Elucidating the role of regulatory T cells in colorectal cancer progression

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SUMMARY

Antigens (Ags) expressed by cancer cells can be specifically recognised by T cells contributing to anti-tumour immunity. For this reason, tumour Ag-specific T helper type 1 (Th1) cells can be detected in cancers and their infiltration is predictive of a prolonged disease-free survival. However, their activity is suppressed by T cells with inhibitory functions i.e. regulatory T cells (Tregs). High infiltration of tumours by these cells often correlates with poor survival. It is therefore crucial to understand their activity. Since Th1 and Treg cells are activated after Ag recognition, it is plausible that tumour Ag-specific Tregs inhibit Th1-mediated tumour immunity. Furthermore, vaccination, rather than inducing effector T cells, may be detrimental by activating Tregs.

Increased numbers of $CD25^{hi}$ Treg cells are found in cancer patients, including CRC. However, in contrast to many cancers, the phenotype of these cells has not been studied in CRC patients. In this thesis, phenotypes of circulating, colon and tumour infiltrating Tregs are described. A high percentage of intratumoural $CD4^{+}CD25^{hi}$ Tregs was found to express immunosuppressive molecules and these cells were enriched in late-stage CRC tumours. In addition, this thesis describes the measurement of Th1 and Treg cells recognising the oncofetal Ag, 5T4. Four immunogenic regions within the oncofoetal tumour Ag 5T4 and 14 peptides able to bind to most commonly found human HLA-DR alleles were identified. $CD4^{+}CD25^{hi}$ cells mediated the control of tumour Ag 5T4-specific Th1 responses in CRC patients and their suppressive activity was not restricted to Th1 cells of the same Ag specificity. Also, responses to some, but importantly not all, immunogenic helper peptides discovered were influenced by Tregs. Thus Tregs may contribute to CRC progression by influencing tumour Ag- specific responses of Th1 cells.

This body of work brings more information regarding the tumour Ag-specificity of Tregs and offers a future rationale to design peptide-based vaccines which exclude Treg peptides.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIRE	Autoimmune regulator
APC	Allophycocyanin
APCs	Antigen presenting cells
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CRC	Colorectal cancer
CSC	Cancer stem cell
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
EBV	Epstein-Barr virus
ELISpot	Enzyme linked immunospot
FACS	Fluorescence activated cell sorting
FITCS	Fluorescein isothiocyanate
FMO	Fluorescence minus one
Foxp3	Forkhead box protein 3
GITR	Glucocorticoid induced tumour necrosis
HLA	Human leukocyte antigen
HPV	Human papillomavirus
IBD	Inflammatory bowel disease
ICOS	TCR-inducible costimulatory molecule
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
LAG-3	Lymphocyte activation gene-3
LAP	Latency-associated peptide
LN	Lymph node
LPS	Lipopolysaccharide
MAGE	Melanoma-associated antigen
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MVA	Modified Vaccinia Ankara
NK	Natural killer
Nrp-1	Neuropilin-1
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed death-1

PE	Phycoerythrin
PECy7	Phycoerythrin-Cy7
PerCpCy5.5	Peridinin chlorophyll protein complex-Cy5.5
PHA	Phytohaemagglutinin
PRR	Pattern recognition receptor
pTreg	Peripherally-induced regulatory T cell
RPMI	RPMI-1640 media
SFC	Spot forming cell
SLO	Secondary lymphoid organ
TaCTiCC	TroVax® and cyclophosphamide treatment in colorectal cancer
T_{CM}	Central memory T
TCR	T cell receptor
Teff	Effector T
T_{EM}	Effector memory T
Th	T helper
Th1	T helper type 1
TIL	Tumour infiltrating lymphocyte
TNM	Tumour / Nodes / Metastasis
Treg	Regulatory T cell
TSA	Tumour specific antigen
tTreg	Thymus derived regulatory T cell
VEGF	Vascular endothelial growth factor
WT1	Wilms tumour antigen 1

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CHAPTER 1. INTRODUCTION

1.1. Cancer cells

1.1.1. Tumour establishment

Tumourigenesis is a gradual process consisting of several steps depending on the acquisition of mutations in cells. Cancers can arise from small numbers of mutations although tumour cells usually exhibit a high number of mutations (1). Indeed, among all mutated genes, mutations in the tumour-suppressor gene p53 are key events in tumourigenesis of many human cancers (2). As a result of p53 mutations, cells are unable to engage apoptosis or senescence and acquire new functions in cell migration (3). Studies have shown that rare cells within tumours are able to drive tumourigenesis suggesting that tumours originate from the transformation of normal stem cells (4). This cancer stem cell (CSC) model has been recently described in different studies with mouse models of brain, skin and intestinal tumours where CSCs that self-renew could produce terminally differentiated tumour cells, reviewed in (5). The intrinsic features of stem cells make them good candidates for acquisition of mutations leading to cancer. Indeed, they are found in several tissues and may persist for a lifetime.

1.1.2. From cancer immune surveillance to immunoediting

Lewis Thomas and Frank Macfarlane Burnet were the first to formally propose that the immune system was able to prevent cancer establishment in immunocompetent hosts by recognizing and killing tumour cells (6). This hypothesis was named cancer immunosurveillance. The immunology community long remained sceptical as strong experimental evidences were lacking. For instance, athymic-nude (not totally

immunodeficient) and normal mice showed no difference in cancer incidence (7). The use of knock-out (gene-targeted) mice revolutionized immunology and clearly showed the link between immunity and cancer suppression. Indeed, *RAG2*^{-/-} mice (lacking adaptive immunity) and *IFNGR1*^{-/-} mice (lacking IFN-γ receptor), were found to be more susceptible to tumour development (8). Similarly, *IFNAR1*^{-/-} mice (lacking IFN-α/β receptor complex) demonstrate increased carcinogen-induced tumourigenesis (9). In humans, the use of immunosuppressive drugs during organ transplantation is also associated with cancers, as shown after kidney transplantation (10). However, slightly conflicting results have emerged which may reflect differences in immunosuppressive regimens. Other evidence supporting the cancer immunosurveillance hypothesis is that high numbers of tumour infiltrating lymphocytes (TILs) predict long-term survival, after surgical intervention, in patients with advanced ovarian carcinoma (11) and CRC (12).

The concept that the immune system is involved in protection against tumours during the early stage of tumourigenesis is seen as too simplistic: if this is the case, cancers should not develop in immunocompetent individuals. Instead, it is now proposed that cancer immunosurveillance is part of a complex process called immunoediting where immune cells also shape tumour immunogenicity. In a study published in 2001, tumours isolated from immunodeficient mice were more immunogenic (no sign of growth in naïve wild type recipient) than tumours from immunocompetent mice (tumour growth in naïve wild type recipient) (3). The first tumours were unedited while the second tumours were edited. The authors postulated that immunoediting is characterized by 3 distinct phases: elimination, equilibrium and escape. The elimination phase corresponds to the cancer immunosurveillance where innate and adaptive cells clear tumours before clinical manifestations. Observing this phase *in vivo* is challenging as tumours remain silent. Tumour cells that are not destroyed enter the equilibrium phase and are subjected to cells

of the adaptive immune system that not only prevent their outgrowth but also shape/edit their immunogenicity. This state of cancer dormancy is the longest (potentially lasting a lifetime) and enables tumour cells to adopt the most immunoevasive mutations. Koebel *et al* confirmed the existence of a long-term cancer control by the adaptive immune system. By using a mouse model, they showed that sarcoma cells held in equilibrium are highly immunogenic (unedited phenotype and rejected in wild-type mice), unlike less immunogenic tumour cells (edited) eventually exiting this phase and entering the escape phase (13). In order to reveal early tumour Ags (before immunoediting) and the effect on immune cells, Matsushita *et al* transplanted 3-methylcholanthrene-induced sarcoma cells into immunodeficient mice, sequenced the sarcoma cell exomes and identified a sequence corresponding to a highly immunogenic mutant (spectrin-β2) peptide recognised by cytotoxic T lymphocytes (CTLs) (14). In immunocompetent mice, only tumour cells lacking expression of this peptide could grow thereby revealing a T cell-dependent mechanism of immunoediting. During the last phase, tumours escape immunity and become invisible. This loss of immune control can be explained by deficient Ag presentation on tumour cell surface, i.e. loss of presentation of spectrin-β2 peptide; or the induction of an immunosuppressive environment [production of vascular endothelial growth factor (VEGF) and indoleamine 2,3-dioxygenase (IDO) enzyme and/or recruitment of regulatory immune cells]. Although many cell types of the innate (NK cells and macrophages) and the adaptive (T and B cells) immune system are involved in cancer control, the relative contribution of these immune cells in the 3 phases of cancer immunoediting is not completely understood (with the exception of CTLs).

In humans, the existence of cancer immunosurveillance (elimination phase) is demonstrated by the higher risk of cancer development in immunosuppressed patients. However, these patients have greater susceptibility to viruses which may reflect an

impairment in anti-viral immunity and not a reduced immunosurveillance of cancer. Yet, immunodeficient individuals have a higher probability of developing cancers with no known viral etiology, reviewed in (15).

1.2. T cell development

1.2.1. TCR gene recombination

All the lineages of the immune system arise from a common lymphoid progenitor, differentiated from a haematopoietic stem cell in the bone marrow. T cell precursors (thymocyte) reach the thymus for maturation whereas B cells develop in the bone marrow. Both organs are indispensable for T and B cell development and are called primary lymphoid organs. The thymus has specific environments (cortex and medulla) allowing T cell receptor (TCR) gene rearrangement and thymocyte selection. These processes respectively generate an enormous repertoire of TCRs that can recognize a very large array of self and foreign peptides and select thymocytes with TCR of intermediate affinity to self-peptide–major histocompatibility complex (MHC) ligands.

Functional TCRs are heterodimers consisting of an α-chain and a β-chain, each presenting a variable (V) amino-terminal region and a constant (C) region (Figure 1.1.A). The organization of the TCRα and TCRβ loci is shown in (Figure 1.1.B). The TCRα locus (similarly to the immunoglobulin light chains) has V and joining (J) gene segments (Vα and Jα). The TCRβ locus (similarly to the immunoglobulin heavy-chain) has diversity (D) gene segments in addition to Vβ and Jβ gene segments. The TCRα locus (chromosome 14) consists of 70–80 Vα gene segments, each having an exon encoding the leader sequence (L). The Jα gene segments are followed by an unique C gene, which contains the exons encoding the constant domain, the hinge, the

A

Antigen combining site

Carbohydrates

Vα Vβ

Cα Cβ

Hinge

Cytoplasmic tail

Transmembrane region

B

TcR α chain locus

L Vα x 70-80

Jα x 61

Cα

TcR β chain locus

L Vβ x 52   Dβ1 Jβ1 x 6   Cβ1   Dβ2   Jβ2 x 7   Cβ2
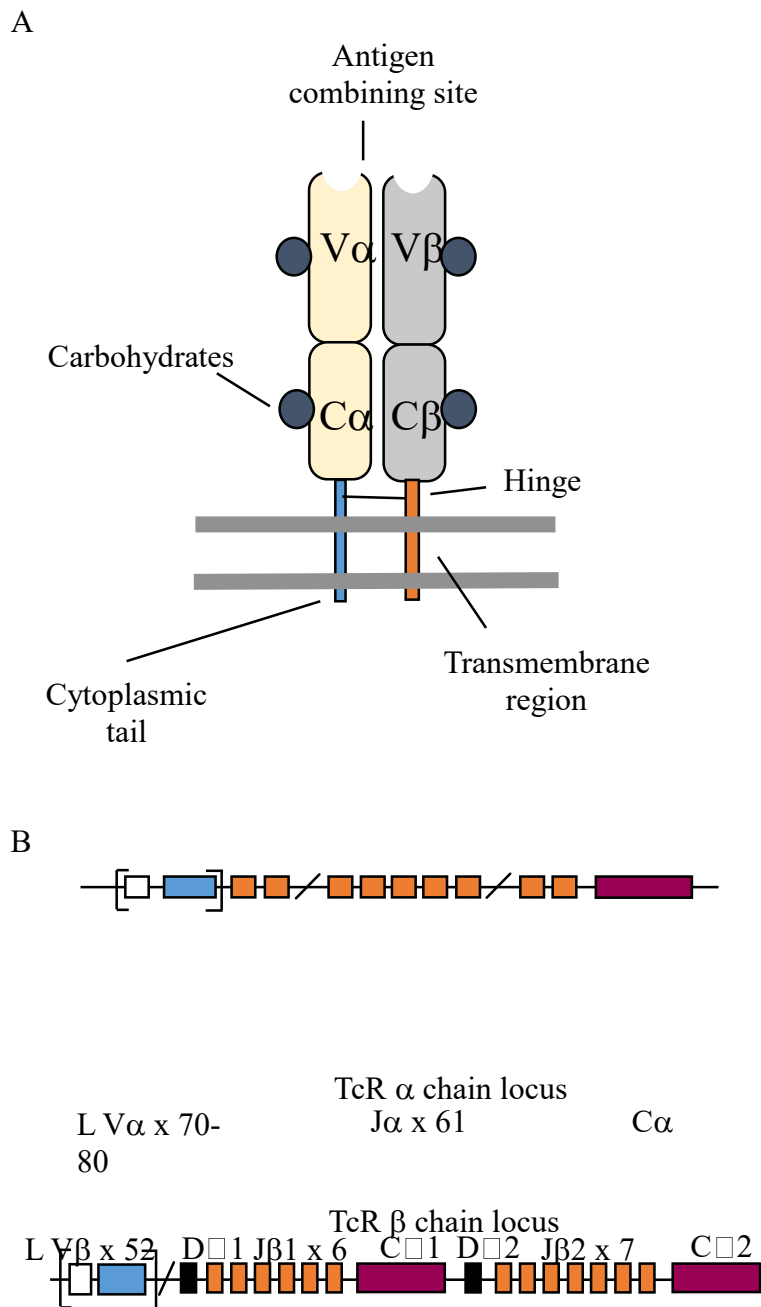
Figure 1.1. Structure of the TCR. A. The TCR heterodimer is composed of two trans-membrane glycoprotein chains, α and β. B. Organization of the human TCR α and β loci.

transmembrane region and the cytoplasmic region. The TCRβ locus (chromosome 7) is characterized by a different organization. A cluster of 52 functional Vβ gene segments is located distantly from two separate clusters each having a single D gene segment, six or seven J gene segments and a single C gene.

The TCR gene segments rearrange in the thymus during T cell development to form a complete V-domain exons. The TCR gene segments are flanked by recombination signal sequences (RSSs). The mechanism of V(D)J recombination involves two proteins, called Recombination Activating Gene (RAG) -1 and RAG-2. The RAG-dependent DNA cleavage phase begins when RAG1 and RAG2 bind at the RSS (Figure 1.2, depicted as filled triangles) adjacent to each V, D or J element (Figure 1.2, depicted as filled rectangles). Then, the two RAG1/RAG 2 complexes bind to each other and bring the V region adjacent to the DJ region (phase 2). The RAG protein cuts the DNA at each signal and the nicks are closed to form a hairpin structure at the end of the V and D regions (phase 3). A number of proteins (e.g. Ku70 and Ku80) and kinases bind to the hairpins and open the hairpinned V and D ends. Then, exonucleases and transferases remove or add random nucleotides to the gap between the V and D region. This junctional diversity accounts for the even greater diversity of TCR specificity. Finally, DNA ligase IV joins the ends of the V and D region to form the coding joint and the two signal-ends to form the signal joint (phase 4).

The TCR alpha chain is generated by VJ recombination, where a Vα gene segment rearranges to a Jα gene segment to create a functional V-region exon. In contrast, the beta chain is generated by VDJ recombination, where Vβ, Dβ, and Jβ gene segments reearrange to create a functional V-region exon. Finally, VJα and VDJβ V-region exons are transcribed and spliced to join to Cα and Cβ, respectively.

RSS RAG

Phase 2

DNA hairpin Signal-ends

Phase 3

Phase 4

Coding joint

Signal
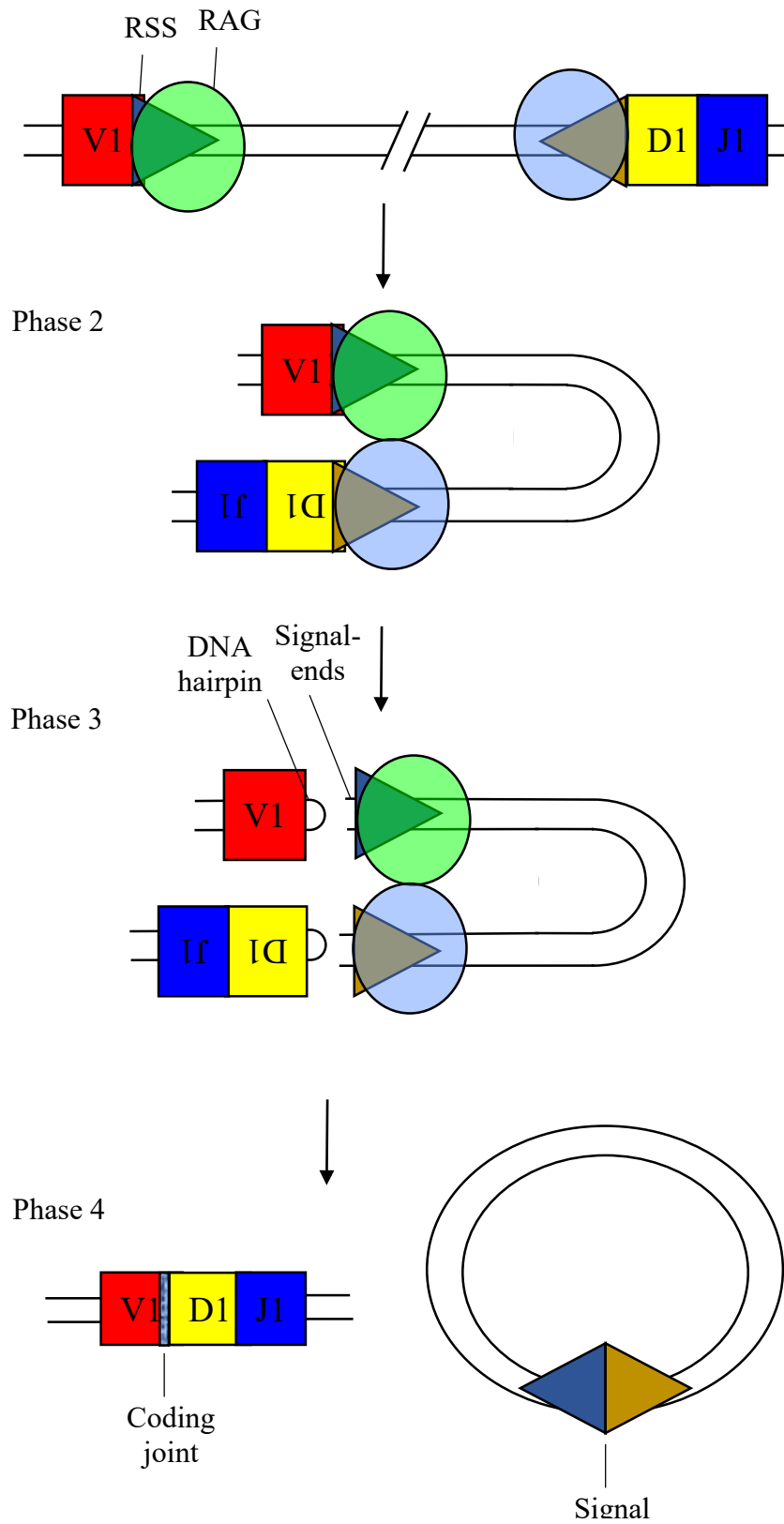
Figure 1.2. Mechanism of TCR gene recombination.

1.2.2. Naïve T cells

In the medulla, double positive $CD4^+CD8^+$ thymocytes further differentiate into CD8 single-positive ($CD4^-CD8^+$) or CD4 single-positive ($CD4^+CD8^-$) lineages and then circulate constantly throughout secondary lymphoid organs (SLOs) via blood circulation as mature naïve T cells. SLOs can be encapsulated (e.g. spleen) or diffuse (mucosa-associated lymphoid tissue) and are the sites of Ag exposure where primary immune responses are initiated. Naïve T cells are found primarily in SLOs as these cells express a unique set of receptors allowing migration and homing in lymph nodes (LNs). For instance, naïve T cells express the receptor CCR7 that binds to CCL21 and CCL19 attracting the cells to LNs and express L-selectin adhesion molecule (also known as CD62L) mediating entry into LNs.

During an infection, Ag-presenting cells (APCs) capture Ags and further process them into peptides. Internalisation of pathogens is mediated by pattern recognition receptors (PRRs) on APCs surface. For naïve $CD4^+$ T cell activation, these peptides are loaded onto MHC class II molecules and peptide-MHC are presented on the APC surface (naïve $CD8^+$ T cells require peptide loaded on MHC class I). APCs displaying Ag taken up in the periphery are then transported to T cell areas of SLOs. *In vivo*, among the cells expressing MHC class II molecules, only dendritic cells (DCs) mediate naïve T cell activation. Indeed, macrophages are mainly localized in nonlymphoid tissues and B cells are inefficient at presenting Ags (16). During migration, pathogen-activated DCs undergo maturation leading to a better ability to interact with naïve T cells in SLOs.

Whilst naïve T cells follow a restricted migratory pattern, some of them can be found in non-lymphoid tissues. During chronic inflammation, naïve T cells can home to the affected tissue following the development of tertiary lymphoid tissue that helps

sustain their activation (17). Also, naïve T cell infiltration and differentiation has been observed at tumour site (18).

DCs provide three signals that are required for the activation of Ag-specific naïve T cells. Depending on the stimulus encountered, DCs can modify these signals in order to differentiate naïve T cells into activated Ag-specific helper T cells (19). In contrast, after priming by DCs, naïve CD8^{+} T cells differentiate into CTLs.

1.2.3. Signal 1

Signal 1, Ag-specific signal, is induced by recognition of peptide-MHC class II or class I complexes by the TCR and is crucial for removing T cells from quiescence (nondividing state). There are three classical class II molecules (isotypes) in Human: human leukocyte antigen (HLA)-DP, -DQ, and -DR. The most highly expressed on APCs is HLA-DR. In parallel, two non-classical molecules, HLA-DM and –DO, are involved in peptide loading in the cells but do not present Ags on APCs.

Crystallography studies have characterised the MHC class II structure (20) (Figure 1.3). They are heterodimers of two polypeptide chains (α and β) made of single transmembrane sequences and short cytoplasmic tails. Terminal $\alpha 1$ and $\beta 1$ regions combine to form a peptide-binding groove that presents peptides to CD4^{+} T cells. Stern *et al* crystallized the HLA-DR1 molecule with influenza peptide hemagglutinin and showed that the peptide bound within 4 distinctive pockets (P1, P4, P6 and P9) in the binding groove whereas the flanking regions extended out of the groove (21). The anchor residues at positions 1, 4, 6 and 9 on MHC class II-bound peptides are crucial for stable interactions. Because MHC class II molecule have an open-ended binding groove theycan present peptides of highly variable lengths (12-24 amino acids) (22). MHC class II-bound peptides have a core of 9
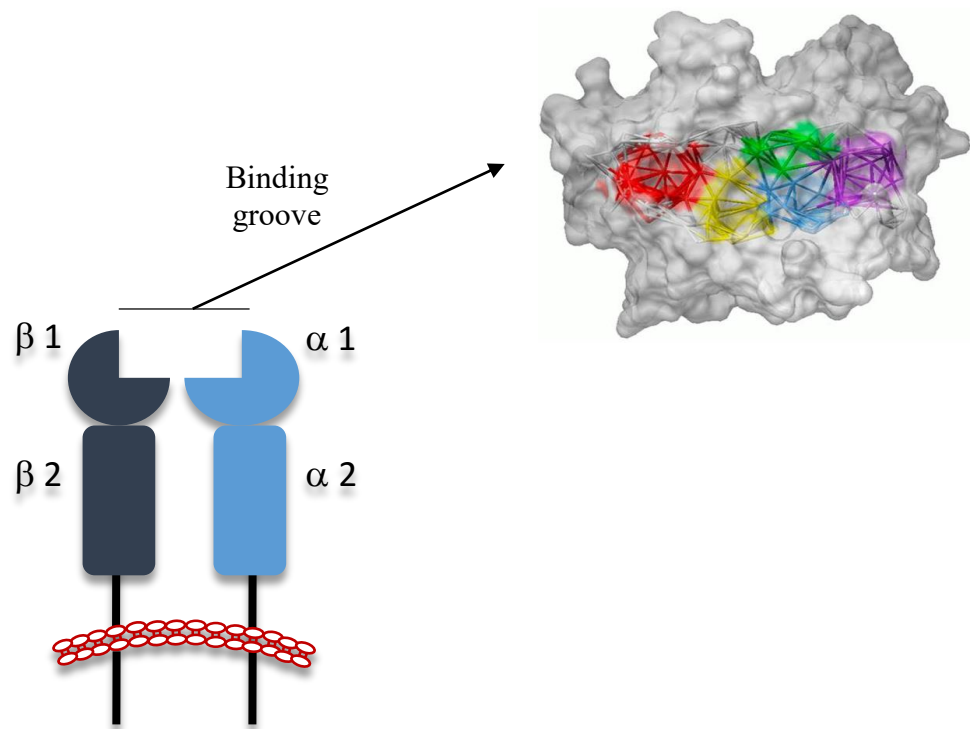
Figure 1.3. Structure of MHC class II molecule. The five pockets in the binding groove of one HLA-DR1 molecule are highlighted (P1 in red; P4 in yellow; P6 in green; P7 in blue and P9 in purple). Figure adapted from *Yeturu et al.*

amino acid and residues outside this nonameric binding region are called peptide flanking residues (PFRs). PFRs have a critical effect on TCR recognition and T cell function (23) and their modifications can result in enhanced CD4$^+$ T cell activation (24). Residues that do not interact with the binding pockets on the MHC class II (mainly residues at positions 2, 5 and 8) are available to interact with the TCR and alterations influence the signal that T cells receive through their TCRs (25).

Upon ligand binding, TCRs cannot transmit signal 1 into the cell without CD3 molecules and CD4/CD8 coreceptors, the latter of which interact directly with MHC class II and class I respectively. Both CD3 molecules and coreceptors serve to translate the binding with MHC class II into intracellular signals (phosphorylations by kinases) required for T cell activation. Furthermore, Huang *et al* characterized the functional sensitivity of CD4$^+$ T cells and showed that a single peptide-MHC could stimulates naive T cells to secrete cytokines (26).

Regarding CD4$^+$ T cells, a close correspondance between the strength of the TCR signal and polarization has been demonstrated (27). *In vivo*, strong TCR signals induce T helper type 1 (Th1) differentiation whereas weak induce Th2 cells. The strength of TCR stimulation *in vivo* is influenced by variables such as Ag concentration, affinity of pepide for MHC and/or stability of T cell-APC interaction.

1.2.4. Signal 2

Although TCR stimulation is necessary, it is not sufficient to support T cell activation. Engagement of co-stimulatory molecules is required to complement and amplify TCR/CD3/CD4- or CD8-generated signals. The most prominent signal is provided by CD80 and CD86, which are highly expressed on activated DCs. These trigger the CD28

receptor expressed by T cells. However, CD28 is not the only receptor capable of delivering a costimulatory signal and other receptors can compensate to a limited extent its absence (28). Indeed, after activation by pathogen or inflammatory molecules, mature DCs upregulate many additional co-stimulatory receptors, classified as members of the B7 or TNF-Receptor families.

Stimulation in the presence of only TCR stimulation in the absence of signal 2 can lead T cell to anergy (nonresponsive to Ag stimulation) or apoptosis. Originally thought to be signal II independent, the activation of memory T cells also requires CD28 costimulation (29). The same costimulatory molecules on DCs can also mediate T cell inactivation through engagement with other receptors expressed on T cells. Indeed, after activation, T cells up regulate cytotoxic T-lymphocyte antigen 4 (CTLA-4), which binds with high affinity to CD80 and CD86 leading to inhibition of T cell function (30). CTLA-4 has a central role in the inhibition of excessive T cells expansion, as shown with CTLA-4-deficient mice developing lethal lymphoproliferative syndrome and patients treated with anti-CTLA-4 therapy developing autoimmune disorders (31, 32).

1.2.5. Signal 3

Signal 3 is the polarizing signal mediated by DC-released cytokines. Already differentiated T cells do not need this third signal. After signals 1 and 2, T cells upregulate receptors, which permit them to receive signal 3. This signal corresponds to the inflammatory cytokines that reflect the nature of the pathogen encountered by DCs and the nature of the infected tissue. For instance, after activation by intracellular pathogens, DCs release IL-12 which promotes Th1 cell development (33).

1.2.6 Memory T cells

Once activated and polarized Ag-specific naïve T cells differentiate into effector T (Teff) cells: T helper (Th) cells (for CD4$^+$ T cells) or CTLs (for CD8$^+$ T cells) that proliferate and produce IL-2, which is required for autonomous clonal expansion. The majority of the Teff cells die by apoptosis after Ag clearance whilst others become long-lived memory cells (central T$_{CM}$ or effector memory T$_{EM}$ cells). T$_{CM}$, expressing both CD62L and CCR7, are located primarily in SLOs and T$_{EM}$ in nonlymphoid tissues (34). Current dogma indicates that memory T cells proliferate in response to lower doses of Ag and are less dependent on costimulatory molecules than naive T cells permitting a faster response when reencountering a pathogen (35). However, recent *in vivo* studies have shown that CD28 costimulation is critical for secondary T cell responses [reviewed in (36)].

1.2.7. T cell tolerance
1.2.7.1. Peripheral tolerance

After TCR recombination (see above) in the thymic cortex, immature T cells (called thymocytes) express an active TCR and both the CD4 and CD8 molecules (double positive). The strength or avidity of the interaction between the TCRs and pMHC molecules is a crucial factor that determines the fate of immature T cells. After interactions with cortical thymic epithelial cells (cTEC), if the TCR – pMHC- interaction is of intermediate affinity the cell undergoes positive selection (Figure 1.4). Those DP thymocytes with a functional TCR will then become single positive cells (expressing either CD4 or CD8) and migrate to the thymic medulla. The others, 90% of the thymocytes, express "useless" TCR with very weak affinity for self pMHC complexes and die by neglect. In the medulla, thymocytes interact with medullary thymic epithelial cells (mTECs) and DCs presenting self-peptides. During this stage, thymocyte that expresses a TCR that has an above threshold affinity to self-peptides will go through

CORTEX   MEDULLA   PERIPHERY

Positive selection   Negative selection

Immature   Mature
T cell   T cell

CD4⁺   cTEC   CD4⁺ or   mTEC   CD4⁺
CD8⁺   CD8⁺   or DC
                        (Intermediate
                        interaction   CD8⁺
                        with pMHC)

Immunoincompetent cells   Self-reactive cells (strong
(weak interaction with pMHC) interaction with pMHC)

Intermediate   Moderate high
avidity (survival)   avidity (Treg selection)

Low avidity   High avidity

TCR affinity for self peptide-MHC

Figure 1.4. Thymic selection of T cells. A. Positive and negative selections. B. Thymic selection of Tregs.

apoptosis (negative selection). Thymocytes that have passed the thymic selection leave the thymus and reach the periphery as mature, naïve T cells.

The generation of natural Treg cells in the thymus is believed to occur as the result of an altered negative selection process. Data from TCR-transgenic mouse studies formed the basis for the current notion that natural Treg development occurs when the TCR avidity for self-antigens lies between the TCR avidities that drive positive selection and negative selection (37). In other words, Treg differentiation is thought to result when their TCR/antigen affinity is just weaker than the affinity needed for negative selection, but high enough to pass the positive selection (38) (Figure 1.4). Therefore, Tregs derived from the thymus are selected on their ability to interact with self-antigens with high TCR affinity, which has great implications for their protective role against autoimmune diseases.

A vital molecule involved in central tolerance is the transcription factor Autoimmune Regulator (AIRE). AIRE plays a critical role in negative selection as mutations are associated with failure to delete autoreactive T cells in the thymus (39) (40). In the thymic medulla, AIRE is expressed by medullary thymic epithelial cells, which also express MHC class II and co-stimulatory molecules (41). During negative selection, these cells present tissue-specific Ags (self Ag only expressed in peripheral organs) to thymocytes.

Besides driving the deletion of autoreactive T cells, AIRE is also required for thymic development of Tregs. As a result, thymus derived Tregs (tTregs) mostly recognize self-Ags encountered during thymic selection. AIRE is also required for tumour-associated Ag specific Tregs (42). In details, Malchow *et al* showed that tTregs, specific for a prostate-associated Ag, are enriched in prostate cancer. These tumour

associated Ag – specific Tregs required AIRE-dependent self Ags during thymus development. These data suggest that tumours recruit tTregs reactive to Aire-dependent self Ags associated with the organ of cancer origin.

1.2.7.2. Peripheral tolerance

Central tolerance is efficient but also incomplete, as not all self-Ags are expressed in the thymus. Therefore, self-reactive T cells are still present in the periphery of normal individuals (43). Peripheral tolerance, or Ag-specific unresponsiveness, is vital to suppress these potentially autoreactive lymphocytes. Several mechanisms are involved in the control of auto-reactive T cells in the periphery avoiding the development of autoimmune diseases. DCs can induce anergy or deletion (apoptotic cell death) by partial activation when, for instance, low doses of tissue Ags are presented to T cells or when Ags are presented in the absence of co-stimulatory molecules. In addition to these mechanisms, Tregs are critical to maintenance of self-tolerance.

1.3. T cells in cancer

1.3.1. CD8+ T cells

One of the key cells for tumour eradication are CD8+ CTLs. Naïve CD8+ T cells are activated upon recognition of tumour Ags in the context of MHC class I on DC surfaces in SLOs and become activated in the presence of costimulatory molecules such as CD80 and CD86 with the help of the CD4+ T cells secreting IL-2 (44). Activated Ag-specific CD8+ T cells traffic from the lymphoid organs to the peripheral site of infection via the blood. CTLs can directly recognize and kill tumour cells provided they express MHC class I molecules (MHC class II molecules being expressed by some tumours only).

CTLs can induce cell death of intracellular pathogen-infected and transformed cells with granules or Fas/Fas-ligand cytotoxic pathways, both triggering cell apoptosis. The granules are made of perforin (pore-forming protein) and granzyme (serine proteinases) that act together to efficiently inducing apoptosis in target cells (45). The role of CTLs in cancer immune surveillance was highlighted in the 1990s when a higher incidence of carcinogen-induced tumours was observed in perforin-deficient mice (46). Fas ligand (FasL or CD95) is upregulated on activated CTLs following Ag stimulation and binds to its receptor expressed on rapidly proliferating cells, such as tumour cells. This interaction results in the apoptosis of the target cell (47). Inactivation of the Fas-mediated cell death by mutation of Fas or FasL was shown to increase the development of B cell lymphomas in mice (48). Recently, one study reported that the FasL-mediated death pathway constitutes a crucial factor of T cell–mediated immune surveillance of murine B cell lymphoma (49).

1.3.2. T helper cells

As described above, the CD4 cell surface protein is expressed by a subset of lymphocytes. After Ag-induced activation in the periphery, naïve CD4^{+} T cells polarize into Teff cells called CD4^{+} Th cells. These lymphocytes can be divided into several subsets with different functions and cytokine signatures (50). This cellular polarization is dependent on the up regulation of transcription factors that are induced by the microenvironment present at the time of the initial Ag encounter by DCs. Indeed, DCs can polarize and promote the development of a specific Th cell subset depending on the pathogen recognized by their PRRs (19).

1.3.2.1. Th1 T cells

The Th1 developmental pathway is driven by IL-12 produced by activated APCs, including DCs (51), as shown by reduced Th1 responses in mice deficient for IL-12 or the IL-12 receptor β1 (52). This critical cytokine activates the transcription factor STAT4, whose absence results in a failure to generate Th1 cells (53). Two other cytokines influence Th1 development, IL-18 (54) and IFN-γ. Signals transmitted via the TCR after Ag recognition also upregulate (in concert with IFN-γ and IL-12) transcription factors such as T-bet, a "critical regulator" to initiate the Th1 differentiation program (Figure 1.5) (55).

Expression of lineage-specific transcription factors induce cytokine production and expression of genes encoding chemokine receptors. IFN-γ is produced predominantly by Th1 cells and is considered the signature cytokine for this subset within the CD4$^+$ T cell population. Other cell types can also however secrete relevant amounts of IFN-γ, including CD8$^+$ T cells and NKT cells (56). IL-2 and tumour necrosis factor-α (TNF-α) are also cytokines used to discriminate Th1 cells.

Th1 cells were first described in 1980s as a determinant of the concept of T cell 'help' where it was observed that CTL activation and expansion required Th1 cells (57). During primary responses, the nature of the 'help' includes local IL-2 production and/or indirect priming through accessory cells. This second alternative is based on the activation by Th1 cells of APCs, through CD40-CD40L interactions, which can subsequently directly activate CTLs (58). Although Th1 cells can be dispensable for primary CTL activation (Th-independent responses), IL-2-secreting Th1 cells are absolutely required for secondary CTL expansion (59) (60).
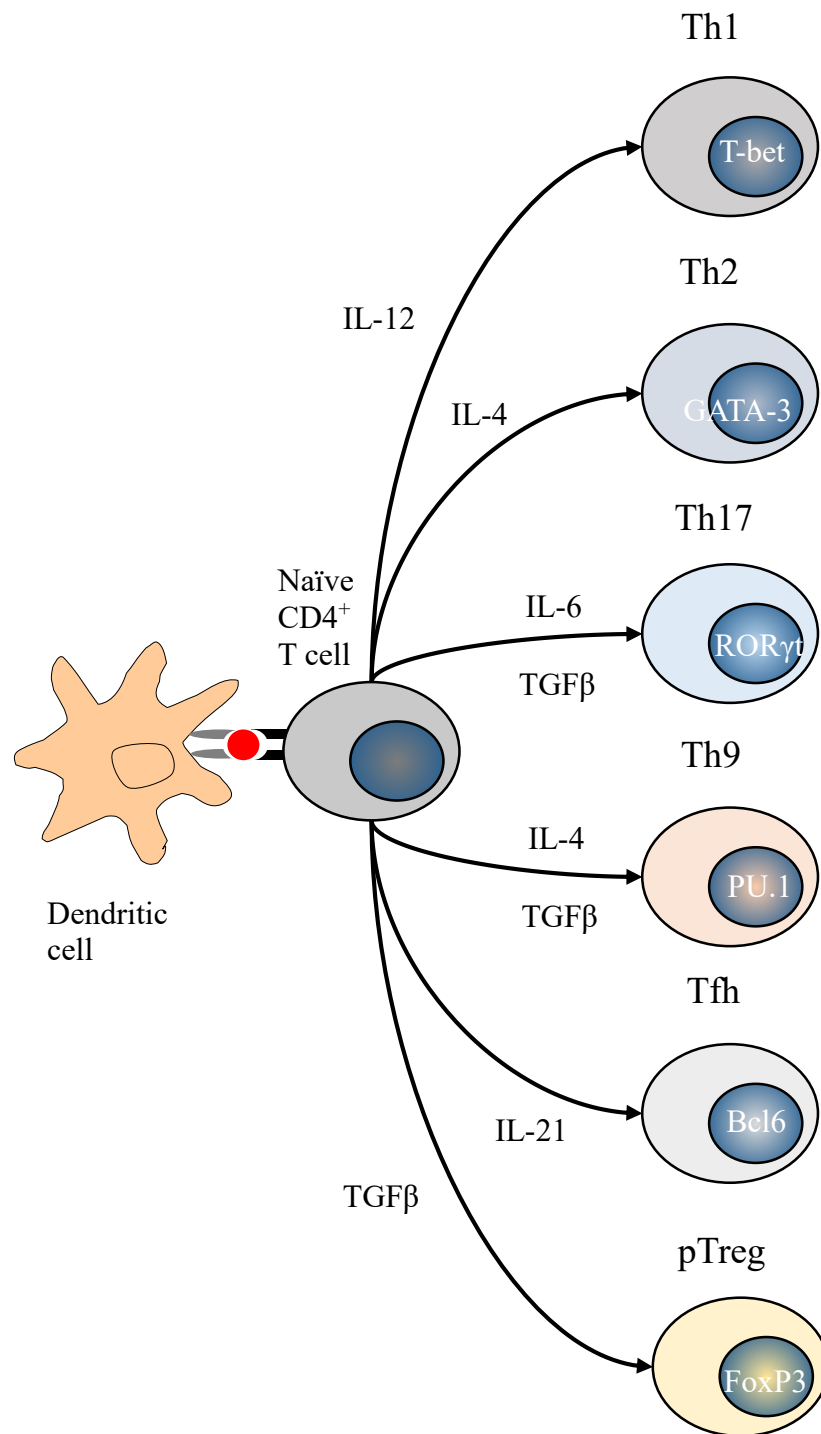
Figure 1.5. Differentiation of CD4$^+$ T cell subsets. After activation by APCs and in the presence of the indicated cytokines, naïve CD4$^+$ T cells can differentiate into 5 different T helper cells or in pTreg. Each differentiated cell expresses a characteristic transcription factor.

Alongside their "indirect" helper activities for cellular immunity against intracellular pathogens and tumour cells, Th1 cells also have "direct" role in anti-tumour immunity. Indeed, before activating CTL, Th1 cells can activate cells of the innate immunity that have anti-tumour activities. IFN-γ released by Th1 cells is the main cytokine involved in this activation and is therefore vital to tumour surveillance, as shown by enhanced tumour growth in mice lacking the IFN-γ receptors (61). IFN-γ allows CD4 Th1 cells to promote anti-tumour responses against tumour cells that do not express MHC class II molecules.

Indeed, IFN-γ derived from tumour Ag-specific Th1 cells activates macrophages, inducing secretions of tumouricidal factors such as reactive oxygen and nitrogen species (62). Furthermore, IFN-γ can induce the expression of the chemokine receptor CXCR3 and its ligands CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC (63) that recruit innate cells (NK cells and type I macrophages) to the tumour site from blood vessels by diapedesis. Observations that blood vessel growth is reduced in tumours of IFN-γ receptor$^{+/-}$ mice demonstrated that Th1 cells can target tumour stroma and inhibit angiogenesis in an IFN-γ–dependent way (64). Also, IFN-γ increases tumour immune surveillance by up regulating MHC class I expression on APCs and tumour cells (65) and thus facilitating CTL recognition and killing.

IFN-γ has also been shown to inhibit cancer cell proliferation by activating STAT1 that increases expression of cyclin dependent kinase inhibitor p21 (66) and to promote tumour cell apoptosis by inducing TRAIL molecule on natural killer (NK) cells (67). More recently, IFN-γ- and TNF-producing Th1 cells have been shown to drive cancer into senescence (68). Therefore, IFN-γ with its pleiotropic effects on multiple cells is key to immune-mediated control of tumour development. Also, IFN-γ has been shown to drive cancer immunoediting, selecting tumour cells with reduced immunogenicity (8).

Even though IFN-γ has been shown to be important in preventing and suppressing the development of cancers, a dual role of this cytokine in tumour immunity has been reported indicating that, paradoxically, IFN-γ may also contribute to cancer development (Figure 1.6). Several mechanisms involved in the tumour-promoting effects of IFN-γ have been proposed. Dong *et al* have shown that IFN-γ up regulates B7-H1 (PD-1 ligand) on the surface of tumour cell lines inducing apoptosis of Ag-specific human T cell clones *in vitro* (69). As tumour infiltrating CD4$^+$ T cells express programmed death-1 (PD-1), B7-H1-induced apoptosis has been described as a mechanism of tumour evasion. In addition, IFN-γ, produced by immune cells may induce expression of IDO in tumour cells (70). IDO catalyses the degradation of tryptophan: the metabolites and reduced tryptophan concentration induce T cell apoptosis in the tumour microenvironment. The development and function of both monocytic and granulocytic myeloid-derived suppressor cells (MDSC) require IFN-γ (71). MDSCs inhibit innate (NK cells) and adaptive anti-tumour immune responses (e.g. induction of cysteine deprivation) and are therefore a component of the pro-tumourigenic functions of IFN-γ.

1.3.2.2. Th2 T cells

Th2 cell differentiation is initiated by IL-4 that activates STAT6 and GATA3 (Figure 1.5). These key transcription factors are crucial for Th2 development and indispensable for Th2 cytokine secretion, including IL-4, IL-5, and IL-13 (72). Although both Th1 and 2 cells have been shown to provide help for Ab secretion by B cells (73), Th2 cytokines are more specialized in immunoglobulin isotype switching (74).
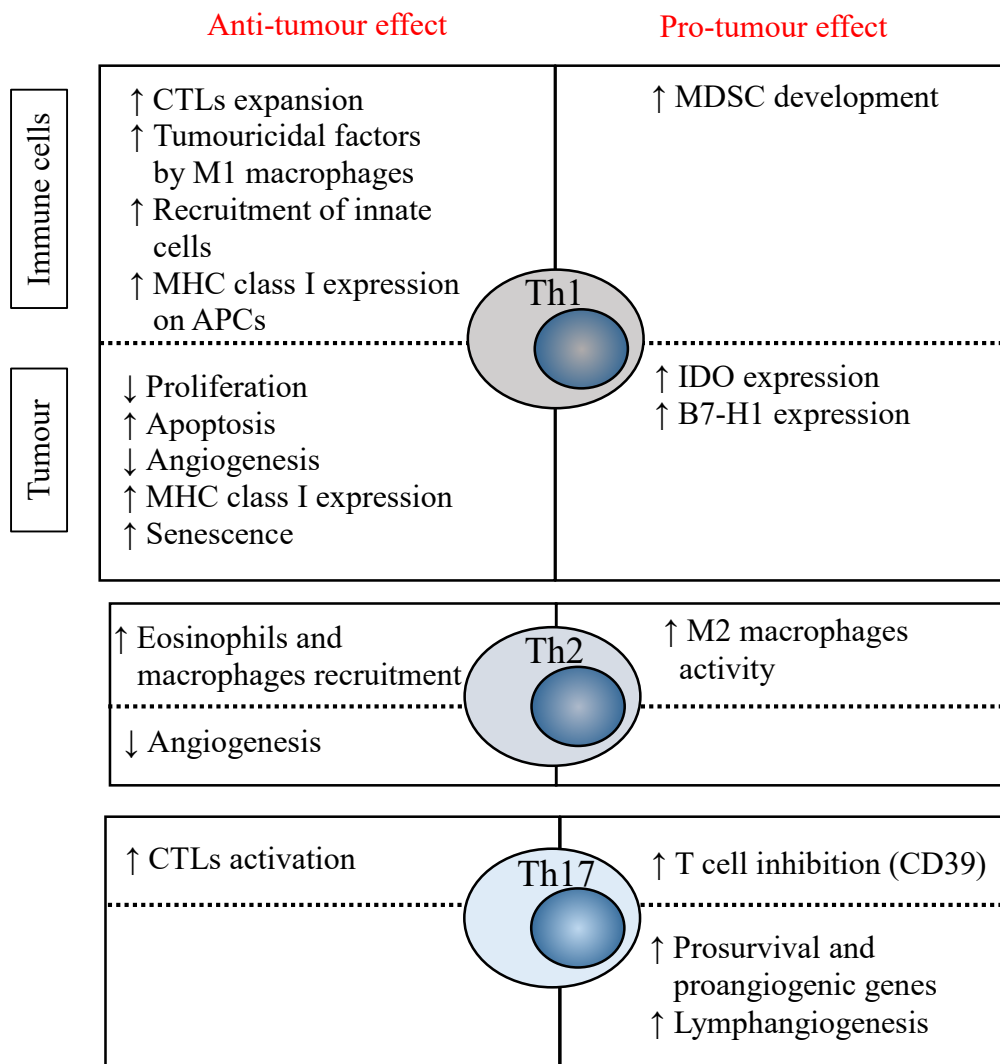
Figure 1.6. Anti- and pro-tumour functions of T helper cells. Even though T helper cells are important in preventing and suppressing the development of cancers, some of these cells may also contribute to cancer development.

There is evidence that specific factors of the Th2-mediated immune response can elicit anti-tumour activity. Indeed, IL-4 can rapidly elicit an inflammatory infiltrate comprising eosinophils and macrophages capable of killing tumour cells (75). In animal studies, the presence of eosinophils in tumour infiltrates has been shown to be driven by Th2 cells and was STAT6-dependant (76). In addition, tumour eradication can be mediated by anti-angiogenic cytokines such as IL-4, arguing for a positive role of Th2 cells in promoting tumour immunity (77). Although Th2 cells can contribute to tumour clearance, there is no evidence that they exert selection pressure arguing that Th1 cells have a more important role in tumour immunity.

In contrast to the above, many studies have demonstrated that Th2 cells have tumour-promoting capabilities (Figure 1.2). DeNardo *et al* revealed that IL-4-expressing T cells promote pulmonary metastasis of mammary carcinomas by eliciting the pro-tumoural activities of macrophages (78). The pro-metastatic Th2 response promoted the activation of M2 tumour-associated macrophages that in return released TGF-β and epidermal growth factor facilitating tumour progression. The finding that the Th2 CD4$^+$ T cells influenced metastasis but not primary tumour development implies opposing effects of T cells on primary carcinogenesis versus metastasis. In mice, IL-4 neutralizing antibodies (Abs) have been shown to limit pulmonary metastasis suggesting that anti-cancer therapies targeting Th2 cells and IL-4 might be relevant for treating late-stage disease.

1.3.2.3. Th17 T cells

The recent discovery of a third Th cell subset updated the Th1-Th2 dichotomy. Th17 cells are different from Th1 and 2 cells by their unique profile of effector cytokines and their development is independent of key cytokines central to Th1 and Th2 cells. The generation

of IL-17-producing T cells is dependent on IL-6 and TGF-β and expression of the transcription factor RORγt is critical for their differentiation (Figure 1.5) (79, 80). Along with IL-17, Th17 cells also produce IL-21, IL-22, and IL-26.

Th17 cells and the cytokines produced by these cells mediate immunity against extracellular bacteria that colonize exposed mucosal surfaces. Indeed, Th17 cells have been shown to mediate clearance of alveolar epithelium colonized by *Klebsiella pneumoniae* (81) and intestinal lumen colonized by *Streptococcus pneumoniae* in order to maintain lung and intestinal homeostasis (82). Mouse models have also indicated a protective effect of Th17 cells against fungal pathogens such as *Candida albicans* infecting oral mucosal (83). One of the mode of actions used by Th17 cells is the recruitment of monocytes/macrophages and neutrophils to sites of infections (82). The chemokine receptor CCR6 is highly expressed on Th17 cells, in contrast to CD62L or CCR7, suggesting that these cells do not home to lymphoid tissues but are recruited to inflamed tissues including tumours (84). Indeed, tumour cells, such as colon cancer cells, and inflamed tissues can express high level of CCL20 (CCR6 ligand), directing the migration of Th17 cells in these tissues (85).

The use of *IL-17a$^{-/-}$* mice demonstrated the role of Th17 in cancer immunosurveillance as these mice were more susceptible to melanoma in the lung (86). This study also showed that IL-17 secretion at the tumour site induced CCL2 and CCL20 production, which in turn attracted DC capable of promoting CTL activation. Moreover, the use of IL-7 as an adjuvant was found to increase Th17 cell activity and enhance vaccine-induced anti-tumour immunity (87). Similarly, IL-17A was reported to elicit the activation of tumour-specific CD8^{+} T cells (86).

Pathologically, Th17 cells have been implicated in promoting inflammation leading to several autoimmune disorders including psoriasis, inflammatory bowel disease or rheumatoid arthritis (88). IL-17, preferentially produced by Th17 cells, can act on various non-immune cells. For instance, IL-17 stimulates epithelial cells to secrete inflammatory cytokines (IL-6 and IL-8) leading to inflammation or matrix metalloproteinase (MMP) by macrophages leading to joint damage (89).

As IL-17 has inflammatory properties it has been suggested that Th17 cells drive inflammation-dependent tumour growth (Figure 1.2). Wu *et al* have tested this hypothesis in mouse and have shown that a commensal bacteria causing colitis promotes colon tumour formation by activating Th17 cells (90). IL-17 can enhance tumour growth directly through activation of STAT3 in tumour cells leading to the up regulating of pro-survival (Bcl-2) and pro-angiogenic (VEGF, MMP9) genes (91). In addition to the induction of blood vessel growth, IL-17 can promote lymphangiogenesis, favoring the spread of tumour metastasis to LNs (92). Recently, tumour-infiltrating Th17 cells from melanoma, colon and lung carcinoma tumour-bearing mice were found to express CD39 and CD73 ectonucleotidases which contribute to immunosuppression through facilitating adenosine production (93). Moreover, adoptive transfer of Th17 cells was shown to drive tumour growth in a CD39-dependent fashion. Furthermore, the expression of Th17-associated genes is associated with poorer outcome in CRC demonstrating the pro-tumour role of Th17 cells (94).

1.3.2.4. Th9 T cells

The latest addition to the list of CD4$^+$ T cell lineages is the Th9 subset. After activation by APCs in the presence of TGF-β and IL-4, naïve CD4$^+$ T cells differentiate *in vitro* into Th9 cells that secrete high levels of IL-9 and IL-10 (Figure 1.1) (95). Th9 development

require signals from both TGF-β and IL-4 receptors. Indeed, the transcription factors PU.1, downstream of the TGF-β signal, and IRF4, expressed in response to IL4-activated STAT6, are essential for the development of IL-9-secreting T cells (96) (97). Furthermore, Th9 cells represent a distinctive subset of T cells, as they express none of the major transcription factors of other Th cells (T-bet, GATA-3, RORγt, and Foxp3) and therefore none of their cytokines, except IL-10 (98).

Depending on the cytokines present in the environment during naïve T cell activation, IL-9 has been found to be secreted by other CD4$^+$ T cells than Th9 cells, including Th17 cells (when activated with TGF-β + IL-6) or Tregs (with TGF-β + IL-2) (99). TGF-β and IL-4 have also been found to be able to reprogram Th2 cells toward a Th9 phenotype (98). Therefore, the IL-9 system provides an example of Teff cell plasticity (at least *in vitro*) and precautions must be taken when evaluating the role of IL-9-secreting Th9 cells.

Licona-Limón *et al* unambiguously addressed the role of IL-9 when secreted by Th9 cells by investigating the influence of IL-9-derived Th9 cells on the type 2 immune responses in the context of parasitic helminth infections (100). The use of IL-9 fluorescent reporter mice identified Th9 cells and type 2 innate lymphoid cells as main source of IL-9 *in vivo*. By using *IL-9−/−* mice, they showed that IL-9 was required for IL-5 and IL-13 expression leading to parasite expulsion. Moreover, adoptive transfer of Th9 cells in Rag2-deficient mice, in contrast to Th2 cells, was associated with worm expulsion. These observations were the first to demonstrate IL-9-secreting Th9 cells as regulators of type 2 immune response *in vivo*. A recent paper characterized the presence of Th9 cells in a model of chronic airway hyperreactivity, driven by type 2 immunity (101). Mice exposed to allergens displayed an accumulation of IL-9-, but not IL-4 and IL-13-, secreting T cells

in their lungs. These cells were classified as Th9 cells. Treatment with anti–IL-9 Abs reduced the inflammation.

A protective effect of Th9 cells has been described in two mouse models of melanoma (102) (103). Purwar *et al* showed that tumour-Ag-specific Th9 cells could suppress subcutaneous melanoma growth and neutralisation of IL-9 abrogated the effect. Also, the percentage of Th9 cells was higher in the skin and blood than metastatic lesions of melanoma patients, supporting that a paucity of Th9 cells promotes tumour growth (102). In addition, Lu *et al* reported that Th9 cells inhibited pulmonary melanoma progression by promoting CD8$^+$ T cells activation through recruitment of DCs into tumour tissues (103).

Recently, Th9 cell-derived IL-9 has been implicated in the development of ulcerative colitis (UC) (104). Authors found that patients with UC had increase numbers of mucosal T cells expressing IL-9 and PU.1 compared to control subjects. Also, these patients had more intestinal epithelial cells expressing IL-9 receptor than control subjects. This study used a mouse model of oxazolone-induced colitis which revealed that IL-9 produced by Th9 cells promoted disease by impairing intestinal barrier function. As ulcerative colitis is associated with an increased risk for the development of CRC, Th9 cells may also play a role in this disease (105).

1.3.2.5. Follicular helper T cells

One way that Th cells offer host protection against specific pathogens is the generation of Abs that neutralize and clear pathogens. In order to differentiate into Ab-secreting plasma cells, B cells need to interact with Th cells. Th2 cells were thought to provide help to B cells, but their roles seem to be restricted to isotype switching as mice lacking

regulators of Th2 differentiation form germinal centres and maintain Ab responses (106). Instead, another CD4 subset was found to express the chemokine receptor CXCR5 and provide help for B cells differentiation in B cell follicles (107). These cells were termed follicular B helper T cells or Tfh cells.

Like their CD4 T cells counterparts, Tfh cells require specific cytokines (IL-21) and transcription factors (Bcl6) (Figure 1.1) (108). Studies have also shown that Tfh cells express a unique association of activation markers (CD40L, PD-1), inducible T cell costimulator (ICOS), CD84 receptor and secrete B helper cytokines such as IL-21 (109). These molecules play a central role in Tfh cell function and the delivery of helper signals for B cell activation and survival. Indeed, CD40L– CD40 interactions induce maturation of DCs, which in return support Tfh cell activation, CXCR-5 expression and migration in B cell follicles (110). PD-1 plays a role in promoting humoral response as PD-1-deficient Tfh cells secrete lower amounts of B-cell-helping cytokines IL-4 and IL-21 leading to reduced numbers of Ab-producing plasma cells (111). ICOS, a CD28 family member, is required for Tfh differentiation as ICOS-deficient mice show poor T cell-dependent B-cell responses (112) and reduced numbers of CXCR5$^+$CD4 T cells (113). The same loss of Tfh cells was observed in ICOS-deficient patients in association with disturbed germinal centre (GC) formation (113).

Tfh cells have been associated with the development of autoimmune diseases. For instance, high levels of CD4$^+$CXCR5$^+$PD1$^+$ T cells have been detected in the peripheral blood of patients with rheumatoid arthritis suggesting a role for Tfh cells in mediating autoimmunity (114). In contrast, loss of Tfh cells has been linked to immunodeficiency as CD40L- or ICOS-deficient individuals have altered humoral responses and lack intact GCs in secondary lymphoid tissues (114).

The role of Tfh cells in tumour immunity is not yet clear. A recent analyses of tumour-infiltrating lymphocytes in breast cancers however revealed a high proportion of Tfh cells. Also, the presence of Tfh cells had a positive prognostic value (115). These data imply that Tfh-dependent humoral immunity is required for an optimal anti-tumour response. Moreover, the same study showed that Tfh cells are an important constituent of tertiary lymphoid structures in breast tumours. It is therefore plausible that Tfh cells influence the formation of a distinct structure to improve tumour immunity. Moreover, these tertiary lymphoid structures have been observed in many cancers and their presence correlates with a better prognosis, reviewed in (116).

Within the follicular lymphoma microenvironment however, Tfh cells have a crucial role in the survival of malignant B cells (117). Gene expression analysis of sorted follicular lymphoma-derived Tfh revealed an over-expression of CD40L and IL4 compared to tonsil-derived Tfh. Importantly, these two molecules, implicated in normal B-cell growth, rescued malignant B cells from anti-CD20 mAb rituximab-induced apoptosis.

1.3.3. Regulatory T cells

Sakaguchi *et al* in 1990s observed that inoculation of CD25 (IL-2 receptor α-chain)-depleted CD4 population to immune-compromised (athymic nude) mice induced autoimmune diseases (118). Also, the pathology could be prevented by co-injection of CD25$^+$ cells. This was the first evidence of CD25-expressing T cells maintaining self-tolerance in the periphery by suppressing autoreactive T cells that escape thymic negative selection. Based on CD25 marker, a new population of Tregs derived from the thymus

and naturally nonproliferative (i.e. anergic) to TCR stimulation *in vitro* was described in the peripheral blood of normal human adults (119).

Although IL-2 receptor is essential for Tregs as IL-2 is critical for their development and peripheral expansion, CD25 is also upregulated by activated T cells. Subsequent studies in humans and mice discovered another marker more specific for Treg: the Forkhead box protein 3 (Foxp3) transcription factor. In humans, mutations in Foxp3 gene is associated with IPEX syndrome, characterized by multiorgan autoimmunity as a result of defective Treg immunosuppressive functions (120). Similar symptoms of autoimmune diseases are observed in scurfy mice with a frameshift mutation in Foxp3 gene (121).

Foxp3 is critical for Treg development and function. Indeed, mice lacking Foxp3 are associated with fatal multi-organ autoimmune pathology driven by uncontrolled autoreactive T cells (122). But Tregs can also mediate immune responses in different contexts such as intestinal homeostasis (123), virus infection (124) or cancer (125). In addition to the thymus-derived Treg (tTregs), other Treg subtypes, with the same ability to regulate the immune system, can be found (depending on environmental conditions), such as peripherally-induced Tregs (pTregs). Because Tregs can also be generated *in vitro* from non-Treg cells (named induced or iTregs), suggestions were made to use the term pTregs to describe the Tregs that differentiate *in vivo* in periphery (126).

1.3.3.1. Thymus-derived Tregs

The large majority of Foxp3$^+$ Tregs that are present in the periphery arise from the thymus (127). The first indication that Tregs arise from the thymus was obtained in 1969 when it was observed that neonatal thymectomy resulted in the development of inflammation of the ovary (128). Whilst this was at first thought to be due to an endocrine function of the

thymus, later experiments indicated that the pathology was in fact due to the absence of Treg-mediated immunosuppression (129)

Strong TCR interactions with self-Ag expressed by MHC class II molecules on thymic epithelial cells in the presence of co-stimulation select immature thymocytes as Foxp3$^-$CD25$^+$ Treg precursors. This high-affinity interaction for self-peptide is crucial for the selection of CD4$^+$CD25$^+$ precursors; thymocytes expressing lower affinity TCRs are not selected to become Tregs (38). The recognition of self-peptides by TCRs expressed by thymic Treg triggers several downstream signalling pathways leading to the induction of Foxp3 expression. After selection, tTregs leave the thymus as mature cells with a defined phenotype in contrast to the other thymus-derived naive T cells where lineage commitment occurs upon Ag exposure.

tTregs can be specific for both self and exogenous Ags expressed by bacteria, virus or tumours demonstrating the diversity of their TCR repertoire. Indeed, although tTregs represent 5–10% of the circulating CD4$^+$CD25$^-$ T cells in humans, their TCR repertoire is as diverse as the CD4$^+$CD25$^-$ T cell repertoire (130). Furthermore, the tTreg TCR repertoire only partially overlaps with CD4$^+$CD25$^-$ T cell TCR repertoire as a broad TCR specificity for a large set of self-Ag is required for tTregs (131).

1.3.3.2. Peripherally-induced Tregs

Even though Foxp3$^+$ tTregs represents a specific T cell lineage, CD4$^+$ T cells can up regulate this transcription factor after Ag stimulation in the periphery. Foxp3 induction and differentiation into immunosuppressive T cells that occur *in vitro* after TCR interaction and co-stimulatory signalling is dependant of TGF-β (Figure 1.1). *In vivo*, the gut environment is a preferential site to generate pTregs because of its ability to secrete

large amount of TGF-β. Sun *et al* showed that this peripheral conversion of Tregs required DCs from the lamina propria, whose action was dependent on TGF-β and retinoic acid (132).

Although commonly accepted, the *in vivo* conversion of Foxp3⁻ T cells into Foxp3⁺ Tregs is difficult to observe by phenotypic analysis as definitive lineage biomarkers distinguishing tTregs and pTregs are lacking. Two markers, the transcription factor Helios (133) and neuropilin-1 (Nrp-1) (134), were found to be expressed at high level on tTregs. However, Helios is considered to be a more reliable marker to define tTregs as Nrp-1 can also be expressed by pTregs during certain circumstances (inflammation, APCs encountered) (135).

There is a small overlap between the TCR repertoires of pTreg and tTreg and hence these populations are believed to recognize different Ags (136). tTregs are most likely to be specific for self-Ags recognized during thymic selection, while pTregs, as derived from CD4⁺ T cells, recognize foreign Ags. Consistent with this, Josefowicz *et al* showed that mice deficient in pTregs spontaneously developed Th2 inflammation at mucosal sites (gastrointestinal tract and lung) (137). Therefore both populations may have distinct functions with tTregs controlling systemic autoimmunity and pTregs suppressing allergic inflammation at mucosal interfaces.

Several studies have investigated the origin of tumour Tregs focusing on Helios expression. In colorectal tumours, the proportion of tumour-infiltrating Tregs expressing Helios was slightly lower than in peripheral blood suggesting that conversion is not the main mechanism that contributes to enrich the intratumoural Treg population (138). Instead, the specific recruitment, from the mucosa and blood, or the local expansion of Tregs may largely contributes to increase the Treg population in tumours.

1.3.3.3. Polarized Treg subtypes

Recent studies have demonstrated that Foxp3$^+$ Treg population is not homogenous but includes subpopulations with specific phenotypes and distinctive functions, reviewed in (139). As described previously, several transcriptional regulators orchestrate differentiation into specific Th CD4$^+$ cells (T-bet for Th1 cells, IFR4 for Th2 cells, RORγt for Th17 cells and Bcl6 for Tfh cells). The same transcription factors can effectively drive Treg differentiation towards one particular subset to control the corresponding helper CD4$^+$ T cells types on the basis of tissue localization.

This "Th class"-specific control suggesting that Tregs can specialize and adapt to particular environments has been described in 5 different studies. Koch *et al* showed that acquisition of T-bet by Tregs induced the expression of the chemokine receptor CXCR3, which enabled the migration of Tregs at sites of Th1 cell-mediated inflammation (lungs of *M*. tuberculosis-infected mice) and the suppression of Th1 cell-mediated responses (140). Mirroring the development of Th1 cells, signaling through the IFN-γ receptor was required for T-bet induction in Tregs as lower frequency of CXCR3$^+$T-bet$^+$ Treg was observed in *IFNGR1*$^{-/-}$ or *STAT1*$^{-/-}$ mice. In another study, Zheng *et al* demonstrated that Foxp3 induce IRF4 expression in Tregs in order to facilitate their control of Th2 responses (141). Indeed, mice containing IRF4-deficient Tregs showed a selective dysregulation of unprovoked pathogenic Th2 responses (high serum concentration of IL-4-dependent IgG1 and IgE and plasma cell tissue infiltration). Similarly, Chaudhry *et al* demonstrated that selective ablation of STAT3 (transcription factor mediating Th17 induction) in Tregs resulted in uncontrolled Th17-dependent pathology characterized by the development of a fatal intestinal inflammation (142). Finally, Tregs expressing Bcl6 have been shown to be essential in the regulation of germinal centre responses, through the expression of CXCR5 (indispensable to enter B-cell follicles) (143). Mice containing *CXCR5*$^{-/-}$ Tregs

and *Bcl6$^{-/-}$* Tregs were inefficient in controlling germinal centre reactions as higher affinity maturation of Abs and plasma cell differentiation were observed in these mice. Consistent with these results, Linterman *et al* showed that the CXCR5^{+}Bcl6^{+} Tregs control germinal centre B cells indirectly through the regulation of Tfh cell numbers (144).

Transcription factors contributing to the gain of specialized suppressive functions in Tregs are known, unlike the molecules mediating these effects. A recent study addressed this point and characterized a distinct Treg subset expressing the coinhibitory molecule TIGIT, which mediated the selective suppression of Th1 and Th17 cells, leaving intact the Th2 cells (145). This inhibition by TIGIT^{+} Tregs was dependent on fibrinogen-like protein 2 secretion, known to inhibit DCs (146).

This concept that a subset of Tregs inhibits a corresponding Th CD4 T cell subset has great implications in human cancer. Indeed, one Treg subtype suppressing a beneficial Th anti-tumour response might be specifically targeted to increase host anti-tumour immunity. In other words, rebalancing Tregs subsets instead of eliminating the Treg population. Identification of surface molecule, such as TIGIT, expressed by Tregs with specialized functions against Th cells may serve as targets in cancer therapy.

Few studies have investigated the role of these polarized Tregs in cancers. CXCR3^{+} T-bet^{+} Tregs are highly enriched in ovarian cancer and selectively suppress Th1 beneficial responses (147). Another study showed that RORγt-expressing Tregs expand in human colon cancer and are associated with disease progression because RORγt^{+} Tregs have impaired anti-inflammatory activities (148). Consistent with this, β-catenin was found to trigger RORγt expression in Tregs in colitis and colon cancer, and could compromise Treg properties by affecting their anti-inflammatory properties (149).

1.3.3.4. Inhibitory cytokines

<u>IL-10</u>

Determining the mechanisms used by Tregs is an area of intense research and should increase our knowledge of the regulation of peripheral tolerance and may improve anti-tumour therapy efficacy by revealing ways in which Tregs can be selectively targeted. Firstly, Tregs can suppress by secretion of inhibitory cytokines, mainly interleukin-10 (IL-10) and TGF-β. Originally thought to be exclusively secreted by Th2 cells to inhibit Th1 responses, IL-10 is also produced by DCs, macrophages and Foxp-3^{-} T cells, including Th1 cells during parasite infections or Teff cells during influenza infections, reviewed in (150) (151). IL-10 has significant anti-inflammatory properties allowing the inhibition of macrophages and DCs. They first lose their ability to secrete pro-inflammatory cytokines and secondly, fail to mature and express co-stimulatory molecules (150). Therefore, the suppressive effects of IL-10 on T cells are predominantly indirect. However, some studies have reported a direct inhibitory effect of IL-10 on T cells, impairing IL-2 production and growth (152) (153).

IL-10 secreted by Tregs can control allergic responses (asthma or eczema) by inhibiting allergen-specific Th2 responses indicating that Tregs can suppress responses to environmental Ags, reviewed in (154). This inhibition is likely to be altered in those who develop allergic diseases. Early observations showed that IL-10 deficient mice suffered from chronic enterocolitis that began after colonization of the gut with the adult microflora (155). Studies relying on adoptive T cell transfers into immunodeficient mice indicated that IL-10 is crucial in the ability of Tregs to control responses against intestinal Ags (156). Furthermore, in addition to the colon, the selective knockout of IL-10 expression in Foxp3^{+} cells was associated with inflammation in lungs and skin

demonstrating that IL-10 secretion is necessary to regulate immune responses at the body's environmental interfaces (157). In the same mouse model, IL-10-deficiency was not followed by the development of spontaneous systemic autoimmunity suggesting that IL-10-mediated Treg function is a mechanism utilised in specific tissues.

In addition to bacterial, parasitic and inflammatory diseases, IL-10 is involved in tumour pathology. Indeed, the presence of IL-10 in advanced metastases and the positive correlation between serum IL-10 levels and cancer progression suggest an important role of this cytokine within the tumour (158). However, no link with Tregs was found in these studies as tumour cells were identified as the main source of IL-10 (159). In contrast, there is evidence that IL-10 secreting Tregs have beneficial effects by controlling tumour-driven inflammation. Indeed, within the tumour of mice with MC38 adenocarcinoma, activated Tregs were the main source of IL-10 that could suppress pro-inflammatory tumour infiltrating Th17 cells (160). Also, IL-10 secreted by Tregs suppresses Th17 cell-driven colitis (161). These data indicate that therapeutic approaches aimed at suppressing tumour infiltrating Treg activity may be harmful when the Tregs are inhibiting Th17-driven inflammation.

TFG-β

TFG-β is a critical regulator of the immune system: $TGF\text{-}\beta1^{-/-}$ mice develop multi-organ inflammatory diseases leading to organ failure and death (162). Like IL-10, different cell types express TGF-β1, thereby making it difficult to analyse its involvement in Treg function. Conflicting *in vitro* studies have shown that Treg suppressor function can occur in the absence of TGF-β1 production (163) while other studies indicated that *in vitro*-stimulated Tregs exert immunosuppression by cell-surface-bound TGF-β1 (164). However, there is clear *in vivo* evidence that TGF-β produced by Tregs is important in

the control of autoreactive CD8$^+$ T cells in type 1- diabetes (165). Further evidence of the direct inhibitory effects of TGF-β is the observation that in mouse models of inflammatory bowel disease (IBD), CD4$^+$ T cells that cannot respond to TGF-β (absence of a functional TGF-β receptor) escape Treg–mediated control and induce colitis (166).

Strauss *et al* investigated Tregs in patients with head and neck squamous-cell carcinoma and reported that Tregs were enriched within the tumour (167). These Tregs were IL-10$^+$TGF-β1$^+$ (revealed by staining of the tumours by immunofluorescence) and mediated stronger suppression than peripheral Tregs. Neutralizing IL-10 and TGF-β abrogated these effects. In a mouse model of colon carcinoma, Tregs were able to abrogate tumour-specific-CD8$^+$ T cell-mediated tumour rejection. This inhibition was TGF-β-dependent as tumour-specific CD8 cells expressing dominant-negative TGF-β receptors were resistant to suppression (168). Therefore, Tregs acting through TGF-β signaling can inhibit anti-tumour immunity.

1.3.3.5. Cytolysis

Cytolysis mediated by the perforin/granzyme B pathway was originally thought to be only used by NK cells and CD8$^+$ T cells to kill target cells. Grossman *et al* showed that Tregs can exhibit cytotoxicity against autologous target cells through the perforin/granzyme-dependent pathway (169). In this study, Tregs could kill a variety of cells, including CD4$^+$ and CD8$^+$ T cells and immature or mature DCs. Further *in vivo* study showed that perforin/granzyme B were involved in Treg cell-mediated suppression of tumour clearance (170). In this model, RMAS lymphoma, B16 melanoma and acute myeloid leukemia tumours provoked granzyme B expression in Tregs to induce NK and CD8$^+$ T cell death (Figure 1.7). Another study revealed by *in vitro* cytotoxic assay and live microscopy that DCs bearing tumour Ags can be killed by Tregs in tumour-draining
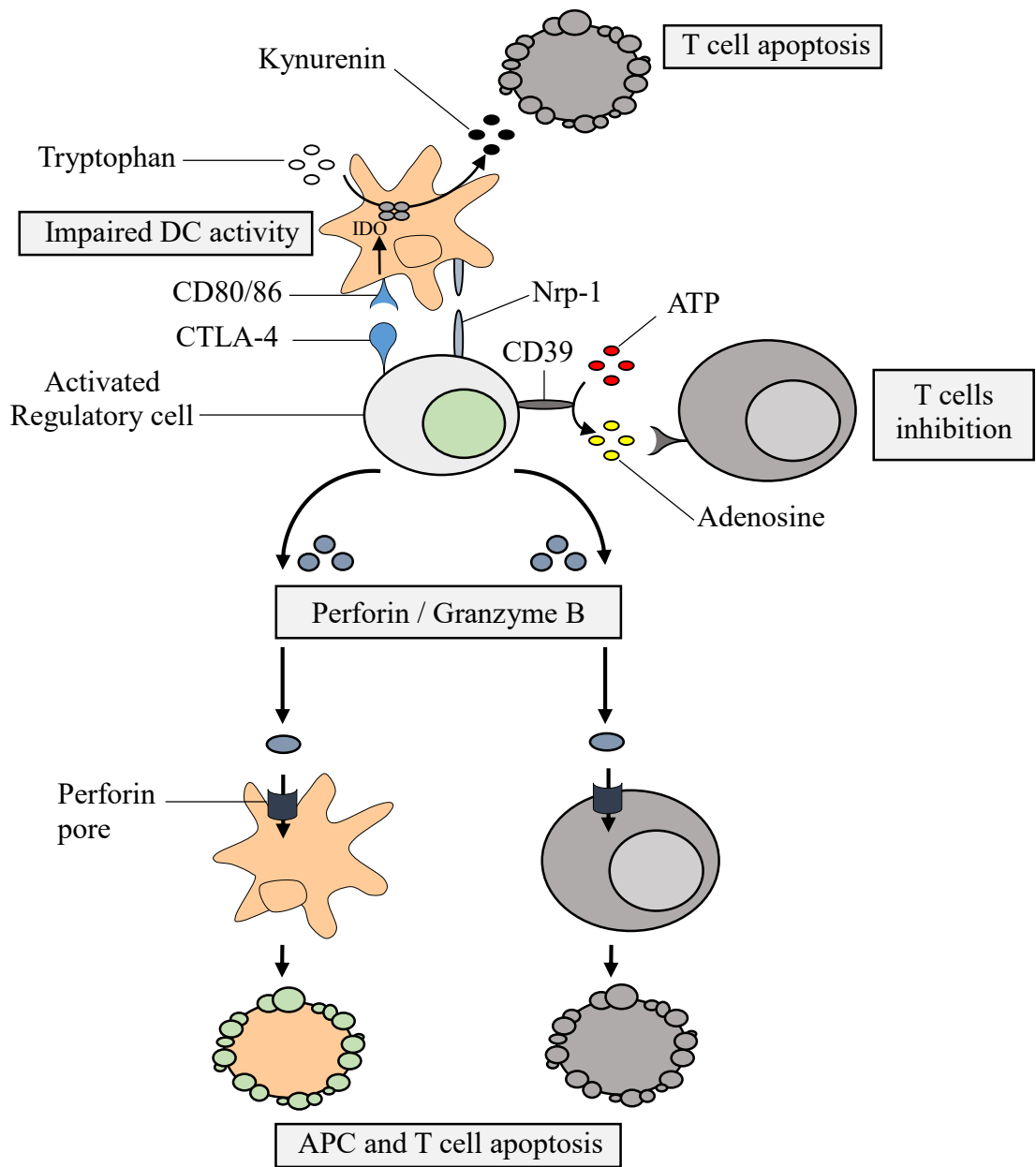
Figure 1.7. Suppressive mechanisms of tumour-infiltrating Tregs. Different mechanisms are used by intratumoural Tregs to inhibit targeted cells. Surface molecule CTLA-4 by binding to CD80/86 can impair DC ability to stimulate T cells and by inducing kynurenine production promotes T cell apoptosis. CD39 ectoenzyme by generating adenosine from ATP induces T cell inhibition. Two other enzymes, perforin and granzyme B, mediate cytolysis.

LNs inhibiting effective CD8$^+$ T cell priming (171). This Treg-mediated DC death was perforin dependent and involved direct contact between the two populations via MHC class II molecules on APCs.

1.3.3.6. Metabolic disruption

IL-2

IL-2 is not essential for Treg development in the thymus but is crucial for maintenance of these cells in the periphery as observed by the absence of peripheral Tregs in IL-2 deficient mice (172). Therefore, although Tregs do not produce IL-2, they require this cytokine for their survival. IL-2 is also an essential cytokine for T cell activation and proliferation. In an *in vivo* study, Pandiyan *et al* showed that Tregs can induce IL-2 deprivation leading to the apoptosis of CD4$^+$ Teff cells (173).

Ectoenzymes

Tregs can suppress through metabolic disruption. The ectoenzymes CD39 and CD73 are expressed by Tregs and facilitate production of pericellular adenosine from extracellular nucleotides, such as ATP and ADP (Figure 1.3). Importantly, the binding of adenosine to adenosine receptors on CD4$^+$ Teff cells inhibits their functions and Tregs from CD39-null mice are compromised in their ability to suppress T cells (174). Stagg *et al* showed that CD73 expression on Tregs is involved in their pro-tumourigenic effect (175). Treg-mediated suppression may also include the direct transfer of inhibitory cAMP into CD4$^+$ T cells via membrane gap junctions during intercellular communication (176).

1.3.3.7. Effects via APCs

CTLA-4

Direct inhibition of T cells is not the only mode of action used by Tregs as they can also directly influence the performance of APCs. Tadokoro *et al* revealed by intravital microscopy that Tregs can attenuate the establishment of stable contact between Ag-specific T cells and Ag-expressing DCs in LNs *in vivo* by direct interaction with DCs thus preventing T cell activation (177). These imaging data suggest that Treg inhibition takes place during the early phase of the immune response (priming) in LNs. Although the mechanism involved in this process is not clear, data suggest the involvement of CTLA-4. Indeed, CTLA-4 expressed in Foxp3$^+$ Tregs has been shown to mediate the down-regulation of CD80 and CD86 on mature DCs limiting the activation of naive T cells via CD28 (Figure 1.3) (178). CTLA-4–deficient mice develop a lethal lymphoproliferative disease, indicating the vital role of CTLA-4 as a negative regulator in lymphocyte homeostasis (31)

Early experiments in murine models using anti-CTLA-4 specific Abs have been shown to enhance anti-tumour immunity, thus revealing the potential of CTLA-4 as a target in cancer therapy (179). This was confirmed with one trial using human anti-CTLA-4 monoclonal Abs in patients with metastatic melanoma where prolongation in overall survival was found (180). Evidence suggests that Tregs may be involved in these beneficial effects. Indeed, Tregs lacking CTLA-4 have impaired *in vivo* and *in vitro* suppressive functions, leading to enhanced tumour immunity (178). While Tregs constitutively express CTLA-4, Teff cells only upregulate this co-stimulatory molecule after activation in order to attenuate the immune response (181). Peggs *et al* showed that blockade of CTLA-4 on both Teff and Treg cells contributes to the anti-tumour effect of administering anti–CTLA-4 Abs (182).

An explanation for CTLA-4-mediated inhibition was provided by Fallarino *et al*. They showed that Tregs can promote the IDO-mediated catabolism of tryptophan in DCs

in a CTLA-4-dependent manner (183). The product of tryptophan catabolism (kynurenin) is pro-apoptotic and leads to Teff cell apoptosis (Figure 1.3). Os *et al* described another mechanism in which CTLA-4 can capture CD80 and CD86 from APCs by trans-endocytosis (184). Subsequently, these ligands were degraded in CTLA-4$^+$ cells impairing costimulation by CD28.

LAG-3

Lymphocyte-activation gene 3 (LAG-3) is another key molecule involved in direct regulation of DCs. This transmembrane protein, with a 30% homology to CD4 (185), was observed in mouse to be expressed on activated Tregs with suppressor activity (186).

LAG-3 is involved in cell-contact interaction between DCs and Tregs by binding to MHC class II molecules expressed on immature DCs (187). This interaction induces the ITAM-mediated inhibitory signaling pathway which requires FcγRγ and ERK for the recruitment of SHP-1 (187). As a result, DC maturation and stimulatory activities are inhibited. As LAG-3 was described as a Treg-associated marker in mice, several studies aimed at identifying LAG-3$^+$ Treg in human tumours have been conducted. Camisaschi *et al* found that LAG-3-expressing Tregs represent a small population within CD4$^+$CD25^{high} (CD25^{hi}) T cells but increased in the peripheral blood mononuclear cells (PBMCs) and tumours of melanoma and CRC patients (188). These cells, from PBMCs of healthy donors, had enhanced suppressor activity as compared with the LAG-3$^{-/low}$ Tregs. A recent study revealed that LAG-3 is not restricted to Foxp3$^+$ Tregs but can be expressed in Foxp3$^-$ T cells with more potent immunosuppressive activity (138).

Neuropilin-1

It has been shown that Nrp-1 expression on Tregs prolongs their interaction with immature DCs during Ag recognition *in vitro* (189). Therefore Nrp-1 molecules promote

TCR signaling on Tregs allowing their preferential stimulation over Teff cells of the same specificity (189). Moreover, the addition of lipopolysaccharide (LPS) restored the long interaction between naïve T cells and DCs suggesting that Nrp-1 provides adhesive features on Tregs in the absence of danger signal (such as LPS) in mice. The restored interaction between naïve T cells and DCs with LPS was most likely caused by changes in DC morphology. However, induction of Nrp-1 expression on naïve T cells was not verified.

Recent work showed that Nrp-1 is involved in the ability of Tregs to inhibit tumour immunity (190). Indeed, mice with Nrp1-deficient Tregs exhibit delayed B16 melanoma tumour growth, a higher percentage of tumour-infiltrating CD8$^+$ T cells, and increased survival compared to those with wild type Tregs. Moreover, blockade of Nrp1 using monoclonal Abs in wild type mice promoted anti-tumour immune responses and significantly decreased tumour growth.

GITR

In order to identify genes encoding unique Treg molecules, a study conducted by McHugh *et al* compared by microarray, gene expression in CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells (191). Several genes were found to be overexpressed by CD4$^+$CD25$^+$, suggesting their involvement in Treg activity. Among the mRNAs induced at high level in the CD25$^+$ subpopulation was that of glucocorticoid-induced tumour necrosis factor-related receptor (GITR). GITR, a member of TNF receptor superfamily, is constitutively expressed on Foxp3$^+$ Tregs and is also upregulated on CD4$^+$ Teff cells following activation (191). The addition of anti-GITR Abs in co-cultures of Treg and Teff cells abrogates the suppressive effects of Tregs suggesting that GITR had a vital role in Treg function (191).

PD-1

Costimulatory interactions between PD-1 receptor and its ligands PD-L1 and PD-L2 are crucial for the regulation of immune tolerance. The first role of PD-1 as negative regulator of immune responses came from the observation that *PD-1$^{-/-}$* mice exhibit lupus-like autoimmune diseases (192). PD-1: PD-L interactions mediate peripheral tolerance in several ways. After T cell activation PD-1 is upregulated and engagement with its ligands on APCs transduces a signal leading to suppression of T cell proliferation (193). PD-1 is also expressed at high level by Tregs, suggesting a role for this molecule in Treg function. There is no clear data showing PD-1-mediated inhibition by Tregs but as PD-1 ligands are expressed by DCs, Tregs may restrain the magnitude of the immune response by suppressing DC activity through PD-1 signaling.

1.4. Tumour antigens

1.4.1. Classification

1.4.1.1. High tumour specificity

Three classes of Ags with high tumour specificity can stimulate immune response against tumour cells: certain viral Ags only expressed in tumour cells, mutated Ags and Ags encoded by cancer-germline genes.

Viral Ag

Some cancers are attributable to viral infections; 15 % of the worldwide cancer incidence (194). Several viruses are associated with human malignancies, including Epstein-Barr virus (EBV) and human papillomaviruses (HPVs). As intracellular pathogens, viruses encode proteins that interfere with intracellular signalling pathways leading to modified cellular activity and tumourigenesis. However, some of the key viral proteins expressed

in tumour cells can be recognized by T cells inhibiting tumour growth. For instance, EBV-specific CTLs have been characterized in the blood and tumours of patients with nasopharyngeal carcinoma (195).

Mutated Ag

Neo-Ags expressed by tumour cells are derived from oncogenic mutations. These tumour-specific Ags (TSAs) can be generated after single amino acid changes, due to mutations which alter reading frames (neoORFs) or modifications of stop codons. A few studies have characterized T cell responses against neo-tumour Ags. For instance, tumour-infiltrating $CD4^+$ T cells were able to recognize a peptide derived from frameshift mutations in TGFβRII in colon cancer patients (196). Even though mutated Ags are difficult to use in large-scale vaccination, as mutations are often spontaneous and unique to individual tumours, RNA sequencing of tumour and normal tissues is currently being used to identify new and potentially more useful targets. In this regard, Schumacher *et al* demonstrated that a vaccine comprising mutated anti-isocitrate dehydrogenase type 1 could be used as a therapeutic treatment for isocitrate dehydrogenase type 1-mutated tumours (197).

Cancer-testis Ag

Cancer-germline (or cancer-testis) genes can encode for many T cell Ags expressed by tumours, the melanoma-associated Ags (MAGE) family being the first described in humans (198). These genes are uniquely expressed in tumours, male germline cells and trophoblastic cells after demethylation. However, the two last cell types do not express HLA class I or class II molecules (they are present in immune privileged sites) and therefore only cancer germline Ags on tumour cells can be targeted by the immune system. These TSAs are expressed by many different tumours, for instance the trophoblast glycoprotein 5T4 is expressed on breast, renal and colon cancers (199).

1.4.1.2. Low tumour specificity

Two classes of Ags with low tumour-specificity (also called tumour-associated Ags) have been found.

<u>Differentiation Ag</u>

Differentiation Ags are encoded by genes expressed in tumour cells and in the normal tissues from where the tumours emerged. Such Ags were originally described in melanoma patients where CTLs specific for MART1 and GP100 were shown to kill both melanoma cells and melanocytes (200). The use of these Ags in immunotherapy can stimulate anti-tumour immunity leading to longer progression-free survival (201).

<u>Overexpressed Ag</u>

In some cases, tissue Ags can be expressed at lower levels on normal cell types than on tumour cells. This overexpression can confer some degree of tumour specificity as T cells require strong TCR signalling to be activated. Indeed, some of these Ags can be recognised by T cells without causing autoimmunity. In a recent study, Chapuis *et al* injected CTLs specific for the overexpressed Wilms tumour Ag 1 (WT1), after hematopoietic cell transplantation, and demonstrated anti-leukemic activity without obvious autoreactivity in patients (202).

1.4.2. Tumour antigen-specific Tregs

The recognition of Ags by Tregs in cancer patients can lead to the suppression of CD4^{+} Teff cell activity (Table 1.1). Wang *et al* established Treg clones from TILs of melanoma patients and found that LAG-1- and ARTC1- (cancer germline Ags) specific Tregs could be identified (203) (204). In both studies, Tregs activated by these Ags could inhibit the ability of CD4^{+} Teff cells to secrete IL-2 after recognition of tumour cells. Another study

Antigen	Antigen feature	Treg feature	Cancer	Ref.
LAGE-1$_{108\text{-}120}$	Cancer germline Ag	Secrete IFN-γ and IL-10, express GITR, cell/cell contact–dependent suppression, isolated from TILs	Melanoma	(203)
ARTC1 (15-mer peptide)	Mutated Ag	Secrete IFN-γ and IL-10, cell/cell contact–dependent suppression, isolated from TILs	Melanoma	(204)
gp100$_{369–383}$ TRP-1$_{449–463}$ Survivin$_{54–67}$ NY-ESO-1	Differentiation Ag Differentiation Ag Overexpressed Ag Cancer germline Ag	Secrete IL-10, cell/cell contact–dependent suppression, proliferation upon Ag stimulation, isolated from blood	Metastatic melanoma	(205)
WT1$_{333\text{-}347}$	Overexpressed Ag	Th2 cytokine profile, secrete granzyme B	Leukemia	(206)
Telomerase CEA Mucin-1 HER2/neu EGFR	Overexpressed Ag Differentiation Ag Cancer germline Ag Overexpressed Ag Overexpressed Ag	Inhibit proliferation of Teff cells	Colon cancer	(207)
E6, E7	Viral Ag	Inhibit proliferation of Teff cells	Cervical cancer	(208)
EBNA1	Viral Ag	Clones express GITR and mediate suppression in soluble factor–dependent or cell/cell contact dependent manners	/	(209)
mam$_{34\text{-}48}$	Overexpressed Ag	Control INF-γ secretion of Ag-specific Teff cells after Ag activation	Breast cancer	(210)

Table 1.1. Regulatory T cells specificity for tumour antigens.

by Vence *et al* characterized circulating Tregs specific for four different tumour Ags in cancer patients without the need to clone TILs (205). It is noteworthy that certain regions of one tumour Ag (NY-ESO-1) that contained Treg epitopes also contained many CD4^{+} helper T cell epitopes (211). Tumour Ag-specific Tregs have also been reported in leukemia patients (206). These Tregs could produce granzyme B after Ag stimulation and selectively induce apoptosis in APCs, but not in leukemia cells, expressing the same tumour Ag. In order to characterize Treg specificity in colon cancer, Bonertz *et al* found circulating Tregs specific for a variety of tumour Ags in CRC patients and that *in vitro* depletion of these cells could unmask tumour Ag-specific T cell responses (207).

Tregs in cancer patients also recognize foreign tumour Ags encoded by tumour viruses. Van der Burg *et al* have demonstrated that HPV-specific Tregs (isolated from LN biopsies of cervical cancer patients) suppress both proliferation and cytokine production (IFN-γ and IL-2) by CD4^{+} T cells of various Ag specificities (HIV-1 or influenza derived peptides) (208). This capacity required activation by HPV-derived Ags (or anti-CD3 Abs). Moreover, tumour Ag-specific Tregs can also be identified in healthy donors as shown with EBNA1, an EBV latent Ag expressed in all EBV-associated tumours. Tregs specific for EBNA1 have been characterized after *in vitro* peptide stimulation of PBMCs from healthy donors with asymptomatic infections (209). These Tregs could recognize the same epitopes as CD4^{+} T cell clones. Tregs activated with these EBNA1-derived Ags could suppresses IL-2 production from EBNA1-specific T cells as well as the proliferative response of naïve CD4^{+} T cells to anti-CD3 Ab stimulation.

Recent progress in peptide-MHC class II tetramer technology has allowed reliable analyses of the presence and frequency of tumour Ag-specific Tregs in cancer patients. Schmidt *et al* generated MHC class II molecules presenting a peptide derived from mammaglobin, a protein overexpressed in all breast tumours. In breast cancer patients,

circulating mam_{34-48}-specific Tregs and $CD4^+CD25^-$ T cells represented 0.2% of their respective populations, which was higher than that observed in healthy donors (0.07%).

1.5. Colorectal Cancer

1.5.1. Pathways controlling colorectal tumorigenesis

CRC is the second leading cause of cancer morbidity and mortality worldwide (212). The 5-year survival expectation for colon cancer patients ranges from 93% for early stages to 8% in fully advanced stages (213). Two kinds of pathways have been shown to drive the process of colorectal neoplasia. 85% of CRCs generally develop following the 'traditional' pathway, also called the chromosomal instability pathway (CIN) or 'suppressor'pathway (214). The 'traditional' pathway is associated with mutation in oncogenes (such as KRAS) and tumor-suppressor genes (such as APC and p53) that directly regulate cell birth and cell death (214). These mutations take place within the tumour in a defined sequence (Figure 1.8). CRC is initiated by inactivation of the adenomatous polyposis coli (APC) tumour-suppressor pathway. Mutation of APC is found in approximately 60% of colon cancers and 82% of rectal cancers (215). The intestinal crypt (where the APC-mutant cell is) becomes dysplastic when abnormal cells accumulate. This slowly forms a polyp. More mutations (e.g. KRAS oncogene) are probably required to develop bigger polyps. Between 10 and 20% of large polyps will lead to cancer. The acquisitions of mutations in genes of other pathways (e.g. p53) is required for their development.

CRC development (from the APC mutation to the metastases) takes between 20 and 40 years. During this time, genetic instabilities increase. They are an integral component of human neoplasia (216). A low percentage of CRC patients (15%) has a genetic instability characterized by mismatch repair (MMR) deficiency. This instability was first
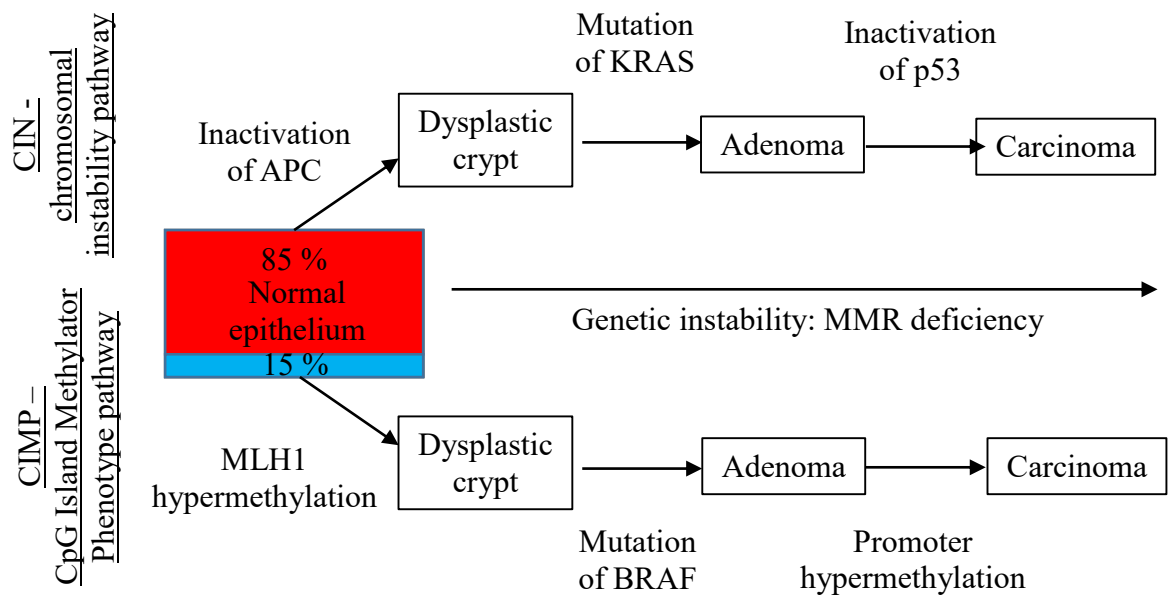
Figure 1.8. Pathways to colorectal cancer.

found in stretches of repetitive DNA, it was thus named microsatellite instability (MSI). 85% of CRC patients do not show a MMR deficiency but have an abnormal chromosomal content (aneuploid).

The CpG Island Methylator Phenotype (CIMP) pathway is the second most common pathway to CRCs. 15% of CRCs generally develop following this pathway. The CIMP pathway provides the epigenetic instability required for cancers to methylate the promoter regions of key tumour suppressor genes. Such hypermethylation leads to the inactivation of the expression of these genes (e.g. MLH1). BRAF is another important factor in the CIMP pathway as showed with the association of CIMP-positive cancers and BRAF mutation in CRC patients (217). The mechanism responsible of this epigenetic instability is uncertain. Environmental factors may contribute to colorectal DNA methylation in CRC patients (218).

1.5.2. Tumour infiltrating immune cells

A number of innate immune cells have anti-tumour effects in CRC. Indeed, in CRC patients, extensive intratumoural infiltration of NK cells is associated with a better prognosis (219). Similarly, dense macrophage infiltration in tumours has been shown to correlate with improved survival (220). The role of unconventional T cells in cancer has also been studied. High number of tumour-infiltrating human invariant natural killer T (NKT) cells is a favourable prognostic factor in CRC (221). NKT cells share surface markers with NK cells, and have a restricted TCR repertoire. Analyses of the tumour infiltrate of CRC revealed that γδ T cells, characterized by MHC-unrestricted cytotoxicity, can also recognize and kill tumour cells (222).

TILs play a central role in the anti-tumour immune response and can be used as prognostic markers. Galon *et al* showed that the type, density and location of immune

cells within tumours are more predictive of overall survival than the standard histopathological methods used to stage CRC (12). In particular, patients with low densities of CD3$^+$ T cells and CD45RO$^+$ memory T cells in the centre of the tumour and the invasive margin have a very poor prognosis. Consistent with this, human CRCs with a high density of infiltrating T$_{EM}$ cells are less likely to disseminate to lymphovascular structures and to regional LNs, processes that characterize early steps in the metastatic processes (223). Altogether, these data suggest that an immune criterion could be used as a biomarker to predict survival in CRC patients following surgery. Pages *et al* used these criteria in stages I and II CRC patients (early-stage tumours) and showed that a high density of memory and cytotoxic T cells at the tumour site could predict tumour recurrence and survival of these patients (224). This finding was then confirmed with a patient cohort encompassing all stages of CRC (225).

Other T cells of the adaptive immune response have been analysed in human CRC. Patients displaying high expression of Th17-related genes in tumoural tissues have a poor prognosis and those with an increased expression of Th1 genes have prolonged disease-free survival (94). In this study, Th2 genes were not predictive of prognosis. However, as these helper T cells can have anti- or pro-tumour effect, it will be relevant to stratify the results according to tumour stages and the type of cancer (sporadic or colitis-associated CRC).

The role of Tregs in CRC is disputed (226). In one study, a high density of tumour-infiltrating Foxp3$^+$ Tregs was shown to be associated with improved survival (227). However, a study by Sinicrope *et al* suggests a negative contribution of Foxp3$^+$ TILs in CRC as indicated by the association of low CD3$^+$/Foxp3$^+$ ratios and poor clinical outcome. These results highlight the need to characterize, for every CRC patient, the type of tumour-associated inflammation as this can affect the influence of Treg. For instance,

Th17-mediated responses are usually pro-tumoural thus inhibition by RORγt[+] Tregs may be beneficial.

1.5.3. Immunotherapy in colorectal cancer

Although 80% of CRC patients have macroscopic clearance of the tumour by surgery, 50% will relapse because of micrometastasis undetected during surgery (228). Therefore, alternative treatments are needed. Better comprehension of interactions between tumour and immune cells has made immunotherapy a possible future option for CRC. This active therapeutic approach aims to stimulate patients' own T cells in order to trigger a tumour-specific immune response.

As DCs can provide the three signals required to induce immunity, several clinical trials based on vaccination with tumour Ag-loaded DC have been conducted in CRC patients. One of these used DCs pulsed with altered peptide sequences from carcinoembryonic antigen (CEA) in an attempt to improve T cell activation (229). Two of 12 patients had dramatic tumour regression with CTL recognizing tumour cells expressing CEA. Vaccination with irradiated autologous tumour cells, expressing known and unidentified tumour Ags, has also been used to treat CRC patients. In one trial, autologous tumour cell-BCG vaccination induced clinical benefit in stage II CRC patients (230).

Peptide-based vaccines represent another approach. Even though many target tumour Ags have been identified (peptide database available on www.cancerimmunity.org/peptide), mouse colon cancer models and clinical trials with CRC patients have not led to major clinical benefits. A growing body of evidence indicate that peptide-based vaccines not only expand desirable Teff cells, but also detrimental

Tregs. Indeed, peptide-based vaccines targeting MAGE-A3 (231), E6/E7 (232) and NY-ESO-1 (233) have been shown to induce Tregs in melanoma, cervical cancer and advanced melanoma cancer patients respectively. Therefore it appears that the choice of peptide sequence used in vaccination may be key to eliciting the appropriate T cell population.

1.5.4. 5T4 glycoprotein

5T4 (also known as trophoblast glycoprotein, TPBG, and Wnt-activated inhibitory factor 1, WAIF1) is a transmembrane glycoprotein expressed in normal human placental tissues, absent in other normal tissues and overexpressed by many carcinomas, e.g. bladder, lung, breast, oesophagus, pancreas and colon (199).

A poorer clinical outcome is observed in CRC patients with 5T4-positive tumours (234). A role for 5T4 proteins in invasion processes was therefore proposed. Carsberg *et al* were the first to observe that 5T4 modifies the adhesion and motility of cultured epithelial cells (235). Later, Kagermeier-Schenk *et al* described the intracellular mechanism regulated by 5T4 (236). They showed that 5T4 augments activation of the β-catenin-independent Wnt signaling pathway in zebrafish embryos. Because Wnt signaling regulates tumour growth and metastasis (237), it is most likely that 5T4 promotes cancer cell invasion by acting on this pathway.

As an oncofetal Ag, 5T4 is a promising candidate for immunotherapy treatment because its expression is restricted to cancer cells. Modified vaccinia Ankara (MVA) encoding the entire 5T4 human protein (trade name TroVax) was shown to induce an anti-tumour immune response in a mouse model of colon cancer (238) and to transiently boost 5T4-specific immune responses in a phase I/II clinical trial (239). Although this trial

revealed a correlation between the development of an anti-5T4 Ab response and patient survival, no long-lived 5T4-specific humoral immunity was observed.

Some HLA-restricted 5T4 epitopes have been identified (Table 1.2) and using these epitopes may be more effective for cancer treatment than the entire protein, as these defined peptides may avoid activating undesirable T cells with nontherapeutic activities. Four HLA class I-restricted peptides derived from 5T4 have been identified. Redchenko *et al* identified a minimal HLA class I-restricted CD8^{+} T cell epitope, PLADLSPFA, using PBMC from healthy individuals (240). Authors identified HLA-Cw7 as the HLA restriction element for this peptide. HLA-Cw7-positive CRC patients vaccinated with TroVax showed strong IFN-γ ELISpot responses to this Ag. Similarly, another study employing the *ex vivo* IFN-γ ELISpot assay, identified 3 different 5T4 peptides from CRC patients vaccinated with TroVax (241). In this study, LTYVSFRNL peptide was HLA-A*01-restricted whereas RLARLALVL and FLTGNQLAV peptides were HLA-A*02-restricted.

Th cells are crucial for facilitating optimal CTL thus helper epitopes should be included in the design of peptide-based vaccines (242). In an attempt to identify 5T4-helper epitopes in healthy donors, Elkord *et al* showed that initial depletion of CD4^{+}CD25^{+} Tregs from PBMCs of healthy donors before *in vitro* stimulation with peptides could generate higher frequencies of 5T4-specific CD4^{+} T cells than observed in unmanipulated cultures (243). In this study, the authors also identified the 15-mer peptide YVSFRNLTHLESLHL as a CD4^{+} T cell epitope in HLA-DR4 positive healthy individuals. When looking at the reactivity of TILs from a regressing renal cell carcinoma, they found that 2 overlapping peptides could stimulate IFN-γ secretion from CD4^{+} T cells and one of these peptides was HLA-DR-restricted. Similarly, anti-tumour immune

Peptide	Position	HLA	Reference
RLARLALVL	17-25	HLA-A*02	(241)
FLTGNQLAV	97-105	HLA-A*02	(241)
PLADLSPFA	153-161	HLA-Cw7	(240)
LTYVSFRNL	249-257	HLA-A*01	(241)
YVSFRNLTHLESLHL	254-265	HLA-DR4	(243)
MADMVTWLK ETEVVQGKDR LTCAYPEKMRNRV	302-333	HLA-DR	(243)

Table 1.2. Reported 5T4 peptides

responses to tumour Ags CEA and 5T4 were suppressed *in vitro* by Tregs in one third of a cohort of 62 CRC patients in Cardiff (244). Interestingly, the team in Cardiff I worked with also showed that selective reduction in the proportion of circulating Tregs by low-dose cyclophosphamide corresponded to an increase in anti-5T4 T cell responses in CRC patients (245). Therefore, these results suggest that in addition to helper T cells, 5T4 peptides can be recognized by suppressive Tregs, which may impair vaccine efficacy.

1.6. Summary

The CD4$^+$ T cell population is made of distinct subsets, each characterised by specific transcription factors and cytokine signatures. Their impact on the immune response, and on tumour immunity in particular, is also very different. Th1 cells promote induction of cellular immunity and have direct anti-tumour functions. They are involved in cancer immunosurveillance by clearing tumours before clinical manifestation and vaccines aim at stimulate these cells to induce their anti-tumour activity. In contrast, Tregs can inhibit host immune responses against tumours and support tumour escape. Although an accumulation of CD4$^+$CD25^{hi} Tregs has been observed in CRC patients, their precise phenotype has not been reported. Furthermore, the oncofoetal Ag 5T4 is overexpressed by a wide variety of human cancers including CRC. Although the entire 5T4 protein is immunogenic in CRC patients, only a few 5T4 peptide Ags recognised by CTLs and Th1 cells have been reported. As Tregs can control responses to 5T4 protein, they are likely to recognise specific 5T4 Ags. Therefore, defining Th1 and Treg cells specificity for 5T4 may profoundly help to design more effective vaccines.

The experiments described in this thesis were performed with the following hypotheses:

1. CD25^{hi} Tregs, enriched in tumour tissues of CRC patients with a distinctive immunosuppressive phenotype, may contribute to CRC progression.

2. 5T4-specific responses are under the control of Tregs in CRC patients.

3. Specific regions and peptides are more immunogenic than others within 5T4 protein.

4. Th1 and Treg cells may recognise the same or different 5T4 peptides in CRC patients

1.7. Objectives

Experiments described in this thesis were performed to fulfil the following objectives:

1. To define the phenotype of CD4$^+$CD25^{hi} cells isolated from the blood, normal and tumour colon of CRC patients.

2. To analyse the effect of Treg-depletion on 5T4-specific responses.

3. To map immunogenic regions within 5T4 protein.

4. To evaluate the frequency of 5T4 peptide-specific Th1 and Treg cells in the blood of healthy donors and CRC patients.

CHAPTER 2. MATERIALS AND METHODS

2.1. Patients

Patients were consented before surgery in order to obtain colorectal tumour and healthy colon specimens as well as 30 mls of blood (see Appendix section for a copy of Patient Information Sheet and Consent form). HLA type was determined, if possible, from blood samples (Welsh Transplantation and Immunogenetics Laboratory, Pontyclun, Cardiff). In total, a cohort of 64 CRC patients was enrolled in this study. Lymphocyte analysis was performed in 30 of these patients (patient characteristics summarized in Table 2.1) and cultured T cell responses were analysed in 34 of these patients (patient characteristics summarized in Table 2.2). A number of blood and tumour samples were analysed from same patients. The Bro Taf Local Research Ethics Committee granted ethical approval for this study.

2.2. Patient specimens

Within 1 hour after resection, CRC specimens were taken to the pathology department for analysis. Autologous colon samples were cut from a normal section of the tissue, approximately 10 cm from neoplastic tissue. Small tumour samples were also resected from the specimen. After dissection, healthy and tumour samples were taken to the lab for further analysis.

Consultant pathologists at the University Hospital of Wales carried out staging of the resected colorectal cancers. Two staging systems were used to define the extent of tumour invasion: Dukes' classification and the international TNM system.

		Male	Female
n		24	6
Age range		33-87	67-81
TNM Stage, 5th Edition (%)	T1	2 (8)	0 (0)
	T2	4 (17)	2 (33)
	T3	10 (42)	2 (33)
	T4	8 (33)	2 (33)
Lymph Node Spread	N0	15 (62)	5 (83)
	N1	4 (17)	0 (0)
	N2	5 (21)	1 (17)
Dukes' Stage (%)	A	4 (17)	2 (33)
	B	11 (46)	3 (50)
	C1	9 (37)	1 (17)
	C2	0 (0)	0 (0)

Table 2.1. CRC patient characteristics whose lymphocytes were analysed by FACS.

		Male	Female
n		21	13
Age range		33-84	47-84
TNM Stage, 5th Edition (%)	T1	2 (10)	1 (8)
	T2	3 (14)	3 (23)
	T3	11 (52)	4 (31)
	T4	5 (24)	5 (38)
Lymph Node Spread	N0	11 (52)	8 (62)
	N1	6 (29)	3 (23)
	N2	4 (19)	2 (15)
Dukes' Stage (%)	A	3 (14)	4 (31)
	B	8 (38)	4 (31)
	C1	10 (48)	5 (38)
	C2	0 (0)	0 (0)

Table 2.2. CRC patient characteristics whose T cell specificity for 5T4 has been analysed.

Dukes' classification:

Dukes' A: Tumour is confined to the bowel wall.

Dukes' B: Tumour go through the bowel wall without lymph nodes involved.

Dukes' C1: Tumour extent into the muscularis propria with less than 4 lymph nodes involved.

Dukes' C2: Tumour completely penetrate the muscularis propria with more than 4 lymph nodes involved.

TNM system:

T refers to the depth the tumour has penetrated the colon wall:

T1: Tumour is ≤ 2 cm across.

T2: Tumour is > 2 cm and ≤ 5 cm across.

T3: Tumour is > 5 cm across.

T4: Tumour of any size growing into adjacent tissues.

N refers to the lymph node involvement:

N0: Tumour has not spread to regional lymph nodes.

N1: Tumour has spread to 1 to 3 axillary lymph nodes.

N2: Tumour has spread to more than 4 lymph nodes.

M refers to the metastases:

M0: no metastatic spread.

<u>M1</u>: Distant metastases.

2.3. Healthy donors

Blood was taken from healthy individuals with no history or clinical evidence of malignancy. All individuals were consented before giving blood. Blood was collected into heparinized tubes.

2.4. Purification of human lymphocytes

PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. Whole blood was layered over lymphoprep (Axis-Shiled, Oslo, Norway) and centrifuged at 2000 rpm for 20 min at 25° C. PBMCs were recovered from the interface using a Pasteur pipette and washed twice in R+ (RPMI-1640 containing Pen-Strep, L-glutamine and sodium pyruvate). Then, they were diluted 1:1 with Trypan blue and counted using a haemocytometer.

2.5. Single cell suspensions from tissues

Healthy colon and tumour tissue samples were mashed with blades in a Petri dish and forced through 70 μm cell strainers using syringe plungers. Strainers were changed when clogged. When a single cell suspension was achieved, cells were washed twice in 10 mls of extraction media (IMDM containing 2 % AB serum, 20 μg/ml gentamicin, 2 μg/ml fungizone and Pen-Strep) and plated for further analyses. In order to preserve the integrity of the cells, no enzymatic digestion with collagenase and DNase was used.

2.6. Depletion of CD25$^+$ regulatory T cells

PBMCs were resuspended in MACS buffer (1×PBS, 0.5% bovine serum albumin, 5 mM EDTA), with 80 µl MACS buffer per 10^7 cells, and anti-human CD25 coated beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added, with 20 µl of CD25 coated beads per 10^7 cells. Then, cells were incubated for 15 minutes at 4°C, washed and resuspended in 500 µl of MACS buffer. MS columns were used for positive selection and effluent CD25^{hi}-depleted and labelled CD25^{hi} cells were collected. The efficacy of depletion confirmed by flow cytometry. Cells were washed twice with R$^+$ before use. Assays were set up using either undepleted or CD25^{hi}-depleted cells.

2.7. Generation and culture of human T cell lines

Isolated PBMCs or CD25^{hi}-depleted fractions were washed in R+ then resuspended at 2×10^6/ml in advanced RPMI supplemented with 5% AB serum (R5). Cells were plated at 100 µl / well in 96 well plates, i.e. 2×10^5/ well. Three PBMC lines were established for each 5T4 peptide pool or 5T4 single peptide and plates were kept in incubator (37°C, 5% CO_2) for 12-14 days. Recall Ags Tetanus Toxoid TT (Statens Serum Institut) or tuberculin purified protein derivative PPD (Statens Serum Institut) and PHA were also used. In order to enrich Ag-specific T cells, 10 µl of CellKine media (Helvetica Healthcare) was added on day 3 and 100 µl of R5 containing 40 IU/ml IL-2 (therefore at 20 IU/ml final concentration) was added on day 6. On day 9, 100 µl of culture supernatant was removed and 100 µl of R5 containing 40 IU/ml IL-2 was added.

2.8. 5T4 antigens

20-mer peptides overlapping by 10 amino acids and spanning the entire 5T4 protein were synthesized with purity > 95% (GLBiochem, Shanghai, China). The sequence of 5T4 protein is shown Figure 2.1. A total of 41 peptides were used to generate peptide pools (Table 2.3). Peptides were dissolved in DMSO at 50 mg/ml and diluted in PBS at 1 mg/ml. 13 peptide pools were created in a matrix system where 1 peptide was present in 2 pools (Table 2.4). Each peptide pool contained 5-7 peptides. Peptides were added at a final concentration of 5 µg/ml/peptide for T cell assays, so the total concentration of peptide was between 25 and 35 µg/ml.

2.9. IFN-γ ELISpot assays

ELISpot plates (Millipore, Moslheim, France) were coated with 50 µl of anti-human IFN-γ capture Ab (Mabtech, Natka, Sweden) and left at 4°C overnight. Wells were extensively washed four times with 150 µl of PBS to remove excess Ab and 100 µl of R5 was added for 1h at 37°C in order to block the wells. The R5 was removed before adding cells. The short term lines cultured in triplicate were pooled, washed and resuspended at $5x10^5$ / ml in R5. 150 µl (75 000 cells) were plated per well in the absence or presence of the corresponding 5T4 peptide pool or single peptide. The plates were subsequently incubated for 18 hours (37°C, 5% CO_2).

Following overnight incubation, cells were removed, plates were washed 5 times with 150 µl of PBS and 50 µl of secondary biotinylated anti-human IFN-γ Ab was added for 1 hour (37°C, 5% CO_2). Wells were washed 4 times with PBS followed by the addition of 50 µl streptavidin-alkaline phosphatase (Mabtech, Sweden) diluted at 1:1000. After 1 hour incubation at room temperature, wells were washed 4 times with PBS and

	10	20	30	40	50

MPGGCSRGPA AGDGRLRLAR   LALVLLGWVS   SSSPTSSASS   FSSSAPFLAS

	60	70	80	90	100

AVSAQPPLPD QCPALCECSE AARTVKCVNR NLTEVPTDLP   AYVRNLFLTG

	110	120	130	140	150

NQLAVLPAGA FARRPPLAEL AALNLSGSRL DEVRAGAFEH LPSLRQLDLS

	160	170	180	190	200

HNPLADLSPF AFSGSNASVS   APSPLVELIL   NHIVPPEDER   QNRSFEGMVV

	210	220	230	240	250

AALLAGRALQ GLRRLELASN HFLYLPRDVL AQLPSLRHLD LSNNSLVSLT

	260	270	280	290	300

YVSFRNLTHL ESLHLEDNAL KVLHNGTLAE LQGLPHIRVF LDNNPWVCDC

	310	320	330	340	350

HMADMVTWLK ETEVVQGKDR LTCAYPEKMR NRVLLELNSA   DLDCDPILPP

	360	370	380	390	400

SLQTSYVFLG IVLALIGAIF LLVLYLNRKG IKKWMHNIRD ACRDHMEGYH

	410	420

YRYEINADPR   LTNLSSNSDV

Figure 2.1. Amino acid sequence of 5T4 protein.

No.	Sequence	No.	Sequence
1	MPGGCSRGPAAGDGRLRLAR	21	AALLAGRALQGLRRLELASN
2	AGDGRLRLARLALVLLGWVS	22	GLRRLELASNHFLYLPRDVL
3	LALVLLGWVSSSSPTSSASS	23	HFLYLPRDVLAQLPSLRHLD
4	SSSPTSSASSFSSSAPFLAS	24	AQLPSLRHLDLSNNSLVSLT
5	FSSSAPFLASAVSAQPPLPD	25	LSNNSLVSLTYVSFRNLTHL
6	AVSAQPPLPDQCPALCECSE	26	YVSFRNLTHLESLHLEDNAL
7	QCPALCECSEAARTVKCVNR	27	ESLHLEDNALKVLHNGTLAE
8	AARTVKCVNRNLTEVPTDLP	28	KVLHNGTLAELQGLPHIRVF
9	NLTEVPTDLPAYVRNLFLTG	29	LQGLPHIRVFLDNNPWVCDC
10	AYVRNLFLTGNQLAVLPAGA	30	LDNNPWVCDCHMADMVTWLK
11	NQLAVLPAGAFARRPPLAEL	31	HMADMVTWLKETEVVQGKDR
12	FARRPPLAELAALNLSGSRL	32	ETEVVQGKDRLTCAYPEKMR
13	AALNLSGSRLDEVRAGAFEH	33	LTCAYPEKMRNRVLLELNSA
14	DEVRAGAFEHLPSLRQLDLS	34	NRVLLELNSADLDCDPILPP
15	LPSLRQLDLSHNPLADLSPF	35	DLDCDPILPPSLQTSYVFLG
16	HNPLADLSPFAFSGSNASVS	36	SLQTSYVFLGIVLALIGAIF
17	AFSGSNASVSAPSPLVELIL	37	IVLALIGAIFLLVLYLNRKG
18	APSPLVELILNHIVPPEDER	38	LLVLYLNRKGIKKWMHNIRD
19	NHIVPPEDERQNRSFEGMVV	39	IKKWMHNIRDACRDHMEGYH
20	QNRSFEGMVVAALLAGRALQ	40	ACRDHMEGYHYRYEINADPR
		41	YRYEINADPRLTNLSSNSDV

Table 2.3. 20-mer 5T4 sequences.

	1	2	3	4	5	6	
1	2	3	4	5	6	**7**
7	8	9	10	11	12	**8**
13	14	15	16	17	18	**9**
19	20	21	22	23	24	**10**
25	26	27	28	29	30	**11**
31	32	33	34	35	36	**12**
37	38	39	40	41		**13**

Table 2.4. 5T4 matrix. Numbers in bold indicate the pools (1 to 13) containing 7 (pools 1 to 6) or 6 (pools 7 to 13) peptides.

spots were developed by the addition of a colorimetric substrate kit (Bio-rad, Hercules, California) consisting of 5% AP development buffer, 1% of substrate A and B in dH_2O. 100 μl of this substrate kit was added into each well and left for 15 minutes at room temperature. To stop the reaction, wells were washed with tap water. After drying the wells, IFN-γ spots were counted using an automated reader (Autoimmun Diagnostika GMBH, A.I.D., Strasberg, Germany). Counting was checked manually by using the ELISpot 5.0 software. The number of spots was normalized to x /10^5 cultured PBMCs. Positive cultured responses were identified as having at least 25 spot forming cells (SFC) per 10^5 cultured PBMC, after subtraction of the background, and as having double background.

2.10. EBV-transformed B cell assays

In order to determine which of the DR serotypes were responsible for Ag presentation, HLA-DR matched EBV-transformed B cells were used as Ag-presenting cells to stimulate 5T4-expanded T cells. HOM2 and HO104, respectively homozygous for HLA-DR*0101 and HLA-DR*1501, were bought from Public Health England. EBV-transformed B cells were incubated overnight with 5T4 peptides (37°C, 5% CO_2) in R5, washed 4 times and added at a 1:1 ratio to 5T4-expanded T cells in an ELISpot assay. After 6 hours of stimulation in R5, IFN-γ spots were revealed as described above.

2.11. Antibody blocking assays

To determine whether T cells recognition of 5T4 peptide was HLA-DR restricted, IFN-γ secretion was measured in ELISpot assays in the absence or presence of blocking Abs. 10 μg/ml of anti-class I (clone w6/32, Biolegend), anti-DQ (clone 1a3, Leinco) and/or

anti-DR (clone L243, Biolegend) Abs were added to 5T4-expanded T cells in ELISpot assays. 5T4 peptides were added after incubation at room temperature for 1h.

2.12. Antibody staining

$2x10^6$ cells were initially stained for viability with aqua amine-reactive viability dye (Invitrogen). The cells were washed twice with PBS and stained with the viability marker previously diluted at 1:10 in PBS. After 15 minutes at room temperature in the dark, cells were washed twice with fluorescence activated cell sorting (FACS) buffer and resuspended in 50 µl FACS buffer for cell surface staining. Several combinations of anti-human Abs were used to stain the cells (Table 2.5). Staining was performed at 4°C for 15 minutes.

After two washes with FACS buffer, cell membranes were permeabilized using Fixation/Permeabilization kit (eBioscience) according to the manufactures' instructions. Before intracellular staining, cells were left in the fridge for 15 minutes in Permeabilization buffer containing 2% rat serum in order to block Fc receptors. Abs specific for various intracellular markers were added and cells were incubated in the dark at 4°C (Table 2.6). Then cells were rinsed in 1X Perm buffer and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich). Stained cells were kept in the dark at 4°C for a maximum of 1 hour before acquisition on a BD FACSCanto II. Representative flow cytometry plots in the $CD4^+CD25^-$, $CD25^{int}$ and $CD25^{hi}$ populations are shown (Figure 2.2). Gates were drawn based on fluorescence-minus-one (FMO) controls (Appendix Figure 1).

Antigen	Conjugate	Clone	Company	Final conc. (μg / mL)
CD4	Pacific Blue	RPA-T4	BD	2
CD25	APCh7	M-A251	BD	6
CD39	PECy7	eBioA1	eBioscience	3
CD45RA	PerCPCy5.5	HI100	eBioscience	3
CD45RO	FITC	UCHL1	BD	3
CD127	PECy7	eBioRDR5	eBioscience	3
HLA-DR	PECy7	L243	BioLegend	2
ICOS	FITC	C398.4A	BioLegend	4
ICOS	PerCPCy5.5	C398.4A	BioLegend	4
LAG-3	PE	Polyclonal	R&D Systems	4
LAP	PerCPCy5.5	TW4-2F8	BioLegend	4

Table 2.5. Human antibodies used for cell surface staining.

Antigen	Conjugate	Clone	Company	Final conc. (μg / mL)
CTLA-4	PE	BNI3	BD	2
Helios	FITC	22F6	BioLegend	2
Foxp3	APC	236A/E7	eBioscience	6

Table 2.6. Human antibodies used for intracellular staining.

2.13. Statistics and graphical analysis

GraphPad Prism Version 5 was used for all statistical analyses. Mean values ± 95 % confidence interval were used for all appropriate results. Unpaired *t*-tests were used to analyse quantitative differences between different groups of CRC patients or when comparing healthy donors and CRC patients. FlowJo version 10 was used to analyse flow cytometry data.
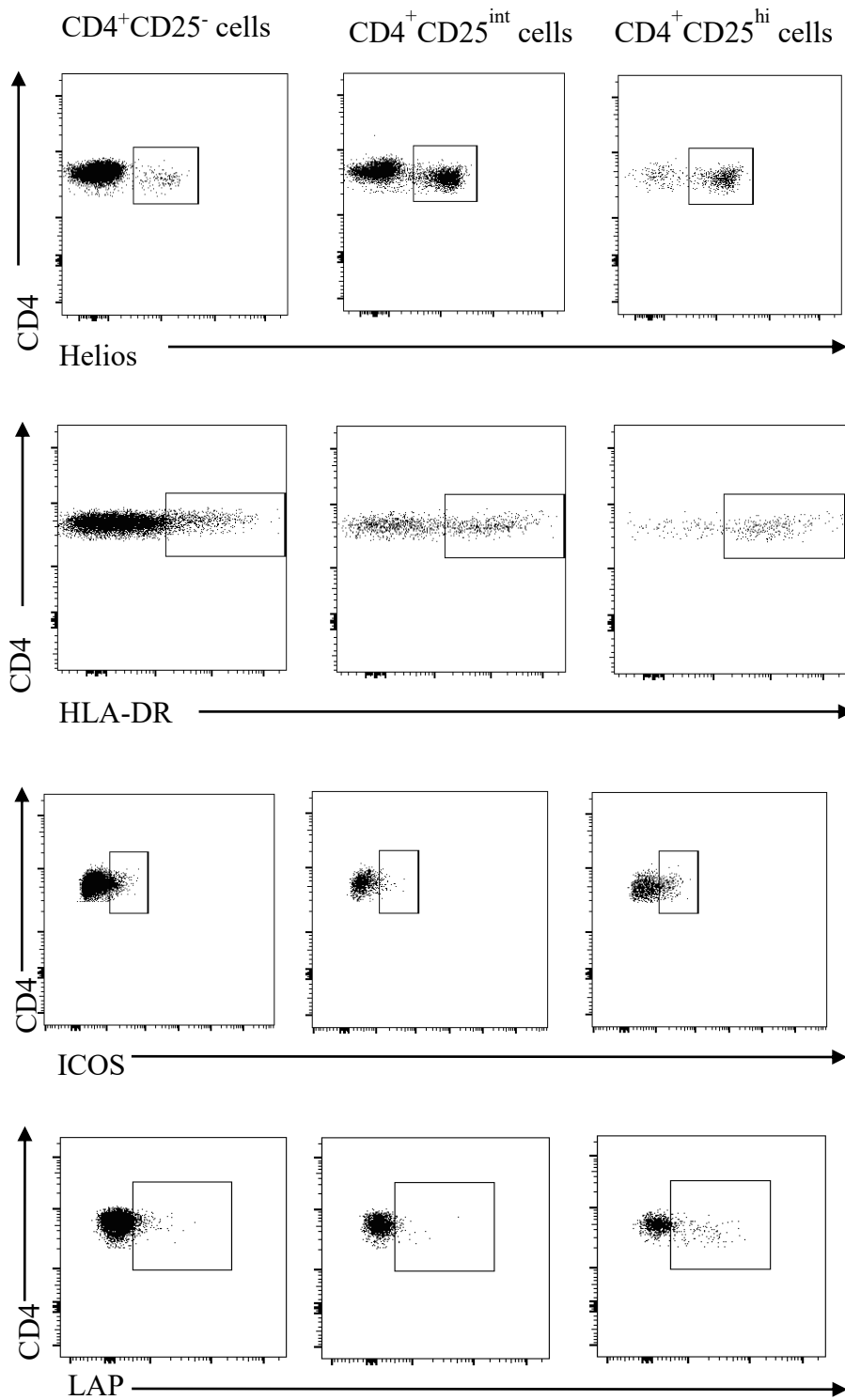
Figure 2.2. Representative flow cytometry plots. The expression of the indicated markers in the different CD25 populations is shown.
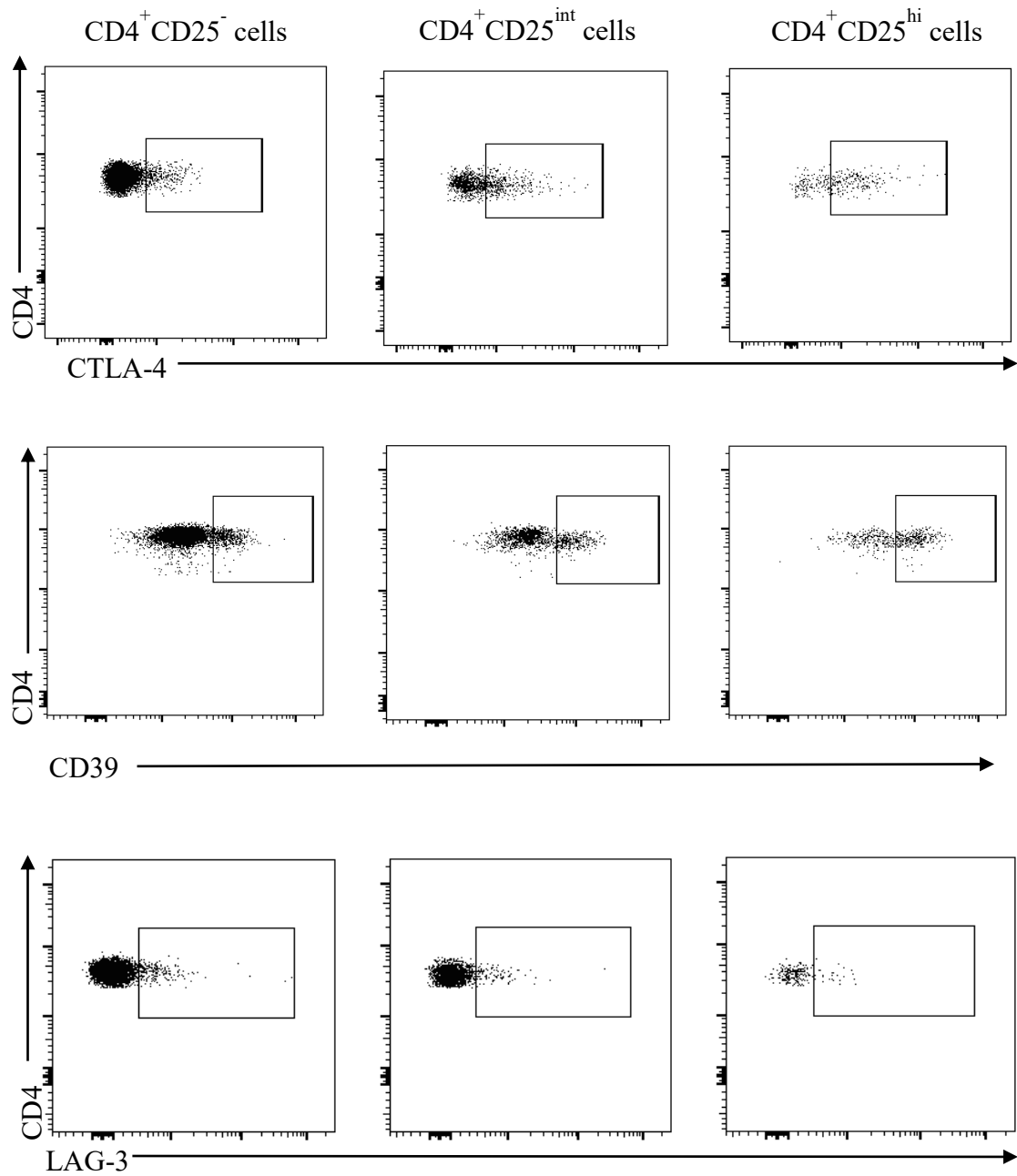
Figure 2.2. Representative flow cytometry plots. The expression of the indicated markers in the different CD25 populations is shown.

CHAPTER 3. ANALYSIS OF HUMAN REGULATORY T CELLS PHENOTYPE

3.1. Introduction

Colorectal cancer is the third most common cancer in the world, with more than 1 million individuals diagnosed every year (246). Recently, immunotherapy treatments have been developed as active therapeutic approaches. Although tumour-specific responses are detected, these effects do not lead, in the vast majority, into improved clinical status. Several mechanisms may account for this limited therapeutic efficacy. In CRC patients, one particular cell type, named Tregs, expressing the transcription factor Foxp3, can suppress anti-tumour immune responses (247). In addition, studies have shown that cancer vaccines can induce tumour-specific regulatory T cells (248). Thus, it is likely that Tregs play a central role in the efficacy of tumour vaccines.

In contrast to mice where $CD4^+CD25^+$ cells are highly suppressive, the CD25 marker in human does not represent a homogenous population (249). The discovery of Foxp3 as a crucial gene for $CD4^+$ Treg development and function confirmed initial findings showing that human $CD4^+CD25^{hi}$ T cells are highly enriched for Tregs (250). However, the use of Foxp3 expression to define Tregs should be used in conjunction with $CD25^{hi}$ cells, as Foxp3 can be transiently expressed on activated $CD4^+CD25^{int}$ T cells, without conferring suppressive activity (251). Indeed, unlike $CD4^+CD25^{hi}$ T cells, FACS-sorted $CD4^+CD25^{int}$ cells do not inhibit the proliferation of co-cultured $CD4^+ CD25^-$ cells (249).

Increased numbers of CD25^{hi} Treg cells have been found in the peripheral blood of patients with different types of cancer, such as CRC patients (247), prostate cancer patients (252) and patients with multiple myeloma (253). Also, the phenotype of tumour-infiltrating Tregs has been investigated in cancers. In renal cell carcinoma patients, intratumoural CD25^{hi} Tregs were found to have marked changes in their phenotype in comparison to the circulating Tregs (254). In CRC, no studies have examined the phenotype of CD4$^+$CD25^{hi} T cells. In order to better understand how colorectal cancers evade tumour immunity, a detailed comparative phenotypic analyses of Treg populations was performed in healthy donors and CRC patients.

3.2. Results

3.2.1. Gating strategy to identify CD25^{hi} cells

In humans, the CD4$^+$CD25$^+$ population is not clearly distinguishable from CD25$^-$ cells and instead a gradation of expression is observed. Therefore, defined parameters must be applied when gating on the CD4$^+$CD25^{hi} Treg population. Previous works in our group and from others have attempted to define these parameters (247) (255). In this study, total lymphocytes, identified according to their SSC/FCS and live cells were gated based on negative Aqua Live/Dead expression (Figure 3.1). Following gating on CD4$^+$ cells, CD25^{hi} or intermediate cells were identified as those where CD25 expression was higher than observed on CD4$^-$ cells (indicated by the dashed line).

3.2.2. CD25^{hi} T cells exhibit a "classic" Treg phenotype

As mentioned above, Tregs cells do not represent a distinct CD25 population and other markers have to be included in their analyses. Expression of naïve (CD45RA) and memory (CD45RO) markers was analysed in the CD25 populations (Figure 3.2). CD45RO was expressed on a significantly higher proportion of CD25^{hi} cells (85.14% ± 5.47) compared to CD25^{int} (58.36% ± 12.4) and CD25$^-$ cells (44.42% ± 17.85). Its reciprocal marker, CD45RA, showed the opposite expression profile. Indeed, CD45RA was predominantly expressed by CD25$^-$ cells (62.36% ± 15.1), compared to less than 40% of CD25^{int} cells. Only a few CD25^{hi} cells expressed CD45RA (12.08% ± 9.65). These data are consistent with an enrichment of naïve cells within the CD25$^-$ population rather than Ag-experienced cells as observed within the CD25$^+$ population. The data also confirm that the vast majority of circulating Tregs are of an activated/memory phenotype, as observed in previous studies (249).
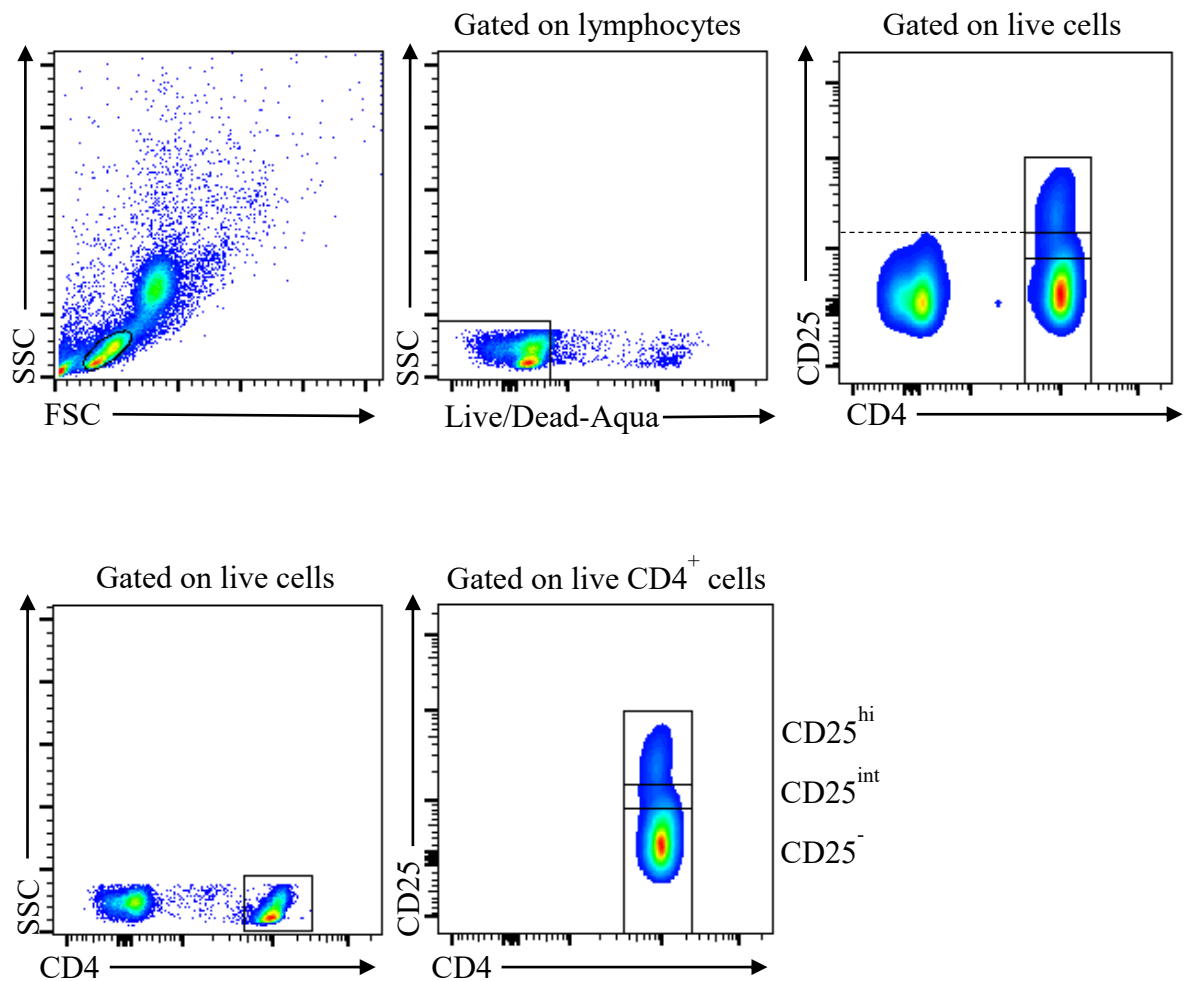
Figure 3.1. Gating strategy to identify T regulatory cells. Lymphocytes were identified based on forward and side scatter characteristics. Then, live cells were gated based on negative Aqua Live/Dead staining. CD4$^+$CD25^{hi} cells were identified as those expressing CD25 at a level higher than observed on CD4$^-$ cells (represented by dashed lines). Finally, CD4$^+$ cells were gated from the live cells and the same gates previously determined were applied to distinguish the three CD25 populations.
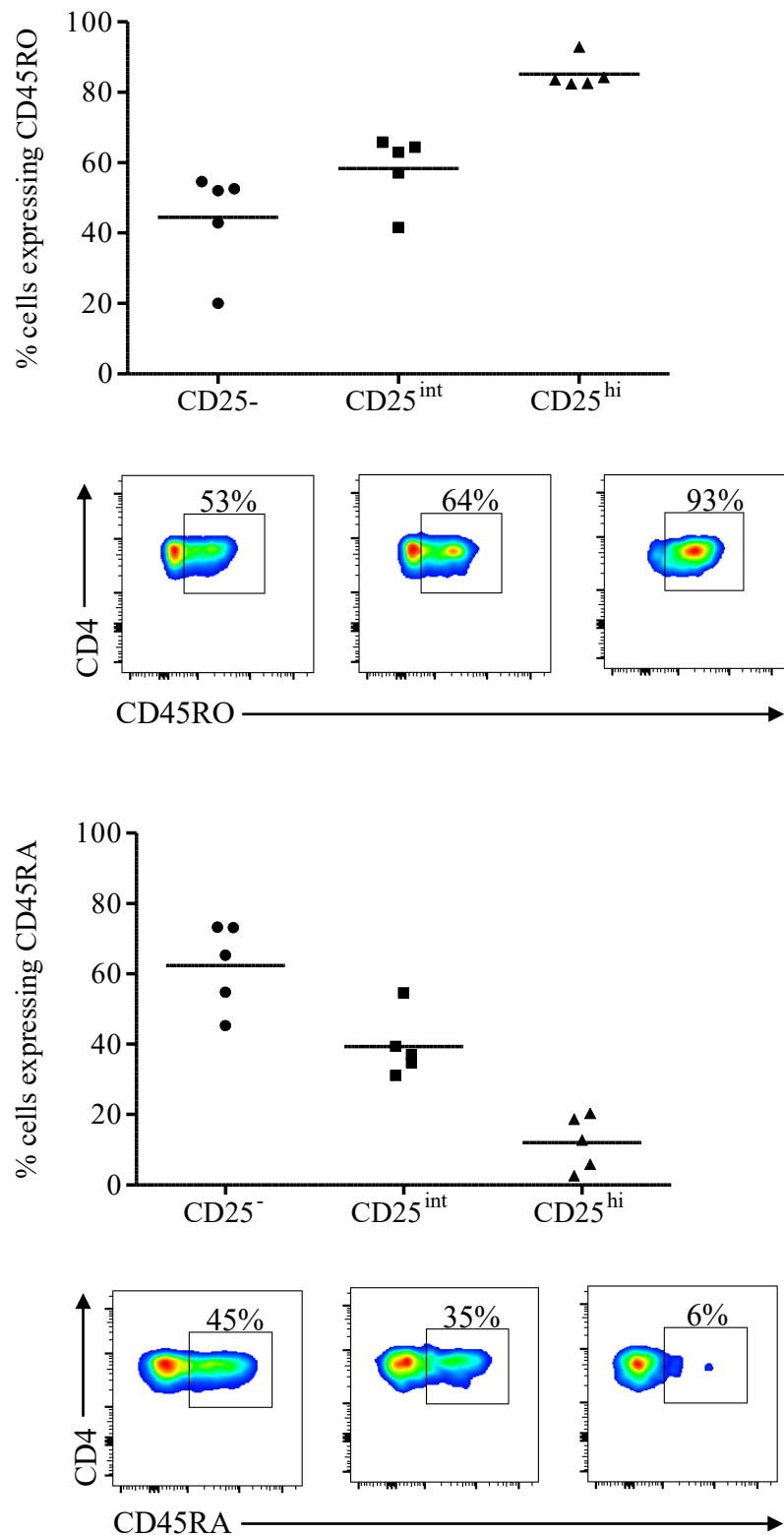
Figure 3.2. CD4+CD25hi cells have a memory phenotype. PBMC isolated from peripheral blood of CRC patients were stained with live/dead, anti-CD4, anti-CD25, anti-CD45RO and RA Abs. Percentage of CD4+CD45RO+ and CD4+CDRA+ T cells within each of the three CD25 populations is indicated. Representative FACS plots are shown.

Liu *et al* have shown that "true" Tregs do not require IL-7 for survival and predominantly exhibit a CD127$^{-/low}$ phenotype (256). CD127 expression was compared in the CD25 populations described herein (Figure 3.3). Over 85% of CD25^{hi} cells within the CD4 population were CD127$^{-/low}$, approximately 40% in the CD25^{int} population whereas the majority of CD25^{-} cells were CD127^{+}. Altogether, these data indicate that the CD25^{hi} population identified has a "classic" Treg phenotype, thus validating the gating strategy used.

3.2.3. Treg identification in blood and colon tissues.

In order to characterise the Treg phenotype in cancer, freshly resected uninfected colon and tumour tissues from CRC patients were mashed, filtered and stained for several markers. This *ex vivo* analyses allowed an accurate determination of the phenotype as tissue-infiltrating Tregs may upregulate or downregulate proteins during *in vitro* incubation. In parallel, when possible, PBMCs were isolated from matched peripheral blood samples and stained for the same markers. One representative FACS plot is given in Figure 3.4. The percentage of live cells was lower in the colon (68%) and in the tumour (28%) tissues than in the blood (96%). It is likely that some cells died during the isolation process but presence of dying cells (especially from the tumour) is also a likely contributing factor. Following the gating strategy described above, CD25^{hi} Tregs were identified within the CD4 population in blood, colon and tumour samples.

3.2.4. Foxp3 expression in peripheral and tissue-infiltrating cells

The transcription factor Foxp3 was originally thought to be a specific Treg marker. However, CD25^{int} T cells can upregulate this factor following activation. In order to
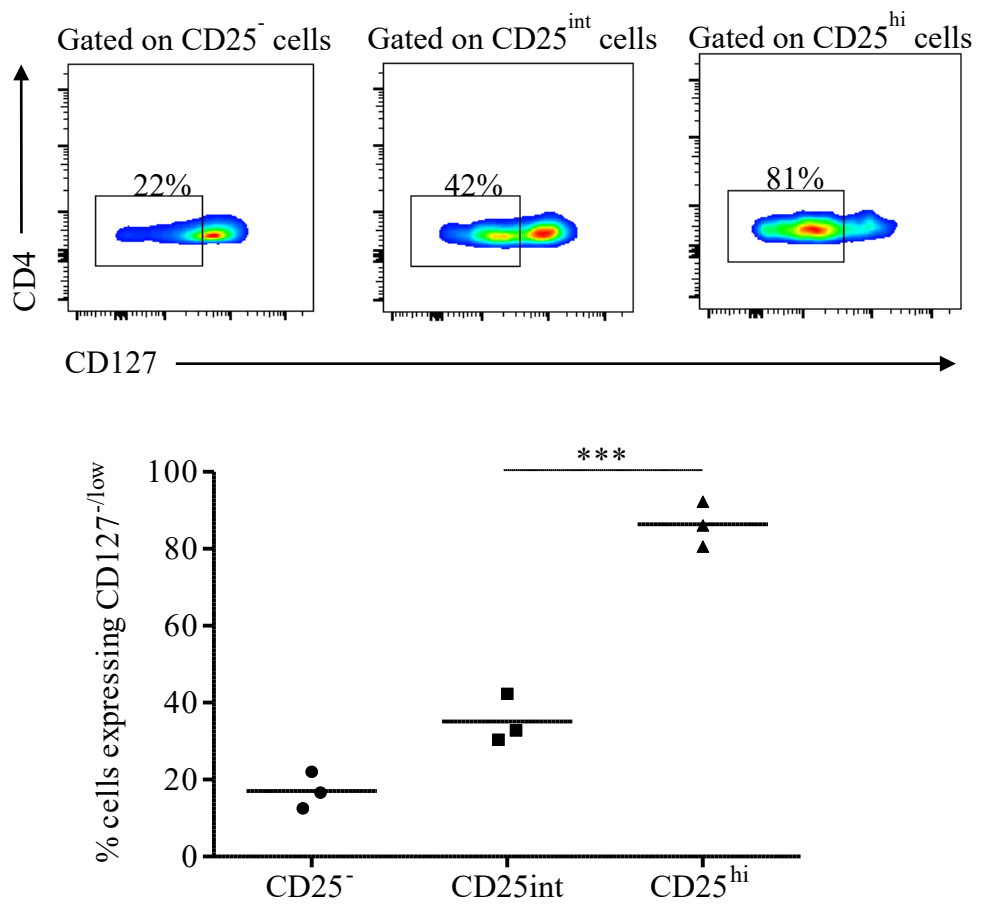
Figure 3.3. Expression of CD127 in CD25 populations. Cells were gated on live CD4^{+} circulating cells from CRC patients and then gated on the indicated CD25^{+} population. FACS plots depicting CD127 staining are shown as representative examples. Significant differences are indicated, ***p < 0.001 (Student's unpaired t test).
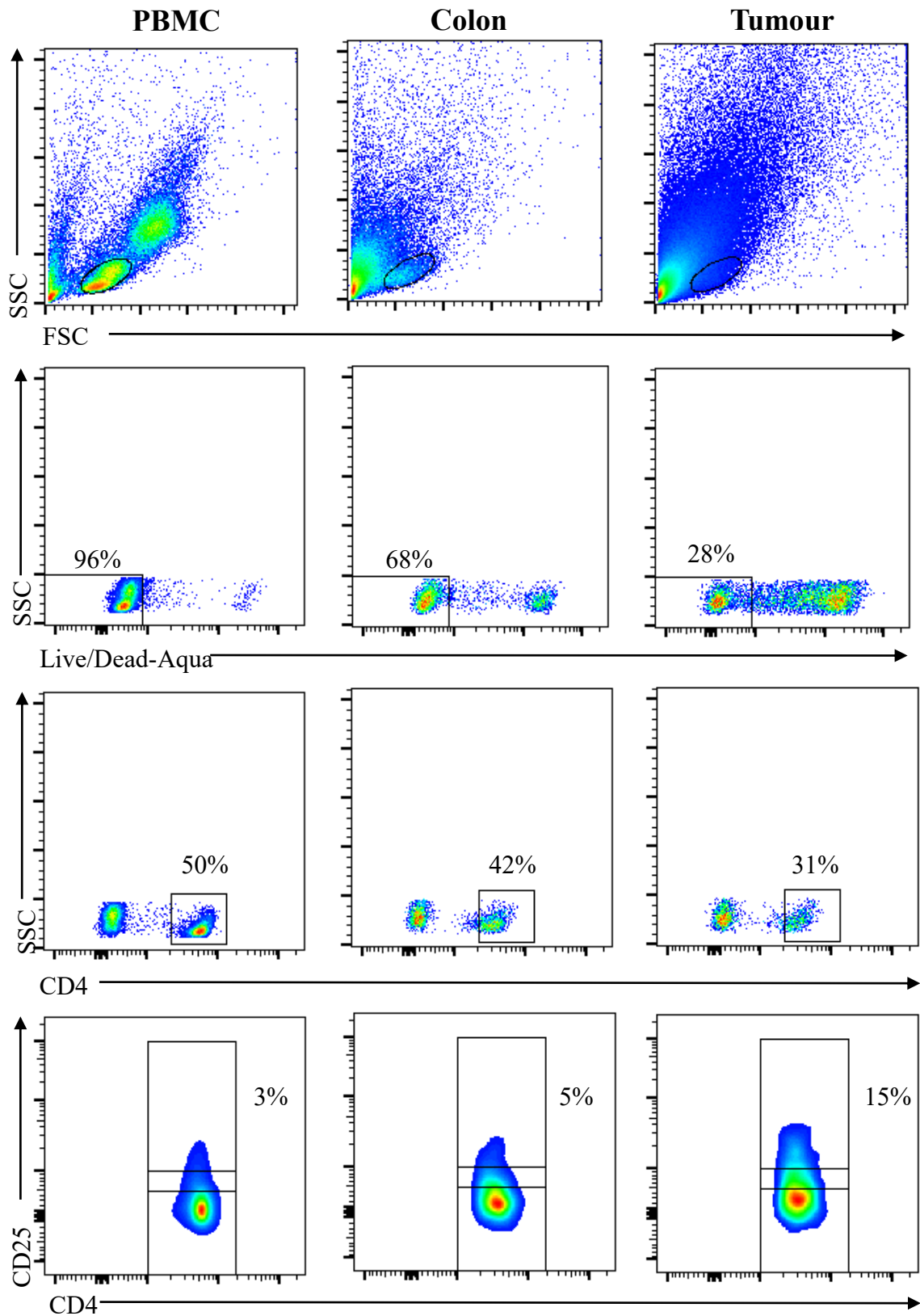
Figure 3.4. *Ex vivo* **phenotype of Tregs in CRC patients.** Representative FACS plots showing CD25 staining on live cells in the peripheral blood, healthy colon and tumour tissues of one CRC patients. Numbers are the percentage of cells in the indicated gates.

verify that the CD4$^+$CD25^{hi} T cells correspond to a homogenous Foxp3$^+$ population, the expression of this marker was assessed in the three CD25 subpopulations present in the live CD4$^+$ population (Figure 3.5.A). In the 25 CRC patients studied, over 85% of circulating CD4$^+$CD25^{hi} T cells expressed Foxp3 whilst 40% of CD25^{int} expressed this marker (Figure 3.5.B). Negligible Foxp3 expression was observed in CD25$^-$ cells. This pattern was the same in CD25^{hi} cells infiltrating unaffected and tumour colon tissues. This predominance of Foxp3 expression in CD4$^+$CD25^{hi} cells is in accordance with other reports (244) and is in line with previous observations showing that these cells are CD45RO$^+$ and CD127$^{-/low}$.

It has been shown that Foxp3 expression correlates with immunosuppressive function (257). Thus the level of Foxp3 expression was determined by calculating the mean fluorescence intensity (MFI) of Foxp3 in the different CD25 populations (Figure 3.6). The level of Foxp3 expression was significantly higher in CD25^{hi} cells compared to the CD25^{int} population (CD25^{hi} 4985 ± 667 vs. CD25^{int} 1338 ± 510, p < 0.0001). Also, the few cells expressing Foxp3 in the CD25$^-$ population exhibited relatively reduced expression levels (CD25$^-$ 552 ± 180 vs. CD25^{int} 1338 ± 510, p = 0.0040).

3.2.5. Tregs are enriched in blood and tumour of CRC patients

Tregs in PBMCs isolated from peripheral blood of healthy controls and CRC patients were analysed by flow cytometry. Frequencies of CD4$^+$CD25^{hi} T cells among CD4$^+$ T cells were increased in the CRC patients (patients 4.64 ± 1.06 vs. controls 1.56% ± 0.79, p = 0.0138; Figure 3.7.A). Next, the proportion of Tregs infiltrating the colon of CRC patients was analysed. There was a higher percentage of CD4$^+$ T cells expressing high levels of CD25 in tumours compared to benign colon tissues (tumour 17.39% ± 3.42 vs. colon 7.02% ± 3.99%, p = 0.0006). Also, this percentage was higher than the frequency

A

B
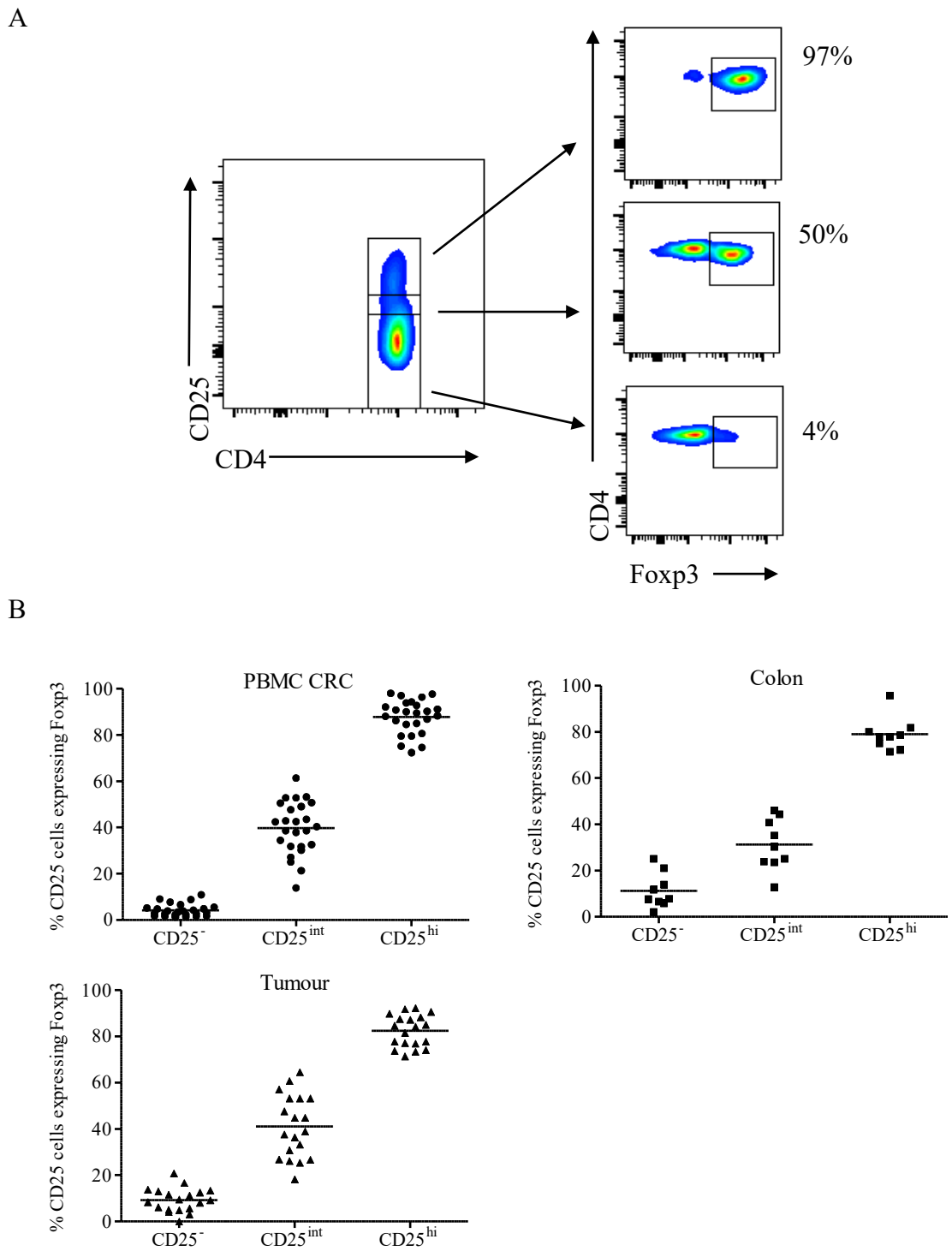
Figure 3.5. Foxp3 expression in the three CD25 populations in CRC patients. A. Representative FACS plots showing Foxp3 expression in CD4$^+$CD25$^-$, CD25^{int} and CD25^{hi} cells. B. Percentage of cells expressing Foxp3 in the indicated CD25 population in blood (n = 25), unaffected colon (n =9) and tumour tissues (n = 19) of CRC patients.
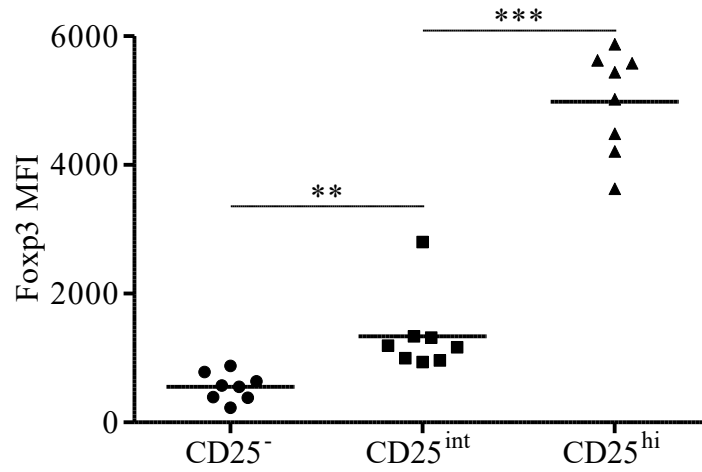
Figure 3.6. Analyse of Foxp3 expression level in CD25 populations. Mean fluorescence intensity (MFI) of Foxp3 in the indicated cells. PBMCs were isolated from CRC patients and stained with live/dead, anti-CD4, anti-CD25 and anti-Foxp3 Abs.
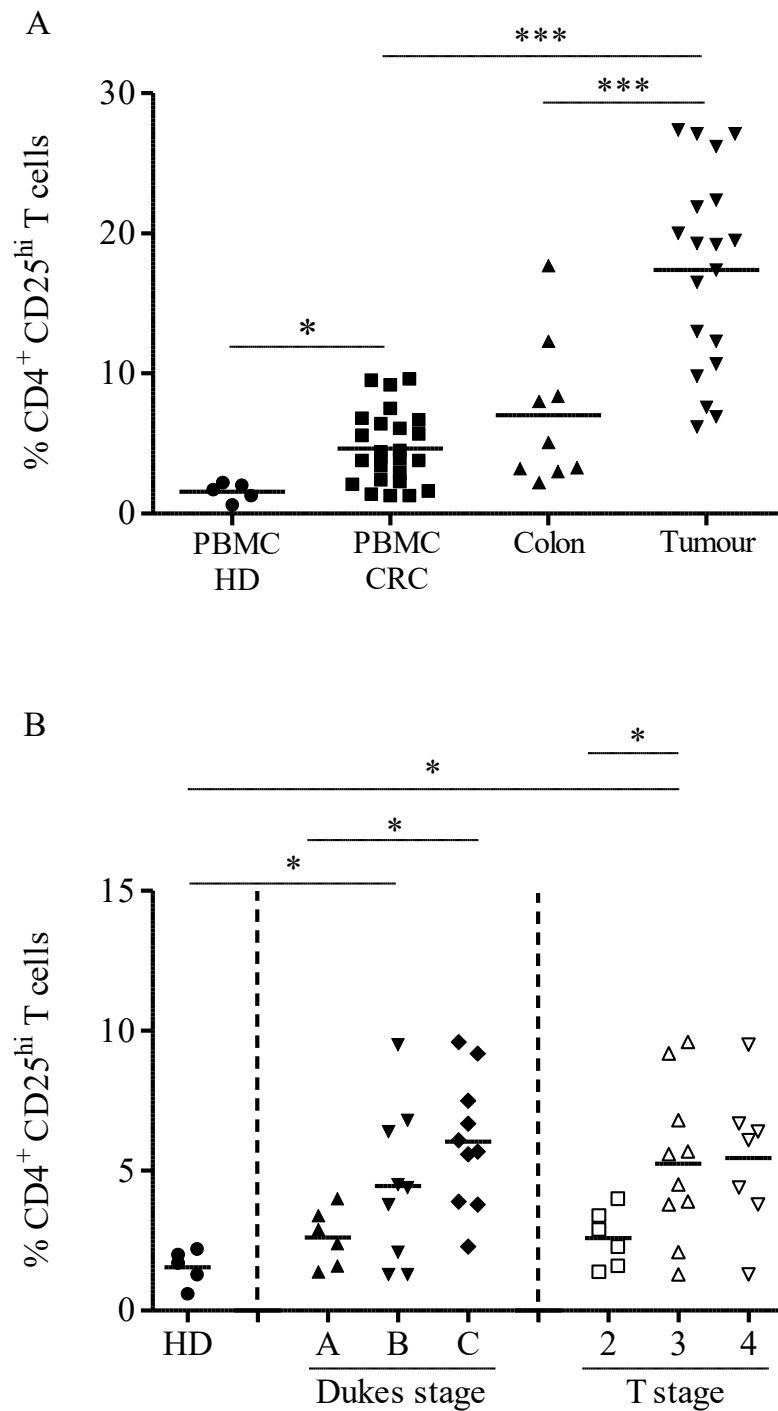
Figure 3.7. Frequency of Tregs in CRC patients. A. Analysis of the percentage of live $CD4^+$ cells expressing CD25 in PBMC isolated from healthy donors (HD, n=5) and CRC patients (n = 25) and from benign colon (n = 9) and tumour tissues (n = 19) from CRC patients. B. Percentage of Tregs in the peripheral blood of patients according to their cancers' Dukes and T stages. Significant differences are indicated, *p < 0.05, ***p < 0.001 (Student's unpaired t test).

of Tregs in the blood of CRC patients (blood vs. tumour, p < 0.0001). These data are in line with numerous reports showing that Tregs accumulate in human carcinomas (252).

Increased levels of suppressive Tregs, defined as CD4$^+$CD25^{hi} T cells, have been reported in the peripheral blood of patients with CRC or with head and neck squamous cell carcinomas (247, 258). The frequency of CD25^{hi} T cells in the CD4 population was next compared in CRC patients with different tumour stages (Figure 3.7.B). Patients with Dukes C cancers had a significantly elevated peripheral level of these cells in comparison to those with Dukes A cancers (Dukes C 6.04% ± 1.67 vs. Dukes A 2.62% ± 1.07, p = 0.0046). Similarly, patients with T3 tumour stage had an increased frequency of circulating CD4$^+$CD25^{hi} T cells (T3 5.25% ± 1.96 vs. T2 2.60% ± 1.07, p = 0.0406). Noteworthy, patients with Dukes B /C and T3/4 tumour stage had higher percentage of circulating Tregs than observed in healthy donors.

In order to better characterise the role of Tregs in tumours, frequencies of intratumoural CD4$^+$CD25^{hi} cells were compared between patients of different Dukes stages (Figure 3.8). Late stage Dukes C tumours were found to have a significant enrichment of Tregs compared to Dukes A (Dukes C 23.88% ± 5.17 vs. Dukes A 13.90% ± 11.06, p = 0.031) and B tumours (Dukes C 23.88% ± 5.17 vs. Dukes B 15.55% ± 4.78, p = 0.0253). These data show that proportions of tumour infiltrating Tregs increase with tumour progression in CRC patients.

3.2.6. Thymus-derived Tregs predominate in colorectal tumours

It has been suggested that two groups of Tregs with different origins can be distinguished based on expression of the transcription factors Helios; the Helios$^+$ thymus-derived Tregs (tTregs) and the Helios$^-$ peripherally induced Tregs (pTregs) (133). Over
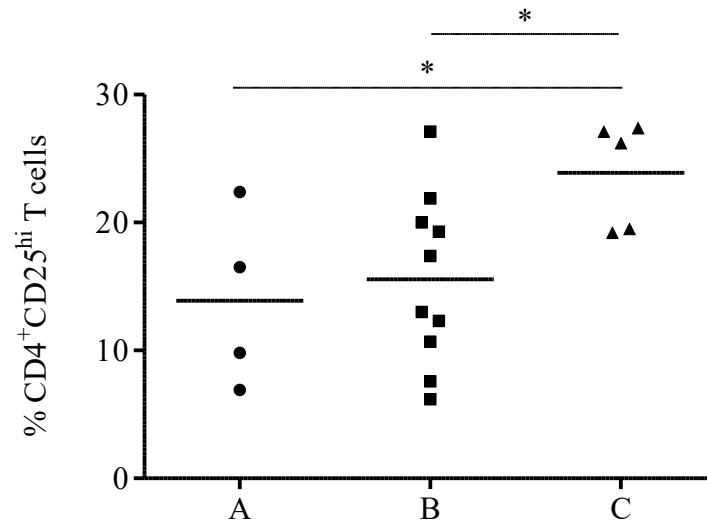
Figure 3.8. Frequency of tumour infiltrating Tregs according to Dukes stages. Percentage of CD4$^+$CD25^{hi} T cells in the tumours of patients with Dukes A, B and C cancers. T cells were isolated from tumours of CRC patients and stained *ex vivo* for FACS analyses. Significant differences are indicated, *$p < 0.05$ (Student's unpaired t test).

70% of circulating CD25^{hi} cells in healthy donors expressed Helios (Figure 3.9.A) as described in the original report detailing Helios expression in CD4$^+$Foxp3$^+$ cells (133). Approximately the same percentage of cells was found to be Helios$^+$ in the blood of CRC patients. Therefore, most of the circulating Tregs are of a thymic origin. Helios expression was then investigated in tissue infiltrating Tregs. There was a significant decrease of CD4$^+$CD25^{hi} cells expressing Helios in unaffected colon tissues compared to the blood of CRC patients (colon 46.94% ± 9.79 vs. blood 73.22% ± 3.68, $p < 0.0001$). Also, even though the majority of intratumoural Tregs expressed Helios, this fraction was significantly decreased compared to the blood (tumour 63.47% ± 7.52 vs. blood 73.22% ± 3.68, $p = 0.0130$). Overall, these data suggest the conversion of naïve T cells into Tregs does not play a significant role in the enrichment of Tregs observed in tumours. Instead, the recruitment and expansion of thymus derived Tregs (tTregs) appears to be the main reason for Treg accumulation in tumours. In line with these results, our group has shown previously in a mouse model of carcinogen-induced tumours that Tregs and conventional T cells do not share the same TCR repertoires (259). The increased number of Helios$^-$ Tregs in unaffected colon is consistent with the increased frequency of Neuropilin-1$^-$ Tregs observed in this tissue by other groups and indicates that the gut is a site where peripherally induced Tregs (pTregs) accumulate (260).

As Helios reflects the Treg pool that emerged from the thymus, thymic involution may affect the frequency of Treg cells expressing Helios. Consistent with this hypothesis, there was an inverse correlation between Helios expression on CD25^{hi} cells in blood and the age of the CRC patients ($p = 0.0328$, $R^2 = 0.41$; Figure 3.9.B).
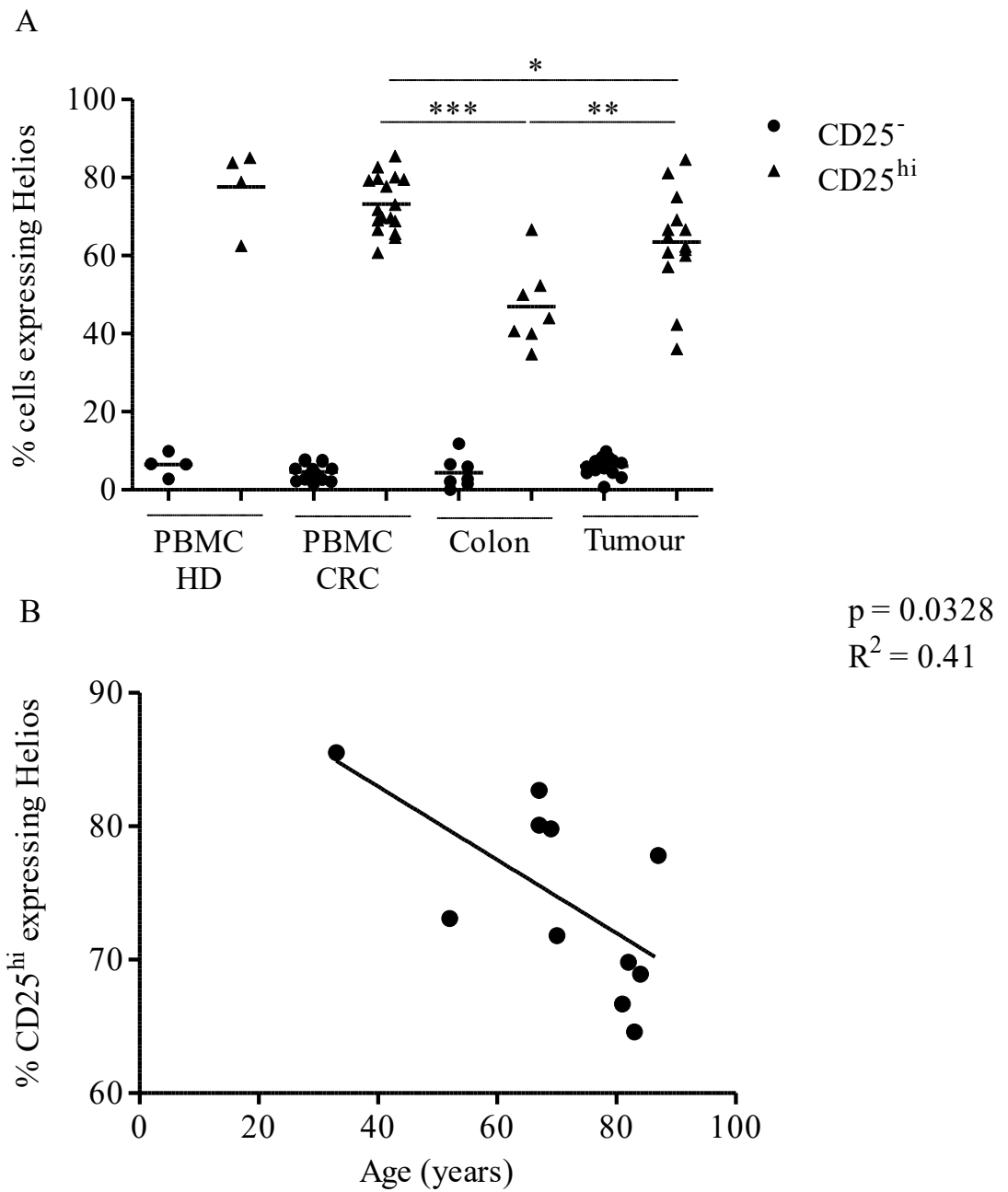
Figure 3.9. Helios expression in CD4$^+$CD25^{hi} cells. A. Intracellular Helios staining was performed on CD25$^-$ and CD25^{hi} cells within the CD4 population of PBMCs and tissue-infiltrating cells. B. Linear regression comparing the percentage of peripheral CD25^{hi} T cells expressing Helios with the age of the CRC patients.

3.2.7. High frequencies of CD25^{hi} cells express MHC-DR molecules

CD4$^+$ T cells upregulate expression of MHC class II molecules after activation. Considered to be late T cell activation markers, these molecules are also functional and can be used to present peptides to other T cells (261). Moreover, studies have shown that MHC class II expression on CD4$^+$CD25^{hi} T cells defines a population of highly proliferative activated Tregs acting through early contact-dependent inhibition (262). We therefore investigated MHC class II expression on Tregs in CRC patients.

The majority of peripheral CD25^{hi} cells within the CD4 population expressed HLA-DR molecules as opposed to CD25$^-$ cells, which did not (CD25^{hi} 53.92% ± 22.49 vs. CD25$^-$ 5.96% ± 3.73, p = 0.0004; Figure 3.10.A). The same was observed with unaffected and colorectal tumour-infiltrating Tregs. Thus, the proportion of activated Tregs is similar in peripheral blood and tumours.

It is noteworthy that compared to peripheral blood more CD25$^-$ cells expressed HLA-DR in colon tissues (healthy and malignant) indicating a differential expression of activation markers in these tissues. Furthermore, about 30% of CD25^{int} cells expressed HLA-DR in the three different samples suggesting that this fraction contains a heterogeneous population of cells expressing early (CD25) and/or late activation markers (Figure 3.10.B).

3.2.8. Intratumoural Tregs overexpress ICOS and CTLA-4

Inducible co-stimulator (ICOS) protein is structurally and functionally similar to CD28 (263). Its interactions with ICOS ligand, on the APC surface, allows an efficient T

Figure 3.10. A high proportion of Tregs are HLA-DR+. Lymphocytes from peripheral blood, colon and tumour tissues were stained for FACS analyses. A. Percentage of CD25- (dots) and CD25hi cells (triangles) expressing HLA-DR. B. Percentage of CD25int cells expressing HLA-DR in the indicated fractions. Significant differences are indicated, ***p < 0.001 (Student's unpaired t test).
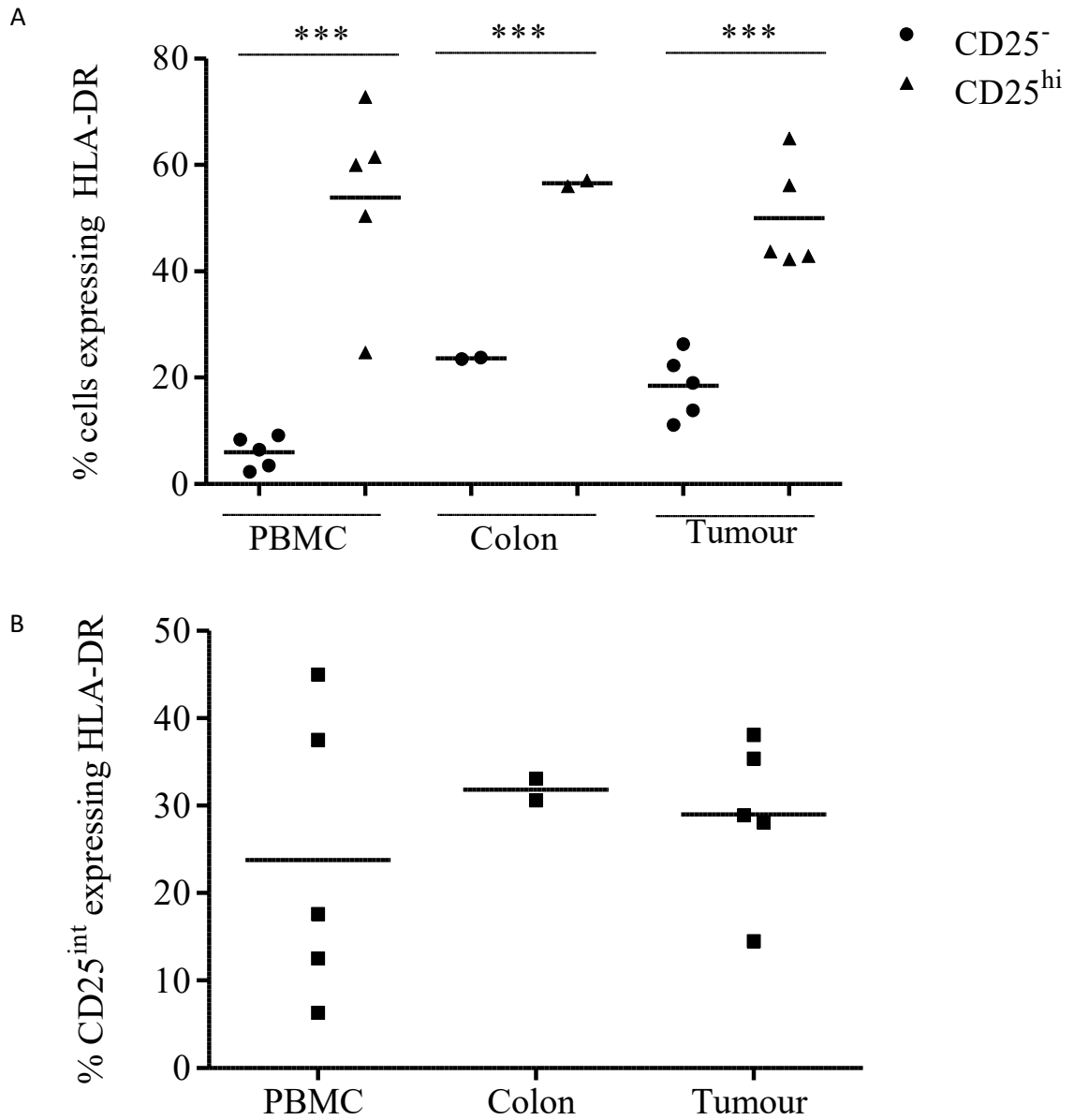
cell response. Unlike CD28, which is constitutively expressed, ICOS is only upregulated after Ag recognition and thus represents an activation marker. Reports have shown that ICOS expression is not restricted to effector T cells but is also found on Tregs. Compared to ICOS⁻ Tregs, $ICOS^+$ Tregs have enhanced suppressor functions and secrete higher levels of the immunosuppressive cytokine IL-10 (264). In order to determine if the suppressive potency of Tregs varies within the tumour, we investigated ICOS expression by $CD4^+CD125^{hi}$ cells.

The percentages of $CD25^{hi}$ $ICOS^+$ cells were comparable in peripheral blood and healthy colon tissue (approximately 50% of cells; Figure 3.11.A). A significantly higher proportion of intratumoural $CD25^{hi}$ cells express ICOS compared to circulating $CD25^{hi}$ cells (tumour 73.60% ± 7.33 vs. blood 44.34% ± 18.68, p = 0.0064). These results are line with previous report showing that higher number of Tregs, defined as $CD4^+Foxp3^+$, isolated from CRC tumours, are enriched for $ICOS^+$ cells (138). As expected, ICOS was found on activated $CD25^{int}$ cells isolated from blood and tissues (Figure 3.11.B) whilst only very low frequencies (less than 8%) of CD25⁻ cells expressing ICOS were observed in blood and colon of CRC patients.

Another cell surface co-receptor, CTLA-4, is involved in the regulation of the immune response. When binding to its ligands B7-1/-2, CTLA-4 functions as a negative regulator of cellular activation (265). Like ICOS, CTLA-4 is marker of activation with its expression restricted to activated T cells. Tregs also express CTLA-4 and is implicated in mediating their suppressive effects.

Intracellular CTLA-4, stored in the cells, can be recruited and be expressed at the cell surface and thus this stock represents the potential that a cell can express. In the blood of CRC patients, intracellular CTLA-4 was detectable in a high proportion (over 70%) of $CD25^{hi}$ cells to less than 5% in the naive CD25⁻ population (Figure 3.12.A), consistent

A

B
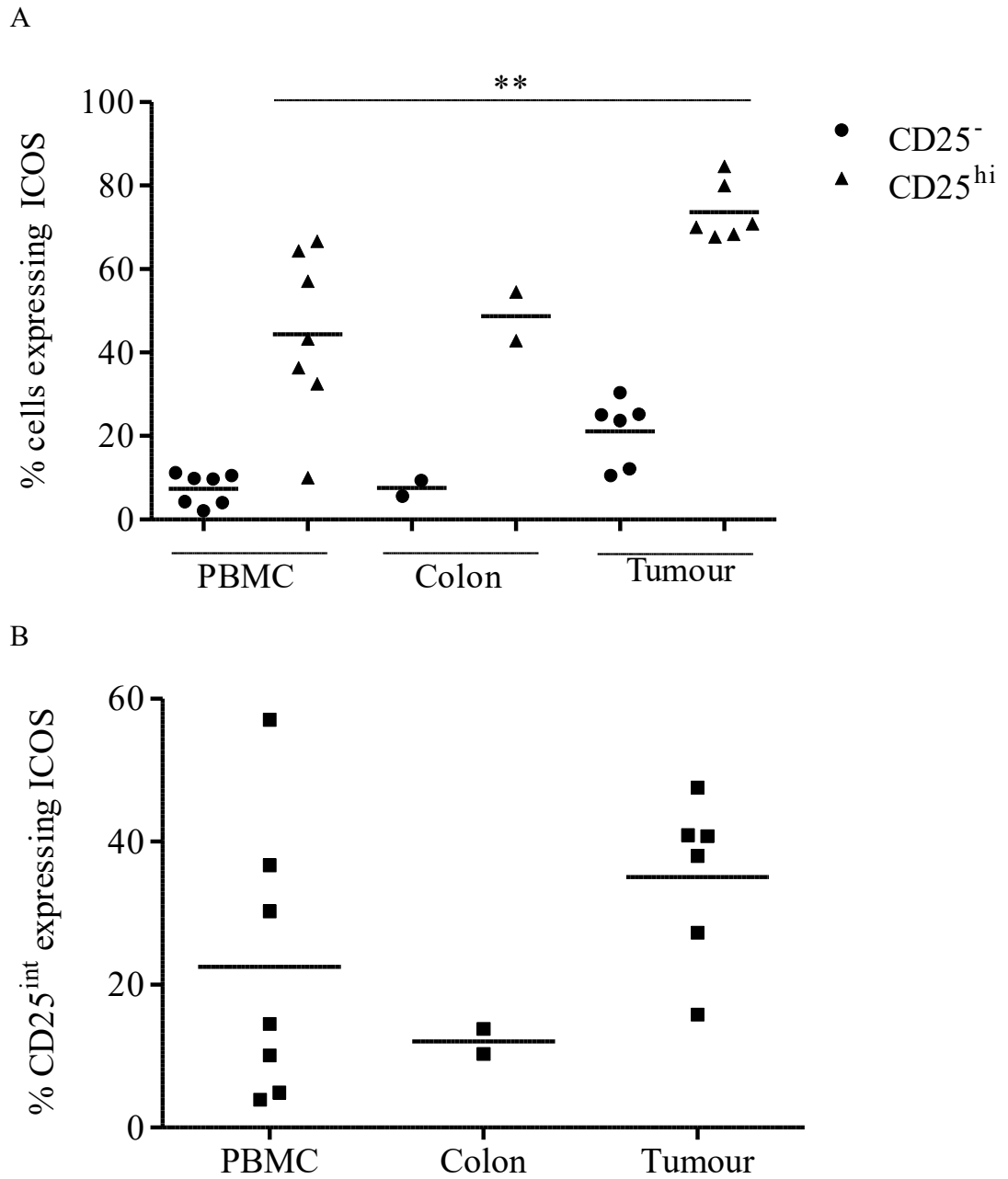
Figure 3.11. CD4+CD25hi T cells expressing ICOS are enriched in CRC tumours. A. Percentages of T cells in the blood, colon and tumour of CRC patients expressing ICOS. B. Percentage of activated CD25int cells expressing ICOS. Significant differences are indicated, ** $p < 0.01$ (Student's unpaired t test).Significant differences are indicated, ***$p < 0.001$ (Student's unpaired t test).

with a central role for CTLA-4 in dictating Treg activity. The same pattern of expression was found in colon and tumour tissues, where a slight but consistent increase in proportions of CD25^{hi} cells expressing CTLA4 was observed compared to blood (tumour 88.58% ± 5.17 vs. blood 71.15% ± 7.15, p = 0.0008). When compared with healthy colon, higher frequencies of tumour-infiltrating Tregs with detectable intracellular CTLA-4 were observed. These data together with those shown in Figure 3.10 show that CD25^{hi} cells in CRC tumours express high levels of costimulatory molecules associated with suppressive functions.

Intracellular stores of CTLA-4 were also observed in CD25^{int} effector T cells, particularly within the intratumoural CD25^{int} cell population (Figure 3.12.B), albeit in a smaller proportion of cells than within the CD25^{hi} population. This may reflect the higher activation status of tumour-infiltrating T cells.

3.2.9. CD39 is upregulated by intratumoural CD4$^+$CD25^{hi} Tregs

CD39 represents another key molecule playing an important role in the regulatory activity of Tregs. This ectonucleotidase facilitates the production of immunosuppressive adenosine from ATP.

The frequency of peripheral CD4$^+$CD25^{hi} cells expressing CD39 was higher than within the CD4$^+$CD25$^-$ population (CD25^{hi} cells 43.2% ± 8.20 vs. CD25$^-$ cells 5.9% ± 2.56, p < 0.0001; Figure 3.13.A). In the blood of CRC patients, the CD4$^+$CD25^{int} subset contained T cells with low CD39 expression (mean 13.4% ± 4.87; Figure 3.13.B). These data are consistent with reports showing that CD39 expression is restricted to CD4$^+$CD25^{hi} Tregs in the blood of healthy people (266).

The proportion of CD39$^+$ cells was significantly higher in CD4$^+$CD25^{hi} cells in the
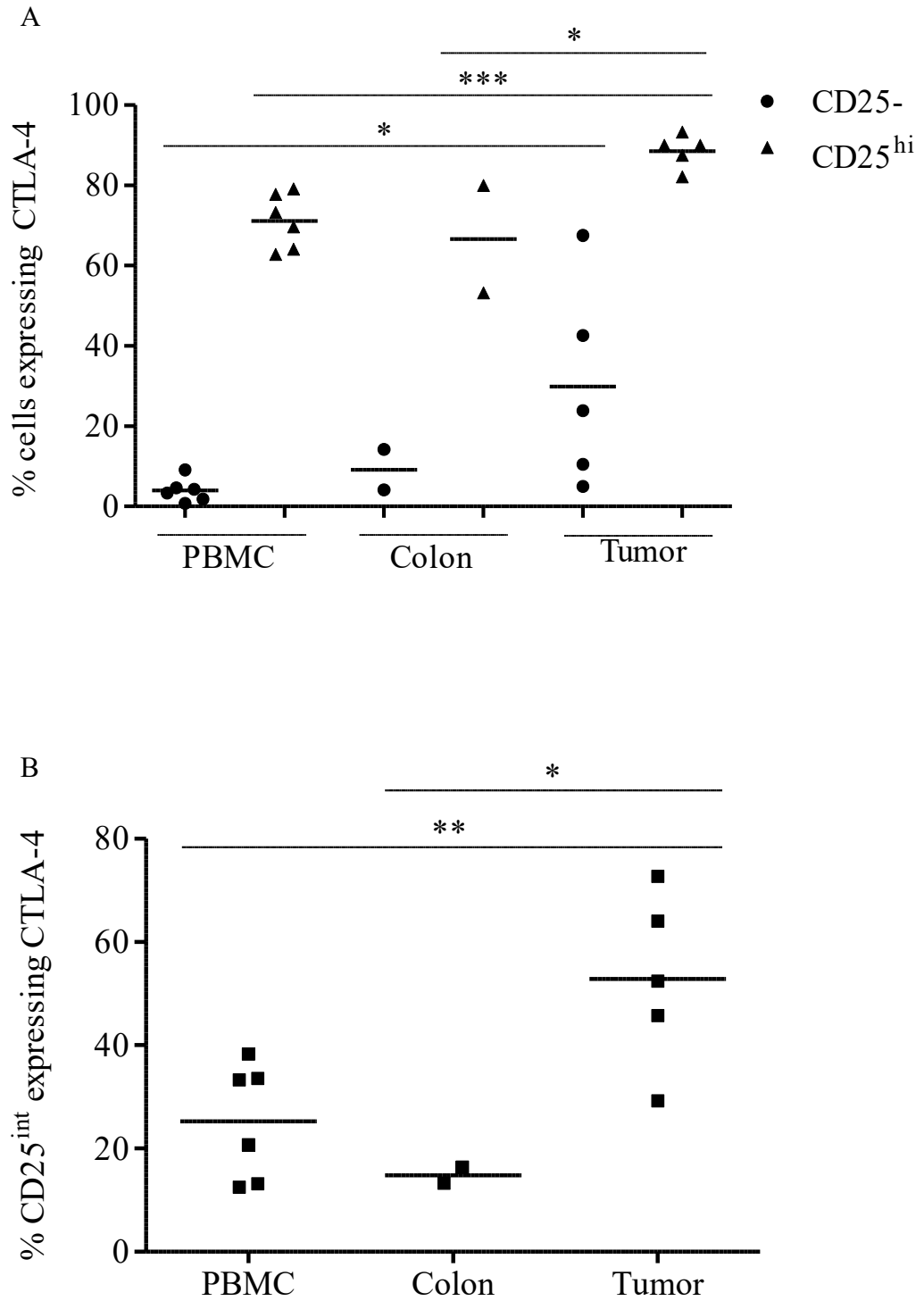
Figure 3.12. Tregs express high levels of CTLA-4. A. Frequencies of T cells expressing intracellular CTLA-4 in blood, colon and CRC tumours. B. Frequencies of activated CD25int cells expressing CTLA-4. Significant differences are indicated, * p < 0.05, ** p < 0.01, ***p < 0.001 (Student's unpaired t test).
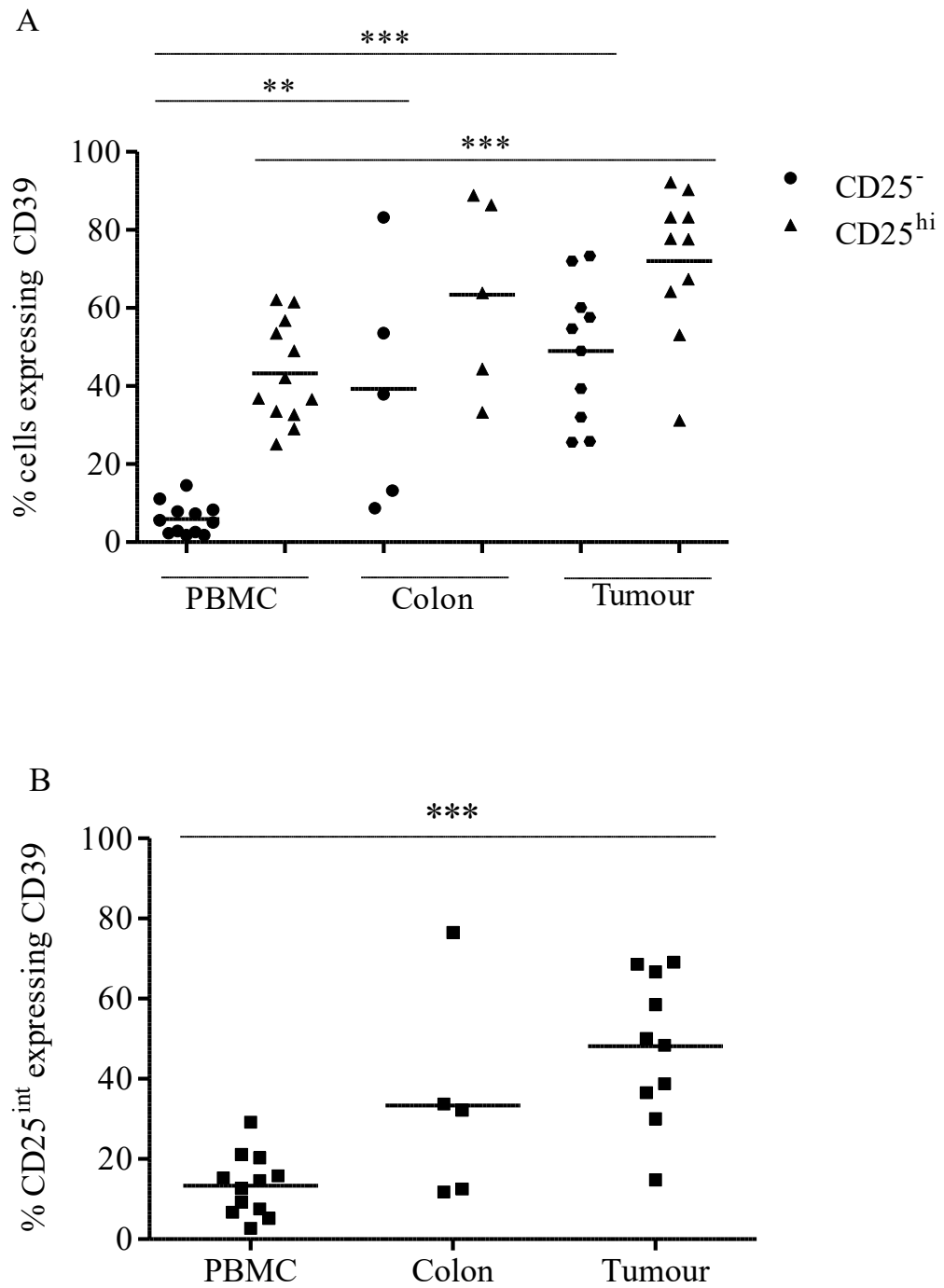
Figure 3.13. Frequency of CD39+ cells is increased in intratumoural CD25hi Tregs.
A. Percentages of CD25- (dots) and CD25hi (triangles) cells expressing CD39 in blood, colon and tumour of CRC patients. B. Percentage of CD25int expressing CD39 in CRC patients. Significant differences are indicated, ** $p < 0.01$, ***$p < 0.001$ (Student's unpaired t test).

tumour compared to blood (tumour 72.04% ± 13.42 vs. blood 43.2% ± 8.20, p = 0.0004; Figure 3.13.A). This up regulation of CD39 in intratumoural Tregs was also observed in head and neck cancer patients (267). The extremely high frequency of intratumoural $CD25^{hi}CD39^+$ Tregs suggests that ATP hydrolysis might an important mechanism used to suppress effector T cells at the tumour sites. These data also demonstrate that CRC tumours are highly immunosuppressive since it has been shown that CD39-expressing Tregs have enhanced suppressive abilities compared to CD39$^-$ Tregs (174).

It is noteworthy that the percentage of CD25$^-$ cells expressing CD39 was higher in the tumours compared to the peripheral blood of CRC patients (tumour 48.97% ± 12.65 vs. blood 5.92% ± 2.57, p < 0.0001; Figure 3.13.A); a similar observation was made in healthy colon. These $CD4^+CD25^-Foxp3^-CD39^+$ cells, may also help counterbalance immune activation in healthy tissues, thereby helping maintain normal immune homeostasis but may contribute to inhibition of anti-tumour immunity in CRC.

3.2.10. CRC patients have increased percentages of CD25^{hi} LAG3$^+$ and LAP$^+$ cells

The transmembrane LAG3 protein represents a marker reported to define Tregs with enhanced suppressor activity (268). Tumour infiltrating-LAG-3$^+$ Tregs have been shown to supress immune responses in Hodgkin lymphoma patients (269).

A discrete population of circulating LAG-3$^+$ cells was detectable in CRC patients, particularly within the CD25^{hi} subset (CD25^{hi} cells 11.01% ± 4.53 vs. CD25$^-$ cells 3.99% ± 2.26, p = 0.0059; Figure 3.14.A). Furthermore, the proportion of tumour-infiltrating $CD4^+CD25^{hi}$ T cells that also express LAG3 was increased in CRC patients compared to
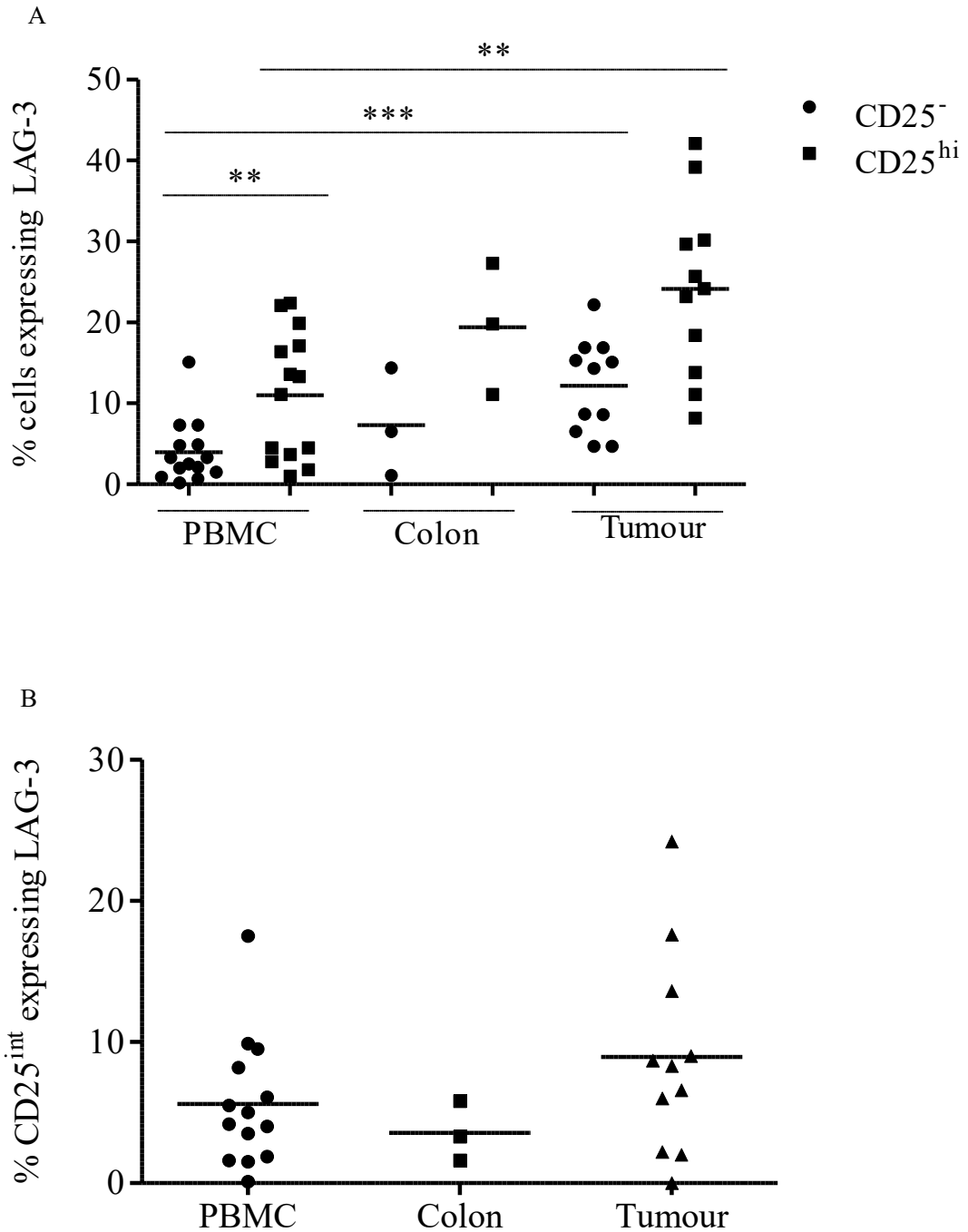
Figure 3.14. Increased frequency of CD4⁺CD25ʰⁱ LAG-3⁺ cells in the tumours of CRC patients. A. Percentages of cells in the indicated CD25 subsets expressing LAG-3 in peripheral blood, colon and tumour of CRC patients. B. Percentage of CD25ⁱⁿᵗ cells expressing LAG-3 in the indicated samples from CRC patients. Significant differences are indicated, ** $p < 0.01$, ***$p < 0.001$ (Student's unpaired t test).

peripheral blood (tumour 24.16% ± 7.31 vs. blood 11.01% ± 4.53, p = 0.0019). This is consistent with the findings of Camisaschi *et al* showing that melanomas are enriched in CD25^{hi}LAG-3^{+} T cells (188). This enrichment of LAG3^{+} T cells within CRC was also seen on the CD4^{+}CD25^{-} T cells (tumour 12.17% ± 3.90 vs. blood 3.99% ± 2.26, p = 0.0003; Figure 3.14.A). Proportions of circulating CD25^{-} and CD25^{int} cells expressing LAG-3 were similar implying that LAG-3 is not a marker of activation (Figure 3.14.B).

TGF-β plays an important role in Treg activity, notably in the suppression of anti-tumour immunity (168). Before being released, TGF-β interacts with Latency Associated Peptide (LAP) on the extracellular matrix. Lately, Chen *et al* have found that, in CRC patients, LAP-expressing Foxp3^{+} Tregs are activated cells with more potent suppressive activity dependent on TGF-β (270). The comparison between circulating and tumour-infiltrating lymphocytes revealed an increase in the frequency of CD25^{hi} cells expressing LAP in the tumours (blood 10.86% ± 1.95 vs. tumour 27.13% ± 7.54, p < 0.0001; Figure 3.15.A). The percentage of CD4^{+}CD25^{hi}LAP^{+} cells was also significantly higher in non-tumoural colon tissues compared with blood (colon 25.20% ± 18.85 vs. blood 10.86% ± 1.95, p = 0.0002). The percentage of CD4^{+}CD25^{-} cells expressing LAP was also increased within the CRC in comparison to blood (tumour 9.12% ± 4.53 vs. blood 4% ± 0.9%, p = 0.0025). Similar observations were found with CD4^{+}CD25^{int} cells.
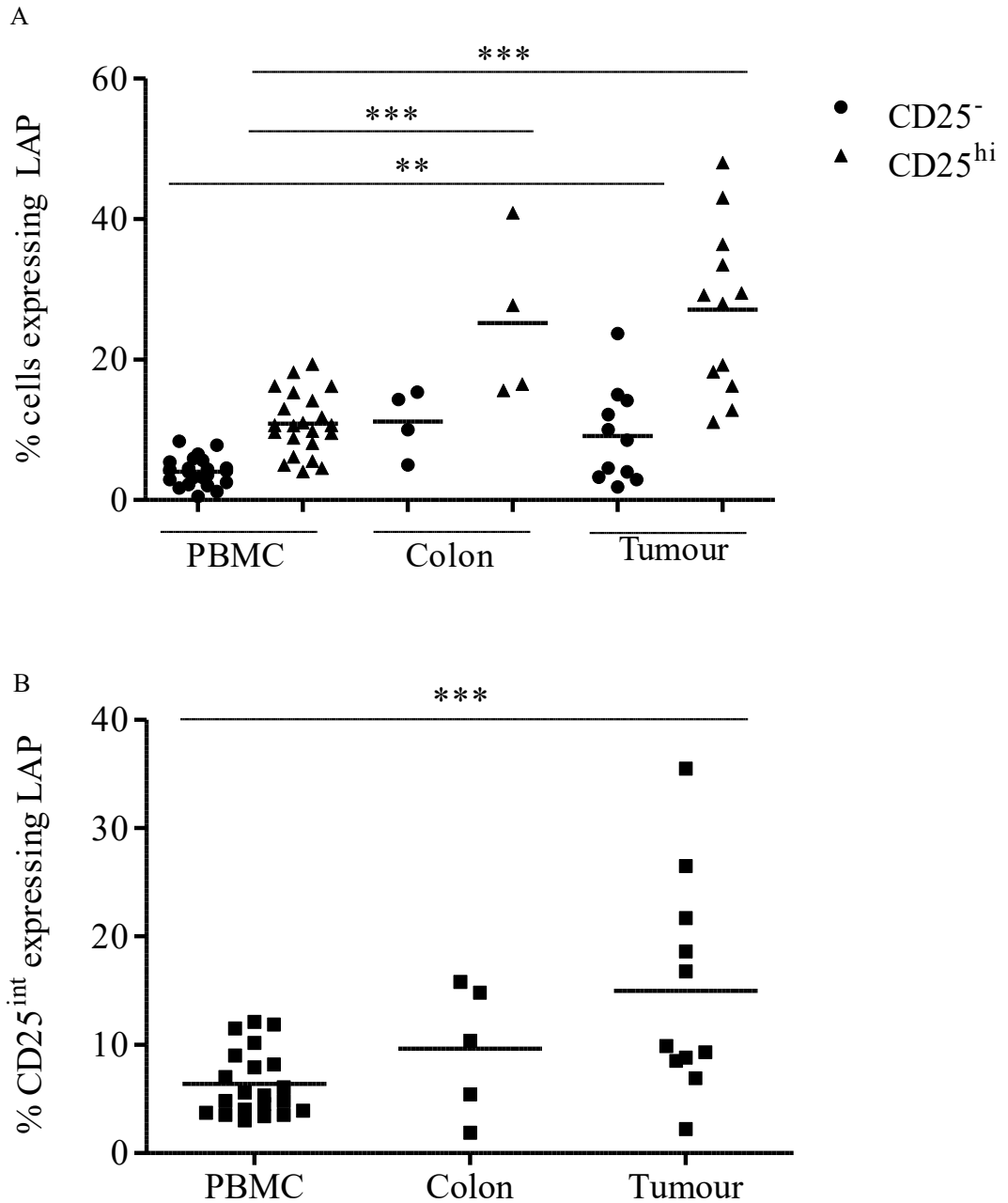
Figure 3.15. Higher frequency of CD4+CD25hi LAP+ cells in colorectal tumours. A. Percentages of CD25- and CD25hi cells expressing LAP in the indicated CRC samples. B. Percentage of CD4+CD25int cells expressing LAP in CRC patients. Significant differences are indicated, ** $p < 0.01$, ***$p < 0.001$ (Student's unpaired t test).

3.3. Discussion

CD4$^+$CD25^{hi} T cells are considered to represent the human Treg population based on their strong *in vivo* and *in vitro* suppressive activity. These cells can inhibit the proliferation of CD4$^+$CD25$^-$ T cells and the maturation of myeloid DC (249, 271). Whilst alterations of CD4$^+$CD25^{hi} T cell functions have been associated with autoimmune diseases (272), increased proportions of these cells are commonly observed in blood of cancer patients (252, 273-275). In CRC, CD4$^+$CD25^{hi} cells have been directly implicated in the inhibition of anti-tumour immunity. By depleting these cells from PBMC of CRC patients, Clarke *et al* showed that new tumour Ag-specific CD4$^+$ T cell responses were generated (247). Although CD4$^+$CD25^{hi} cells are enriched in blood and tumours of CRC patients (247, 276), their phenotype has not been comprehensively studied. This study reveals that intratumoural CD4$^+$CD25^{hi} cells overexpressed immunomodulatory molecules in CRC patients implying significant ability to impact negatively on anti-tumour immunity.

Peripheral CD4$^+$CD25^{hi} cells invariably expressed high levels of Foxp3 and were CD127^{low}, which are to date the best markers to define Tregs, when suppressive assays cannot be performed (256). These cells had the same phenotype (CD45RA$^-$CD45RO$^+$) as the suppressive human CD4$^+$CD25^{hi} cells originally reported by Baecher-Allan *et al* (249). The frequency of CD4$^+$CD25^{hi} cells was increased in the blood and at tumour site of CRC patients, confirming previous reports. This study showed that patients with late stage CRC had elevated percentage of circulating and tumour-infiltrating CD4$^+$CD25^{hi} cells compared to early stage. Also, this study demonstrates that recruitment or expansion of thymus-derived Tregs are the main mechanisms contributing to CD4$^+$CD25^{hi} enrichment in tumours. Factors supporting this accumulation, such as IDO and VEGF-a (277), represent good alternative therapies. In this respect, VEGF-a blockage has been

shown to decrease the number of intra-tumoural $CD4^+CD25^{hi}$ in preclinical mouse models (278).

In this study, 6 Treg-associated markers were used to define the $CD4^+CD25^{hi}$ cell phenotype. HLA-DR was the only protein that was expressed by similar proportions on peripheral and tumour-infiltrating $CD4^+CD25^{hi}$ cells (approximately 50%). This marker defines activated mature $CD4^+CD25^{hi}$ cells involved in contact-dependent suppression. (262). Therefore, $CD4^+CD25^{hi}HLA-DR^+$ cells may not be the main suppressive cells within the tumour of CRC patients. In contrast, ICOS, CTLA-4, CD39, LAG-3 and LAP were significantly expressed by more tumour-infiltrating $CD4^+CD25^{hi}$ cells compared to circulating $CD4^+CD25^{hi}$ cells in CRC patients. Some of these markers have been reported to be expressed with higher frequency on tumour-infiltrating $CD4^+CD25^{hi}$ cells; ICOS (264), CTLA-4 (279) and LAG-3 (188), in melanoma patients. There is evidence that intratumoural $CD4^+CD25^{hi}$ cells exhibiting a predominant immunosuppressive phenotype have enhanced suppressive activity (267). In this study, a striking high number, more than 70%, of $CD4^+CD25^{hi}$ cells expressed ICOS, CTLA-4 and CD39. These molecules may be key modulators of $CD4^+CD25^{hi}$ cell activity and promote a strong immunosuppressive environment in tumours of CRC patients.

Although blockade of ICOS, CTLA-4 and CD39 may enhance anti-tumour immunity, such treatment is likely to induce immune-related adverse events. Indeed, these proteins were also expressed, albeit at lower level, on $CD4^+CD25^{int}$ cells either isolated from blood, colon or tumour tissues. These cells correspond to non-suppressive effector T cells (249), and 40% were found to express Foxp3; an event that may take place to attenuate T cell activation (280). Moreover, this Foxp3 expression in non-Treg cells may explain the high percentage of $CD4^+Foxp3^+$ cells, compared to $CD4^+CD25^{hi}$ cells, observed in CRC patients (Appendix Figure 2).

Therefore, since Tregs share many markers with CD4$^+$CD25^{int} cells, precautions must be taken in order to target the first population without impacting the second. This absence of specific Treg markers explains the immune-related adverse events during mAb-based therapies. For instance, autoimmune disorders are often associated with anti-CTLA-4 mAbs (e.g. ipilimumab) treatment in metastatic melanoma patients (32). In addition to impacting Treg activity, anti-CTLA-4 mAbs are likely to prevent cell-intrinsic down-regulation of T cell activation resulting in harmful uncontrolled immune responses. Since high expression of CD25 is an unique feature of suppressive CD4$^+$CD25^{hi} cells, drugs specifically targeting this molecule on Tregs are less likely to lead to immune disorders. Using low dose of DAB$_{389}$IL-2, a recombinant IL-2-diphtheria toxin conjugate, Dannull *et al* could selectively eliminate human CD4$^+$CD25^{hi} cells without impacting the function of CD4$^+$CD25^{int} cells (281). This approach has been reported to decrease the percentage of circulating Tregs in cancer patients (282) and to induce tumour regression in some melanoma patients in a recent phase II clinical trial (283).

In conclusion, this study showed that the majority of CD4$^+$CD25^{hi} cells express immunomodulatory molecules at tumour sites of CRC patients. In parallel, these molecules were also detected on CD4$^+$CD25^{int} effector cells. These results give insights into the mechanisms potentially mediating Treg suppressive activity in CRC patients. They also highlight the absence of unique Treg markers which has implications for the development of safe cancer immunotherapy.

CHAPTER 4. EFFECT OF TREGS ON 5T4-SPECIFIC RESPONSES

4.1. Introduction

The aim of the work described in this Chapter is to characterise T cell responses to the 5T4 Ag in CRC patients and healthy controls. As described in Chapter 1, there is evidence that 5T4 is an immunogenic protein but 5T4 immunogenicity has never been characterized in detail. Therefore, responses to peptide pools spanning the sequence of the 5T4 protein were analyzed (Table 2.4 in Material and Methods) in order to compare magnitude of T cell responses, and percentage of responders between healthy donors and CRC patients. Peptide pools, rather than the whole 5T4 protein, were used so that precise immunogenic regions and peptides recognised by CD4$^+$ T cells could be identified. These peptides were overlapping by 10 amino acids to include all potential T cell epitopes. As cancer patients have high numbers of Tregs relative to healthy individuals, anti-tumour Ag responses are more likely to be controlled by these cells. Thus, the impact of Tregs on 5T4 responses was also studied.

5T4-specific T cell responses were measured by IFN-γ production in ELISpot assays following overnight stimulation with 5T4 peptide pools. Even though ELISpot is a very sensitive functional assay, the frequency of 5T4-specific T cells in peripheral blood is too low to be consistently measured *ex vivo*. Therefore, a 12-day stimulation period was required to expand the 5T4-specific T cells in order to detect cognate T cells. In the assays used here, peptides were directly added to the PBMCs for loading onto MHC molecules and presentation to T cells.

4.2. Results

4.2.1. ELISpot assay variability

The first report of the use of ELISpot assay, to enumerate Ab-secreting cells, was published thirty years ago (284). Since then, the assay has evolved to measure Ag-specific T cell responses. It is an extremely sensitive assay (detection of frequencies as low as $5/10^6$ cells) but data on its variability is limited. I initially set out to examine this feature in the context of 5T4-specific T cell responses. All analyses done in the course of this PhD were performed with standard laboratory procedures in order to allow reliable comparisons. All assays were performed with freshly isolated PBMCs.

Intra-assay precision

In order to evaluate the level of intra-assay variability, PBMC from one healthy donor were stimulated with 5T4 single peptides in IFN-γ ELISpot assays. One representative example, after stimulation with peptide 2, is shown in Figure 4.1.A. Very low variation was found between the replicates in both control and stimulated wells. The same observations were obtained with two other peptides that elicited a response (Figure 4.1.B). For instance, the mean response to peptide 20 was 166 spots / 10^5 cultured cells with 95% confidence interval of 126 to 206. This absence of substantial variability in the assay was observed throughout the study.

Variation in T cell responses over time

The variation in T cell responses was assessed at three different time points using PBMCs from the same healthy control. Reponses against five different candidate peptides were measured in duplicate at intervals of not less than two weeks. Positive responses to 3 peptides (peptides 2, 20 and 26) were detected at two or more time points; a responses to

A

B
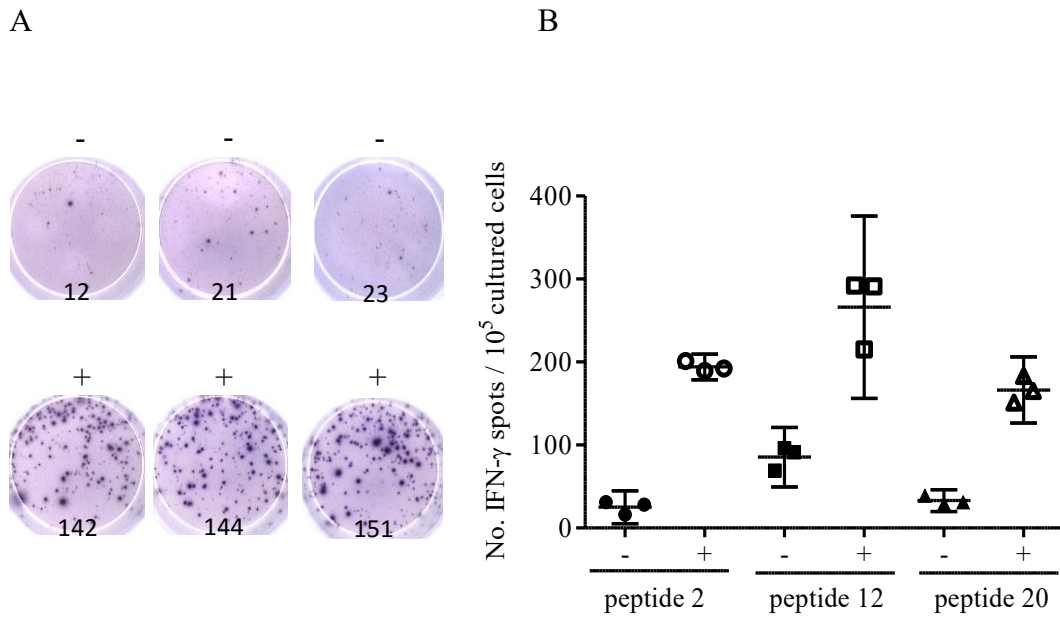
Figure 4.1. Intra-assay variability. PBMC from one healthy donor were stimulated with 5T4 peptides for 12 days. Cells from three cell lines stimulated by the same peptides were then pooled, washed and added in ELISpot well. Cells were plated in triplicate wells. A. Example of IFN-γ ELISpot wells in the absence (-) or presence (+) of peptide 2. Numbers indicate the frequency of IFN-γ spots / 75000 cells. B. Data indicate the number of IFN-γ spots normalised to per 10^5 cultured cells. Circles: peptide 2; squares: peptide 12; triangles: peptide 20. Closed symbols represent background controls (no peptide restimulation) and open symbols represent 5T4-specific responses (peptide restimulation). Lines indicate means with 95% confidence interval. All experiments were performed in triplicate.

peptide 12 was only seen at the final time point; no response was found to peptide 38 at any time point. The responses to peptide 26 were consistently lower in frequency. Each positive assay at any particular time point demonstrated tight error bars suggesting, as shown in Figure 4.1, that the actual assays conducted at each time point were reliable, and that there is a natural variation in T cell responses over time. These results are summarised in Figure 4.2.

It is therefore important to appreciate that an assay performed on an individual at only one time point may not reflect the full possible repertoire of cognate T cell responses to 5T4 peptides; this variation in 5T4 responses has been subsequently observed in an on-going clinical trial in our laboratory where patients are tested 6-8 times over 15 weeks (Andrew Godkin and Martin Scurr: personal communication). This temporal variation has also been seen to other Ags such as influenza haemagglutinin (Andrea Schauenburg: personal communication).

4.2.2. Broad 5T4-specific responses in healthy donors

The T cell specificity for 5T4 peptides was explored in healthy individuals. A summary of donors' details is shown Table 4.1. PBMCs from 9 healthy donors (HD) were stimulated with 5T4 peptides (utilising pools 7 - 13) representing adjacent regions of the protein (Table 2.4 in Material and Methods). Specific T cells were quantitated by IFN-γ ELISpot assay and were scored as spots per 10^5 cultured cells.

Two representative examples are illustrated in Figure 4.3. HD205 was found to be positive for peptide pools 9, 11 and 13; and HD215 positive for pools 8, 10, 11 and 12. The robustness of these responses is illustrated with examples of IFN-γ ELISpot wells shown in Figure 4.3.B. A range in frequencies of peptide pool-specific T cells was
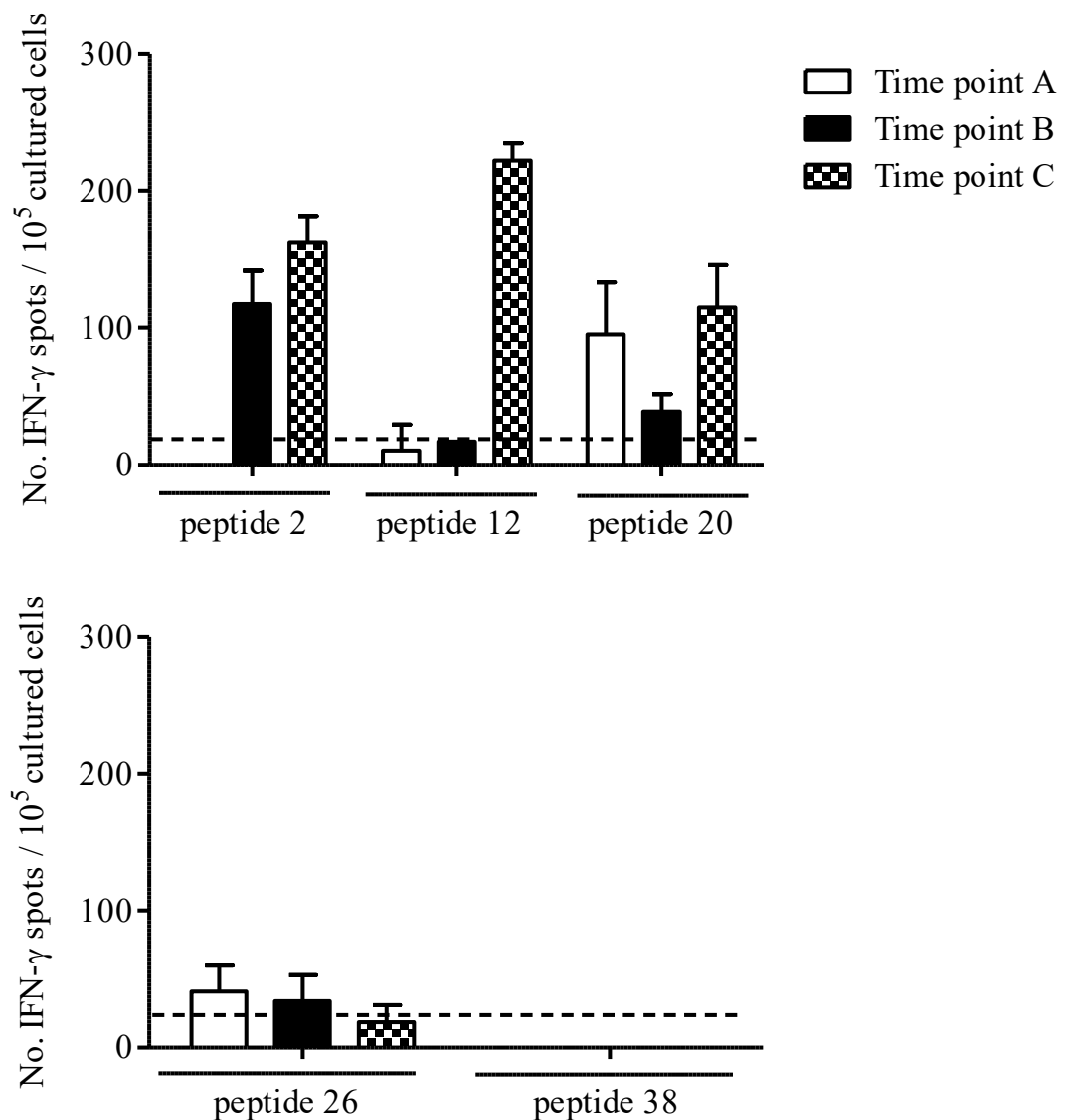
Figure 4.2. IFN-γ responses measured by ELISpot assay change over time. PBMC from one healthy donor were stimulated with five 5T4 peptides in duplicate. T cell responses were monitored at three different time points, represented by bars. At least two weeks separated each time point. Data represent mean frequencies of responses with 95% confidence interval, after subtracting control background. A line was considered positive if there was >25 spots / 10^5 cultured cells (represented by dashed horizontal lines).

Healthy donor	Gender	HLA-DRB1	HLA-DQB1
HD204	m	*01, *15	*05, *06
HD205	f	*07, *13	*06
HD206	f	*03, *07	*02
HD210	m	*04, *15	*06, *03
HD215	m	*04, *07	*02, *03
HD216	m	*04	*03
HD218	m	*15, *10	*05, *06
HD220	m	*09, *14	*05, *03
HD222	m	*01, *07	*02, *05

Table 4.1. Characteristics of the 9 healthy donors included in the IFN-γ ELISpot analysis

A

HD205

HD215

B

PP 8   PP 10   PP 11   PP 12
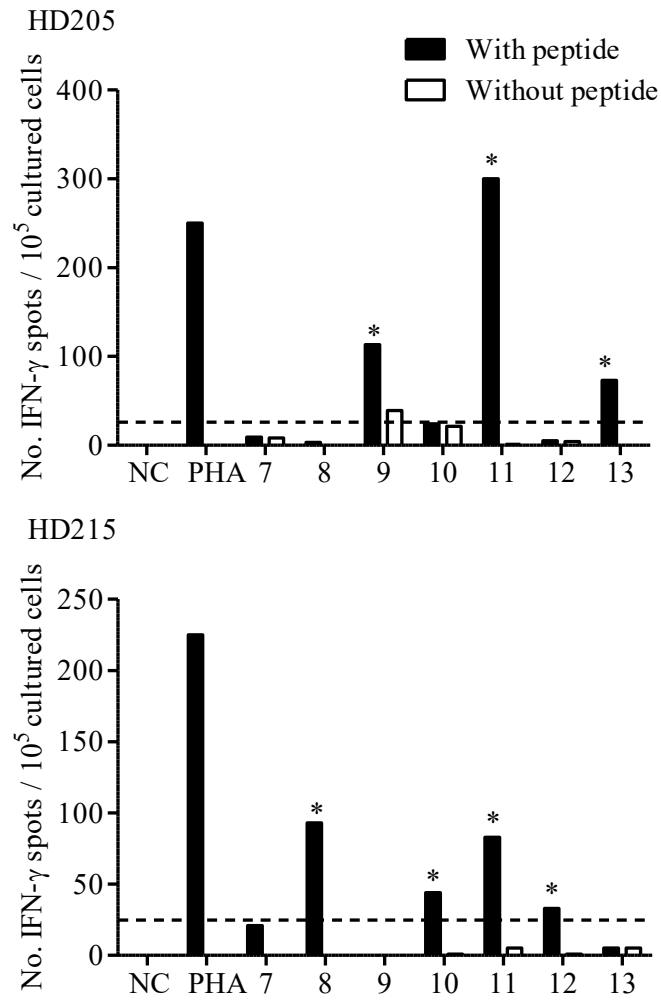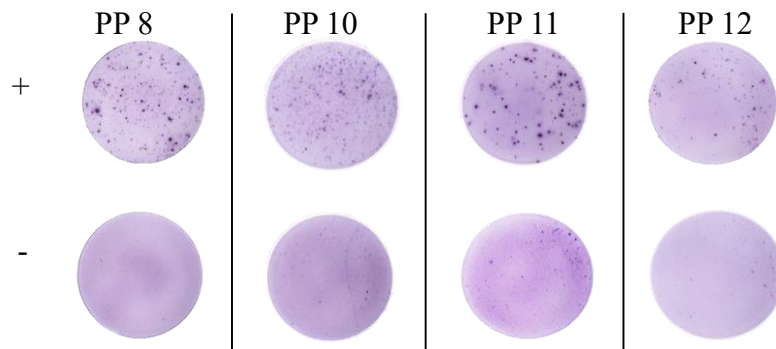
Figure 4.3. ELISpot assay permits the detection of T cells specific for 5T4 in healthy donors. A. PBMCs from HD205 and HD215 were stimulated for 12 days with the indicated peptide pools. Black bars indicate stimulation with peptides and white bars indicate the negative background (stimulation in culture medium only). Phytohemagglutinin (PHA) was used as a positive control. Negative control (NC) indicates responses with unstimulated cells. Asterisks on the top of bars point to positive responses. B. Wells illustrating positive IFN-γ responses to 4 5T4 peptide pools in HD215. Each spot corresponds to a single T cell producing IFN-γ after stimulation.

observed in these 2 patients. For instance, HD205 had a frequency of pool-specific T cell that ranged from 1/1369 (peptide pool 13 with 73 IFN-γ spots / 10^5 cells) to 1/250 (peptide pool 11 with 399 IFN-γ spots / 10^5 cells). Similarly, in a second healthy donor the frequency of pool-specific T cell ranged from 1/3125 (peptide pool 12 with 32 IFN-γ spots / 10^5 cells) to 1/1075 (peptide pool 8 with 93 IFN-γ spots / 10^5 cells). Furthermore, several pools (3 for HD205 and 4 for HD215) stimulated IFN-γ secretion indicating that the 5T4-specific response is broad and not focused on one particular peptide.

The same IFN-γ ELISpot assay was next applied to determine the frequencies of peptide-specific T cells in a further 9 healthy donors. Results are summarized in Table 4.2. Responses to pools spanning the entire 5T4 protein were observed. Indeed, across all donors, we observed responses to each of the 7 pools. With the exception of HD220, all healthy donors responded to at least 3 peptide pools confirming that the response to 5T4 is broad in these subjects. Individuals most frequently exhibited positive responses to pool 13 (67%), followed by pools 8, 10 and 11 (56%) and then pools 9 and 12 (33%).

The frequencies of 5T4 peptide-pool specific T cells in healthy donors are summarized in Figure 4.4. The magnitude of IFN-γ responses was also different between pools. Overall, the strongest response was observed against pool 11 with a mean response of 1/448 (i.e. 223 spots /10^5 cells) among responders. The weakest response was directed against pool 12 with mean response of 1/2222 (45 spots / 10^5 cells). From these experiments, pool 11 appears to be the most immunogenic on the basis that it is recognized by most donors, and often with high frequencies of specific T cells. With similar reasoning, pool 12 is the least immunogenic pool.

| | Peptide Pools | | | | | | | | | | | | | | | | | | | | |
| | 7 | | | 8 | | | 9 | | | 10 | | | 11 | | | 12 | | | 13 | | |
	+	-	=	+	-	=	+	-	=	+	-	=	+	-	=	+	-	=	+	-	=
HD210	0	0	0	363	0	363	279	143	136	212	4	208	328	3	325	77	0	77	320	3	317
HD222	36	7	29	235	9	225	25	7	19	61	25	36	204	132	72	31	3	28	47	23	24
HD206	45	0	45	0	0	0	47	0	47	32	5	27	9	3	7	215	172	43	1	1	0
HD204	27	0	27	0	0	0	400	0	400	3	0	3	400	127	273	4	0	4	121	7	115
HD215	21	0	21	93	0	93	0	0	0	44	1	43	83	5	77	33	1	32	5	5	0
HD205	9	8	1	3	0	3	113	39	75	24	21	3	400	1	399	5	4	1	73	0	73
HD216	209	7	203	93	3	91	47	27	20	36	9	27	19	16	3	27	17	9	117	19	99
HD220	29	16	13	21	19	3	24	12	12	37	15	23	51	32	19	32	15	17	105	3	136
HD218	107	8	99	48	21	27	31	16	15	37	29	8	75	32	43	7	7	0	116	16	100
Responders	5/9			5/9			3/9			5/9			5/9			3/9			6/9		

Table 4.2. 5T4-specific IFN-γ responses in 9 healthy donors. PBMCs were stimulated with 5T4 peptides assembled in peptide pools 7 to 13. ELISpot results from stimulated (symbolized with +) and unstimulated wells (symbolized with -) are shown. After subtracting the background (=), positive responses were underlined. All responses are per 10^5 cultured cells.
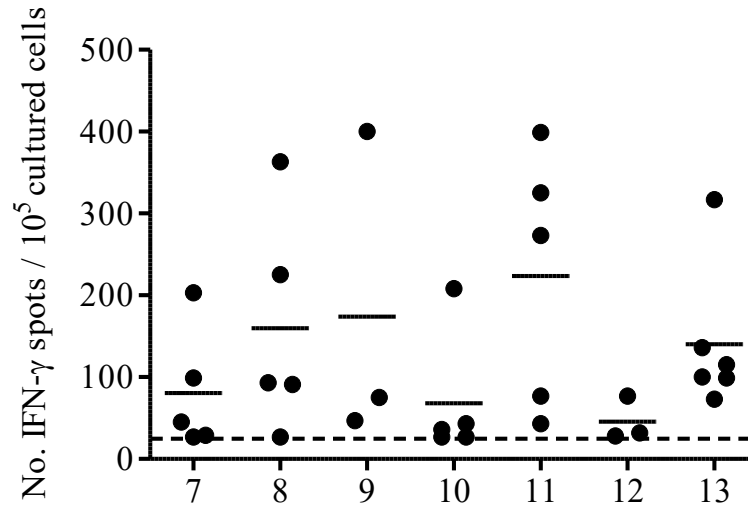
Figure 4.4. Frequencies of specific T cells for each 5T4 pool in healthy donors. Only positive responses are shown. Positive responses had at least twice the number of spots in unstimulated wells and at least 25 SFC/10^5 cells (threshold indicated by dotted line).

4.2.3. 5T4-specific T cells in CRC patients

Using PBMCs isolated from CRC patients' blood taken before surgery, IFN-γ ELISpot assays were performed to measure T cell responses to 5T4. A summary table of patients' details is shown in Table 4.3. Results of ELISpot assays for two representative examples (patient MB2 and MB3) are shown in Figure 4.5. MB2 responded to pools 7, 10, 12 and 13 with a frequency that varied between 1/2777 (peptide pool 12) to 1/751 (peptide pool 7), whilst MB3 responded to pools 7, 11 and 13 with a range of 1/1818 (peptide pool 13) to 1/303 (peptide pool 11). Straight away, the frequencies can be seen to be within a similar range to the healthy controls, and individuals demonstrate responses to particular pools.

I proceeded to test the pools in 16 patients pre-operatively before they underwent surgery for CRC. Table 4.4 summarizes the T cell responses to 5T4 in these 16 CRC patients. Each pool induced IFN-γ secretion in at least 3 out of 16 patients, with the top pools being pool 13 (9 out of 16 patients responded); pool 7 (8 out of 16); and pool 11 (7 out of 16). In term of the mean magnitude of responses, overall, pool 11 induced the highest mean number of IFN-γ secretion with a frequency of 1/287 (348 spots / 10^5 cells) (Figure 4.6). The weakest mean response was observed with pool 12: frequency of 1/1613 (62 spots / 10^5 cells).

4.2.4. Comparison of 5T4-specific responses between healthy donors and CRC patients

The overall summation of 5T4-specific T cells in the pools scored as positive (defined by ≥25 specific spots / 10^5 cells), for pools 7 to 13 were calculated for each individual e.g. MB2 (Table 4.4) responded to pools 7, 10, 12 and 13 with 133, 107, 36 and 80 spots,

CRC patient	Gender	Age	HLA-DRB1	TNM Stage	Dukes' Stage
MB01	M	67	n/a	T3, N1	C1
MB2	m	74	n/a	T3. N2	C1
MB3	m	63	*15, *04	T3, N2	C1
MB4	m	62	n/a	T1, N0	A
MB6	m	67	*01, *07	T4, N1	C1
MB7	m	56	*03, *04	T3, N2	C1
MB8	m	81	*13, *15	T4. N0	B
MB9	m	74	*04, *07	T3, N0	B
MB11	m	84	*13, *15	T0, N2	C1
MB12	m	70	*13	T3, N0	B
MB14	f	67	*01, *14	T3, N0	B
MB15	m	33	*15, *04	T2, N0	A
MB16	m	60	*03	T4, N0	B
MB19	f	68	*01, *04	T1, N0	A
MB21	m	79	*04, *08	T3, N0	B
MB23	f	69	*07	T2, N0	A

Table 4.3. Characteristics of the 16 CRC patients included in the INF-γ ELISpot analysis. n/a: not available
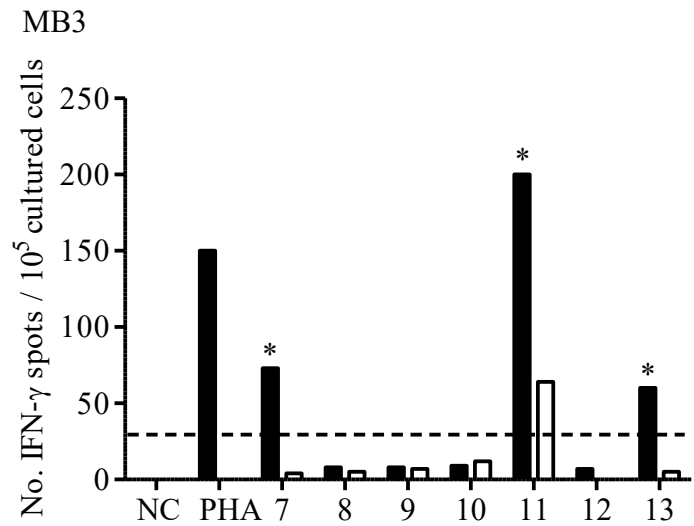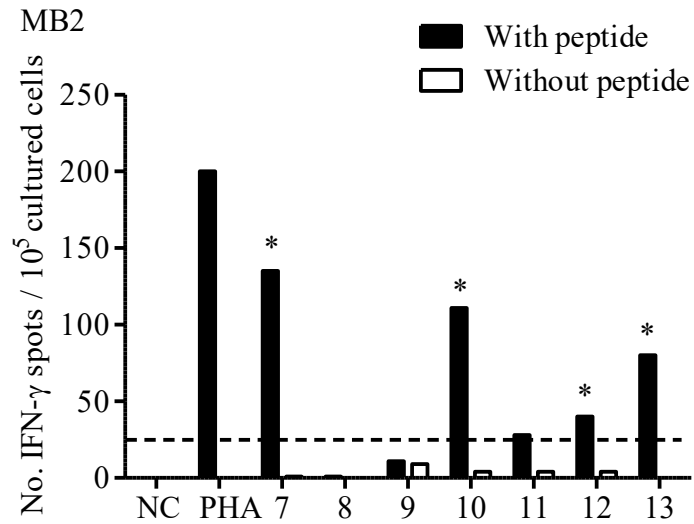
Figure 4.5. Representative IFN-γ ELISpot in two CRC patients. PBMCs from MB2 and MB3 were stimulated for 12 days with peptide pools and restimulated in ELISpot plates with the same pools (black bars) or in medium only (white bars). Asterisks indicate positive responses.

	Peptide Pools																				
	7			8			9			10			11			12			13		
	+	-	=	+	-	=	+	-	=	+	-	=	+	-	=	+	-	=	+	-	=
MB2	135	1	_133_	1	0	1	11	9	1	111	4	_107_	28	4	24	40	4	_36_	80	0	_80_
MB3	73	4	_69_	8	5	3	8	7	1	9	12	0	393	64	_329_	7	0	7	60	5	_55_
MB4	81	5	_76_	12	3	9	7	7	0	8	7	1	425	47	_379_	11	1	9	80	4	_76_
MB6	16	0	16	36	12	24	237	7	_231_	280	4	_276_	439	3	_436_	97	0	_97_	1	0	1
MB7	28	1	_27_	261	11	_251_	199	16	_183_	176	13	_163_	36	12	24	93	3	_91_	27	5	21
MB8	3	0	3	40	0	_40_	7	5	1	8	3	5	1	1	0	8	3	5	400	1	_399_
MB9	400	1	_399_	47	0	_47_	8	0	8	1	0	1	3	3	0	43	0	_43_	28	0	_28_
MB12	217	13	_204_	23	12	11	9	4	5	8	0	8	155	1	_153_	15	21	0	400	9	_391_
MB14	3	0	3	1	0	1	0	0	0	3	1	1	20	0	20	3	3	0	164	8	_156_
MB15	83	0	_83_	320	0	_320_	197	5	_192_	249	1	_248_	416	71	_345_	12	0	12	111	0	_111_
MB16	0	0	0	1	1	0	3	3	0	4	0	4	16	4	12	0	0	0	3	0	3
MB19	68	0	_68_	71	5	_65_	5	0	5	3	3	0	11	0	11	12	9	3	100	97	3
MB23	21	11	11	0	0	0	0	0	0	76	1	_75_	400	0	_400_	1	0	1	31	4	_27_
MB01	36	47	0	60	60	0	116	125	0	44	53	0	104	127	0	69	23	_47_	97	111	0
MB11	0	0	0	75	0	_75_	3	0	3	1	0	1	1	0	1	1	1	0	8	1	7
MB21	19	19	0	0	0	0	7	7	0	4	4	0	400	0	_400_	16	8	8	7	0	7
Responders	8/16			6/16			3/16			5/16			7/16			5/16			9/16		

Table 4.4. 5T4-specific IFN-γ responses in 16 CRC patients. ELISpot results from stimulated (symbolized with +) and unstimulated wells (symbolized with -) are shown. After subtracting the background (=), positive responses were underlined. All responses are per 10^5 cells.
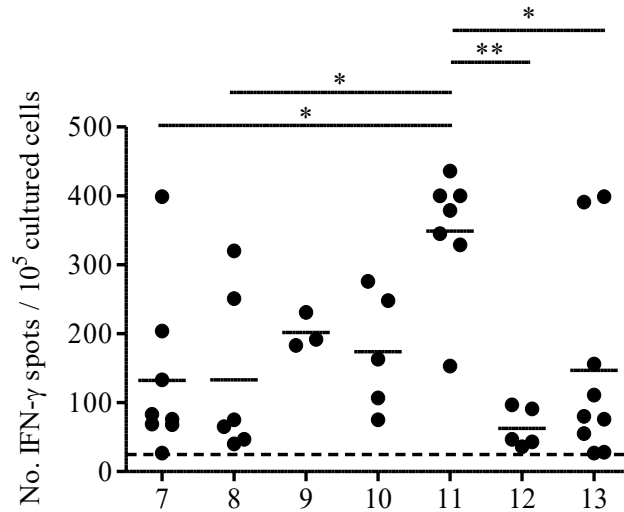
**Figure 4.6. Frequency of 5T4-specific IFN-γ-producing cells in CRC patients.** Dots indicate the individual frequencies of specific T cells for each pool in responders. Threshold of 25 SFC/10^5 PBMC is indicated by dotted line. N= 16. Significant differences were identified using ANOVA; *p<0.05 and ** p<0.01.

respectively; the total specific T cell response was therefore 356. The results for all subjects are shown Figure 4.7.A. No significant difference was observed between healthy donors and CRC patients, as the mean of the total number of 5T4-specific T cells was the same for each group (500 spots / 10^5 cells).

When comparing mean responses against each of the pool, a strikingly similar pattern of reactivity was seen in both groups (Figure 4.7.B). Pool 11 was associated with the highest mean number of responding T cells (223 and 348 specific spots /10^5 cells for HD and CRC patients, respectively) and pool 12 was the least immunogenic pool (45 and 62 specific spots / 10^5 cells for HD and CRC patients). There was no statistical difference between the mean IFN-γ responses for each pool between healthy donors and CRC patients.

The number of responders to a given peptide pool represents another parameter of immunogenicity, and is shown as a percentage of responders to each pool in controls and patients (Figure 4.7.C). Compared to CRC patients, higher percentages of positive donors within the healthy group were observed for all the pools albeit the overall pattern was similar.

In addition, there were more non-responders in the CRC patient group (6%) than in healthy donors (0%). Also, cancer patients recognized fewer pools than healthy individuals. Indeed, 33% of healthy persons responded to 1 to 3 peptide pools compared to 63% in the CRC group. The percentage of individuals that could recognize more than 4 peptide pools was higher in the healthy group (67%) than in the CRC group (31%). These results are summarised in Table 4.5.

A

B

C
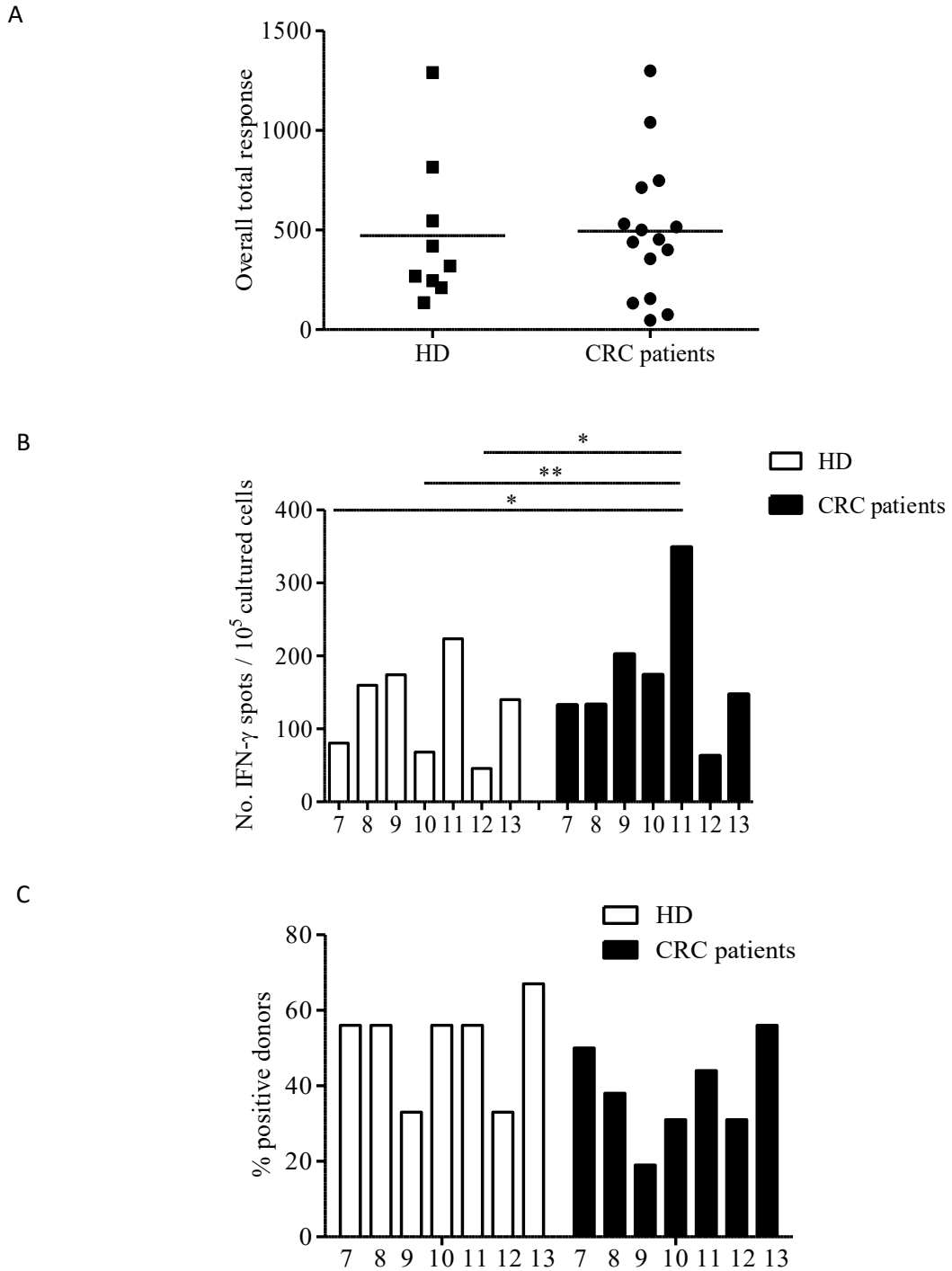
Figure 4.7. Magnitude and percentage of responders within healthy donors and CRC patients. A. Overall total response of 5T4-specific T cells in HD and CRC patients. For each individual, the sum of positive responses to peptide pools 7 to 13 is shown. B. Mean number of 5T4-specific T cells in healthy donors and CRC patients. Only donors recognizing at least 1 pool were included in A and B. C. Percentages of responders to peptide pools 7 to 13 in healthy donors (n=9) and CRC patients (n=16). Significant differences are indicated, * p < 0.5, ** p < 0.01 (1 way Anova).

Number of PP recognized	% of healthy donor	% of CRC patient
0	0	6
1 to 3	33	63
>4	67	31

Table 4.5. Diversity of responses between healthy donors and CRC patients. The percentage of non-responders (0 PP recognized) and percentages of individual recognizing 1 to 3 PP and more than 4 PP among the 7 pools (PP7 to 13) are indicated for healthy donors (n=9) and CRC patients (n=16).

4.2.5. Depletion of CD25^{hi} T cells

In order to identify epitopes that might be influenced by Tregs, CD25^{hi} cells were depleted from PBMCs before evaluating 5T4-specific T cell responses in 12-day cultures. Previous work in the lab empirically established that using CD25 MicroBeads II and a positive selection MS column allows deletion of mainly CD25^{hi} cells. The CD25 marker allows detection of 3 different populations (Figure 4.8.A). Using CD25 beads, depletion of the CD25^{hi} Tregs was consistently achieved (Figure 4.8.A). The percentage of CD4$^+$CD25^{hi} Tregs within the CD4$^+$ population (5.20% ± 1.18) decreased significantly after depletion (0.49% ± 0.16) as shown in Figure 4.8.B. This was confirmed by a decrease of Foxp3$^+$ cells (Figure 4.8.C). This significant decrease of CD4$^+$Foxp3$^+$ cells was confirmed in several individuals (before: 11.16% ± 1.28 vs. after: 3.79% ± 0.80) (Figure 4.8.D).

However, as the CD25 marker does not clearly differentiate between Foxp3$^+$ Tregs and activated Foxp3$^-$ T cells, it is clear that some CD25^{int} cells, comprising the Foxp3$^-$ population, are also lost (Figure 4.8.E). However, the degree of loss is far less in the CD25^{int} population compared to CD25^{hi} which is almost completely removed.

4.2.6. Effect of CD25^{hi} T cell depletion

The effect of the depletion of CD25^{hi} cells on T cell responses has been observed previously using the same method (247). I designated the possible effects (on the magnitude of the T cell responses in peptide-pool driven short term T cell lines after depleting CD25^{hi} cells) to five groups: i) increase by >50%; ii) decrease by >50%; iii) new response not seen in whole undepleted PBMCs; iv) loss of response only seen in whole undepleted PBMCs; v) neutral i.e. no marked effect- these are illustrated in Figure 4.9.
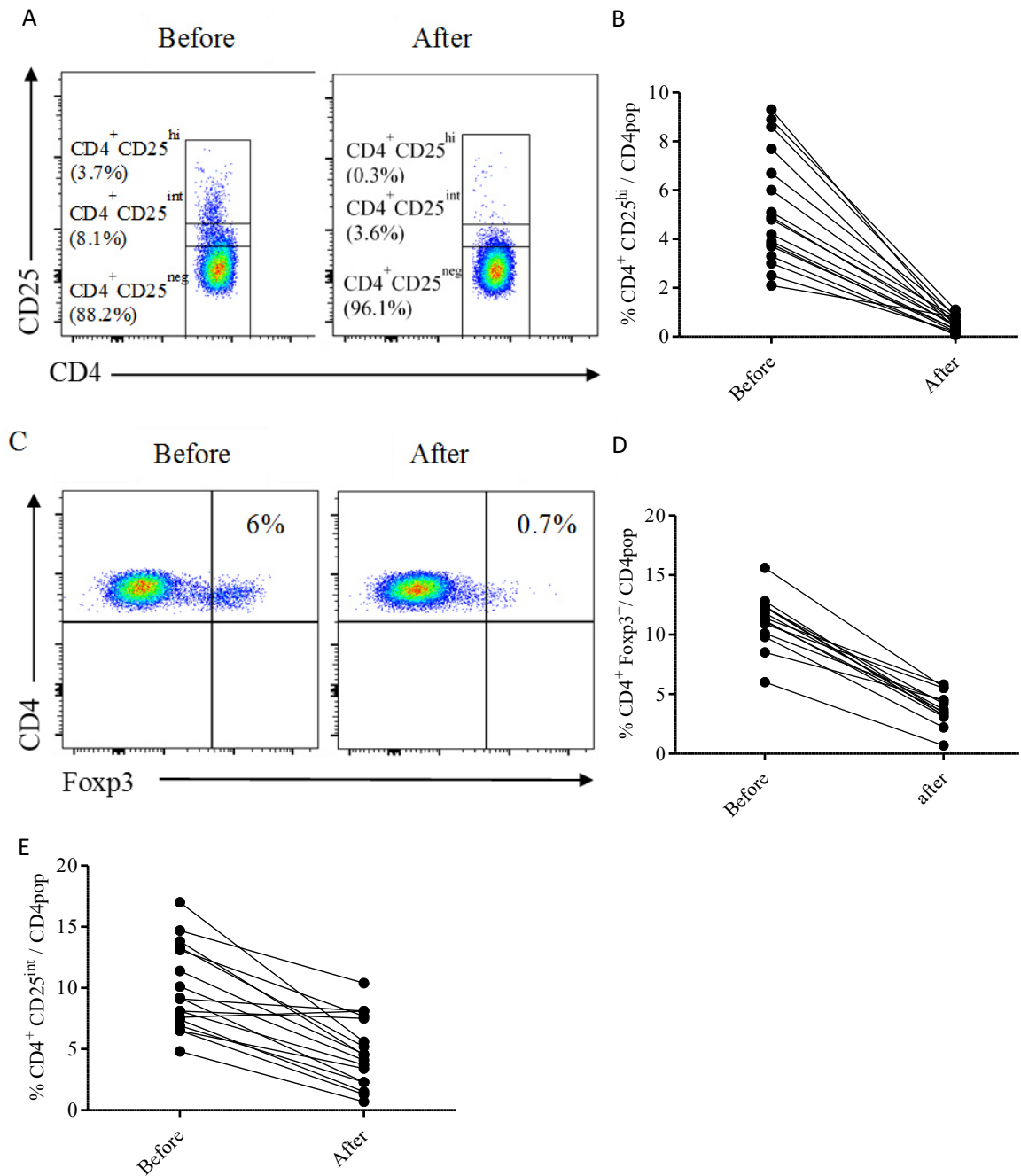
Figure 4.8. Depletion of CD4+CD25hi Treg cells with anti-CD25 Ab-coated magnetic MicroBeads. A. Representative example FACS plots showing levels of CD25 expression on $CD4^+$ T cells before and after depletion. B. Percentages of $CD25^{hi}$ T cells in the $CD4^+$ T cell population before and after depletion in 17 subjects. C. Representative example of FACS plots of Foxp3 expression in $CD4^+$ T cells before and after depletion. D. Percentages of $Foxp3^+$ T cells in the $CD4^+$ T cell population before and after depletion in 17 subjects. E. Percentages of $CD25^{int}$ T cells in the $CD4^+$ T cell population before and after depletion.
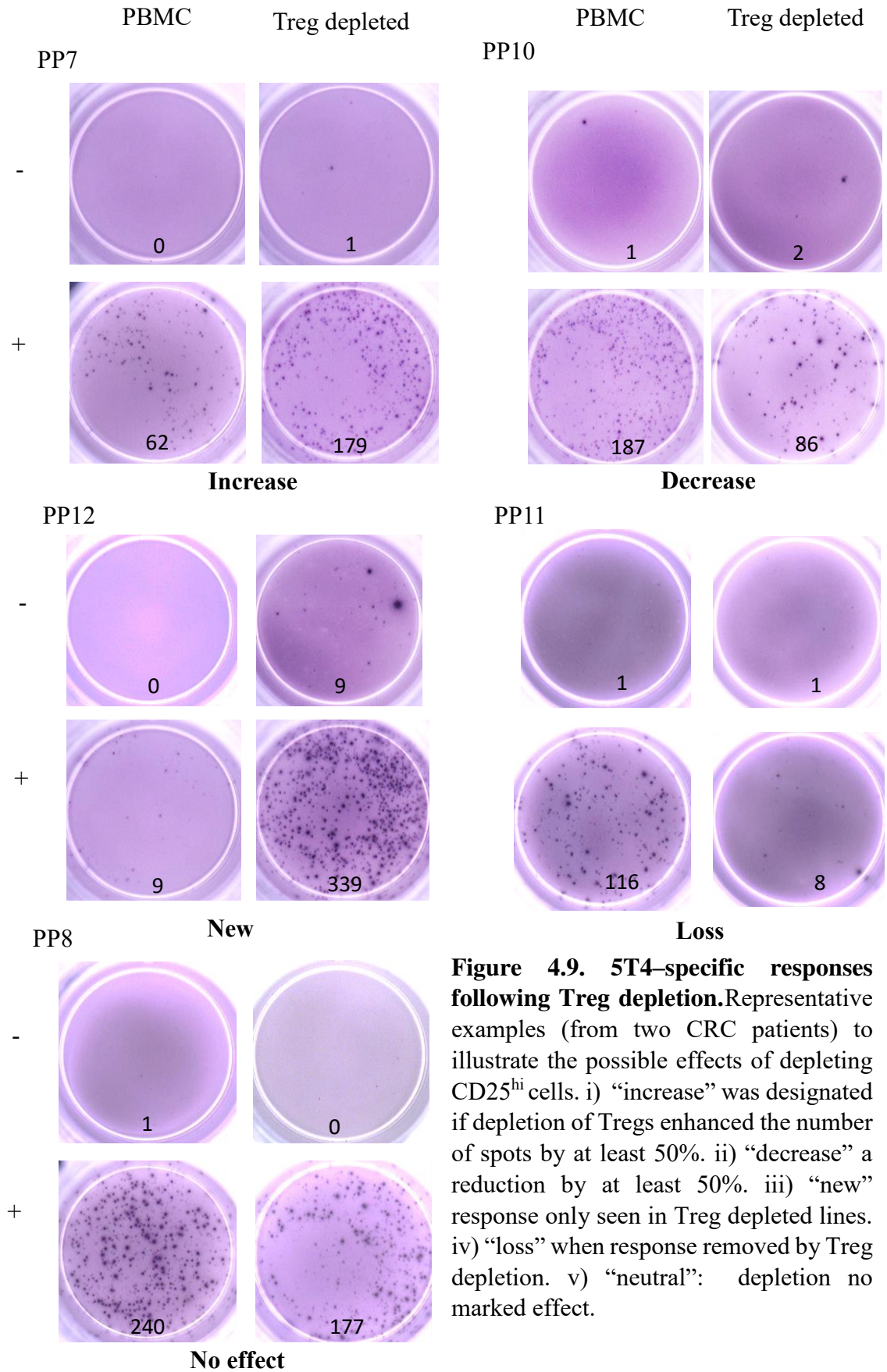
Figure 4.9. 5T4–specific responses following Treg depletion. Representative examples (from two CRC patients) to illustrate the possible effects of depleting CD25^{hi} cells. i) "increase" was designated if depletion of Tregs enhanced the number of spots by at least 50%. ii) "decrease" a reduction by at least 50%. iii) "new" response only seen in Treg depleted lines. iv) "loss" when response removed by Treg depletion. v) "neutral": depletion no marked effect.

4.2.7. Influence of Tregs on 5T4-specific responses in HD and CRC patients

Global effect of Treg depletion

The impact of Treg-depletion was subsequently compared in the HD and CRC groups. The overall summation of responses to the pools 7 to 13 (that cover all 5T4) before and after Treg depletion were similar in both groups (Figure 4.10). No effect of Tregs was observed on the summed of responses against pools in these donors. However, overall summation pre- vs post- depletion may not be the most relevant parameters to evaluate the impact of the Tregs as differential effects amongst peptide pools may mask Treg-mediated suppression.

Individual-specific effect of Treg depletion

I next analysed individual frequencies of 5T4 peptide pool-specific T cells before and after Treg depletion. The data are summarised in Table 4.6. Effects of Treg depletion are indicated in blue (increase response) or red (new response). In all healthy donors (HD in Table 4.6) the response to at least one peptide pool was affected by CD25^{hi} removal. Responses from HD204 were the most influenced by CD25^{hi} depletion as new responses against three peptide pools were unmasked and one peptide pool-specific response increased. In 9/13 CRC patients (MB in Table 4.6) responses to at least one peptide pool per patient was influenced by CD25^{hi} depletion. 5T4 responses from MB9 and MB19 were the most affected by CD25^{hi} cells as responses against five pools were either increased or unmasked in these patients.

Figure 4.11 shows the frequencies of responses to pools 7 to 13 (symbolized by dots) in MB9 and MB19. The lines link the frequencies obtained with and without Tregs for the same peptide pool. When stimulated by pools 7-13, MB9 and MB19 demonstrated
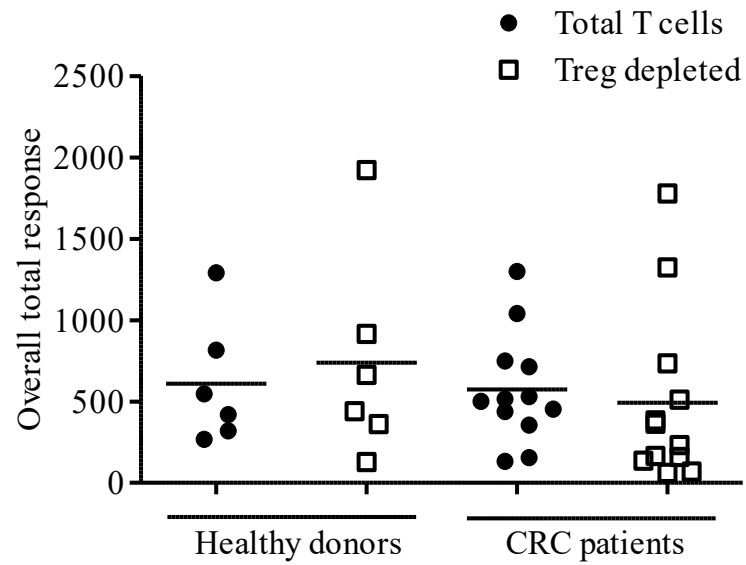
Figure 4.10. Effect of Tregs on 5T4-specific T cell frequencies. Whole PBMC and CD25^{hi}-depleted PBMC from 6 controls and 13 CRC patients were stimulated with 7 different peptide pools (PP7 to 13). Each symbol represents the sum of responses to all the positive pools in one donor before and after Treg depletion.

	7		8		9		10		11		12		13		Number of pool	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HD210	0	97	363	397	136	100	208	312	325	389	77	399	317	227	5	7
HD222	29	0	225	259	19	11	36	75	72	9	28	15	24	28	4	3
HD204	27	64	0	31	400	3	3	291	273	349	4	25	115	156	4	6
HD205	1	37	3	12	75	8	3	28	399	393	1	0	73	232	3	3
HD216	203	96	91	161	20	51	27	87	3	5	9	19	99	47	4	5
HD218	99	37	27	55	15	13	8	15	43	36	0	8	100	0	4	3
MB2	133	25	1	168	1	5	107	25	24	116	36	25	80	25	4	6
MB3	69	27	3	28	1	72	0	0	329	39	7	0	55	0	3	4
MB4	76	33	9	36	0	63	1	0	379	27	9	0	76	1	3	4
MB6	16	4	24	1	231	0	276	0	436	0	97	0	1	5	4	0
MB7	27	28	251	41	183	1	163	0	24	3	91	4	21	0	5	2
MB8	3	25	40	12	1	25	5	4	0	1	5	7	399	312	2	3
MB9	399	293	47	263	8	175	1	7	0	380	43	297	28	371	4	6
MB12	204	25	11	1	5	0	8	0	153	9	0	0	391	111	3	2
MB14	3	0	1	0	0	1	1	1	20	8	0	5	156	232	1	1
MB15	83	237	320	236	192	25	248	112	345	35	12	440	111	239	6	7
MB16	0	3	0	0	0	0	4	36	12	25	0	0	3	0	0	2
MB19	68	157	65	56	5	3	0	105	11	93	3	93	3	229	2	6
MB23	11	0	0	85	0	5	75	5	400	400	1	1	27	25	3	3

Table 4.6. Magnitude of IFN-γ secretion in healthy donors and CRC patients before and after depleting Tregs. Undepleted and CD25^{hi} -depleted PBMC obtained from healthy donors (HD) and CRC patients (MB) were stimulated by 7 different pools. The numbers represent the spot-forming cells /10^5 cultured PBMC after subtraction of background spots, in the presence (+ sign) or absence (- sign) of CD25^{hi} cells. Underlined numbers indicate positive responses. Blue numbers indicate an increase of at least 50% in depleted cells and red new responses, compared to undepleted cells.
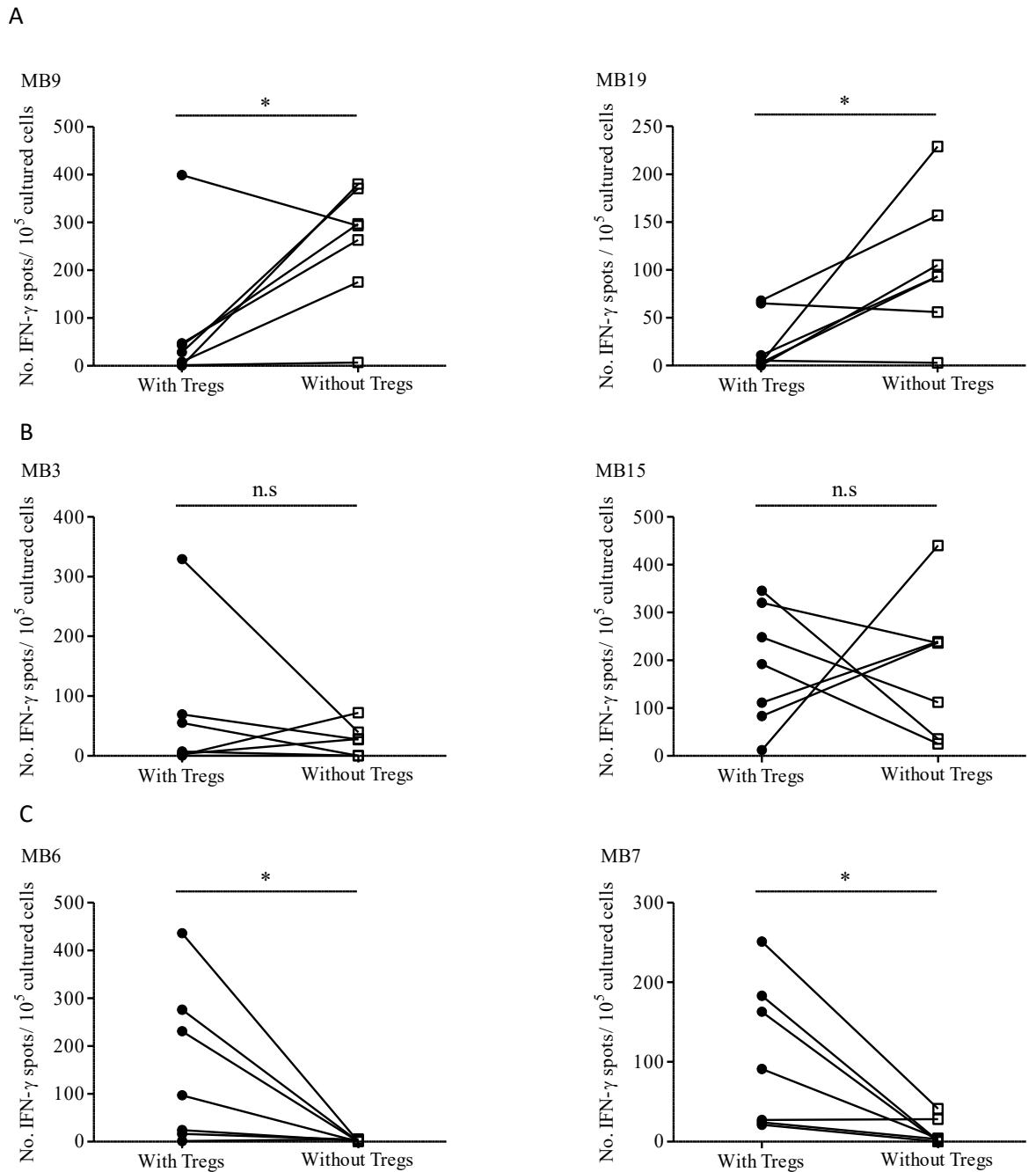
Figure 4.11. Responses from CRC patients pre and post CD25^{hi} depletion.
Increased (A), unchanged (B) and decreased (C) mean responses after Treg depletion.
Each dot represents the number of IFN-γ spots / 10^5 cells in response to one of the 7
pools. The lines indicate the changes after CD25^{hi} depletion. The p values were
calculated using an unpaired student's *t*-test. n.s = non-significant.

means of 75 and 22 specific spots / 10^5 cultured cells respectively. Both responses were significantly increased after Treg depletion to a mean of 255 and 10^5 specific spots / 10^5 cells. Therefore, when analysing certain individual healthy donors and CRC patients, an influence of Tregs can be observed. Similarly, mean responses remained unchanged in some patients (Figure 4.11.B) or were decreased after Treg depletion (Figure 4.11.C).

As showed in the previous part, responses against 5T4 is broad in healthy donors and CRC patients, but most of them recognised more peptide pools in the absence of CD25^{hi} cells (last two columns of Table 4.6). Even though variations were observed, as described above, most healthy donors and CRC patients only had 2 pools affected by CD25^{hi} cells suggesting that Tregs only control responses to certain regions in the 5T4 protein. By increasing the total number of pools that can be recognised, CD25^{hi} depletion reveals that other regions of the 5T4 protein are actually immunogenic in certain subjects, but these responses are often masked by Tregs.

Peptide pools influenced by Tregs in healthy donors and CRC patients

Since CD25^{hi} cell-depletion unmasked or increased responses to 5T4 pools, I aimed to identify whether some pools were more influenced than others by CD25^{hi} cells. In healthy donors, peptide pool 11 was the only one that was not associated with Treg effects. In contrast, pools 7 and 10 were the most influenced by CD25^{hi} cells with 3/6 donors showing new or increased responses after depletion (Table 4.6). In CRC patients, pool 10 was the less influenced by CD25^{hi} cells with only 2/13 patients having new responses to this pool in the CD25^{hi}-depleted PBMC fraction (Table 4.6). On the other hand, pool 8 was more likely to be recognised after depletion as 5/13 patients had new or increased responses to this pool. These data suggest that different parts of 5T4 proteins are

influenced and potentially recognised by Tregs in healthy donors and CRC patients. However, there is no consistent pattern either within or between the two cohorts studied.

4.2.8. Correlating Treg effect and HLA types of donors

After the HLA-types of patients and healthy donors were obtained, responses pre- and post-CD25^{hi} cell depletion were stratified based on the HLA class II genotype; Table 4.7 shows results from all donors who expressed HLA-DRB1*0101, -DRB1*1501, -DRB1*0301, -DRB1*0401, -DRB1*0701 and -DRB1*1301. Responses from healthy individuals and patients were grouped as low numbers of donors were tested.

Responses against peptide pools 9 and 11 from HLA-DR1$^+$ and –DR15$^+$ donors were unaffected by CD25^{hi} cells in contrast to the other DR groups. Almost all responses to pool 7 from HLA-DR15$^+$ donors were either increased or only detected after CD25^{hi}-depletion. Similarly, high number of DR4$^+$ donors had responses against pool 9 only in the absence of CD25^{hi} cells. These results suggest that certain HLA class II alleles are associated with Treg-mediated inhibition of specific 5T4 sequences.

		7		8		9		10		11		12		13	
DR1+		+	-	+	-	+	-	+	-	+	-	+	-	+	-
HD222	*01, *07	29	0	225	259	19	11	36	75	72	9	28	15	24	28
HD204	*01, *15	27	64	0	31	400	3	3	291	273	349	4	25	115	156
MB6	*01, *07	16	4	24	1	231	0	276	0	436	0	97	0	1	5
MB14	*01, *14	3	0	1	0	0	1	1	1	20	8	0	5	156	232
MB19	*01, *04	68	157	65	56	5	3	0	105	11	93	3	93	3	229
DR15+															
HD210	*04, *15	0	97	363	397	136	100	208	312	325	389	77	399	317	227
HD204	*01, *15	27	64	0	31	400	3	3	291	273	349	4	25	115	156
MB3	*15, *04	69	27	3	28	1	72	0	0	329	39	7	0	55	0
MB8	*13, *15	3	25	40	12	1	25	5	4	0	1	5	7	399	312
MB15	*15, *04	83	237	320	236	192	25	248	112	345	35	12	440	111	239
DR3+															
MB7	*03, *04	27	28	251	41	183	1	163	0	24	3	91	4	21	0
MB16	*03	0	3	0	0	0	0	4	36	12	25	0	0	3	0
DR4+															
HD210	*04, *15	0	97	363	397	136	100	208	312	325	389	77	399	317	227
HD216	*04, *--	203	96	91	161	20	51	27	87	3	5	9	19	99	47
MB3	*15, *04	69	27	3	28	1	72	0	0	329	39	7	0	55	0
MB7	*03, *04	27	28	251	41	183	1	163	0	24	3	91	4	21	0
MB9	*04, *07	399	293	47	263	8	175	1	7	0	380	43	297	28	371
MB15	*15, *04	83	237	320	236	192	25	248	112	345	35	12	440	111	239
MB19	*01, *04	68	157	65	56	5	3	0	105	11	93	3	93	3	229
DR7+															
HD205	*07, *13	1	37	3	12	75	8	3	28	399	393	1	0	73	232
HD222	*01, *07	29	0	225	259	19	11	36	75	72	9	28	15	24	28
MB6	*01, *07	16	4	24	1	231	0	276	0	436	0	97	0	1	5
MB9	*04, *07	399	293	47	263	8	175	1	7	0	380	43	297	28	371
MB23	*07	11	0	0	85	0	5	75	5	400	400	1	1	27	25
DR13+															
HD205	*07, *13	1	37	3	12	75	8	3	28	399	393	1	0	73	232
MB8	*13, *15	3	25	40	12	1	25	5	4	0	1	5	7	399	312
MB12	*13	204	25	11	1	5	0	8	0	153	9	0	0	391	111

Table 4.7. Effect of Treg depletion in HLA-DR1, -DR15 (-DR2), -DR3, -DR4, -DR7 and -DR13 individuals. The numbers represent the spot-forming cells /10^5 cultured PBMC after subtraction of background spots, in the presence (+ sign) or absence (- sign) of CD25^{hi} cells. Underlined numbers indicate positive responses. Blue numbers indicate an increase of at least 50% in depleted cells and red new responses, compared to undepleted cells.

4.3. Discussion

T cells from healthy subjects recognise 5T4 tumour Ag

The first finding showed that healthy donors were more likely to respond to 5T4 peptides than CRC patients. Indeed, higher percentages of positive donors were observed within the healthy group for each of the 7 peptide pools. Moreover, in most cases, intensities of 5T4-specific responses were comparable in healthy donors and cancer patients after peptide pool stimulation. These observations show the remarkable efficiency of 5T4 Ag to induce T cell responses. Such a high immunogenicity in healthy subjects has also been found for a few other tumour Ags, such as CEA (285).

As 5T4 is a highly glycosylated protein, the use of synthetic peptides allowed us to exclude the detection of glycan-specific T cells. Therefore, responses monitored as described in this chapter were strictly directed against unmodified 5T4 peptides. However, studies have shown that glycosylation can affect the immunogenicity of glycoproteins. Such effect is thought to be mediated by protease, such as asparaginyl endopeptidase (286). For instance, Surman *et al.,* showed after epitope mapping that immunogenic gp140 epitope hotspots are bordered by regions of heavy glycosylation (287). Similarly, Housseau *et al.,* showed that T cells can recognise tyrosinase tumour antigen when expressed constitutively in melanoma cells but not when overlapping peptides are used in culture (288). It is possible that 5T4 peptide pools that were not associated with high immunogenicity contain peptides requiring glycosylation for T cell recognition. Stern *et al.,* have characterised seven N-linked glycosylation sites in the extracellular domain of 5T4 (289). One of them (NASVSAPS) is found on peptide 17 present in peptide pool 9. The low percentage of HD and CRC patients responding to peptide pool 9 may be caused by the absence of glycosylation on peptide 17.

When these Ag are not tumour specific and are expressed at low level in healthy tissues, it is likely that the cellular reactivity in donors is caused by auto-reactive T cells. The thymic deletion of T cells is not totally efficient and a low-avidity, self-reactive T cell population is present in the periphery (290). These self (tumour) Ag-specific T cells are controlled in the periphery by several mechanisms including Tregs. My data showed that Treg depletion can increase the number of healthy donors responding to 5T4 peptide pools demonstrating the role of these immunosuppressive cells in the regulation of self (tumour) Ag-specific T cells.

Other studies performed in the lab have shown that 5T4-specific responses are restricted to the CD45RO^{+} memory compartment. This rules out the possibility that naïve 5T4-specific T cells are expanded in culture but raises questions regarding how a pool of memory T cells specific for 5T4 is established. Although originally thought to be restricted to tumour cells, 5T4 expression was also observed on normal oral mucosa and inflamed gut [(291) and lab data]. Therefore, 5T4 expression in non-malignant cells may prime autoreactive T cells and maintain a pool of Ag-experienced T cells.

Effect of Tregs on 5T4-specific responses

Previous results from our group showed that depletion of CD25^{hi} cells, which contains the vast majority of Tregs (as showed in the previous Chapter), unmasked new responses to 5T4 (247). These results were obtained using the whole 5T4 protein and they raised questions regarding which peptides within 5T4, if any, stimulated Tregs. In this chapter, stimulation of Treg-depleted and untouched cells with 5T4 peptide pools was carried out.

First, in each individual, Treg depletion never affected responses to each of the 7 pools. Instead, increased or new responses were regularly seen in response to only a few

pools. Second, most donors could respond to peptide pools even in the presence of Tregs. One interpretation of these results is that, in one individual, only a few 5T4 sequences are control by Tregs. Distinct patterns of suppression were observed in different individuals, implying that Treg-mediated inhibition of responses vary between individuals of different MHC type and TCR repertoires. Overall, the data presented in this Chapter indicate that Treg depletion does unmask T cell responses to 5T4-derived peptides: for the most part this appears to reflect total suppression of a given T cell response rather than a partial effect.

The present results argue that Treg depletion can ameliorate 5T4-specific T cell responses in patients as long as effector T cells are not depleted at the same time. Treatments specifically targeting Tregs would be more appropriate than unspecific drugs such as anti-CD25 monoclonal Ab (daclizumab). Alternatively, in the case that the homeostatic system senses the loss of Tregs and generates more cells, functional Treg inactivation could prove a more efficient approach.

Immunogenic 5T4 peptide pool in CRC patients

In CRC patients, peptide pool 11 was found to be the most immunogenic pool in term of magnitude of responses with a mean of 348 spots / 10^5 cells (or 1/287). It also induced responses in a large proportion of patients (44%). This pool may contain one or several immunogenic 5T4 epitopes and is the most interesting for vaccination purpose. However, new responses to pool 11 were observed in some patients following CD25^{hi} depletion. Both Th1 and Treg cells may thus recognise the same or different peptides present in pool 11. This hypothesis is addressed in the next Chapter.

CHAPTER 5. MAPPING THE IMMUNOGENIC REGIONS OF 5T4

5.1. Introduction

The results of experiments described in Chapter 4 point to highly immunogenic regions within the 5T4 protein but also suggest that peptide epitopes can be found throughout the entire protein. Since the number of reported 5T4-derived peptide epitopes is thus far very limited [4 MHC-class I and 2 MHC-class II restricted peptides (240, 241, 243)] comprehensive mapping of T cell epitopes was conducted in order to further assess the usefulness of 5T4 as a vaccine Ag.

For this purpose, the same peptides were used as in the previous section (41 peptides overlapping by 10 amino acids and spanning the entire sequence of 5T4; Table 2.4 in Materials and Methods). However, these peptides were now grouped into 13 different pools that were arranged in a matrix where each peptide was present in 1 row and 1 column. Thus "horizontal" pools were used as before (pools 7-13) with the addition of "vertical" pools 1-6 (Table 2.4 in Materials and Methods). Therefore, the intersection of two positive pools in the matrix should theoretically correspond to the immunogenic peptide containing the T cell epitope. My objective was to identify peptide epitopes from across 5T4 and identify the restricting MHC molecule. The advantages of the strategy however, compared to the use of peptides predicted by bioinformatics is that it does not require identification of the donors' HLA types before screening. Indeed, this was particularly important in this study, as the HLA-types of the donors were not available prior to the ELISpot assays due to logistical reasons. Ideally, peptide pools should be used for preliminary screening, followed by another screen based on the use of individual peptides present in the positive pools. However, due to the limited number of PBMCs and

the high number of pools eliciting responses in donors, this was not always possible. Responses from seven donors were verified by stimulating pool-reactive T cell lines with individual peptides deduced using the matrix.

5.2. Results

5.2.1. Determining candidate 5T4 peptides using a peptide matrix

Two representative examples of ELISpot assays using the peptide matrix are shown in Figure 5.1. In the first example (CRC patient, MB3), the 6 positive pools (PP1, 3, 6, 7, 11 and 13) are highlighted in grey in the matrix (Figure 5.1.A). Peptides found in the intersections of these pools are in red and correspond to the candidate peptides recognized in the assay. Similarly, screening revealed that CRC patient MB12 was positive for pools 1, 4, 6, 7, 11 and 13, which lead to the identification of 8 candidate peptides (Figure 5.1.B).

When several multiple pools are positive, results are harder to interpret in terms of peptide specificity. For instance, in MB3, three different peptides were potentially recognized within pool 1 and it is possible that all or just one peptide was recognised by T cells.

Correlating HLA types of patients and healthy donors with peptide-specific T cell responses

After the HLA-types of patients and healthy donors were obtained, responses were stratified based on the HLA class II genotype; Figure 5.2 shows results from all donors who (A) expressed HLA-DRB1*0101, (B) expressed -DRB1*1501 and (C) expressed -DRB1*0301; Figure 5.3 shows results from all donors who (A) expressed HLA-DRB1*0401, (B) -DRB1*0701 and (C) -DRB1*1301. The heat map shown beneath the Figures indicate the percentage of responders per individual peptide where different colours symbolize different response rates.
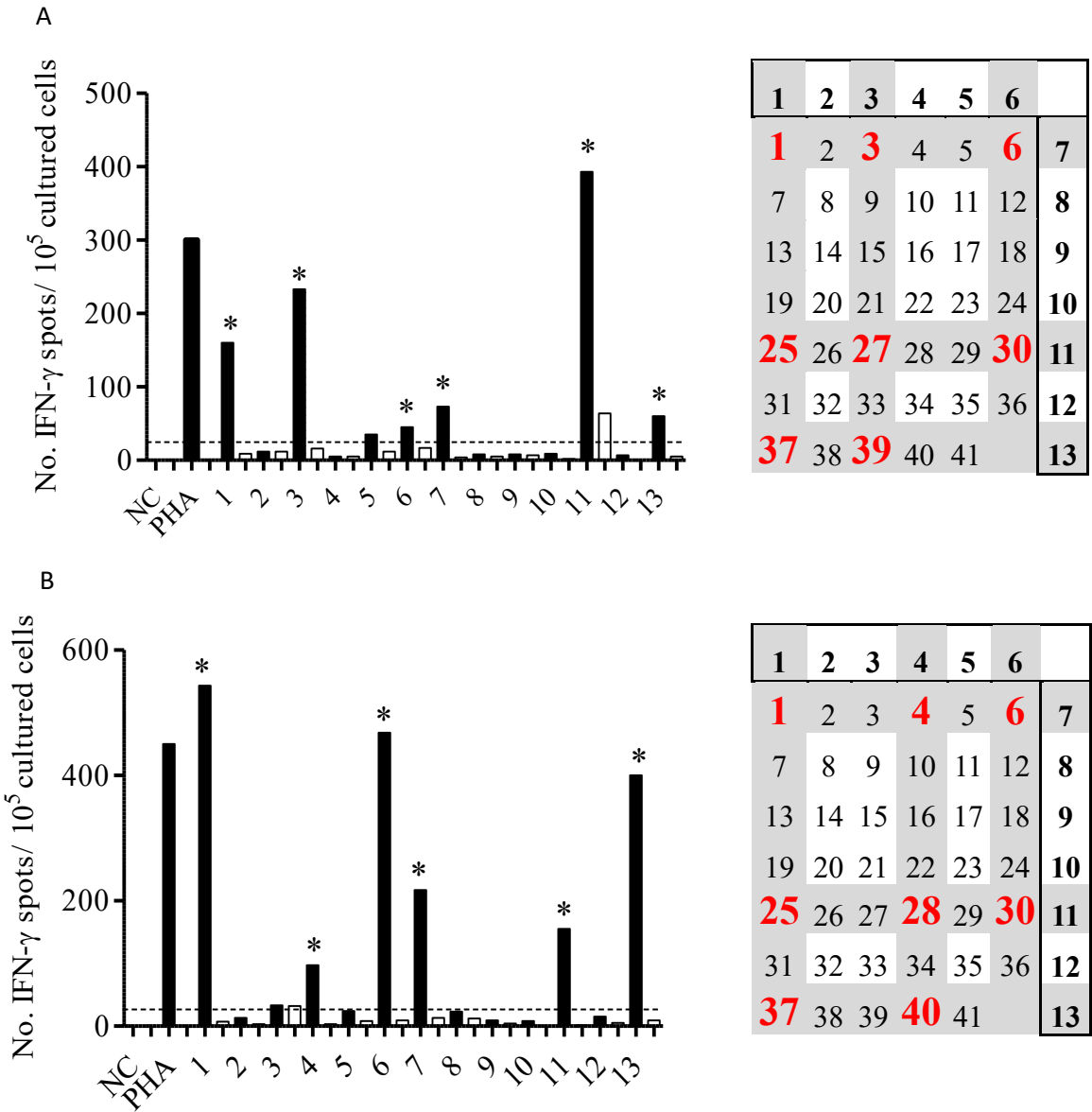
Figure 5.1. Determining candidate 5T4 peptides using a peptide matrix. After 12 days of stimulation, specific responses were measured in an IFN-γ ELISpot assay. The matrix allows the rapid identification of the peptide recognized. Two representative example are given with CRC patients MB3 (A) and MB12 (B).

A. **HLA-DR1**
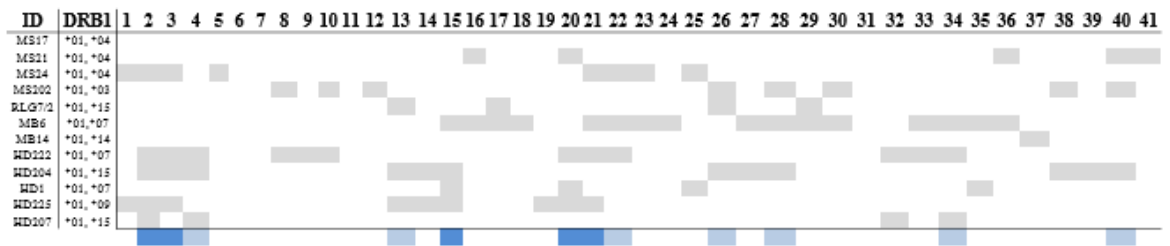
B. **HLA-DR15**

C. **HLA-DR3**
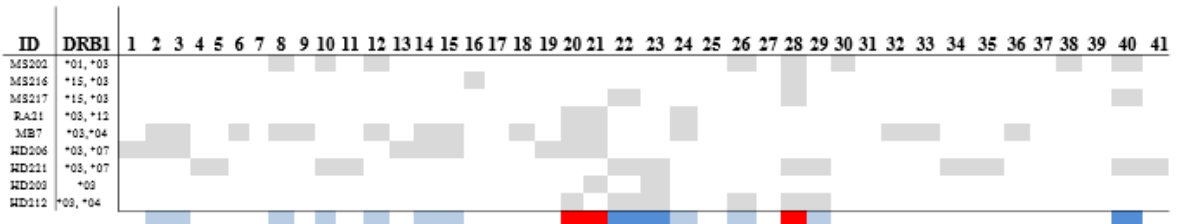
<20	Peptide recognised in less than 20% of subjects	
20-30	Peptide recognised in 20-30% of subjects	
30-40	Peptide recognised in 30-40% of subjects	
> 40	Peptide recognised in more than 40% of subjects	

Figure 5.2. 5T4 candidate peptides in HLA-DR1, -DR15 (-DR2) and -DR3 individuals. Candidate 5T4 peptides that induced responses in PP were deduced by using the 5T4 matrix. These data include responses from both CRC patients (MS, MB) and HD. HLA-DR1 (A), n=12; -DR15 (B), n=15; and -DR3 (C), n=9. Beneath is a summary with each colour indicating the proportion of positive responder.

A. **HLA-DR4**
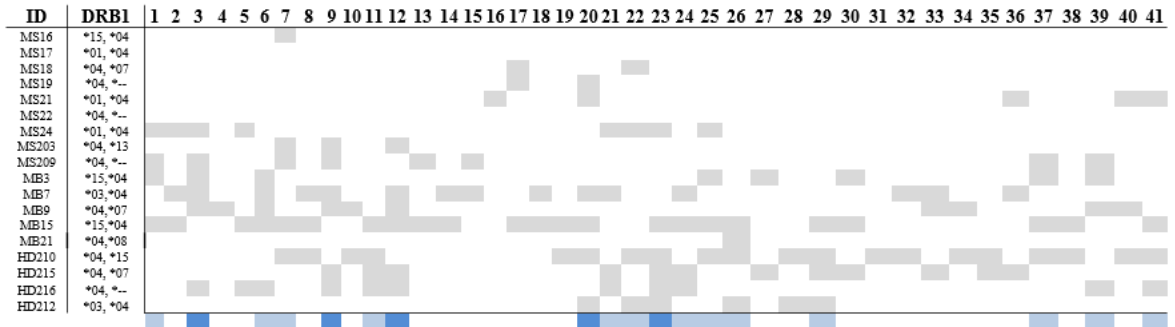
B. HLA-DR7
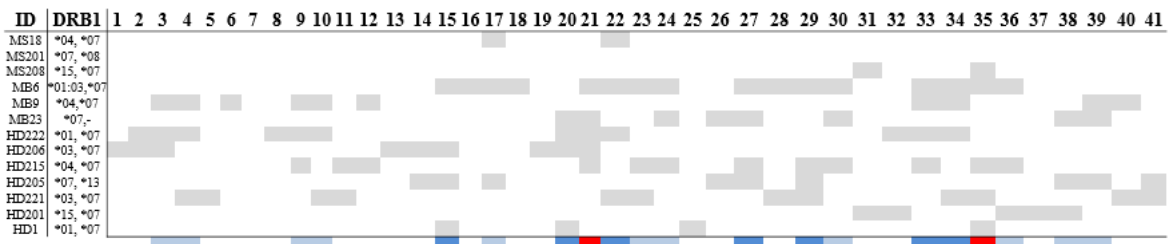
C. **HLA-DR13**
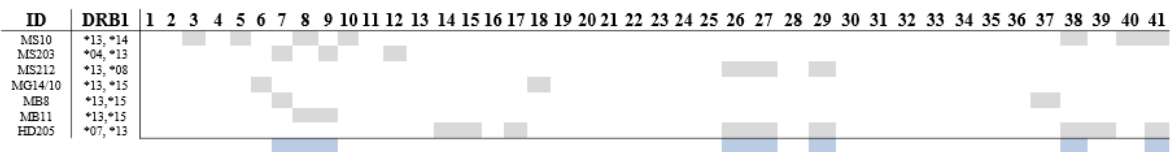
<20	Peptide recognised in less than 20% of subjects
20-30	Peptide recognised in 20-30% of subjects
30-40	Peptide recognised in 30-40% of subjects
> 40	Peptide recognised in more than 40% of subjects

Figure 5.3. 5T4 candidate peptides in HLA-DR4, -DR7 and -DR13 individuals. Candidate 5T4 peptides that induced a response in PP were deduced by using the 5T4 matrix. These data include responses from both CRC patients (MS, MB) and HD. HLA-DR4 (A), n=18; -DR7 (B), n=13; and -DR13 (C), n=7. Beneath is a summary with each colour indicating the proportion of positive responder.

5.2.2. Candidate 5T4 peptides

Figure 5.4 summarizes the reactivity of the peptides in each of the 6 DR groups. These 6 molecules are the most commonly expressed class II alleles encoded by the HLA-DRB1 genes in our local population in Wales (as described on www.allelefrequencies.net). In line with previous observations examining the reactivity of the pools, heat maps revealed that 5T4 peptides are overall highly immunogenic. Indeed, screening of the entire amino acid sequence of 5T4 revealed 4 immunogenic regions (squares delimited by a black borders). These regions contain peptides potentially recognized by individuals with different HLA-DR genotypes with a reactivity of 30-40% (blue squares) in at least one HLA-DR type.

The 5T4 region between amino acids 191 and 300 seems to be the most immunogenic as it comprises 10 peptides (peptides 20 to 29 within pools 10 and 11) with high reactivity for different HLA-DR molecules. In detail, peptides 20 and 21 are highly reactive in HLA-DR3^{+} donors where they were recognized by more than 40% of donors. Several peptides derived from other tumour Ags, such as NY-ESO-1, are broadly presented by multiple different HLA-DRαβ glycoproteins (292). Other immunogenic peptides were found between amino acids 11 and 40 (peptides 2 and 3 within pool 7), 61 to 100 (peptides 7 to 9 within pool 8) and 371 to 410 (peptides 38 to 40 within pools 13).

Only 4 peptides (5, 16, 18 and 19) did not induce responses in the tested donors. However, as responses to individual peptides, deduced from use of peptide pools, were not confirmed (as discussed above), it is possible that the number of peptide epitopes has been over-estimated. It is clear however, that amino acid regions 131 to 200 (peptides 14 to 19) and 301 to 370 (peptides 31 to 36) contain fewest epitopes as indicated by the relatively low proportions of individuals responding to pools 9 and 12.
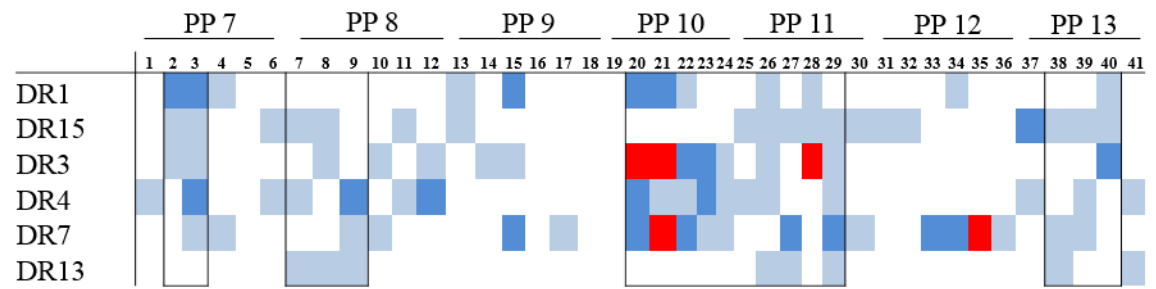
Figure 5.4. 5T4 T cell epitope heat map. Visual illustration depicting the reactivity of each peptide for the DR groups present in the cohort. Four colours are used to characterize the responsiveness to the peptides. Regions of 5T4 which are commonly recognised are enclosed in a black box.

Figure 5.4 also highlights the differential peptides. Whereas the HLA-DR7^{+} group could potentially recognize 20 peptides, only 12 5T4 candidate peptides were immunogenic in the HLA-DR1^{+} group. This difference was not caused by skewing towards HLA-DR7^{+} donors as the number of individuals in each group was comparable (HLA-DR1^{+} group contained 12 individuals and the -DR7^{+} group 13). Therefore, it is possible that HLA-DR7 molecules are more likely to present 5T4 peptides than DR1 molecules.

Detailed analysis of individual peptide responses

Several peptides were selected for further investigations based on:

- potentially presented by ≥ 3 HLA-DR types

and

- recognised by T cells in at least 20% of individuals in each HLA-DR groups

Based on the above, 14 candidate peptides were selected: peptides 2, 3, 15, 20 to 29 and 38. Each of these peptides (except peptide 15) was found in the immunogenic regions of 5T4. As predicted, these peptides are contained in pools that induced high frequency responses in many donors (Figure 4.7).

Furthermore, 3 peptides were also added based on the use of NetMHC2.2 and IEDB prediction algorithms: Peptides 10 and 12 predicted to be good binders to different HLA-DR molecules by both algorithms, peptide 11 predicted to bind to fewer HLA-DR molecules (Appendix Table 1). These 3 peptides were used in order to evaluate the accuracy of prediction algorithms.

5.2.3. Response to individual peptides

Based on the results of the initial screening of the peptide pools, PBMCs from healthy donors and CRC patients were stimulated with individual 5T4 peptides, selected due to their ability to induce responses in a large proportion of donors. When enough PBMCs were available, they were tested against each of the 17 candidate peptides (peptides 2, 3, 10, 11, 12, 15, 20 to 29 and 38).

Figure 5.5 indicates the maximum number of spots in response to one peptide counted for each donor. The number of single peptides used ranged from 3 to 17 in healthy donors and from 7 to 16 in CRC patients. Positive IFN-γ spots were detected in 80% (8/10) of healthy donors and in 67% (12/18) of CRC patients. The tumour stage did not influence reactivity in cancer patients.

As observed previously, T cell responses against peptides were variable between donors. In healthy donors, all peptides, with the exception of peptide 15, were able to induce IFN-γ secretion (Figure 5.6). However, in CRC patients all peptides other than 11 and 29 induced a response, albeit responses to some peptides were seen only in very few individuals. No conclusion can be drawn with respect to peptide 29 due to low numbers of donors tested (n=2). These results validate the mapping strategy used previously as numerous strong responses were detected using peptides selected from the heat maps i.e. peptides 2, 3, 15, 20-29 and 38 (Figure 5.4). Also, as predicted by the algorithms, peptides 10 and 12 were more immunogenic than peptide 11.

The most common peptides recognized in healthy subjects were p12 (78%), p38 (67%), p20 (63%) and p2 (56%). In CRC patients, responses to p38 (35%), p2 (33%), p3 (28%) and p28 (28%) were the most often detected (Figure 5.6).
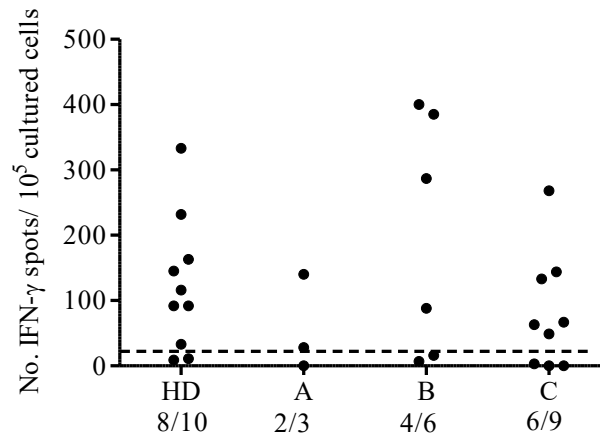
Figure 5.5. IFN-γ secretion induced by 5T4 peptides in healthy donors and CRC patients. Cytokine secretion in healthy donors, n=10, and in stage A, n=3, stage B, n=6, and stage C, n=9, CRC patients. Each dot indicates the chosen highest responder peptide in each donor. The number of peptide-specific spots was calculated by subtracting the number of spots in the negative well. Horizontal lines represent the set threshold of positivity (25 spots / 10^5).
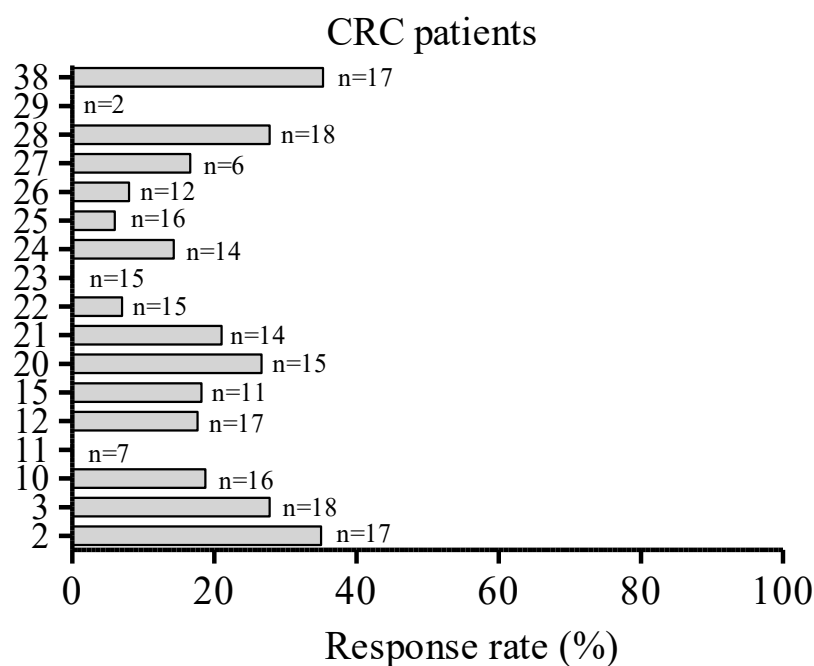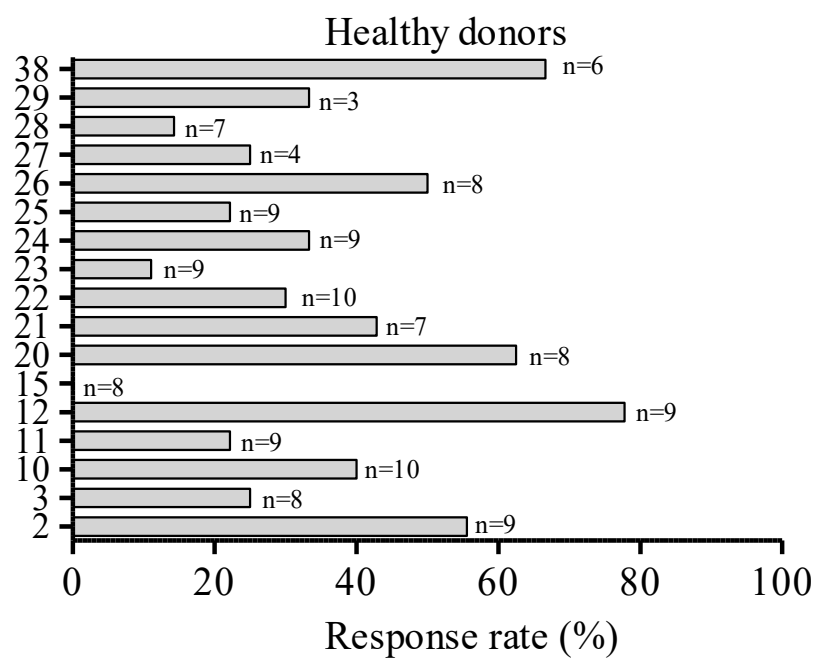
Figure 5.6. Response rates to individual 5T4 peptides. The percentage of healthy donors and CRC patients who had positive responses to 5T4 peptides is displayed. The number of individual tested for each peptide is indicated.

Alongside the number of responders, the magnitude of responses varied between individual 5T4 peptides (Figure 5.7). In healthy donors, the lowest mean response was induced by peptide 22 with 62 specific spots / 10^5 cultured cells (range 45-73) and the highest by peptide 21 with 135 specific spots / 10^5 cultured cells (25-333). In CRC patients, the lowest mean response was induced by peptide 24 with 40 specific spots / 10^5 cultured cells (range 37-43) and the highest by peptide 12 with 258 specific spots / 10^5 cultured cells (79-400). Mean responses to peptide 2 were similar in healthy donors and cancer patients (79 and 69 specific spots / 10^5 cultured cells, respectively). Also, both groups had equivalent mean responses to peptide 38 (79 and 70 specific spots / 10^5 cultured cells in healthy and cancer individuals, respectively).

Altogether these data indicate that peptides 2 and 38 are able to stimulate high frequencies of T cells in elevated proportions of subjects, independently of their health status and without restriction to specific HLA class II molecules. Indeed, many donors possessing T cells specific for these peptides expressed various class II alleles. This confirms data obtained after extrapolation of the matrix where p2 and p38 were found within 2 regions of high reactivity in different DR groups. Moreover, a low response rate to some candidate peptides was expected because the matrix may have overestimated the number of peptide recognized when several pools were positive. This is particularly true for peptides 20 to 29 which were described as immunodominant after the first screen. Mapping the fine specificity with single peptides showed that the immune response was mainly directed against only a few of these. Also, for all peptides tested (except for peptides 15 and 3), the
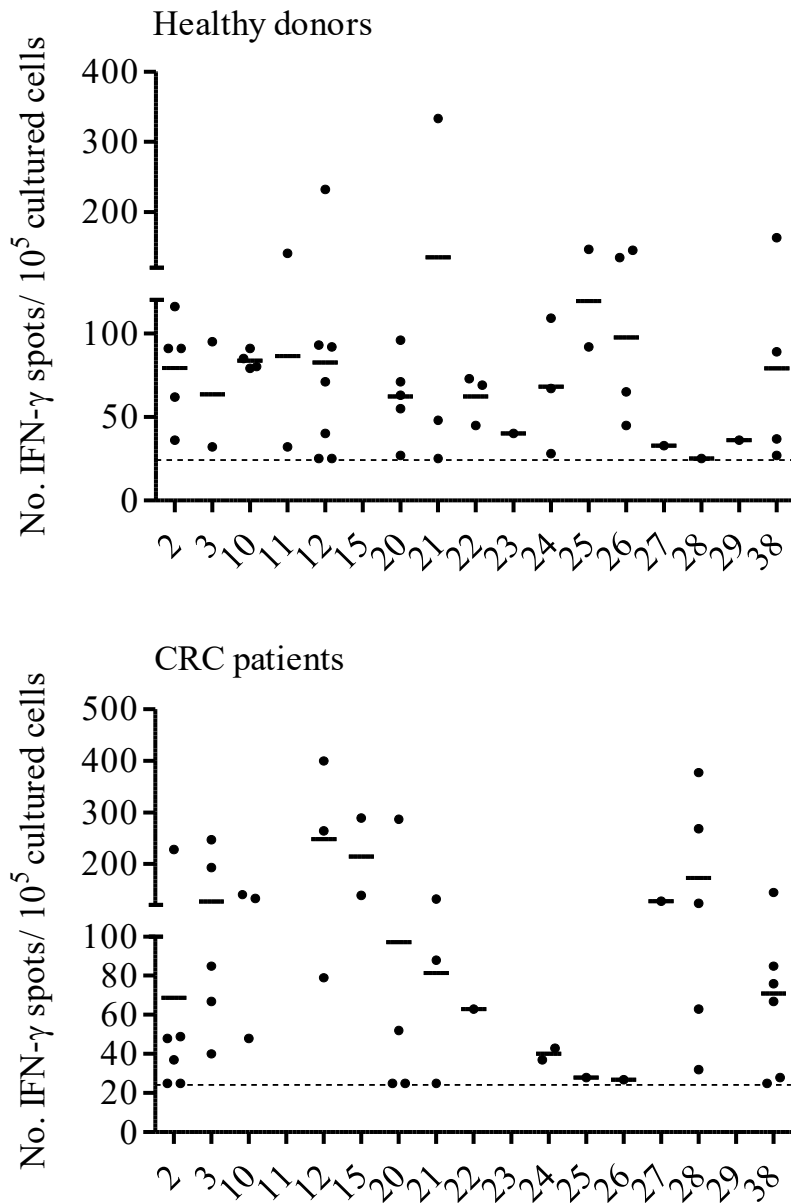
Figure 5.7. Specific immune responses against individual 5T4 peptides. Magnitude of positive IFN-γ ELISpot responses against candidate 5T4 peptides. Each dot represents the magnitude of the T cell response from one donor against the indicated peptide in healthy donors and CRC patients. Only responses superior to 25 / 10^5 PBMC (symbolized by dash lines) are indicated in both groups.

percentage of responders was higher in healthy donors than in CRC patients. This is consistent with early observations with peptide pools (Figure 4.7.C).

5.2.4. Immunodominant peptides

It is striking that some peptides appear to be recognised mainly by healthy donors including 11, 23 and possibly 29 (although the latter was only tested in 2 patients), and others are favoured by CRC patients such as peptide 15. However, the key information is how large the cognate T cell response is to a peptide, and what proportion of individuals actually recognise these peptides? Based on the magnitude of positive responses and response rates, some peptides have emerged as candidates for immunodominant regions of 5T4. The data shown in Figures 5.6 and 5.7 are re-organised in Figure 5.8, in order to displays the mean magnitude of peptide-specific IFN-γ positive responses coupled to the response rate in each population (healthy donors and patients) to each of the 17 candidate peptides. This graph allows a grouping of some of responses into the following: i) peptides which are seen with high frequency responses in many healthy donors and patients (i.e. upper outer quadrant of the graph) e.g. peptide 2, 20, and 38 (in green); ii) peptides which move from the lower inner quadrant (low frequency responses) to the upper outer quadrant from healthy donors to CRC patients e.g. peptides 3 and 28 (in blue) ; iii) peptides which move from the upper outer quadrant to the lower inner quadrant from healthy donors to patients (loss of responses) e.g. peptides 11 and 23 (in red).
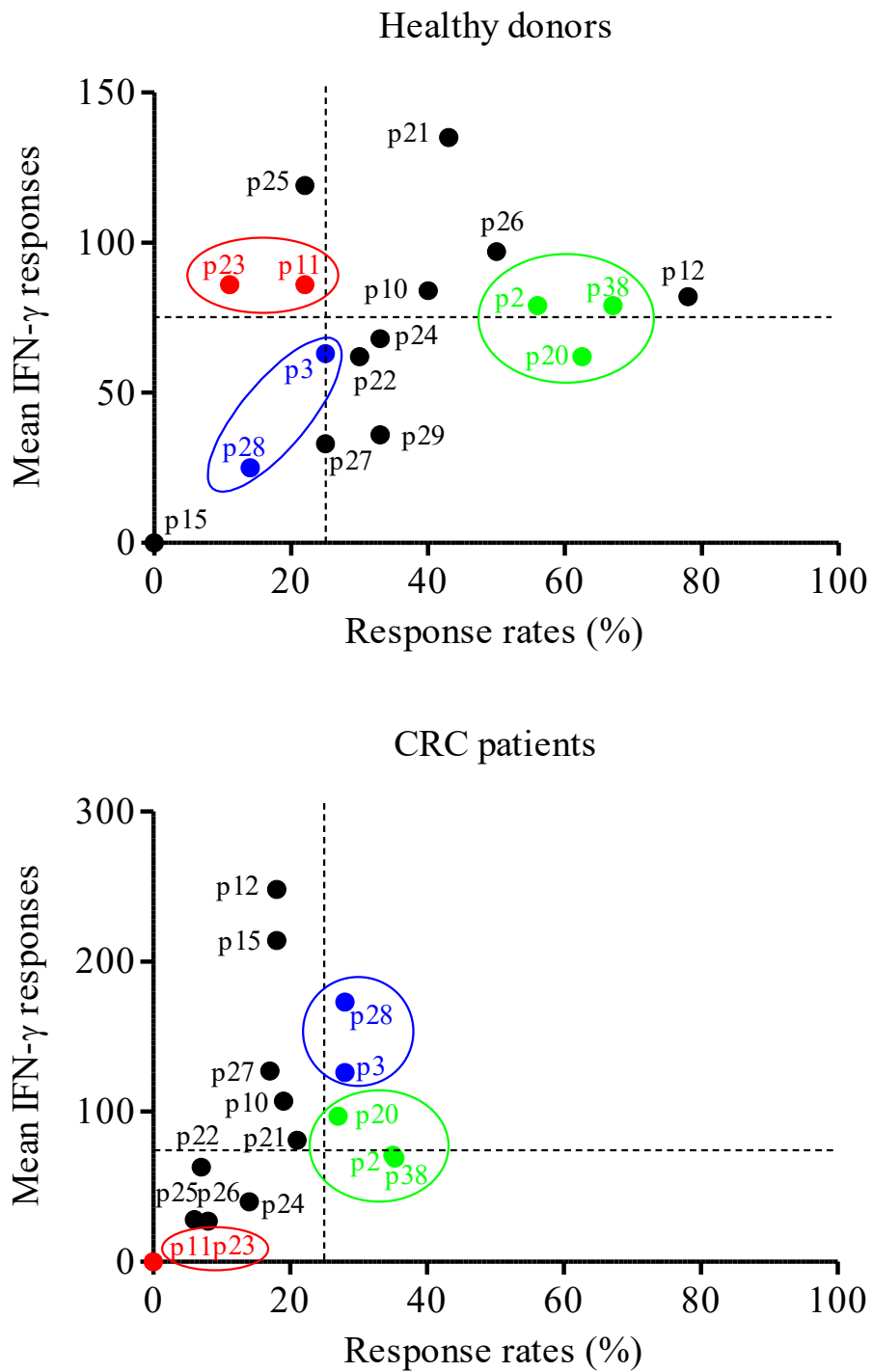
Figure 5.8. Immunodominant 5T4 peptides. Two-dimensional graph displaying the mean of IFN-γ response as a function of response rate for each candidate peptide. The peptides were classified in different groups depending on 2 arbitrary parameters: 75 IFN-γ spots / 10^5 PBMCs defining high frequency response and 25% defining a high prevalence of responders.

5.3. Discussion

Among the Ags expressed by germ cells, trophoblasts and tumours (cancer testis Ags), 5T4 is a good target for immunotherapy (293). However, the number of immunogenic 5T4 peptides reported is very limited and mostly restricted to a set of peptides presented by MHC class I molecules (240, 241, 243). Given the crucial role of Th1 cells in anti-tumour immunity (see Chapter 1), 20-mer peptides were used to stimulate T cells in order to identify MHC-class II peptides eliciting IFN-γ production.

Four immunogenic regions within 5T4 tumour Ag (residues 11-40, 61-100, 191-300 and 371-410) were identified using peptide pools for further studies. 14 peptides (11/14 previously unreported) were selected due to their potential for presentation by several HLA-class II molecules and because to > 20% of individuals with a given MHC class serotype responded. These 2 parameters are crucial in the context of vaccine design where a few peptides are used to induce or boost immune responses in a large population with different HLA-types. Three additional peptides were also chosen using predictive algorithms. Single peptide stimulation revealed that magnitude of responses and number of responders (i.e. immunoprevalence) were different between healthy donors and CRC patients.

ELISpot assay to map antigenic peptide

Whilst some ELISpot assays rely on initial *in vitro* T cell expansion with Ag-pulsed DCs and HLA matched or autologous PBMCs, I enriched specific T cells by pulsing APCs already present in the PBMC fraction. This quick and efficient protocol allowed me to significantly extend the list of immunogenic 5T4 peptides with a limited amount of donors' PBMCs. For these reasons, the same protocol is currently used in TaCTiCC (TroVax® and Cyclophosphamide Treatment in Colorectal Cancer) clinical trial

monitoring 5T4 responses in CRC patients. IFN-γ ELISpot assay was used to map 5T4 peptide Ags. The ability of T cells to secrete IFN-γ was evaluated after short-term peptide stimulation. Memory T cells are preferentially detected in cultured ELISpot assays whilst effector T cell responses are generally measured by *ex vivo* ELISpot (294). As memory cells induce strong and long lasting immune responses, identifying their specificity for tumour Ags is of crucial importance for vaccination.

Pooling peptides in a matrix format minimized the number of assays required to identify candidate peptides. However, one limitation of using peptide pools (when several peptides are restricted through the same HLA allele) is the possible competition between peptides from the same pool for binding to HLA molecules. However, in order to reduce potential competition and compare results between pools, all peptides within the pools were at equimolar concentrations. The heat map (Figure 5.4) suggests that some peptides, e.g. peptide 19, do not induce T cells responses. But it is possible that, within the same pool, some peptides with stronger binding affinity were preferentially detected causing absence of T cell responses to peptide 19. This may also explain why some peptides used individually to stimulate T cells were more immunogenic than predicted by the heat map, e.g. peptide 12. Therefore our strategy may have favoured the recognition of peptides with high affinity for DR molecules.

Immunogenicity of peptides selected from the heat map

There was a good correlation between candidate peptides expected to bind to several DR molecules (heat map Figure 5.4) and the *in vitro* assays. Indeed, when used individually to stimulate T cells, almost all 14 peptides could elicit responses in at least 3 different HLA-DR groups, albeit number of responders varied between DR serotypes (Appendix Table 2). Therefore, the use of a matrix to interpret results obtained with peptide pools

permits the accurate identification of peptides able to bind to HLA-DRB1 molecules of various serotypes. There were only two exceptions; whilst the paucity of responses to peptide 29 may simply reflect the low number of donor tested, the same is not true for peptide 23. Only one donor responded to this peptide although it was tested in 9 healthy donors and 15 cancer patients. Using peptide pools, peptide 23 was expected to be recognised by a high number of DR3$^+$ and DR4$^+$ donors (heat map Figure 5.4).

Responders identified using the matrix or obtained with single peptide stimulation did not always align. For instance, according to the matrix, peptides 2 and 20 are potentially recognised by 30 to 40% of HLA-DR1$^+$ donors (Figure 5.4), whilst in fact when single peptides were used, they were respectively recognised by 75% (6/8) and 88% (7/8) of donors (Appendix Table 2). Furthermore, the previous Chapter showed that CD4$^+$CD25^{hi} cells could influence specific responses to all 5T4 pools. Therefore, it cannot be excluded that Tregs were stimulated by other peptides present in the pools and in return suppressed responses against peptides 2 and 20. On the contrary, the matrix indicated that peptide 25 is recognised by 20 - 30% of HLA-DR4$^+$ donors but using single peptides, these were immunogenic in only 11% (1/9). Thus matrix and peptide pools give good indications regarding the ability of a peptide to bind to different DR molecules but do not provide accurate information regarding their immunoprevalence. This is likely to be caused by an overestimation of the number of peptides recognised when several positive pools are recognised. These results point the importance of the single peptide stimulation for validation of candidate peptides deduced using a matrix.

Interestingly, individual peptide stimulation showed that all peptides were poorly immunogenicity in HLA-DR4$^+$ CRC patients. In comparison, some peptide (e.g. 2, 3 and 20) were more immunogenic in HLA-DR1$^+$ patient. These data suggest that HLA-DR1 serotype is protective in CRC patients. In contrast, HLA-DR4 may be associated with bad

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prognosis as this allele is not associated with high reactivity for 5T4 peptides. Unfortunately, studies analysing the association between HLA and cancer risk are limited in number and mainly restricted to HLA A*2 (295). Only the HLA-DR17 gene (DRB1*0301 allele) is more frequent in individuals with ulcerative colitis-associated colorectal cancer (296).

Immunogenic peptides in healthy donors and CRC patients

By comparing magnitude of responses and percentage of responders in healthy donors and CRC patients, 3 groups of peptides were observed. In the first group, 3 peptides (2, 20 and 38) had high immunoprevalence in both healthy donors and CRC patients; e.g. up to 67% and 35% positive responses to peptide 38 in healthy donors and patients, respectively. These peptides can be good candidates to monitor 5T4-specific immunity in CRC patients. Only a few tumour Ags with such high immunoprevalence have been reported; NYESO-1 (292), MELOE-1 (297) and Survivin (298). Given the relative long length of the peptides used (20 amino acids), it is possible that several epitopes of different 9-mer core sequences (with distinct HLA-DR restriction) are present within these peptides. As a results, many donors of different HLA types would respond. However, they may well be promiscuous peptides able to bind to various HLA-alleles. For instance, Wen *et al* showed that a short epitope (14-mer) derived from HCA587 tumour Ag can stimulate T cells in the context of more than one HLA class II allele (299). Alternatively, recognition of these peptides may be caused by TCR cross-reactivity. This T cell degeneracy allows the recognition of potential pMHC complex by the limited diversity of αβ-TCRs in one individual at a time (300). Provided molecular similarities, a T cell could thus recognise peptides 2, 38 and 20 which could explain high response rates observed in individuals.

In the second group, 2 peptides (3 and 28) had a low immunogenicity in healthy donors but were highly immunogenic in cancer patients. In addition to their high immunoprevalence in patients, the magnitude of responses elicited by these peptides were particularly high. Peptide 38 as mentioned above had a high immunoprevalence but was associated with low magnitude of IFN-γ responses. Peptides 3 and 28 may induce better T cell responses if used as peptide vaccine in CRC patients. The difference in immunogenicity of these peptides in healthy donors and CRC patients indicates that 5T4 overexpression in tumour cells leads to specific T cell priming. This raises the question of natural de novo peptide reactivity in cancer patients. It is possible that chemotherapy has influenced recognition of some peptides that are non immunogenic in healthy donors.

Also, these peptides were (with peptide 20) the most immunogenic in patients. In comparison, healthy donors had 6 peptides of high immunogenicity. It is possible that CRC patients have a more limited TCR repertoire of 5T4-specific T cells, as this has been shown for TRAG-3 tumour Ag in melanoma and breast cancer patients (301). Furthermore, change of TCR repertoire diversity is associated with overall condition of CRC patients (patients in remission have a broader diversity of TCR repertoire) (302). As all patients were in phase of cancer progression (T cells assays were performed with blood samples obtained before tumour resection), this could explain the low diversity of their TCR repertoire.

Despite the presence of these immunogenic peptides in CRC patients, healthy donors had better responses. This can be attributed to a loss of expression of relevant 5T4 Ag peptides on the surface of cancer cells, defects in antigen presentation or release of immunosuppressive substances (e.g. from myeloid-derived suppressor cells) affecting T cell function. Also, the role of the peripheral tolerance to self Ag (e.g. 5T4) in cancer

cannot be excluded. In the previous chapter, FACS analysis showed that Tregs were present at high percentage in blood of CRC patients which may explain the low immunogenicity of the candidate peptides. In contrast, healthy donors seem to have high degree of reactivity against 5T4 suggesting that peripheral tolerance does not prevent spontaneous T cell activity against 5T4.

In the last group, specific responses against 2 peptides (#11 and #23) were seen in healthy donors but were lost in cancer patients. Responses against these peptides may be beneficial to prevent cancer in healthy donors. When a tumour is established however, cancer cells may suppress responses to these peptides by supporting Treg activity. CRC patients vaccinated with 5T4 vaccine and drugs targeting Tregs may regain responses to these peptides. Furthermore, studies in mice suggest that Tregs can selectively inhibit responses to the most immunodominant CD8 determinants (303). Peptides 11 and 23 may be immunodominant peptides in CRC patients preferentially suppressed by Tregs.

CHAPTER 6. HLA-DR RESTRICTED TH1 AND TREG 5T4 PEPTIDES

6.1. Introduction

Several groups have reported the existence of 5T4 peptides recognised by CTLs. Originally reported in healthy donors (240), they were subsequently observed in CRC patients vaccinated with TroVax (recombinant vaccinia viral vector encoding 5T4) (241). Despite the crucial role of Th1 cells for the optimal induction of CTL responses (242), the specificity of CD4$^+$ T cells for 5T4 Ag remains poorly defined (243). In the previous Chapter, I provided evidence that 17 20-mer 5T4 peptides induce IFN-γ secretion by T cells. It is reasonable to suppose that these peptides are presented by MHC class II due to the culture conditions used (12 days, low dose IL-2), which my lab has shown previously to favour expansion of CD4$^+$ T cells (304) (305). Also the majority of MHC class II-restricted tumour Ags are between 15-20 amino acid long (cancerimmunity.org). The possibility that CTL epitopes were identified cannot be excluded. It has been shown that 20mer peptides may contain overlapping CTL and CD4$^+$ T cell epitopes as has been shown in two KIF20A tumour Ag-derived peptides bearing T helper and CTL epitopes (306). Thus, in this chapter, I describe experiments set up to determine whether the 5T4 peptides previously found do indeed elicit CD4$^+$ T cell responses and which HLA class II molecules are responsible for peptide presentation. Furthermore, previous findings described in this thesis revealed that Tregs critically affect responses to 5T4-derived peptide epitopes. Thus, this Chapter describes an investigation of Treg specificity for 5T4 peptide Ags to gain new insights into their mechanism of activation.

6.2. Results

6.2.1. Evaluation of the HLA restriction

In order to investigate the HLA restriction of the immunogenic peptides identified, ELISpot assays were carried out as described previously but in the presence of HLA class I and II blocking Abs. Where available, each of the anti-HLA class I, anti-(HLA)-DQ and anti-(HLA)-DR blocking Abs were added into ELISpot cultures before peptide restimulation. As shown in the example in Figure 6.1, IFN-γ secretion was totally abolished by the addition of anti-DR Abs. In detail, anti-MHC class I Abs (HD18) or anti-DQ Abs (MB26) had no effect and responses to 5T4 were similar to those obtained without Abs.

MHC-restriction was assessed, in most cases, with PBMCs from several donors where responses to the peptides were abrogated in the presence of anti-HLA-DR Abs (Figure 6.2). Again, class I blocking Abs had no effect, demonstrating that these polyclonal responses are not HLA class I-restricted.

Recognition of two peptides, i.e. peptides 11 and 21, was reduced but not totally abolished by blocking anti-DR molecules (more than 50% reduction). Use of anti-class I Abs did not alter the responses, thus it is likely that these peptides can be presented to T cells by MHC class II molecules other than HLA-DR. However, strong responses associated with > 200 spots / 10^5 cells (observed against peptides 2, 3, 15 and 26) were totally abrogated with anti-DR Abs demonstrating the HLA-DR restriction of these peptides.

In addition, Table 6.1 indicates the HLA-types of the donors whose 5T4 responses were restricted *in vitro* by anti-DR Abs (as described in Figure 6.2). Donors with different
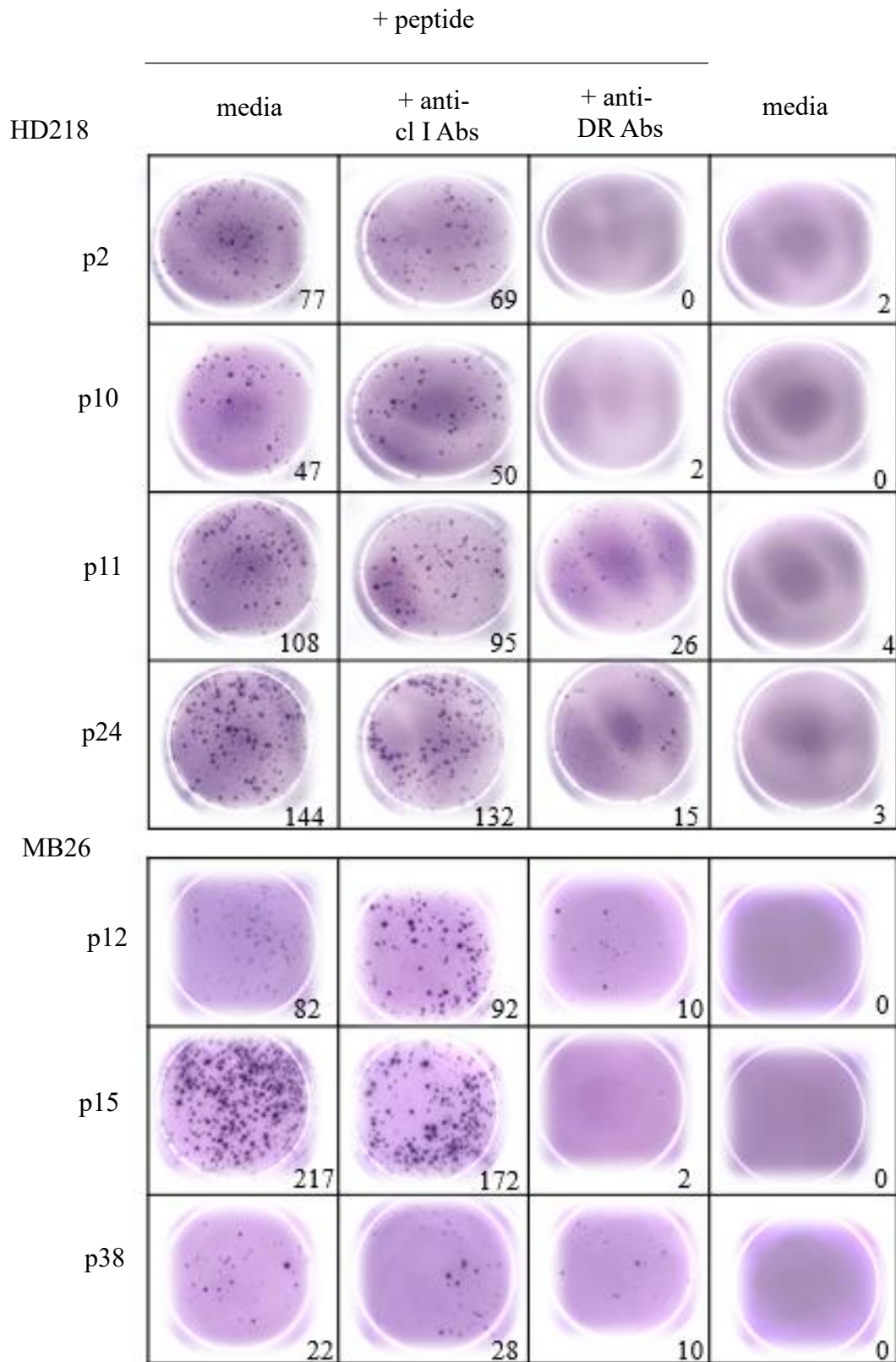
Figure 6.1. Representative examples of MHC-blocking experiments. PBMCs obtained from donors HD18 and MB26 were incubated in ELISpot plates for 16 hr in the absence or presence of either anti-HLA class I or class II Abs. Number of spot-forming cells / 7.5 x10^4 cultured cells is indicated. PBMC were also plated in media only (background control indicated by media).
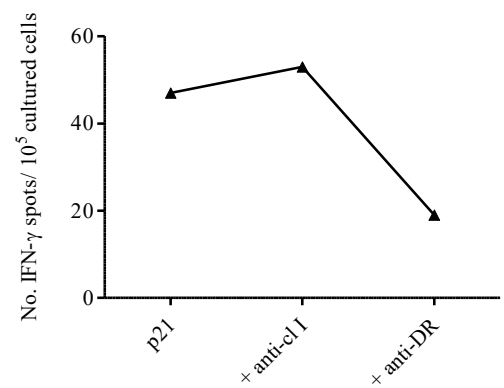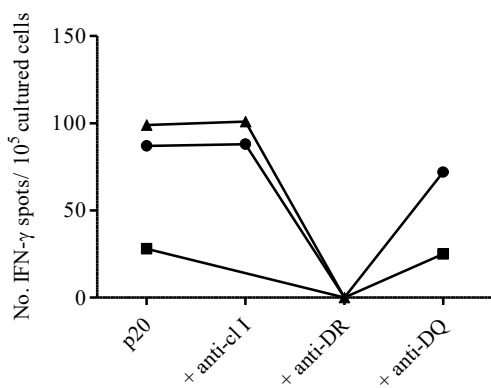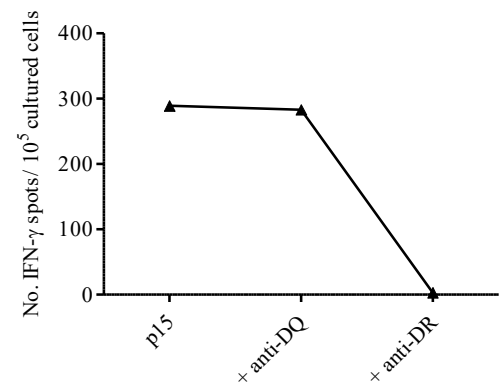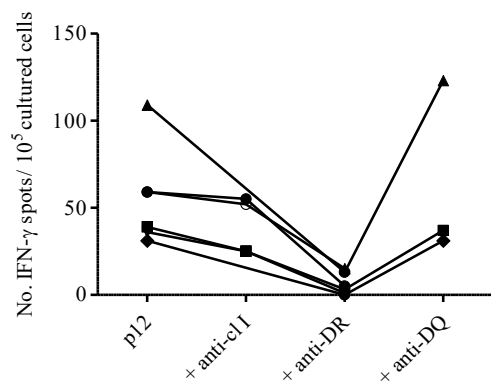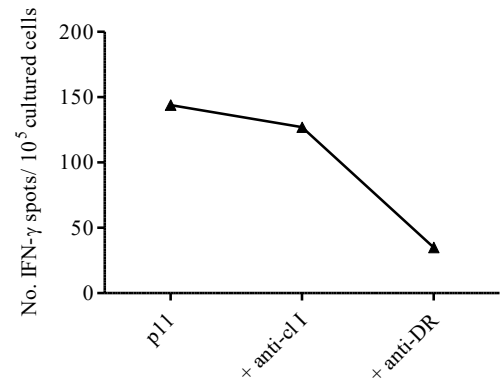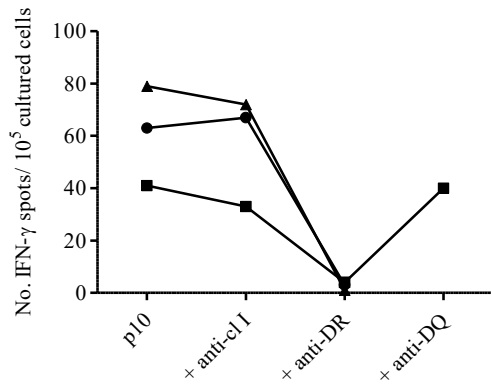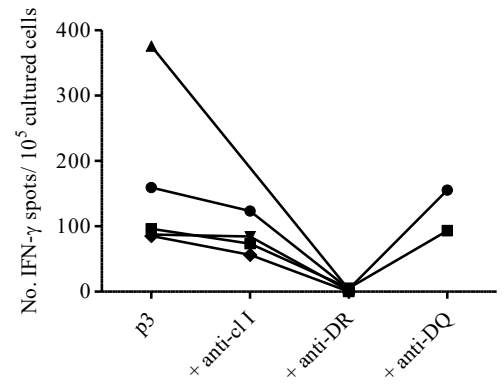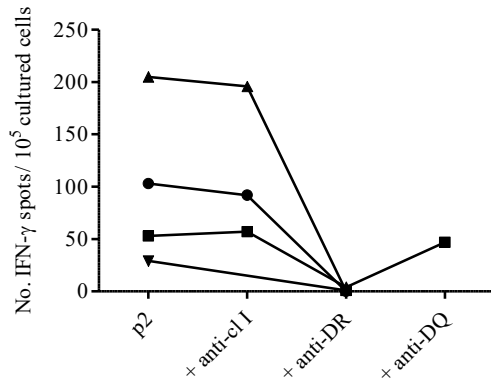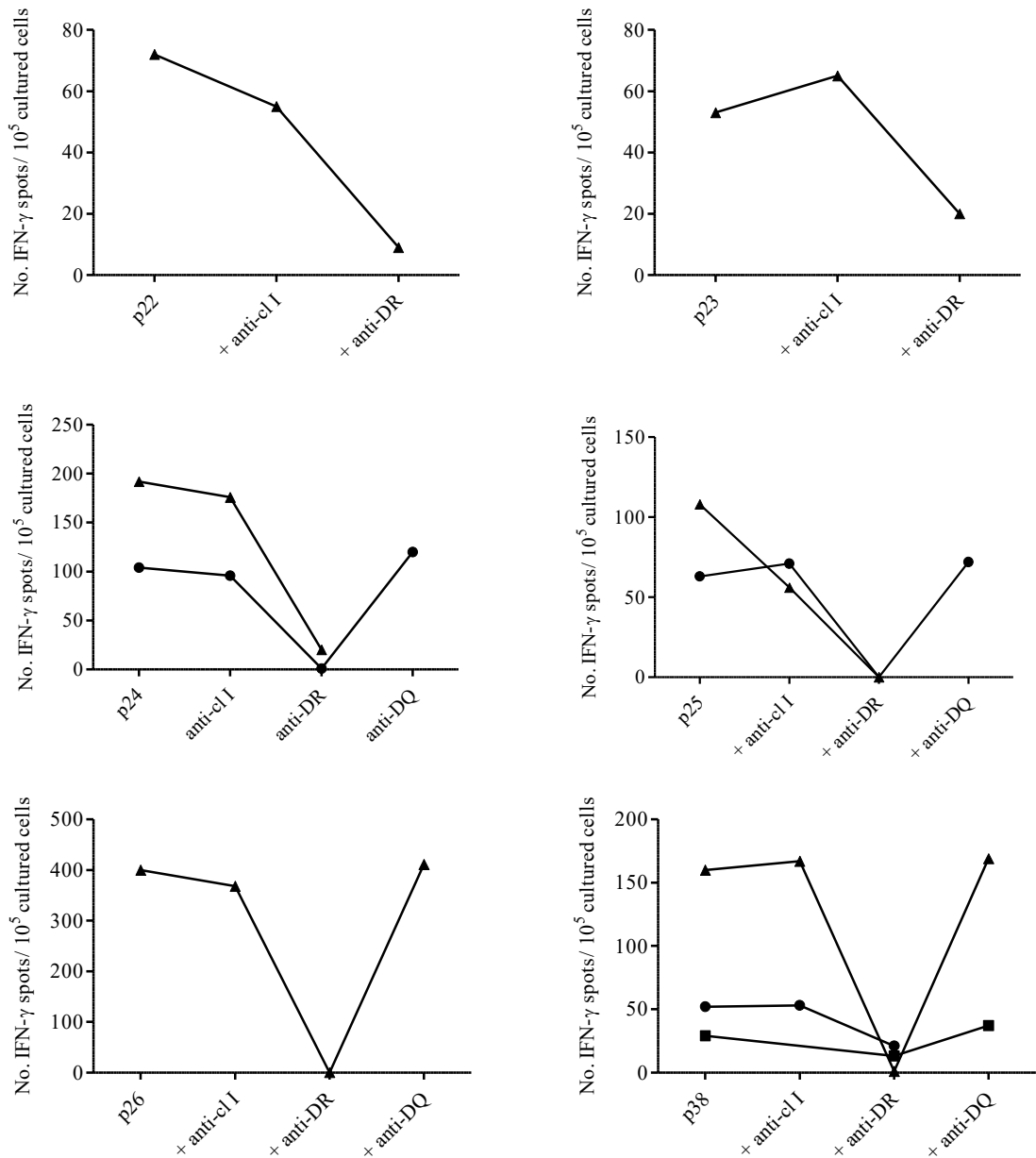
Figure 6.2. Immunogenic 5T4 peptides induce MHC-class II restricted responses. PBMCs were stimulated with the indicated peptides in the presence of MHC-blocking Abs. Results are expressed as the number of IFN-γ spots / 10^5 cultured cells after subtracting the background. When several donors were tested, each symbol represents one individual.

Donor	HLA-DRB1	DR-restricted peptide
HD18	*15, *10	p2
HD214	*01, *12	p2
HD207	*01, *15	p2
MB29	*03, *04	p2
HD214	*01, *12	p3
HD207	*01, *15	p3
MB29	*03, *04	p3
MB27	*08, *14	p3
MB49	*03, *15	p3
HD18	*15, *10	p10
HD207	*01, *15	p10
HD204	*01, *15	p10
HD18	*15, *10	p11
HD210	*04, *15	p12
HD204	*01, *15	p12
HD222	*01, *07	p12
MB26	*15, *04	p12
MB27	*08, *14	p12
MB41	*03, *04	p12
MB26	*15, *04	p15
HD225	*01, *09	p20
HD207	*01, *15	p20
HD222	*01, *07	p20
MB49	*03, *15	p21
HD204	*01, *15	p22
MB41	*03, *04	p23
HD18	*15, *10	p24
HD207	*01, *15	p24
HD207	*01, *15	p25
HD210	*04, *15	p25
HD207	*01, *15	p26
HD18	*15, *10	p38
HD207	*01, *15	p38
MB26	*15, *04	p38

Table 6.1. HLA-DRB1 molecules potentially involved in peptide presentation. Only peptides whose reactivities were abrogated with anti-DR Abs are indicated.

HLA-DRB1 types could respond to the same peptides. For instance, HLA-DR-restriction for peptide 2 was observed in 4 donors with 4 different HLA-DRB1 alleles. The absence of a unique DR-subtype restriction for these 5T4-derived peptides most likely reflects the 20mer length peptides employed, facilitating the binding to several HLA-DR subtypes.

6.2.2. Fine HLA-DR restriction analysis

After characterizing DR as the restriction element of the immunodominant peptides, I next conducted experiments to determine which of the two HLA-DRαβ heterodimers (in heterozygotes) of a given donor was involved in the peptide presentation. Moreover, analysis of patients' HLA types revealed that 3 donors were homozygous for 3 different DRB1 alleles. Thus their responses against DR-restricted 5T4 peptides gave indications of the HLA-DR subtypes involved.

Table A (Figure 6.3) shows that peptide 2, 20 and 38 were not restricted to one particular HLA-DR subtype but can be presented by multiple -DR molecules. Indeed, p38 induced IFN-γ secretion in HLA-DRB1*03 and –DRB1*15 homozygous donors (MB62 and MB64) and peptides 2 and 20 in HLA-DRB1*01 and –DRB1*15 homozygous patients (MB62 and MB64). These results are in line with the above observations and confirm that multiple HLA-DR subtypes are involved in presenting the same 5T4-derived peptides.

The HLA-DR restriction of a peptide can easily be determined if the responder is HLA homozygous, as described above. In heterozygotes, more experiments are required to determine which individual HLA-DR subtypes are involved in peptide presentation. As T cell clones were not available, cell lines were maintained in culture. For this purpose, irradiated allogeneic PBMCs and PHA were added to provide feeder cells and

A

Patient	HLA-DRB1* allele	Peptide recognized
MB62	03, -	38
MB64	15, -	2, 20, 28 , 38
MB65	01, -	2, 3, 10, 20

B   HD204, HLA-DRB1*01*15
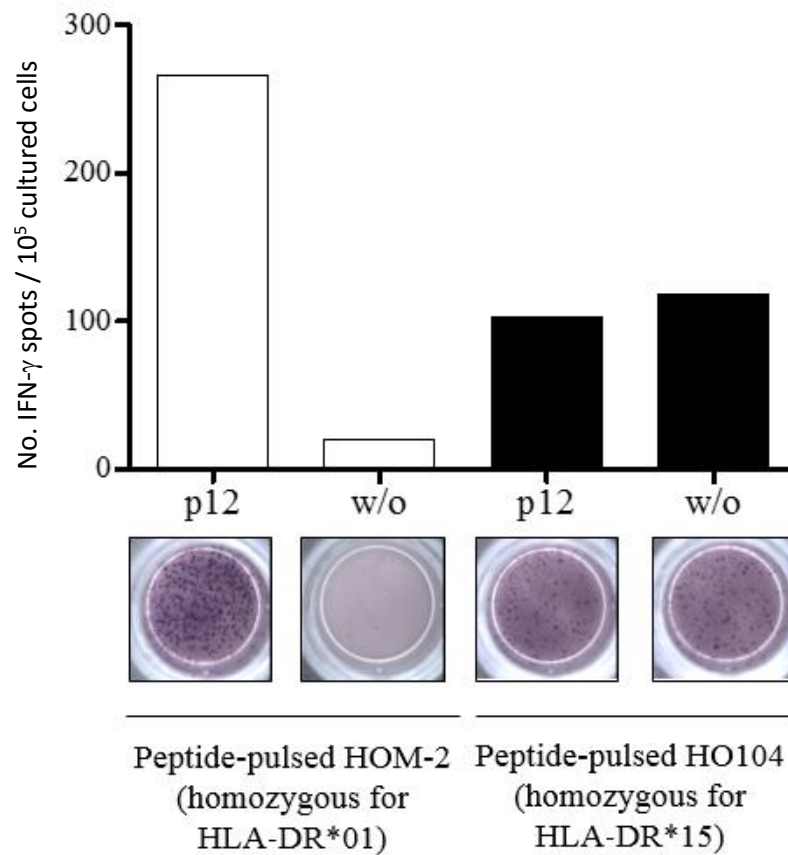
Figure 6.3. Fine HLA-DR restriction of 5T4-specific T cells. A. HLA-DRB1 types of donors who recognized the indicated peptides. B. HLA-DR restriction determinants of 5T4 peptide 12. After 4 weeks of expansion, T-cells were incubated with peptide-pulsed HOM-2 and HO104 EBV-transformed B cells, homozygous for the indicated HLA-DR molecules, for 5h in IFN-γ ELISPOT wells.

non-specific mitogens, respectively. When the cell number required was reached, cells were restimulated in ELISpot plates with HLA-DR-homozygous B cell lines used as APCs. Due to allo-reactivity against EBV-transformed B cells, peptide stimulation was shortened to 5h in ELISpot plates, in contrast to the normal 16 hours. PBMCs from donor HD204 were reactive for p12 and were restimulated with p12-pulsed HOM-2 and HO104 cell lines, each expressing one of the two HLA-DRB1 alleles of the donor. As shown in Figure 6.3.B, p2 was only recognized in association with HLA-DR*01 expressed by HD204 and not the HOM-2 cell-line.

Altogether these data suggest that most 5T4 peptides are presented by HLA-DR molecules, confirmed using anti-DR blocking Abs and HLA-matched/mismatched EBV-transformed B cells to present the peptides.

6.2.3. 5T4 Treg epitopes

Indirect evidence for Treg-mediated inhibition of 5T4-specific responses

Evidences shows that Tregs can inhibit the reactivity of T cells of the same Ag specificity (207). I therefore asked whether responses to the immunogenic 5T4 peptides identified above could be influenced by Tregs of the same peptide specificity. In these experiments, CD25^{hi} T cells were depleted and IFN-γ ELISpot assays were conducted with total T cells and Treg-depleted PBMC. Then, cells of both fractions were stimulated with single candidate peptides. The potential effect of Tregs was classified as either: i) no effect: no enhancement or reduction; ii) Treg effect: a response was either unmasked or increased (by least 50%) post depletion of Tregs.

In CRC patients, responses to 17 peptides were assessed in ELISpot assays using 12-day cultures pulsed with single peptides. Cultures were established in the presence and

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absence of CD25^{hi} cells. The data are summarized in Table 6.2. Responses to 8 of the peptides (peptide 11, 15, 20, 21, 25, 26, 27 and 29) were comparable in cultures established in the presence or absence of CD25^{hi} cells, a finding which may reflect the inability of these peptides to stimulate activation of Tregs. Interestingly, the highly immunogenic peptide p20 was one of them. Responses were altered in the case of the other 9 peptides tested. Following depletion of CD25^{hi} cells, new or increased responses were observed against each peptide in at least one donor (Figure 6.4), a finding which may reflect the ability of these peptides to concurrently stimulate Tregs and Th1 cells. Variations were observed between donors. Peptides 3 and 12 were the most associated with Treg effects as 19% and 21% of donors, respectively, either had new or increased responses to these peptides after CD25^{hi} depletion. These data indicate that Tregs can either fully or partially suppress responses to 5T4-derived peptide epitopes. A relatively high percentage of CRC patients (39%) had responses against at least one 5T4 peptide subjected to these effects. The low number of healthy controls tested (n=3) did not allow for a representative analysis. Therefore, Tregs affect responses to some, but not all, immunogenic peptides in a high number of CRC patients.

Direct observation of Treg-mediated inhibition of 5T4-specific response

The data above and those obtained with the use of peptide pools (Chapter 4) indicate that Tregs can influence 5T4-specific responses. The implication of the experiment described above is that where Tregs affect responses, these Tregs are of the same specificity as the effector T cells. It is also possible that Tregs may be stimulated by a given peptide but suppress in an Ag non-specific manner. An experiment was therefore designed to determine whether those 5T4 peptides, which rarely stimulated IFN-γ production (as indicated in Figure 5.4, Chapter 5), might in fact represent Treg epitopes. Peptides 5, 16,

	2		3		10		11		12		15		20		21		22		23		24		25		26		27		28		29		38	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
MB26			24	4			0	0	**400**	15	**289**	4			8	0													**123**	8			**85**	3
MB27	**25**	177	13	264			4	4	**264**	**25**	1	0			1	1													**377**	12			**25**	144
MB28	**1**	0	1	0	3	1	0	0	0	0			0	0	1	0	9	0	0	0	0	0	0	0	1	0	4	0	7	0	5	0	0	0
MB29	**48**	0	**247**	**33**	11	**37**	0	8	4	0			0	7	**131**	9	0	4	0	3	**43**	0	4	0	1	7	3	0	**268**	5	7	0	0	**27**
MB30	1	0	0	0	0	0	7	0	0	0	**139**	0	**25**	1	**25**	0	8	0	4	0	0	0	0	4	**27**	0	0	7	0	**28**			**144**	**55**
MB31	**37**		0		0				0		0		3		11		**63**		12		0		0		9		0		0				0	
MB41	0	0	0	0	0	0	3	0	16	**47**	0	0			0	0	0	0	0	**39**	0	0	1	0	0	0			0	0			0	0
MB46	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0	0	0	0			0	0			0	0
MB48	0	0	0	0	0	0			0	0	0		0	0			0	0	0	0	0	0	0						0	0			0	0
MB49	0	0	**85**	0	0	0			0	0	0	0	0	0	**88**	**32**	0	0	0	0	0	0	0	0					0	0			4	0
MB51	0	0	1	0	11	0			1	0	0	0	0	0	0	0	**3**	0	0	0	0	0	0	0	3				0	0			0	
MB52	0		0	0	0				0		0		0		0		0		0		0		0						0				0	
MB53	0		0	0	**133**				0		0		0		0		0		0		0		0						0				0	
MB56	**228**		**193**		13								**287**				0						**28**		0		**127**		**63**				4	
MB62	0	9	3	1	0	**29**			4	**63**			1	19			9	4					11	9	1	0			5	3			**28**	**27**
MB64	**25**	1	3	7	3	0	0		1	0			**52**	0	13	5	1	0	3	1	1	3	3	0	5	8	3	1	**32**	1			**67**	8
MB65	**49**	11	**40**	160	**48**	0			0	4			**25**	0	1	9	1	0	1	1	1	**81**	0	0	3	8	3	1	1	1				0
MB66	9	**31**	**67**	148	**140**	11		5	**79**	264			3	1	20	9	4	**388**	8	5	**37**	1	13	1	5	0			8	13			**76**	**93**

Table 6.2. Magnitude of IFN-γ secretion in CRC patients before and after depleting Tregs. Undepleted and Treg-depleted PBMC from CRC patients were stimulated with each of the candidat peptides. The numbers represent the spot-forming cells /10^5 cultured PBMC after subtraction of background spots, in the presence (+ sign) or absence (- sign) of CD25^{hi} cells. Underlined numbers indicate positive responses. Blue numbers indicate an increase of at least 50% in depleted cells and red new responses, compared to undepleted cells.

	2	3	10	12	22	23	24	28	38
■ Treg effect	2 (16%)	3 (19%)	2 (17%)	3 (21%)	1 (9%)	1 (8%)	1 (9%)	1 (7%)	2 (16%)
☐ No Treg effect	11 (84%)	13 (81%)	10 (83%)	11 (79%)	10 (91%)	11 (92%)	10 (91%)	13 (93%)	11 (84%)
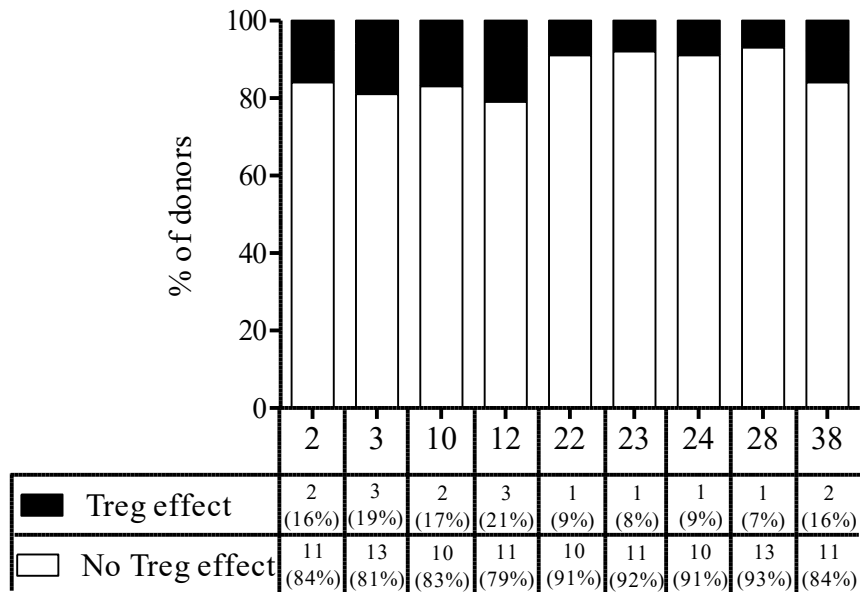
No Treg effect with peptides :11 (n=9), 15 (n=7), 20 (n=11), 21 (n=11), 25 (n=11), 26 (n=9), 27 (n=4) and 29 (n=2)

Figure 6.4. Effect of Tregs on responses against 5T4 candidate peptides. Undepleted and Treg-depleted PBMC from CRC patients were stimulated with each of the candidat peptides in different IFN-γ assays.

18 and 19, which induced IFN-γ responses in less than 20% of donors were selected, and added together to make a putative "Treg pool".

PBMCs from one healthy donor were either depleted of the CD25^{hi} cells or untouched. Both fractions were stimulated for 12 days with the individual candidate peptides described above and with and without the "Treg pool". This *in vitro* stimulation was followed by IFN-γ ELISpot assay to evaluate the effect of the putative Treg epitopes on 5T4 responses.

As shown in Figure 6.5, both fractions could respond to the recall Ag PPD, indicating that the cells were functional. When using the PBMC, a strong response to peptide 2 was detected but this was suppressed when cells were activated by peptide 2 in the presence of "Treg pool". This effect was not apparent in cultures depleted of CD25^{hi} cells indicating that Tregs can be activated by 5T4 peptides to exert a suppressive effect.

Response to PPD

PBMC
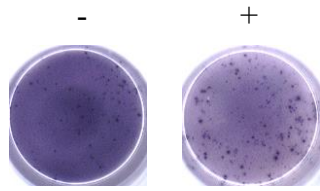
Treg-depleted PBMC
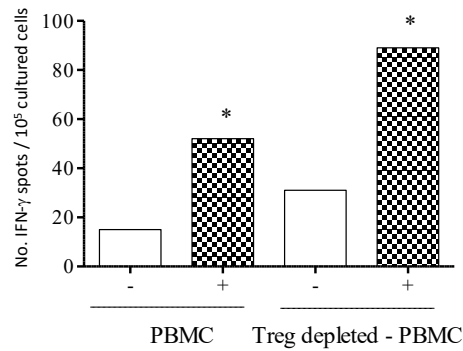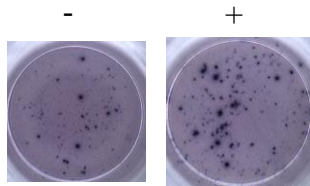
Response to peptide 2

PBMC
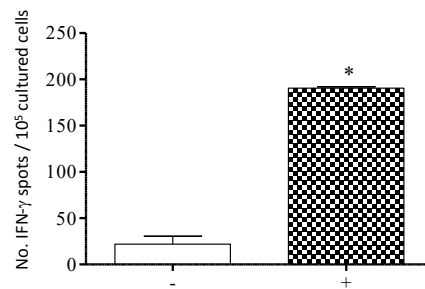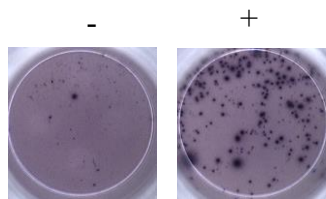
PBMC with Treg pool
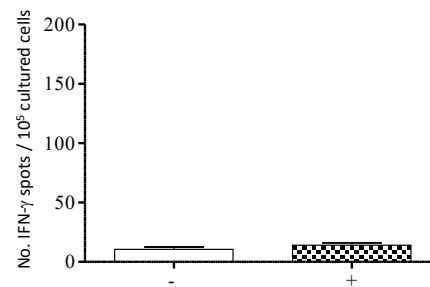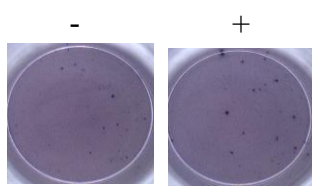
Treg-depleted PBMC
with Treg pool
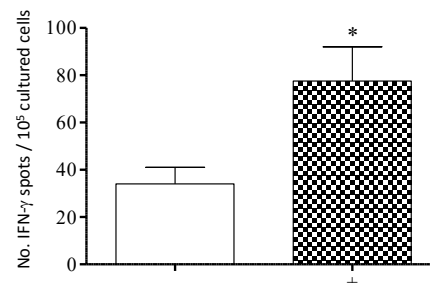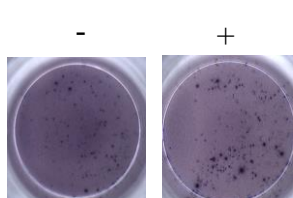
Figure 6.5. 5T4-specific Tregs can suppress 5T4-specific Th1 cells. PBMC and Treg-depleted PBMC were stimulated with PPD in IFN-γ ELISpot assays. They were also stimulated with peptide 2 with or without Treg peptides. Experiment with peptide 2 were performed in duplicate. Asterisks on the top of the bars indicate positive responses.

6.3. Discussion

In this chapter, the MHC-class II restriction of the majority of the most immunoprevalent 5T4 peptides was proven. Also, using anti-MHC Abs their restriction elements were found to be mainly HLA-DR molecules.

Several studies have investigated the specific responses to 5T4 and four MHC-class I restricted peptides have been delineated; $5T4_{17-25}$, $5T4_{97-105}$, $5T4_{153-161}$ and $5T4_{249-257}$ (240, 241). Three of these epitopes are encompassed within peptides 2, 10 and 25 respectively. The use of anti-class I Abs did not abrogate specific responses to these peptides (Figure 6.2). The absence of $CD8^+$ responses is most likely due to the lack of the relevant restricting class I molecule in the tested donors. Peptide 26 encompassed one reported MHC-class II 5T4 epitope (residues 251- 265) (243). This peptide was found to be HLA-DR restricted confirming the $CD4^+$ T cell- priming capacity of $5T4_{254-265}$.

Furthermore, although specific responses to peptides 11 and 21 were unchanged using anti-class I Abs, they were not entirely abrogated using anti-DR Abs (50% decrease), suggesting that either HLA-DP or –DQ molecules can also present these peptides. $CD4^+$ T cells can recognise the same epitope in the context of multiple HLA-DR and DP molecules (cross-reactivity of TCR-peptide-MHC recognition). This has been shown with $CD4^+$ T cell clones recognising NY-ESO-$1_{119-143}$ presented by HLA-DR and -DP molecules (307). It is possible that within the polyclonal population present in a cell line, a T cell clone recognised the same epitopes, derived from peptides 11 or 21, in the context of HLA-DP and –DQ. It is also likely that peptides 11 and 21 comprise epitopes presented by several MHC-class II molecules and recognised by different T cell clones.

Possible MHC-DR-binding residues in 5T4 peptides

The binding of peptides on MHC class II molecules relies on major anchor residues at positions 1, 4, 6 and 9 in the 9mer core sequence (Chapter 1). The anchor residues of several HLA-DR subtypes have been defined (308) and are indicated in the Appendix (Table 3). As 12/14 5T4 peptides were strictly restricted by HLA-DR (Table 6.1), it follows that these peptides must have specific HLA-DR- binding residues. The use of the published binding motifs revealed the residues involved in the HLA-DR binding of all 12 peptides. Moreover, the majority of the peptides contain binding motifs for several different HLA-DR molecules explaining their immunogenicity in responders of different HLA types (Appendix Table 4). Interestingly, the presence of all and/or certain determinant motifs was associated with high immunogenicity in the relevant HLA-DR groups. For instance, peptide 26, which elicited responses in 50% of HLA-DR1^{+} donors, (Appendix Table 2) contains each of the 4 reported HLA-DR1 motifs within the core sequence LTHLESLHL.

In contrast, although peptides 10 and 38 did contain all 4 HLA-DR15 anchor residues, they induced specific responses in a high number of HLA-DR15^{+} donors; 57% for peptide 10 and 86% for peptide 38 (Appendix Table 2). This immunogenicity must be caused by the presence of distinctive bulky hydrophobic residues at position 4 in their core sequences (phenylalanine in peptide 10 and tyrosine in peptide 38), important primary anchors for binding to HLA-DR15 (309). Similarly, peptide 12 was highly immunoprevalent in HLA-DR1^{+} donors with 63% responders (Appendix Table 2). This immunogenicity may be explained by the presence of a small amino acid residue (alanine) at position 6 in its core sequence. This amino acid is a determinant residue binding within pocket 6 of HLA-DR1 molecules (308).

Furthermore, peptide 20 is particularly interesting as it has 2 core sequences that enable binding to HLA-DR1 molecules, i.e. EGM<u>V</u>V<u>A</u>AL<u>L</u> and <u>F</u>EG<u>M</u>VVAA<u>L</u> (anchor

residues underlined). However, it is most likely that <u>F</u>EG<u>M</u>VVAA<u>L</u> binds to HLA-DR1 since position P1 is the most important HLA-DR1 anchor residue (with a preference for tyrosine or phenylalanine at this position) (310). In line with these observations, peptide 20 was recognised in 88% of HLA-DR1$^+$ donors.

Safe 5T4 peptides

It is well established that Treg activation is Ag-specific and that they are capable of recognising different types of Ag including tumour and viral Ags (204). The findings described in this chapter showed that Tregs and Th1 cells share the same specificity for some, but importantly not all, 5T4 peptides (Figure 6.4). Responses to six immunogenic HLA-DR restricted peptides (peptides 15, 20, 21, 25, 26 and 27) were unaffected by Treg depletion, i.e. no increase or new responses after CD25^{hi} depletion. Among them, 5T4 p20$_{191\text{-}210}$ is the most promising because of its high immunoprevalence, especially in HLA-DR1$^+$ and –DR15$^+$ donors (Appendix Table 2). These Treg-independent peptides represent promising "safe" vaccine components, as they should not induce Tregs in patients. This is a crucial parameter as Tregs may either be induced or reactivated following peptide vaccination (232).

Tumour peptides binding various HLA-DR molecules, i.e. universal cancer peptides, may represent the most useful for vaccinating large populations of diverse HLA types (311). However one potential problem is that these peptides may be presented to both Th1 cells and Tregs. Such peptides have been identified in the Study described here. For instance, peptide 12 was immunogenic in several HLA-DR groups but was recognised by Tregs in 3 patients with 3 different HLA-DR alleles. Thus, due to the likely presence of deleterious Treg peptides within tumour Ags, the use of agents modulating the Treg population may be essential to prevent Treg activation post-vaccination.

Moreover, some studies have reported that Tregs do not need to recognise the same Ag as Th1 cells in order to suppress them. For instance, HPV-specific Tregs can suppress influenza specific-Th1 cell activity when both cells are activated by their respective Ags (208). However, no studies have yet reported unspecific Treg suppression of tumour Ags. Bonertz *et al* showed that Tregs can recognise several tumour Ags but without evaluating their ability to suppress in an Ag non-specific manner (207). *In vitro* Treg assays performed in this Chapter showed that the addition of a pool of Treg peptides to a 5T4 peptide-specific T cell line resulted in the inhibition of IFN-γ secretion (Figure 6.5). Importantly, the peptide used to stimulate the T cell line was not found in the Treg pool. Although this experiment needs to be repeated, it suggests that Tregs activated by their related tumour Ag peptide can functionally inhibit Th1 cells of a different tumour Ag specificity.

Melief *et al* have shown that vaccination with HPV-derived long peptides induce HPV-specific CD4$^+$CD25$^+$Foxp3$^+$ T cells in patients with different type of cancers (232, 312). Notably, patients with large vulvar intraepithelial neoplasia lesions displayed strong HPV-specific Treg responses after vaccination and did not have complete regression. The use of this vaccine would require agents modulating Treg activity. A 5T4 vaccine based on some of the T helper peptides described in this Study would not require modulation of Treg activity / numbers as they do not appear to stimulate Tregs. It is therefore possible that such a vaccine, specifically designed to lack Treg epitopes, may best serve to increase the magnitude of the anti-tumour immune response in CRC patients. In conclusion, the results presented in this Chapter indicate the potential for the design of such vaccines.

CHAPTER 7. FINAL DISCUSSION

In this thesis, I investigated the phenotype of CD4$^+$CD25^{hi} cells in CRC patients and examined the immunogenicity of 5T4 tumour Ag. Collectively, these data support the need to target the CD25^{hi} cells as they exhibit a strong immunosuppressive phenotype (Chapter 3) and inhibit responses to 5T4-derived peptides (Chapter 4). It is the first time that both Th1 and Treg cells specificities for an entire tumour antigen are studied. Mapping using a two-step approach (first screen using peptide pools and second screen using individual peptides) revealed different degrees of immunogenicity of 5T4 peptides in healthy donors and CRC patients (Chapter 5), confirming my original hypothesis that specific regions and peptides are more immunogenic than others within 5T4 protein. Furthermore, 5T4 peptides recognized by Th1 cells and / or by CD25^{hi} cells have been found confirming my hypothesis that TCRs expressed by Th1 and Treg cells share similar specificity for 5T4 tumour antigen. This is the most important finding of this work as it has great implications for immunotherapy and the design of 5T4-based vaccine. Although these data presented in this thesis followed my hypothesis, they also raise questions regarding the mode of action used by Tregs and TCR specificity for tumour antigens. These questions are discussed in this Chapter. These findings have implications for the design of more effective 5T4-based peptides.

7.1. *Ex vivo* phenotype of CD4$^+$CD25^{hi} cells

Staining PBMCs isolated from peripheral blood and cells isolated from colon and tumor of CRC patients offered the opportunity to characterize the CD25 population in these samples. The percentage of CD25$^-$ cells expressing the markers studied (HLA DR, ICOS, CTLA-4, CD39, LAG-3 and LAP) was higher in tumours than in the blood of CRC

patients. Whilst these observations were expected with the circulating cells as they are on the majority of a naïve phenotype (Figure 3.2), they may indicate the presence of either activated or immunosuppressive $CD25^-$ cells in CRC tumours. Characterizing these eventual $CD25^-$ suppressive cells, classified as non-conventional Tregs, is of a great importance in cancers where lots of attention have been put on $CD25^{hi}$ Tregs (justified by the poor prognostic of patients with high infiltration of $CD25^{hi}$ T cells). It would be interesting to look at the effect of these IL-2 independent cells on other CD25- that do not express Treg markers. Alternatively, these cells may represent intratumoral Tregs that have temporally lost Foxp3 and CD25 expression. These cells have been shown in mice to reacquire Treg suppressive activity upon TCR stimulation (313).

Furthermore, although at different levels, all markers studied were expressed by $CD25^{hi}$ cells (independently of the samples they were isolated from). This reflects the diversity of the immunosuppressive arsenal available by Tregs and their ability to affect a very large range of immune cells. Based on the high percentages of cells expressing these markers, it is likely that not one but a combination of suppressive mechanisms is used by intratumoral Tregs. However, it remains unclear whether all Treg-associated proteins can be used by all Tregs or by unique Treg subsets that have specific functions in different environments.

7.2. 5T4 tumour peptides

It is the first time that immunogenic sites within the oncofetal Ag 5T4 have been mapped. New HLA-DR restricted peptides derived from 5T4 tumour Ag were identified. These immunogenic peptides may serve to develop tools such as soluble MHC-peptide complexes called tetramers. Their use in immunology, especially in cancer research, has

been proven to be highly useful to gain insight into the frequencies, phenotypes and TCR of tumour Ag-specific T cells (314). However, obstacles such as the low yield of stable monomers that can be obtained and the low affinity of TCR/tumour peptide MHC-class II have limited their broad use. Six tetramers of DRB1*01 allele incorporating different 5T4 peptides (P2, P3, p12, P20, P26 and P38) identified in this study have been generated in the laboratory (by Bruce MacLachlan). Current experiments are focussed on optimising the use of these tetramers. Many important factors are under consideration including time/temperature of incubation and the peptide-MHC tetramer concentration.

Tetramers can be used to monitor the frequencies of tumour Ag-specific T cells mediating spontaneous or vaccine-induced immune responses in patients. *Ex vivo* tetramer staining of PBMCs of non-vaccinated ovarian cancer patients revealed low frequencies of NY-ESO-1-specific circulating $CD4^+$ Th1 cells (314). Reports using *ex vivo* tetramer staining have shown that the frequency of tumour Ag-specific T cells was increased in patients vaccinated with NY-ESO-1-based vaccines (315). Tetramers can also serve to phenotypically characterise T cells used in the 12-day culture followed by IFN-γ ELISpot assays. To evaluate the nature of responses detected by IFN-γ ELISpot after 10-day *in vitro* culture, Todryk *et al* stained cultured T cells with tetramers comprising HLA molecules and viral peptide epitopes (294). Costaining with CCR7 and CD62L revealed that these T cells exhibit a central memory phenotype and their role in IFN-γ ELISpot was confirmed by depleting CCR7 cells at the start of the culture period.

Tetramers may also be used to isolate live cells with a defined Ag specificity. Obtaining clones in this way would enable further functional assays to be conducted or characterisation of TCR for subsequent use in cell-based immunotherapies. Indeed, after detection and isolation of 5T4-specific T cells with tetramers, cDNA coding for TCR α

and β chains that are specific for 5T4 could be amplified, cloned into a viral vector and used to transduce T cells isolated from the blood or tumour of cancer patients. T cells genetically modified in this way have been expanded and reinfused into patients with metastatic melanoma patients. Cancer regression was observed supporting the feasibility and potential of this approach for cancer therapy (316).

7.3. Improving the immunogenicity of 5T4 peptides

Both in the cohort of healthy donors and CRC patients studied, a number of 5T4 immunodominant peptides were found. These peptides were defined as those inducing the highest numbers of IFN-γ producing cells and those recognised by the highest number of donors. Thus, they were defined as immunodominant based on both the intensity and frequency of responses (Chapter 5, Figure 5.8). Several factors can affect the immunogenicity of a given peptide such as effective Ag processing and presentation, a high binding affinity to MHC molecules and the precursor frequency of peptide-specific T cells. For therapeutic peptides used in vaccination, extrinsic factors are added such as the route of administration (intramuscular or intravenous administration).

The relationship between MHC binding affinity and immunogenicity of peptides has long since been described, at least for MHC class I molecules (317). More recently, by determining the crystallographic structure of gp100$_{209-217}$ tumour Ag, Borbulevych *et al* showed that one amino acid substitution in the Ag was associated with an enhanced affinity for HLA-A2 molecules leading to an increased immunogenicity (318). Whether or not increasing the affinity of peptides for MHC class II molecules results in a better immune response has not been studied. It would be interesting therefore to define the

binding characteristics of the identified 5T4 immunogenic peptides to HLA molecules by crystallography in order to design superior vaccine candidates.

Only a few crystal structures of tumour-Ag/MHC class II complexes have been reported thus little is known about the chemical properties permitting optimal peptide binding to MHC-class II molecules. The crystal structure of HLA-DR4 with $gp100_{44-59}$ epitope has recently been reported (319). This study revealed the identity of the anchor residues binding to the pocket of HLA-DR4 molecules. Substitution of these residues by certain other amino acids was shown to enhance the MHC–peptide affinity underlining the potential of this approach. However, such substitutions in the peptide core sequence may prove counterproductive as these have been shown to alter the TCR specificity (320). An alternative option to improving $CD4^+$ T cell responses might be to alter amino acid residues in the nonbinding terminal regions of peptides, named flanking regions. Cole *et al* have shown that C-terminal modifications in the influenza hemagglutinin $HA_{305-320}$ epitope can lead to enhanced $CD4^+$ T cell activation (24). More precisely, the presence of an Arginine at position 10 or 11 in the C-terminal flanking region could enhance TCR/pMHC-II affinity. Surprisingly, despite the clear utility of this approach, no attempt has ever been made to improve the affinity of TCRs to tumour Ag/MHC class II by altering peptide flanking residues. The 14 MHC-class II-restricted 5T4 peptides identified in this study represent good candidates for such experiments.

7.4. Tumour Ag-specific Tregs

One aim of this PhD was to define 5T4-derived peptides recognised by Tregs. These suppressive cells had a negative impact on 5T4-specific responses as the majority of CRC patients could recognise more peptide pools after depletion of $CD25^{hi}$ cells (Chapter 4,

Figure 4.6). One striking observation is that all pools were susceptible to be affected by Treg depletion in CRC patients. These results raise questions regarding the advantage of a broad Treg tumor Ag specificity. Since Tregs only exert a suppressive activity after antigenic stimulation through their TCRs (321), they may have a broad TCR repertoire to keep regulatory functions in case one peptide is made unavailable.

Also, single peptide-stimulation assays revealed that Tregs can recognise the same 5T4 peptides as Th1 cells and suggested that they can suppress Th1 cells of a different Ag specificity (Chapter 6, Figures 6.4 and 6.5). This results confirm our hypothesis that 5T4-specific Th1 and Treg cells can be identified in CRC patients.

Although human Tregs were discovered approximately 20 years ago, their TCR specificity has not been extensively studied. The lack of tools allowing quick and direct identification and quantification of Ag-specific Tregs is, in part, responsible. Instead, several approaches, which have proven laborious and difficult to reproduce, have been reported for the identification of tumour-specific Tregs. For instance, several studies found tumour Ag-reactive Tregs by cloning and subsequent immune profiling (203, 206). Whilst informative, such methods are not applicable for routine detection of Tregs. Of note, the assays carried out during the course of this PhD were based on functional assays where Tregs were depleted or "Treg peptides" added to reactive T cell lines and are thus more effective than reported assays. Indeed, only 14 days were required to evaluate whether or not T cell responses against a 5T4 peptide were controlled by Tregs. This high throughput screening allowed the analysis of Treg activity in a high number of donors.

Beckhove *et al* showed that HLA-class II tetramers can be used to assess the presence and quantify tumour Ag-reactive T cells in cancer patients and healthy donors (210). Using this approach they revealed *ex vivo* the presence of effector and Treg cells

specific for mam$_{34-48}$-derived mammaglobin protein. Mammaglobin (mam) is a tumour associated Ag overexpressed in breast tumours. Both populations were expanded in breast cancer patients in comparison to healthy subjects. Since data presented in Chapter 6 showed that Tregs can recognise the same 5T4 peptides as Th1 cells, it will be interesting to compare frequencies of both populations using tetramers. Also, CD4$^+$CD25^{hi} cells are enriched in tumours of CRC patients (Chapter 3, Figure 3.7); thus it will be of interest to determine if the proportion of 5T4-specific Tregs is also highly increased within the tumours. To the best of my knowledge, there are no studies using HLA-class II tetramers, which have so far revealed the presence of Ag-specific Tregs in tumours.

An alternative approach for the detection of Ag-specific Tregs without the need to generate tetramers has been reported. It is based on the observed down-regulation of CD3/TCR complexes following TCR recognition (322). Using this approach, Ebert *et al* concluded that the NY-ESO-1/ISCOMATRIXTM peptide-based vaccine was able to induce new or increase pre-existing Treg responses against NY-ESO-1 in advanced melanoma patients (233). However, this finding has not been validated by other groups thus precaution must be taken when assessing Treg activity based on CD3 downregulation. Furthermore, Ebert *et al* defined Tregs as CD25$^+$Foxp3$^+$ cells in their study. Even though the advantage of this approach lies in the simplicity of its routine use, 40% of peripheral CD4$^+$CD25^{int} effector T cells express Foxp3 (Chapter 3, Figure 3.5), so suppressive Tregs may not be the population down regulating CD3. It would be of interest to evaluate the ability of CD4$^+$CD25^{hi} cells to down regulate CD3 after activation.

7.5. Implications for immunotherapy

Stimulating pre-existing anti-5T4 T cells

Memory T cells are preferentially detected in cultured ELISpot assays whilst Teff cell responses are generally measured by ex vivo ELISpot [our group and (294)]. Therefore the 5T4-reactive T cells measured in the cultured ELISpot assays described herein are likely derived from pre-existing memory T cells in the blood of donors. This pre-existing CD4 memory Th1 cell repertoire specific for 5T4 tumour Ag in cancer patients is of major importance for the development of cancer vaccines. Indeed, vaccination with such peptides may allow a fast, strong and long-lasting secondary Th-mediated anti-tumour immune response (Figure 7.1). In contrast, epitopes recognised by naïve T cells will only elicit slow and weak primary Th responses when injected into cancer patients (Figure 7.1). Another reason for the failure of vaccines may be the use of peptides recognised by both Th1 and Treg cells considerably limiting the effectiveness of the vaccine (Figure 7.1).
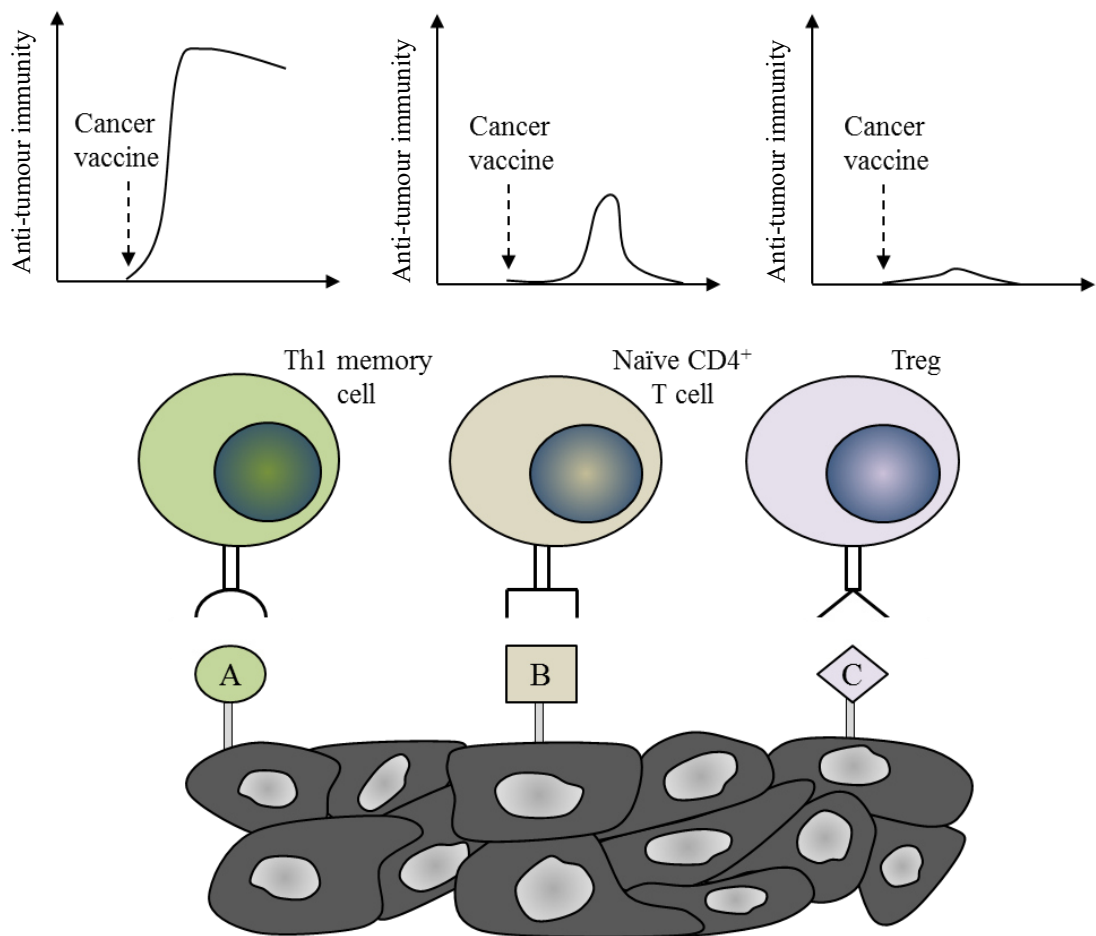
Figure 7.1. T cell populations induced by peptide-based vaccines and consequences. Anti-tumour immunity is either rapidly enhanced, slowly increased or inhibited depending on the tumour peptide used in vaccination and the nature of the cells activated.

T helper peptide-based vaccine with checkpoint inhibitors

Kenter *et al* showed that a HPV vaccine containing long overlapping peptides was safe and immunogenic in cervical cancer patients but could not exert effective therapeutic action (323). Further analysis of the T cell responses post vaccination in cervical cancer patients showed that not only did the vaccine induce CD4$^+$ Th and CD8$^+$ cells but also Tregs (232). Due to the presence of Treg epitopes, the use of Treg modulating agents would therefore be required for such vaccines in cervical cancer. Nevertheless, this HPV vaccine could induce clinical responses (including complete regression) in a majority of vulvar intraepithelial neoplasia patients (324). The therapeutic activity of the vaccine may be caused by the absence of HPV-specific Tregs in these patients.

The six HLA-DR restricted peptides (15, 20, 21, 25, 26 and 27) identified in this study represent good candidates for vaccines as i) they were immunogenic in CRC patients of different MHC background and ii) were not recognised by Tregs. These T helper peptides can therefore be used in a multipeptide-vaccine overcoming the need to modify Treg activity.

Targeting inhibitory receptors that normally reduce the immune response represent another strategy to enhance anti-tumour immunity. Checkpoint inhibitors, such as anti-CTLA-4 and anti-PD-1 mAbs, have been reported to enhance anti-tumour immunity in cancer patients (180, 325). However, clinical trials have so far only showed low objective response rates. Following vaccination with anti-CTLA-4 Abs (Ipilimumab), objective responses were observed in 5% of melanoma (326) and in 12.5% of renal cell carcinoma (327) patients. Low objective response rates are also observed following anti–PD-L1 Abs treatments; 11.8% in renal-cell cancer and 5.9% in ovarian cancer patients (328). Furthermore, such treatments are often associated with severe grade III/IV side effects (329). Their use as monotherapies or as adjuvants is thus not entirely safe but they are so

far the most promising drugs in immunotherapy. It will be of interest to determine the clinical benefit of such drugs in association with vaccines, including vaccines inducing 5T4-specific T cell responses.

Engineered 5T4 neoAntigens

Vaccination with mutated tumour Ags represent a promising approach alongside to native Ags. These neoAgs are not affected by central T cell tolerance and their expression is strictly tumour specific. Since somatic mutations take regularly place in CRC (330), it is likely that 5T4 mutated peptides are presented by tumour cells. Introducing mutations in the 5T4 Th peptides reported in this Study may create neoAgs which could induce specific responses *in vivo* in cancer patients. Also, as checkpoint inhibitors, at least anti-PD Abs, work better against mutated Ags (331), such treatment would make CRC more sensitive to 5T4 neoAg therapy.

After exome sequencing, potential neoAgs are selected on the basis of their predicted strong affinity for MHC molecules (332). The 5T4 peptides described in this study are immunogenic in CRC patients which may reflect, among other factors, their good affinity for MHC class II molecules. Therefore, modification should be limited to amino acid substitution at TCR contact residues (position 2, 3, 5, 7 and 8) within their core sequences, which would not modify their binding properties to MHC molecules. Furthermore, the immunogenicity and thus the clinical potential of the engineered neoAgs could easily be assessed in *in vitro* IFN-γ ELISpot assays with PBMCs of CRC patients.

7.6. Conclusion

I have shown that a strategy, based on use of overlapping peptide pools and validation with single peptides, can be successfully used to identify CD4 epitopes on the entire 5T4 tumour Ag. Four immunodominant regions within 5T4 were unveiled, which contained MHC-class II peptides presented by different HLA-DR alleles. This approach, purely based on *in vitro* assays, was independent of computational prediction and thus allowed a, previously undescribed, T cell mapping of peptides present on the entire 5T4 protein. Tregs from CRC patients were phenotypically characterised and their influence on 5T4-specific responses studied. The high expression of inhibitory molecules on their membrane and the shared Ag specificity with helper T cells argue for the redesign of cancer vaccines to specifically exclude peptides commonly recognised by Tregs. The identification of immunogenic helper peptides not recognised by Tregs will, not only be useful to track T cells responses during vaccination, but also should help to make cancer vaccines more efficient in patients with 5T4$^+$ tumours.

APPENDIX

Appendix Figure 1. Controls for commonly used fluorescently labelled antibodies.

Helios - FITC

HLA-DR - PECy7

ICOS - FITC

CTLA-4 - PE

CD39 - PECy7

Foxp3 - APC

CD45RO - FITC

CD45RA - PerCPCy5.5

CD4 – Pacific Blue

LAP - PerCPCy5.5
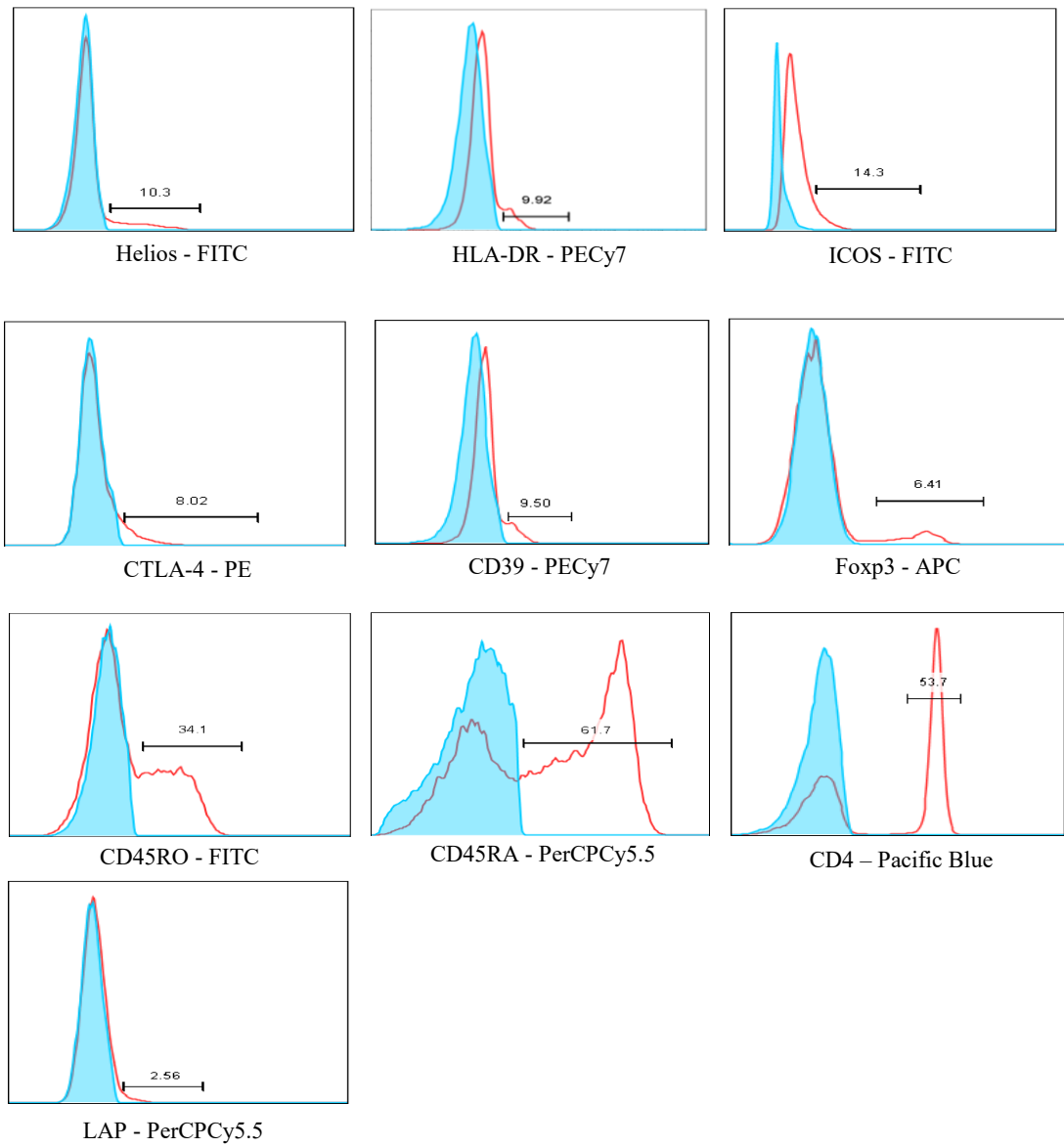
Appendix Figure 2. Proportions of circulating and intratumoural CD4$^+$CD25^{hi} and CD4$^+$Foxp3$^+$ cells within the CD4$^+$ population in CRC patients.
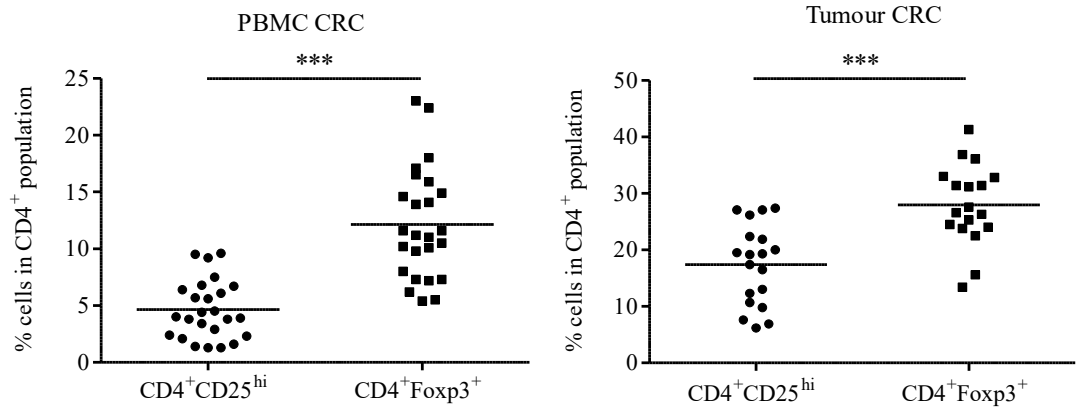
Appendix Table 1. *In silico* predicted MHC class II-restricted peptides of 5T4 protein. Grey boxes indicate predicted strong binders and white boxes non-binders. Default prediction thresholds were used for both algorithms to predict strong binders; IC50 <500 nM for NetMHC2.2, percentile rank below 10% for IEDB.

NetMHC2.2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
*0101		■	■	■	■					■	■	■	■				■			■			■			■		■						■	■	■		■			■
*1501		■								■															■									■							
*0301																																						■			■
*0401					■										■	■								■	■				■					■							
*0701				■						■		■											■		■	■															
*1302																		■							■				■												

EIDB

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
*0101		■	■	■						■	■	■		■											■	■									■	■	■	■			■
*1501		■	■	■						■	■								■			■		■	■	■	■	■										■			■
*0301		■		■			■	■	■	■					■				■				■		■	■	■		■	■	■	■	■	■	■	■		■			■
*0401		■	■	■				■				■	■	■	■	■			■	■	■				■														■		
*0701		■	■								■	■								■	■				■																■
*1302		■	■	■																																					■

Algorithm	Nb peptide binding to 3 HLA-DR alleles	Nb peptide matching heat map	Nb peptide not matching heat map	Nb peptide missed
NetMHC2.2	6	5	1	14
IEDB	20	13	7	6

Appendix Table 2. Responses to individual 5T4 peptides stratified by HLA-DR serotype. Each peptide was used to stimulate T cells from healthy donors (HD) and CRC patients (MB) The numbers represent the spot-forming cells / 10^5 cultured PBMC after subtraction of background spots. Underlined bold numbers indicate positive responses. Absence of numbers indicate peptides not tested

	Donor	HLA-DRB1	2	3	10	11	12	15	20	21	22	23	24	25	26	27	28	29	38
DR1	HD207	*01, *15	**62**	**95**	**85**	**32**	**40**	0	**55**	**25**	**45**	1	**67**	**147**	**135**		0		**163**
	HD214	*01, *12	**91**	**32**	0	0	**232**	0	**63**		0	0	0	7	0				5
	HD222	*01, *07	**36**	8	0	5	**93**	0	**96**	**333**	0	0	**28**	0	**65**	3	0	**36**	5
	HD225	*01, *09	**116**	0	**91**	0	**25**	0	**71**	1	4	1	1	0	**45**	0	1		0
	HD204	*01, *15			**79**		**92**				**69**								
	MB30	01, 13	1	0	0	7	0	**139**	**25**	**25**	8	4	0	0	**27**	0	0		**144**
	MB51	01,03	0	1	11		1	0	0	0	3	0	0	0	3		0		0
	MB56	01,08	**228**	**193**	13				**287**		0			**28**	0	**127**	**63**		4
	MB65	01, -	**49**	**40**	**48**		0		**25**	1	1	1	1	0	3	3	1		
DR15	HD218	*15, *10	**91**	0	**80**	**141**	0	0	**27**	4	3	0	**109**	3	**145**	1	**25**	4	**89**
	HD210	*04, *15	9	1	1	1	**71**	0	13	**48**	**73**	**40**	0	**92**	24		3		**37**
	HD204	*01, *15			**79**		**92**				**69**								
	HD207	*01, *15	**62**	**95**	**85**	**32**	**40**	0	**55**	**25**	**45**	1	**67**	**147**	**135**		0		**163**
	MB26	15, 04		24		0	**400**	**289**		8				0			**123**		**85**
	MB49	03, 15	0	**85**	0		0	0	0	**88**	0	0	0	0			0		4
	MB64	15, -	**25**	3	3		1		**52**	13	1	3	1	3	5		**32**		**67**
	MB66	04. 15	9	**67**	**140**		**79**		3	20	4	8	**37**	13	5		8		**76**
DR3	HD203	*03, *--	0	0	9	0	0		0	0	0	0	0	0			0		
	MB29	03, 04	**48**	**247**	11	0	4		0	**131**	0	0	**43**	4	1	3	**268**	7	0
	MB41	03,04	0	0	0	3	16	0			0	0	0	1	0		0		0
	MB48	03, 13	0	0	0		0	0	0		0	0	0	0			0		0
	MB49	03, 15	0	**85**	0		0	0	0	**88**	0	0	0	0			0		4
	MB51	01,03	0	1	11		1	0	0	0	3	0	0	0	3		0		0
	MB62	03, -	0	3	0		4		1			9		11	1		5		**28**
DR4	HD215	*04, *07	1		1	3		0			8	0	1	11	5				
	HD210	*04, *15	9	1	1	1	**71**	0	13	**48**	**73**	**40**	0	**92**	24		3		**37**
	MB26	15, 04		24		0	**400**	**289**		8				0			**123**		**85**
	MB28	04, 07	1	1	3	0	0		0	1	9	0	0	0	1	4	7	5	0
	MB29	03, 04	**48**	**247**	11	0	4		0	**131**	0	**0**	**43**	4	1	3	**268**	7	0
	MB31	04, 13	**37**	0	0		0	0	3	11	**63**	12	0	0	9	0	0		0
	MB41	03,04	0	0	0	3	16	0			0	0	0	1	0		0		0
	MB46	04,13	0	0	0	0	0	**0**	0	0	0	0	0	0	0		0		0
	MB53	04,07	0	0	**133**		0	0	0	0	0	0	0	0			0		0
	MB66	04. 15	9	**67**	**140**		**79**		3	20	4	8	**37**	13	5		8		**76**
DR7	HD215	*04, *07	1		1	3		0			8	0	1	11	5				
	HD222	*01, *07	**36**	8	0	5	**93**	0	**96**	**333**	0	0	**28**	0	**65**	3	0	**36**	5
	MB28	04, 07	1	1	3	0	0		0	1	9	0	0	0	1	4	7	5	0
	MB53	04,07	0	0	**133**		0	0	0	0	0	0	0	0			0		0
DR13	MB30	01, 13	1	0	0	7	0	**139**	**25**	**25**	8	4	0	0	**27**	0	0		**144**
	MB31	04, 13	**37**	0	0		0	0	3	11	**63**	12	0	0	9	0	0		0
	MB46	04,13	0	0	0	0	0	0	0	0	0	0	0	0	0		0		0
	MB48	03, 13	0	0	0		0	0	0		0	0	0	0			0		0
	MB52	13,-	0	0	0		0	0	0		0	0	0	0			0		0

Appendix Table 3. MHC binding preferences of most common HLA-DR subtypes in Wales. Motifs were found using the Syfpeithi database.

HLA-DRB1*0101

1	2	3	4	5	6	7	8	9
Y			L		A			L
V			A		G			A
L			I		S			I
F			V		T			V
I			M		C			N
A			N		P			F
M			Q					Y
W								

HLA-DRB1*1501

1	2	3	4	5	6	7	8	9
L			F			I		
V			Y			L		
I			I			V		
						M		
						F		

HLA-DRB1*0301

1	2	3	4	5	6	7	8	9
L			D		K			Y
I					R			L
F					E			F
M					Q			
V					N			

HLA-DRB1*0401

1	2	3	4	5	6	7	8	9
F			P		N	D		D
Y			W		S	E		E
W			I		T	H		H
I			L		Q	K		K
L			V		H	N		N
V			A		R	Q		Q
M			D		R	R		R
			E		S	S		S
					T	T		T
					Y	Y		Y
					A	A		A
					C	C		C
					I	I		I
					L	L		L
					M	M		M
					V	V		V

HLA-DRB1*0701

1	2	3	4	5	6	7	8	9
F			D		N			V
Y			E		S			I
W			H		T			L
I			K					Y
L			N					F
V			Q					
			R					
			S					
			T					
			Y					

Appendix Table 4. 9mer core sequences (red) and anchor residues (underlined) of HLA-DR restricted 5T4 peptides. The analysis only includes the DR subtypes of patients from whom peptides were found DR-restricted using blocking Abs. Absence of sequence indicates that less than 3 residues matched the published motifs (weak binders). Since only 2 anchor residues are reported for HLA-DR15, core sequences with 1 determinant motif defined strong DR15 binders.

Peptide 2

HLA-DRB1	Sequence
DR1 DR15	AGDGRLR**LARLALVLL**GWVS
DR4 DR3	AGDGRLR**LARLALVLL**GWVS

Peptide 3

HLA-DRB1	Sequence
DR1 DR15 DR3	LALVL**LGWVSSSSP**TSSASS
DR4	LALVLLG**WVSSSSPTS**SASS

Peptide 10

HLA-DRB1	Sequence
DR1	AY**VRNLFLTGN**QLAVLPAGA
DR15	AYV**RNLFLTGNQ**LAVLPAGA

Peptide 12

HLA-DRB1	Sequence
DR1 DR15 DR3	FARRPP**LAELAALNL**SGSRL
DR4 DR7	FARRPP**LAELAALNL**SGSRL

Peptide 15

HLA-DRB1	Sequence
DR15 DR4	LPS**LRQLDLSHN**PLADLSPF

Peptide 20

HLA-DRB1	Sequence
DR1 DR1 DR15 DR7	QNRS**FEGM<u>V</u>VA<u>A</u>L<u>L</u>**AGRALQ QNRSF**EGM<u>V</u>V<u>A</u>ALL**AGRALQ

Peptide 22

HLA-DRB1	Sequence
DR1 DR15	GLRR**LEL<u>A</u>SNHFL**YLPRDVL

Peptide 23

HLA-DRB1	Sequence
DR3 DR4	HFLY**LPR<u>D</u>VLAQL**PSLRHLD HFLY**LPR<u>D</u>VLAQL**PSLRHLD

Peptide 24

HLA-DRB1	Sequence
DR1 DR15	AQLPS**LRH<u>L</u>DLSN<u>N</u>**SLVSLT

Peptide 25

HLA-DRB1	Sequence
DR1 DR4 DR15	LSNNSLVS**LTY<u>V</u>SFRNL**THL LSNNSLVSLT**YVSFRNLTH**L LSNNSLV**SLT<u>Y</u>VSFRN**LTHL

Peptide 26

HLA-DRB1	Sequence
DR1 DR15	YVSFRN**LTHLESLHL**EDNAL

Peptide 38

HLA-DRB1	Sequence
DR1 DR15 DR4	L**LVLYLNRKG**IKKWMHNIRD

Patient Information Sheet:
An Investigation into the role of lymphocytes on the generation of tumour immunity in humans.
You are being invited to take part in a research study. Please ask us if anything is unclear or if you need more information. Take time to decide whether or not you wish to take part.

Purpose of the Study:

Your immune system is involved in fighting infections such as bacteria and viruses using white blood cells. However, there is strong evidence that the immune system is important in causing inflammatory conditions such as Crohn's disease or ulcerative colitis, and also in fighting abnormal cancer cells, which might arise in normal or inflamed tissue. The purpose of this study is to examine the way your white blood cells recognise and attack cells of the body, including malignant tumour cells.

The Volunteers

We are interested in patients who are about to undergo a resection of their colon for malignancy or a complication of inflammatory bowel disease.

Do I have to take part?

It is up to you whether or not to take part. If you do decide to take part, you will keep this information sheet and be asked to sign a consent form. If you decide not to take part, it will **not** affect the standard of your care in any way.

What does it involve?

To carry out this research, we would require a small sample of blood (30-40 mls, i.e. one syringe full) before your operation. After your operation, it is usual for the resected bowel to be sent down to a pathologist for examination. In the course of this standard examination, the pathologist will remove a small sample of the abnormal bowel and adjacent lymph nodes, required for use in this study. The white cells in these samples will also be analysed. After you have made a full recovery from your operation, 2-3 further blood samples would be analysed over a 12 month period. These samples will usually be obtained by us when you are attending outpatients.

Are there any risks?

There are no extra risks in taking part. **This study in no way impinges or alters your treatment**. There is only the inconvenience of an extra blood sample.

What are the benefits of taking part?

This is a valuable study in allowing us to understand how and why our white blood cells attack our own tissues. This knowledge would potentially allow new therapies to be designed in the future that manipulate this response to treat certain cancers or inflammatory conditions.

What happens when the research is over?
When we have all the results, we will aim to present the data at local, national and international meetings. At these meetings, experts in the field will be able to review and discuss the new information. The work will also be published in journals so that the information can reach a wide audience.

Patient Confidentiality
All information collected during the study will be kept confidential. Any information about you that leaves the hospital will have your name and address removed so that it cannot be recognised. All results obtained from blood or tissue samples that you provide will be completely anonymous.

Who is organising the research?
This study is supervised by Dr Andrew Godkin (Consultant Gastroenterologist) in close liason with Mr Mike Davies, Miss Rachel Hargest or Mr Simon Phillips (Consultant Surgeons) who is performing your operation and looking after you whilst you are in hospital. The research is carried out in the Henry Wellcome Building (Cardiff University) at the Heath Hospital site.

Who has reviewed the study?
This study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Committee and the Local Research Ethics Committee. The study is being funded by a grant from Cancer Research Wales and was initially sent out for external review.

Who to contact
If you wish to discuss any issues, please ask the houseman looking after you on the ward. If issues arise that he / she cannot deal with, Mr Davies or Dr Godkin will be able to discuss these further

Last Updated: 3/2/2011

CONSENT FORM

An Investigation into the role of lymphocytes on the generation of tumour immunity in humans.

Name of Researcher: Matthieu Besneux (PhD Student)

Please tick box

Research Supervisor: Dr Andrew Godkin (Consultant Gastroenterologist)

1. I confirm that I have read and understand the information sheet dated 3/2/2011 for the above study and have had the opportunity to ask questions.

   ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

   ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by responsible individuals from Cardiff University or the Cardiff and Vale NHS Trust. I give permission to these individuals to have access to my records. All information will remain confidential.

   ☐

4. I consent for my anonymized results of this study to be published in scientific / medical journals.

   ☐

5. I consent for obtained samples to be stored for future research.

   ☐

6. I agree to take part in the above study.

   ☐

Name of Patient	Signature	Date
Name of Person taking consent	Signature	Date

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes / histopathology.

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