UPPSALA
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UPTEC X 15 019
Examensarbete 30 hp
December 2015

Immune responses in urogenital cancer

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UPPSALA UNIVERSITET

Degree Project in Molecular Biotechnology

Masters Programme in Molecular Biotechnology Engineering,
Uppsala University School of Engineering

UPTEC X 15 019	**Date of issue 2015-12**

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Title (English)

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Title (Swedish)

Abstract

Urinary bladder cancer and renal cancer are two cancer types belonging to the urogenital cancer category and together cause hundreds of thousands of deaths each year worldwide. A new approach for treating these types of cancer is the use of immunotherapy by checkpoint inhibitors. Two molecules that have been blocked in this type of treatment are the immune inhibitory molecules CTLA-4 and PD-1, which both are expressed on T cells at different degrees. This work investigates the immune response when culturing cells derived from patients with urinary bladder or renal cancer while adding blocking antibodies towards CTLA-4 and PD-1. No significant trends or differences were seen when studying CD4+ T cells and regulatory T cells cultured with different blocking antibodies. Interestingly, in cultures derived from a single sentinel node from one patient that underwent cystectomy an increase in regulatory T cells was seen when culturing with CTLA-4 blocking antibodies. Additional cultures are needed to see if CTLA-4 blockage actually leads to increased expansion of regulatory T cells.

Keywords

Urinary bladder cancer, renal cancer, CTLA-4, PD-1, blockade, T-cells, regulatory T-cells

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **45**

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Immunolgiska svar vid urogenital cancer

Christian Lundgren

Populärvetenskaplig sammanfattning

Urogenital cancer är ett stort problem i dagens samhälle och inkluderar cancer i urinblåsan, njurarna samt de manliga och kvinnliga könsorganen. Omkring 770 000 nya fall av njur- och urinblåsecancer var registrerade 2012 i världen och samma år dog runt 310 000 patienter på grund av dessa sjukdomar. Behandling av dessa sjukdomar består normalt av cellgifter eller kirurgi eller en kombination av dessa. En ny typ av behandling som fått stort intresse är immunterapi vilket baserar sig på att använda kroppens egna celler och immunförsvar för att behandla sjukdomar. Molekylerna CTLA-4 och PD-1, vilka båda har en hämmande funktion på kroppens immunsystem, har båda visat sig hjälpa tumörer att undvika immunförsvaret. Därför undersöks nu om immunterapier som går ut på att blockera dessa hämmande molekyler med hjälp av specifika antikroppar kan påverka prognosen. Blockeringen av dessa molekylar kan leda till att immunförsvaret åter igen kan attackera tumören och optimalt leda till att tumören försvinner. Dock har blockeringen av dessa molekyler visat sig ha vissa biverkningar och har i några fall resulterat i att patienter avlidit. Dessa biverkningar kommer från att dessa molekyler fyller en viktig funktion i det normala immunförsvaret.

Detta arbete beskriver effekten av blockerande antikroppar gentemot molekylerna CTLA-4 och PD-1 i kulturer med celler från patienter med njur- och urinblåsecancer. Syftet var att undersöka om någon av de blockerande antikropparna eller kombinationen av de båda skulle ha störst potential som immunterapi gentemot cancer.

Examensarbete 30 hp

Civilingenjörsprogrammet i Molekylär bioteknik

Uppsala universitet, december 2015

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Reprinted section

Section 1.2.4 is, to high degree, resembling literature written by me as part of a research training course taken at Uppsala University (Lundgren, 2015)

Abbreviations

APC	Antigen presenting cell
BCR	B cell receptor
CBA	Cytometric bead assay
CD	Cluster of differentiation
CPM	Counts per minute
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAMP	Danger-associated molecular pattern
FACS	Fluorescent-activated cell sorting
FASCIA	Flow-cytometric assay of specific cell-mediated immune response in activated whole blood
FOXP3	Forkhead Box P3
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IrAEs	Immune related adverse events
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
nSN	non-Sentinel node
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PD-1	Programmed death-1
PRR	Pattern-recognition receptors
SEB	Staphylcoccus Enterotoxin B
SN	Sentinel node
TCR	T cell receptor

Th	T helper cell
TGF	Transforming growth factor
TILs	Tumor infiltrating lymphocytes
TNF	Tumor Necrosis Factor
Treg	Regulatory T lymphocyte
TUR-B	Transurethral resection of the bladder
UBC	Urinary bladder cancer

1. Introduction

1.1. Project description

The aim of this thesis is to evaluate the effect of antibodies against the immune checkpoint molecules CTLA-4 and PD-1 when culturing cells derived from patients with urogenital cancer. These inhibiting antibodies are of interest because of their usage in immune therapy which is a treatment based on using the body's own immune system as a way to treat diseases such as cancer. However, the blockade of these specific molecules has shown some side effects and in rare cases even resulted in death. In this study we have set out to evaluate if one check-point inhibiting antibody or the combination of them has the greatest potential as immune therapy against cancer. In addition, the study aimed to get an improved insight in what might be the cause to the side effects seen with the ultimate goal of attempting to improve the efficiency and safety of this type of treatment in future patients. *In vitro* studies were performed using flow cytometry to study the effects of these antibodies.

1.2. Innate and adaptive immune system

1.2.1. The innate immune system

The immune system is comprised of two arms working together, the more primitive in the evolutionary perspective, the innate system and the more evolved adaptive system. The innate immune system is the body's first line of defense against pathogens and it is able to take care of a wide range of pathogens. The immediate defense against pathogens are the biological, chemical and physical barriers such as the epithelial cells in the skin, the gut flora, tears and mucosal surfaces. When a pathogen is able to penetrate this outer defense, the innate system quickly initiate a primary response when encountering pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). The recognition of these molecule patterns by pattern-recognition receptors (PRRs) expressed on the surface of innate immune cells leads to phagocytosis, the production of antimicrobial peptides and proteins such as interferons and cytokines, and to the activation of the complement system. The immune cells associated with the innate immune system are the monocytes, dendritic cells, macrophages, mast cells, basophils, eosinophils, neutrophils and natural killer (NK)-cells. The innate system is normally activated within minutes to hours after it encounters a pathogen.

1.2.2. The adaptive immune system

Not all pathogens are discovered and destroyed by the innate immune system due to mutations and evolutionary adaptations allowing the pathogens to evade the innate system. It is here the second arm of the immune system also known as the adaptive immune system takes over. The adaptive immune system unlike the innate immune system is highly specific towards antigens. This high specificity comes from the generation of highly random antigen receptors through rearrangement of different gene segments during the development of the cells belonging to the adaptive system. The cells belonging to this arm of the immune system are named T- and B cells and they develop

in either the thymus (T cells) or in the bone marrow (B cells). The gene rearrangement of B cell receptors (BCRs) and T cell receptors (TCRs) during their development allows for a huge amount of different receptors to be created, each unique for a different antigen. Only one type of receptor can be expressed on a single cell which means the chance of this cell encountering its unique antigen is rather small. But when an antigen is recognized by the receptor this will lead to proliferation and further survival of this cell and its antigen specificity and it will result in an immunological memory. The adaptive immune system not only differs from the innate immune system regarding its specificity but also in the time it takes for it to become effective. The amount of time it takes for the adaptive immune system to produce an effective response towards an antigen is generally a few days. Despite the difference in the pathogen recognition and the response time between the two different arms of the immune system they work in collaboration with each other. This is very important especially for the adaptive arm since before the B cells or T cells can recognize the antigens it has to be presented to them. This presentation is done by antigen presenting cells such as macrophages and dendritic cells.

1.2.3. Th1/Th2 and Th17 cell linages

The T cells, which together with the B cells represent the adaptive arm of the immune system in the body, can broadly be divided into two linages, the cytotoxic $CD8^+$ T cells and the $CD4^+$ helper T cells. Here the abbreviation CD stands for cluster of differentiation and is a protocol used for identification of cell surface molecules while the number seen behind the abbreviation represent specific molecules. The $CD8^+$ T cells are the effector cells in a cell-mediated immune response and mainly target cells that have been infected by viruses and other intracellular pathogens. $CD4^+$ T cells helps the immune system by releasing different cytokines upon activation which helps activate other immune cells. The $CD4^+$ subset can be classified further into a number of T helper (Th) subsets, with the most studied ones being the Th1/Th2 and Th17 subsets based on their cytokine production (Yamane and Paul, 2012). Th1 cells mainly produce the cytokines interferon gamma (IFN-γ) and interleukin 2 (IL-2; Interleukin are a group of cytokines and normally shortened as IL followed by a number representing the specific interleukin. Interleukin will from this point on be abbreviated as IL.) and are regulated by IL-12 through the transcription factor T-bet and are associated with the activation of cellular and pro-inflammatory responses necessary for killing intracellular pathogens (Bedoya et al., 2013; Zhang et al., 2014). The Th2 subset is characterized by the secretion of IL-4, IL-5, IL-10 and IL-13 and is regulated by the transcription factor GATA3 and results in an antibody dependent immune response towards extracellular pathogens and parasites including helminthes. The function of IL-10 production by Th2 cells are to suppress Th1 proliferation to make sure excessive pro-inflammatory responses does not lead to tissue damage (Yamane and Paul, 2012; Zhu and Paul, 2008). The third major subset is Th17 which is named from its secretion of the cytokine IL-17. Th17 cells mediate responses towards extracellular bacteria and fungi. Besides IL-17, Th17 cells also produce IL-21, IL-22 and tumor necrosis factor alpha (TNF-α; a cytokine involved in systematic inflammation and cell death) (Eyerich and Zielinski, 2014).

1.2.4. Regulatory T cells

Regulatory T cells (Tregs), another subset of the T-helper linage, are known for their ability to down regulate immune-mediated inflammation and maintaining peripheral T cell tolerance (Levings et al., 2006; Loser and Beissert, 2012). Tregs can broadly be divided into two main types; the naturally occurring Tregs (nTregs) that are developed in the thymus as part of the normal T cell maturation process and the induced Tregs (iTregs), which are generated from naïve T cells found in the periphery in an environment with a high concentration of regulatory cytokines or by interactions with nTregs. The nTregs are defined as $CD4^{+}CD25^{high}$ cells that lack the IL-7 receptor CD127 while expressing the transcription factor Forkhead Box P3 (FOXP3) (Levings et al., 2006; Liu et al., 2006; Yuan and Malek, 2012). The function of Tregs are mainly to suppress the immune system, especially T cells using a number of different mechanisms. One of these mechanisms is that they induce a cytokine-derived apoptosis in T cells by depleting the IL-2 together with many other cytokines. IL-2 is interesting because it is necessary for Tregs for their growth and proliferation but they can not produce it themselves. This leads to a surrounding depleted of IL-2 around the Tregs which results in induction of anergy (unresponsiveness to specific antigens) and apoptosis of T effector cells following TCR signaling (Pandiyan et al., 2007). Besides depleting IL-2 to cause immunosuppression Tregs can also secrete transforming growth factor beta (TGF-β) and IL-10 resulting in a modulated cytokine composition and immunosuppression (Bilate and Lafaille, 2012). Tregs not only rely on using cytokines to suppress the immune system but are also able to directly kill off other cells by the use of granzyme/perforin or the FasL molecule (Yolcu et al., 2008). One other important mechanism which is very interesting as part of this degree project is that Tregs continuously express high amounts of CTLA-4 molecules both on the surface and intracellularly (Sakaguchi et al., 2009). The mechanism behind suppression using CTLA-4 will be described further in a later chapter.

1.3. Urogenital cancer

Urogenital cancer includes cancer in the urinary bladder, kidneys and the male and female genital organs. In this thesis urinary bladder cancer patients have mainly provided material for the research done but one case of kidney cancer was included and therefore they will be the two types of cancer studied.

1.3.1 Bladder cancer

Urinary bladder cancer (UBC) is a big concern in today's society with approximately 429,800 new cases and 165,100 deaths due to the disease occurring during 2012 worldwide. Of these new cases of Urinary bladder cancer the majority occurs in men and internationally there is a high variation in the incidence rates. The highest incidence rates of this disease is in Europe, North America and Northern Africa while the lowest rates can be seen in Central America, Eastern, Middle, and Western Africa (Torre et al., 2015). Here in Sweden more than 2,700 persons got newly diagnosed with Urinary Bladder Cancer during 2013 and out of those patients 75 % were male (Socialstyrelsen, 2014). Of the UBCs approximately one-fourth is muscle invasive (stage T2, T3 and T4; T stands for the primary tumor while the numbers describe the size of the tumor and the amount of spreading) and these individuals account for around 80 % of the deaths related

to this disease (Sherif et al., 2010). Some causes to bladder cancer are smoking, urinary tract infections, STDs, chemical irritants and as seen in Egypt, Schistosomiasis. All these causes suggests that chronic inflammation plays a big role in the development of bladder cancer (Michaud, 2007; Mostafa et al., 1999).

1.3.2. Renal cancer

Kidney cancer includes renal cell carcinoma and renal pelvis carcinoma and 338,000 new cases were reported worldwide during 2012. During the same period 144,000 deaths occurred due to this disease (Ferlay et al., 2015). Some of the causes to development of renal cancer shown are urinary tract infections and viruses such as the hepatitis C virus (Alibek et al., 2012).

1.4. Tumor immune escape mechanisms

The reason we are getting cancer is that somehow the tumor cells manage to evade the immune cells that are constantly circulating the body looking for and protecting the body from cells that show cancer like properties (Dunn et al., 2004). This immune escape is the result of a number of mechanisms which includes a suppressive microenvironment, altered antigen presentation, co-inhibition and induction of tolerance towards apoptosis. The tumor releases factors into their microenvironment that inhibits innate and adoptive cells anti-tumor activity. One such factor is IL-10 that inhibits tumoricidal activity in NK-cells and granulocytes by inducing the Stat3 pathway. The induction of this pathways also seems to occur in dendritic cells (DCs) within the tumor making them tolerogenic (immunologically tolerant) and able to induce anergy in T cells. Another factor that has been associated with the tumor environment and suppression is TGF-β which is an inhibitor of cell proliferation and causes cell cycle arrest in the G1 phase (Drake et al., 2006). The suppression of T cells in the close environment around the tumor can also be done by the metabolic enzyme Indoleamine 2,3-dioxygenase (IDO) which has been shown to be expressed by tumor cells. IDO is a key enzyme that helps with catabolism of the amino acid tryptophan. This depletion of tryptophan and creation of tryptophan metabolites has a negative effect on proliferation, survivability and activity of T cells and the metabolites also have the possibility to induce apoptosis in lymphocytes. IDO has also shown to be able to enhance the Treg phenotype in $CD4^+$ T cells which can help suppress the immune system (Godin-Ethier et al., 2011). It is widely known that tumors differ from normal cells in their antigenic composition mainly due to their genetic instability. To avoid that these different antigens get presented to immune cells and activate them, the tumor cells downregulate some mechanisms behind antigen processing especially the pathways associated with major histocompatibility complex (MHC) class I. The most common way for the tumor to downregulate MHC class I is through mutation or deletion of β2-microglobulin genes (Drake et al., 2006; Rabinovich et al., 2007). One other way for the tumor to suppress the immune system is through the expression of co-inhibitory molecules such as CTLA-4 and PD-1's ligand PD-L1 which both have the ability to suppress cells through a number of mechanisms that will be explained more in detail in later chapters (Contardi et al., 2005; Kim et al., 2013). The programmed cell death by apoptosis works as a natural barrier towards cancer

development but the tumor cells have evolved a variety of strategies to avoid this. These include increased expression of antiapoptotic regulators and survival signals and the downregulation of proapoptotic signals (Hanahan and Weinberg, 2011). Recruitment and/or induction of Tregs by the tumor could also be a possible way for the tumor to evade the immune system.

1.5. Immune checkpoints

Immune checkpoints refers to a number of pathways that are crucial for the maintenance of self-tolerance (in other words, preventing autoimmunity) and to protect the tissue from damage during immune responses to pathogenic infections. Two checkpoints pathways that has got a lot of attention with regards to cancer immunity are the CTLA-4 and PD-1 pathways.

1.5.1. CTLA-4

The cytotoxic T lymphocyte antigen 4 (CTLA-4) or CD152 as it also is known as is a member of the immunoglobulin superfamily. CTLA-4 has a high homology to the costimulatory receptor CD28 and like this receptor binds to the ligands CD80 (B7-1) and CD86 (B7-2). But where CD28 function is to bind CD80/CD86 to provide the second costimulatory signal that together with the primary signal from the binding of the TCR to its specific antigen allows the activation of the T cell, CTLA-4 binding to CD80/CD86 results in suppression of the T cell. CTLA-4 is not found on resting T cells or even recently activated T cell but is primarily found after 48 hours on activated T cells and the expression seems to be higher in $CD4^+$ T cells and Tregs compared to $CD8^+$ T cells (Chan et al., 2014; Murakami and Riella, 2014). Presynthesized CTLA-4 molecules are stored inside intracellular vesicles and upon activation transported to the cell surface of the activated T cell. This transport of CTLA-4 to the surface is not completely without restriction and is regulated by a number of signal molecules (Linsley et al., 1996). The CTLA-4 molecule has a number of suggested mechanisms by how it allows for suppression but the main one is that it outcompetes the CD28 molecule. CTLA-4 binds CD80/CD86 with a higher affinity than CD28 and this difference in affinity has been reported to be 500 to 2500 times higher in CTLA-4 compared to CD28. This difference in affinity results in that CD28 is not able to bind CD80/CD86 and thus the T cell does not receive the second costimulatory signal necessary for activation of the cell (Figure 1) (Wolchok and Saenger, 2008). Another suggested mechanisms for how CTLA-4 inhibits activation of T cells is that CTLA-4 delivers a negative signal through its cytoplasmic tail that inhibits TCR and/or CD28 signaling. There has also been suggested that by binding B7 on antigen presenting cells (APCs) during the antigen presentation it up-regulates the catabolizing enzyme IDO, resulting in the depletion of tryptophan in its microenvironment and inhibition of T cell responses. The CTLA-4 is continuously expressed on Tregs and is further expressed as its TCR binds its antigen, similar to effector T cells and is one of the ways by which Tregs keep the immune system under control (Murakami and Riella, 2014; Tai et al., 2012).

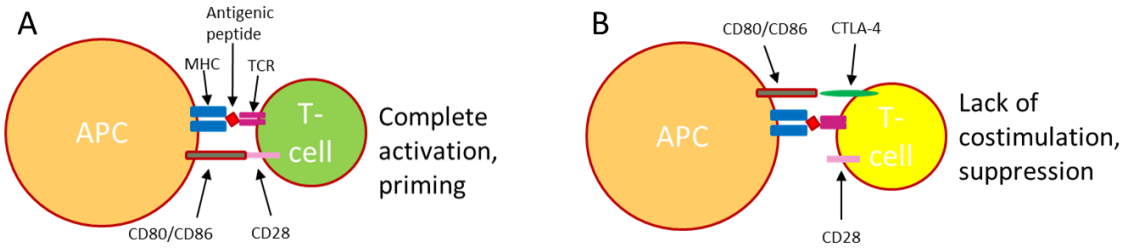


Figure 1: *The "outcompeting" mechanism of CTLA-4.* ***(A)*** *The T cell receives its primary signal between the TCR and its antigen presented by the APC and its secondary costimulatory signal from CD28 binding to CD80/CD86 thus resulting in complete activation of the T cell.* ***(B)*** *Here CTLA-4 has bound to CD80/CD86. Despite receiving the primary signal the T cell is not able to receive the secondary co-stimulatory signal resulting in the suppression of the T cell.*

1.5.2. PD-1

PD-1 (programmed death-1), or CD279, is like the CTLA-4 molecule a member of the immunoglobulin superfamily and shares homology with CD28. PD-1 is a type 1 transmembrane protein containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail and is inducibly expressed on T cells, B cells, monocytes and dendritic cells upon activation. The PD-1 molecule has 2 ligands it can bind, the programmed death ligand-1 (PD-L1 also known as CD274) and programmed death ligand-2 (PD-L2 or CD273) (Latchman et al., 2001). As the name suggest the molecule is associated with programmed cell death (apoptosis) and it has been shown that PD-1 expression on activated T cells results in T cell death. This programmed cell death is initiated by interaction with apoptotic genes. One of these genes is the anti-apoptotic Bcl-xL gene which during normal T cell activation is expressed. PD-1 inhibits the expression of this gene by inhibition of the enzyme PI3K which is essential for the up-regulation of Bcl-xL. PD-1 mainly acts on activated T cell in contrast to CTLA-4 that primarily regulates the earlier stages of the T cell activation. The expression of PD-L1 has besides on activated immune cells such as DCs, natural killer (NK) cells, T cells, B cells, macrophages and monocytes also as mentioned earlier been seen on human tumor cells where it is either continuously expressed or induced. Some of the cancers where it has been seen expressed are renal, bladder, urothelial, cervical and breast (Ostrand-Rosenberg et al., 2014). The expression of the PD-L1 is induced by a number of molecules like IL-10, IL-4, IFNs, TNF-α, lipopolysaccharide (LPS), granulocyte-macrophage colony-stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF) with INF-γ being the most effective inducer (Ostrand-Rosenberg et al., 2014; Sznol and Chen, 2013). As with the CTLA-4 molecule there is a number of different mechanisms by how PD-1 and PD-L1 manage to suppress T cells. The binding of the ligand to PD-1 blocks downstream signals triggered by the antigen presented on MHC to the TCR and the secondary costimulation signal through CD28 and CD80/CD86. This results in an impaired T cell activation and IL-2 production. One way that it impairs the TCR signal is by reducing the phosphorylation of the tyrosine kinase ZAP70 which is necessary for signaling through the TCR through activation of

downstream adapter molecules and enzymes. The ligation to its ligands also prevents the phosphorylation of PKC-θ, which is essential for the production of IL-2 and the activation of Smad3 by PD-1 and makes the T cell stop in the G_1 phase of its cell cycle thus preventing further proliferation (Sheppard et al., 2004). The binding between PD-1 and its ligands also results in a down-regulation of TCR on T cells. Normally during T cell activation TCR are down-regulated and this is mediated by the E3 ubiquitin-protein ligase Cbl-b. The ligation leads to an increase in Cbl-b resulting in a down-regulation of the TCR (Karwacz et al., 2011). The PD-1 pathway also suppresses immune responses by increasing the amount of Tregs. This is done by APCs expressing PD-L1 which induces natural Tregs in the thymus and converts peripherally naïve $CD4^+$ T cells to inducible Tregs. Also mature Th1 $CD4^+$ T cells can be converted to $FOXP3^+$ Tregs by engagement of the PD-1 molecule (Ostrand-Rosenberg et al., 2014).

1.5.3. CTLA-4 and PD-1 in clinical trials

Blocking antibodies against the CTLA-4 and PD-1 pathways have been used in a number of clinical studies ranging from phase 1 to phase 3 studies. These studies have been targeting several different cancers such as advanced melanoma, bladder cancer, renal cell cancer, gastric cancer, and head and neck cancer. In a number of these studies the use of a single blocking antibody towards either the CTLA-4 pathway or PD-1 pathway has shown clear anti-tumor response thus showing the possibilities with this type of treatment (Callahan et al., 2015). Of the PD-1 blocking antibodies, the ones that has progressed the furthest in clinical trials and showed most success are nivolumab and pembrolizumab which both have shown clinical activity (Philips and Atkins, 2015). When it comes to PD-1 pathway blocking antibodies there is also the possibility of blocking the PD-1 binding ligand PD-L1, which also has been done in some clinical studies. The idea behind blocking the ligand instead of the receptor is that it might lead to less toxicity since it would lead to a more selectively modulation of the immune response in the tumor microenvironment. Two examples of blocking antibodies towards PD-L1 that has been tested in clinical studies for treatment of solid cancers are MEDI4736 and MPDL3280a which both have shown positive results (Philips and Atkins, 2015). For the CTLA-4 pathway two blocking antibodies targeting CTLA-4, ipilimumab and tremelimumab, managed to enter the clinical trials based on preclinical studies. Both of these showed good results during the early studies but tremelimumab did not provide a statistically significant improvement of the overall survivability. Ipilimumab on the other hand was able to improve the overall survivability in patients with advanced melanoma leading to its approval by the US Food and Drug Administration (FDA) together with the anti-PD-1 antibody pembrolizumab as part of the treatment for advanced melanoma (Callahan et al., 2015; Postow et al., 2015). To improve the number of patients that will benefit from the immune checkpoint blockade the CTLA-4 and PD-1 blocking antibodies are being combined with each other or in combination with other therapies such as chemotherapy, radiotherapy and other types of immunotherapies. The usage of blocking antibodies has shown promising results but it has also been shown that blocking these pathways can lead to some irAEs and even death since these pathways are part of the normal immune system and the use of the antibodies might disrupt its normal function (Postow et al., 2015). Examples of irAEs are dermatologic, hepatic, endocrine and other inflammatory events but the most relevant consists of diarrhea/colitis which seems more common in CTLA-4 blockade than PD-1

blockade and has even resulted in some treatment related deaths (Postow et al., 2015; Sznol and Chen, 2013).

1.6. Surgical techniques for bladder cancer

The material from Urinary Bladder cancer patients used for this degree project has come from two types of surgical operations, either a transurethral resection of the bladder (TUR-B) or from a cystectomy.

1.6.1. TUR-B

During the TUR-B, peripheral blood and tumor samples were received together with microscopically healthy bladder tissue from the patient. When it was possible different transurethral resection samples were taken from the tumor. When possible different samples taken were obtained from the central part (CP), transitional zone (TZ) and invasive front (IF) and their positioning on the tumor can be seen in Figure 2.

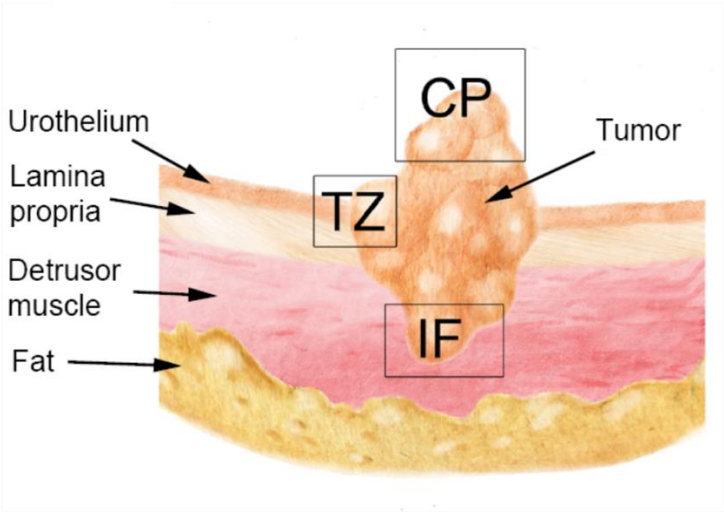


Figure 2: *The positioning of the different transurethral resection samples possible to get during a TUR-B. Central Part (CP), Transitional zone (TZ) and Invasive Front (IF). Picture used with permission of creator, Malin E. Winerdal.*

1.6.2. Cystectomy and sentinel node detection

Some of the patients that undergo TUR-Bs will have muscle invasive bladder cancer and will therefore proceed to cystectomy as part of their treatment. A cystectomy means that part or all of the urinary bladder is surgically removed. As part of these cystectomies the patients also went through sentinel node detection according to Sherif et al., (2001). The concept of sentinel node is that lymphatic drainage from a primary tumor first passes through a specific regional lymph node called the sentinel node (SN) and thereafter continue to other lymph nodes. These sentinel nodes are important because they work as a primary site for interaction between the tumor and the immune system allowing for expansion of tumor specific effector cells. In short the procedure involves the patient being injected with a radioactive tracer divided into 4 equal parts into 4 different

locations surrounding the tumor or residual tumor base. After the bladder is removed a handheld Geiger meter is used for detecting the lymph nodes. The nodes found during operation are carefully dissected from the operation area allowing for optimal control regarding if they are sentinel nodes or non-sentinel nodes. Based on the individual radioactivity, measured in counts per minute (CPM), of the nodes they are either defined as sentinel or non-sentinel lymph nodes. The CPM of each node is registered and the node is divided in two, with one piece going to the lab and the other part being send for pathological analysis.

1.7. Markers used for staining of cells

1.7.1. Viability dye

The function of the viability dye is to be able to distinguish between dead and living cells during the fluorescent-activated cell sorting (FACS). The principle behind this dye is that living cells will react with the reactive dye but only on the surface which will result in cells with a weak fluorescent signal when running them through the FACS machine. The cells with a compromised membrane (dead cells) will react with the dye throughout their whole volume which will result in a higher fluorescent signal than for the living cells.

1.7.2. CD45

PTPRC (protein tyrosine phosphatase receptor type C) also more known as CD45 or leukocyte common antigen (LCF) is a transmembrane protein expressed on all nucleated cells belonging to the hematopoietic linage except erythrocytes and platelets (Hermiston et al., 2003; Tchilian and Beverley, 2006). This receptor has an essential role in the antigen receptor signal transduction and the activation of T- and B cells. It has also been shown to modulate signals coming from integrins and cytokine receptors (Hermiston et al., 2003, p. 45; Jacobsen et al., 2002). CD45 occurs in 8 different isoforms, which are all generated based on the alternative splicing of its exons numbered 4, 5 and 6 (Jacobsen et al., 2002). One of these isoforms is termed CD45RO and it is also one of the markers used for this experiment and it will be explained more in detail below.

1.7.3. CD3

CD3 (cluster of differentiation 3) is a T cell co-receptor belonging to the immunoglobulin superfamily and it consist of 4 different chains (CD3γ, δ, ε, and ζ) and forms a complex with the TCR and helps with its intracellular assembly and surface expression (Dave, 2009). The CD3 chains together forms two different signaling modules where the CD3γε and CD3δε heterodimers forms one module and the CD3ζζ homodimer represents the second (Kuhns and Badgandi, 2012). These signaling modules allow the TCR to transduce signals that result in thymocyte differentiation and the generation of mature T lymphocytes (Dave, 2009). In these panels the CD3 marker is used to differentiate T lymphocytes from other leukocytes separated identified by the CD45 gating.

1.7.4. CD4

CD4 is a transmembrane protein found expressed on most thymocytes, one of the two major T cells linages (helper T cells), monocytes, macrophages and dendritic cells (Cameron et al., 1992; Ellmeier et al., 2013). The function of the CD4 molecule in monocytes and macrophages is not well understood but its function in T cells has been widely described. The function of CD4 in T cells is that of a coreceptor and helps the TCR with signaling with APCs. The CD4 corecepetor specifically helps with the binding to MHC-II molecules on APCs. Besides helping with the binding to MHC-II molecules the CD4 molecule also helps with the activation of the signal cascade following TCR stimulation. This is done with the help of lymphocyte cell kinase (lck). The lck interacts with the cytoplasmic tail of the CD4 molecule and during MHC binding it is brought close to the TCR allowing for phosphorylation of ITAMs and further downstream signaling (Egawa, 2015; Nyakeriga et al., 2012).

1.7.5. CD8

CD8 is like CD4 expressed on most thymocytes but instead it is expressed on the second major T cell linage, the cytotoxic T cells (Ellmeier et al., 2013). The CD8 molecule can also be found on dendritic cells (Qiu et al., 2009). CD8 is also a transmembrane protein but it is composed of two subunits, α and β, that form a heterodimer (Hennecke and Cosson, 1993). CD8 specifically binds to MHC-I molecules. Like CD4, CD8 is a coreceptor helping with the TCR signaling and uses the same lck pathway to help with the signaling (Egawa, 2015; Nyakeriga et al., 2012).

1.7.6. FOXP3

Forkhead box protein 3 (FOXP3) is one out of four transcription factors belonging to the FOXP family and it is important for the development and function of Tregs (Ma et al., 2013; Wu et al., 2006). FOXP3 regulates the development of the Tregs by repressing and trans-activating specific genes but the mechanisms behind these regulations are not well defined. What is known is that during the repression of the genes some of the interactions involve histone acetyltransferases, histone deacetylases and histone linkers (McMurchy et al., 2013).

1.7.7. HLA-DR

In humans, MHC molecules are referred to as human leukocyte antigen (HLA) and the MHC class II molecule HLA-DR is the most highly expressed isoform belonging to this family (Wilkinson et al., 2012). The function of HLA-DR is to present antigen peptides to T-helper cells (Kanakoudi-Tsakalidou et al., 2001).

1.7.8. CTLA-4

The CTLA-4 molecule is described in detail in section 1.5.1.

1.7.9. CD69

The very early activation marker CD69 is a transmembrane protein belonging to the C type lectin superfamily and one of the earliest molecules expressed on activated T- and

B cells. CD69 acts as a costimulatory molecule resulting in proliferation, secretion and cytotoxicity (Borrego et al., 1999).

1.7.10. CD45RO

CD45RO is one of the 8 isoforms that CD45 can occur in and works as a marker for memory T cells. CD45RO is the isoform with the lowest molecular weight since it lacks the exons 4-6. This lack results in that CD45RO more effectively supports signal transduction and activation compared with the other isoforms containing any of the exons 4-6 (Jacobsen et al., 2002).

2. Material and methods

2.1. Preparing material from TUR-Bs

The tumor material from TUR-Bs are directly transported from the site of operation to the lab located at Karolinska Hospital, Solna in 50 ml plastic tubes containing 10 ml RPMI 1640 medium and are kept cold with cooling blocks through the whole transport. The blood from the same patient (taken during the operation) is transported in 9 ml tubes containing heparin and unlike the tumor samples kept in room temperature during transport to the lab. At the arrival at the lab the material is immediately prepared for isolation of cells and tumor homogenate.

2.1.1. Tumor/non-tumor:

First the tumor or the non-tumor was divided into two parts in a petri dish under sterile conditions. During this procedure a GentleMACs was used, which is an instrument used for automated tissue dissociation and homogenization.

Isolation of tumor infiltrating lymphocytes:

1. One part of the tumor was cut (IF,TZ and CP are all treated separately) into small pieces and put into a GentleMACS tube together with 9.9 mL of AIM-V medium and 100 µL collagenase.
2. GentleMACS (C-tube) program hTumor cycle 1 was used and then the tubes were incubated in 37ºC for 1h. The tubes were inverted every 15 min.
3. After the incubation the GentleMACS cycle 2 was used and the tubes were once again incubated for 1h in 37ºC and inverted every 15 min.
4. The GentleMACS cycle 3 was started and the cell suspension was filtered through a 40 µm cell strainer into a 50 ml test tube.
5. The tube was centrifuged at 300xg for 10 min and the supernatant was removed and the cells resuspened in 5 mL AIM-V medium
6. Finally the cells were counted.

To stimulate the cells during the culturing, tumor homogenate was used to activate tumor specific cells.

Tumor homogenate (for FASCIA setup):

1. Part 2 of the tumor was weighted (here TZ, IF and CP are considered as one and thus weighed together) and 5 volumes (weight/volume) of 2x PBS was added.
2. The tumor was homogenized with an Ultra Turrax, which is a dispersing instrument.
3. The homogenate was boiled for 5-15 min in water bath and used immediately after or stored in a -20ºC freezer (The homogenate stored in the freezer was used during culturing material received from cystectomies)

The boiling step of the tumor homogenate procedure were there because this protocol originated from making tumor homogenate from colorectal tumors. Since in these cancers the risk was big to get contamination from *Escherichia coli* boiling was necessary when making tumor homogenate. During this degree project it has also been tried to not boil the tumor homogenate when the amount of tumor sample has been great to try to compare the two different homogenates during culture.

2.1.2 Blood

As mentioned previously during the TUR-B the group also receives heparin tubes with peripheral blood from which peripheral blood mononuclear cells (PBMCs) was extracted.

1. 2 tubes were spun at 300xg for 10 min
2. The plasma layer was removed from these tubes and transferred to a new tube and stored in a -20ºC freezer (This plasma is used for experiments not associated with this degree project)
3. Remaining samples were then pooled with the remaining blood from the heparin tubes in a 1:1 ratio with room temperatured PBS
4. This diluted blood was layered onto Ficoll-Paque PLUS (GE Healthcare) and centrifuged at 400xg for 30 min at the lowest acceleration and without brakes.
5. The visible layer containing PBMC was collected and transferred to a new tube. The PBMC was then washed with 1x PBS and centrifuge at 400xg for 10 min.
6. Supernatant was removed and cells were resuspended in 10 mL AIM-V medium before counting them.

2.2. Preparing material from cystectomies

As with the TUR-Bs the material from the cystectomies was also transported directly from the location of the operation to the lab at Karolinska Hospital, Solna. The patients that had undergone cystectomies had already undergone a TUR-B and therefore normally did not have any tumor left to be sent back to the lab. The lymph nodes were transported in 15 ml plastic tubes containing 5 mL pure RPMI 1640 medium while possible normal tissue and tumor were transported in 50 mL plastic tubes with 10 mL pure RPMI 1640 medium. The blood was, like during TUR-Bs, transported in 9 ml blood tubes containing heparin.

2.2.1 Lymph nodes

The lymph nodes were all treated separately during the extraction of cells but could afterwards be pooled if necessary. If the nodes were pooled, cells from sentinel nodes and non-sentinel nodes were not pooled together since they have different properties and tumor response.

1. Lymph nodes were put in a petri dish and if possible the fat from it was removed.
2. The lymph node was transferred to a 100 µm cell strainer inside another petri dish and 5 mL AIM-V medium was added.

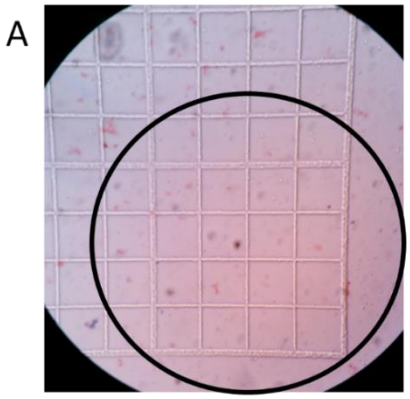
3. Homogenization of the lymph node through the cell strainer was performed using the backside of a syringe.
4. The medium containing the cells from the homogenized lymph nodes was transferred to a tube and if it seemed necessary an additional 5 mL AIM-V medium was added.
5. Counting of the cells then took place and the sample was centrifuged at 300xg for 10 min.

2.2.2 Blood

The blood samples from the cystectomies were processed exactly according to the procedure seen in chapter 2.1.2.

2.3. Counting cells

All the cells extracted from the different materials were counted microscopically using a normal light microscope. Trypan blue was added to a small sample taken from the extracted cells and then transferred to a cell counting slide. The counting slide is divided into 10 A-squares in a 2 by 5 formation. Each A-square is further divided into 16 squares in a 4 by 4 pattern (see Figure 3A). During the counting of the cells 3 A-squares were counted (see Figure 3B and 3C) and the average of these 3 squares were used for determining the amount of cells in the sample.



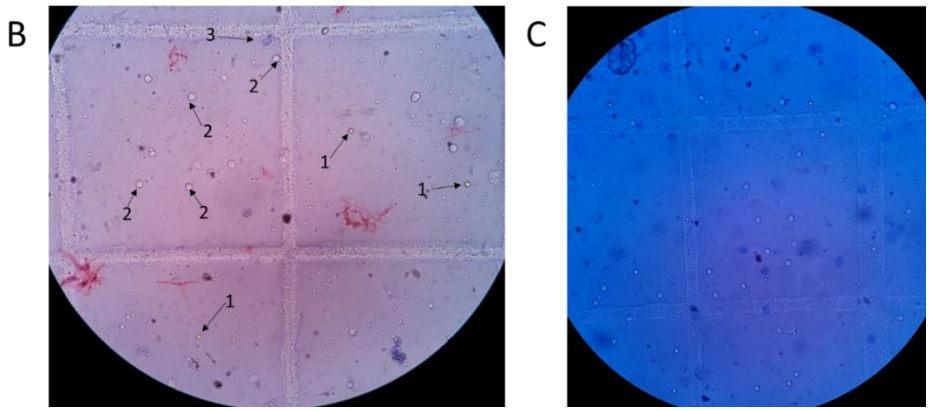


Figure 3: ***(A)*** *Counting plate at 10x magnification with the black circle showing what is called an A-square. The A-square is surrounded by a wider border and further divided into 16 smaller squares allowing for easier counting.* ***(B)*** *PBMC at 40x magnification and the numbers seen represent different cell types. The number 1 shows the location of red blood cells which when looking through the microscope has a yellow color and are normally smaller than the lymphocytes and monocytes. '2' marks some of the mononuclear cells that will be counted to determinate the cell concentration. The blue cell marked with the number 3 is a dead cell that has been colored blue by the trypan blue.* ***(C)*** *Cells isolated from a SN at 40x magnification.*

The following equation was used to calculate the concentration of the cells in the samples

$$C\left(\frac{cells}{ml}\right) = \left(\frac{A_1 + A_2 + A_3}{3}\right) \times dilution \times 10000 \quad (1)$$

Here C represents the final concentration, A_x represents the A-square numbered x and dilution means the dilution of the sample that occurred with the addition of trypan blue to it (for example 75 µL trypan blue was added to 25 µL sample resulting in a dilution of 4).

To get the total amount of cells the concentration was multiplied with the volume in mL that the cells were suspended in.

2.4. Setting up cultures

The major part of this Degree project is based on culturing cells with different combinations of the anti-CTLA-4 blocking antibody and the anti-PD-1 blocking antibody. As a stimuli for these cultures the tumor homogenate made from TUR-Bs were used or SEB in the case of the positive control. After counting the isolated cells they were either diluted or concentrated to a final concentration of 5 x 10^6 cells.

Then culturing of cells has been taking place in 96-well plates where one plate was used for a single type of material such as blood, SNs or nSNs (non-Sentinel nodes). If possible a single SN or nSN was cultured on a single plate but pooled SNs or nSNs could also be plated but seperatly. The amount of cells cultured in a single well were 0.5 x 10^6 cells (equal to 100 µL of the final cell concentrations). To these wells either IL-2, IL-12, SEB, α-CTLA-4, α-PD-1 or a combination of these were added. In the case of negative controls only AIM-V medium was added to the cell culture.

The combination of these molecules to the 0.5 x 10^6 cells were as following:

1. Medium
2. Medium + IL-2
3. Tumor + αCTLA-4
4. Tumor + αPD-1
5. Tumor + αCTLA-4 + αPD-1

6. Tumor + IL-2 + IL-12
7. Tumor + IL-2 + IL-12 + αCTLA-4 + αPD-1
8. Tumor + IL-2 + αCTLA-4 + αPD-1
9. SEB
10. Tumor boiled
11. Tumor unboiled (if enough tumor was received to be able to make unboiled tumor homogenate)

The final concentrations of αCTLA-4 and αPD-1 in the cultures were 10 µg/mL and for IL-2 10 ng/mL. The IL-12 concentration in the wells were 1/100 of the concentration it was stored at. The SEB concentration in the positive control was set to 5 ng/mL. For the tumor homogenate a volume of 2 µL were added to each well where it was needed. AIM-V medium was then added to the wells so that the final volume in each well was 200 µL. In Figure 4 an example of a plate setup can be seen. The empty wells were filled with 200 µL PBS and the plates were put into an incubator at 37ºC and 6% CO_2 for 7 days.

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B		1	9	11	13	15	17	19	24	26	28		p1
C		2						20					p2
D		3	10	12	14	16	18	21	25	27	29		p3
E		4	7 (0)					22					p4
F		5									30		p5
G		6	8 (0 fix)		32 (p1)	33 (p3)	34 (p5)	23 (0 fix)			31		p6
H													

Medium
Medium + IL-2
Tumor + αCTLA-4
Tumor + αPD-1
Tumor + αCTLA-4 + αPD-1
Tumor + IL-2 + IL-12
Tumor + IL-2 + IL-12 + αCTLA-4 + αPD-1
Tumor + IL-2 + αCTLA-4 + αPD-1
SEB
Tumor Boiled
Tumor Unboiled

Figure 4: *An example of the culture setup using a 96-well plate. On the plate each color represents one of the different stimulation setups used during the culturing. A list explaining each color can be seen below the plate setup. The p1-p6 seen to the right of the plate setup represent different antibody panels that will be used during the FACS staining of the cultures on day 7. The wells containing "0" or "0 fix" are the cultures that will be used as controls during the staining and FACS analysis. The wells represented by 0 will be controls for the extracellular staining while the ones marked with 0 fix will be controls for the intracellular staining. The numbers seen ranging from 1 to 34 are there to help distinguish between the different samples when they are transferred to FACS tubes which also have numbers ranging from 1 to 34. In this way each tube is associated with a specific well on the plate.*

2.5. FACS staining

Analysis of all the samples, both at day 0 and 7, during this degree project has been done using the FACS machine BD LSRFortessa.

2.5.1. FACS staining in tubes

For the day 0 staining of the different samples 0.5 x 10^6 cells from each sample were transferred to separate FACS tubes to allow for easier staining.

1. First 2 ml of PBS were added to the cells and they were centrifuged at 300xg for 5 min and the supernatant discarded.
2. The pellets were resuspended and dissolved in 1000 µL Live/Dead viability dye blue (diluted 1:1000 in PBS). They were then incubated at 4ºC and dark for 30 min before washed with 2 mL PBS.
3. Once again the tubes were centrifuged at 300xg for 5 min, the supernatant discarded and pellets dissolved.
4. The addition of 100 µL antibody master mix to each tube was done according to what cells had be stained. For the control tubes only 100 µL PBS was added. All tubes were then incubated for 30 min at 4ºC and dark.
5. 2 mL of PBS were then added to each tube and they were centrifuge for 5 min at 300xg. The supernatant was discarded and the pellets dissolved.
6. If only surface staining was to be done: the cells were dissolved in 300 µL PBS to be ready for FACS analysis.
   For intracellular staining the pellet was dissolved in 1000 µL Fixation/Permeabilization solution (1 part concentrate was diluted with 3 parts diluent, eBioscience). The cells were incubated for 60 min at 4ºC and dark while vortexing the tubes every 30 min.
7. The cells were centrifugated for 5 min at 300xg and the supernatant discarded. After discarding the supernatant the pellets were dissolved and washed with 2 mL 1x Permabilization buffer (eBioscience, Diluted from 10x with Milli-Q water).
8. The cells were centrifugated again at 300xg for 5 min and the supernatant discarded. The pellets were dissolved and 100 µL of intracellular staining antibodies were added (Either a Foxp3 antibody or an Isotype control antibody diluted in 1x Permabilization buffer. To the fixation control tube only 100 µL 1x Permabilization buffer was added). The cells were incubated at 4ºC and darkness for 30 min.
9. 2 mL of 1x Permabilization buffer were added to each tube and they were centrifugated for 5 min at 300xg. The supernatant was discarded and the pellets dissolved.
10. 2 mL of 1x Permabilization buffer were used to wash the cells. The cells were centrifugated for 5 min at 300xg and the supernatant discarded.
11. The pellets were dissolved in 300 µL PBS/tube and were ready for FACS analysis

2.5.2. FACS staining in 96-well plates

The staining of the 96-well plate differed a little bit from the staining of cells in tubes because the wells only could contain a certain amount of liquid and that the supernatant from the cultures needed to be kept for CBA analysis.

1. The plates were centrifuges at 300xg for 5 min and the supernatant from each well belonging to panel 1 was transferred to 1.5 mL Eppendorf tubes and stored in the -20ºC freezer until CDB analysis could be done
2. The pellets were loosened by gently tapping the sides of the plate. The cells were dissolved in 200 µL Live/Dead viability dye blue (diluted 1:1000 in PBS) and incubated at 4ºC and dark for 30 min. The wells were then washed with 200 µL PBS.
3. The plate was centrifuged at 300xg for 5 min and the supernatant discarded. As with the previous step the plate was tapped on the sides to be able to dissolve the pellets and later being washed with 200 µL PBS.
4. Once again the plates were centrifuged at 300xg for 5 min and the supernatant discarded.100 µL of the appropriate antibody master mix was added to each tube according to what cells were to be stained. For the control tubes only 100 µL PBS was added instead of an antibody solution. After the addition of the antibodies the plates were incubated for 30 min at 4ºC and dark.
5. After the incubation 100 µL of PBS was added to each well and the plates centrifuged for 5 min at 300xg. As normal the supernatant was discarded and the plate tapped on the sides to loosen the pellets.
6. The plate was washed again with 200 µL of PBS and centrifuged for 5 min at 300xg. The supernatant was removed and the pellets dissolved again.
7. If only surface staining was to be done on a well the cells were dissolved in 175 µL PBS and transferred to FACS tubes and an additional 150-200 µL of PBS was added to each tube for it to be ready for FACS analysis.
   For doing the intracellular staining in a well the pellets were dissolved in 200 µL Fixation/Permeabilization solution (dilute 1 part concentrate with 3 parts diluent, eBioscience) and the plate incubated 60 min at 4ºC and dark while tapping the sides every 30 min.
8. The plate was then centrifuged for 5 min at 300xg. After discarding the supernatant and tapping on the sides the wells were washed with 200 µL 1x Permabilization buffer (eBioscience, Diluted from 10x with Milli-Q water).
9. The plate was centrifuged at 300xg for 5 min and the supernatant discarded. After loosening the pellets by taping the sides 100 µL of intracellular staining antibodies (Either FOXP3 antibody or Isotype control antibody diluted in 1x Permabilization buffer) was added to the appropriate well. For wells with the fixation control sample, only 100 µL of 1x Permabilization buffer was added. After the addition of the intracellular antibodies the plates were allowed to incubate at 4ºC and dark for 30 min.
10. An additional 100 µL of 1x Permabilization buffer was then added to each well. After the centrifugation for 5 min at 300xg and discarding of supernatant as well

as loosening the pellets. This step was then repeated once more but with the addition of 200 µL 1x Permabilization buffer to each well instead of 100 µL

11. The cells were then transferred to FACS tubes by dissolving the pellets in 175 µL of PBS. When transferred to tubes, an additional 150-200 µL of PBS was added to each tube to make them ready for FACS analysis.

To each of the FACS tubes, before they were analyzed 10 µL (equal to 10000 beads) of counting beads were added. This was to allow for the evaluation of the total number of cells in each tube and thus the cells in the culture by dividing the total amount of beads added by the amount of beads that got analyzed together with the cells in the FACS machine. This multiplier could then be used to determine the total amount of each cell population in the tube by multiplying it with the number of cells that were actually analyzed. The data accumulated from the FACS analysis was analyzed using the software FlowJo (FlowJo.com, 2015) and the gating strategies used for separating the different cell populations from each other can be seen in supplement 1. The gating strategies consist of separating different populations within the sample based on their different properties. Some examples of such differences could be size of the cell, its internal complexity as well as the expression of certain markers. All data presented in this degree project was done using the software GraphPad Prism (GraphPad.com, 2015).

2.5.3. FACS antibodies and fluorophores

The antibodies with their respective fluorophore used for staining the cells for FACS analysis at both day 0 and after 7 days of culturing can be seen in Table 1. The mouse IgG2b κ and IgG1 κ antibodies are isotype controls for the antibodies with the same fluorophores as the isotypes.

Table 1: *Antibodies with their respective fluorophore used for staining of cells for FACS analysis. Contains both intracellular and extracellular antibodies.*

Antibody	Fluorophore	Antibody	Fluorophore
Viability dye	Blue (life tech)	FOXP3	Alexa Fluor 647
CD4	Pacific blue	CD3	Alexa Fluor 700
CD45	Horizon V500	CD45RO	APC-Cy-7
CD25	Brilliant violet 605	CD8	APC
HLA-DR	FITC	Mouse IgG2b κ	FITC
CTLA-4	PE	Mouse IgG1 κ	PE
CD127	PE-CF594	Mouse IgG1 κ	PerCp-eFluor710
CD39	PerCp-eFluor710	Mouse IgG1 κ	Alexa Fluor 647
CD69	PE-Cy7		

2.6. CBA Human Th1/Th2/Th17 Cytokine analysis

Besides looking at the cell composition using antibodies towards specific markers on the surface and intra cellular, the supernatant from the cell cultures has been used to look at differences in cytokine composition. The CBA Human Th1/Th2/Th17 Cytokine kit (BD) used for this analysis could measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF), Interferon-γ (IFN-γ), and Interleukin-17A (IL-17A) protein concentration in the samples and give the concentration in pg/mL. The procedure for the CBA analysis was done according to the manufacturer's instruction delivered together with the CBA Human Th1/Th2/Th17 Cytokine kit. In short the procedure consisted of making a series of dilutions ranging from 0 to 5000 pg/mL of Th1/Th2/Th17 Cytokine Standard to create a standard curve for each cytokine. These dilutions were added to tubes containing 50 µL mixed Capture beads. 50 µL of each unknown sample were also transferred to properly labeled tubes containing 50 µL mixed Capture beads. To all tubes 50 µL of human Th1/Th2/Th17 PE Detection reagent was added. The tubes were then incubated at room temperature for 3 hours and washed with Wash buffer. The tubes were centrifuged and the supernatant carefully aspirated and discarded from each tube. 300 µL Wash buffer was added to each tube and the tubes were ready for flowcytometric analysis. To differentiate between the different Capture beads they all have a different fluorophore intensity resulting in 7 different peaks when looking at histograms where the Y-axis represents counts and the X-axis the intensity in the APC channel. The capturing beads intensity starting from the lowest to the highest were IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A. The amount of each cytokine could then be calculated by the intensity of PE seen on each peak when making a dot plot with intensity of APC on the Y-axis and intensity of PE on the X-axis. The median intensity of PE could be put in the equation received from its respective standard curve and be converted to a concentration in the form of pg/mL.

3. Results and discussion

3.1. FOXP3 in cell cultures

For the cultures we used cells received from TUR-Bs and cystectomies which leads to a problem when analyzing them. What can not be done during the analysis is to group data from both types of operations together and treat them as one group. This is because sometimes the patients that undergo cystectomies will have received chemotherapy before they do the operation which can have an effect on the immune system (Hu et al., 2013). To see if there actually was a difference between the samples received from TUR-Bs and cystectomies from UBC, the $CD4^+FOXP3^+/CD4^+$ ratio in PBMC (blood is the only sample that is received from both type of operations) from both types of surgery were plotted next to each other and can be seen in Figure 5. By studying the $CD4^+FOXP3^+/CD4^+$ ratio it is possible to see the percentage of $CD4^+FOXP3^+$ cells, which can be defined as Tregs, in the total population of $CD4^+$ cells in order to see if the amount of Tregs changes between the different samples.

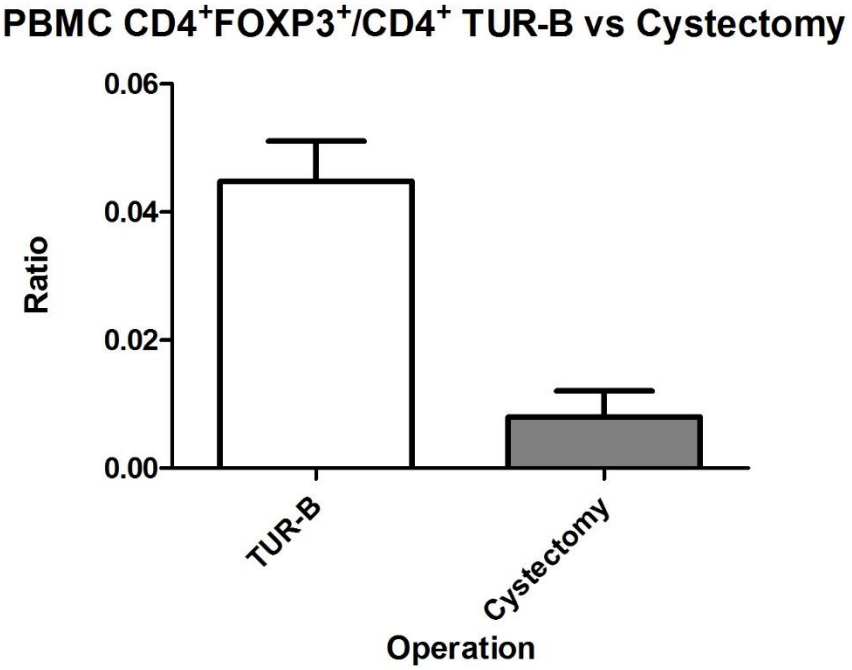


Figure 5: *Proportion of $CD4^+FOXP3^+$ from $CD4^+$in PBMC from either TUR-Bs or cystectomies. The error bars seen above each bar represent the error, or uncertainty in the shown measurement. The more the original data values differs from the mean value shown (both above and below), the wider the error bars will be.*

Here it can be seen that there seems to be a difference between the proportions when first looking at the PBMC taken from different operations and that the ratio of $CD4^+FOXP3^+$ cells seems to be higher in the TUR-Bs. The problem with looking at ratios is the question; why have the proportion changed? Did the amount of $CD4^+FOXP3^+$ cells decrease or did maybe the amount of $CD4^+$ cells increase. The patients that underwent cystectomies and from whom material was given to this degree

project all had received chemotherapy before the surgery. Regarding the change in $CD4^+$ $FOXP3^+/CD4^+$ ratio there has been suggested that in cases of advanced esophageal cancer some chemotherapies inhibit the expression of FOXP3 at transcriptional levels (Xu et al., 2011). Since FOXP3 is the key regulatory factor of Treg development, the decreased FOXP3 mRNA expression inhibits the proliferation of Tregs, leading to a decreased number of Treg cells in the blood. Some cytostatic drugs also targets actively dividing cells and Tregs are known to undergo rapid turnover at steady state compared to other T cell subsets. This could lead to that the cytotoxic drugs selectively depletes the Tregs due to their increased proliferation resulting in the decrease of the proportion between $CD4^+$ $FOXP3^+$ cells and $CD4^+$ cells seen in the blood taken during cystectomies.

During day 0 analyzing this proportion of $CD4^+$ $FOXP3^+$ was also looked at in the different locations that were possible to receive from operation. The nephrectomy was the only operation where both blood, lymph nodes and tumor was taken at the same time and in this patient there could be seen that the proportion of Tregs clearly was higher in TILs compared with both PBMC and SNs (Figure 6A). Since there only was one patient no statistical analysis could be done but this increase has been demonstrated earlier in UBC patients (Winerdal et al, 2015). From UBC material TILs were only able to be isolated from material coming from TUR-Bs so it was only able to compare between PBMC and TILs (Figure 6B). There is a significant difference in the proportions of $CD4^+$ $FOXP3^+$ between the PBMC and the TILS. This increase may be due to tumor recruitment or induction of Tregs in its surrounding as a way to evade the immune system.

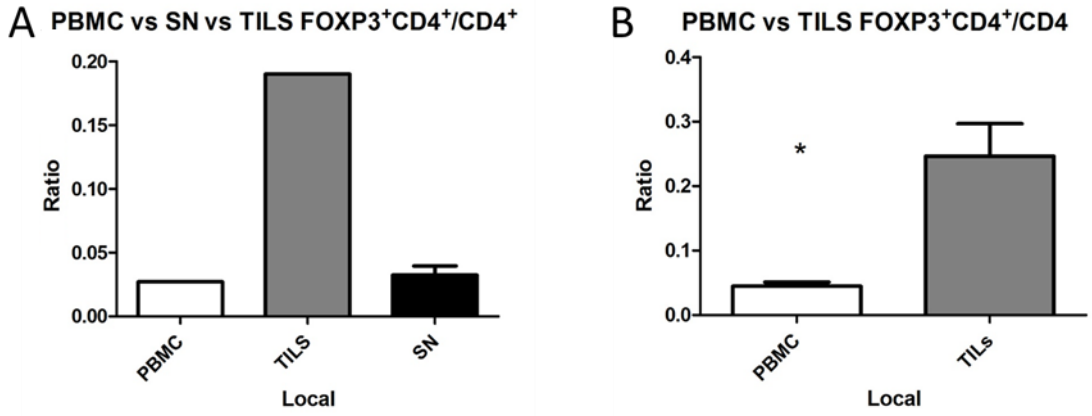


Figure 6: *Bar diagrams showing the proportion of $CD4^+$ $FOXP3^+$cells among $CD4^+$ T cells in different locals from either **(A)** nephrectomy (renal cancer) or **(B)** Tur-B (bladder cancer). The PBMC and TILs sacks error bars since there is only one data sample for each local.*

The main part of this degree project has been the culturing of cells from different locals together with CTLA-4 blockade antibodies, PD-1 blockade antibodies or the combination of them both to see if they have different effect on the cells since the CTLA-4 and PD-1 uses different pathways to suppress the immune system normally in the body. When looking at cultures with cells from either PBMC or lymphnodes from UBC

patients (see Figure 7A and 7B respectively) with regard of the proportion of $CD4^+$ $FOXP3^+$ cells from the total $CD4^+$ T cell population tit seems like there is no difference between the different culture setups in PBMC.

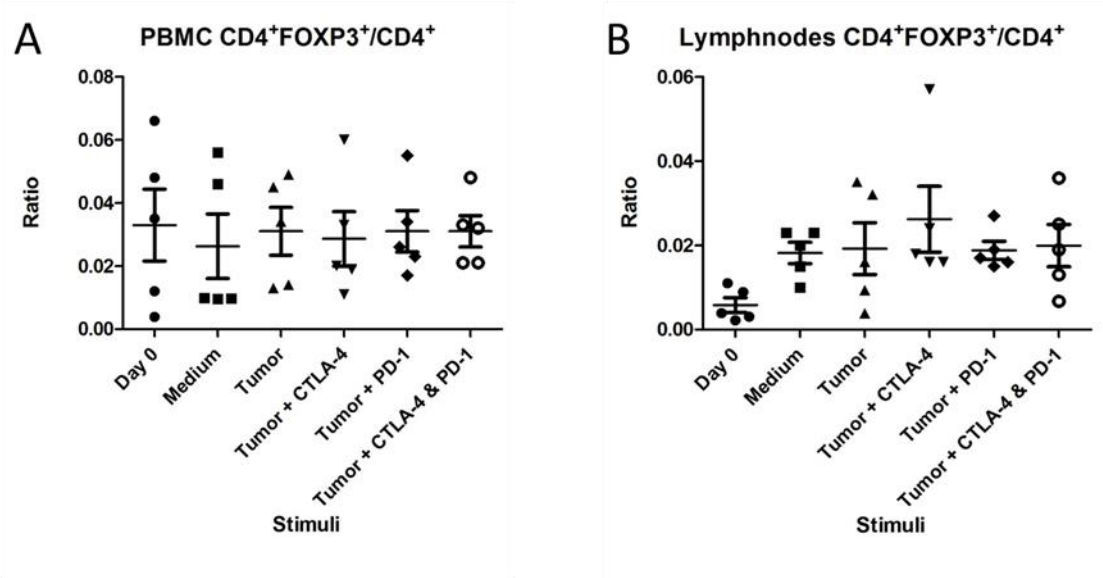


Figure 7: ***(A)*** *Proportion of $CD4^+$ $FOXP3^+$cells of $CD4^+$cells seen in PBMC at day 0 or 7 days of culturing. In the error bars seen the line in the middle shows the mean value of the original data values which have all been plotted. The difference seen in width of the error bars represent how much the original values differs from the mean values.* ***(B)*** *Proportion of $CD4^+$ $FOXP3^+$cells of $CD4^+$cells seen in cells isolated from lymphnodes on day 0 or from day 7 cultures.*

Even though there is a big variety within each culture setup these variations seem to be the same when comparing the different culture setups. The high variety seen in PBMC is mainly because of the high heterogeneity seen when working with human material and especially with cancer patients. So even though there is a variation in proportion within the same culture setup these are consistent between the different setups, so the patients with high proportion of $CD4^+$ $FOXP3^+$ cells in cultures with only medium also have high proportions in the rest of the culture setups. The cultures with cells isolated from the lymphnodes also do not seem to show any difference between the different culture setups except for one of the sentinel nodes. In the culture done on cells isolated from one sentinel node it can be seen that the proportion of the $CD4^+$ $FOXP3^+$ cells is clearly higher in the culture where besides tumor homogenate also the CTLA-4 blocking antibody was added compared to the other culture setups. A slight increase can also be found in the culture with the addition of both types of blocking antibodies while the addition of only the PD-1 blocking antibody does not result in any difference compared with the addition of only tumor homogenate or medium. So in other words the cultures with the addition of the CTLA-4 blocking antibody seem to at least for this specific sentinel node result in a higher proportion of $CD4^+$ $FOXP3^+$ cells.

To see if the reason for these higher proportions seen in the cultures with CTLA-4 blockade was due to an increase in the actual numbers of the Tregs in the culture, the total count of $CD4^+$ $FOXP3^+$ cells was plotted for each culture (Figure 8).

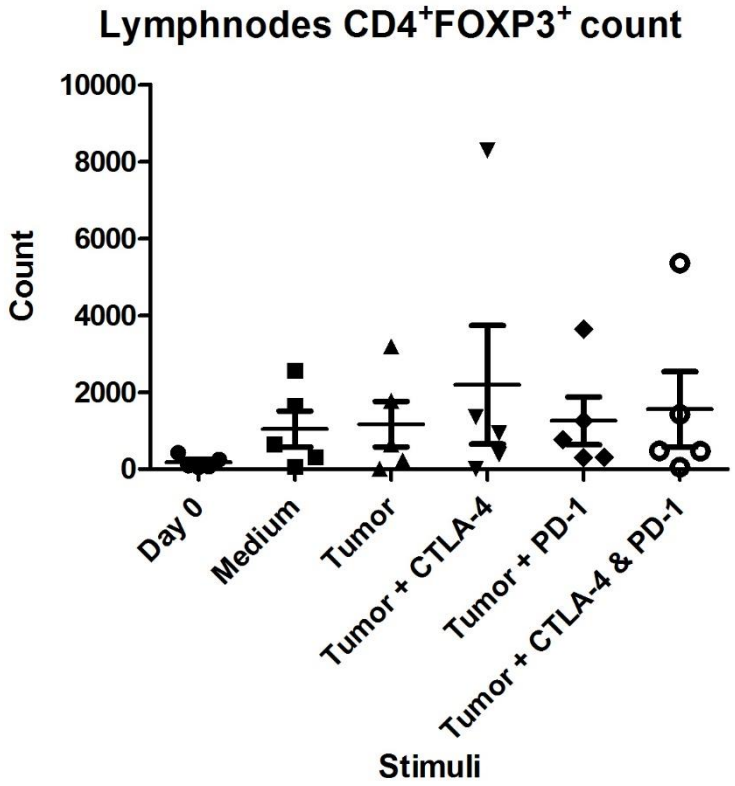


Figure 8: *Total amount of $CD4^+$ $FOXP3^+$ cells in each culture. Total count seen on the Y-axis while the stimulation for the cultures can be seen on the X-axis.*

Once again there does not seem to be any clear difference between the different stimulations used for culturing if looking at all the lymphnodes. But as with the plot showing the proportions of $CD4^+$ $FOXP3^+$ cells there are some cultures that differs from the rest. When looking through the data it showed that also these points in the plots came from the cultures with cells isolated from the same sentinel node that showed a difference in the plot with $CD4^+$ $FOXP3^+$ proportions. Here it can be seen that the total number of Tregs has increased in the cultures containing CTLA-4 compared with the rest of the cultures. It also shows that the increase is higher in the culture which only received the CTLA-4 blocking antibody compared with the culture that got both CTLA-4 and PD-1 blocking antibodies. This concludes that the differences seen in the cultures with cells from this SN was mainly due to an increase in the number of total Tregs in the cultures with CTLA-4. Since this is only one SN from one patient not much can be said about the actual function of the CTLA-4 blocking antibody and if it increases the amount of Tregs. To draw a conclusion regarding this, further cultures would need to be set up. Here also the heterogeneity associated with human material and cancer also provides a problem. If these results would be seen in other samples that could show that CTLA-4 blocking antibodies increases the amount of Tregs which could be an

explanation to why there seems to be an higher increase of immune related adverse events in patients treated with CTLA-4 blockade compared with patients treated with PD-1 blockade, and why there sometimes is severe adverse events as a result of the blockade treatment.

Besides looking at the proportion of $CD4^+$ $FOXP3^+$ in the cultures I also looked more directly on the $CD4^+$ $FOXP3^+$ population with regards to the expression of CD45RO, CD69, HLA-DR and CTLA-4 on them to see if any of these markers differed between the different cultures. By looking at the expression of these molecules it is possible to see the amount of activated cells since all of these molecules work as markers for activated cells. Looking at the expression of CD45RO also allows the detection of memory T cells. The expression of these markers was measured in the form of mean fluorescence intensity (MFI) which despite its name actually is the median of the fluorescent intensity seen for each marker. Looking at these markers there were no differences between the culture setups. Since no differences was seen when looking at all lymph nodes as one local we decided to see it there were any difference between SNs and nSNs since these can have different immunological responses when cultured. No clear differences were seen when looking at the MFI for the CD45RO, HLA-DR and CTLA-4 expression. The only trend seen was that the MFI of the early activation marker CD69 was increased in the cultures with cells isolated from SNs compared with cells isolated from nSNs (Figure 9). Since this difference was seen even in the cells cultured with only medium it does not seem to be a result of culturing with tumor homogenate. Instead it could be a result of the properties of the different types of nodes *in vivo* or that something was transferred with the cells to the cultures when isolating the cells which explains the differences. The reason CD69 MFI is higher at day 0 compared with day 7 for nSNs could be the results of that day 0 shows a snapshot of how the cells looks *in vivo* under normal conditions. Since these cells are cultured *in vitro* they will not have access to all the normal molecules and signals that they get *in vivo*.

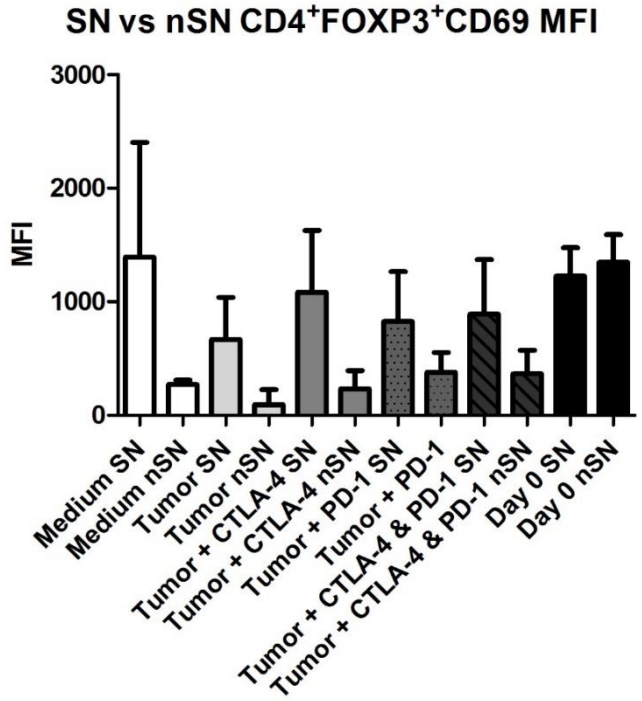


Figure 9: *Differences in MFI for CD69 between cells isolated from SNs and nSNs and then cultured for 7 days with different stimuli.*

3.2. $CD4^+$ T cells in cultures

Since both CTLA-4 and PD-1 are associated with T cell suppression I also looked at $CD4^+$ and $CD8^+$ T cells in the different cultures. No differences could be seen between the different cultures when looking at the ratio between $CD4^+$ and $CD8^+$ T cells when looking at cultures derived from PBMC. Because some variation had been seen in the lymphnodes when looking at FOXP3 it was decided to look more into the lymphnodes. When looking at the $CD4^+$ /$CD8^+$ ratio in the lymphocyte population of cells isolated from lymphnodes no difference was seen between the cultures (Figure 10A). When instead looking at the blasting population there seems to be an increase in the ratio of $CD4^+$ cells when CTLA-4 was added to the culture (Figure 10B). One reason for this change could be that the CTLA-4 blockade activates $CD4^+$ cells more than $CD8^+$ cells but there could also be that since there is normally more $CD4^+$ cells than $CD8^+$ cells there just happens to be by chance that more $CD4^+$ cells get activated. Since no significant difference was seen it can not be told if these differences seen is a result of the blockade or that it is only the result of heterogeneity and these patients just happen to be affected by the blockade.

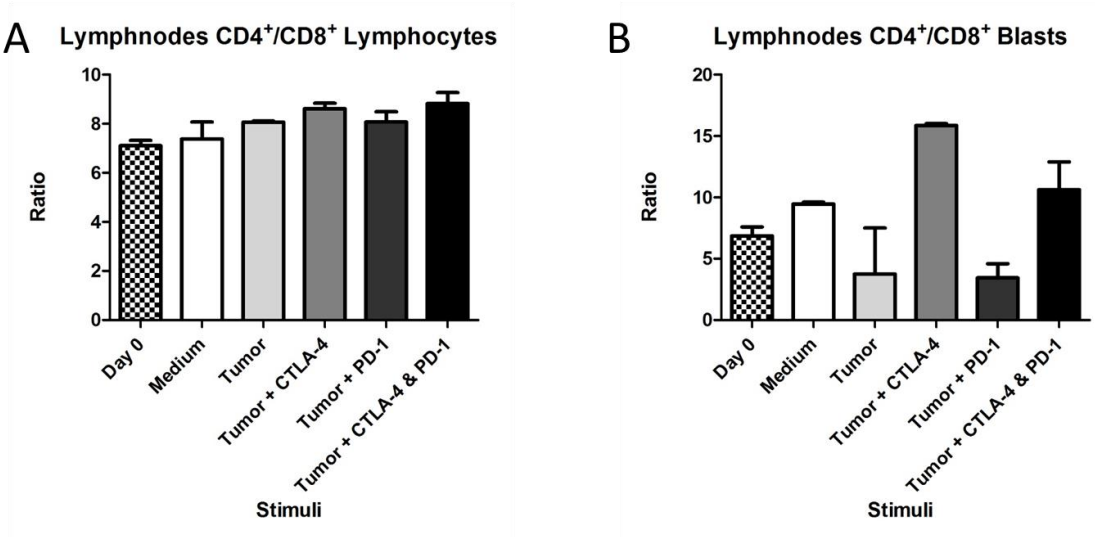


Figure 10: *The ratio between $CD4^+$T cells and $CD8^+$ T cells seen in the **(A)** lymphocyte population and **(B)** blasting population.*

Similar to the FOXP3 population in lymphnodes we decided to look at the MFI of CD69, CD45RO and HLA-DR in the $CD4^+$ population and compare them between SNs and nSNs. No consistent trends or differences were seen when looking at CD45RO or CD69 but when looking at the MFI for HLA-DR it seems to show a trend of increased MFI in the sentinel nodes compared with the non-sentinel nodes (Figure 11). This difference does not seem to be a result of the different culture setups since this increase can be seen in all of them.

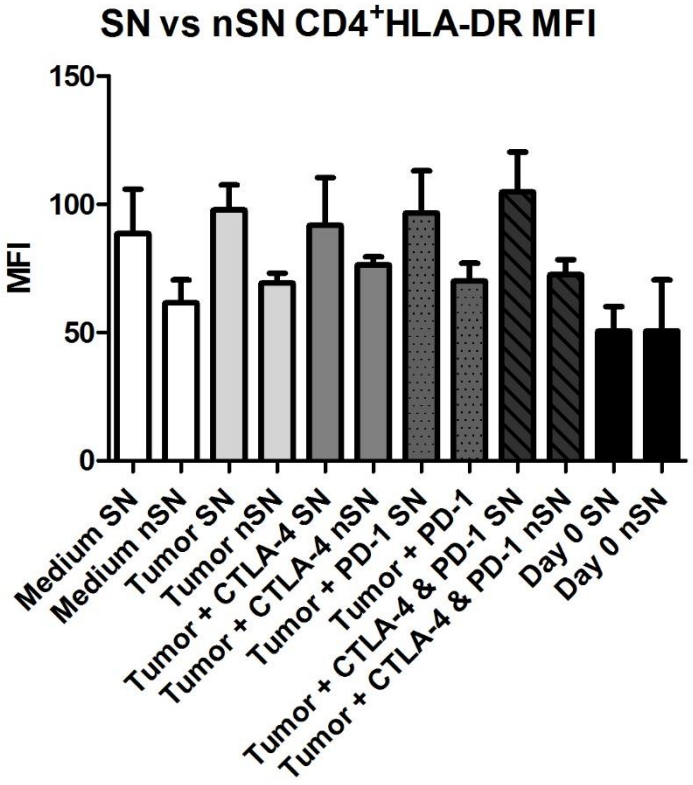


Figure 11: *MFI for the HLA-DR antibody on $CD4^+$ T cells isolated from either SNs or nSNs after culturing for 7 days.*

3.3. CBA

The CBA analysis of the supernatant from the cell cultures was mainly done to be able to see if the addition of the blockade antibodies led to any skewing towards the Th1, Th2 or Th17 cell linages based on the detection of different cytokines seen in the supernatant. From the analysis data of the supernatants no significant differences were seen when it came to any of the different cytokines when comparing the cultures with cells extracted from lymph nodes and grown together with only medium, boiled tumor homogenate, boiled tumor homogenate together with CTLA-4 blocking antibodies, boiled tumor homogenate together with PD-1 blocking antibodies or boiled tumor homogenate with both CTLA-4 and PD-1 blocking antibodies. This suggest that the addition of the blocking antibodies does not lead to any shift of the cells towards either cell linage. In most of the cultures no cytokine production was seen at all except for IL-6 where it seems that the addition of tumor to the cells leads to an increase in IL-6 production (not-significant) compared with the cells only cultured in medium.

3.4. Ethical discussion

Research with material derived from human patients makes it necessary to take into account some ethical concerns about participant safety, informed consent, and confidentiality. Concerns about participant safety could be that the operations would be prolonged but due to the little amount of sample that was taken from the patients participating in this research the prolonged operation time was minimal. The sentinel node detection also did not involve any specific risks. All participants had also been informed about the overall plan about the research, the purpose of the research as well as possible risks with the research. There should also be known that all participation was voluntarily and could be cancelled at all times by the participant. Regarding concerns about confidentiality all participants' personal information was coded in order to achieve anonymity and only a selected few had access to the codekey.

4. Conclusions

Not many conclusions can be drawn from the data analyzed and presented here in this degree project. It seems that there is a possibility that in some patients the CTLA-4 blocking antibody could result in an increase of Tregs in lymph nodes. But to actually see if the addition of the different blocking antibodies had any effect on the cell populations and if these could explain why some people treated with these antibodies as part of their cancer treatment get some severe side effects it will be necessary to perform more cultures with material from new patients. If possible, it would also be interesting to do these type of cultures on TILs since these are the cells that are actually in the direct environment of the tumor and should be the cells that are the most specific towards the tumor cells and also the cells that are being the most suppressed. There could also be some interest in trying to extend the culturing time since most patients that receive these types of blocking antibodies as treatment for their cancer normally get injection of these multiple times during a long period and the IrAEs normally takes some time to manifest.

5. Acknowledgement

I would first of all like to thank my supervisor Ola Winqvist for arranging this interesting degree project and allowing me to take part of it and for taking his time to share his vast knowledge of immunology. I would also like to thank Magnus Essand for taking his time and agreeing to become the subject reviewer for this project. Further I would like to thank the main surgeon Amir Sherif for taking a chance with me and introducing me to Ola and his group almost a year ago and for allowing me to see the actual operations. I also wants to thank Ciputra, Emma, Ali, Malin and David, the people from Ola's group that I have mainly been working together with on the UBC material and the renal cancer. Ludvig, Kurt, Lu, Jin and the rest of Ola's group for interesting talks and discussions and helping the time just fly away.

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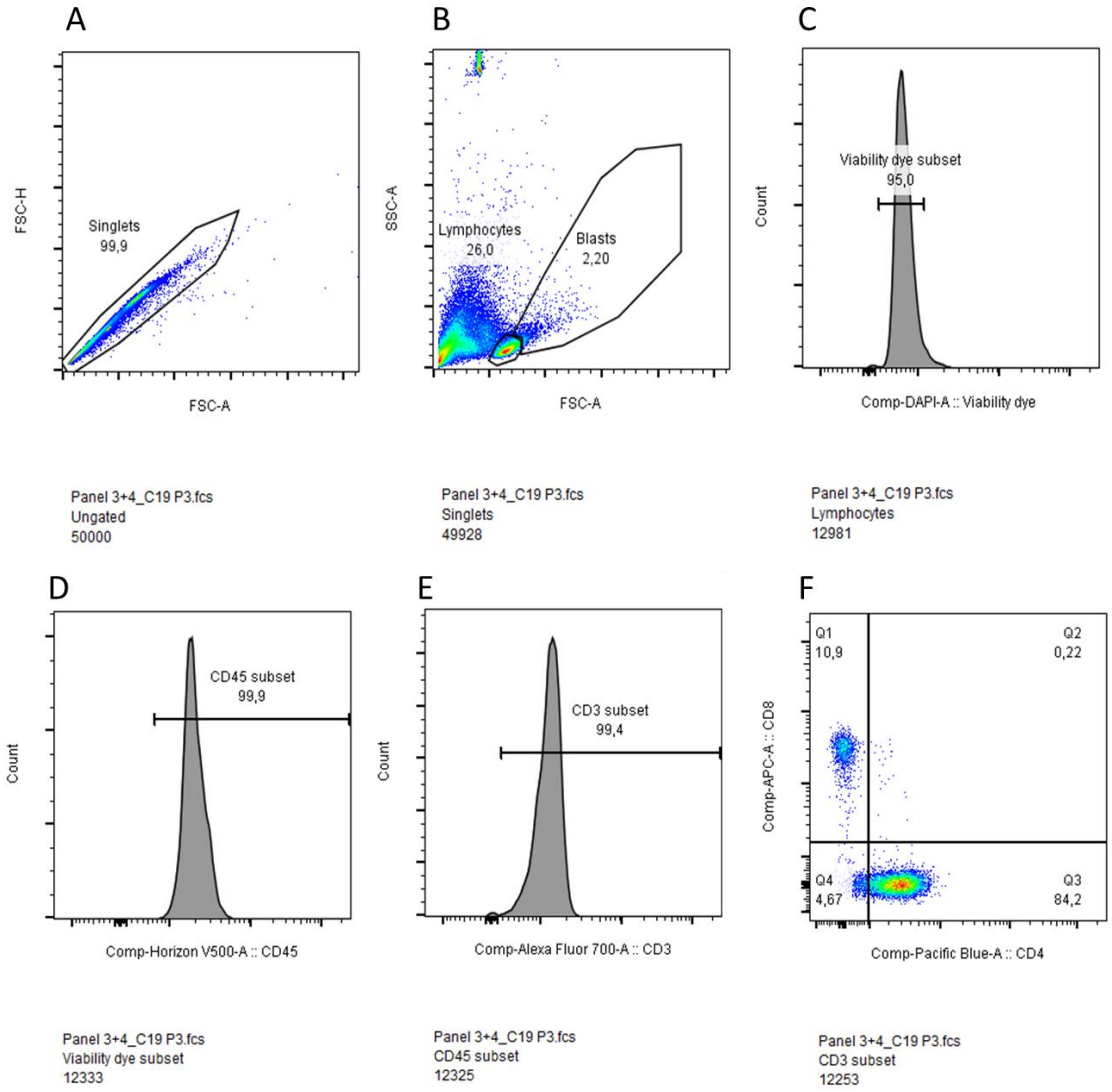
7. Supplements

Supplement 1: Gating strategies for extracellular and intracellular panels



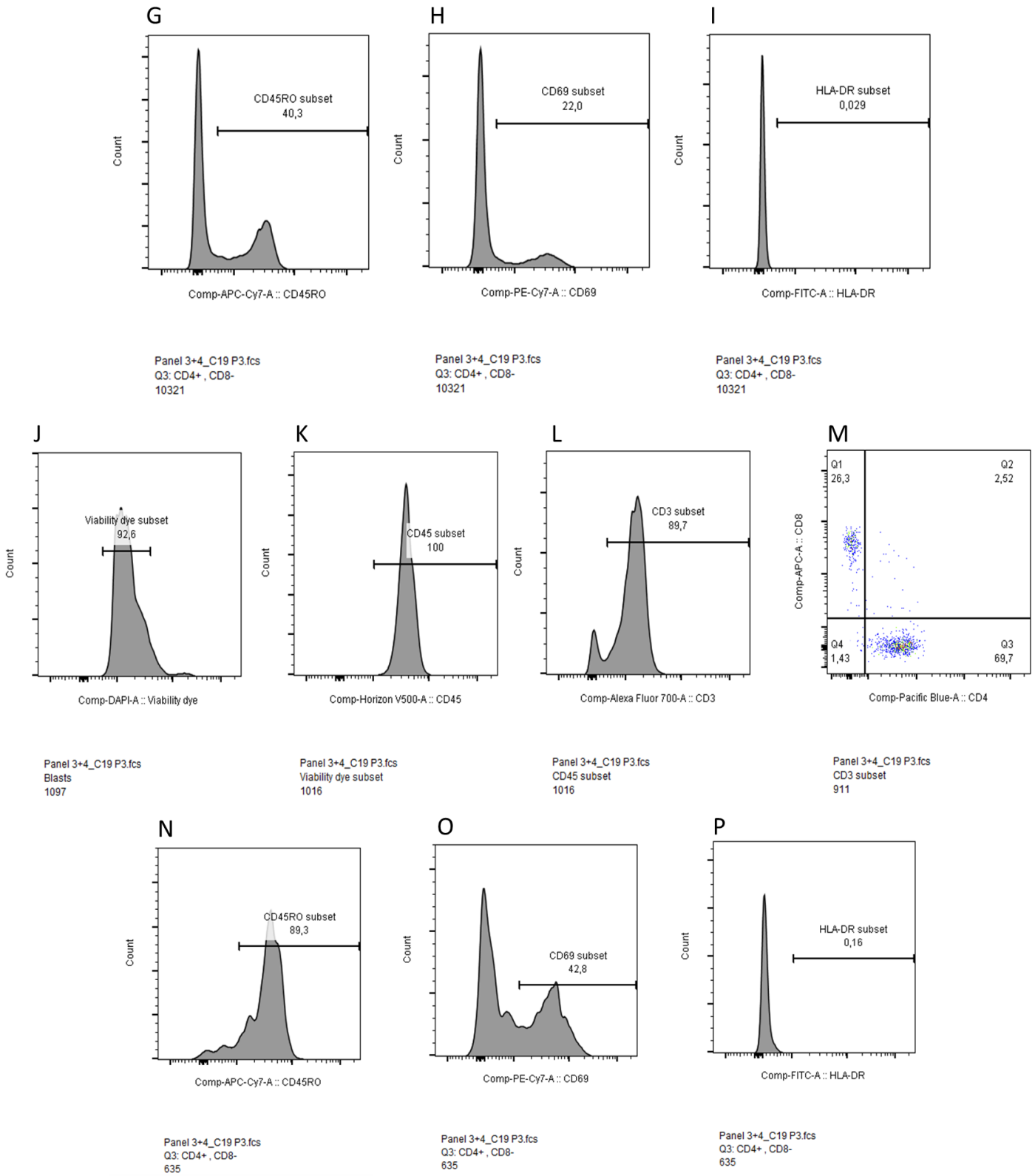
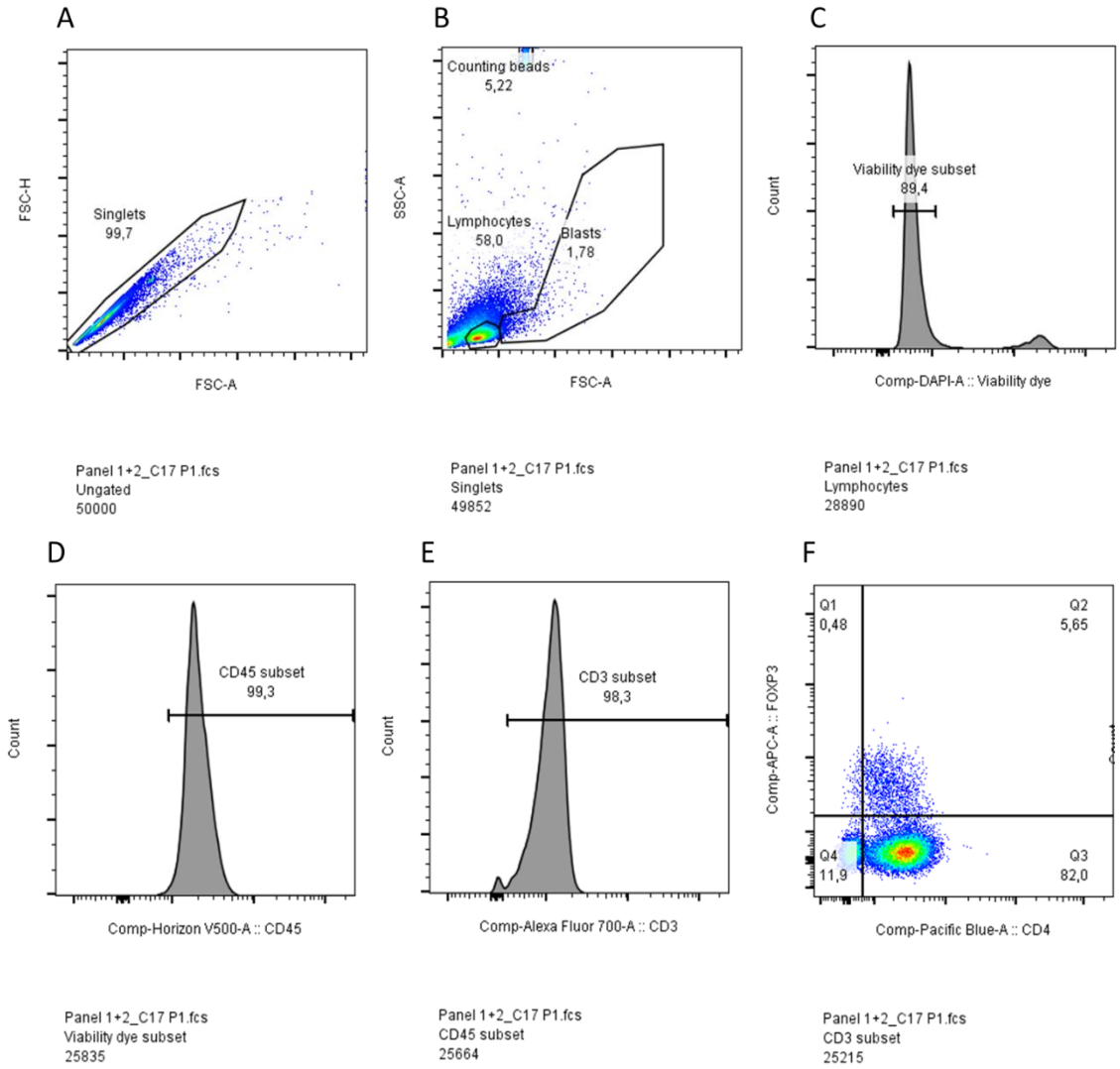


Figure 12: ***Extracellular panel (A)*** *In order to separate single cells from doublets the cells are gated based on their forward scatter (size) in terms of height and area.* ***(B)*** *During this gating step the lymphocyte and blasting population are separated out from the debris and other cell types such as granulocytes and neutrophils. This gating is*

done based on forward scatter and side scatter (internal complexity). The population seen in the upper left corner consist of the counting beads. ***(C)-(E)*** *The viability, CD45 and CD3 subsets for the lymphocyte population. Each gating is done on the subset gated previously, so the CD45 subset is gated from the selected cells from the viability dye gating and the CD3 subset is then selected from the CD45 subset.* ***(F)*** *Here the $CD4^+$ subset is separated from the $CD8^+$ subset as well as any cells not expression either of these markers.* ***(G)-(I)*** *Gating strategies for CD45RO, CD69 and HLA-DR subsets respectively. Each gating is done on the $CD4^+$subset selected in (F).* ***(J)-(P)*** *The gating done in these figures are done the same as the gating seen in the figures (C)-(I) with the difference that these subsets exist within the blast population seen in figure (B) instead of the lymphocyte population.*



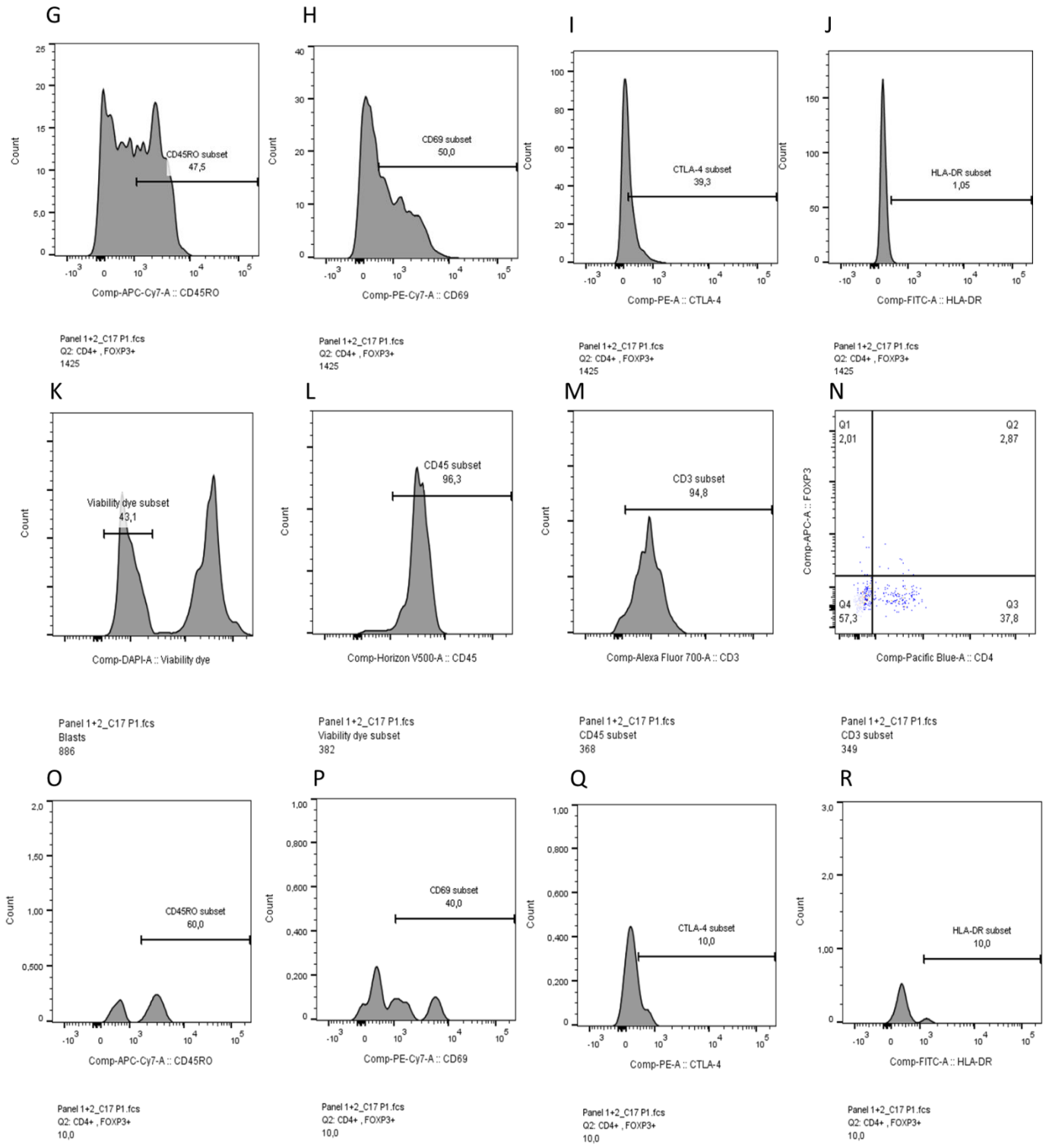


Figure 13: ***Intracellular panel (A)*** *Gating for retrieval of singlet subset.* ***(B)*** *Separating the lymphocyte and blast subsets from debris and unwanted cells.* ***(C)-(E)*** *Sorting out the viable, CD45 and CD3 subsets respectively from the lymphocyte population as described in figure 12.* ***(F)*** *Using the intracellular marker FOXP3 and the extracellular marker CD4 to find the $FOXP3^{+}CD4^{+}$ (Treg) subset. This subset is located in the upper right quadrant.* ***(G)-(J)*** *Gating for the CD45RO, CD69, CTLA-4 and HLA-DR subsets individually from the $FOXP3^{+}CD4^{+}$ subset.* ***(K)-(R)*** *Same gating as seen in (C)-(J) but from the blast subset seen in (B)*