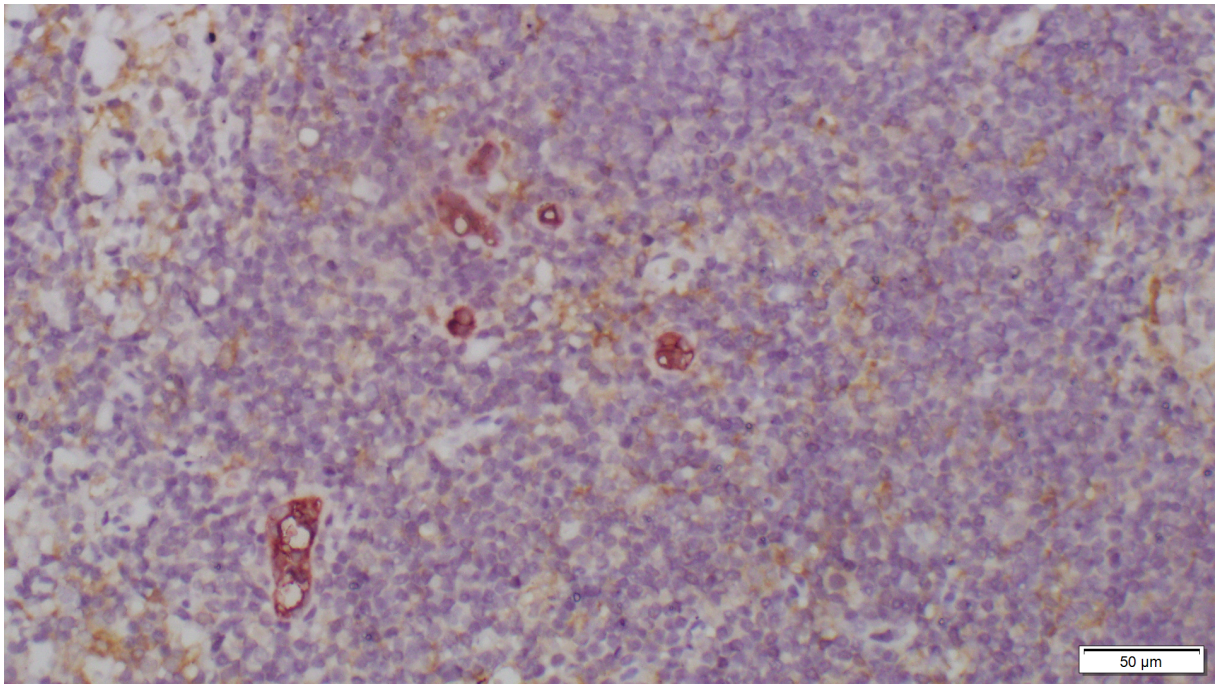
Immunohistochemistry – principles and methods

A literature study combined with practical experiments



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Abstract

Immunohistochemistry (IHC) is a method used in the daily diagnostic routine by all departments of pathology in Norway, and uses the principal of antibodies recognizing antigens in different tissues. It is an approved supplemental examination of the tissue in addition to basic Hematoxylin/Eosin-staining. The method is used to look for specific cells or markers of interest, and to conclude whether these are present or not. These cell markers can be of vital importance, especially in cancer diagnostics to make a definite diagnosis. It is also of great importance in the case of metastatic tissue, to simply clarify from which organ a tumor originates. IHC is also a method for discriminating which patients who can benefit from specific cancer therapy, and many markers are also important for suggesting prognosis. The method can be performed on both formalin-fixed-paraffin-embedded (FFPE) tissue samples and on frozen sections.
This thesis will give an introduction to the principles of antigens and antibodies, and relate this to our immune system. In addition the basic of different procedures within immunohistochemical staining will be presented. This is all based on available literature. A protocol for performance of IHC with step-by-step explanation will be given. Finally, practical experiments with implementation of a new antibody based on my own work will be presented.

Introduction

Antigens and antibodies

An antigen is any substance that gives an immunological response. Most antigens are proteins from a foreign organism. The part of an antigen that interacts with an antibody is called an *epitope.* An antigen may have several epitopes, and a specific antibody recognizes each of these epitopes. *(Figure 1)*

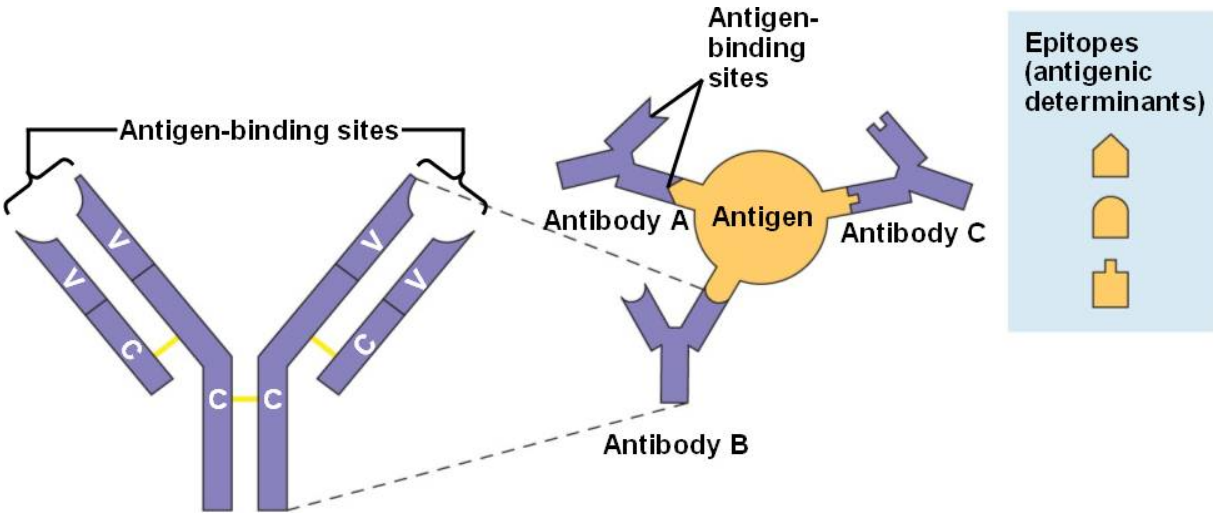

Figure 1: How antigens interact with antibodies (1).

Antibodies are synthesized exclusively by B-lymphocytes, and can be in a soluble form as serum proteins or membrane-bound to the surface of mast cells or B-lymphocytes (see chapter about the immune system, page 12). When the immune system detects foreign antigens, it starts to produce antibodies. The antibodies can with very high specificity bind to a particular antigen. A microbe may have several antigenic binding sites (epitopes) where different antibodies can bind. Antibodies belong to a family of proteins called gamma globulins, and are also called immunoglobulins. The basic structures of the immunoglobulins are the same for all antibodies: a Y-shaped protein that comprises two light-chains and two heavy-chains connected by disulfide-bonds.

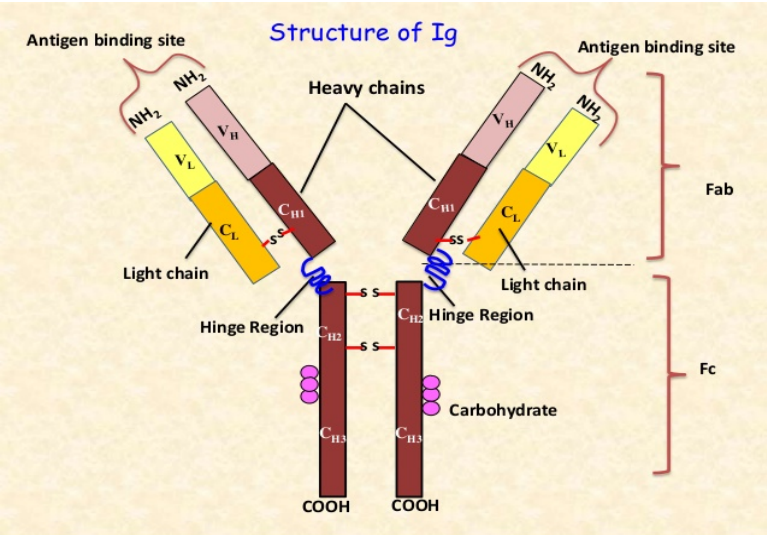

Figure 2: The antibody structure of IgG as an example (2).

Both heavy chains and light chains are identical to each other, and each chain contains a constant and a variable region. The variable regions contain the action-site of the immunoglobulin, binding the antigen to the antibody. The action-sites are also known as *paratopes.*

The constant region of the heavy chain is divided into domains, also known as regions of sequence homology. Between the first and second domains of the constant region of the heavy chain, there is an interdomain region known as the hinge region. The hinge region makes the antibody more stretchable and flexible. However, this region is only found in IgD, IgG and IgA (3)*(Figure2).*

The antibody structure can also be divided into two different fragments. The antigen-binding (Fab) fragment is the region that binds to antigens, whereas the fragment crystallisable (Fc) region is the tail of the antibody. The Fc-region interacts with cell surface receptors called Fc-receptors found on macrophages, neutrophils and natural killer (NK)-cells. This allows the antibody not just to interact with antigens, but also to activate the immune system (4).

Based on the structure of the constant region, immunoglobulins are divided into five major classes; IgG, IgA, IgM, IgD and IgE. It is the structure of the heavy chains that decides which class an antibody belongs to.

Subclasses of immunoglobulins

IgG – gamma heavy chains

IgG accounts for about 10-20% of plasma protein, and is the major class of the five classes of immunoglobulins. We also find it in lymph fluid, cerebrospinal fluid and peritoneal fluid. It can bind different types of pathogens; bacteria, viruses and fungi and neutralize them through the process of phagocytosis and ADCC (antibody dependent cell mediated cytolysis). IgG also activates the complement system. Another important feature of IgG is that it is the only antibody that can pass through the placenta to provide protection to the fetus in utero. There are four subtypes of IgG, all playing different parts in the immune response. Determination of subclasses may be useful to diagnose a potential antibody deficiency (4, 5).

IgA – alpha heavy chains

Of all immunoglobulins in secretions, such as saliva, tears and gastric fluids, IgA is the most important. IgA is a neutralizing antibody, and can attach to and penetrate surfaces of invading pathogens, mainly protecting us against gastrointestinal and respiratory microorganisms. In humans, there are two subtypes of IgA: IgA1 and IgA2. IgA1 makes up for approximately 85% of total IgA concentration in serum. It has a good immune response to protein antigens, whereas IgA2 plays an important role in the mucosa of the eyes, airways and GI-tract fighting against polysaccharide- and lipopolysaccharide-antigens. In blood and tissue fluids IgA can be found as a monomer, but in secretions, such as saliva, tears, milk and gastric fluids IgA is dimeric. , In these secretions, IgA is actively secreted where it effectively aggregate antigens and prevent their penetration of the body surface (6).

IgM – mu heavy chains

IgM is constructed by five monomers, and can in theory have 10 antigen binding sites. These units each comprise two heavy chains and two light chains, and IgM is the largest antibody in the circulatory system. Due to the size, IgM cannot transverse blood vessels, and is restricted to the blood stream. IgM is the first antibody produced in response to an antigen, and is the most effective immunoglobulin in the early stages of disease, being responsible for agglutination and cytolytic reactions. This makes IgM very efficient in neutralizing microorganisms, and prevents further spread of disease. Because IgM is found in the serum without any prior contact with an antigen it is also called a “natural antibody” (7, 8).

IgD – delta heavy chains

As a part of an ancestral surveillance system, IgD is involved in microbial sensing and immune activation. IgD is expressed in the membrane of immature B-lymphocytes, also known as B-cells. IgD participate in the activation of B-cells, a role IgD share with IgM. The functional difference between IgM and IgD action on B-cells is not clear. Similar to IgA, IgD is also produced in a secreted form, which can be found in blood plasma. It has

been found that a class switch from IgM to IgD takes place in the upper respiratory tract where IgD activates basophilic granulocytes and mast cells, which gives a layer of mucosal protection. The total function of IgD is not completely understood, and research is ongoing (9, 10).

IgE – epsilon heavy chains

This antibody controls allergic reactions, and can only be found in mammals. It is the least abundant isotype of all antibodies, but can trigger the most powerful immune reactions. IgE secretion leads to activation of mast cells and basophilic granulocytes. These cells have specialized surface receptors, called Fc-epsilon-receptors. When IgE-molecules attach to these receptors, the cells become activated. This again leads to the release of different substances such as histamine, serotonin, proteases and different cytokines, which are stored inside the cells. This is called degranulation, and leads to a wide spread allergic reaction in the vascular system and surrounding tissue with vasodilation and increased permeability of the blood vessels. This reaction is called anaphylaxis, and can give circulatory shock within a few minutes. IgE also plays an important role in the host defense against parasites, as it induce phagocytic cells to localize and neutralize pathogens (11, 12).

The antibody – antigen reaction

Antibodies bind to antigens via non-covalent forces. These forces are dependent on a short distance between the sites that interact. A good 3D fitting between the antigen-antibody binding sites is therefore essential. Another important feature is that a non-covalent force is reversible . That means that an antibody-antigen complex also can dissociate (13).
This equilibrium reaction can be illustrated as followed:

$$antigen + antibody \leftrightarrows antigen\text{-}antibody\ complex$$

This equilibrium presupposes a continuous association and dissociation between the epitopes and the paratopes. Several factors, such as temperature, pH, ionic strength, enzyme treatment, concentration of antibodies and antigens, number of antigen sites per cell and duration of incubation can affect the reaction between an antigen and an antibody (8).

The strength of the binding and the affinity depends on in which direction the equilibrium is pushed. It can either be pushed towards the formation of an immune complex, or the dissociated single components. The concentration of immune complexes and the single components at equilibrium can be expressed by the equilibrium constant, K (Figure 7) (8).

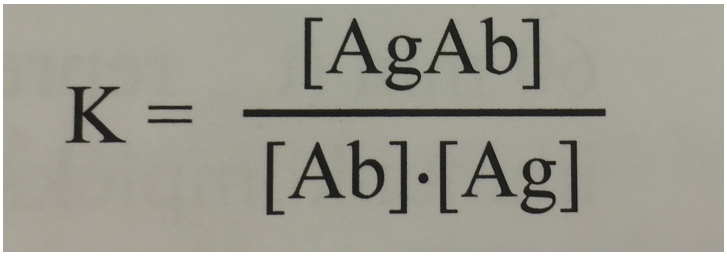

Figure 7: The expression of the equilibrium constant, K (8).

Temperature is a factor of great importance when it comes to affecting the equilibrium constant. In practice, we can see this when performing immunohistochemistry (8). If we have a problem with the antibody binding, a good solution to the problem might be to incubate the sections with antibodies at a lower temperature over a longer period of time.

The immune system

The most important function of the humoral immune system is the production of antibodies. The system is organized into primary and secondary lymphoid organs. The primary lymphoid organs comprise the red bone marrow and the thymus gland. In the bone marrow, the formation of lymphocytes takes place, and progenitor cells differentiate into mature B-lymphocytes, or B-cells (bone marrow derived lymphocytes). Some of the progenitor cells travel to the other primary lymphoid tissue, the thymus, to differentiate and mature. These cells are called the T-lymphocytes, or T-cells (thymus-

derived lymphocytes) (14). Both the B- and the T-lymphocyte is derived from the lymphoid progenitors, which represents the adaptive immunity *(Figure 8)* (15). Lymph nodes, tonsils and the spleen are major secondary lymphoid organs (SLOs). This is where the lymphocytes interact with each other and other non-lymphoid cells. In addition, we have tertiary lymphoid structures (TLSs). In TLSs, lymphoid cells accumulate and organize themselves in structures, which are similar to the SLOs, without being encapsulated. Immune cells have been found to form TLSs during chronic inflammatory reactions. It is well established that TLSs can be found in chronic inflammatory diseases, such as rheumatoid arthritis, and recently also in cancers (16, 17).

Before the lymphocytes are released into the blood- and lymph, they undergo a strict process of selection. This process tests and selects cells that can recognize foreign material from the body itself. If the lymphocytes were to attack and destroy healthy body tissue by mistake, the consequences would be severe. That is why most lymphocytes die soon after development without ever functioning.

The B- and T-lymphocytes carry out the two broad classes of adaptive immune responses, the antibody responses and T-cell mediated immune responses, respectively (8, 12).

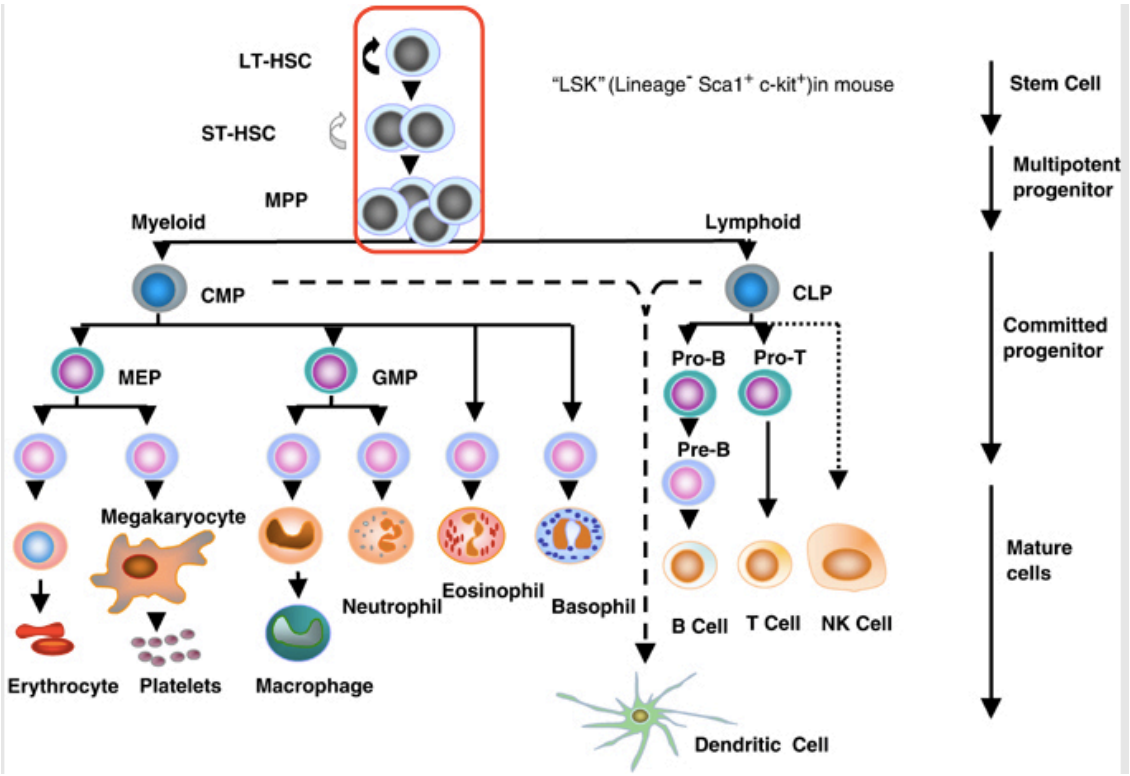

Figure 8: The hierarchy of hematopoietic cells (15).

T-lymphocytes

The T-lymphocytes produced in the bone marrow leave the bone marrow at an early stage, and continue their development in the thymus gland. During the process in the thymus, the T-lymphocytes (or thymocytes, or T-cells) develop a T-cell receptor (TCR). The development of the TCRs is antigen-independent.

The development of the receptor is a multi-step process that includes positive selection in the cortex of the thymus, where the T-cells with moderate or strong binding of their TCR to epithelial cells moves on to the next round, whereas T-cells with weak or no binding dies. In the next round the binding to self-antigen is tested. This happens with help from dendritic cells, macrophages and other cells in the thymus. T-cells with strong binding of their TCR to self-antigen die and T-cells with moderate or weak binding to self-antigen live. This is called negative selection. Both the epithelial cells in the cortex, and the other cells helping in this process contain a Major Histocompatibility Complex (MHC) that presents antigen, binds to, and test the TCR (13, 18).

A special feature with the T-cells is that they only react when foreign material is localized in the membrane of other cells. As opposed to the B-cells, the T-cells cannot react to antigens in soluble form. To develop their effector functions, the T-cells have to cooperate with other types of cells called professional antigen presenting cells (pAPC). The pAPC includes macrophages, dendritic cells and B-cells. The pAPCs constantly sample the environment for antigens. If they find an antigen, their intracellular machinery breaks down the molecules and makes them recognizable to T-lymphocytes by presenting them on MHC. In addition they shuttle antigens from tissues to SLOs (19).

All T-cells have the co-receptors CD4 and CD3. During the development in the thymus, the T-cells also become either CD4-positive or CD8-positive. These are also co-receptors giving the T-cell different effector functions. CD8+-T-cells are also called cytotoxic T-cells. They contain stored lytic granules, filled with cytotoxins. When a CD8+-T-cell arrives at a site of infection, they recognize peptides presented via MHC by infected cells. When the CD8+-T-cell binds to the MHC-molecule, it releases the cytotoxins from the lytic granules, and the target cell starts to die. The CD8+-T-cell then moves on, produces more cytotoxins and is ready to kill the next target.

CD4+-T-cells are subdivided into two groups, called CD4+Th1 cells and CD4+Th2-cells. These cells are distinguished by the sets of cytokines they make and which effect they have on the immune response. The Th1-cell develops a cell-mediated immune response, working with macrophages. They help the macrophages to become more efficient in the killing of pathogens at the infection site. The Th2-cells develops an antibody-mediated immune response, working with B-cells. They help the B-cells to proliferate and form plasma cells. This is why these cells are also called helper Th2-cells (18, 20). *(Figure 9)*

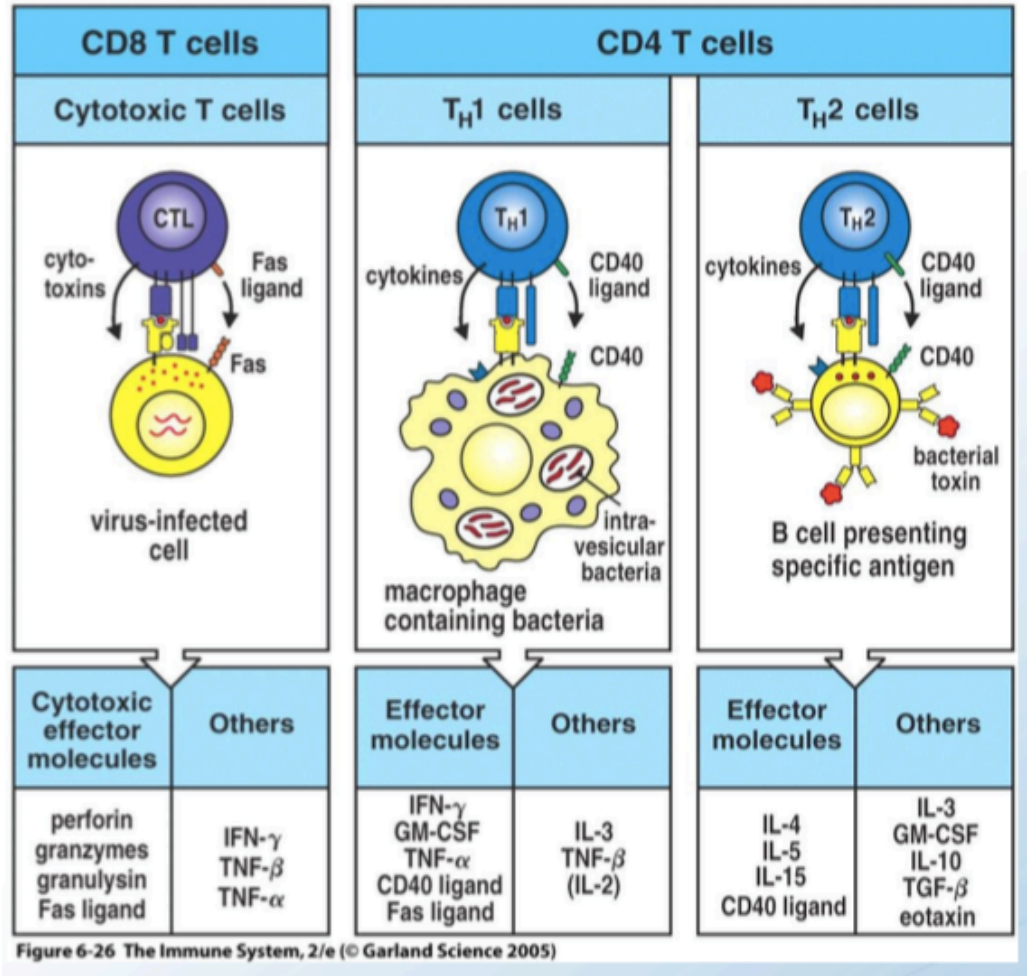

Figure 9: The three types of effector T-cells (20)

B-lymphocytes

The B-lymphocytes are produced in the red bone marrow, and finish their maturation here,. They are called B-lymphocytes, or B-cells because they finish the maturation in the bone marrow. During the maturation the immature B-cells develop an antigen-receptor called B-cell receptor (BCR). The BCR is different in each B-cell clone, and can be produced in millions of forms. The development of the BCR is (similar to the TCR) independent of antigens, but have the ability to interact with antigens when the B-cell has settled in a secondary lymphoid organ. The BCR may be better known as immunoglobulin, or antibodies!

During the maturation of B-cells the BCR are rearranged through a series of events including rearranging of the immunoglobulin genes. B-cells are also tested, and the cells that bind to self-surface antigens are removed by negative selection. When tested and approved, immature B-cells migrate to the peripheral lymphoid organs, to see if they find any cells to interact with (20).

In the lymph node (or any other peripheral lymphoid tissue such as TLSs), the B-cell interacts with follicular dendritic cells (fDC), antigens and cytokines. This drives the maturation of B-cells. Mature B-cells that interact with antigens activate and start producing daughter cells that share the same antigen specificity. This is called clonal expansion and leads to the formation of germinal centers in the lymph node. The B-cell differentiates to plasma- or memory cells. Plasma cells are examples of B-effector cells, and the only function they have is to secrete large amounts of antibodies. One can distinguish between an immature and a mature B-cell by biomarkers. Immature B-cells typically expresses CD10 and CD34, whereas mature B-cells express CD20 and CD22 (21).

Antibodies are not in themselves toxic or dangerous to pathogens. The mission of the antibodies is simply to bind tightly and hold on to the antigens of the pathogens. By doing this, several things could happen:

1. Neutralizing viruses and toxins by binding to the surface of the pathogen and cover it up. In this way the pathogen cannot bind to human cells and cause an infection.
2. Opsonization of the pathogen by binding to it with the antigen binding sites, and to a phagocytic cell with the Fc region promotes phagocytosis. When the antigen

is marked with an antibody, phagocytic cells get a signal to "eat" them. In this way the antigen gets degraded and can not cause any harm.

3. Activate the complement system through the classical pathway which leads to further promoting of phagocytosis by activating the complement receptors of phagocytes (20).
4. Antibody dependent cellular cytotoxicity (ADCC): A process where cytotoxic effector cells kill antibody-coated targets via a non-phagocytic pathway. The process is characterized by the release of the cytotoxic granules or by expression of cell death-inducing molecules. NK- cells, monocytes, macrophages, neutrophils, eosinophils and dendritic cells are cell types that mediate ADCC. B-cells and B-cell-tumors are destroyed by this mechanism when treated with the therapeutic monoclonal antibody Rituximab (20, 22).

After the termination of an infection, regulatory mechanisms stop the immune response. This allows damaged tissue to be repaired by reducing the inflammation. Effector T-cells get signals to die, but plasma cells are allowed to persist. This means that pathogen-specific antibodies can maintain in the circulation for years. The next time the same pathogen comes along, the antibodies are ready to target them right away, and the body can get rid of the infection in the most efficient way.
Long-lived memory B-cells have the power of immunological memory. This ensures a faster and stronger response during a second infection (20).

Secondary lymphoid orrans (SLOs) and Tertiary lymphoid structures (TLSs)

In SLOs, the B and T-cells settle in different areas. These are called the B- and T-cell areas. When explaining how the cells are organized, the lymph node is a good example. In lymph nodes, the B-cell area is located in the cortex, whereas the T-cell area is in the paracortical region.
The SLOs are the sites where mature, naive B-cells encounter specific antigen. When this happens, the B-cells are in the T-cell area, and become activated by antigen-specific Th2 helper cells. The T-cells give the B-cells signals that activate them and make them

proliferate and differentiate further. Naive B-cells can also become activated in "their own" zone by meeting fDCs in the germinal centers (20).

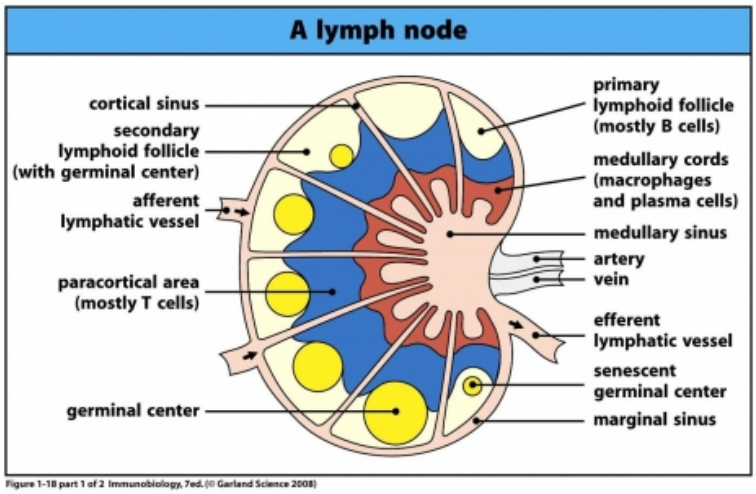

Figure 10: Schematic structure of the lymph node (20).

The clusters of lymphocytes are surrounded by specialized blood vessels called high endothelial venules (HEV), which through most of the lymphocytes enter. The lymphocytes recognize and adhere to the HEVs via cytokines, and squeeze through them and arrive at the cortical regions of the node. HEVs are in these manner key players in the process of giving an appropriate immune response by sampling lymphocytes from the blood stream so that the lymphocytes can meet other cells and become activated. The entry of the naïve lymphocytes is controlled by dendritic cells, which modulate the phenotype of the HEVs (23).

In TLSs, the lymphocytes also settle in different areas. Typically, TLSs comprise aggregates of B-cells in a network of follicular dendritic cells (fDCs). T-cells and HEVs surround these aggregates *(Figure 11).* Because TLSs are not encapsulated, they get a direct antigenic stimulation from the surrounding environment. This difference can be of importance in cancer. In solid tumors such as breast-, ovarian-, non-small-cell-lung- and colorectal cancer, TLSs have been described (17, 24-26). The presence of these structures is supposed to be associated with a better patient outcome, but the data on

TLS development and correlation with clinical outcome in cancer are still limited (17, 27).

HEVs have recently also been found in relationship with stroma in the vicinity of human cancers. This is of great importance because it raises the possibility that naive lymphocytes can be recruited into the tumor site, where the pro-inflammatory environment could generate cancer-destroying effector lymphocytes without redistribution of the lymphocytes from draining lymph nodes (28).

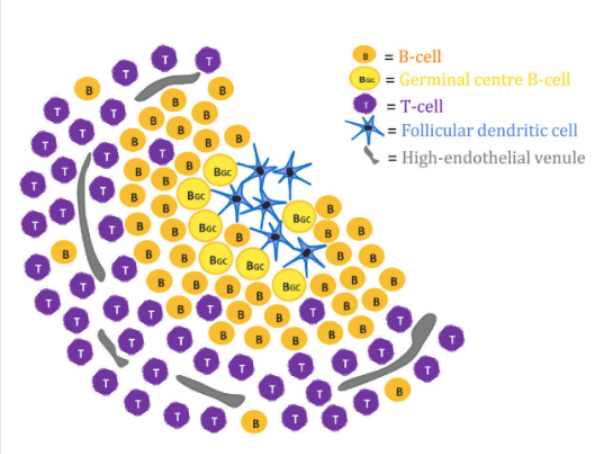

Figure 11: Schematic structure of TLSs (17)

Immunohistochemistry can be used to visualize how the lymphocytes are organized in TLSs *(Figures 12 and 13).*

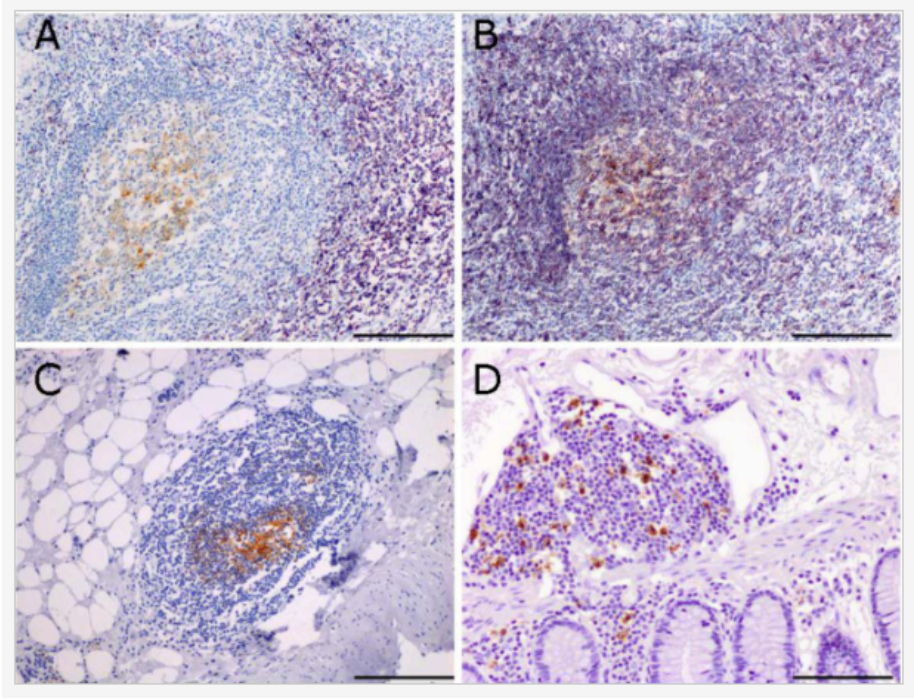

Figure 12: Immunohistochemistry of TLSs in human colorectal cancer. In figure A, T-cells are stained purple; in figure B CD20+ B-cells are purple. In figure A-C, CD21+ follicular dendritic cells are stained brown. Figure D shows brown-stained DC-LAMP+ dendritic cells. The figure visualizes the compartmentalization of the different cells. The Scale bar indicates 100μm (29).

Principles of immunohistochemistry

The principle of immunohistochemistry (IHC) has been known since the 1930. Coons et al. reported the first IHC study in 1942, in which they identified pneumococcal agents in infected tissue (30). Today, IHC is a widely used method for localizing antigens in biologic cells or tissues, based on interaction between antigens and antibodies. It is used for disease diagnosis, drug development and biological research (31). Also, antibodies are very selective in recognizing their antigens, which makes them a perfect tool for IHC. Under natural conditions, antigens induce the production of antibodies. This process is imitated when the antibodies for IHC are made. Synthesized antigens with the structure of a naturally occurring protein are injected into host animals (rat, sheep, goat etc.), which then produce antibodies against the synthesized antigen (32).

Direct-, indirect- and sandwich method

The IHC-technique is a multi-step process, and there are three main methods: direct, indirect or enzymatic (sandwich) method. First, a primary antibody must bind to the tissue antigen one wants to identify. This is the most critical antibody because without any binding, there will be no result. Then, a secondary antibody must bind to the primary antibody. Depending on the type of experiment, one can also add a tertiary antibody. In the indirect method, the secondary, or tertiary antibody has to be bound to a reporter-label in order to visualize the antigen of interest. The primary antibody is always unlabeled in the indirect method as opposed to the direct method, where the label is attached to the primary antibody (33).

Direct methods are one-step, where the direct-labeled antibody binds to the tissue of interest. The antibodies can be purchased already labeled, or the researcher can label them with the label of choice (31).

The direct method is pretty straightforward and not so time-consuming as the other methods, but is also usually less sensitive, and cannot amplify weak signals. It addition, the antibodies may be tagged with a label not suitable for future applications. This is why the direct method is less used compared to the other methods (33).

The indirect method the unlabeled primary antibody is added to react with the tissue antigen. When the secondary antibody is added, it binds to and reacts with the primary antibody. This method is more sensitive, and also more economic because the secondary antibody can be used with many primary antibodies in future applications (34).

The sandwich method uses enzymatic complexes to amplify the signal and detection of the primary antibody instead of labeled antibodies. This is by definition also a kind of indirect method. In this method the secondary antibody acts like a bridge between the primary antibody and the enzymatic complex. Just like the cheese between the bread in a sandwich. The main sandwich methods are called the avidin-biotin-complex (ABC) method, the peroxidase-anti-peroxidase (PAP) method and the alkaline phosphatase anti alkaline phosphatase (APAAP) method (33).

In addition, polymers are also used in the polymer based two-step method. The methods will be discussed in detail later.

Enzymatic chromogenic and fluorescent labels

The type of label used in the direct and indirect method will be dependent on the expression of the protein of interest. If the protein is expressed in low levels, the label needs to be more sensitive. There are two main types of labels: fluorescent and chromogenic.

The fluorescent method uses fluorochrome labels that are conjugated to antibodies. This is a type of luminescence. Two commonly used fluorochromes are fluorescin isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC). Molecules with luminescent properties emit light when excited by light of a shorter wavelength. It is the atomic structure of the fluorescent materials that makes them give off light. When the electrons absorbs energy from a photon, they become "excited" and jumps to a higher, unstable level of energy. During this jump, the electron loses some amount of energy to heat, and the excess energy is given off as a photon as the electron jumps back to the lower level of energy. Many wavelengths of light can excite the electrons of a fluorochrome, but every fluorochrome has an optimal wavelength, which is called the excitation peak. The excitation peak is different for each fluorochrome, which means that different fluorochromes can be excited by the same wavelength. The fluorescence labeled antibodies bind to the antigen and allows antigen detection through fluorescent methods. For example confocal microscopy (35). The result can be quite beautiful. *(Figure 13)*

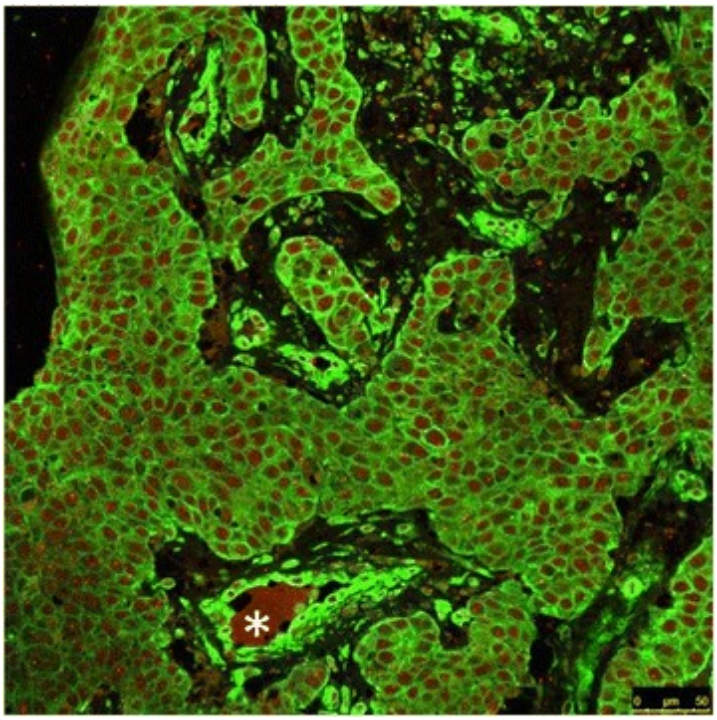

Figure 13: Immunofluorescence staining of oral squamous cell carcinoma tissue. The cells positive for plectin are stained green, and the cells positive for uPAR are stained red. The mark indicates a blood vessel, in which plectin is highly expressed. The antibodies used was rabbit anti-plectin antibody (ab83497, Abcam, Cambridge, MA) diluted and anti-human uPAR antibody (#3936, Sekisui Diagnostica, Carpintera, CA, USA) (36).

The chromogenic detection is based on enzymes and their chromogenic substrates. The antibodies have an enzymatic label that through chemical reactions converts colorless substrates into insoluble particles with color. These particles can be observed under the microscope. The four most commonly used enzymatic labels are horseradish peroxidase (HRP), alkaline phosphatase, glucose oxidase and beta-D-galactosidase. The most frequently used enzyme is HRP, due to its properties. Its small size gives a good intracellular penetration. It also has a fast conversion rate of chromogenic substrates. DAB (3,3'-diamino-benzidine) which gives a brown color, AEC (3-amino-9-ethyl-carbazole) which gives a red color and CN (4-chloro-1-naphthol) which gives a blue color, and are examples of chromogenic substrates often used with HRP (37). For strongly expressed antigens the DAB-substrate will give a long-lasting staining allowing the slides to be stored (35).
Both the enzymatic chromogenic- and the fluorescent method have benefits and detractions. The fluorescent detection has a shorter procedure and good resolution to the finest details but will fade over time (photobleaching). The enzymatic chromogenic detection is more time-consuming, has lower sharpness and contrast, but gives more stable colors that won't fade. In addition there are no limitation to the tissue type because the chromogenic signals are not affected by endogenous autofluorescence (32).

Avidin -biotin-complex (ABC) method

Avidin (naturally found in egg whites) and streptavidin (naturally found in the bacterium *streptomyces avidinii)* are structurally similar proteins that both have four binding sites for biotin (38, 39). Therefore, these proteins bind strongly and with high specificity to biotin. Both avidin and streptavidin are used, but streptavidin is a recent

innovation for substitution of avidin. When streptavidin is used in the ABC-method, it sometimes is called the Labeled StreptAvidin Biotin (LSAB) method. The ABC method for antigen detection exploits the properties of avidin or streptavidin by using antibodies conjugated to biotin. A biotin molecule is conjugated to antibodies and enzymes, and in the ABC-method the secondary antibodies are conjugated to biotin. We say that the secondary antibodies are biotinylated. When conjugated to the antibodies, biotin functions as a bridge between the tissue-bound primary antibodies and the avidin-biotin-peroxidase complex. In this way, the signal from the primary antibody is amplified (39) (40) *(Figure 14).*

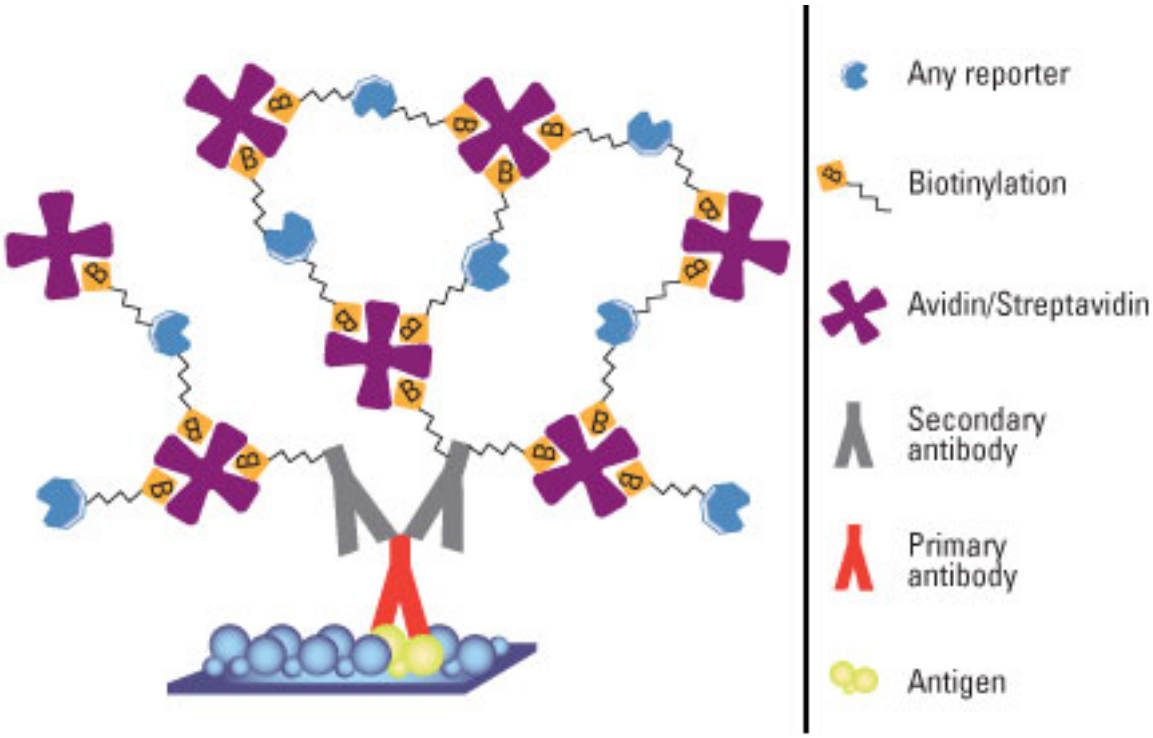

Figure 14: Schematic representation of the ABC-staining method (41).

The peroxidase-anti-peroxidase (PAP) method and the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) method

The PAP-method is considered very sensitive and gives good results even with low concentration of antibody. It is on the other hand time-consuming and complicated (8).

First, a primary antibody is added to bind to the tissue antigen. Then, an unconjugated secondary antibody is added. This makes it possible for multiple secondary antibodies to bind to one primary antibody. A tertiary antibody in complex with peroxidase is added on top of this. And again, many tertiary antibodies will bind to one secondary antibody. This gives a level of amplification that is 100-1000 times greater than secondary antibody amplification (39). The PAP complex comprises three molecules of enzyme peroxidase and two molecules of anti-peroxidase (42) *(Figure 15).*

The APAAP method is the companion of the PAP-method, and works in the same way. The difference is that the APAAP complex comprises two molecules of the enzyme (alkaline phosphatase) and one molecule of the antibody. The increased number of enzyme per antigen binding site gives increased sensitivity (42). The APAAP method is not always as successful, as background staining can be a problem compared to the PAP method (43).

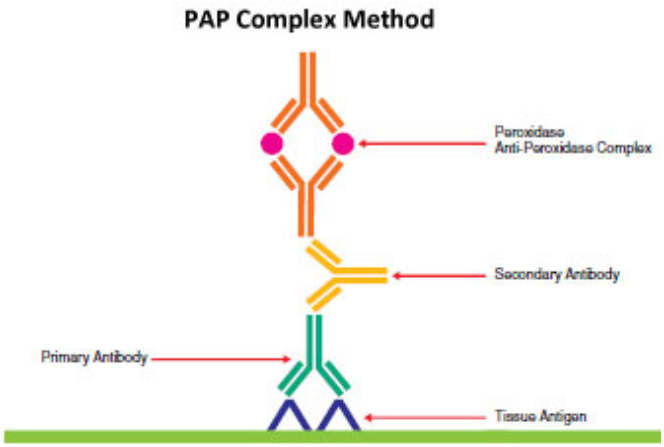

Figure 15: The PAP complex method (44).

Polymer based two-step method

In this method, the secondary antibody is conjugated to a polymer. This method is newer than the previously described methods as it was developed in the 1990s. The polymer molecule of dextran or synthetic peptide is coupled to an enzyme and can carry up to 40 molecules of HRP, which gives more sensitivity, efficiency and reliability compared to the other methods. However, the dextran molecule occupies a larger

volume, which reduces the penetrative ability of the detection reagent in aqueous environments (45) *(Figure 16).*

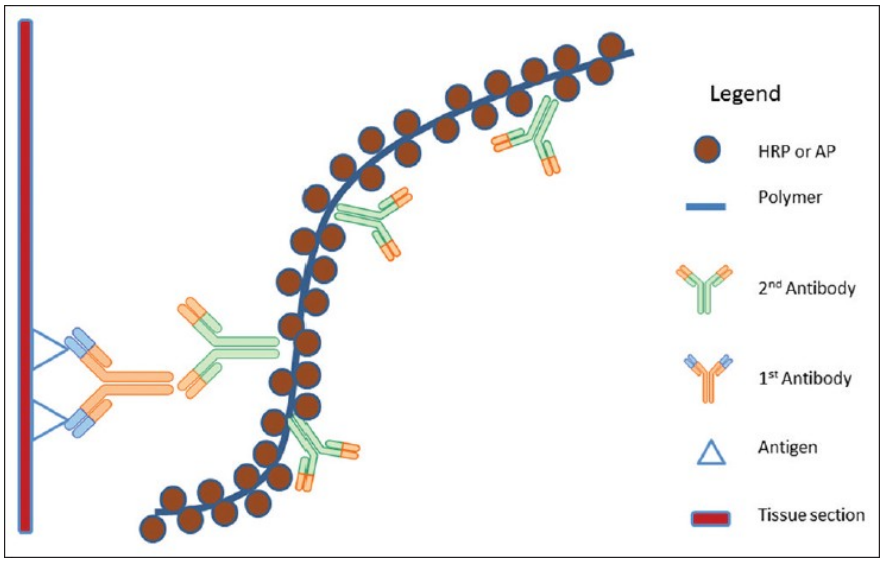

Figure 16: Scheme of polymer-based technique in immunohistochemistry (45).

Working with antibodies

There are several things to keep in mind when working with antibodies. One of the things is clonality. In the Oxford Dictionary clonality is defined as "the fact or condition of being genetically identical, as to a parent, sibling or other biological source" (46). This is also a common used term in the world of IHC, as antibodies may be either poly- or monoclonal.

Polyclonal antibodies are generated by different B-cell clones, but are directed against various epitopes on the same antigen (47). The antibodies are collected from several different B-cell clones that have been activated by the immune system of the host animal. Animals like rabbit, sheep, goat, rat etc. are injected with a specific antigen that makes the B-cell clones produce antibodies against it. When the sera of the host animals are collected, it contains a heterogeneous mixture of high affinity polyclonal antibodies.

Monoclonal antibodies are generated by a single B-cell-clone. Hence, they are a homogenous population. These antibodies are identical, and recognize a single epitope on the antigen. If the antibody-producing B-cell is fused with a myeloma cell, the unit is immortalized and can give a constant supply of antibodies (48).

Choosing mono- or polyclonal antibodies in IHC depends on the tissue or protein the researcher wants to examine. Since monoclonal antibodies are highly specific, examples of recommended use are detecting a specific antigen, a single protein or to stain cells with less background staining. They are in other words great to use in IHC. Polyclonal antibodies tend to have more non-specific reactivity, and the variability from batch to batch is high. The polyclonal antibodies are recommended to use when detecting low levels of a particular antigen, targets in solutions and denatured proteins. When used in IHC, the probability for background staining is increased compared to monoclonal antibodies (49). On the other hand are polyclonal antibodies more robust when used on routinely processed tissue specimens (47).

Mono- and polyclonal antibodies also differ in what is called cross-reactivity. The term "cross-reactivity" describes a reaction that can happen when an antigen reacts with several different antibodies. The common case is that several antigens share at least one common epitope. Cross-reactivity can also occur during antigen retrieval (see later) when changes in one or several epitopes can be induced by accident (47).

A third, and perhaps the most important example of cross reactivity in IHC, are cross-species cross reactivity.

The species the primary antibody is generated in should always be different from the species of the tissue or protein one wants to detect. If you are going to detect a rat protein, the primary antibody must not be generated in a rat. If the primary antibody also is from a rat, the secondary antibody, which is added later, could cross react with both the primary antibody and endogenous immunoglobulins in the rat tissue. Needless to say, the IHC-staining will not be successful. A primary antibody from goat would be a more appropriate choice.

When choosing a secondary antibody, it should be against the host species of the primary antibody. In the example above, the secondary antibody would be anti-goat (50).

A final thing to take into consideration when working with antibodies is the dilution. When the antibodies are received from the producers they are highly concentrated. This

means that the antibodies have to be diluted and adjusted to every experiment to get the optimal contrast. The recommended dilution range is always written in the data-sheet that follows each antibody, which makes the job a bit easier.

Method and work process

There are several steps in the procedure for immunohistochemical staining. These steps may have to be adjusted for every lab, and for every antibody one is working with. The method presented is based on my own work in the laboratory.
Before I could start my experiments, I spent some time reading about the method and learning how to perform IHC. This was done during the spring/summer of 2015. My supervisor helped me to find the correct antibodies and the antibodies were ordered in September 2015. The experiments were performed during the fall of 2015 and the project was finished on estimated time and according to the project plan.
In my first experiment, I used the enzymatic chromogenic indirect method; with HRP as the enzymatic label, and in my second experiment I used the polymer-based two-step method with HRP as the enzymatic label. My third and final experiment was a double staining. All experiments were performed in the laboratory at the Tumorbiology Research Group, Institute of Medical Biology, Faculty of Health sciences, UiT – The Arctic University of Norway.
In my first experiment (experiment nr.1), I used the general procedure for single staining of formalin-fixed-paraffin-embedded (FFPE) human tissue (51). I performed the staining on human lymph nodes, aiming to stain HEVs, which has earlier been performed successfully by several research groups on different tissues (52) (53). Because of physiology earlier described, a human lymph node would be a positive control, and I would expect to find HEVs in the lymph node. I also included a negative control, which were not incubated with the primary antibody. It was important to validate this procedure ahead of the later doublestaining.
When working with formalin-fixed sections, they have to be boiled in citrate-buffer with pH 6 for 20 minutes. This boiling will break the protein cross-links formed by formalin fixation, and uncover hidden antigens. This is called antigen retrieval. This step is not necessary if the sections are fixed in zinc or other mediums (step 2) (54).

For my primary antibody I used rat-anti-peripheral node adressin (PNAd), clone MECA-79, Biolegend, San Diego with a dilution of 1:25. This antibody recognizes PNAd that is expressed on HEV of lymphoid tissues, chronic inflamed tissues and rheumatoid synovia (55). For my secondary antibody, I used HRP-labeled goat anti-rat light chain antibody, #AP202P, Millipore, Temecula, CA with a dilution of 1:250. To dilute the antibodies, one can use different blocking solutions. Depending on the origin of the secondary antibody, the blocking solution can be made of different sera. These sera carry antibodies that bind to reactive sites, and prevent non-specific binding of the secondary antibodies. The serum has to be from the species that the secondary antibody was generated in. In my experiment, the secondary anti body was generated in goat, so I used 1,5% goat serum diluted in PBS for my blocking solution (51, 56).

In the protocol, there are several steps where the sections are washed in phosphate-buffered saline (PBS). This is an isotonic buffer that matches the osmolality and ion-concentration of the human body, and helps to maintain a constant pH (57). For detection, I used Vectastain elite ABC-Kit, Vector Laboratories, #PK-6100, Burlingame CA, which gives a red color. Finally, the sections were counterstained with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO).

In my second experiment (experiment nr. 2) I aimed to stain myeloid dendritic cells positive for the biomarker CD11c, also known as integrin alpha X. CD11c is not only expressed by dendritic cells but also on tissue macrophages (58). Research groups have successfully stained CD11c+ dendritic cells using IHC (59). A human lymph node would serve as a positive control for the CD11c+ dendritic cells too and a negative control was also included.. In this experiment I also used the general procedure for single staining, working with FFPE-tissue. For my primary antibody I used rabbit anti-human CD11c, #AHP1226, AbD Serotec, North Carolina with a dilution of 7,5 micrograms/ml. For the secondary antibody I used labeled polymer HRP-anti Rabbit from the Dako EnVision+ System-HRP (DAB), Dako North America inc., Carpinteria, CA for use with rabbit primary antibodies. For detection, diaminobenzidine (Dako EnVision + System-Horseradish Peroxidase, Dako) were used, which gives a brown color.

It was not random that both experiments 1 and 2 had the same positive control. In my third experiment (experiment nr. 3), the double staining, the aim was to detect both HEVs and CD11c+ dendritic cells in a human lymph node at the same time. I developed a

protocol combining the protocol from the two experiments above to perform this specific staining (see below).

Protocol for single staining

This is the general protocol for FFPE-tissue described step-by-step.

Before staining, all sections have to be incubated overnight at 60°C to melt the paraffin.

1. To deparaffinize further, the sections are sent through a series of baths with xylene and alcohol, and finally, distilled water.
2. Boil in citrate buffer for 20 minutes
3. After the boiling, the sections have to gradually cool down. Let the sections cool down in the hot citrate-buffer for 20 minutes. Then, add running distilled water, for 10 minutes.
4. Incubate the sections with 3% H2O2 diluted 1:100. This is to block endogenous peroxidase activity in other cells than the ones of interest. By doing this, the background staining is reduced.
5. Wash in 3 x 5 minutes in PBS
6. Incubate with blocking solution.
7. Incubate with the primary antibody for 30 minutes-1hour in room temperature
8. Wash 3 x 5 minutes in PBS
9. Incubate with the secondary antibody for 30 minutes in room temperature
10. Wash 3 x 5 minutes in PBS
11. Incubate with the chromogenic substrate solution for 10 minutes
12. Wash 3 x 5 minutes in PBS
13. Wash in hematoxylin for 30 seconds. The hematoxylin has to be filtered to remove precipitates
14. Wash in Scotts solution for 15 seconds
15. Dehydrate the sections by sending them through a series of baths with alcohol and xylene, in the opposite order from when the sections were deparaffinized.
16. Mount with histokit.

Double staining protocol for CD11c+ dendritic cells/ PNAd+ HEVs

The protocol is based on the previously described protocol for single staining, with some changes to get a good enough contrast between the two cell types. It turned out to be a two-day procedure.

I started with the staining of the HEVs and followed the single staining protocol down to step seven using the rat-anti- PNAd primary antibody. In step seven I changed the incubation of the primary antibody, from 30 minutes – 1 hour in room temperature to overnight in the refrigerator. The next day I continued with step 8-12 using the goat anti-rat light chain secondary antibody. Then, I added an extra step with 3% H2O2 diluted 1:100 to block endogenous peroxidase activity and washed 3x5 minutes in PBS. To stain the CD11c+ dendritic cells, step 7-12 was then repeated using the rabbit anti-human CD11c primary antibody and the labeled polymer HRP-anti Rabbit secondary antibody. Finally, step 13-16 was performed. Step 15 was replaced with dehydration in heat cabinet at 60°C for 10 minutes.

Multiple staining

Multiple staining is of interest when the researcher wants to look at the localization or presence of two or more antigens in the same tissue sample or even in the same cell. There are several protocols to perform multiple staining, and in my experience, the procedure is very time-consuming. In general, every multiple staining protocol is a combination of single staining protocols for each antibody. One must be very aware of cross-reactions and the blocking steps as the researcher now works with more than two antibodies, and it may take several failed experiments to find the right incubation times and dilutions. Another problem is to find color combinations that give a crisp contrast (60).

Results

Experiment nr. 1:

The PNAd+ HEVs were stained successfully and were easy to spot in the microscope. As figure 17 shows, the red color gave a good contrast to the surrounding cells and the HEVs were clear and well defined. The picture is taken at 40x enlargement and shows three HEVs surrounded by lymphocytes, which have a purple color from the hematoxylin counterstaining.

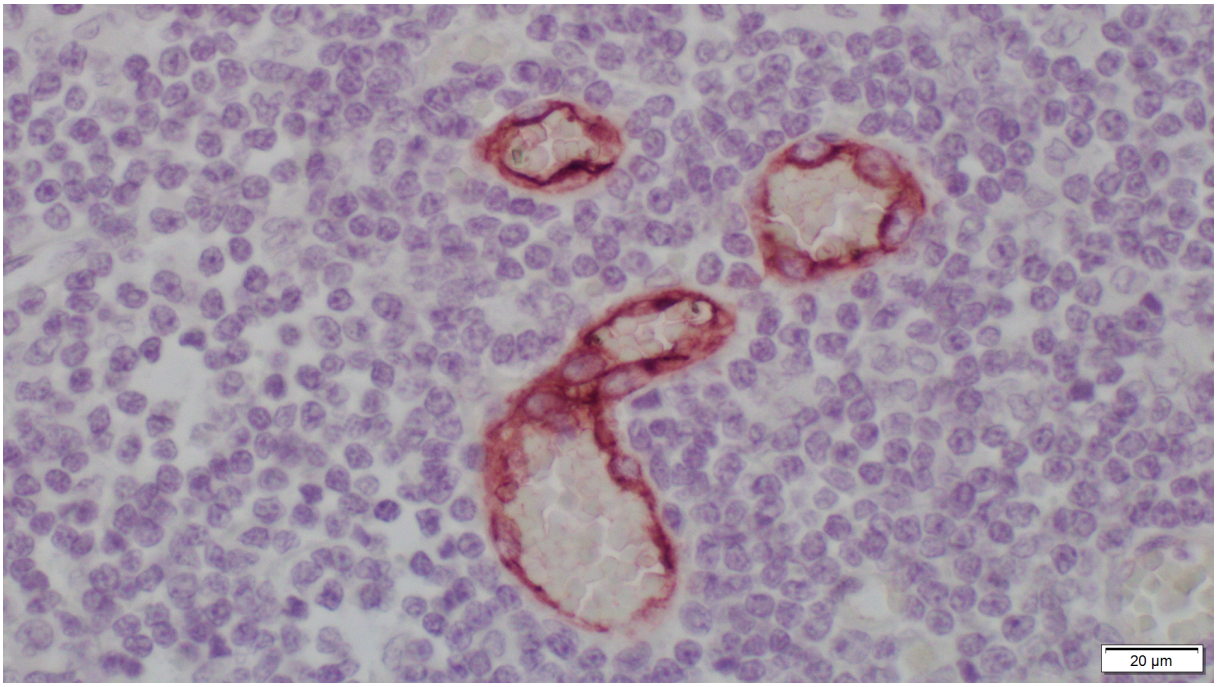

Figure 17: Representative IHC staining for HEVs surrounded by lymphocytes in a human lymph node at 40 x enlargement. PNAd+ vessels are stained red and cell nuclei are stained blue by hematoxylin. The scale bar indicates 20µm.

Experiment nr. 2:

The CD11c+ dendritic cells were stained successfully, and they appeared really clear and defined with their branches in the microscope as shown in figure 18. The DAB-chromogen gave a really good contrast to the surrounding cells and the dendritic cells

were easy to spot. Figure 18 is a picture taken at 40 x enlargement and shows a cluster of CD11c+ dendritic cells surrounded by lymphocytes, which are purple from the hematoxylin counterstaining.

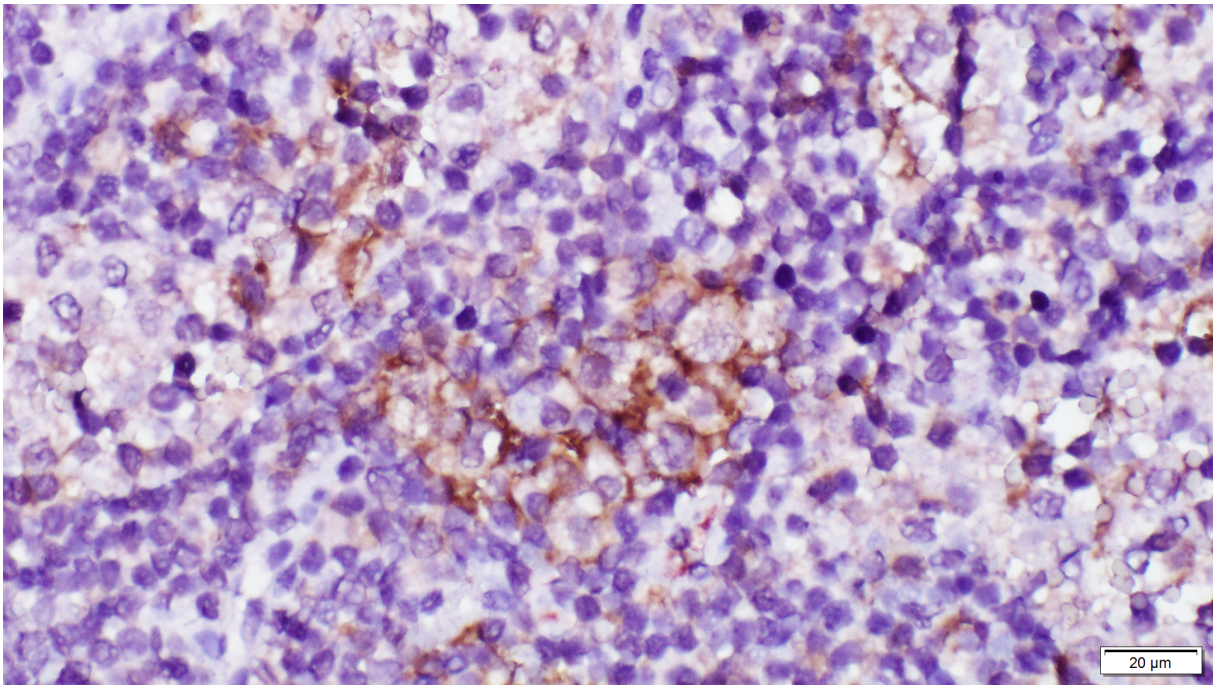

Figure 18: Representative IHC staining of a cluster of CD11c+ dendritic cells surrounded by lymphocytes in a human lymph node at 40 x enlargement. CD11c+ dendritic cells are stained brown and cell nuclei are stained blue by hematoxylin. The scale bar indicates 20µm.

Experiment nr. 3:

The double staining experiment was successful and the HEVs and the dendritic cells were easy distinguishable. The chromogens worked well together and gave clear defined colors with little background staining. Figure 19 is taken at 20 x enlargement and shows six HEVs in a cluster of dendritic cells with their branches. Figure 20 is from the same area as figure 19 but is taken at 40 x enlargement. The figure shows five HEVs in a cluster of dendritic cells. In both figure 19 and figure 20 the CD11c+ dendritic cells and the HEVs are surrounded by lymphocytes, which are purple from the hematoxylin counter staining.

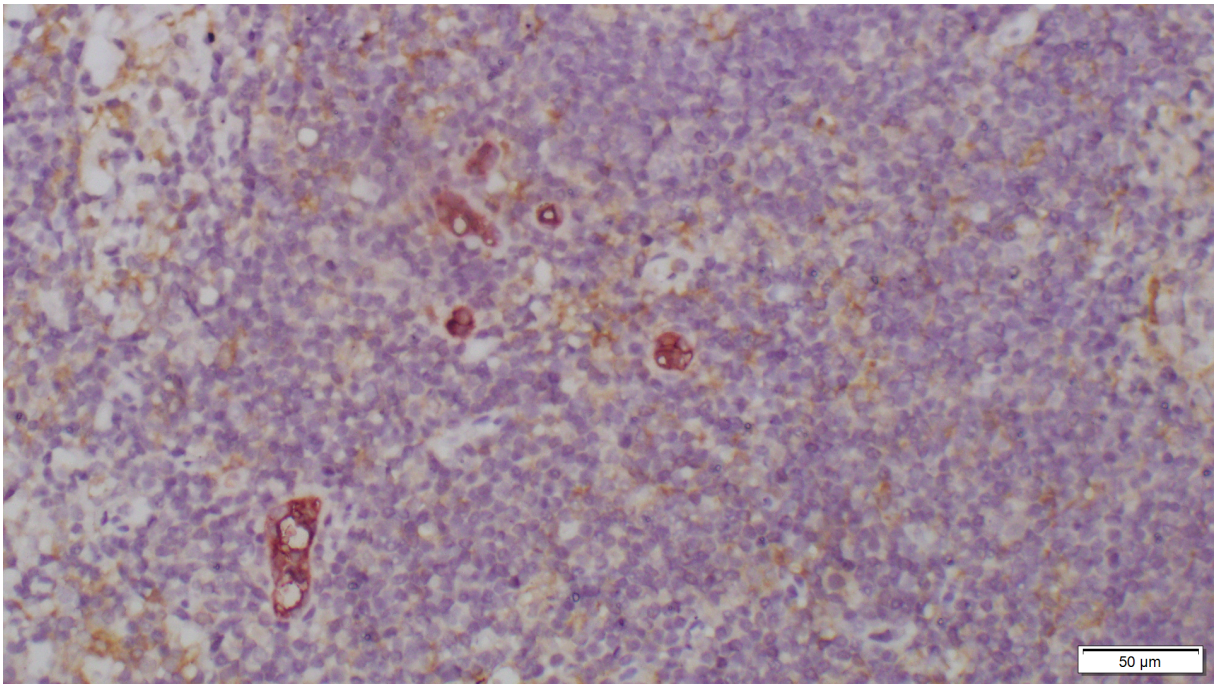

Figure 19: Double staining of CD11c+ dendritic cells and HEVs in a human lymph node at 20 x enlargement. PNAd+ vessels are stained red and CD11c+ dendritic cells are stained brown. Cell nuclei are stained blue by hematoxylin. The scale bar indicates 50µm.

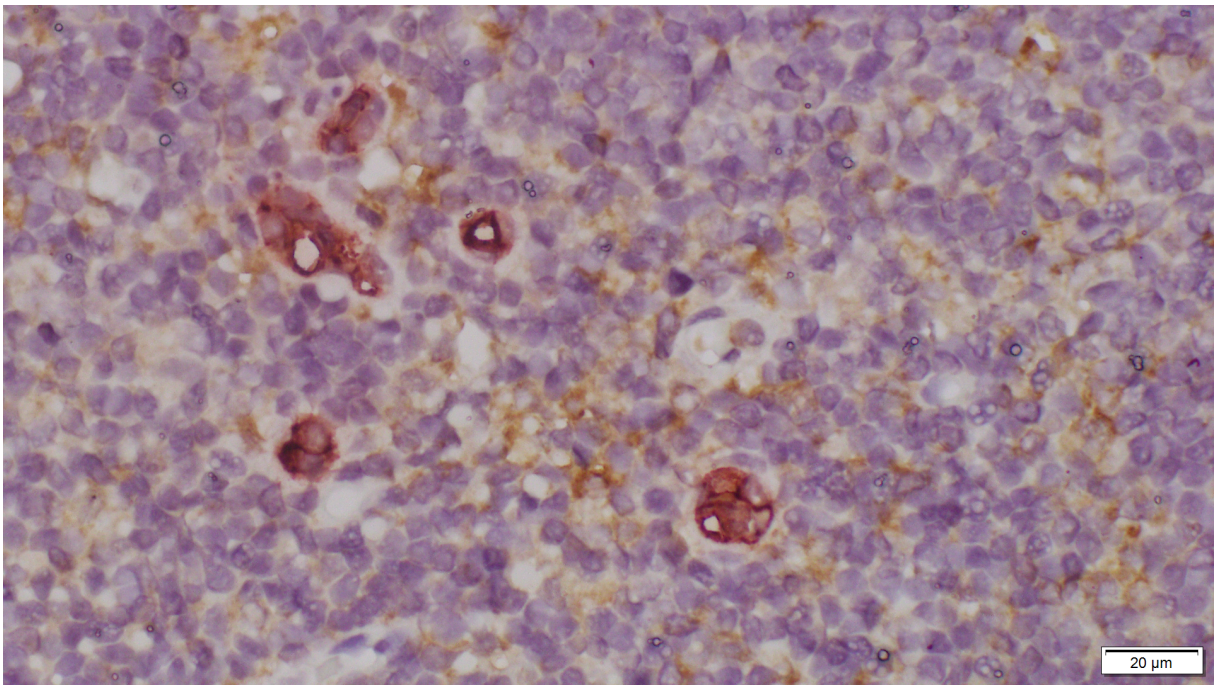

Figure 20: Double staining of CD11c+ dendritic cells and HEVs in a human lymph node at 40 x enlargement. PNAd+ vessels are stained red and CD11c+ dendritic cells are stained brown. Cell nuclei are stained blue by hematoxylin. The scale bar indicates 20µm.

Discussion

The immune system is complex, and vital for production of antibodies. Immunohistochemistry combines two disciplines; immunology and chemistry and is now one of the most used special procedures in pathology departments. The visualization of antibodies that have bounded to specific antigens can be utilized in the aim to make a correct diagnosis, and also for the sake of prognostic evaluation.
In diagnostic pathology, automatic machines have more or less standardized protocols for IHC. Even though IHC is a well-established method, there is no standard protocol that will work for all antibodies when doing manual staining with antibodies in the laboratory. This leads to a lot of trying and failing before the right antibody-dilutions and incubation times are settled. Factors as temperature, equipment and internal procedures are also playing a role and every lab has to do a test-stain with different dilutions to find what works best in their lab. There can also be difficulties with the antibodies, as different batches may give different result. There is also the risk of contamination of the antibody mixture. This makes IHC a time-consuming and sometimes difficult procedure. On the other hand, IHC represents in most cases a robust and specific method to detect tissue markers and plays an important role in diagnostics.
In my experiments I faced some difficulties and spent some time troubleshooting. I had to do several tests with different dilutions to get the strength of the staining strong enough, but not so strong that the slide was overstained. Also with the double staining several trials had to be completed before both colors could be visualized at the same time. It is important to work together with experienced technicians and also to have the benefit to evaluate slides together with well-trained pathologists. When I first found the correct dilutions and incubation-times, the procedures were pretty straightforward and it was very satisfying to see the color reaction develop before my eyes, and the project was finished on the estimated time. The protocols are understandable, but it requires lab training and some background information to understand what happens when incubating with the different solutions.
All in all I experienced IHC as a reliable method and I think IHC always will play an important role in research and diagnostics.

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