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B LYMPHOCYTES IN SOLID HUMAN MALIGNANCIES

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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There are no incurable diseases, only the lack of will.

There are no worthless herbs, only the lack of knowledge.

-*Avicenna*

To my beloved grandfather, Mohammadreza Najafi (R.I.P.); uncle, Nasir Zirakzadeh (R.I.P.); and to my family.

ABSTRACT

Cancer remains one of the leading causes of death worldwide. Modern treatments such as immunotherapy and neoadjuvant chemotherapy exhibit promising results. However better understanding of the treatments may lead to optimizations and consequently better prognosis.

In this thesis, we begin to categorize subpopulations of B cells in patients with malignancies. We demonstrate increased proportions of plasmablasts, switched memory and $CD86^+$ B cells in tumor associated tissues. Noticeable skewing of Igλ/Igκ ratio of tumor infiltrating B lymphocytes (TIL-Bs) indicates tumor specific B cell expansion. Monoclonal expansion is confirmed by spectrartype analysis and sequencing the clonally expanded heavy chain, reveals somatic hypermutation. We conclude that B cells in cancer patients, display signs of $CD4^+$ T cell dependent response against cancer.

Furthermore, we investigate the effect of three conventional chemotherapeutic drugs on human B cells. We expose increased CD86 expression on B cells upon doxorubicin treatment which explains their escalated antigen presenting ability. In addition, doxorubicin results in decreased production of the cytokines, IL-10 and TNF-α. Investigation of B cells in urinary bladder cancer patients treated with neoadjuvant chemotherapy containing doxorubicin, reveals increased CD86 expression. Thus we argue, optimization of time and dose schedules may increase the potency of a chemotherapy and immunotherapy combination.

TILs have increasingly been correlated with patient survival. TIL-Bs are reportedly a significant part of TILs. We illustrate that urinary bladder cancer patients, exhibit tumor associated follicular-like structures (FLSs) in the proximity of tumor where B cells and T cells interact. We demonstrate that FLSs and tumor-associated $CD38^+$ plasma cells, in addition to IL-10 produced by non-B cells, may contribute to positive patient prognoses. On the contrary, IL-10 produced by tumor-associated Bregs, may affect the prognosis negatively.

It is acknowledged that B cells undergo allelic exclusion. On the contrary to this understanding, we describe rare $Ig\lambda^+$ $Ig\kappa^+$ B cell populations in cancer patients which are increased in tumor associated tissues and present in activated B cell populations, as well as among switched memory B lymphocytes. Furthermore we demonstrate that they do not undergo somatic hypermutation and receptor editing. We conclude their emergence is due to the influence of tumor environment which is confirmed by $Ig\lambda^+$ $Ig\kappa^+$ B cell induction in culture with supernatant from a tumor cell line.

Due to highly specific antigen receptors, B cells may be very effective antigen presenting cells (APCs). Further studies can reveal their role as APCs in cancer patients which may be exploited for $CD4^+$ T cell adoptive immunotherapy.

LIST OF SCIENTIFIC PAPERS

I. **Zirakzadeh AA**, Marits P, Sherif A, Winqvist O.
Multiplex B cell characterization in blood, lymph nodes, and tumors from patients with malignancies.
J Immunol. 2013 Jun 1;190(11):5847-55.

II. **Zirakzadeh AA**, Kinn J, Krantz D, Rosenblatt R, Winerdal ME, Hu J, Hartana CA, Lundgren C, Bergman EA, Johansson M, Holmström B, Hansson J, Sidikii A, Vasko J, Marits P, Sherif A, Winqvist O.
Doxorubicin enhances the capacity of B cells to activate T cells in urothelial urinary bladder cancer.
Clin Immunol. 2016 Dec 24;176:63-70.

III. **Zirakzadeh AA**, Rosenblatt R, Ahlén Bergman E, Winerdal M, Yang D, Cederwall J, Sherif A, Winqvist O, Marits P.
Tumor-associated B cells in Urinary Bladder Cancer.
Manuscript

IV. **Zirakzadeh AA**, Hartana CA, Krantz D, Winerdal ME, Johansson M, Holmström B, Hansson J, Sidikii A, Vasko J, Riklund K, Marits P, Sherif A§, Winqvist O§.
Rare B cell population with dual expression of Ig κ and λ light chains in cancer patients.
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Marits P, **Zirakzadeh AA**, Sherif A, Winqvist O
The many flavors of tumor-associated B cells.
Oncoimmunology. 2013 Aug 1;2(8):e25237.

Marits P, **Zirakzadeh AA**, Sherif A, Winqvist O.
Response to comment on "multiplex B cell characterization in blood, lymph nodes, and tumors from patients with malignancies".
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LIST OF ABBREVIATIONS

AID	Activation-induced cytidine deaminase
ALL	Acute lymphoblastic leukemia
APC	Antigen presenting cell
Bregs	Regulatory B cells
CC	Colon cancer
CTL	Cytotoxic T lymphocytes
CD	Cluster of differentiation
CD40L	CD40 ligand
CDR3	Complementarity determining region 3
CLL	Chronic lymphatic leukemia
CRP	C reactive protein
CSR	Class switch recombination
FASCIA	Flow cytometric assay of specific cell mediated immune response
FLSs	Follicle-like structures
FR3	Frame 3
GALT	Gut-associated lymphoid tissues
HER-2	Human epidermal growth factor 2
HLA	Human leukocyte antigen
HPV	Human papillomavirus
IFN-γ	Interferon gamma
IL-2	Interleukin 2
MHC	Major histocompatibility complex
MIBC	Muscle invasive urinary bladder cancer
MLN	Metastatic lymph node
MM	Malignant melanoma
NAC	Neadjuvant chemotherapy
nSN	Non-sentinel node
NK	Natural killer
NMLN	Non-metastatic lymph node

PC	Pancreatic cancer
PCR	Polymerase chain reaction
PrC	Prostate cancer
RAG	Recombination activating gene
RPII	RNA polymerase II
RSSs	Recombination signal sequences
SEB	Staphylococcus aureus enterotoxin B
SN	Sentinel node
TGF	Tumor growth factor
TILs	Tumor infiltrating lymphocytes
TIL-Bs	Tumor infiltrating B cells
Tregs	Regulatory T cells
UBC	Urinary bladder cancer

1 INTRODUCTION

Immunology is a relatively new science compared to other scientific fields. It is believed to originate from Edward Jenner who discovered the vaccine against smallpox in 1796. Later, scientists from the 19th century such as Robert Koch and Louise Pasteur continued with the field by proving that infectious disease was caused by microorganisms and by developing rabies vaccine respectively. The immune system comprises of molecules, cells and organs, which recognize and help clearing potential threats. It is divided into evolutionarily indigenous innate immune system which provides immediate defense against a pathogen and specialized, long lasting adaptive immune system, which is developed during lifetime of an individual and divides into humoral and cell-mediated immunity.

All the cells of the immune system originate from hematopoietic stem cells in the bone marrow. The progenitor of the innate immune cells, myeloid progenitors, enter the blood from the bone marrow and when needed, migrate to infected or damaged tissues before they differentiate into dendritic cells, macrophages, mast cells, etc. Organized tissues containing cells of the adaptive immune system comprise of central lymphoid organs, such as bone marrow and thymus where the cells are generated, and peripheral lymphoid organs, such as blood, lymph nodes and spleen where the cells of the adaptive immune system, the lymphocytes, reside and where the adaptive immune response is initiated.

Tumors are recognized and rejected by the immune system with the help of expressed tumor antigens on the surface of tumor cells. There are multiple types of tumor antigens to be named, including point mutated oncogenes or tumor suppressors such as Caspase-8 (Kim, Lee et al. 2003, Fulda 2009), proteins which are normally expressed only in male germline cells, MAGE-1 and 3 (Boon, Coulie et al. 1997), and antigens only expressed in specific tissues, such as enzymes involved in synthesis of black pigment, melanin. In addition, over expression of self-antigens in tumors compared to non-malignant cells, such as Her-2/neu (Slamon, Godolphin et al. 1989), proteins that exhibit abnormal posttranslational modifications e.g. MUC-1(Hollingsworth and Swanson 2004), and oncoviral antigens, such as the human papillomavirus (HPV) type 16 proteins, E6 and E7 have been detected(Walboomers, Jacobs et al. 1999). However, successful tumors in cancer patients use multiple mechanisms by which they can escape the immune system and survive.

This thesis investigates B cell responses in cancer patients (papers I and III) as well as the effect of the tumor environment on rare subtype of B cells, Ig κ^+ Ig λ^+ $CD19^+$ cell (paper IV). In addition, it reflects on the influence of chemotherapy on B cells and their ability to enhance T cell responses (paper II).

1.1 ADAPTIVE IMMUNE RESPONSE

The adaptive immune response comprises of mainly B and T cells, both of which originate in the bone marrow. However, T cell progenitors leave the bone marrow to reside in the thymus and continue with their full development into naïve T cells whereas B cell development occurs in the bone marrow. Several T cell subsets have been described which develop into effector cells and have distinct functional properties. Two major subsets are $CD4^+$ and $CD8^+$ T cells. Naïve $CD4^+$ T cells develop into T helper cells and orchestrate different immune cell responses by producing cytokines, whereas naïve $CD8^+$ T cells develop into cytotoxic T lymphocytes (CTL) which can directly eliminate infected cells and tumor cells. B cells produce antibodies and contribute to the humoral immune response. In addition, both T and B lymphocytes are necessary for the development of long-term immunological memory.

Naïve $CD4^+$ T cells require interaction with antigen presenting cells (APCs) to get activated and function as T helper cells. They recognize foreign and mutated self-antigens as peptides in the context of MHC class II molecules, displayed by professional APCs (Ashwell, DeFranco et al. 1984).

1.2 ANTIGEN PRESENTING CELLS

There are three distinct classes of professional APCs, namely macrophages, dendritic cells and B cells (Ashwell, DeFranco et al. 1984). Upon encountering an antigen, they internalize it by endocytosis or in the case of B cells by receptor-mediated endocytosis (Figure 1). Endosomes containing the antigen, acidify as they travel inside the cell and fuse with lysosomes. Acidification activates proteases inside the vesicles, which in turn can degrade the antigens into smaller peptide fragments. The vesicles containing peptide fragments fuse with vesicles containing MHC class II molecules where peptides bind to the MHC class II pockets and are brought up to the surface of the APCs for presentation to T cells. Upon encountering a peptide specific $CD4^+$ T cell, there are three signals needed for both cells to be fully activated. The first signal is the specific binding of the T cell receptor with MHC class II-antigen complex, after which several costimulatory molecules on both cells are upregulated. The second signal is binding of the costimulatory molecules on both cells. CD86 expressed on APCs bind to CD28 on T cells whereas CD40 on APCs bind to CD40 ligand (CD40L) on T cells which contributes to further activation. Finally, production and secretion of inflammatory cytokines, such as interleukin 2 (IL-2), interferon gamma (IFN-γ) or IL-4 leads to binding of the cytokines to their respective receptors on the cells and hence the third signal and full cell activation (Jenkins, Khoruts et al. 2001).

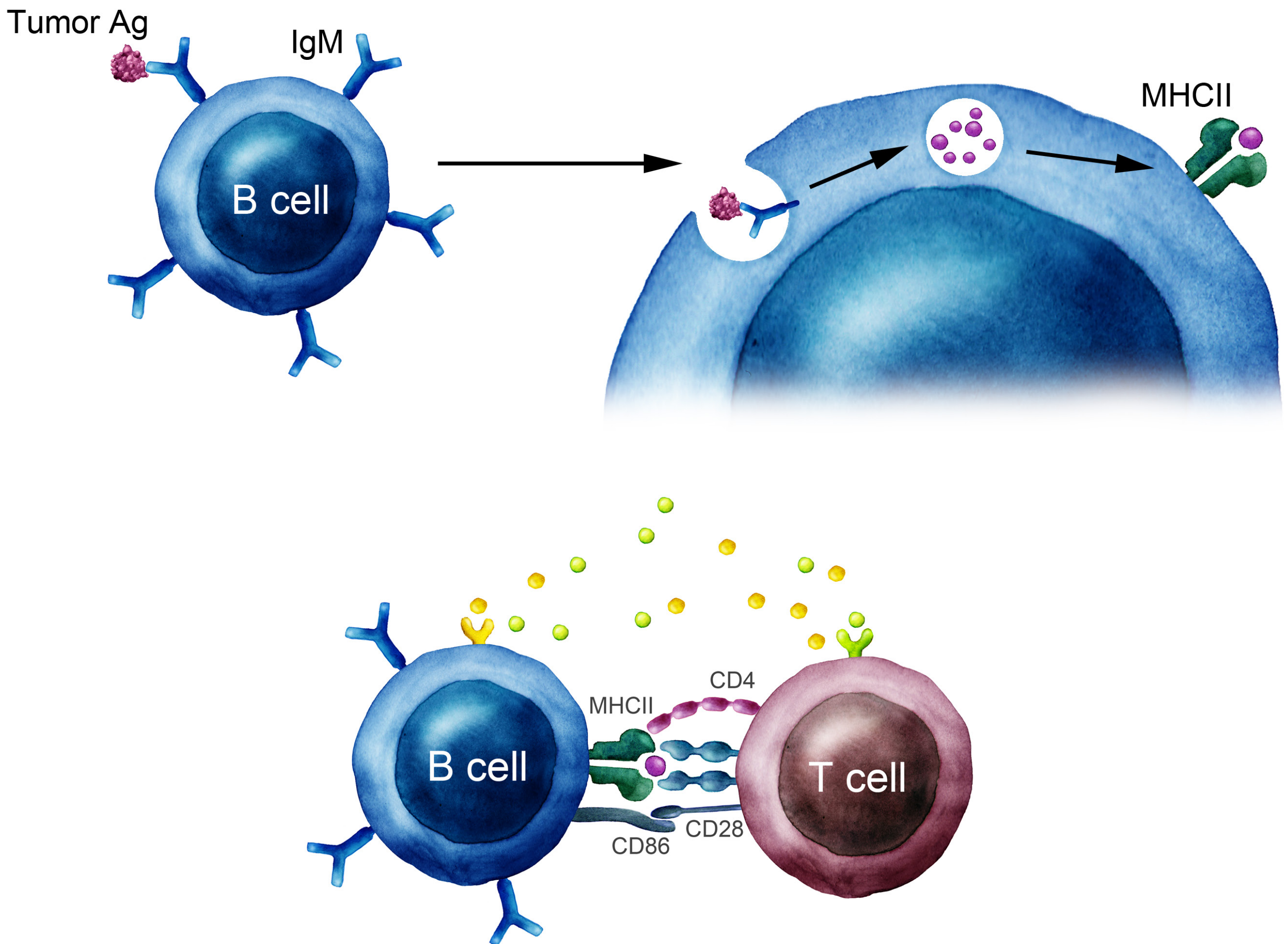

Figure 1. Schematic overview of a B cell antigen presentation. After encountering antigen via BCR, the B cell internalize it, process it and present it to T cells using MHC class II. Three signals contribute to full activation of both B and T cells; 1. MHC class II-peptide interaction with T cell receptor (TCR) complex. 2. Costimulatory binding on both cells. 3. Cytokine secretion. Figure courtesy of Malin Winerdal.

1.3 B LYMPHOCYTES

1.3.1 Development

B cell progenitors reside and develop in the bone marrow from hematopoietic cells. Their development involves receptor gene rearrangements, which is defined into three developmental stages (Carsetti, Rosado et al. 2004). In the first stage, pro-B cells go through D and J recombination of the heavy chain (H-chain), which is then followed by rearranging the V region to join the previously recombined DJ segment. The next step is to rearrange the μ-H-chain. A complete heavy chain is then fused with the surrogate light chains which consist of two proteins, λ5 and VpreB. Together they form the pre-B cell receptor. If a functional receptor is achieved, the cell enters the second stage of the B cell development, the pre-B cell stage. It

is in this stage when light chain rearrangements occur. The heavy and light chain recombination of the variable regions at this stage, occur when two recombination activating gene (RAG) 1 and 2 complexes, bind to the conserved recombination signal sequences (RSSs) which exist on the flanks of the gene segments. RAG complexes then cleave the DNA with their endonuclease activity. At this stage, DNA repair enzymes begin to modify the open ends of the DNA segments, removing nucleotides by exonuclease activity and adding random new nucleotides, using the enzyme, terminal deoxynucleotidyl transferase (TdT). Finally DNA ligase IV joins the modified open DNA strands together, reconstructing new rearranged gene (Schlissel 2003).

In the third stage, Combination of a μ chain and either a κ or λ light chain gives rise to an IgM molecule, which is expressed, on the cell surface, creating a massive repertoire of B cells with $>10^{14}$ different antigen specificities (Yaari and Kleinstein 2015). At this stage, the cells are designated immature B cells. B cells with non-functional or autoreactive receptors, which do not survive the bone marrow, are deleted (Spanopoulou, Roman et al. 1994) and only positively selected B cells leave the bone marrow as transitional B cells ($CD38^{hi}$ IgM^{hi}) to enter the spleen and complete their development to mature naïve B cells. It has been reported in a study with mice that only 10-20% of the immature B cells reach the spleen (Rolink, Andersson et al. 1998).

1.3.2 Receptor editing

B cell progenitors, which produce non-functional, or autoreactive receptors in the bone marrow, can rearrange their receptor gene segments in an attempt to make their receptors more functional or less self-reactive and thus evade apoptosis. After a functional heavy chain is expressed, the light chain segments are set to be tested in order (Korsmeyer, Hieter et al. 1982). Firstly, Ig κ light chain RAG dependent rearrangement on one allele occurs. If a functional receptor is not achieved, new rearrangements on the second allele is tested. In case of failure, Ig λ light chain on one allele and then again on the second allele is tried. Final outcome of a B cell with a functional receptor leads to downregulation of further receptor rearrangements leading to survival of the B cell with one single receptor in a process called allelic exclusion.

However, in violation of allelic exclusion, B cells with dual Ig κ and Ig λ light chain have been encountered in different studies (Giachino, Padovan et al. 1995, Xu 2006). In a study with anti-DNA heavy chain transgenic mice, Ig κ^+ Ig λ^+ B cells were detected in the marginal zone and were revealed to be partially autoreactive (Li, Jiang et al. 2001, Li, Li et al. 2002). In addition dual light chain positive B cells have been observed in a patient with chronic lymphatic leukemia (CLL) (del Senno, Gandini et al. 1987). Ig κ^+ Ig λ^+ B cells have essentially been studied in mice and in patients with malignant B cells. Relatively, little is known about B cells with dual positive light chains in patients with solid tumors.

1.3.3 B cell subsets

The phenotype of naïve B cell subsets has been described in different studies. According to the Freiburg classification, they express IgM and IgD but lack the tumor necrosis factor (TNF) receptor superfamily molecule, CD27 (Figure 2) (Wehr, Kivioja et al. 2008). Upon activation B cells upregulate the early activation marker CD69 (Hara, Jung et al. 1986) followed by the

costimulatory molecules CD80 and CD86 (Hathcock, Laszlo et al. 1994). Activated B cells enter germinal centers around day 3-5 after T cell dependent activation (Liu, Johnson et al. 1992). In germinal centers B cells go through class switch recombination (CSR), somatic hyper mutation (SHM) and clonal expansion (MacLennan 1994). CSR and SHM are initiated by activation-induced cytidine deaminase (AID), which converts the cytidine base (C) into uracil (U) by deamination of the former base. During DNA replication, U is converted to thymine (T) which then leads to an overall gene conversion from C:G to T:A (Petersen-Mahrt, Harris et al. 2002, Peled, Kuang et al. 2008, Stavnezer 2011).

Additional T cell signals, such as CD40 ligand (CD40L) can contribute to the survival of B cells and allow them to develop into memory B cells (IgD^{-} CD27^{+}), whereas the cytokines IL-10 and IL-2 can cause differentiation into class switched plasmablasts (CD38^{+++} IgM^{-}) and later antibody secreting plasma cells (Figure 2) (Arpin, Dechanet et al. 1995, Liu and Banchereau 1997). Plasma cells travel to the site of infection or return to the bone marrow and produce antigen specific antibodies.

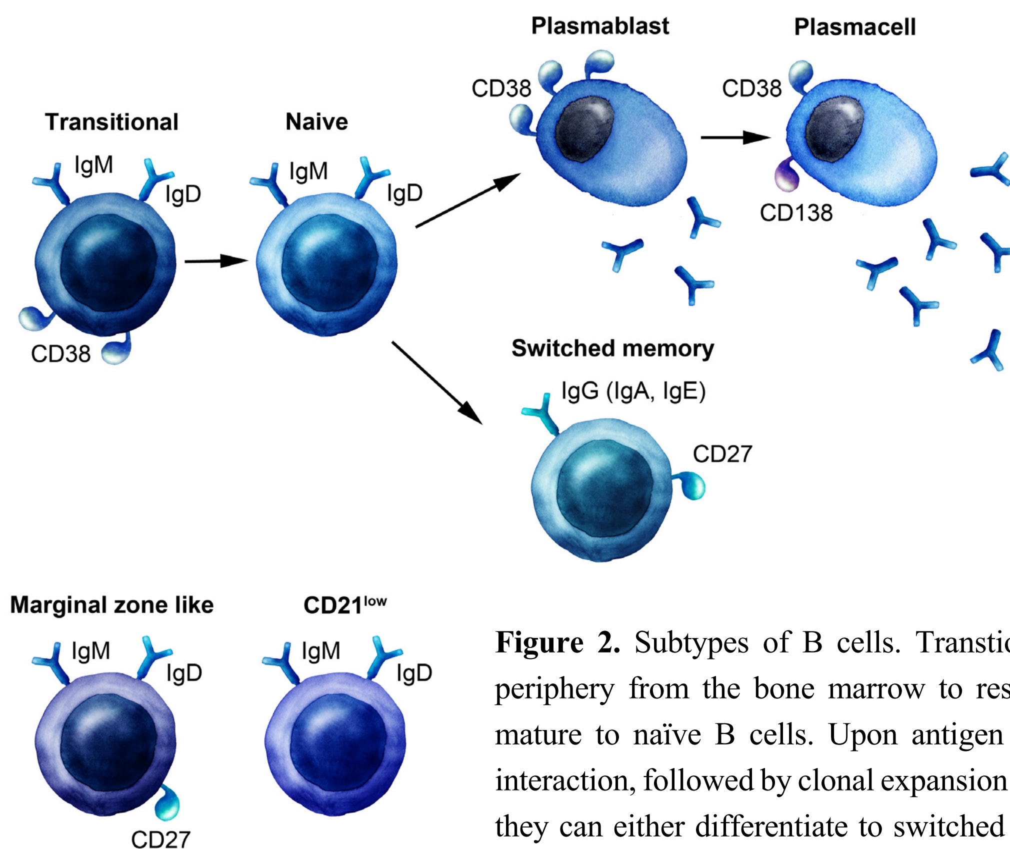

Figure 2. Subtypes of B cells. Transtional B cells enter the periphery from the bone marrow to reside in the spleen and mature to naïve B cells. Upon antigen encounter and T cell interaction, followed by clonal expansion in the germinal center, they can either differentiate to switched memory B cells or to plasmablasts and finally antibody secreting plasma cells.

Marginal zone-like (MZ) and CD21^{low} B cells have also been detected in human. MZ express IgM and exhibit memory B cell properties. CD21^{low} also express IgM but show signs of previous activation. Figure courtesy of Malin Winerdal.

In addition to the mentioned B cell sub-classes, another subtype, marginal zone (MZ) B cells (IgM^+ IgD^+ $CD27^+$) can be noticed (Figure 2). They are IgM producing ($CD27^+$ IgD^+) B cells that in mice reside in marginal zone of the spleen. There are distinct differences between murine and human MZ B cells. Unlike in mice, human MZ circulate in the body and in addition to blood and spleen, they have been encountered in lymph nodes, tonsils and under the dome epithelium of Peyer's patches in gut-associated lymphoid tissues (GALT) (Stein, Bonk et al. 1980, Spencer, Finn et al. 1985, Liu, Barthelemy et al. 1995, Spencer, Perry et al. 1998, Dono, Zupo et al. 2000). Furthermore, the presence of somatic mutations in the IgM receptors of human MZ B cells, as opposed to their counterparts in rodents (Dammers, Visser et al. 2000), have prompted authors to call them marginal zone-like or IgM memory B cells. In general, MZ B cells are described to develop and respond to antigen, in a T cell-, germinal center-independent manner (Weller, Braun et al. 2004).

$CD21^{low}$ B cells have been encountered in patients with common variable immunodeficiency (Wehr, Kivioja et al. 2008). Their origin and function has been debated. Some studies describe it to resemble innate-like tissue homing B cells and suggest they exhibit previous activation and proliferation but are unable to proliferate in response to B cell receptor stimulation (Rakhmanov, Keller et al. 2009). Their role depends on the underlying condition.

1.3.4 B cell receptor isotypes

In general interaction between CD40 and CD40L on B and T cells respectively, generate class switching. The IgM receptor of naïve B cells is then switched to the isotypes, IgG, IgA or IgE, depending on the environment and location in the body. Each isotype is characterized by a distinct function. IgG is the most abundant isotype in human serum. It has been demonstrated to be induced by Interleukin-6 (IL-6) (IgG1) and a combination of IL-6 and IFN-γ (IgG2) (Kawano, Noma et al. 1994). IgA is mostly produced by B cells residing in intestinal and urogenital tracts. IgA in the mucosal secretion is found as dimers and oligomers in both mice and humans whereas circulating IgA antibodies exist predominantly as monomers in humans and largely as polymers in mice (Macpherson, McCoy et al. 2008). IgA class switching is induced by tumor growth factor beta (TGF-β) as well as a combination of CD40L, IL-4, IL-6 and IL-10. The effector function of IgA is to bind to type I Fc receptor for IgA (FcαRI) expressed on innate immune cells, such as monocytes, macrophages, dendritic cells and granulocytes. In addition, IgA neutralizes toxins and pathogens in the mucosa (Cerutti, Zan et al. 1998, Cerutti 2008). IgE antibodies are associated with allergic reactions and asthma but are also involved in defense mechanisms against parasitic infections. The class switching recombination is induced by CD40L, IL-4 and IL-13 (Geha, Jabara et al. 2003). IgE is mainly produced under the skin or epithelial surfaces of the airways.

1.3.5 Cytokines

1.3.5.1 IL-2

IL-2 is a cytokine with effects on many different cells, the most pronounced of which is the T cell. T cells synthesize IL-2 upon activation through their antigen peptide receptor, which is

then followed by upregulation of the high affinity IL-2 receptor (CD25). Thus, IL-2 allows T cell to undergo rapid, specific expansion (Lenardo, Chan et al. 1999, Gaffen and Liu 2004) and it is also needed for their long-term survival (Rathmell, Vander Heiden et al. 2000, Frauwirth and Thompson 2004). Additionally, IL-2 can promote upregulation of IL-2 receptor on B cells and consequently induce differentiation to plasma cells (Miyawaki, Suzuki et al. 1987).

In addition to activated $CD4^+$ T cells and B cells, regulatory $CD4^+$ $CD25^+$ T cells (Tregs) also express CD25, which is a subunit of the IL-2 receptor, but the latter produce the molecule in higher magnitude (Kuniyasu, Takahashi et al. 2000, Baecher-Allan, Brown et al. 2001). IL-2 signaling is needed for development of Tregs in the thymus as well as their expansion and maintenance in the periphery (Nelson 2004).

1.3.5.2 IFN-γ

IFN-γ is a pro-inflammatory cytokine, important in innate and adaptive immune response against viral and intracellular bacterial infections. In addition, it is involved in tumor control and enhancing tumor immune response (Ikeda, Old et al. 2002, Rosenzweig and Holland 2005). It contributes to differentiation of $CD4^+$ T cells into Th1 effector cells. In addition of helper T cells, it affects natural killer (NK) cells and $CD8^+$ T cells. It can be produced in large amounts by Th1 effector cells (Dong 2006, Harrington, Mangan et al. 2006).

1.3.5.3 IL-6

Different cells such as, macrophages, dendritic cells and B cells express IL-6. IL-6 is involved in differentiation of CD4 effector T cells, in addition to B cells and macrophages (Diehl, Anguita et al. 2000). In addition, IL-6 is the major cytokine, inducing the production of the acute phase reactant, C reactive protein (CRP) from liver innate cells as a response and a sign of systemic inflammation (Schmidt-Arras and Rose-John 2016).

1.3.5.4 Tumor necrosis factor alpha (TNF-α)

TNF-α is generally regarded as a pro-inflammatory cytokine produced by different cells, e.g. macrophages and T cells. Moreover, TNF-α deficient mice exhibit lack of splenic B cell follicles (Pasparakis, Alexopoulou et al. 1996). However, TNF-α has been proved to have dual functions, promoting Treg expansion and increasing suppressive Treg function in addition to the cytokine's pro-inflammatory properties (Grinberg-Bleyer, Saadoun et al. 2010).

1.3.5.5 IL-10

IL-10 can be produced by different leukocytes but also by epithelial cells. IL-10 has a dual function both as a pro-inflammatory and an anti-inflammatory cytokine. Deficiency in IL-10 or the IL-10 receptor has been suggested to cause infantile inflammatory bowel disease (IBD) (Kotlarz, Beier et al. 2012) whereas IL-10 treatment in mice increased anti-tumor immunity and tumor specific $CD8^+$ T cell activation (Fujii, Shimizu et al. 2001, Emmerich, Mumm et al. 2012), which illustrate the dual functionality of the cytokine.

1.3.6 Regulatory B cells

Bregs are B cells with immune suppressive function. They have mainly been identified as producers of IL-10 (Fillatreau, Sweenie et al. 2002, Mizoguchi, Mizoguchi et al. 2002, Mauri, Gray et al. 2003). In recent years IL-10 producing Bregs with multiple phenotypes have been described both in human and in mouse models. $CD5^+$ $CD1d^{hi}$ B (B10) cells (Yoshizaki, Miyagaki et al. 2012), $CD138^+$ plasma cells (Shen, Roch et al. 2014), $CD19^+$ $CD24^{hi}$ $CD38^{hi}$ $CD1d^{hi}$ cells (Blair, Norena et al. 2010) and $CD19^+$ $CD24^{hi}$ $CD27^+$ cells (Iwata, Matsushita et al. 2011) are examples on such Bregs. Whether the differences are because of existence of a specific Breg lineages or due to immunological environment is not yet known (Rosser and Mauri 2015).

It has been demonstrated in different studies that IL-10 producing Bregs also express other regulatory cytokines, such as TGF-β and IL-35. TGF-β produced by lipopolysaccharide (LPS)-activated B cells can induce apoptosis in $CD4^+$ effector cells (Tian, Zekzer et al. 2001) and chimeric mice with B cell specific IL-35 deficiency, developed more severe experimental autoimmune encephalomyelitis (EAE) and better protected against Salmonella-induced sepsis. In the Salmonella case, B cells with lack of IL-35, resulted in induction of Th1 cell responses and increased number of macrophages in the spleen (Shen, Roch et al. 2014).

1.4 CANCER

Despite modern therapeutic strategies, cancer continues to be one of the main causes of death worldwide. Further understanding of cellular and molecular events behind development of cancer, as well as the body's immune alteration and response during the progression of the disease will help the ongoing human battle against cancer.

1.4.1 Development

There are several different capabilities that cells need to acquire in order to develop into cancer cells. As described by Hanahan *et al*. sustaining proliferative signaling is one of the main attributes. Normal tissues have controlled production and secretion of growth-promoting signals that regulate the cell proliferation and number and can therefore keep the tissues architecture and function intact. By ungoverned production of these growth signals, cancer cells lose control over their destiny (Hanahan and Weinberg 2011). In addition to sustaining growth signals, cancer cells must escape the body's negative regulation of cell proliferation. Furthermore, they must resist cell death by apoptosis. p53 is a protein which can stop cells cycle progression in case of growth-promoting signals or glucose are produced in abnormal levels. In addition, it can cause apoptosis if irreparable damage in the genome has occurred. It has been demonstrated that mice lacking p53 develop leukemia and sarcoma (Ghebranious and Donehower 1998). Thus, p53 is as an example of the mentioned regulators of cell proliferation, which cancer cells must evade to develop. Another requirement, is unlimited replicative potential. There are verifications that telomeres protecting the end of chromosomes are involved in the capability for unlimited cell divisions (Shay and Wright 2000, Blasco 2005). Telomerase is a DNA polymerase that adds telomere to the end of telomeric DNA. Non-

immortalized cells almost lack telomerase whereas approximated 90% of spontaneously immortalized cells produce it at functional levels.

In order to survive, tumors are in need of nutrients and oxygen, like normal tissues. Thus, induction of angiogenesis is another requirement for tumor growth, as well as activating invasion and metastasis. In some aggressive carcinomas cell-to-extracellular matrix and cell-to-cell adhesion molecules are expressed in abnormal levels. Metastasis is believed to begin with local invasion followed by penetration of tumor cells into the blood or lymphatic vessels. Then the cells escape from the vessels into distant tissues where they become micro-metastases and thereupon grow to macroscopic tumors (Hanahan and Weinberg 2011).

1.4.2 Cancer immune response

Tumor infiltrating lymphocytes (TILs) are accepted as components of tumors. Many TILs, in particular T cells have been correlated with positive prognosis in patients with different malignancies such as melanoma, breast cancer, ovarian cancer, esophageal carcinoma, and urothelial carcinoma. (Clemente, Mihm et al. 1996, Schumacher, Haensch et al. 2001, Zhang, Conejo-Garcia et al. 2003, Pages, Galon et al. 2010). Cytotoxic T cells and NK cells can kill malignant cells directly and contribute to increased patient survival (Camus, Tosolini et al. 2009, Marcus, Gowen et al. 2014), hence they are usually thought as the most important cells with anti-tumoral activity. However, other immune cells have emerged as strong contributors in tumor immune response, such as B cells.

1.4.2.1 B cell immune response against cancer

B cells can contribute in cancer immune responses in multiple ways. They can present tumor antigen to $CD4^+$ T cells, using MHC class II. (Rodriguez-Pinto 2005, Yanaba, Bouaziz et al. 2008). Notably the high-affinity binding of the Ig receptor to its specific antigen makes B cells unique compared to other APCs. Furthermore, they can produce tumor antigen specific antibodies (Reuschenbach, von Knebel Doeberitz et al. 2009). In addition, they can produce granzyme B (Hagn, Schwesinger et al. 2009) that upon secretion with perforin may trigger apoptosis in tumor cells. They can also produce cytokines and chemokines that regulate other immune cells. Notably, B cells promote the formation of $CD4^+$ memory T cells (Whitmire, Asano et al. 2009) and facilitate survival of proliferation of activated $CD8^+$ T cells through CD27-CD70 binding (Deola, Panelli et al. 2008).

It has been demonstrated that B cells represent a significant portion of TILs and exhibit anti-tumoral immune responses in colorectal cancer (Shimabukuro-Vornhagen, Schlosser et al. 2014). Moreover, B cells are detected in approximately 25% of breast cancers and B cells may constitute up to 20% of the TIL population (Chin, Janseens et al. 1992, Marsigliante, Biscozzo et al. 1999, Coronella-Wood and Hersh 2003). Furthermore, TIL-Bs in collaboration with tumor infiltrating $CD8^+$ T cells correlated with survival in ovarian cancer (Milne, Kobel et al. 2009). As we demonstrated in our previous studies, B cell immune response against tumor antigen is not restricted to TIL-Bs and could be detected in tumor associated lymph nodes (Zirakzadeh, Marits et al. 2013). Investigations with a murine mouse model by others have

confirmed our observations and identified tumor B cell immune response in tumor draining lymph nodes (TDLN). CD40 stimulation of TDLN isolated B cells and dendritic cells, in combination with CD3 stimulation of T cells, induced antitumor response. However, combination of either B cells or dendritic cells with T cells, exhibited a decreased antitumor response, which signifies the importance of the combination (Li, Teitz-Tennenbaum et al. 2009). Thus, B cells are important participants of the anti-tumor immune response and they are beneficial for patient survival.

In addition to B effector cells, tumor infiltrating Bregs have been encountered but on the contrary to the former, they appear to be disadvantageous to patient prognosis. They have been detected in tumors and metastasis with advanced colorectal cancer, in considerable numbers (Shimabukuro-Vornhagen, Schlosser et al. 2014).

1.4.3 Cancer immune escape mechanisms

Cancer immune escape starts after two other immune editing processes namely elimination and equilibrium. Elimination is the starting point of immunosurveillance. Tumor cells are recognized by the immune system and finally get eliminated. Equilibrium occurs when pressure of immune defense on tumor cells is enough to contain them but not enough to fully eradicate them. Tumor cells at that point are genetically unstable and rapidly mutating. During this process, the tumor cells acquire additional immune escape mechanisms, making them more resistant to the immune defense. This phase is likely the longest of the three processes and may continue during many years. Immune escape represents the final phase when the tumor cells acquire insensitivity to be recognized by the immune defense and/or to get eradicated. Thus, they begin to expand in an uncontrolled way. (Dunn, Bruce et al. 2002).

There are many mechanisms by which tumor can evade immune recognition. One of them is by producing immune suppressive cytokines and/or by attracting regulatory immune cells, in addition to down regulating pro-inflammatory cytokines, such as IFN-γ. Among the cytokines, which have shown to exhibit immune suppression in the tumor, IL-10 and TGF-β can be mentioned. Increased levels of the cytokines, correlated with loss of CD3-ζ expression on TILs in pancreatic cancer patients. (von Bernstorff, Voss et al. 2001). CD3-ζ is part of the T cell receptor complex and lack of it, inhibits tumor specific antigen signaling, which in turn contributes to immune tolerance. One way for tumor cells to be recognized by the immune system is presenting tumor specific antigens to cytotoxic T cells, by human leukocyte antigen (HLA) class I. However, tumors can downregulate HLA class I as a frequent mechanism to evade the immune system. 96% of HLA class I loss have been reported in cervical and breast carcinomas (Cabrera, Angustias Fernandez et al. 1996, Koopman, Corver et al. 2000). Another mechanism, by which tumor escape can occur, is down-regulation of immunogenic tumor specific antigen by the tumor. In addition, tumors in many malignancies overexpress self-proteins that makes it difficult for the immune system to recognize the tumor as foreign. In addition, APCs capture tumor antigen and present it to T cells. In the absence of pro-inflammatory environment, caused by the tumor suppressive mechanism, APCs fail to mature,

up-regulate costimulatory molecules and get activated; hence potent tumor antigen presentation fails which leads to immunological tolerance (Pinzon-Charry, Ho et al. 2005).

The tumor may also upregulate proteins, leading to negative stimulatory effects, such as CTLA-4. CTLA-4 has been demonstrated to be expressed by tumor cells and trigger apoptosis in target cells (Contardi, Palmisano et al. 2005). In addition, programmed death receptor 1 (PD1) and programmed death receptor ligand 1 (PDL-1) contribute to suppression of the immune system. It has been revealed that PDL-1 expression on tumor cells have a negative impact on the malignant cells and have a negative prognosis for cancer patients (Blank and Mackensen 2007).

1.4.4 Cancer treatments

1.4.4.1 Chemotherapeutic drugs

Chemotherapy is one of the primary treatments for cancer patients. Doxorubicin (Adriamycin), cisplatin and irinotecan are among the conventional chemotherapeutic drugs used for chemotherapy today. According to national cancer institute (NCI) as part of national institutes of health (NIH), Doxorubicin is used for treatment of malignancies such as, acute lymphoblastic leukemia (ALL), breast cancer, gastric cancer, ovarian cancer, small cell lung cancer, thyroid cancer, urinary bladder cancer, etc. It is one of the most potent chemotherapeutic drugs approved (Carvalho, Santos et al. 2009). Its ability to engage rapidly dividing cells and to induce slow disease progression has been widely known and acknowledged for decades. It is limited only by its toxicity on normal cells in the body (Tacar, Sriamornsak et al. 2013).

The role of Topoisomerase II is to cut and religate DNA strands in order to unknot DNA tangles and supercoils and make the strands ready for replication. Doxorubicin at low concentrations (<1μM), functions by blocking DNA religation during the activity of topoisomerase II, hence preventing DNA replication. At high concentrations (>10μM), doxorubicin intercalates between base pairs in the DNA helix, preventing topoisomerase II from binding to the DNA, thereby prohibiting DNA replication (Pommier, Leo et al. 2010). In highly proliferating cells, such as cancer cells, topoisomerase II activity is high which makes doxorubicin an effective drug to combat cancer.

Cisplatin is used to treat severe forms of bladder cancer, ovarian cancer, testicular cancer, etc. In addition to doxorubicin, it is one of the most potent chemotherapeutic drugs used to treat cancer patients. There are two chloride ligands included in the molecule, one of which is spontaneously displaced with a water molecule in the body. This so called aquation process leads in turn, to the water molecule to be replaced with a DNA base, and hence the molecule binds to the DNA. The other chloride ligand can in turn be replaced with another DNA based leading to crosslinking the DNA. Different repair mechanisms arise for the damaged DNA but when repair proves impossible, cell apoptosis will be induced (Siddik 2003).

Irinotecan is mainly used for treating colorectal cancer with metastasis. It is a prodrug which is hydrolyzed to its active form, SN-38, in the body. It functions by binding the interface of the

cleaved DNA during topoisomerase I activity (Pommier 2013). Thus, the enzyme gets inactivated, leading to prevention of DNA replication and transcription.

MVAC is a chemotherapeutic regimen based on cisplatin, which in addition to the mentioned drugs, involves three other immunotherapeutic agents, methotrexate, vinblastine and doxorubicin (Adriamycin) (Meeks, Bellmunt et al. 2012). It is widely used in neoadjuvant chemotherapy for advanced bladder cancer patients (Witjes, Comperat et al. 2014) when the regimen is administered before cystectomy.

Despite the side effects the chemotherapeutic drugs can exhibit on normal cells, including the cells of the immune system, there have been reports about increased immune antigen response as an effect of cell treatment with chemotherapeutic drugs and induction of activation phenotypes upon drug treatment (Moschella, Valentini et al. 2011, Hu, Kinn et al. 2013). In addition, it has been demonstrated that chemotherapy in combination with presence of TILs contributes to an improved prognosis in breast cancer (Denkert, Loibl et al. 2010). One explanation for the synergistic effect of the drugs and the immune system is that in addition to DNA, cellular molecules and proteins may act as potential targets which could have an activating effect on the immune cells (Hato, Khong et al. 2014). Further studies are needed to dissect the mechanism of chemotherapeutic agents on the immune cells.

It has been illustrated that immunotherapy may have synergistic effects with chemotherapy (Godet, Fabre et al. 2012). However, optimizing the administration plans might be crucial to achieve a successful response as administration of the drugs in a vulnerable phase of the leucocyte activation and proliferation may have an adverse effect.

1.4.4.2 Immunotherapy

Immunotherapy has increasingly become attractive to treat cancer patients. IFN-α2 and IL-2 have been used to treat melanoma (Kirkwood and Ernstoff 1985, Kirkwood, Butterfield et al. 2012). Another way to stimulate the immune system against cancers is to use identified tumor antigens as vaccines. Tumor antigens stimulate a cellular and/or a humoral immune response in cancer patients (Sahin, Tureci et al. 1995). They can promote epitopes that are presented by the tumor cells using MHC class I to activate $CD8^+$ T cells. In addition they can give rise to fragments presented to $CD4^+$ T cells by APCs, using MHC class II. Dendritic cells (DCs) have an important role in immunotherapy. A way to make sure that tumor antigen is delivered and proper signal for immunization is induced, is loading patient DCs with tumor antigen ex vivo before transfusion in their host (Schuler, Schuler-Thurner et al. 2003). In addition, we have previously demonstrated that $CD4^+$ T cell adoptive immunotherapy is a promising treatment for muscle-invasive bladder cancer and colon cancer patients (Karlsson, Marits et al. 2010, Sherif, Hasan et al. 2015). Ex vivo expansion of Tumor infiltrating $CD8^+$ T cells, using IL-2, is another method used by others to treat melanoma (Rosenberg, Yang et al. 2011). Hybridoma technology has facilitated production of highly specific antibodies against tumor antigens. Monoclonal antibodies, such as rituximab (anti-CD20) and trastuzumab (anti-human epidermal growth factor 2 [HER-2]) are routinely used for treatments against lymphoma and breast cancer

respectively (Campoli, Ferris et al. 2010). Another treatment being tested on patients with high scale, is adoptive T cell therapy with chimeric antigen receptors (CARs). The idea is that T cells are removed from the patients and genetically engineered to produce single chain fragment variable (scFv) of a monoclonal antibody, targeted against tumor associated antigens. From the inside the cells, scFvs are connected to the signal transduction machinery of the T cells. The therapy has shown promising results although some toxicity has been encountered, especially when the expression of targeted tumor antigen is shared with healthy tissues (Casucci and Bondanza 2011). Further studies are needed to optimize immunotherapy and increase a tumor specific immune response by multiple immune mediators working in combination to combat cancer.

2 AIMS OF THE THESIS

The overall aim of this thesis was to study B cells in patients with solid malignancies and to investigate their immune response against tumors, in order to address their antigen presenting ability. Tumor specific B cells as potent APCs may be beneficial to utilize in $CD4^+$ T cell adoptive immunotherapy.

The specific aims for each study were:

Paper I. To characterize subpopulations of B cells in blood, metastatic lymph nodes and tumor of patients with malignancies and to examine their phenotypes.

Paper II. To investigate the effect of chemotherapeutic drugs on human B cells with respect to their antigen presenting ability.

Paper III. To study the impact of tumor associated B cells, $CD38^+$ cells and IL-10 on survival of patients with urinary bladder cancer.

Paper IV. To examine the existence of Ig κ^+ Ig λ^+ B cells in cancer patients and reveal their phenotype and properties.

3 MATERIALS AND METHODS

This chapter contains a summary of the patients and methods used in the studies included in the thesis. For more detailed information please refer to the respective study.

3.1 PATIENTS

3.1.1 Patient characteristics

Over all 20 patients were included in paper I and in group 1 in paper IV. 5 colon cancer (CC), 9 Urinary bladder cancer (UBC), 4 malignant melanoma (MM), 1 pancreatic cancer (PC) and 1 patient with prostate cancer (PrC), between ages 47-86. 16 of the patients were male whereas 4 were females.

In paper II, 15 patients with muscle invasive urinary bladder cancer (MIBC), between the ages 55-86, were included. They were 11 males and 4 females with clinical tumor stages between T2-T4. In group 2 from paper IV, 19 UBC patients, 14 males and 5 females were included. The patients underwent TUR-B operation after which 8 patients received neoadjuvant chemotherapy (NAC) containing the chemotherapeutic drug, Doxorubicin. Depending on post NAC clinical data and response of the patients, they underwent cystectomy.

In paper III, 33 muscle invasive UBC patients were included in the retrospective study, where paraffin embedded tumor sections from 24 males and 9 females between the ages of 46-81 were studied. The patient had been cystectomized at the Karolinska University hospital during 1999-2002. Last updated patient survival data was received on 18th of October 2015.

3.1.2 Specimens

Metastatic and non-metastatic lymph nodes (papers I and IV), in addition to blood, primary tumor and in some cases macroscopically non-malignant urinary bladder tissue (paper I) were collected for the prospective studies. Furthermore, blood (papers II and IV) and tumor (paper IV) were collected at the time of TUR-B operations. Blood (papers II and IV), sentinel and non-sentinel nodes (paper IV) were collected at cystectomies. In addition, blood samples from healthy donors were obtained for papers I and IV.

4 µm paraffin embedded tumor sections on glass slides were obtained for a retrospective study in paper III.

3.1.3 Identification of metastatic and sentinel lymph nodes

Metastatic lymph nodes were detected by either pathological examination or by using flow cytometry as described in our previous studies (Karlsson, Nilsson et al. 2008, Hartana, Kinn et al. 2016). Sentinel nodes were identified by radioactive isotope injection around the tumor and measuring the radioactive signal by a handheld Geiger meter (Sherif, De La Torre et al. 2001, Rosenblatt, Johansson et al. 2016).

3.1.4 Cell preparation

Cells from the specimens in paper I, II and IV, were obtained within 2 hours after each operation. PBMC were isolated from blood using density centrifugation (Ficoll-Paque plus; GE Healthcare). Single-cell suspensions from the lymph nodes were collected by firstly cutting the specimens into smaller pieces. Then by either using a glass homogenizer (paper I and group 1 in paper IV) or by gentle pressure on 100 μ cell strainers (group 2 in paper IV), single cell suspensions were isolated. Primary tumors and non-malignant tissues were homogenized, using GentleMACS dissociator (Miltenyi biotec) in 10 ml RPMI 1640 (Sigma) for paper I and group 1 in paper IV, or 10 ml AIM V (GIBCO; Life Technologies) for paper II and group 2 in paper IV, containing 1% collagenase/Hyaluronidase solution (Stem cell technologies).

3.2 IMMUNOLOGICAL ANALYSIS

3.2.1 Flow cytometry

3.2.1.1 Surface staining

Single cell suspensions were freshly used for flow cytometry. In paper I and group 1 in paper IV, the cells were washed and stained in FACS buffer, containing PBS, 2.5% bovine growth serum and 0.05% NaN_3. In paper II and group 2 in paper IV, the cells were firstly washed with PBS before they were stained with blue LIVE/DEAD Fixable blue dead cell stain kit (Life technologies) according to the manufacturer's protocol. The cells were then washed with PBS again and stained with surface antibodies in PBS. Flow cytometry was carried out with either FACS Aria (BD biosciences) or with FACS Fortessa (BD biosciences). The data were analyzed using FACSDiva software (BD biosciences).

3.2.1.2 Flow cytometry and assay of specific cell mediated immune response

To analyze T cell lymphoblasts in paper II, the cells were stained with surface antibodies according to the above mentioned procedure. FACS Aria was used to acquire the cells. Smaller non-granular lymphocytes and larger lymphoblasts were identified based on their position on dot-plots showing forwards-scatter and side-scatter. T cell lymphoblasts ($CD3^+$, $CD4^+$, $HLA\text{-}DR^+$, $CD45RO^+$) were gated and analyzed using a flow cytometric assay of specific cell mediated immune response (FASCIA) (Svahn, Linde et al. 2003). The ratio of the T cell lymphoblasts were calculated according to the following formula: $n = \frac{T\ cell\ lymphoblasts}{\text{Total T cells}}$

3.2.1.3 Intracellular staining

Doxorubicin treated and untreated B cells, sorted from 5 healthy blood donors were treated with PMA, Ionomycin and Golgiplug. The cells were then fixed and permeabilized using Cytofix/Cytoperm solution kit (BD biosciences) according to the manufacturer's protocol.

Anti-human IL-10 antibody (allophycocyanin; JES3-19F1; Biolegend) was used to stain the cells. Rat IgG2a, κ antibody (allophycocyanin; RTK2758; Biolegend) was used as isotype control, before cytometry analysis was carried out (paper II).

3.2.2 Spectratype analysis

In paper I, DNA was extracted from PBMC, lymph nodes, tumor and nonmalignant tissue from a 61 year old female UBC patient, using DNA lysis buffer pH 8.0 (10 mM Tris, 1 mM EDTA, 1% SDS) and proteinase K. The DNA samples were incubated in 56º C for 10 minutes before they were purified using phenol:chloroform:isoamyl alcohol 25:24:1, saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma-Aldrich). Following centrifugation, the upper phases were separated from the rest of the samples before 3 M sodium acetate (NaOAC) and 100% ethanol were added. Stepwise rehydration followed resuspension in ultrapure water.

PCR was then performed based on 7 B cell Vh subgroups using 7 forward primers against frame 3 region in each subgroup and a universal 6-FAM-conjugated reverse primer against Jh region. GOTaq master mix (Promega) was used to perform the PCR according to the manufacturer’s protocol.

Spectratype analysis was then carried out using Gene Scan 400 HD size standard, Hi-Di formadide and ABI 3730 DNA Analyzer (Applied Biosystems). The software Peak Scanner (Applied Biosystems) was used to analyze the data.

3.2.3 Sequencing

PCR products for the above mentioned spectratype analysis, were purified with QIAquick PCR purification kit (Qiagen) before they were cloned using TOPO TA Cloning Kit for Sequencing with TOP10 chemically competent cells (Invitrogen). The plasmids were then purified with Plasmid Mini Kit (Qiagen) and then sequenced for paper I, using ABI 3730 DNA analyzer (Applied Biosystems).

3.2.4 Cell sorting

In Paper II, PBMCs were isolated from blood of healthy donors as described above. Magnetic beads were then used to isolate B cells and $CD4^+$ T cells from the PBMCs with $CD19^+$ B cell isolation kit (positive selection) and CD4+ T cell isolation kit II (negative selection) (Miltenyi Biotec, Bergisch Gladbach, Germany) respectively, and an autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany). For figure 2 in paper II, B cells were isolated from the PBMCs with CD19+ B cell isolation kit (positive selection) (Stem cell). All the sorting experiments were carried out using manufacturer’s protocol.

3.2.5 *In vitro* cell culture media and reagents

In Figure 1, paper II, Roswell Park Memorial Institute (RPMI)-1640 cell culture medium (Sigma-Aldrich, St Louis, MO, USA) in addition to 1% L-glutamin, 1%

penicillin/streptomycin and 10% FCS (Hyclone, Logan, UT, USA), whereas in figure 2 and 4D, AIM V medium (GIBCO; Life Technologies) were used.

The cells in paper II were treated with one of the three conventional chemotherapeutic drugs, Irinotecan, Cisplatin or Doxorubicin in the concentrations 125 µM, 25 µM or 0.2 µM respectively. The concentrations of the drugs were obtained by a titration in our previous study where LD50 of the treated cells were achieved.

3.2.6 *In vitro* cell culture

Following the isolation of $CD19^+$ cells and $CD4^+$ T cells in paper II (figure 1), B cells were treated with one of the three mentioned chemotherapeutic drugs in RPMI medium. $CD4^+$ T cells were incubated in RPMI medium alone. To prepare the negative control, the isolated B cells were cultured in RPMI alone whereas $CD4^+$ T cells were treated with the three chemotherapeutic drugs. All cells were incubated in +37° C, overnight. The next day, the B cells and the T cells were washed twice and cultured together at a 1:2 ratio respectively. In addition, Staphylococcal enterotoxin B (SEB) was added (5 µg/ml) and the cultures were incubated in +37° C for the indicated number of days before they were harvested for FASCIA analysis.

In figure 2, paper II, isolated $CD19^+$ cells were cultured in AIM V medium and with or without doxorubicin, at a concentration of $1x10^6$ cells/ml. The cells were then incubated at 37° C for 12 hours.

In paper 4, the muscle invasive cell line, 5637 (ATCC), was cultured in AIM V medium. The supernatant after 70% cell confluence was gathered and stored in -70° C. PBMCs from a healthy donor were then cultured in the 5637 supernatant in the concentration gradients, 0%, 25%, 50%, 75% and 100%, in addition to AIM V medium, in +37° C. The cells were then collected for flow cytometry analyses at day 2, 3 and 4.

3.2.7 CD86 blocking experiment (paper II)

Sorted healthy human B cells from peripheral blood were cultured with or without Doxorubicin in cell culture medium and incubated in +37° overnight. The following day, the cells were washed and incubated with anti-CD86 blocking antibody (1 µg/ml) for 30 minutes before they were washed again. The cells were then cultured with untreated $CD4^+$ T cells. Finally SEB (5 µg/ml) was added to the cultures.

3.2.8 Cytokine analyses

Supernatants from day 2 culture of the CD86 blocking experiment mentioned above, were collected and stored in -20° C. Before the cytokine experiment, the supernatants were thawed and sent to Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, for Luminex 14-plex cytokine analysis.

3.2.9 Reverse transcription-quantitative PCR (paper IV)

Ig κ^+, Igλ^+ single positive and double positive Ig κ^+ Igλ^+ B cells from a sentinel node of a UBC patients were sorted using FACS Aria. Messenger RNA (mRNA) was then extracted from each population and quantified by NanoDrop 1000 before cDNA synthesis was carried out using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). QT PCR was performed with primers for the activation-induced cytidine deaminase (AID) gene, fwd: 5'-CACCACTATGGACAGCCTCTTG-3', rev: 5'-ACTGTCACGCCTCTTCACTAC-3' and 2x SYBR Select Master Mix (Life Technologies), using CFX96 Real-Time System (BioRad). The data were analyzed with CFX Manager Software (Bio-Rad). The expression level of AID transcript was calculated with respect to RNA polymerase II (RPII) as a housekeeping gene. The expression levels were normalized to Ig κ as control.

3.2.10 Immunohistochemistry

In paper III, paraffin embedded tumor sections were incubated in 60º C for 1 hour and deparaffinized in xylene following rehydration in ethanol dilution series, 100%, 95% and 70%. Heat-based antigen retrieval was carried out using Tris-EDTA buffer (pH 9.0). The primary antibodies used in this paper were monoclonal mouse anti-human CD20 (Abcam, Cambridge, UK, 1:20 dilution), rabbit polyclonal anti-human IL-10 (Abcam, 1:400 dilution) and monoclonal rabbit anti-human CD38 (clone EPR4106; Abcam, Cambridge, UK, 1:100 dilution). To stain the secondary antibody, the VECTASTAIN Elite ABC kit was used according to the manufacturer's protocol and visualization was performed using diaminobenzidine (DAB) kit (SK-4100, Vector Laboratories). The sections were then counter stained with Mayer's haematoxylin (1 minute).

Following deparaffinization, rehydration and antigen retrieval as mentioned above, endogenous peroxidase and alkaline phosphatase activity were blocked with BLOXALL Blocking solution (Vector Laboratories) for double staining of the sections. Unspecific protein binding was blocked with 2.5% normal horse serum following incubation with the first primary antibody CD20 (Abcam, Cambridge, UK). The first secondary antibody, ImPRESS Alkaline Phosphatase Reagent kit (Vector Laboratories) was stained according to the manufacturer's protocol. Visualization was performed using VECTOR Blue Alkaline Phosphatase substrate kit (Vector Laboratories). Blocking unspecific protein binding as mentioned above, was then followed by second primary and secondary antibodies, rat anti-human CD3 (Bio-Rad) and ImPRESS HRP Reagent kit (Peroxidase): Goat anti-Rat IgG, mouse adsorbed (Vector laboratories), respectively. ImPACT NovaRED Peroxidase substrate kit (Vector laboratories) was used to visualize the second staining. For negative control, the primary antibodies were not added.

3.3 EVALUATION AND STATISTICAL ANALYSIS

In paper I, the comparisons were made using Student two-tailed unpaired *t*-test and Mann–Whitney *U* test using the GraphPad Prism software. The Ig λ/Ig κ L chains proportions were analyzed using Fisher's F-test.

In paper II, the data were compared using paired t-test and Mann-Whitney *U* test. P-values < 0.05 were considered significant.

When evaluating CD20 and IL-10 stained sections in paper III, 3 random field with 4x magnifications were selected and photographed in consecutive slides from each patient. The photographs were then evaluated by 2 independent observers. When evaluating CD38 stained sections, 5 fields with highest numbers of positive cells were photographed. The positive cells were then counted by 2 independent observers. Finally Kaplan Meier curves were plotted with survival data and the data were analyzed using Log-rank (Mantel-Cox) test. Data from patients with non-cancer specific death before 5 years of survival were excluded. The data in figure 3, paper III were analyzed using Mann-Whitney *U* test in Graphpad prism software.

In paper IV, the data were analyzed using unpaired *t*-test and Mann-Whitney *U* test in GraphPad Prism software.

4 RESULTS AND DISCUSSION

4.1 MULTIPLEX B CELL CHARACTERIZATION IN BLOOD, LYMPH NODES AND TUMOR FROM PATIENTS WITH MALIGNANCIES

B cells can contribute to the immune defense against cancer in multiple ways. In particular their high affinity binding Ig receptor can bind to low amount of antigens in the surroundings which makes them unique compared to other professional APCs. Thus, the role of B cells as APCs may prove to be important in cellular cancer immunotherapy. Before this study was conducted, B cells were comparatively scarcely investigated in cancer patients, which constituted one of the triggering factors to begin the study. An ideal beginning was to examine subpopulations of B cells in blood, lymph nodes and tumor from patients with solid tumor, using the Freiburg B cell classification (Wehr, Kivioja et al. 2008).

We found no difference between the proportion of blood-borne CD19$^+$ cells in patients and healthy donors. However, in metastatic lymph nodes (MLNs), we found increased transitional B cells compared to non-metastatic lymph nodes (NMLNs), which suggests recruitment of the B cell subpopulation to the MLNs. In addition, plasmablasts and CD86$^+$ expressing proportions of CD19$^+$ B cells, were increased in MLNs compared to NMLNs, which demonstrates B cell activation. This hypothesis strengthened when we studied B cell subpopulations in the tumor and compared them with their counterparts in non-malignant tissues. We observed increased proportions of switched memory B cells and plasmablasts among tumor infiltrating lymphocytes (TIL-Bs). In addition, TIL-Bs exhibited distinct skewing of Igλ/Igκ ratios among TIL-Bs demonstrating clonal B cell expansion. To investigate further, we performed a spectratype analysis of the complementarity determining region 3 (CDR3) of the B cell heavy chain receptor, IgH, in a UBC patient (Figure 3). We demonstrated a monoclonal expansion of the Vh7 family in the tumor, thus strengthening our previous Igλ/Igκ ratio observation. The CDR3 length of the expanded IgH in the tumor could also be observed as the highest peak in a tumor-associated lymph node, demonstrating that the same clonal expansion was seen in a tumor draining lymph node. To further analyze the single clonal expansion in the tumor, we cloned the CDR3 region and sequenced 3 clones. In frame 3 (FR3), we observed a WRCY hotspot motif where a serine in the corresponding germline region had been changed to glycine. Furthermore, one of the clones displayed a mutation in the CDR3 region, switching glycine into glutamic acid, which suggests ongoing somatic hyper mutation in the tumor.

Thus, our investigation revealed B cell activation in tumor-associated-tissues, which suggest tumor antigen encounter by the B cells. Increased proportions of switched memory B cells and plasmablasts suggest CD4$^+$ T cell dependent responses strongly supported by the observation of somatic hyper mutation and clonal expansion in the tumor.

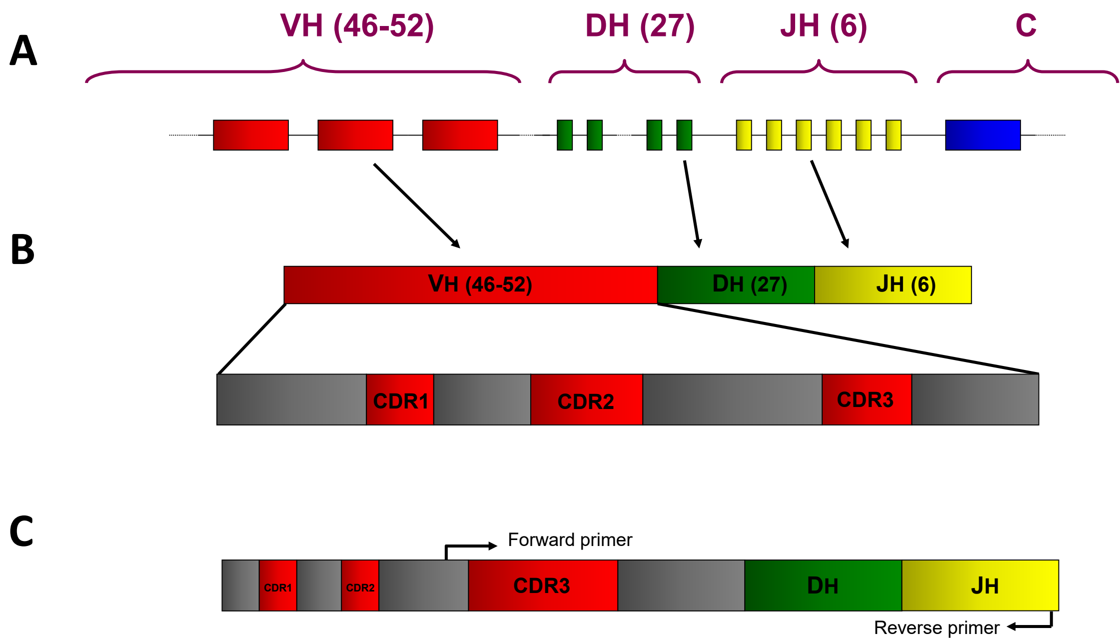

Figure 3. The schematic overview of heavy chain variable region. (A) The variable region in the genome consists of 46-52 V segments, 27 D segments and 6 J segments. (B) During receptor gene rearrangements, a V, a D and a J segment fuse together randomly to constitute a variable region, ready to be transcribed. Highly variable CDR1, CDR2 and CDR3 are included in the V segment, which comprise B cell heavy chain specificity. (C) The position of the primers used in paper I for polymerase chain reaction (PCR) and the following spectratype analysis.

4.2 DOXORUBICIN ENHANCES THE CAPACITY OF B CELLS TO ACTIVATE T CELLS IN UROTHELIAL URINARY BLADDER CANCER

Chemotherapy is currently the conventional treatments for cancer patients. However, chemotherapy gives rise to multiple side effects, including suppression of the immune system. On the contrary to this belief, we demonstrated in a previous study that chemotherapeutic drugs could enhance the immune system (Hu, Kinn et al. 2013). In addition, a combination of chemotherapy and immunotherapy has shown to be beneficial for patients with non-small cell lung cancer (Zhong, Teng et al. 2011).

In this study, we set out to investigate the effect of three conventional chemotherapeutic drugs, doxorubicin, cisplatin and irinotecan on human B cells. We demonstrated that B cells treated with doxorubicin, exhibited increased expression of the costimulatory molecule CD86 and subsequently leading to increased $CD4^+$ T cell activation in presence of the super antigen, staphylococcus aureus enterotoxin B (SEB). In addition, this effect could be inhibited by using a CD86 blocking antibody. We suggest that doxorubicin can induce CD86 expression that in turn, contributes to increased T cell activation.

Furthermore, we demonstrated that treating B cells with doxorubicin could inhibit secretion of anti-inflammatory cytokines, IL-10 and TNF-α ($p = 0.040$ and $p = 0.026$ respectively) in the co culture of B cells and T cells. When we compared IL-6/IL-2 secretion ratios, we found out increased relative contribution of IL-6 when B cells were treated with doxorubicin (two folds) further suggesting pro-inflammatory effect of doxorubicin.

To address the effect of doxorubicin on regulatory B cells (Bregs), isolated $CD19^+$ B cells were treated with doxorubicin and studied with respect to intracellular IL-10 expression. We demonstrated that doxorubicin contributed to decreased IL-10 expression. We suggest that doxorubicin inhibits Bregs and contributes to decreased total secretion of IL-10 in the B cell T cell culture.

When investigating circulating B cells from UBC patients, we observed increased CD86-expression in patients treated with neoadjuvant doxorubicin containing chemotherapy strengthening our *in vitro* findings.

We conclude that chemotherapeutic drugs, especially doxorubicin, may contribute to enhance the immune system by increasing the antigen presenting ability of B cells. Further studies are needed to dissect the effect of doxorubicin and other chemotherapeutic drugs on the immune system.

4.3 TUMOR-ASSOCIATED B CELLS IN URINARY BLADDER CANCER

Tumor infiltrating lymphocytes (TILs) have been correlated with survival in cancer patients. B cells and plasma cells are important elements of these infiltrates. Tumor infiltrating B cells (TIL-Bs) have notably correlated with survival in different malignancies (Milne, Kobel et al. 2009). However, their role in UBC patients remains obscure. In addition, we studied the expression of IL-10, a cytokine with both immunostimulatory and anti-inflammatory attributes.

In this study, we used paraffin embedded tumor sections from 33 UBC patients to investigate the existence of B cells, $CD38^+$ cells and IL-10 producing cells, in and around the tumor. Their correlation to patient survival was the next step in our investigation.

CD20 staining of the sections, revealed follicle-like structures (FLSs) where the B cells had been accumulated in the proximity of the tumor. In addition, tendency towards increased patient survival with mean number of ≥ 1 FLS was demonstrated, suggesting the importance of B cell accumulation in the tumor. Double staining with $CD20^+$ and $CD3^+$ antibodies, unfolded $CD3^+$ cell accumulation in the center of FLSs, with B cells coexisting both in the

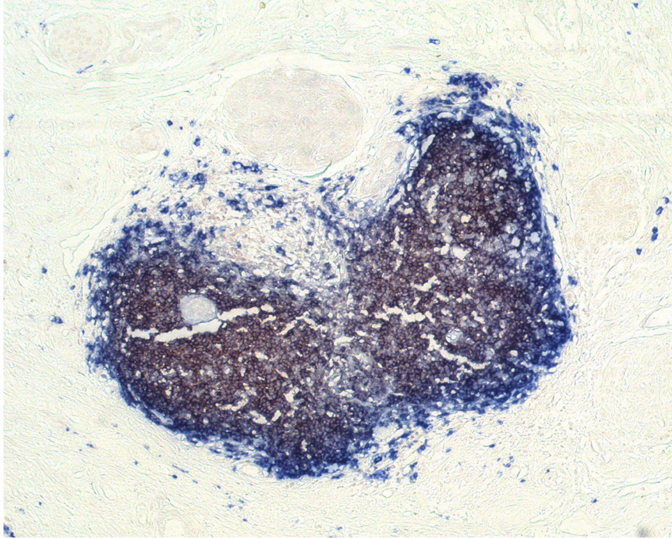

Figure 4. Representative example of a follicle-like structure. The structure comprise of B cells (blue) and $CD3^+$ cells (red/brown) which accumulate in the proximity of the tumor.

center and around the FLSs (Figure 4). This suggests B cell and T cell interaction in the proximity of the tumor of UBC patients.

Furthermore, we demonstrated that in 38% of the patients, IL-10 was expressed, whereas in 62%, no IL-10 expression was detected. Kaplan-Meier analysis illustrated a tendency towards increased survival for patients with IL-10 expression in their tumors. Median survival time of patients with no IL-10 expression was 3.0 years, whereas median survival time for IL-10 positive group was 7.39 years. Thus, in addition to FLSs, IL-10 may be important for patient survival. When we examined both $IL\text{-}10^+$ and $IL\text{-}10^-$ groups, increased mean numbers of FLSs in $IL\text{-}10^+$ sections were exposed, which supports the above mentioned survival data for both phenotypes.

We compared CD20 stained sections with consecutive IL-10 stained sections and discovered that IL-10 expression co-localized with B cells in 13% (n=3) of the patients. This suggests existents of Bregs in these tumor sections. Survival time of the patients with tumor-associated Bregs revealed to be poor compared to patients with lack of IL-10 expression (n=15) or patients with detached IL-10 expression (n=5).

Finally, we demonstrated tumor-associated $CD38^+$ cells in the tumor sections. The patients were divided into two groups; a high $CD38^+$ cell group (mean number ≥200) and a low $CD38^+$ group (mean number <200). We demonstrated a tendency towards increased survival for the high $CD38^+$ cell group. CD38 is prominently expressed on plasmablasts and plasma cells. We suggest CD38 expression has a favorable effect in survival for UBC patients.

We conclude, B cells and plasma cells in and around tumor, may contribute to survival in UBC patients. In addition, expression of IL-10 by tumor-associated Bregs may affect the survival negatively whereas IL-10 expression by other cells in the tumor may have a positive prognosis. More detailed studies are needed to dissect the role of B effector cells as well as regulatory B cells in cancer patients and to utilize the knowledge for designing more effective cellular immunotherapies.

4.4 RARE B CELL POPULATION WITH DUAL EXPRESSION OF IG K AND Λ LIGHT CHAINS IN CANCER PATIENTS

Since B cells rearrange their Ig receptors, it is generally accepted that they go through allelic exclusion, where they express a single unique heavy chain and a single unique light chain. However, in violation of allelic exclusion, B cells with dual Ig κ^+ Ig λ^+ light chain receptors have been encountered in anti-DNA transgenic mice and in human malignant B cells, e.g. in chronic lymphatic leukemia (CLL) (del Senno, Gandini et al. 1987, Li, Li et al. 2002).

Comparatively, little is known about healthy human dual light chain positive B cells and even less about Ig κ^+ Ig λ^+ B cells in patients with solid tumors. In this study, we reported the prevalence of dual light chain positive B cells (Figure 5) and characterize their phenotypes in patients with solid malignancies.

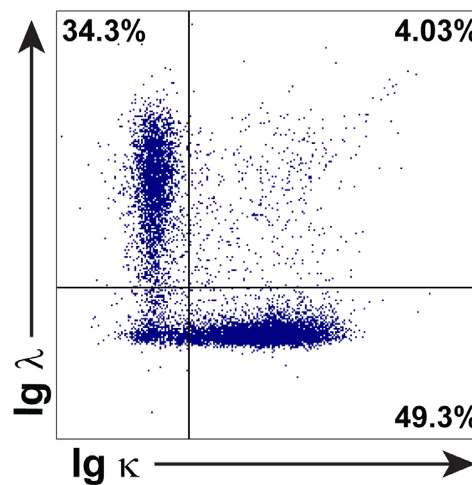

Figure 5. Representative example of Ig κ^+ Ig λ^+ B cells in a lymph node from a patient with malignant melanoma. Flow cytometry analysis demonstrates $CD19^+$ cells in the dot plot. The gate is set to illustrated Ig κ+, Ig λ+ and Ig κ+ Ig λ+ B cells.

We demonstrated increased Ig κ^+ Ig λ^+ B cell proportion of $CD19^+$ cells in MLNs compared to proportion of B cells with dual light chains in NMLNs. In addition, increased Ig κ^+ Ig λ^+ B cell proportion in sentinel nodes (SNs) compared to their counterparts in non-sentinel nodes (NSN) was observed which suggests emergence of Ig κ^+ Ig λ^+ B cells is due to the tumor environment. This hypothesis was strengthened by our observation of TIL-Bs where increased proportions of B cells with dual light chains compared to Ig κ^+ Ig λ^+ B cells in non-sentinel nodes, were detected ($p<0.001$).

Ig κ^+ Ig λ^+ B cell population was present in both switched memory and activated B cell populations and they exhibited increased proportion of respective B cell subpopulations, in tumor associated lymph nodes compared to non-metastatic and non-sentinel lymph nodes respectively. This further supports our previous data about emergence of dual light chain B cells being due to the tumor environment.

Furthermore, we detected no expression level of RAG2 in Ig κ^+ Ig λ^+ B cell RNA from a SN of a UBC patient, but demonstrated decreased expression of AID transcript in B cells with dual light chain receptor, compared to single light chain B cells from the same SN. Lack of RAG2 and low levels of AID expressions, confirms inability of Ig κ^+ Ig λ^+ B cells to undergo somatic hypermutation, receptor rearrangements.

To investigate whether tumor environment is a contributing factor behind the increased prevalence of Ig κ^+ Ig λ^+ B cells, we cultured PBMCs from a healthy donor with supernatant from the UBC cell line, 5637 (70% confluence). We demonstrated that the proportion of Ig κ^+

Ig λ^+ B cells increased with increased concentration of the supernatant, during 4 days of culture. Thus, we revealed tumor environment to be an important contributing element in increasing the Ig κ^+ Ig λ^+ B cell population.

We conclude the emergence of Ig κ^+ Ig λ^+ B cells in tumor associated specimen, is likely due to tumor environment. Many questions about the function of the B cells with dual light chain expression in cancer patients still remain to be answered. For example, whether they are effector cells or Bregs. More studies are needed to understand the role of Ig κ^+ Ig λ^+ B cells in cancer patients.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Previously we have demonstrated that activation and expansion of sentinel node derived, tumor reactive $CD4^+$ T cells are promising for adoptive immunotherapy in cancer patients (Karlsson, Marits et al. 2010). For activation and subsequently expansion, T helper cells require encountering antigen in major histocompatibility complex (MHC) class II pocket of antigen presenting cells (APCs). Activation and expansion of APCs may therefore prove to streamline our immunotherapy setup further. There are three major APCs in lymph nodes, macrophages, dendritic cells and B cells (Itano and Jenkins 2003). B cells are the most abundant of the three APCs in the lymph nodes. In addition, their highly specific antigen binding receptor makes them unique in comparison to the other two APCs. Thus, tumor specific B cells may be effective APCs in the context of our $CD4^+$ T cell based immunotherapy.

In our studies, we have demonstrated increased plasmablast and switched memory B cell populations, in addition to signs of somatic hypermutation and clonal expansion in tumor associated tissues suggesting a tumor antigen presenting ability of B cells. Furthermore, we confirmed the existence of B cells and plasma cells in tumor sections and demonstrated B cell-T cell interaction in follicle like structures (FLSs) in the proximity of the tumor. Moreover, we observed that increased FLSs and plasma cells in and round the tumor contributed to positive patient prognoses whereas Bregs seemed to be disadvantageous in patient survival. In addition to our studies, others have demonstrated B cell immune response against solid tumors (Shimabukuro-Vornhagen, Schlosser et al. 2014). However, B cells need further studies to dissect their APC abilities. The existence of regulatory B cells (Bregs) among sentinel node derived tumor specific B cells and the depletion of them from the culture intended for T cell expansion, need to be addressed.

The next step is to set up an effective combination of cytokines and growth factors to expand the tumor specific B cells and keep them alive in culture during a longer time period. Candidates to start with could be IL-4 and CD40L (Banchereau 2015).

Chemotherapy is a conventional treatment for cancer patients but can suppress the immune system as a side effect. This can lead to a destructive immuno-chemotherapy combination as chemotherapy can have harmful effect on immunotherapy if administered in a sensitive stage of *in vivo* lymphocyte activation and proliferation. On the contrary, we demonstrated that doxorubicin increases the antigen presenting ability of human B cells. There are also reports about improved survival time of patients who received both chemotherapy and immunotherapy (Zhong, Teng et al. 2011). We suggest an optimized plan for time and dose of drug administration, may improve combination of both treatments.

As a next step, APCs isolated from patients treated with neoadjuvant chemotherapy should be treated with autologous tumor extract and co-cultured with $CD4^+$ T cells from the same patients, to investigate tumor antigen presenting properties of the APCs. Cells from the patients with no chemotherapy treatment can be used for comparison. Furthermore, screening the

immune cells with other chemotherapeutic drugs is necessary to have a more detailed scheme of the drugs with a positive effect on the immune system. To broaden this detailed scheme, other cell types should be studied. The effect of chemotherapeutic drugs on the tumor specific properties of cytotoxic T cells, regulatory T and B cells, natural killer (NK) cells and macrophages are highly important to investigate. Furthermore, studying different concentrations of the drugs with moderate effect on the immune cells may result in finding an optimized setup in which they can be more effective on the immune system. Finally, the mechanism with which the chemotherapeutic drugs enhance tumor antigen presenting ability of the APCs should be examined to increase the understanding of the drug properties.

Among B cell subpopulations isolated from patients with solid malignancies, we found rare populations with dual light chains. A finding that contradicts the theory behind allelic exclusion. Interestingly, increased Ig κ^+ Ig λ^+ B cells proportions of $CD19^+$ cells were detected in tumor and tumor associated lymph nodes, suggesting contribution of tumor environment in the increased emergence of the B cell subpopulation. To increase our understanding of B cells as APCs in the context mentioned immunotherapy, the role and function of Ig κ^+ Ig λ^+ B cells need to be studied further. Based on our study, we know that Ig κ^+ Ig λ^+ B cells isolated from tumor associated tissues, can be found among activated, as well as IgG^+ B cells, suggesting tumor antigen engagement. Furthermore, we demonstrated lack of RAG II production and decreased levels of AID transcript in sentinel node isolated Ig κ^+ Ig λ^+ B cells in a UBC patient, which illustrates the inability of the dual receptor B cells to undergo somatic hypermutation and class switching. There are reports about Ig κ^+ Ig λ^+ B cells in mice, which possess one, self-reactive and one non-autoreactive receptor, making the B cells partially autoreactive (Liu, Velez et al. 2005). In this case, B cells with dual receptors may not be very optimal in our immunotherapy setup and should be depleted in *in vitro* cultures. In addition, Ig κ^+ Ig λ^+ B cells with possible regulatory properties will not be responding in synergy with the desired effector B cells in our $CD4^+$ T cell based adoptive immunotherapy.

Thus, further studies are needed to dissect the role of Ig κ^+ Ig λ^+ B cells and their properties in cancer patients. The next step is to examine tumor antigen presenting characteristic of dual positive B cells in cancer patients, by sorting the cells and treating them with autologous tumor extract before co-culturing with $CD4^+$ T cells as responder cells. B cells with single light chains can be used as control in this setup. In addition, the proportion of Ig κ^+ Ig λ^+ B cells in cancer patients with neoadjuvant chemotherapy should be compared with their counterparts in patients without chemotherapy to address the effect of chemotherapeutic drugs on the emergence of B cells with dual receptors. Additionally, mRNA screening of the Ig κ^+ Ig λ^+ B cells are needed to further understand their properties.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kroppens immunförsvar består bland annat av vita blodkroppar som försvarar oss mot främmande mikroorganismer. De känner igen främmande strukturer som finns på ytan av bland annat virus, bakterier och parasiter. Immunförsvaret kan delas in i en medfödd och en adapativ del. Immunceller som hör till det medfödda immunförsvaret kan snabbt agera vid mot en inkräktare. De adaptiva immuncellerna tar längre tid på sig att reagera, men kan bilda så kallade, minnesceller och bidra till ett långvarigt, specifikt och starkt immunförsvar.

Cancer bildas när fel uppstår i arvsanlaget (DNA) hos kroppens celler. Detta gör att de kan snabbt föröka sig och så småningom bilda tumör. Dessa celler kan i många fall uppvisa proteiner på sin yta som uppfattas av immuncellerna som främmande. Immuncellerna tar då hand om cancern hos friska människor. Cancercellerna kan dock i vissa fall lära sig att undgå kroppens immunförsvar genom att till exempel gömma de proteiner som uppfattats som främmande av immunförsvaret. Detta leder till tumörtillväxt och metastasering.

Immunterapi handlar om att förstärka immunförsvaret så att tumörceller kan bli igenkända och eliminerade av de medfödda och adaptiva immuncellerna. Ett sätt, är att ta ut celler från patientvävnader som tagits vid operation och odla en variant av de adaptiva immuncellerna som kallas T celler. Man ser till att de blir fler och effektivare mot tumören innan de ges tillbaka till patienten via blodet.

T cellers roll i kroppen är bland annat att producera signal-molekyler, så kallade cytokiner med vilka de kan leda och dirigera uppgifter till andra immunceller som i sin tur kan döda cancercellerna. För att en T cell ska kunna skicka tumörspecifika signaler till de andra immuncellerna, behöver den blir presenterad till främmande tumörämnen, så kallade tumörantigen. Det finns en del immunceller som tar upp och presenterar tumörspecifika proteiner till T cellerna. De kallas för antigenpresenterande celler. En av de antigenpresenterande cellerna kallas för B celler, som också hör till det adaptiva immunsystemet.

Denna avhandling handlar om hur B celler påverkas av tumör men även av cytostatikabehandling hos cancer patienter. I det första arbetet studerade vi olika utvecklingsstadier av B celler i cancer patienter. Vi visade att fler B celler i sina aktiverade och mogna tillstånd existerade i tumör och metastaserade lymfkörtlar som tydde på att de genomgått en tumörspecifik respons. Vidare visade vi att en specifik B cell i tumören hade förökat sig, troligtvis på grund av att den hade hittat tumörantigen och visat det till T cellerna som in sin tur hade signalerat B cellerna att expandera. Vi drar slutsatsen att B celler hos cancerpatienter aktiveras mot deras tumör och troligtvis presenterar antigen till T cellerna.

Cytostatika behandling är en terapi som ofta leder till cancer förstörelse men samtidigt kan också orsakar många biverkningar hos patienter. Bland annat är det allmänt accepterat att de försvagar immunförsvaret. I det andra arbetet utredde vi hur tre sedvanliga cytostatika som cancer patienter ofta får som behandling, nämligen cisplatin, doxorubicin och irinotecan, påverkar B celler. B celler isolerades från friska bloddonatorer och behandlades med

ovannämnda cytostatika. Vi avslöjade att B celler, till skillnad mot den allmänna uppfattningen om cytostatika, blev mer aktiverade av doxorubicin. Vår studie framhävde även att antigenpresenterande förmågan av B celler som var behandlade med doxorubicin ökade. Vidare visade vi att B celler i blodet hos patienter med cancer i urinblåsan, som var behandlade med doxorubicin-innehållande cytostatikapaket, hade ökad antigenpresenterande förmåga. Vi drar slutsatsen att med rätt tids och dosplanering av cytostatika, kan både cytostatika behandling och immunterapi kombineras som en framtid strategi att behandla cancerpatienter.

I flera studier har forskare visat att vita blodkroppar som finns in i tumörvävnaden, har stor positiv betydelse för patienternas överlevnad. I arbete III, rapporterar vi att B celler runtom tumör hos patienter med urinblåsecancer, bildar speciella strukturer tillsammans med T celler som visar täcken på ett samspel mellan dem i tumören. Vidare belyste vi att antal B/T cellstrukturer verkade ha en positiv verkan på patientprognosen. Cytokiner som nämndes ovan, produceras av immunceller för att väcka immunförsvaret. Det finns dock cytokiner som produceras för att hämma immunförsvaret. Detta är en funktion hos så kallade, regulatoriska immunceller, som skyddar kroppen från att ta skada av för stor aktivitet av immunförsvaret. En sådan cytokin kallas interleukin 10 (IL-10). IL-10 kan dock både hämma och stimulera immunförsvaret. Vi studerade även denna cytokin i tumören hos urinblåsecancer-patienterna. Vi såg att IL-10 som var producerat av regulatoriska B celler, hade en negativ inverkan på patienternas överlevnad medan IL-10 producerat av andra celler i tumören än B celler hade en positiv effekt. Vi drar slutsatsen att B celler i tumör är positivt för patientöverlevnaden men inte när de producerar IL-10. IL-10 producerat av andra celler är dock gynnsamt.

B celler kan känna igen tumörantigen med hjälp av receptorer som finns på sina cellytan. Den allmänna uppfattningen är att när en B cell kommer ut från benmärgen har den endast en typ receptor, specifikt mot en enda antigen. I sista arbetet detekterade vi B celler med två olika receptorer på sin yta hos cancer patienter, nämligen Igκ och Igλ. Dessa B celler fanns i ökat antal i tumörassocierade lymfkörtlar jämfört med icke-tumörassocierade lymfkörtlar. Detta väckte misstanken att tumören och dess miljö orsakade deras uppkomst. Vi kunde påvisa att de fanns bland aktiverade B celler men hade inte förmågan att utvecklas vidare eller att bli effektivare mot tumörantigen. När vi odlade B celler i medium från en tumörcellslinje, ökade antalet B celler med dubbla receptorer. Vi drar slutsatsen att $Ig\lambda^{+}$ $Ig\kappa^{+}$ B celler samlas I tumörassocierande vävnader, troligtvis på grund av tumörmiljön. Mer forskning är nödvändigt för att förstå rollen av B celler med dubbla receptorer hos cancer patienter.

Med denna avhandling har vi visat att B celler har stor potential att användas som antigenpresenterande celler i en T cellsbaserad immunterapi. Framtida projekt bör handla om hur man bäst kan optimera denna typ av immunterapi genom att odla B cellerna tillsammans med T cellerna för att förstärka patienterna immunförsvar.

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