhanced migration and persistence. *Nature Communications* **8** (2017).
<https://doi.org:10.1038/s41467-017-01867-9>

- 227 Sallusto, F., Lenig, D., Förster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708-712 (1999). <https://doi.org/10.1038/44385>
- 228 Veiga-Fernandes, H., Walter, U., Bourgeois, C., McLean, A. & Rocha, B. Response of naïve and memory CD8⁺ T cells to antigen stimulation in vivo. *Nature Immunology* **1**, 47-53 (2000). <https://doi.org/10.1038/76907>
- 229 Croft, M., Bradley, L. M. & Swain, S. L. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J Immunol* **152**, 2675-2685 (1994).
- 230 Zhang, Y., Joe, G., Hexner, E., Zhu, J. & Emerson, S. G. Host-reactive CD8⁺ memory stem cells in graft-versus-host disease. *Nature Medicine* **11**, 1299-1305 (2005). <https://doi.org/10.1038/nm1326>
- 231 Cieri, N. *et al.* IL-7 and IL-15 instruct the generation of human memory stem T cells from naïve precursors. *Blood* **121**, 573-584 (2013). <https://doi.org/10.1182/blood-2012-05-431718>
- 232 Gattinoni, L. *et al.* Wnt signaling arrests effector T cell differentiation and generates CD8⁺ memory stem cells. *Nature Medicine* **15**, 808-813 (2009). <https://doi.org/10.1038/nm.1982>
- 233 Lugli, E. *et al.* Identification, isolation and in vitro expansion of human and nonhuman primate T stem cell memory cells. *Nature Protocols* **8**, 33-42 (2013). <https://doi.org/10.1038/nprot.2012.143>
- 234 Gattinoni, L. *et al.* A human memory T cell subset with stem cell-like properties. *Nature Medicine* **17**, 1290-1297 (2011). <https://doi.org/10.1038/nm.2446>
- 235 Lugli, E. *et al.* Superior T memory stem cell persistence supports long-lived T cell memory. *Journal of Clinical Investigation* (2013). <https://doi.org/10.1172/jci66327>

- 236 Clement, L. T. Isoforms of the CD45 common leukocyte antigen family: Markers for human T-cell differentiation. *Journal of Clinical Immunology* **12**, 1-10 (1992).
<https://doi.org:10.1007/BF00918266>
- 237 Shiow, L. R. *et al.* CD69 acts downstream of interferon- α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* **440**, 540-544 (2006).
<https://doi.org:10.1038/nature04606>
- 238 Pham, T. H. M., Okada, T., Matloubian, M., Lo, C. G. & Cyster, J. G. S1P1 Receptor Signaling Overrides Retention Mediated by G α i-Coupled Receptors to Promote T Cell Egress. *Immunity* **28**, 122-133 (2008). <https://doi.org:10.1016/j.immuni.2007.11.017>
- 239 Mackay, L. K. *et al.* Cutting Edge: CD69 Interference with Sphingosine-1-Phosphate Receptor Function Regulates Peripheral T Cell Retention. *The Journal of Immunology* **194**, 2059-2063 (2015). <https://doi.org:10.4049/jimmunol.1402256>
- 240 Masopust, D., Vezys, V., Marzo, A. L. & Lefrançois, L. Preferential Localization of Effector Memory Cells in Nonlymphoid Tissue. *Science* **291**, 2413-2417 (2001).
<https://doi.org:10.1126/science.1058867>
- 241 Reinhardt, R. L., Khoruts, A., Merica, R., Zell, T. & Jenkins, M. K. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* **410**, 101-105 (2001).
<https://doi.org:10.1038/35065111>
- 242 Okla, K., Farber, D. L. & Zou, W. Tissue-resident memory T cells in tumor immunity and immunotherapy. *Journal of Experimental Medicine* **218** (2021).
<https://doi.org:10.1084/jem.20201605>
- 243 Jiang, X. *et al.* Skin infection generates non-migratory memory CD8⁺ TRM cells providing global skin immunity. *Nature* **483**, 227-231 (2012). <https://doi.org:10.1038/nature10851>
- 244 Beura, L. K. *et al.* T Cells in Nonlymphoid Tissues Give Rise to Lymph-Node-Resident Memory T Cells. *Immunity* **48**, 327-338.e325 (2018).
<https://doi.org:10.1016/j.immuni.2018.01.015>

- 245 Mackay, Laura K. *et al.* T-box Transcription Factors Combine with the Cytokines TGF- β and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* **43**, 1101-1111 (2015). <https://doi.org/10.1016/j.immuni.2015.11.008>
- 246 Strutt, T. M. *et al.* IL-15 supports the generation of protective lung-resident memory CD4 T cells. *Mucosal Immunology* **11**, 668-680 (2018). <https://doi.org/10.1038/mi.2017.101>
- 247 Adachi, T. *et al.* Hair follicle-derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma. *Nature Medicine* **21**, 1272-1279 (2015). <https://doi.org/10.1038/nm.3962>
- 248 Zhao, Y. *et al.* Clonal expansion and activation of tissue-resident memory-like TH17 cells expressing GM-CSF in the lungs of patients with severe COVID-19. *Science Immunology* **6**, eabf6692 (2021). <https://doi.org/10.1126/sciimmunol.abf6692>
- 249 Gebhardt, T. *et al.* Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* **477**, 216-219 (2011). <https://doi.org/10.1038/nature10339>
- 250 Tian, Y. *et al.* Unique phenotypes and clonal expansions of human CD4 effector memory T cells re-expressing CD45RA. *Nature Communications* **8** (2017). <https://doi.org/10.1038/s41467-017-01728-5>
- 251 Blank, C. U. *et al.* Defining 'T cell exhaustion'. *Nature Reviews Immunology* **19**, 665-674 (2019). <https://doi.org/10.1038/s41577-019-0221-9>
- 252 Siddiqui, I. *et al.* Intratumoral Tcf1+PD-1+CD8+ T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity* **50**, 195-211.e110 (2019). <https://doi.org/10.1016/j.immuni.2018.12.021>
- 253 Miller, B. C. *et al.* Subsets of exhausted CD8+ T cells differentially mediate tumor control and respond to checkpoint blockade. *Nature Immunology* **20**, 326-336 (2019). <https://doi.org/10.1038/s41590-019-0312-6>
- 254 Jansen, C. S. *et al.* An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. *Nature* **576**, 465-470 (2019). <https://doi.org/10.1038/s41586-019-1836-5>

- 255 Han, J. *et al.* Resident and circulating memory T cells persist for years in melanoma patients with durable responses to immunotherapy. *Nature Cancer* **2**, 300-311 (2021). <https://doi.org/10.1038/s43018-021-00180-1>
- 256 Park, S. L. *et al.* Tissue-resident memory CD8+ T cells promote melanoma-immune equilibrium in skin. *Nature* **565**, 366-371 (2019). <https://doi.org/10.1038/s41586-018-0812-9>
- 257 Tallón De Lara, P. *et al.* CD39+PD-1+CD8+ T cells mediate metastatic dormancy in breast cancer. *Nature Communications* **12** (2021). <https://doi.org/10.1038/s41467-021-21045-2>
- 258 Savas, P. *et al.* Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. *Nature Medicine* **24**, 986-993 (2018). <https://doi.org/10.1038/s41591-018-0078-7>
- 259 Park, S. L., Gebhardt, T. & Mackay, L. K. Tissue-Resident Memory T Cells in Cancer Immunosurveillance. *Trends in Immunology* **40**, 735-747 (2019). [https://doi.org:https://doi.org/10.1016/j.it.2019.06.002](https://doi.org/https://doi.org/10.1016/j.it.2019.06.002)
- 260 Braumüller, H. *et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494**, 361-365 (2013). <https://doi.org/10.1038/nature11824>
- 261 Menares, E. *et al.* Tissue-resident memory CD8+ T cells amplify anti-tumor immunity by triggering antigen spreading through dendritic cells. *Nature Communications* **10** (2019). <https://doi.org/10.1038/s41467-019-12319-x>
- 262 Luoma, A. M. *et al.* Tissue-resident memory and circulating T cells are early responders to pre-surgical cancer immunotherapy. *Cell* **185**, 2918-2935.e2929 (2022). <https://doi.org/10.1016/j.cell.2022.06.018>
- 263 Weber, E. W. *et al.* Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. *Science* **372**, eaba1786 (2021). <https://doi.org/10.1126/science.aba1786>
- 264 Bowers, J. S. *et al.* Th17 cells are refractory to senescence and retain robust antitumor activity after long-term ex vivo expansion. *JCI Insight* **2** (2017). <https://doi.org/10.1172/jci.insight.90772>

- 265 Corgnac, S. *et al.* CD103+CD8+ TRM Cells Accumulate in Tumors of Anti-PD-1-Responder Lung Cancer Patients and Are Tumor-Reactive Lymphocytes Enriched with Tc17. *Cell Reports Medicine* **1**, 100127 (2020). <https://doi.org:10.1016/j.xcrm.2020.100127>
- 266 Martin-Orozco, N. *et al.* T Helper 17 Cells Promote Cytotoxic T Cell Activation in Tumor Immunity. *Immunity* **31**, 787-798 (2009). <https://doi.org:10.1016/j.immuni.2009.09.014>
- 267 Lv, L. *et al.* The Accumulation and Prognosis Value of Tumor Infiltrating IL-17 Producing Cells in Esophageal Squamous Cell Carcinoma. *PLoS ONE* **6**, e18219 (2011). <https://doi.org:10.1371/journal.pone.0018219>
- 268 Tosolini, M. *et al.* Clinical Impact of Different Classes of Infiltrating T Cytotoxic and Helper Cells (Th1, Th2, Treg, Th17) in Patients with Colorectal Cancer. *Cancer Research* **71**, 1263-1271 (2011). <https://doi.org:10.1158/0008-5472.can-10-2907>
- 269 Chen, X. *et al.* Increased IL-17-producing cells correlate with poor survival and lymphangiogenesis in NSCLC patients. *Lung Cancer* **69**, 348-354 (2010). <https://doi.org:10.1016/j.lungcan.2009.11.013>
- 270 Majchrzak, K. *et al.* β -catenin and PI3K δ inhibition expands precursor Th17 cells with heightened stemness and antitumor activity. *JCI Insight* **2** (2017). <https://doi.org:10.1172/jci.insight.90547>
- 271 Bengsch, B. *et al.* Human Th17 Cells Express High Levels of Enzymatically Active Dipeptidylpeptidase IV (CD26). *The Journal of Immunology* **188**, 5438-5447 (2012). <https://doi.org:10.4049/jimmunol.1103801>
- 272 Picelli, S. *et al.* Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nature Methods* **10**, 1096-1098 (2013). <https://doi.org:10.1038/nmeth.2639>
- 273 Chi, X. *et al.* ROR γ t expression in mature TH17 cells safeguards their lineage specification by inhibiting conversion to TH2 cells. *Science Advances* **8** (2022). <https://doi.org:10.1126/sciadv.abn7774>
- 274 Hall, J. A. *et al.* Transcription factor ROR α enforces stability of the Th17 cell effector program by binding to a Rorc cis-regulatory element. *Immunity* **55**, 2027-2043.e2029 (2022). <https://doi.org:10.1016/j.immuni.2022.09.013>

- 275 Kumar, B. V. *et al.* Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Reports* **20**, 2921-2934 (2017). <https://doi.org/10.1016/j.celrep.2017.08.078>
- 276 Doi, Y. *et al.* Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. *Proceedings of the National Academy of Sciences* **105**, 8381-8386 (2008). <https://doi.org/10.1073/pnas.0803454105>
- 277 Raveney, B. J. E., Oki, S. & Yamamura, T. Nuclear Receptor NR4A2 Orchestrates Th17 Cell-Mediated Autoimmune Inflammation via IL-21 Signalling. *PLoS ONE* **8**, e56595 (2013). <https://doi.org/10.1371/journal.pone.0056595>
- 278 Hasan, Z. *et al.* JunB is essential for IL-23-dependent pathogenicity of Th17 cells. *Nature Communications* **8** (2017). <https://doi.org/10.1038/ncomms15628>
- 279 Carr, T. M., Wheaton, J. D., Houtz, G. M. & Ciofani, M. JunB promotes Th17 cell identity and restrains alternative CD4⁺ T-cell programs during inflammation. *Nature Communications* **8** (2017). <https://doi.org/10.1038/s41467-017-00380-3>
- 280 Sebzda, E., Zou, Z., Lee, J. S., Wang, T. & Kahn, M. L. Transcription factor KLF2 regulates the migration of naive T cells by restricting chemokine receptor expression patterns. *Nature Immunology* **9**, 292-300 (2008). <https://doi.org/10.1038/ni1565>
- 281 Fries, A. *et al.* Differentiation of IL-26⁺ TH17 intermediates into IL-17A producers via epithelial crosstalk in psoriasis. *Nature Communications* **14** (2023). <https://doi.org/10.1038/s41467-023-39484-4>
- 282 Loyal, L. *et al.* SLAMF7 and IL-6R define distinct cytotoxic versus helper memory CD8⁺ T cells. *Nature Communications* **11** (2020). <https://doi.org/10.1038/s41467-020-19002-6>
- 283 Ciric, B., El-Behi, M., Cabrera, R., Zhang, G.-X. & Rostami, A. IL-23 Drives Pathogenic IL-17-Producing CD8⁺ T Cells. *The Journal of Immunology* **182**, 5296-5305 (2009). <https://doi.org/10.4049/jimmunol.0900036>
- 284 Mair, F. *et al.* Extricating human tumour immune alterations from tissue inflammation. *Nature* **605**, 728-735 (2022). <https://doi.org/10.1038/s41586-022-04718-w>

- 285 Lujun, C. *et al.* IL1R2 increases regulatory T cell population in the tumor microenvironment by enhancing MHC-II expression on cancer-associated fibroblasts. *Journal for ImmunoTherapy of Cancer* **10**, e004585 (2022). <https://doi.org:10.1136/jitc-2022-004585>
- 286 Colotta, F. *et al.* Interleukin-1 Type II Receptor: A Decoy Target for IL-1 That Is Regulated by IL-4. *Science* **261**, 472-475 (1993). <https://doi.org:10.1126/science.8332913>
- 287 Sefik, E. *et al.* Individual intestinal symbionts induce a distinct population of ROR γ + regulatory T cells. *Science* **349**, 993-997 (2015). <https://doi.org:10.1126/science.aaa9420>
- 288 Ohnmacht, C. *et al.* The microbiota regulates type 2 immunity through ROR γ t+ T cells. *Science* **349**, 989-993 (2015). <https://doi.org:10.1126/science.aac4263>
- 289 Yang, B. H. *et al.* Foxp3+ T cells expressing ROR γ t represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunology* **9**, 444-457 (2016). <https://doi.org:10.1038/mi.2015.74>
- 290 Britton, G. J. *et al.* Microbiotas from Humans with Inflammatory Bowel Disease Alter the Balance of Gut Th17 and ROR γ t+ Regulatory T Cells and Exacerbate Colitis in Mice. *Immunity* **50**, 212-224.e214 (2019). <https://doi.org:10.1016/j.immuni.2018.12.015>
- 291 Zhou, L. *et al.* TGF- β -induced Foxp3 inhibits TH17 cell differentiation by antagonizing ROR γ t function. *Nature* **453**, 236-240 (2008). <https://doi.org:10.1038/nature06878>
- 292 Gagliani, N. *et al.* Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* **523**, 221-225 (2015). <https://doi.org:10.1038/nature14452>
- 293 Beriou, G. *et al.* IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* **113**, 4240-4249 (2009). <https://doi.org:10.1182/blood-2008-10-183251>
- 294 Komatsu, N. *et al.* Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nature Medicine* **20**, 62-68 (2014). <https://doi.org:10.1038/nm.3432>

- 295 Ogawa, C. *et al.* Blimp-1 Functions as a Molecular Switch to Prevent Inflammatory Activity in Foxp3+ROR γ t+ Regulatory T Cells. *Cell Reports* **25**, 19-28.e15 (2018).
<https://doi.org:10.1016/j.celrep.2018.09.016>
- 296 Nicolet, B. P., Guislain, A. & Wolkers, M. C. CD29 Enriches for Cytotoxic Human CD4+ T Cells. *The Journal of Immunology* **207**, 2966-2975 (2021).
<https://doi.org:10.4049/jimmunol.2100138>
- 297 Nicolet, B. P. *et al.* CD29 identifies IFN- γ -producing human CD8+ T cells with an increased cytotoxic potential. *Proceedings of the National Academy of Sciences* **117**, 6686-6696 (2020). <https://doi.org:10.1073/pnas.1913940117>
- 298 Wolf, T. *et al.* Dynamics in protein translation sustaining T cell preparedness. *Nature Immunology* **21**, 927-937 (2020). <https://doi.org:10.1038/s41590-020-0714-5>
- 299 Araki, K. *et al.* Translation is actively regulated during the differentiation of CD8+ effector T cells. *Nature Immunology* **18**, 1046-1057 (2017). <https://doi.org:10.1038/ni.3795>
- 300 Kokubo, K. *et al.* Thioredoxin-interacting protein is essential for memory T cell formation via the regulation of the redox metabolism. *Proceedings of the National Academy of Sciences* **120** (2023). <https://doi.org:10.1073/pnas.2218345120>
- 301 Huang, T.-Y. *et al.* Phosphoenolpyruvate regulates the Th17 transcriptional program and inhibits autoimmunity. *Cell Reports* **42**, 112205 (2023).
<https://doi.org:10.1016/j.celrep.2023.112205>
- 302 Lu, Y. *et al.* MondoA–Thioredoxin-Interacting Protein Axis Maintains Regulatory T-Cell Identity and Function in Colorectal Cancer Microenvironment. *Gastroenterology* **161**, 575-591.e516 (2021). <https://doi.org:10.1053/j.gastro.2021.04.041>
- 303 Gattinoni, L., Speiser, D. E., Lichterfeld, M. & Bonini, C. T memory stem cells in health and disease. *Nature Medicine* **23**, 18-27 (2017). <https://doi.org:10.1038/nm.4241>
- 304 Qin, Z. *et al.* TCR signaling induces STAT3 phosphorylation to promote TH17 cell differentiation. *Journal of Experimental Medicine* **221** (2024).
<https://doi.org:10.1084/jem.20230683>

- 305 Purvis, H. A. *et al.* Low-strength T-cell activation promotes Th17 responses. *Blood* **116**, 4829-4837 (2010). <https://doi.org:10.1182/blood-2010-03-272153>
- 306 Wyatt, M. M. *et al.* Augmenting TCR signal strength and ICOS costimulation results in metabolically fit and therapeutically potent human CAR Th17 cells. *Molecular Therapy* **31**, 2120-2131 (2023). <https://doi.org:10.1016/j.ymthe.2023.04.010>
- 307 Yamazaki, S. *et al.* The AP-1 transcription factor JunB is required for Th17 cell differentiation. *Scientific Reports* **7** (2017). <https://doi.org:10.1038/s41598-017-17597-3>
- 308 Lu, D. *et al.* The phosphatase DUSP2 controls the activity of the transcription activator STAT3 and regulates TH17 differentiation. *Nature Immunology* **16**, 1263-1273 (2015). <https://doi.org:10.1038/ni.3278>
- 309 Qin, H. *et al.* TGF- β Promotes Th17 Cell Development through Inhibition of SOCS3. *The Journal of Immunology* **183**, 97-105 (2009). <https://doi.org:10.4049/jimmunol.0801986>
- 310 Paulos Chrystal, M. *et al.* The Inducible Costimulator (ICOS) Is Critical for the Development of Human TH17 Cells. *Science Translational Medicine* **2**, 55ra78-55ra78 (2010). <https://doi.org:10.1126/scitranslmed.3000448>
- 311 Guedan, S. *et al.* ICOS-based chimeric antigen receptors program bipolar TH17/TH1 cells. *Blood* **124**, 1070-1080 (2014). <https://doi.org:10.1182/blood-2013-10-535245>
- 312 Park, S. L. *et al.* Divergent molecular networks program functionally distinct CD8⁺ skin-resident memory T cells. *Science* **382**, 1073-1079 (2023). <https://doi.org:doi:10.1126/science.adi8885>
- 313 Cepek, K. L. *et al.* Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the α E β 7 integrin. *Nature* **372**, 190-193 (1994). <https://doi.org:10.1038/372190a0>
- 314 Lo, J. W. *et al.* Immune checkpoint inhibitor-induced colitis is mediated by polyfunctional lymphocytes and is dependent on an IL23/IFN γ axis. *Nature Communications* **14** (2023). <https://doi.org:10.1038/s41467-023-41798-2>

- 315 Di Pilato, M. *et al.* CXCR6 positions cytotoxic T cells to receive critical survival signals in the tumor microenvironment. *Cell* **184**, 4512-4530.e4522 (2021).
<https://doi.org:10.1016/j.cell.2021.07.015>
- 316 Wein, A. N. *et al.* CXCR6 regulates localization of tissue-resident memory CD8 T cells to the airways. *Journal of Experimental Medicine* **216**, 2748-2762 (2019).
<https://doi.org:10.1084/jem.20181308>
- 317 Hou, L. & Yuki, K. CCR6 and CXCR6 Identify the Th17 Cells With Cytotoxicity in Experimental Autoimmune Encephalomyelitis. *Frontiers in Immunology* **13** (2022).
<https://doi.org:10.3389/fimmu.2022.819224>
- 318 Eschweiler, S. *et al.* JAML immunotherapy targets recently activated tumor-infiltrating CD8+T cells. *Cell Reports* **42** (2023). <https://doi.org:10.1016/j.celrep.2023.112040>
- 319 Clarke, J. *et al.* Single-cell transcriptomic analysis of tissue-resident memory T cells in human lung cancer. *Journal of Experimental Medicine* **216**, 2128-2149 (2019).
<https://doi.org:10.1084/jem.20190249>
- 320 Duhen, T. *et al.* Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nature Communications* **9** (2018). <https://doi.org:10.1038/s41467-018-05072-0>
- 321 Simoni, Y. *et al.* Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates. *Nature* **557**, 575-579 (2018). <https://doi.org:10.1038/s41586-018-0130-2>
- 322 Van Den Bulk, J. *et al.* CD103 and CD39 coexpression identifies neoantigen-specific cytotoxic T cells in colorectal cancers with low mutation burden. *Journal for ImmunoTherapy of Cancer* **11**, e005887 (2023). <https://doi.org:10.1136/jitc-2022-005887>
- 323 Talhouni, S. *et al.* Activated tissue resident memory T-cells (CD8+CD103+CD39+) uniquely predict survival in left sided “immune-hot” colorectal cancers. *Frontiers in Immunology* **14** (2023). <https://doi.org:10.3389/fimmu.2023.1057292>

- 324 Kishton, R. J., Sukumar, M. & Restifo, N. P. Metabolic Regulation of T Cell Longevity and Function in Tumor Immunotherapy. *Cell Metabolism* **26**, 94-109 (2017).
<https://doi.org:10.1016/j.cmet.2017.06.016>
- 325 Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences* **102**, 15545-15550 (2005). <https://doi.org:10.1073/pnas.0506580102>
- 326 Szabo, P. A. *et al.* Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease. *Nature Communications* **10** (2019).
<https://doi.org:10.1038/s41467-019-12464-3>
- 327 Asano, M., Toda, M., Sakaguchi, N. & Sakaguchi, S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *The Journal of experimental medicine* **184**, 387-396 (1996). <https://doi.org:10.1084/jem.184.2.387>
- 328 Liu, W. *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *The Journal of Experimental Medicine* **203**, 1701-1711 (2006).
<https://doi.org:10.1084/jem.20060772>
- 329 Liu, J. *et al.* Tumor-Associated Macrophages Recruit CCR6+ Regulatory T Cells and Promote the Development of Colorectal Cancer via Enhancing CCL20 Production in Mice. *PLoS ONE* **6**, e19495 (2011). <https://doi.org:10.1371/journal.pone.0019495>
- 330 Suan, D. *et al.* CCR6 Defines Memory B Cell Precursors in Mouse and Human Germinal Centers, Revealing Light-Zone Location and Predominant Low Antigen Affinity. *Immunity* **47**, 1142-1153.e1144 (2017). <https://doi.org:10.1016/j.immuni.2017.11.022>
- 331 Pawlak, M. *et al.* Induction of a colitogenic phenotype in Th1-like cells depends on interleukin-23 receptor signaling. *Immunity* **55**, 1663-1679.e1666 (2022).
<https://doi.org:https://doi.org/10.1016/j.immuni.2022.08.007>
- 332 Wertheimer, T. *et al.* IL-23 stabilizes an effector Treg cell program in the tumor microenvironment. *Nature Immunology* **25**, 512-524 (2024).
<https://doi.org:10.1038/s41590-024-01755-7>

- 333 Jacobse, J. *et al.* Interleukin-23 receptor signaling impairs the stability and function of colonic regulatory T cells. *Cell Reports* **42**, 112128 (2023).
<https://doi.org/10.1016/j.celrep.2023.112128>
- 334 Yang, X. O. *et al.* STAT3 Regulates Cytokine-mediated Generation of Inflammatory Helper T Cells. *Journal of Biological Chemistry* **282**, 9358-9363 (2007).
<https://doi.org/10.1074/jbc.c600321200>
- 335 Brüstle, A. *et al.* The development of inflammatory TH-17 cells requires interferon-regulatory factor 4. *Nature Immunology* **8**, 958-966 (2007).
<https://doi.org/10.1038/ni1500>
- 336 Schraml, B. U. *et al.* The AP-1 transcription factor Batf controls TH17 differentiation. *Nature* **460**, 405-409 (2009). <https://doi.org/10.1038/nature08114>
- 337 Xu, H. *et al.* The induction and function of the anti-inflammatory fate of TH17 cells. *Nature Communications* **11** (2020). <https://doi.org/10.1038/s41467-020-17097-5>
- 338 Iezzi, G. *et al.* CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4⁺ T cells. *Proceedings of the National Academy of Sciences* **106**, 876-881 (2009).
<https://doi.org/10.1073/pnas.0810769106>
- 339 Pappu, B. P. *et al.* TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated autoimmune disease. *The Journal of Experimental Medicine* **205**, 1049-1062 (2008).
<https://doi.org/10.1084/jem.20071364>
- 340 Kimura, M. Y. *et al.* IL-7 signaling must be intermittent, not continuous, during CD8⁺ T cell homeostasis to promote cell survival instead of cell death. *Nature Immunology* **14**, 143-151 (2013). <https://doi.org/10.1038/ni.2494>
- 341 Koenen, P. *et al.* Mutually exclusive regulation of T cell survival by IL-7R and antigen receptor-induced signals. *Nature Communications* **4**, 1735 (2013).
<https://doi.org/10.1038/ncomms2719>

- 342 Li, Y. *et al.* Id2 epigenetically controls CD8⁺ T-cell exhaustion by disrupting the assembly of the Tcf3-LSD1 complex. *Cellular & Molecular Immunology* **21**, 292-308 (2024). <https://doi.org/10.1038/s41423-023-01118-6>
- 343 Omilusik, K. D. *et al.* Sustained Id2 regulation of E proteins is required for terminal differentiation of effector CD8⁺ T cells. *Journal of Experimental Medicine* **215**, 773-783 (2018). <https://doi.org/10.1084/jem.20171584>
- 344 Ramesh, R. *et al.* Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *Journal of Experimental Medicine* **211**, 89-104 (2014). <https://doi.org/10.1084/jem.20130301>
- 345 Skepner, J. *et al.* Pharmacologic Inhibition of ROR γ t Regulates Th17 Signature Gene Expression and Suppresses Cutaneous Inflammation In Vivo. *The Journal of Immunology* **192**, 2564-2575 (2014). <https://doi.org/10.4049/jimmunol.1302190>
- 346 Lin, C.-C. *et al.* IL-1-induced Bhlhe40 identifies pathogenic T helper cells in a model of autoimmune neuroinflammation. *Journal of Experimental Medicine* **213**, 251-271 (2016). <https://doi.org/10.1084/jem.20150568>
- 347 Zitti, B. *et al.* Human skin-resident CD8⁺ T cells require RUNX2 and RUNX3 for induction of cytotoxicity and expression of the integrin CD49a. *Immunity* **56**, 1285-1302.e1287 (2023). <https://doi.org/https://doi.org/10.1016/j.immuni.2023.05.003>
- 348 Goto, N. *et al.* Lymphatics and fibroblasts support intestinal stem cells in homeostasis and injury. *Cell Stem Cell* **29**, 1246-1261.e1246 (2022). <https://doi.org/https://doi.org/10.1016/j.stem.2022.06.013>
- 349 Hirota, K. *et al.* Plasticity of TH17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nature Immunology* **14**, 372-379 (2013). <https://doi.org/10.1038/ni.2552>
- 350 Skon, C. N. *et al.* Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8⁺ T cells. *Nature Immunology* **14**, 1285-1293 (2013). <https://doi.org/10.1038/ni.2745>

- 351 Mackay, L. K. *et al.* The developmental pathway for CD103⁺CD8⁺ tissue-resident memory T cells of skin. *Nature Immunology* **14**, 1294-1301 (2013). <https://doi.org:10.1038/ni.2744>
- 352 Cambier, S., Gouwy, M. & Proost, P. The chemokines CXCL8 and CXCL12: molecular and functional properties, role in disease and efforts towards pharmacological intervention. *Cellular & Molecular Immunology* **20**, 217-251 (2023). <https://doi.org:10.1038/s41423-023-00974-6>
- 353 Allaoui, R. *et al.* Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers. *Nature Communications* **7**, 13050 (2016). <https://doi.org:10.1038/ncomms13050>
- 354 Shimaoka, T. *et al.* Molecular Cloning of a Novel Scavenger Receptor for Oxidized Low Density Lipoprotein, SR-PSOX, on Macrophages *. *Journal of Biological Chemistry* **275**, 40663-40666 (2000). <https://doi.org:10.1074/jbc.C000761200>
- 355 Hojo, S. *et al.* High-Level Expression of Chemokine CXCL16 by Tumor Cells Correlates with a Good Prognosis and Increased Tumor-Infiltrating Lymphocytes in Colorectal Cancer. *Cancer Research* **67**, 4725-4731 (2007). <https://doi.org:10.1158/0008-5472.Can-06-3424>
- 356 Zundler, S. *et al.* Hobit- and Blimp-1-driven CD4⁺ tissue-resident memory T cells control chronic intestinal inflammation. *Nature Immunology* **20**, 288-300 (2019). <https://doi.org:10.1038/s41590-018-0298-5>
- 357 Wang, C., Kang, S. G., Lee, J., Sun, Z. & Kim, C. H. The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal Immunology* **2**, 173-183 (2009). <https://doi.org:10.1038/mi.2008.84>
- 358 Yamazaki, T. *et al.* CCR6 Regulates the Migration of Inflammatory and Regulatory T Cells. *The Journal of Immunology* **181**, 8391-8401 (2008). <https://doi.org:10.4049/jimmunol.181.12.8391>
- 359 Fergusson, J. R., Fleming, V. M. & Klenerman, P. CD161-Expressing Human T Cells. *Frontiers in Immunology* **2** (2011). <https://doi.org:10.3389/fimmu.2011.00036>

- 360 Cook, D. N. *et al.* CCR6 Mediates Dendritic Cell Localization, Lymphocyte Homeostasis, and Immune Responses in Mucosal Tissue. *Immunity* **12**, 495-503 (2000).
[https://doi.org:10.1016/s1074-7613\(00\)80201-0](https://doi.org:10.1016/s1074-7613(00)80201-0)
- 361 Gounaris, E. *et al.* Mast cells are an essential hematopoietic component for polyp development. *Proceedings of the National Academy of Sciences* **104**, 19977-19982 (2007). <https://doi.org:doi:10.1073/pnas.0704620104>
- 362 Blatner, N. R. *et al.* Expression of ROR γ t Marks a Pathogenic Regulatory T Cell Subset in Human Colon Cancer. *Science Translational Medicine* **4**, 164ra159-164ra159 (2012).
<https://doi.org:doi:10.1126/scitranslmed.3004566>
- 363 Kim, B.-S. *et al.* Generation of ROR γ t+ Antigen-Specific T Regulatory 17 Cells from Foxp3+ Precursors in Autoimmunity. *Cell Reports* **21**, 195-207 (2017).
<https://doi.org:10.1016/j.celrep.2017.09.021>
- 364 McGeachy, M. J. *et al.* TGF- β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nature Immunology* **8**, 1390-1397 (2007).
<https://doi.org:10.1038/ni1539>
- 365 Zehn, D., Lee, S. Y. & Bevan, M. J. Complete but curtailed T-cell response to very low-affinity antigen. *Nature* **458**, 211-214 (2009). <https://doi.org:10.1038/nature07657>
- 366 Lynn, R. C. *et al.* c-Jun overexpression in CAR T cells induces exhaustion resistance. *Nature* **576**, 293-300 (2019). <https://doi.org:10.1038/s41586-019-1805-z>
- 367 Yoshida, N. *et al.* ICER is requisite for Th17 differentiation. *Nature Communications* **7**, 12993 (2016). <https://doi.org:10.1038/ncomms12993>
- 368 Lin, C.-C. *et al.* Bhlhe40 controls cytokine production by T cells and is essential for pathogenicity in autoimmune neuroinflammation. *Nature Communications* **5** (2014).
<https://doi.org:10.1038/ncomms4551>
- 369 Yasuda, K. *et al.* Satb1 regulates the effector program of encephalitogenic tissue Th17 cells in chronic inflammation. *Nature Communications* **10** (2019).
<https://doi.org:10.1038/s41467-019-08404-w>

- 370 Sekiya, T. *et al.* Nr4a Receptors Regulate Development and Death of Labile Treg Precursors to Prevent Generation of Pathogenic Self-Reactive Cells. *Cell Reports* **24**, 1627-1638.e1626 (2018). <https://doi.org/10.1016/j.celrep.2018.07.008>
- 371 Sekiya, T. *et al.* Regulation of peripheral Th/Treg differentiation and suppression of airway inflammation by Nr4a transcription factors. *iScience* **24**, 102166 (2021). <https://doi.org/10.1016/j.isci.2021.102166>
- 372 Pepper, M. *et al.* Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. *Nature Immunology* **11**, 83-89 (2010). <https://doi.org/10.1038/ni.1826>
- 373 Hendriks, J. *et al.* CD27 is required for generation and long-term maintenance of T cell immunity. *Nature Immunology* **1**, 433-440 (2000). <https://doi.org/10.1038/80877>
- 374 Laurence, A. *et al.* Interleukin-2 Signaling via STAT5 Constrains T Helper 17 Cell Generation. *Immunity* **26**, 371-381 (2007). <https://doi.org/10.1016/j.immuni.2007.02.009>
- 375 Amezcua Vesely, M. C. *et al.* Effector TH17 Cells Give Rise to Long-Lived TRM Cells that Are Essential for an Immediate Response against Bacterial Infection. *Cell* **178**, 1176-1188.e1115 (2019). <https://doi.org/10.1016/j.cell.2019.07.032>
- 376 Kirchner, F. R. & Leibundgut-Landmann, S. Tissue-resident memory Th17 cells maintain stable fungal commensalism in the oral mucosa. *Mucosal Immunology* **14**, 455-467 (2021). <https://doi.org/10.1038/s41385-020-0327-1>
- 377 Hong, H. S. *et al.* OXPHOS promotes apoptotic resistance and cellular persistence in TH17 cells in the periphery and tumor microenvironment. *Science Immunology* **7**, eabm8182 (2022). <https://doi.org/10.1126/sciimmunol.abm8182>
- 378 Künzli, M. & Masopust, D. CD4⁺ T cell memory. *Nature Immunology* **24**, 903-914 (2023). <https://doi.org/10.1038/s41590-023-01510-4>
- 379 Ukita, M. *et al.* CXCL13-producing CD4⁺ T cells accumulate in the early phase of tertiary lymphoid structures in ovarian cancer. *JCI Insight* **7** (2022). <https://doi.org/10.1172/jci.insight.157215>

- 380 Zhao, H., Wang, H., Zhao, Y., Sun, Q. & Ren, X. Tumor-Resident T Cells, Associated With Tertiary Lymphoid Structure Maturity, Improve Survival in Patients With Stage III Lung Adenocarcinoma. *Frontiers in Immunology* **13** (2022). <https://doi.org:10.3389/fimmu.2022.877689>
- 381 Mori, T. *et al.* Tertiary lymphoid structures show infiltration of effective tumor-resident T cells in gastric cancer. *Cancer Science* **112**, 1746-1757 (2021). <https://doi.org:10.1111/cas.14888>
- 382 Helmink, B. A. *et al.* B cells and tertiary lymphoid structures promote immunotherapy response. *Nature* **577**, 549-555 (2020). <https://doi.org:10.1038/s41586-019-1922-8>
- 383 Cabrita, R. *et al.* Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature* **577**, 561-565 (2020). <https://doi.org:10.1038/s41586-019-1914-8>
- 384 Overacre-Delgoffe, A. E. *et al.* Microbiota-specific T follicular helper cells drive tertiary lymphoid structures and anti-tumor immunity against colorectal cancer. *Immunity* **54**, 2812-2824.e2814 (2021). <https://doi.org:10.1016/j.immuni.2021.11.003>
- 385 Del Prete, A. *et al.* Dendritic cell subsets in cancer immunity and tumor antigen sensing. *Cellular & Molecular Immunology* **20**, 432-447 (2023). <https://doi.org:10.1038/s41423-023-00990-6>
- 386 Pittet, M. J., Michielin, O. & Migliorini, D. Clinical relevance of tumour-associated macrophages. *Nature Reviews Clinical Oncology* **19**, 402-421 (2022). <https://doi.org:10.1038/s41571-022-00620-6>
- 387 Mantovani, A., Allavena, P., Marchesi, F. & Garlanda, C. Macrophages as tools and targets in cancer therapy. *Nature Reviews Drug Discovery* **21**, 799-820 (2022). <https://doi.org:10.1038/s41573-022-00520-5>
- 388 Network, T. C. G. A. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487**, 330-337 (2012). <https://doi.org:10.1038/nature11252>
- 389 Newman, A. M. *et al.* Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nature Biotechnology* **37**, 773-782 (2019). <https://doi.org:10.1038/s41587-019-0114-2>

- 390 Newman, A. M. *et al.* Robust enumeration of cell subsets from tissue expression profiles. *Nature Methods* **12**, 453-457 (2015). <https://doi.org:10.1038/nmeth.3337>
- 391 Hänzelmann, S., Castelo, R. & Guinney, J. GSEA: gene set variation analysis for microarray and RNA-Seq data. *BMC Bioinformatics* **14**, 7 (2013). <https://doi.org:10.1186/1471-2105-14-7>
- 392 Fridman, W. H. *et al.* Tertiary lymphoid structures and B cells: An intratumoral immunity cycle. *Immunity* **56**, 2254-2269 (2023). <https://doi.org:10.1016/j.immuni.2023.08.009>
- 393 Togashi, Y., Shitara, K. & Nishikawa, H. Regulatory T cells in cancer immunosuppression — implications for anticancer therapy. *Nature Reviews Clinical Oncology* **16**, 356-371 (2019). <https://doi.org:10.1038/s41571-019-0175-7>
- 394 Gangaplara, A. *et al.* Type I interferon signaling attenuates regulatory T cell function in viral infection and in the tumor microenvironment. *PLOS Pathogens* **14**, e1006985 (2018). <https://doi.org:10.1371/journal.ppat.1006985>
- 395 Overacre-Delgoffe, A. E. *et al.* Interferon- γ Drives Treg Fragility to Promote Anti-tumor Immunity. *Cell* **169**, 1130-1141.e1111 (2017). <https://doi.org:10.1016/j.cell.2017.05.005>
- 396 Efremova, M., Vento-Tormo, M., Teichmann, S. A. & Vento-Tormo, R. CellPhoneDB: inferring cell-cell communication from combined expression of multi-subunit ligand-receptor complexes. *Nature Protocols* **15**, 1484-1506 (2020). <https://doi.org:10.1038/s41596-020-0292-x>
- 397 Jin, S. *et al.* Inference and analysis of cell-cell communication using CellChat. *Nature Communications* **12** (2021). <https://doi.org:10.1038/s41467-021-21246-9>
- 398 Browaeys, R., Saelens, W. & Saeys, Y. NicheNet: modeling intercellular communication by linking ligands to target genes. *Nature Methods* **17**, 159-162 (2020). <https://doi.org:10.1038/s41592-019-0667-5>
- 399 Wilk, A. J., Shalek, A. K., Holmes, S. & Blish, C. A. Comparative analysis of cell-cell communication at single-cell resolution. *Nature Biotechnology* **42**, 470-483 (2024). <https://doi.org:10.1038/s41587-023-01782-z>

- 400 Kara, E. E. *et al.* CCR2 defines in vivo development and homing of IL-23-driven GM-CSF-producing Th17 cells. *Nature Communications* **6**, 8644 (2015).
<https://doi.org:10.1038/ncomms9644>
- 401 Hoshino, K. *et al.* Cutting Edge: Generation of IL-18 Receptor-Deficient Mice: Evidence for IL-1 Receptor-Related Protein as an Essential IL-18 Binding Receptor. *The Journal of Immunology* **162**, 5041-5044 (1999). <https://doi.org:10.4049/jimmunol.162.9.5041>
- 402 Hyun Kim, S. *et al.* Functional Reconstitution and Regulation of IL-18 Activity by the IL-18R β Chain. *The Journal of Immunology* **166**, 148-154 (2001).
<https://doi.org:10.4049/jimmunol.166.1.148>
- 403 Wu, C. *et al.* IL-18 Receptor β -Induced Changes in the Presentation of IL-18 Binding Sites Affect Ligand Binding and Signal Transduction. *The Journal of Immunology* **170**, 5571-5577 (2003). <https://doi.org:10.4049/jimmunol.170.11.5571>
- 404 Tsutsumi, N. *et al.* The structural basis for receptor recognition of human interleukin-18. *Nature Communications* **5**, 5340 (2014). <https://doi.org:10.1038/ncomms6340>
- 405 Harrison, O. J. *et al.* Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3⁺ Treg cell function in the intestine. *Mucosal Immunology* **8**, 1226-1236 (2015).
<https://doi.org:10.1038/mi.2015.13>
- 406 Tomura, M. *et al.* Differential Capacities of CD4⁺, CD8⁺, and CD4[−]CD8[−] T Cell Subsets to Express IL-18 Receptor and Produce IFN- γ in Response to IL-18. *The Journal of Immunology* **160**, 3759-3765 (1998). <https://doi.org:10.4049/jimmunol.160.8.3759>
- 407 Kohyama, M. *et al.* Direct Activation of Human CD8⁺ Cytotoxic T Lymphocytes by Interleukin-18. *Japanese Journal of Cancer Research* **89**, 1041-1046 (1998).
<https://doi.org:10.1111/j.1349-7006.1998.tb00494.x>
- 408 Nakamura, K., Okamura, H., Wada, M., Nagata, K. & Tamura, T. Endotoxin-induced serum factor that stimulates gamma interferon production. *Infection and Immunity* **57**, 590-595 (1989). <https://doi.org:10.1128/iai.57.2.590-595.1989>

- 409 Freeman, B. E., Hammarlund, E., Raué, H.-P. & Slifka, M. K. Regulation of innate CD8+ T-cell activation mediated by cytokines. *Proceedings of the National Academy of Sciences* **109**, 9971-9976 (2012). <https://doi.org/doi:10.1073/pnas.1203543109>
- 410 Rose-John, S., Jenkins, B. J., Garbers, C., Moll, J. M. & Scheller, J. Targeting IL-6 trans-signalling: past, present and future prospects. *Nature Reviews Immunology* **23**, 666-681 (2023). <https://doi.org/10.1038/s41577-023-00856-y>
- 411 Heise, D. *et al.* Selective inhibition of IL-6 trans-signaling by a miniaturized, optimized chimeric soluble gp130 inhibits TH17 cell expansion. *Science Signaling* **14**, eabc3480 (2021). <https://doi.org/10.1126/scisignal.abc3480>
- 412 Harbour, S. N. *et al.* TH17 cells require ongoing classic IL-6 receptor signaling to retain transcriptional and functional identity. *Science Immunology* **5**, eaaw2262 (2020). <https://doi.org/10.1126/sciimmunol.aaw2262>
- 413 Zheng, Y., Saftig, P., Hartmann, D. & Blobel, C. Evaluation of the Contribution of Different ADAMs to Tumor Necrosis Factor α (TNF α) Shedding and of the Function of the TNF α Ectodomain in Ensuring Selective Stimulated Shedding by the TNF α Convertase (TACE/ADAM17). *Journal of Biological Chemistry* **279**, 42898-42906 (2004). <https://doi.org/10.1074/jbc.m403193200>
- 414 Toy, D. *et al.* Cutting Edge: Interleukin 17 Signals through a Heteromeric Receptor Complex. *The Journal of Immunology* **177**, 36-39 (2006). <https://doi.org/10.4049/jimmunol.177.1.36>
- 415 Chong, W. P. *et al.* The Cytokine IL-17A Limits Th17 Pathogenicity via a Negative Feedback Loop Driven by Autocrine Induction of IL-24. *Immunity* **53**, 384-397.e385 (2020). <https://doi.org/10.1016/j.immuni.2020.06.022>
- 416 Cao, W. *et al.* Leukemia Inhibitory Factor Inhibits T Helper 17 Cell Differentiation and Confers Treatment Effects of Neural Progenitor Cell Therapy in Autoimmune Disease. *Immunity* **35**, 273-284 (2011). <https://doi.org/10.1016/j.immuni.2011.06.011>
- 417 Baumgartner, F. *et al.* Activation of gp130 signaling in T cells drives T _H 17-mediated multi-organ autoimmunity. *Science Signaling* **17** (2024). <https://doi.org/10.1126/scisignal.adc9662>

- 418 Verstockt, B. *et al.* IL-12 and IL-23 pathway inhibition in inflammatory bowel disease. *Nature Reviews Gastroenterology & Hepatology* **20**, 433-446 (2023).
<https://doi.org:10.1038/s41575-023-00768-1>
- 419 Hasegawa, H. *et al.* IL-23p19 and CD5 antigen-like form a possible novel heterodimeric cytokine and contribute to experimental autoimmune encephalomyelitis development. *Scientific Reports* **11** (2021). <https://doi.org:10.1038/s41598-021-84624-9>
- 420 Hatscher, L. *et al.* Select hyperactivating NLRP3 ligands enhance the TH1- and TH17-inducing potential of human type 2 conventional dendritic cells. *Science Signaling* **14**, eabe1757 (2021). <https://doi.org:10.1126/scisignal.abe1757>
- 421 Persson, E. K. *et al.* IRF4 Transcription-Factor-Dependent CD103+CD11b+ Dendritic Cells Drive Mucosal T Helper 17 Cell Differentiation. *Immunity* **38**, 958-969 (2013).
<https://doi.org:10.1016/j.immuni.2013.03.009>
- 422 Schlitzer, A. *et al.* IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. *Immunity* **38**, 970-983 (2013).
<https://doi.org:10.1016/j.immuni.2013.04.011>
- 423 Hildner, K. *et al.* Batf3 Deficiency Reveals a Critical Role for CD8 α + Dendritic Cells in Cytotoxic T Cell Immunity. *Science* **322**, 1097-1100 (2008).
<https://doi.org:doi:10.1126/science.1164206>
- 424 Spranger, S., Bao, R. & Gajewski, T. F. Melanoma-intrinsic β -catenin signalling prevents anti-tumour immunity. *Nature* **523**, 231-235 (2015). <https://doi.org:10.1038/nature14404>
- 425 Sánchez-Paulete, A. R. *et al.* Cancer Immunotherapy with Immunomodulatory Anti-CD137 and Anti-PD-1 Monoclonal Antibodies Requires BATF3-Dependent Dendritic Cells. *Cancer Discovery* **6**, 71-79 (2016). <https://doi.org:10.1158/2159-8290.cd-15-0510>
- 426 Salmon, H. *et al.* Expansion and Activation of CD103+ Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity* **44**, 924-938 (2016). <https://doi.org:10.1016/j.immuni.2016.03.012>

- 427 Enamorado, M. *et al.* Enhanced anti-tumour immunity requires the interplay between resident and circulating memory CD8⁺ T cells. *Nature Communications* **8** (2017). <https://doi.org:10.1038/ncomms16073>
- 428 Mayer, C. T. *et al.* Selective and efficient generation of functional Batf3-dependent CD103⁺ dendritic cells from mouse bone marrow. *Blood* **124**, 3081-3091 (2014). <https://doi.org:10.1182/blood-2013-12-545772>
- 429 Stuart, T. *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-1902.e1821 (2019). <https://doi.org:10.1016/j.cell.2019.05.031>
- 430 Qi, J. *et al.* Single-cell and spatial analysis reveal interaction of FAP⁺ fibroblasts and SPP1⁺ macrophages in colorectal cancer. *Nature Communications* **13** (2022). <https://doi.org:10.1038/s41467-022-29366-6>
- 431 McDonald, B. D., Jabri, B. & Bendelac, A. Diverse developmental pathways of intestinal intraepithelial lymphocytes. *Nature Reviews Immunology* **18**, 514-525 (2018). <https://doi.org:10.1038/s41577-018-0013-7>
- 432 Broz, P. & Dixit, V. M. Inflammasomes: mechanism of assembly, regulation and signalling. *Nature Reviews Immunology* **16**, 407-420 (2016). <https://doi.org:10.1038/nri.2016.58>
- 433 Arnold, I. C. *et al.* CD11c⁺ monocyte/macrophages promote chronic *Helicobacter hepaticus*-induced intestinal inflammation through the production of IL-23. *Mucosal Immunology* **9**, 352-363 (2016). <https://doi.org:10.1038/mi.2015.65>
- 434 Corbin, A. L. *et al.* IRF5 guides monocytes toward an inflammatory CD11c⁺ macrophage phenotype and promotes intestinal inflammation. *Science Immunology* **5**, eaax6085 (2020). <https://doi.org:10.1126/sciimmunol.aax6085>
- 435 Asano, K. *et al.* CD169-Positive Macrophages Dominate Antitumor Immunity by Crosspresenting Dead Cell-Associated Antigens. *Immunity* **34**, 85-95 (2011). <https://doi.org:10.1016/j.immuni.2010.12.011>
- 436 Zaitsev, K., Bambouskova, M., Swain, A. & Artyomov, M. N. Complete deconvolution of cellular mixtures based on linearity of transcriptional signatures. *Nature Communications* **10** (2019). <https://doi.org:10.1038/s41467-019-09990-5>

- 437 Sutton, G. J. *et al.* Comprehensive evaluation of deconvolution methods for human brain gene expression. *Nature Communications* **13** (2022). <https://doi.org/10.1038/s41467-022-28655-4>
- 438 Elgueta, R. *et al.* CCR6-Dependent Positioning of Memory B Cells Is Essential for Their Ability To Mount a Recall Response to Antigen. *The Journal of Immunology* **194**, 505-513 (2015). <https://doi.org/10.4049/jimmunol.1401553>
- 439 Deteix, C. *et al.* Intra-graft Th17 Infiltrate Promotes Lymphoid Neogenesis and Hastens Clinical Chronic Rejection. *The Journal of Immunology* **184**, 5344-5351 (2010). <https://doi.org/10.4049/jimmunol.0902999>
- 440 Peters, A. *et al.* Th17 Cells Induce Ectopic Lymphoid Follicles in Central Nervous System Tissue Inflammation. *Immunity* **35**, 986-996 (2011). <https://doi.org/10.1016/j.immuni.2011.10.015>
- 441 Rangel-Moreno, J. *et al.* The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nature Immunology* **12**, 639-646 (2011). <https://doi.org/10.1038/ni.2053>
- 442 Barone, F. *et al.* IL-22 regulates lymphoid chemokine production and assembly of tertiary lymphoid organs. *Proceedings of the National Academy of Sciences* **112**, 11024-11029 (2015). <https://doi.org/10.1073/pnas.1503315112>
- 443 Berghuis, D. *et al.* Pro-inflammatory chemokine–chemokine receptor interactions within the Ewing sarcoma microenvironment determine CD8+ T-lymphocyte infiltration and affect tumour progression. *The Journal of Pathology* **223**, 347-357 (2011). [https://doi.org:https://doi.org/10.1002/path.2819](https://doi.org/https://doi.org/10.1002/path.2819)
- 444 Whitley, S. K. *et al.* Local IL-23 is required for proliferation and retention of skin-resident memory TH17 cells. *Science Immunology* **7**, eabq3254 (2022). <https://doi.org/doi:10.1126/sciimmunol.abq3254>
- 445 Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. & Stockinger, B. TGF β in the Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of IL-17-Producing T Cells. *Immunity* **24**, 179-189 (2006). <https://doi.org/10.1016/j.immuni.2006.01.001>

- 446 Bergsbaken, T., Bevan, M. J. & Fink, P. J. Local Inflammatory Cues Regulate Differentiation and Persistence of CD8⁺ Tissue-Resident Memory T Cells. *Cell Reports* **19**, 114-124 (2017). <https://doi.org/10.1016/j.celrep.2017.03.031>
- 447 Hu, B. *et al.* Augmentation of Antitumor Immunity by Human and Mouse CAR T Cells Secreting IL-18. *Cell Reports* **20**, 3025-3033 (2017). <https://doi.org/10.1016/j.celrep.2017.09.002>
- 448 Milner, J. J. *et al.* Runx3 programs CD8⁺ T cell residency in non-lymphoid tissues and tumours. *Nature* **552**, 253-257 (2017). <https://doi.org/10.1038/nature24993>
- 449 Fonseca, R. *et al.* Runx3 drives a CD8⁺ T cell tissue residency program that is absent in CD4⁺ T cells. *Nature Immunology* **23**, 1236-1245 (2022). <https://doi.org/10.1038/s41590-022-01273-4>
- 450 Massagué, J. TGF β signalling in context. *Nature Reviews Molecular Cell Biology* **13**, 616-630 (2012). <https://doi.org/10.1038/nrm3434>
- 451 Mani, V. *et al.* Migratory DCs activate TGF- β to precondition naïve CD8⁺ T cells for tissue-resident memory fate. *Science* **366**, eaav5728 (2019). <https://doi.org/10.1126/science.aav5728>
- 452 Iborra, S. *et al.* Optimal Generation of Tissue-Resident but Not Circulating Memory T Cells during Viral Infection Requires Crosspriming by DNGR-1⁺ Dendritic Cells. *Immunity* **45**, 847-860 (2016). <https://doi.org/10.1016/j.immuni.2016.08.019>
- 453 Villani, A.-C. *et al.* Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science* **356**, eaah4573 (2017). <https://doi.org/10.1126/science.aah4573>
- 454 Yu, C. *et al.* Human CD1c⁺ Dendritic Cells Drive the Differentiation of CD103⁺ CD8⁺ Mucosal Effector T Cells via the Cytokine TGF- β . *Immunity* **38**, 818-830 (2013). <https://doi.org/10.1016/j.immuni.2013.03.004>
- 455 Bourdely, P. *et al.* Transcriptional and Functional Analysis of CD1c⁺ Human Dendritic Cells Identifies a CD163⁺ Subset Priming CD8⁺CD103⁺ T Cells. *Immunity* **53**, 335-352.e338 (2020). <https://doi.org/10.1016/j.immuni.2020.06.002>

- 456 Dutertre, C.-A. *et al.* Single-Cell Analysis of Human Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating Inflammatory Dendritic Cells. *Immunity* **51**, 573-589.e578 (2019). <https://doi.org:10.1016/j.immuni.2019.08.008>
- 457 Garrido-Martin, E. M. *et al.* M1 hot tumor-associated macrophages boost tissue-resident memory T cells infiltration and survival in human lung cancer. *Journal for ImmunoTherapy of Cancer* **8**, e000778 (2020). <https://doi.org:10.1136/jitc-2020-000778>
- 458 Iijima, N. & Iwasaki, A. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* **346**, 93-98 (2014). <https://doi.org:10.1126/science.1257530>
- 459 Hirai, T. *et al.* Competition for Active TGF β Cytokine Allows for Selective Retention of Antigen-Specific Tissue- Resident Memory T Cells in the Epidermal Niche. *Immunity* **54**, 84-98.e85 (2021). <https://doi.org:10.1016/j.immuni.2020.10.022>
- 460 Malenica, I. *et al.* Integrin- α V-mediated activation of TGF- β regulates anti-tumour CD8 T cell immunity and response to PD-1 blockade. *Nature Communications* **12** (2021). <https://doi.org:10.1038/s41467-021-25322-y>
- 461 Watanabe, R. *et al.* Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. *Science Translational Medicine* **7**, 279ra239-279ra239 (2015). <https://doi.org:doi:10.1126/scitranslmed.3010302>
- 462 Cheuk, S. *et al.* CD49a Expression Defines Tissue-Resident CD8 + T Cells Poised for Cytotoxic Function in Human Skin. *Immunity* **46**, 287-300 (2017). <https://doi.org:10.1016/j.immuni.2017.01.009>
- 463 Klicznik, M. M. *et al.* Human CD4+ CD103+ cutaneous resident memory T cells are found in the circulation of healthy individuals. *Science Immunology* **4**, eaav8995 (2019). <https://doi.org:10.1126/sciimmunol.aav8995>
- 464 Mackay, L. K. *et al.* Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proceedings of the National Academy of Sciences* **109**, 7037-7042 (2012). <https://doi.org:doi:10.1073/pnas.1202288109>

- 465 Koprak, S., Matheravidathu, S., Springer, M., Gould, S. & Dumont, F. J. Down-regulation of cell surface CXCR6 expression during T cell activation is predominantly mediated by calcineurin. *Cellular Immunology* **223**, 1-12 (2003).
[https://doi.org/https://doi.org/10.1016/S0008-8749\(03\)00130-8](https://doi.org/https://doi.org/10.1016/S0008-8749(03)00130-8)
- 466 Ganesan, A.-P. *et al.* Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer. *Nature Immunology* **18**, 940-950 (2017).
<https://doi.org/10.1038/ni.3775>
- 467 Malik, B. T. *et al.* Resident memory T cells in the skin mediate durable immunity to melanoma. *Science Immunology* **2**, eaam6346 (2017).
<https://doi.org/doi:10.1126/sciimmunol.aam6346>
- 468 Kitakaze, M. *et al.* Cancer-specific tissue-resident memory T-cells express ZNF683 in colorectal cancer. *British Journal of Cancer* **128**, 1828-1837 (2023).
<https://doi.org/10.1038/s41416-023-02202-4>
- 469 Herrera-De La Mata, S. *et al.* Cytotoxic CD4⁺ tissue-resident memory T cells are associated with asthma severity. *Med* **4**, 875-897.e878 (2023).
<https://doi.org/https://doi.org/10.1016/j.medj.2023.09.003>
- 470 Wang, P. *et al.* CD103⁺CD8⁺ T lymphocytes in non-small cell lung cancer are phenotypically and functionally primed to respond to PD-1 blockade. *Cellular Immunology* **325**, 48-55 (2018).
<https://doi.org/https://doi.org/10.1016/j.cellimm.2018.02.002>
- 471 Djenidi, F. *et al.* CD8⁺CD103⁺ Tumor-Infiltrating Lymphocytes Are Tumor-Specific Tissue-Resident Memory T Cells and a Prognostic Factor for Survival in Lung Cancer Patients. *The Journal of Immunology* **194**, 3475-3486 (2015).
<https://doi.org/10.4049/jimmunol.1402711>
- 472 Nizard, M. *et al.* Induction of resident memory T cells enhances the efficacy of cancer vaccine. *Nature Communications* **8**, 15221 (2017). <https://doi.org/10.1038/ncomms15221>
- 473 Murray, T. *et al.* Very Late Antigen-1 Marks Functional Tumor-Resident CD8 T Cells and Correlates with Survival of Melanoma Patients. *Frontiers in Immunology* **7** (2016).
<https://doi.org/10.3389/fimmu.2016.00573>

- 474 Gálvez-Cancino, F. *et al.* Vaccination-induced skin-resident memory CD8⁺T cells mediate strong protection against cutaneous melanoma. *OncoImmunology* **7**, e1442163 (2018). <https://doi.org:10.1080/2162402x.2018.1442163>
- 475 Chang, C.-H. *et al.* Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis. *Cell* **153**, 1239-1251 (2013). <https://doi.org:10.1016/j.cell.2013.05.016>
- 476 Menk, A. V. *et al.* Early TCR Signaling Induces Rapid Aerobic Glycolysis Enabling Distinct Acute T Cell Effector Functions. *Cell Reports* **22**, 1509-1521 (2018). <https://doi.org:10.1016/j.celrep.2018.01.040>
- 477 Hamid, M. H. B. A. *et al.* Unconventional human CD61 pairing with CD103 promotes TCR signaling and antigen-specific T cell cytotoxicity. *Nature Immunology* **25**, 834-846 (2024). <https://doi.org:10.1038/s41590-024-01802-3>
- 478 Luo, Y. *et al.* Immune-infiltrating signature-based classification reveals CD103⁺CD39⁺ T cells associate with colorectal cancer prognosis and response to immunotherapy. *Frontiers in Immunology* **13** (2022). <https://doi.org:10.3389/fimmu.2022.1011590>
- 479 Van Der Kraak, L. A. *et al.* Genetic and commensal induction of IL-18 drive intestinal epithelial MHCII via IFN γ . *Mucosal Immunology* **14**, 1100-1112 (2021). <https://doi.org:10.1038/s41385-021-00419-1>
- 480 Sidhom, J.-W. *et al.* Deep learning reveals predictive sequence concepts within immune repertoires to immunotherapy. *Science Advances* **8** (2022). <https://doi.org:10.1126/sciadv.abq5089>
- 481 Croce, G. *et al.* Deep learning predictions of TCR-epitope interactions reveal epitope-specific chains in dual alpha T cells. *Nature Communications* **15** (2024). <https://doi.org:10.1038/s41467-024-47461-8>
- 482 Tan, C. L. *et al.* Prediction of tumor-reactive T cell receptors from scRNA-seq data for personalized T cell therapy. *Nature Biotechnology* (2024). <https://doi.org:10.1038/s41587-024-02161-y>

- 483 Hudson, D., Fernandes, R. A., Basham, M., Ogg, G. & Koohy, H. Can we predict T cell specificity with digital biology and machine learning? *Nature Reviews Immunology* **23**, 511-521 (2023). <https://doi.org:10.1038/s41577-023-00835-3>
- 484 Ng, K. W. *et al.* Antibodies against endogenous retroviruses promote lung cancer immunotherapy. *Nature* **616**, 563-573 (2023). <https://doi.org:10.1038/s41586-023-05771-9>
- 485 Lin, W. *et al.* Human CD4 cytotoxic T lymphocytes mediate potent tumor control in humanized immune system mice. *Communications Biology* **6** (2023). <https://doi.org:10.1038/s42003-023-04812-3>
- 486 Oh, D. Y. *et al.* Intratumoral CD4+ T Cells Mediate Anti-tumor Cytotoxicity in Human Bladder Cancer. *Cell* **181**, 1612-1625.e1613 (2020). <https://doi.org:10.1016/j.cell.2020.05.017>
- 487 Beresford, P. J., Xia, Z., Greenberg, A. H. & Lieberman, J. Granzyme A Loading Induces Rapid Cytolysis and a Novel Form of DNA Damage Independently of Caspase Activation. *Immunity* **10**, 585-595 (1999). [https://doi.org:10.1016/s1074-7613\(00\)80058-8](https://doi.org:10.1016/s1074-7613(00)80058-8)
- 488 Shresta, S., Graubert, T. A., Thomas, D. A., Raptis, S. Z. & Ley, T. J. Granzyme A Initiates an Alternative Pathway for Granule-Mediated Apoptosis. *Immunity* **10**, 595-605 (1999). [https://doi.org:10.1016/s1074-7613\(00\)80059-x](https://doi.org:10.1016/s1074-7613(00)80059-x)
- 489 Martinvalet, D., Zhu, P. & Lieberman, J. Granzyme A Induces Caspase-Independent Mitochondrial Damage, a Required First Step for Apoptosis. *Immunity* **22**, 355-370 (2005). <https://doi.org:10.1016/j.immuni.2005.02.004>
- 490 Darmon, A. J., Nicholson, D. W. & Bleackley, R. C. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* **377**, 446-448 (1995). <https://doi.org:10.1038/377446a0>
- 491 Mahuron, K. M. *et al.* Layilin augments integrin activation to promote antitumor immunity. *Journal of Experimental Medicine* **217** (2020). <https://doi.org:10.1084/jem.20192080>

- 492 Yamauchi, T. *et al.* CX3CR1–CD8+ T cells are critical in antitumor efficacy but functionally suppressed in the tumor microenvironment. *JCI Insight* **5** (2020).
<https://doi.org:10.1172/jci.insight.133920>
- 493 Yamauchi, T. *et al.* T-cell CX3CR1 expression as a dynamic blood-based biomarker of response to immune checkpoint inhibitors. *Nature Communications* **12** (2021).
<https://doi.org:10.1038/s41467-021-21619-0>
- 494 Duquette, D. *et al.* Human Granzyme K Is a Feature of Innate T Cells in Blood, Tissues, and Tumors, Responding to Cytokines Rather than TCR Stimulation. *The Journal of Immunology* **211**, 633-647 (2023). <https://doi.org:10.4049/jimmunol.2300083>
- 495 Bensussan, A. *et al.* BY55 monoclonal antibody delineates within human cord blood and bone marrow lymphocytes distinct cell subsets mediating cytotoxic activity. *Proceedings of the National Academy of Sciences* **91**, 9136-9140 (1994).
<https://doi.org:10.1073/pnas.91.19.9136>
- 496 Cachot, A. *et al.* Tumor-specific cytolytic CD4 T cells mediate immunity against human cancer. *Science Advances* **7**, eabe3348 (2021). <https://doi.org:10.1126/sciadv.abe3348>
- 497 Boulch, M. *et al.* Tumor-intrinsic sensitivity to the pro-apoptotic effects of IFN- γ is a major determinant of CD4+ CAR T-cell antitumor activity. *Nature Cancer* **4**, 968-983 (2023). <https://doi.org:10.1038/s43018-023-00570-7>
- 498 Rijnders, M. *et al.* Gene-expression-based T-Cell-to-Stroma Enrichment (TSE) score predicts response to immune checkpoint inhibitors in urothelial cancer. *Nature Communications* **15** (2024). <https://doi.org:10.1038/s41467-024-45714-0>
- 499 Carstens, J. L. *et al.* Spatial computation of intratumoral T cells correlates with survival of patients with pancreatic cancer. *Nature Communications* **8**, 15095 (2017).
<https://doi.org:10.1038/ncomms15095>
- 500 Xiao, Z. *et al.* Desmoplastic stroma restricts T cell extravasation and mediates immune exclusion and immunosuppression in solid tumors. *Nature Communications* **14** (2023).
<https://doi.org:10.1038/s41467-023-40850-5>

- 501 Grout, J. A. *et al.* Spatial Positioning and Matrix Programs of Cancer-Associated Fibroblasts Promote T-cell Exclusion in Human Lung Tumors. *Cancer Discovery* **12**, 2606-2625 (2022). <https://doi.org:10.1158/2159-8290.cd-21-1714>
- 502 Peranzoni, E. *et al.* Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti-PD-1 treatment. *Proceedings of the National Academy of Sciences* **115**, E4041-E4050 (2018). <https://doi.org:10.1073/pnas.1720948115>
- 503 Kisakol, B. *et al.* Identification of unique rectal cancer-specific subtypes. *British Journal of Cancer* (2024). <https://doi.org:10.1038/s41416-024-02656-0>
- 504 Hong, S. P. *et al.* The differential impact of microsatellite instability as a marker of prognosis and tumour response between colon cancer and rectal cancer. *European Journal of Cancer* **48**, 1235-1243 (2012). <https://doi.org:https://doi.org/10.1016/j.ejca.2011.10.005>
- 505 Stintzing, S. *et al.* Consensus molecular subgroups (CMS) of colorectal cancer (CRC) and first-line efficacy of FOLFIRI plus cetuximab or bevacizumab in the FIRE3 (AIO KRK-0306) trial. *Annals of Oncology* **30**, 1796-1803 (2019). <https://doi.org:10.1093/annonc/mdz387>
- 506 Hu, X. *et al.* In Vitro Priming of Adoptively Transferred T Cells with a ROR γ Agonist Confers Durable Memory and Stemness In Vivo. *Cancer Research* **78**, 3888-3898 (2018). <https://doi.org:10.1158/0008-5472.can-17-3973>
- 507 Poon, M. M. L. *et al.* Tissue adaptation and clonal segregation of human memory T cells in barrier sites. *Nature Immunology* **24**, 309-319 (2023). <https://doi.org:10.1038/s41590-022-01395-9>
- 508 Brischwein, K. *et al.* MT110: A novel bispecific single-chain antibody construct with high efficacy in eradicating established tumors. *Molecular Immunology* **43**, 1129-1143 (2006). <https://doi.org:https://doi.org/10.1016/j.molimm.2005.07.034>
- 509 Gulden, G. *et al.* CAR-T Cells with Phytohemagglutinin (PHA) Provide Anti-Cancer Capacity with Better Proliferation, Rejuvenated Effector Memory, and Reduced Exhausted T Cell Frequencies. *Vaccines* **11**, 313 (2023). <https://doi.org:10.3390/vaccines11020313>

- 510 Supimon, K. *et al.* Anti-mucin 1 chimeric antigen receptor T cells for adoptive T cell therapy of cholangiocarcinoma. *Scientific Reports* **11**, 6276 (2021). <https://doi.org:10.1038/s41598-021-85747-9>
- 511 Carneiro, B. A. & El-Deiry, W. S. Targeting apoptosis in cancer therapy. *Nature Reviews Clinical Oncology* **17**, 395-417 (2020). <https://doi.org:10.1038/s41571-020-0341-y>
- 512 Oh, D. Y. & Fong, L. Cytotoxic CD4⁺ T cells in cancer: Expanding the immune effector toolbox. *Immunity* **54**, 2701-2711 (2021). <https://doi.org:10.1016/j.immuni.2021.11.015>
- 513 Alspach, E. *et al.* MHC-II neoantigens shape tumour immunity and response to immunotherapy. *Nature* **574**, 696-701 (2019). <https://doi.org:10.1038/s41586-019-1671-8>
- 514 Rodig, S. J. *et al.* MHC proteins confer differential sensitivity to CTLA-4 and PD-1 blockade in untreated metastatic melanoma. *Science Translational Medicine* **10**, eaar3342 (2018). <https://doi.org:10.1126/scitranslmed.aar3342>
- 515 Linnemann, C. *et al.* High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4⁺ T cells in human melanoma. *Nature Medicine* **21**, 81-85 (2015). <https://doi.org:10.1038/nm.3773>
- 516 Kreiter, S. *et al.* Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* **520**, 692-696 (2015). <https://doi.org:10.1038/nature14426>
- 517 Ott, P. A. *et al.* An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **547**, 217-221 (2017). <https://doi.org:10.1038/nature22991>
- 518 Platten, M. *et al.* A vaccine targeting mutant IDH1 in newly diagnosed glioma. *Nature* **592**, 463-468 (2021). <https://doi.org:10.1038/s41586-021-03363-z>
- 519 Cattaneo, C. M. *et al.* Identification of patient-specific CD4⁺ and CD8⁺ T cell neoantigens through HLA-unbiased genetic screens. *Nature Biotechnology* **41**, 783-787 (2023). <https://doi.org:10.1038/s41587-022-01547-0>
- 520 Kalaora, S. *et al.* Identification of bacteria-derived HLA-bound peptides in melanoma. *Nature* **592**, 138-143 (2021). <https://doi.org:10.1038/s41586-021-03368-8>

- 521 Naghavian, R. *et al.* Microbial peptides activate tumour-infiltrating lymphocytes in glioblastoma. *Nature* **617**, 807-817 (2023). <https://doi.org/10.1038/s41586-023-06081-w>
- 522 Roberts, N. J. *et al.* Intratumoral injection of *Clostridium novyi*-NT spores induces antitumor responses. *Science Translational Medicine* **6**, 249ra111-249ra111 (2014). <https://doi.org/doi:10.1126/scitranslmed.3008982>
- 523 Routy, B. *et al.* Gut microbiome influences efficacy of PD-1–based immunotherapy against epithelial tumors. *Science* **359**, 91-97 (2018). <https://doi.org/10.1126/science.aan3706>
- 524 Vétizou, M. *et al.* Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* **350**, 1079-1084 (2015). <https://doi.org/doi:10.1126/science.aad1329>
- 525 Sivan, A. *et al.* Commensal *Bifidobacterium* promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* **350**, 1084-1089 (2015). <https://doi.org/doi:10.1126/science.aac4255>
- 526 Romero, A. I. *et al.* Regulation of CD4+NKG2D+ Th1 Cells in Patients with Metastatic Melanoma Treated with Sorafenib: Role of IL-15R α and NKG2D Triggering. *Cancer Research* **74**, 68-80 (2014). <https://doi.org/10.1158/0008-5472.can-13-1186>
- 527 Dighe, A. S., Richards, E., Old, L. J. & Schreiber, R. D. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN γ receptors. *Immunity* **1**, 447-456 (1994). [https://doi.org/https://doi.org/10.1016/1074-7613\(94\)90087-6](https://doi.org/https://doi.org/10.1016/1074-7613(94)90087-6)
- 528 Bhat, P., Leggatt, G., Waterhouse, N. & Frazer, I. H. Interferon- γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death & Disease* **8**, e2836-e2836 (2017). <https://doi.org/10.1038/cddis.2017.67>
- 529 Kruse, B. *et al.* CD4+ T cell-induced inflammatory cell death controls immune-evasive tumours. *Nature* **618**, 1033-1040 (2023). <https://doi.org/10.1038/s41586-023-06199-x>
- 530 Corgnac, S., Boutet, M., Kfoury, M., Naltet, C. & Mami-Chouaib, F. The Emerging Role of CD8+ Tissue Resident Memory T (TRM) Cells in Antitumor Immunity: A Unique Functional

- Contribution of the CD103 Integrin. *Frontiers in Immunology* **9** (2018).
<https://doi.org/10.3389/fimmu.2018.01904>
- 531 Zhang, Q. *et al.* Integration of single-cell RNA sequencing and bulk RNA transcriptome sequencing reveals a heterogeneous immune landscape and pivotal cell subpopulations associated with colorectal cancer prognosis. *Frontiers in Immunology* **14** (2023).
<https://doi.org/10.3389/fimmu.2023.1184167>
- 532 Fraietta, J. A. *et al.* Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nature Medicine* **24**, 563-571 (2018). <https://doi.org/10.1038/s41591-018-0010-1>
- 533 Jung, I.-Y. *et al.* Tissue-resident memory CAR T cells with stem-like characteristics display enhanced efficacy against solid and liquid tumors. *Cell Reports Medicine* **4** (2023).
<https://doi.org/10.1016/j.xcrm.2023.101053>
- 534 Cao, J. *et al.* The single-cell transcriptional landscape of mammalian organogenesis. *Nature* **566**, 496-502 (2019). <https://doi.org/10.1038/s41586-019-0969-x>