Follicular T helper cells and their potential roles in autoimmune pathology

Kitty de Leur (3257738)
Bachelor Thesis Biomedical Sciences
Utrecht June 2011

Universiteit Utrecht

About picture on the front cover: *'A germinal centre is like a bag of marbles. Different immune cells are constantly entering and leaving the germinal centre, like the stream of different marbles coming in and out the marbles bag of a playing child.'*

Kitty de Leur (3257738)
Bachelor Thesis Biomedical Sciences
Utrecht, June 2011
k.deleur@students.uu.nl

Supervisor: T. van den Broek
Laboratory of Translational Immunology,
Wilhelmina Children Hospital Utrecht

Table of contents

1. Abstract

Follicular T helper (Tfh) cells are essential in the formation of high-affinity antibody producing plasma cells and memory B cells. After antigen encounter naive Tfh cells start to upregulate the chemokine receptor CXCR5. This results in homing of the Tfh cells to lymph node follicles where the Tfh cells specifically localise in germinal centres (GCs). In the GCs the Tfh cells stimulate B cells to differentiate resulting in the production of antibodies. In systemic lupus erythematosus (SLE) an increased amount of autoantibodies is seen. Recent studies postulate that an increased amount of autoantibodies can be related to Tfh cells. The exact role of Tfh cells in the production of autoantibodies in SLE remains elusive, but some research papers provide information from which potential roles can be drawn. The essence of these research papers is the increased level of the chemokine ligand CXCL13 in SLE. CXCL13 levels correlate with disease severity. Further knowledge concerning the mechanism of antibody production in SLE could provide new targets for more specific treatments.

Key words: Tfh cell, CXCR5, CXCL13, B cell, Germinal Centre, autoantibody, SLE.

2. Brief introduction to the immune system

This literature study describes one subject out of the highly developed and complex network of cells working together as the immune system, namely the potential roles of Tfh cells in autoimmune pathology. This introduction gives a brief overview of the immune system and clarifies the aim of this literature study.

The study of immunology has been extensively studied in recent years and is still an ongoing topic. This field of science found his fundamentals in the 20th century which makes immunology a relative young topic. The immune system is an expert in protecting our body against different invaders. During our life we are constantly exposed to a wide range of different microorganisms. These microorganisms can cause diseases, but with the help of the immune system we become rarely ill. The wide variety of cells and molecules comprising the immune system form a dynamic network capable of specifically recognise and combat foreign invaders [1]. The immune system is able to recognise different molecular patterns in a more general way, but also in a highly specific way[2]. Moreover, the immune system possesses ingenious mechanism to discriminate between self and nonself molecules. In response to pathogen recognition different immune effector functions are put forward to attack the pathogen, for example activation of the complement system, phagocytosis by different immune cells, cytotoxic activity of immune cells and the production of antibodies. The immune system is able to self regulate these effector functions. This immune regulation is of high importance to prevent against over expression of the effector functions that can cause allergy or autoimmune diseases [1].

There are two main types of immune responses, namely the innate and the adaptive immune response. The innate immune response is immediately able to attack the pathogenic invader in an a-specific way and without causing any immunological memory. On the other hand, the adaptive immune response is a more long-term response, which in the end forms a lifelong protective immunity against specific pathogens. Antibodies are key players in the formation of lifelong immunity. Antibodies are able to recognise small molecular substances that are part of the microorganism. These antibodies are produced by differentiated B cells, called plasma cells[1].

Immature immune cells develop in the bone marrow followed by migration into the periphery. The immune cells are than able to settle in a particular tissue or they will circulate in the bloodstream or in the lymphatic system. White blood cells form the majority of the immune cells, they are important for both innate and adaptive immunity. Two main types of white blood cells can be distinguished, the myeloid lineage and lymphoid lineage. The cells belonging to the myeloid lineage are progenitor cells that in the end will belong to the innate immune system. On the other hand, the lymphoid lineage contributes to the formation of lymphocytes belonging to the adaptive immune system[1]. This literature study will focus on a particular type of lymphocytic cells in the adaptive immune system.

Two types of lymphocytes can be distinguished, B lymphocytes and T lymphocytes. B lymphocytes are derived from the bone marrow while T lymphocytes leave the bone marrow to have their final education in the thymus. Both types of immune cells have different functions but to come to an efficient immune response their collaboration is essential. One of the main differences between B and T lymphocytes is the difference in antigen receptor. B lymphocytes have a B-cell receptor (BCR) on their cell membrane. After an antigen has bound this BCR the B lymphocyte will proliferate and differentiate into a plasma cell.
T lymphocytes have a T-cell receptor (TCR) on their membrane. After an antigen has bound the TCR the T lymphocyte differentiates in a particular effector T lymphocyte. The three most important functions of T lymphocytes – killing pathogens and activate and regulate other immune cells- are performed by three different lineages: Cytotoxic T cells, Helper T cells and Regulatory T cells respectively. These T-lymphocytes can again be divided in several subsets[1].

One way to identify the different T cell lineages is by looking for their cluster of differentiation (CD) molecules. Different lineages and maturational stages of lymphocytes express their own composition of membrane proteins. These membrane proteins are recognised by their own monoclonal antibodies. This group of monoclonal antibodies together is called CD. Different molecular patterns are connected to the lymphocyte lineages by giving lymphocytes different CD designations, for example all T cells are CD3+, in combination with CD4+ these cells are known as T helper cells, whether CD8+ cells are known as cytotoxic T cells[2]. Besides CD designations there are many other surface markers to identify cell types.

This literature study describes the characteristics of Follicular T helper cells (Tfh cells). Tfh cells are localised in germinal centres (GCs). Tfh cells are essential for the activation and production of antibodies by B cells [3]. As will be discussed in this literature study, many things are already known about the Tfh cells. On the other hand, there are still doubts about the way these Tfh cells differentiate and activate B cells. Moreover, it is still not known if these Tfh cells are a subtype of other T helper cell lineages or if they are a distinct cell lineage. In addition, little is known about the plasticity of Tfh cells. Whether these Tfh cells are able to alter or modify their function after they have reached their committed stage remains elusive. Many recent studies have focused on this plasticity versus commitment of Tfh cells.

This literature study first describes the position of the Tfh cell in the immune system. With a general introduction of CD4+ T cells in chapter 3, a framework is formed to describe the position of the Tfh cell in this group of immune cells. Chapter 4 describes the characteristics of Tfh cells that distinguish them from other T lymphocytes. Than a detailed description of the way the Tfh cell localises in the GC is given in chapter 5. Followed by the explanation of the Tfh cell activation and the role of the Tfh cell during the GC reactions in chapter 6. Chapter 7 describes the different opinions about Tfh cell plasticity in relation to Tfh cell commitment. Different models of Tfh cell differentiation are described and the plasticity of Tfh cells is discussed. In order to develop new vaccines, it is of high importance to understand Tfh cell differentiation and their role in the production of antibodies.

Since the majority of vaccines triggers a T-cell dependent antibody response. In addition, Tfh cells can be involved in autoimmune diseases, for instance systemic lupus erythematosus (SLE) and rheumatoid arthritis[4]. Currently many patients suffer from immune disorders without having adequate medication. Chapter 8 describes what is known so far about the function of Tfh cells in autoimmune pathology.

Tfh cells are essential for the stimulation of B cells to differentiate into antibody producing plasma cells. When this process is disturbed a switch to autoantibody production may appear. The autoimmune disease SLE is characterised by the high number of autoantibodies circulating through the body. In chapter 9 the potential roles of Tfh cells in the production of autoantibodies are described using SLE. In addition, potential approaches are discussed to restore this immune disturbance. Taking previous points in consideration, the main aim of this literature study is formulated using the following research question:

What are the potential roles of follicular T helper cells in autoimmune pathology and which potential targets can be used to develop new therapeutics to restore this immune disturbance?

3. The CD4+ T cell family

3.1 Discriminate between self and non-self

T lymphocytes mature in the thymus. One of the key features of this T cell maturation is providing immunological tolerance preventing the T cells to become autoreactive. First elimination of autoreactive lymphocytes occurs in the thymus, this leads to the so called central tolerance. Via different mechanisms that influence cell survival and cell death, a selection is made between autoreactive and non-autoreactive cells. After the central tolerance in the thymus is obtained a second elimination called peripheral tolerance regulates cell death mechanisms in the periphery to maintain the lymphoid homeostasis[5]. An autoimmune disease may appear when these self-tolerance obtaining mechanisms are not working in a right way. Autoimmune diseases affect approximately 5% of the population of Western countries[1]. This relatively low number illustrates the efficient mechanisms of the immune system to produce immune cells only acting against non-self molecules. Nevertheless, the discrimination between self and non-self molecules remains a complex process.

3.2 Activation of the naïve T cell

During the maturation of T cells a membrane bound receptor called the T-cell receptor (TCR) is expressed on the T cell membrane. All TCRs share some constant regions but because of some variable regions they are highly polymorphic and only bind unique antigens. These antigens have to be presented on membrane molecules called major histocompatibility complex (MHC) molecules. Two types of MHC complexes can be distinguished: MHC I molecules are expressed on membranes of all nucleated cells, whereas MHC II molecules are only expressed on membranes of antigen-presenting cells (APCs). APCs include macrophages, dendritic cells, B cells and a collection of tissue specific APCs, such as dendritic cells in the epidermis, known as langerhans cells [2]. After peptide-MHC recognition the T cell is able to interact with the peptide-MHC complex. This interaction does not only consist of TCR binding to the peptide-MHC complex. In addition, a conformational change occurs and a different number of cell adhesion molecules increases the strength of the binding between the T cell and the APC. This increase of membrane adhesion molecules facilitates a closer contact between the T cell and the APC, which promotes the transport of cytokines and other substances contributing to the T cell activation[2].

The activation of a naive T cell to become an effector T cell needs at least two other signals, called co-stimulatory signals (Figure 1). These co-stimulatory signals are mostly given by the same APC. The first co-stimulatory signal is given via the CD4 or CD8 co-receptor.

The B7 molecule, a member of the immunoglobulin superfamily, gives the second co-stimulatory signal. These B7 molecules are only expressed on the surface of cells that stimulate naïve T cell proliferation, for example dendritic cells. CD28 is the receptor on the T cell membrane that can be bound by B7. Several other co-stimulators for naïve T cells have been reported among which only the B7 molecules have been validated to perform co-stimulatory signals to naïve T cells during an immune response. After CD4 or CD8 and B7-CD28 co-stimulation the naïve T cell is activated and starts to produce cytokines. When CD28 binds the B7 molecule the production of the transcription factors (AP-1 and NFκB) is increased which then increases transcription initiation of interleukin-2 (IL-2). IL-2 drives the proliferation and differentiation of naïve T cells. Resting T cells express an incomplete form of the IL-2 receptor. After encounter with the peptide-MCH complex and co-stimulation this incomplete receptor will transform to an active receptor. Thus, after peptide-MHC binding to the TCR and co-stimulation the activated T cell matures and starts to produce IL-2. IL-2 then stimulates the progression through the cell cycle and continues this process. In the end one activated T cell can divide several times a day for more days in a row, which gives rise to thousands of effector and memory T cells bearing exactly the same antigen receptor[1]. Figure 1 illustrates the T cell activation in a schematic way.

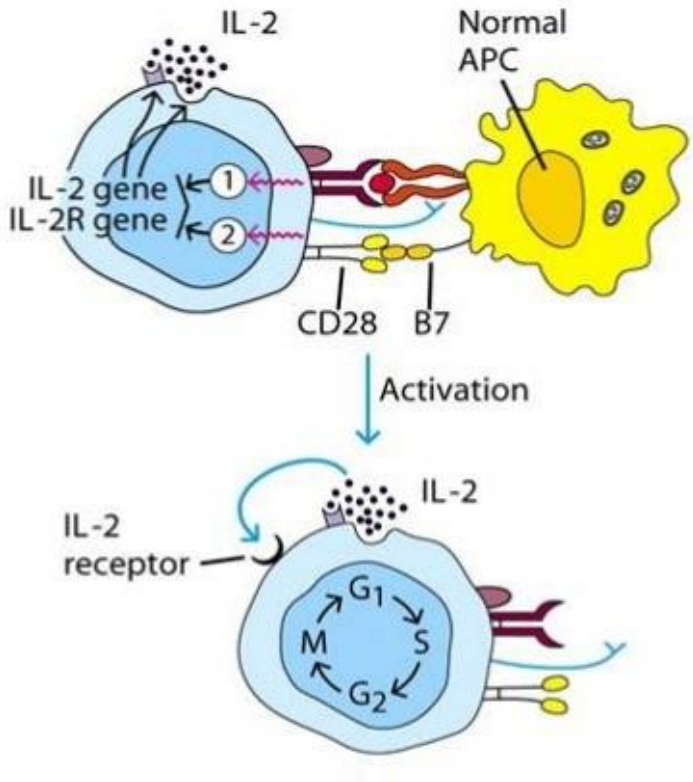

Figure 1. Schematic drawing of the T cell activation process[2]. The naïve T cell in blue needs at least two signals before it is able to differentiate and proliferate. First activation via binding of the specific peptide-MHC complex to the TCR occurs in combination with at least two co-stimulatory bindings (CD4 or CD8 co-stimulation and B7-CD28 co-stimulation). After successful binding the T cell starts to produce IL-2 and starts to express the high affinity IL-2 receptor that triggers progression of the cell cycle and stimulates itself to divide several times a day. A large number of progeny appears and differentiates into effector T cells[1].

3.3 Different CD4+ T cell lineages

So far the general T cell mechanisms are described. This literature study focuses on the CD4+ T cell family containing the follicular T helper (Tfh) cell. The CD4+ T cell family consists of several T helper (Th) cell subsets. Th cells are only able to recognise antigenic peptides presented on MHC class II molecules. Th cells trigger different responses through the cytokines they produce after Th activation. They are involved in both the humoral and cell-mediated immune response, where they play a key role in the production of antibodies and the activation of cytotoxic T cells. Without this 'T helper' function, no adaptive immunity is formed. When looking at the different subsets of Th cells, there are a couple of subsets. These subsets are described as Th1, Th2 and Th17 cells and they show overlap with Tfh cell (see Table 1). The extent of this overlap and the question whether Tfh cell differentiation is dependent on these subsets or a subset on itself is discussed later on[4]. A brief description of these closely related T helper cell lineages is given below.

3.4 T helper 1 cells

Th1 cells are mainly characterised by their production of interferon- γ (IFN-γ) and IL-2, the development of these cells is regulated by the transcription factors T-bet, STAT4 and STAT1. After their activation Th1 cells are able to stimulate the immunity against intracellular pathogens such as *Leishmania major* and *Mycobacterium spp*[6].

The activity of phagocytic cells is induced by the Th1 production of IFN-γ. After this activation the phagocytic cells start to produce reactive oxygen species (ROS), nitric oxide (NO) and tumor necrosis factor-α (TNF- α) leading to the destruction of the intracellular pathogen. This Th1 function belongs to the cell-mediated immune response. Besides the role of Th1 cells in the cell-mediated immune response Th1 cells are also involved in the production of antibodies against extracellular pathogens. The Th1 cells are able to produce co-stimulatory signals for naïve B lymphocytes which are already antigen-activated[6].

3.5 T helper 2 cells

When looking at the Th2 lineage the most important substances involved in the development of the Th2 cells are the cytokine IL-4 and the transcription factors GATA-3 and STAT6. Th2 cells are involved in the humoral immune response where the Th2 cells stimulate eosinophil activation and trigger differentiation of naïve B cells into the formation of antibody producing plasmacells. This humoral immune response inhibits phagocytic functions, meaning that the humoral immune response gives a phagocyte-independent protection. Th2 cells especially promote the class switching to immunoglobulin E (IgE), the antibody responsible for the combat against parasitic infections. When an increased amount of IgE is present after exposure to a certain substance an allergic reaction may appear and in this way the development of an atopic disease may appear as well. The high amount of IgE is connected to a more Th2-like profile in atopic patients while on the other hand non-atopic controls show a more Th1-like profile. Thus, it is thought that Th2 cells play a key role in the development of allergic diseases and asthma[6].

3.6 T helper 17 cells

Th17 cells are strong producers of IL-17. Their development is regulated by the transcription factors RORc and STAT3. By producing IL-17 and other cytokines the Th17 cells are able to attract neutrophils and macrophages to the site of inflammation which on their turn deliver protection against extracellular bacteria and fungi[7]. Th17 cells are thought to play an important role in the development of autoimmune diseases and inflammatory diseases. A potential explanation for this contribution to autoimmune and inflammatory diseases might be the pro-inflammatory way of acting[8].

3.7 Regulatory T cells

Regulatory T cells are best known by the presence of CD4 and CD25 on their cell membrane. Also the transcription factors FOXP3 and STAT5 are highly associated with the Treg cells. Among the Treg cells two different types can be distinguished with different sources of origin. The first type is natural regulatory T (nTreg) cells that fully develop in the thymus under FOXP3 influence. The second type is induced regulatory T (iTreg) cells which also produce FOXP3 but are generated in the periphery under the presence of among others transforming growth factor (TGF)-β and retinoic acid[7]. The most important function of Treg cells is the regulation of effector T cells by suppressing them; this explains the anti-inflammatory character of Treg cells. A shift has been recognised during immune responses from effector T cells in the beginning of the immune response to more Treg cells in the end of the immune response. When this shift to Treg cells is absent there will be a higher risk of developing autoimmune diseases since there will be more effector T cells which may also produce autoantibodies[9].

	Th1	**Th2**	**Th17**	**Treg**	**Tfh**
Target	Microbes in macrophages, extracellular bacteria	Helminth parasites	Extracellular bacteria/fungi	T cells	Lymph node B cells
Secreted effector molecules	IL-2, IFN-γ	IL-4. IL-5, IL-13, IL-10	IL-17, IL-21, IL-22	IL-10 TGF-β, IL-35	IL-21, IL-6, IL-27, IL-4, IFN-γ
Surface phenotype	TCR, CD3, CD4, IFN-γR, IL-12R, CXCR3	TCR, CD3, CD4, IL-4R, IL-33R, CCR4	TCR, CD3, CD4, IL-23R, IL-1R, CCR6, CD161	TCR, CD3, CD4, CD25, CTLA4, GITR	TCR, CD3, CD4, CXCR5, SLAM, OX40L, CD40L, ICOS, IL21R, PD1
Transcription factors	T-bet, STAT4, STAT1	GATA3, STAT6	RORc, STAT3	FOXP3, STAT5	BCL-6, STAT3

Table 1. Brief overview of different T helper cell lineages and their characteristics. The different T cell lineages can be distinguished based on molecules expressed on their cell surface, effector molecules they secrete and the transcription factors stimulating their development. Although these T cell markers identify different T cell lineages, recent studies demonstrate the plausible dynamics of the expression of these characteristics[10].

3.8 T helper cell plasticity and the Tfh cell

The previous descriptions of the most important Th-cell subsets emphasise the mediating role of all Th cells. Moreover, all Th-cell lineages are involved in coordinating other cellular immune system processes. The various transcription factors involved in the development of the Th-cell subsets represent the gene-expression pattern for the different Th-cell lineages. In this way Th cells are able to differentiate independently in a certain subset both in vitro and in vivo[8]. Although these are defined features of different Th-cell subsets, there is an upcoming presumption that the different lineages are able to convert into another lineage, the so called T cell plasticity[10]. This T cell plasticity has been studied extensively during the past years and could for example be involved in the balance between anti- and pro-inflammatory Th cells. After a detailed description about the Tfh cells in the following chapters, this Th-cell plasticity is discussed in relation to the Tfh cell differentiation. The Th-cell plasticity might play an important role in the development of Tfh cell dependent autoimmune pathology.

4. Place of the follicular T helper cell in the CD4+ T cell family

4.1 Tfh cells first seen in the tonsils

As described in the previous chapter, T helper cells are essential for the regulation of other cellular immune components involved in adaptive immune responses. They play an important role by inducing macrophage activity, recruit immune cells like neutrophils and produce cytokines and chemokines that are able to regulate the immune response. Besides of the previous capacities, the Th cell contains another important property: the ability of Th-cell stimulation to differentiate B cells into plasma cells and memory B cells. In the beginning of immunology research the Th1 and Th2 lineage where the most well characterised Th-cell lineages giving help to activate B cells. A new Th-cell lineage was first described by different researchers in 2000 and 2001, this lineage was called the follicular T helper (Tfh) cell lineage[11, 12]. This Tfh cell lineage is also able to stimulate B cell activity, and in this way it gives a humoral immune response.

The first phenotypic feature which distinguished the new discovered Tfh-cell lineage from other Th-cell lineages was the expression of chemokine CXCR5. The CXCR5+ Tfh cells were first discovered in tonsils. These secondary lymphoid organs are constantly exposed to antigens via the throat which makes the tonsils a suitable organ to study the T helper activity to B cells[11, 12]. The chemokine CXCR5 is responsible for the adhesion of lymphocytes to a particular site of action. The chemokine receptor CXCR5 is predominantly expressed on the membrane of B cells and is required for the development of follicles (B cell zones) and the localisation of B cells in these follicles[11]. The follicles are part of secondary lymphoid tissues, inside the follicles the germinal centres (GCs) are formed during antigen elimination. The expression of CXCR5 by Tfh cells provides the Tfh cells to enter the B cell follicles and GCs. In this way the Tfh cells may play a crucial role in the stimulation of B cell reactions in germinal centres (GCs) located in several secondary lymphoid tissue structures[11]. A detailed description about the localisation of the Tfh cells in the GC and the activation of B cells is given in respectively chapter 5 and 6.

4.2 Transcription factor Bcl6 distinguishes Tfh differentiation

Although the designation that a new CXCR5+ Tfh-cell was found, these Tfh cells were not immediately recognised in the scientific literature as a distinct cell subset. The differentiation of the most well known Th-cell subsets (Th1, Th2, Th17, Treg) is strongly regulated by some master regulator transcription factors (T-bet, GATA3, RORγt, Foxp3), this master regulator transcription factor was not yet known for the Tfh cell[13]. However, researchers recently discovered a master regulator transcription factor responsible for Tfh cell differentiation, shown to be B cell lymphoma 6 (Bcl6). The expression of Bcl6 is regulated by IL-6 and IL-21. Bcl6 expression is important for both *in vitro* Tfh cell differentiation and Tfh cell activity to B cells in mice[14]. When Bcl6 expression is increased, Tfh-related gene expression is seen in combination with the inhibition of other Th-cell subsets. Bcl6 deficiency resulted in a disturbed Tfh cell development[15].

On the contrary, following experiments performed in mice, the transcription factor Blimp-1 acts has an antagonistic effect on Bcl6 (Fig. 2). After Blimp-1 activation, Tfh differentiation and activity are inhibited, meaning that the formation of B cell GCs and antibody responses decreases[16]. In the presence of Bcl6 in the Tfh cell environment, Blimp-1 is suppressed by Bcl6. Furthermore, Bcl6 also binds the promoters of the T-bet and RORγt genes, which represses the production of IFN-γ and IL-17 resulting in a decreased Th1 and Th17 development[16]. Also the RORγt activity and GATA3 protein expression are blocked by Bcl6. The transcription factor Foxp3 is still produced under Bcl6 circumstances. It has been demonstrated that Foxp3+ T cells are able to convert into Tfh cells in mouse Peyer's patches (PPs). Apparently the PPs can have particular environmental condition in which Foxp3+ T cells are forced to release their Foxp3 and differentiate into Tfh cells. When immunoglobulin production is needed in the PPs a change from Foxp3+ T cells to Tfh cells appears[17].

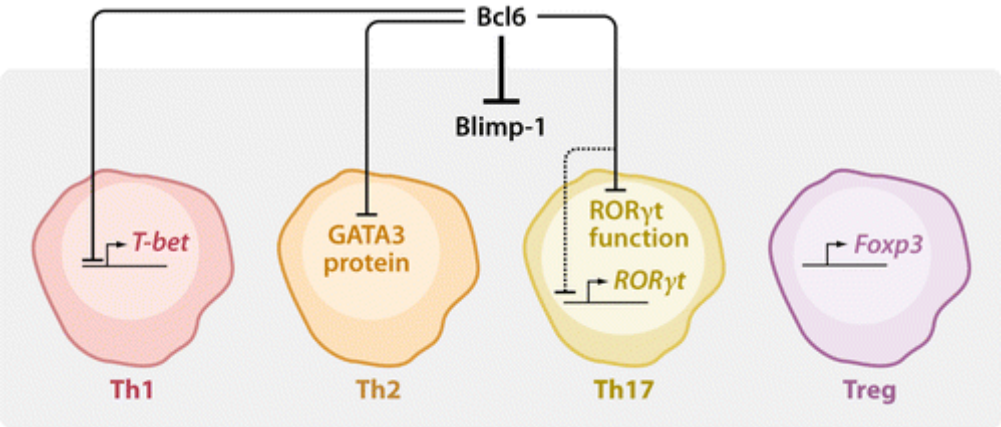

Figure 2. Bcl6 is the master transcription factor of Tfh cell[4]. In contrast, Bcl6 suppresses different stages involved in pathways of CD4+ T cell differentiation. A general inhibition of other CD4 T cell differentiation stages is performed by the inhibition of the Bcl6 antagonist Blimp-1. Bcl6 is able to bind the T-bet which suppresses Th1 differentiation. The GATA3 protein is blocked by Bcl6 which inhibits Th2 differentiation and suppression of RORγt activity in combination with RORγt promoter binding by Bcl6 inhibits the differentiation of Th17. Foxp3+ T cells are able to convert into Tfh cells, explaining the maintenance of Foxp3 expression under Bcl6 circumstances[4,16,17].

4.3 Several surface and effector molecules compose the Tfh pattern

A collection of cytokines and other molecules are responsible for the development and effector function of Tfh cells (Figure 3). These molecules are not by definition restricted to Tfh cells, they are produced by other Th lineages as well. However, Tfh cells show an increased expression of a particular subset of molecules that correlate with increased GC B cell activity and antibody production[3].

Besides the general TCR present on the membrane of the Tfh cell the co-stimulatory molecule CD40L is seen on Tfh cells. CD40 co-stimulation is present on activated CD4 T cells and for Tfh cells this co-stimulation is important for activation of B cells to produce antibodies. After Tfh cell activation high levels of inducible co-stimulator (ICOS) are expressed on the Tfh cell membrane. ICOS upregulates the synthesis of different cytokines by the Tfh cell like IL-10, IL-4, IL-21 and IFN-γ. These cytokines are necessary for the maintenance of Tfh cells and an effective help to stimulate B cells[18]. IL-21 receptor (IL-21R) is also present on the Tfh cell membrane. IL-21 in combination with IL-6 are currently the two most important cytokines responsible for Tfh differentiation[19]. The increased expression of OX40 by activated Tfh cells acts as a marker for Tfh cells but the role of OX40 in Tfh cell function is currently unresolved. Two other important molecules correlated with the Tfh cell are signalling lymphocytic activation molecule (SLAM) and its adaptor molecule SLAM-associated protein (SAP). SAP binds to SLAM and is able to modulate the TCR signalling and modulate Th2 cell differentiation. In human X-linked lymphoproliferative disease there is mutation in the gene coding for SAP. In a mouse model the same gene was mutated which created an analogous situation as in the disease. These mice show defects in GC formation indicating a role for SAP in this process. The exact role of SAP in this process is still unknown[20]. Programmed death-1 (PD-1) is an inhibitory receptor described to play a role in different circumstances during T cell responses. PD-1 is highly expressed on Tfh cells. GC B cells show upregulation of the PD-1 ligands PD-L1 and PD-L2. A study with mice deficient in PD-1, PD-L2, or PD-L1 and PD-L2 showed that these mice had fewer quantities of long-lived plasma cells. In the absence of PD-1 less Tfh cell cytokine production and more GC cell death was noticed. Thus, PD-1 seems to affect the selection and survival of GC cells[21]. As described previously the chemokine CXCR5 is present as well on the Tfh cell membrane, controlling the homing process of Tfh cells.

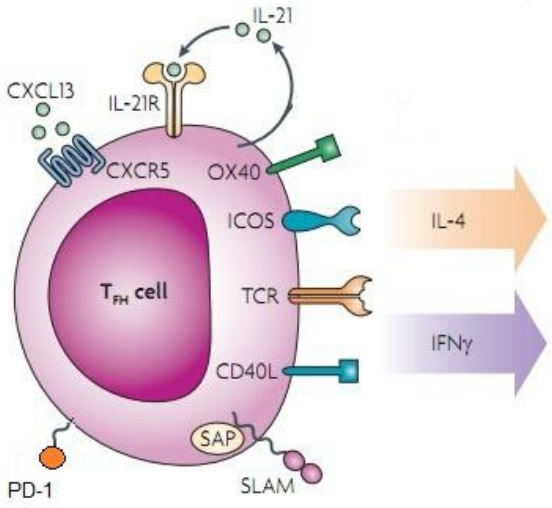

Figure 3. Schematic drawing of a Tfh cell and its important surface and effector molecules[3]. The TCR and co-stimulatory molecule CD40L are responsible for the activation of the Tfh cell after peptide recognition on MHC II. After Tfh activation ICOS increases the production of different effector cytokines (*e.g.* IL-4, IFNγ). The IL-21 receptor is important for maintenance of the Tfh cell. OX40 is associated with Tfh cells but its function is still unresolved. SLAM and its adaptor molecule SAP are able to modulate TCR modelling and Th2 differentiation. PD-1 is an inhibitory receptor affecting the survival and selection of GC cells, especially the quantity of plasma cells. CXCR5 is a chemokine mediating the localisation of the Tfh cell in the GC.

4.4 Collaboration between IL-21 and IL-6

Different factors are important when looking at the Tfh cell differentiation. When the naive CD4+ T cells are surrounded by molecules, that form a particular environment, they are able to differentiate into effector Tfh cells. The way Tfh differentiation occurs exactly is still unknown. However, several cytokines are thought to play an important role during the differentiation of the Tfh cell. IL-6 and IL-21 are currently the most important candidate cytokines when looking at the Tfh cell development[19]. These two cytokines are able to drive Tfh differentiation when they are situated in the correct environment. When IL-6 and IL-21 are kept outside this environment these interleukines are insufficient. In splenic CD4 T cells from C57BL/6 (B6) mice researchers demonstrated that a combined absence of IL-6 and IL-21 resulted in a significant decrease in Tfh frequency. Figure 4 demonstrates this lowered Tfh frequency in splenocytes of B6 mice infected with LCMV which are IL-21 and IL-6 deficient[19].

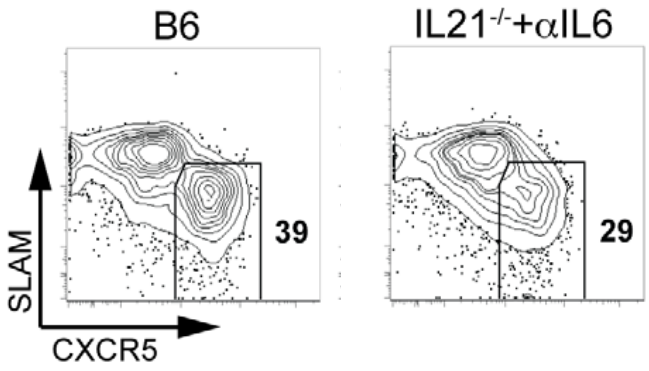

Figure 4. IL-21 and IL-6 are important for Tfh cell differentiation[19]. Two dotplots of splenocytes with LCMV infection followed for 8 days in C57BL/6 (B6) mice. B6 are the healthy controls and $IL21^{-/-}$ +αIL6 are the B6 mice with an IL-21 knockout and antibodies against IL-6. In this dotplot Tfh cells are gated ($CXCR5^{+}SLAM^{low}$) in the box on activated CD4 T cells. Tfh frequency is lower in the absence of IL-21 and IL-6 (29% vs. 39%).

5. Tfh cell localisation

5.1 The lymphatic system: source of immune cells

Lymphocytes, including CD4+ T cells, are moving through the body via lymphatic vessels. These lymphatic vessels contain fluid called lymph and are able to deliver this lymphocyte containing fluid to the blood circulation via the thoracic duct. Besides the lymphatic vessels, there are several lymphatic organs distributed through the body. Examples of lymphatic organs are the lymph nodes, bone marrow, the thymus, the spleen but also lymph nodules located in the digestive system (tonsils, Peyer's patches). Around the lymphatic vessels and inside the lymphatic organs lymph nodes are located. These kidney-shaped structures mainly contain lymphocytes, APCs, reticular cells, and plasma cells. These cells are arranged in the lymph node and form an outer cortex mainly containing T cells and APCs and an inner medulla where B cells are primary located[22]. When T and B cells circulating in the blood want to localise in lymph nodes, they first have to enter special high endothelial venules (HEVs) or afferent lymphatic vessels. CC chemokine receptor 7 (CCR7) is essential for migration of naïve T cells through these HEVs to lymphoid tissue[12].

However, the Tfh cells have their own homing markers. When the naive T cells are settled in the lymph nodes, they are primary activated by dendritic cells. Dendritic cells are able to take up an antigen for example in the skin, present the antigen on their MHC II complex and migrate to lymph nodes to provide T cell activation. After naïve T cell activation by dendritic cells and other APCs the naïve T cells start to proliferate and differentiate. The naïve T cells transform to effector T cells and when the naïve T cells have become Tfh cells they migrate to B-cell follicles to take part in the formation of GCs[12].

Germinal centres are areas of high mitotic activity located in B cell follicles in the lymph nodes (Figure 5). The GCs are formed in the B-cell follicles after B cell antigen encounter and are mainly situated in the cortex. Afterwards, the B cells migrate to the boundary between the B-cell follicle and the outer area where a high number of T cells is localised. At this T-B border, the B-cells search for particular T-cell help. The Tfh cells dispose the characteristics to help the follicular B-cells by developing GCs and stimulate the GC reactions[23].

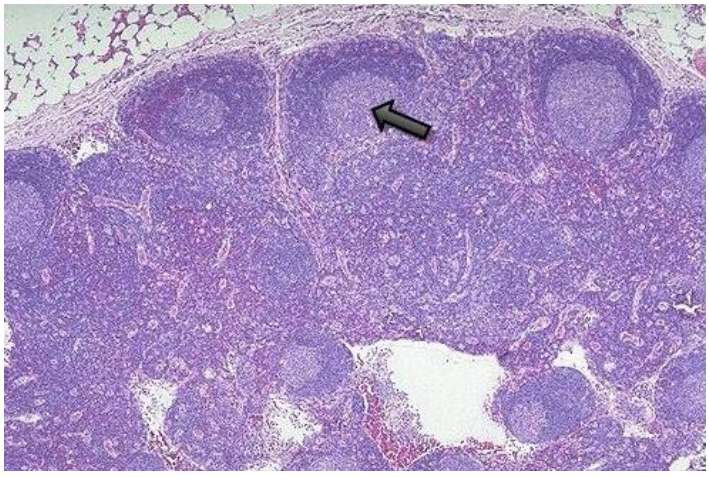

Figure 5. histological staining of a lymph node[36]. The black arrow is pointing to a germinal centre in a secondary follicle located in the paracortex.

5.2 CXCR5 required for Tfh homing

After B cells have encountered an antigen, the B cells are able to home in the B-cell follicles in response to B cell-attracting chemokine 1 (BCA-1, also called CXCL13) which binds the receptor CXCR5, present on mature B cells. After antigen recognition naïve Th cells start to upregulate the expression of CXCR5. At the same time the expression of CCR7, receptor for chemokines CCL19 (ELC) and CCL21 (SLC) localised in the T-cell zone, is reduced. In this way the Tfh cell also migrates in response to CXCL13[24]. Haynes and collegues tested whether CXCR5 presentation on Tfh cells is required for homing to the B-cell follicles (Figure 6). CXCR5-deficient T-cells and wild-type OT-II transgenic T cells were transferred into mice and immunized with OVA adjuvant. After a follow up of 5 days Immunohistochemistry was performed on the peripheral lymph nodes of the mice. B-cell areas were defined with B-cell markers anti-IgD. Anti CD45.1 was used to mark the transferred OT-II T cells. These data show a high amount of OT-II CXCR5+/+ T cells within the IgD+/CD35+ B-cell follicles. On the contrary, OT-II CXCR5-/- T cells failed to migrate into the follicles. Thus, the chemokine CXCR5 upregulation on Tfh cells is essential for homing into the B-cell follicles. In addition, the CXCR5 ligand CXCL13 seems to play an important role in autoimmune pathology. This is described in detail in chapter 8.

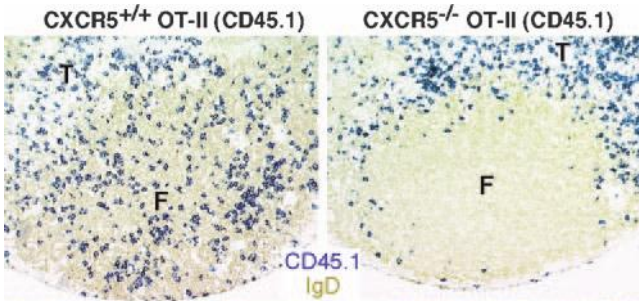

Figure 6. CXCR5 is essential for Tfh cell homing in GCs[24]. CXCR5-deficient T-cells and wild-type OT-II transgenic T cells were transferred into mice and immunized with OVA adjuvant. After a follow up of 5 days Immunohistochemistry was performed on the peripheral lymph nodes of the mice. Anti-IgD defines the follicular B-cell zone. Anti CD45.1 defines the transferred OT-II T cells. OT-II CXCR5+/+ T cells are migrating to the IgD+/CD35+ B-cell follicles. OT-II CXCR5-/- T cells failed to migrate into the B-cell follicles. CXCR5 expression on Tfh helper cells is essential for homing in B-cell follicles. Abr. T: T zone, F: follicle[24].

5.3 GC cell distribution

By the use of histologic analysis, a general organisation of the GC has been clarified[23]. Two different zones are visible in the GC, which can be distinguished on the basis of their histologic appearance. These two GC zones are termed the dark zone and light zone. The dark and light zones are surrounded by a mantle of naive follicular B cells. The GCs are B cell rich and developed after B cell antigen encounter in the B-cell follicles. Within these GC different stages of B cell maturation can be distinguished within the GC dark and light zone (Figure 7).

The dark zone contains a high density of B cells. B cells in the GC dark zone are also called centroblasts. Centroblasts are large B cells with a high rate of proliferation and with downregulated expression of surface immunoglobulins. The GC light zone is characterised by a low density of B cells and the presence of follicular dendritic cells (FDC) and Tfh cells. B cells in the GC light zone, also called centrocytes, are small non-proliferating cells with expression of surface immunoglobulins. The FDC present in the GC light zone present native antigens and provide selection for high-affinity B cells. The Tfh cells represent a minor population in the GC light zone, namely 5% to 20 % of the GC cells[23]. The distribution of centroblasts and centrocytes within the different GC compartments depends on the alternating expression of chemokines[23]. The expression of the chemokine receptor CXCR4 is higher on centroblasts compared to centrocytes. CXCL12 is the ligand for CXCR4 and this ligand is expressed in the GC dark zone. In contrast, centrocytes express low rates of CXCR4 and high rates of CXCR5. In this way the CXCR5 present on the centrocytes binds its ligand CXCL13 present in the GC light zones. The presence of CXCL13 in GC light zones explains the localisation of CXCR5+ Tfh cells in this area of the GC. CXCR5 is also expressed on centroblasts but CXCR4 dominates and achieves the localisation of these B cells in the GC dark zone[23].

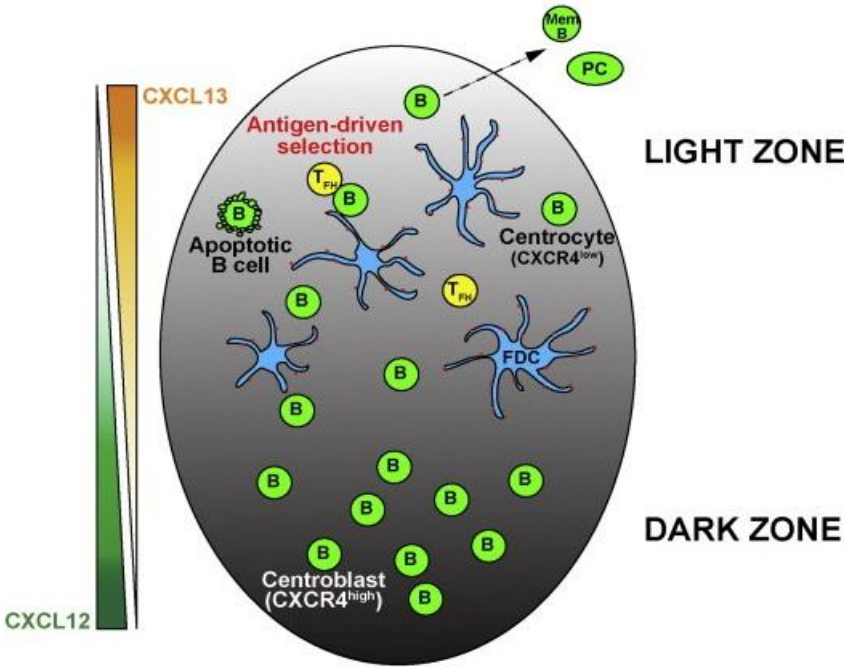

Figure 7. Schematic draw of the GC organization [23]. The CXCR4^{high} centroblasts are localised in the GC dark zone via the chemokine receptor-ligand bond CXCR4-CXCL12. A lower density of centrocytes in combination with Tfh cells and follicular dendritic cells (FDC) are localised in the GC light zone. The CXCR4^{low} CXCR5^{high} centrocytes are localised in the GC light zone via the chemokine receptor-ligand bond CXCR5-CXCL13. CXCR5 is expressed on FDC and Tfh cells as well explaining their localisation in the GC light zone.

6. Role of Tfh cell in germinal centre (GC) reactions

6.1 GC reactions are crucial for a diverse antibody production

So far the different characteristics of the Tfh cell are described. Since the discovery of the Tfh master regulator transcription factor Bcl6 more evidence appeared for Tfh cells of being a distinct cell lineage. Although, there is a high amount of other molecules playing a role in the Tfh cell regulation showing overlap with other Th cell lineages (e.g. Th1, Th2, Th17, Treg). Only the chemokine CXCR5 is authentic for homing of the Tfh cell at the T-B boundary on the edge of the B-cell follicle. Here the initial contact between the Tfh cell and the follicular B cell appears. This initial contact is of high importance for the maturation of the B cell into high affinity antibody producing plasma cells and memory B cells, this maturation occurs mainly via the germinal centre (GC) reactions. This high affinity antibody production generated by the GC reactions is one of the main events during an adaptive immune response. In addition, the efficacy of vaccines is highly dependent on a correct progress of these GC reactions.

The Two major reactions that can be distinguished during the GC reactions are somatic hypermutation (SHM) and class-switch recombination (CSR) (Figure 8)[23]. SHM is the process in which antibody variable regions undergo somatic mutations in the GC to increase their affinity. These somatic hyper mutations are not inherited[23]. During CSR the Ig heavy chains organize the expression of different Ig isotypes. The first antibody produced by B cells during an immune response is IgM isotype. When IgM producing B cells encounter antigens they are able to switch to B cells producing secondary isotypes like IgG, IgE or IgA. Chromosomal rearrangements are involved during this CSR to form a variety of Ig isotypes. The Ig isotypes share the Ig heavy chain variable regions generated during B cell VDJ recombination but the CSR provides diversity in Ig heavy chain constant regions.

Cytokines derived from the Tfh cell and CD40-CD40L co-stimulation are responsible for upregulation of the intracellular enzyme activation-induced cytidine deaminase (AID). AID binds a switch recombination sequence present at the 5’ end of one of the immunoglobulin heavy chain constant region genes (γ, ε, and α). AID mediates demethylation of deoxycytidine in the targeted switch recombination sequence and afterwards deletion of the resulting deoxyuracil bases by uracil DNA glycosylase occurs. This process results in a variety of daughter cells originating from the same B cell but producing high affinity antibodies of different isotypes[23].

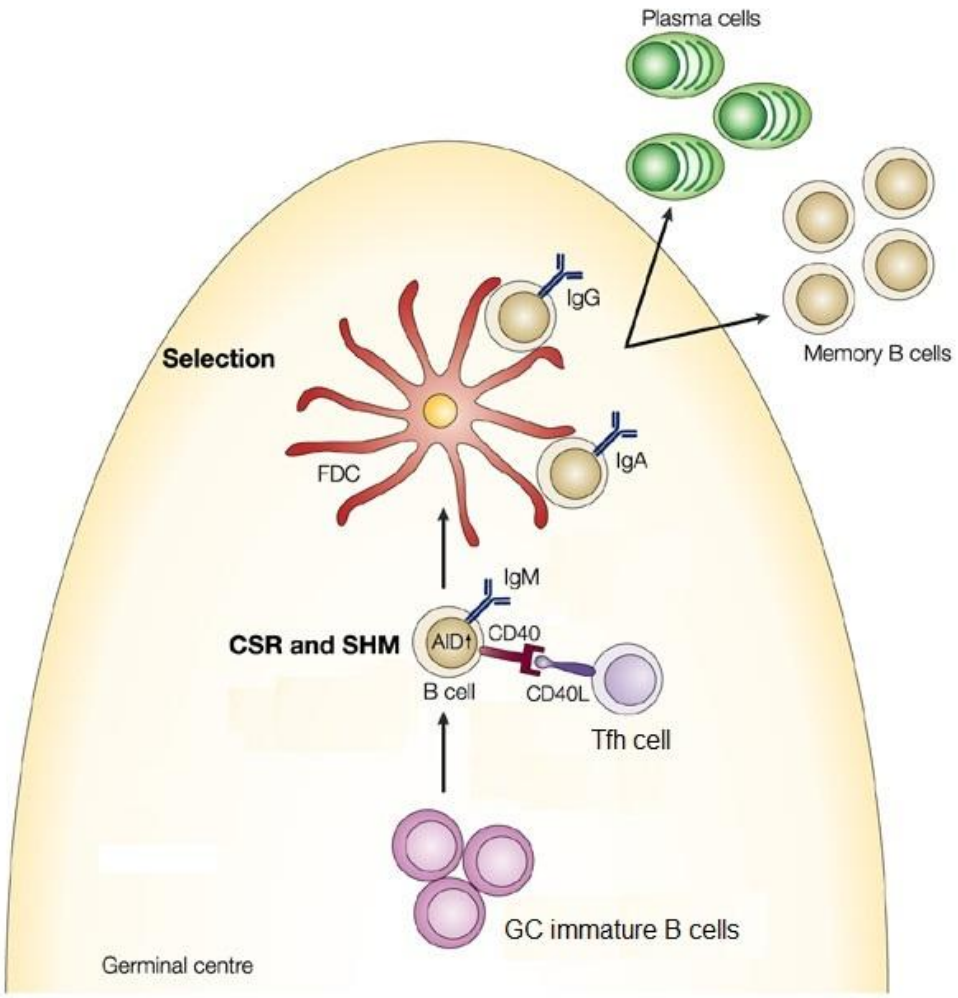

Figure 8. Schematic drawing of GC B cell maturation leading to the production of plasma cells and memory B cells [25]. Tfh cell CD40L binds to CD40 on the B cell. This co-stimulation in combination with the secretion of different cytokines by the Tfh cell is needed to upregulate activation-induced cytidine deaminase (AID). AID is necessary for B cell somatic hypermutation (SHM) and class switch recombination (CSR). After SHM and CSR the B cells start producing different Ig isotypes. FDC are present to select for high-affinity B cells which in the end leave the GC and become plasma cells and memory B cells.

7. Tfh cell plasticity versus commitment

7.1 CD4+ T helper cell plasticity

As described in chapter 3 different CD4+ T helper cell subsets are known. A recent topic of discussion is whether these Th cell lineages are able to convert into another lineage or if they are terminally differentiated into one committed state. Th cells are able to change their cytokine profile *in vitro*, but whether this change occurs with the same intensity *in vivo* remains elusive[10]. Because of recent studies the classical image about terminally differentiated Th cell lineages is changing drastically which makes the view on Th subset differentiation more dynamic. Knowing the molecular mechanisms of Th cell differentiation and the underlying organisation of gene regulation might help explaining the change in cytokine profiles that are related to the Th cell subset specification. Eventually, these findings about Th cell plasticity might be associated with the development of autoimmune diseases.

7.2 Tfh differentiation models

Although the Tfh cell has been described as a distinct cell line distinguished by its master transcription factor Bcl6, there are still a lot of doubts about the exact differentiation of the Tfh cell[14]. Does Tfh cell differentiation occurs directly after the first antigen encounter of the naive Th cell or is Tfh differentiation a secondary process where another Th subset forms the basis of the Tfh differentiation. Based on previous research different models have been put forward. The exact way of Tfh differentiation remains elusive, but the three most plausible models up till now are described below.

Model 1 (Figure 9) is based on the cytokine dependent differentiation of Th subsets. When CD4 T cells are cultured together with APCs, IL-21 and IL-6 they start to produce CXCR5 mRNA and Bcl6 mRNA. After transferring these cells into host mice they have a higher capacities to help B cells compared to unstimulated CD4 T cells. The same experiment was performed with *in vitro* stimulation of CD4 T cells with IL-6 or IL-21. Both cytokines are able to induce CXCR5 and Bcl6 mRNA expression. IL-6 and IL-21 are both activated by STAT3 (signal transducer and activator of transcription 3). STAT3-deficient CD4 T cells are not able to differentiate into Tfh cells[15]. These results support the model of Tfh cells being a distinct cell subset. After exposure to certain cytokines (IL-6 or IL-21) the naive CD4 T cell differentiates into a Tfh cell independent of Th1, Th2 or Th17 differentiation. But there are still some controversial points when looking at this model. IL-21 is also produced by Th17 cells and Th2 cells *in vitro*, it is unclear how Tfh differentiation specificity accomplished is in this situation. This specificity might be accomplished by the difference in TCR affinity, since the Tfh cells bear the highest affinity TCRs on their membrane. Furthermore, mice with IL-21 or IL-21R deficiency are still able to accomplish almost normal levels of Tfh cells. However, IL-21 may not be required for Tfh development but the production of IL-21 remains required for GC B-cell regulation[26].

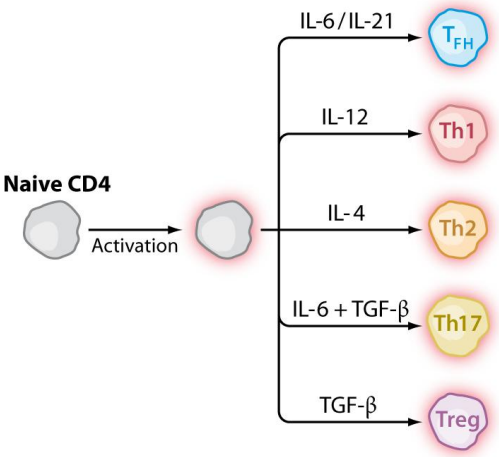

Figure 9. Tfh cell differentiation model 1[4]. The naive CD4 T cells are activated by a peptide-MHCII complex. Afterwards the different CD4 T cell subsets are formed independently under the presence of their own cytokines. Tfh cells differentiate under the presence of IL-6 or IL-21.

Model 2 (Figure 10) follows the idea of Tfh cells being a distinct subset independent of Th1, Th2 and Th17. But this model is characterised by the fact that maintenance of Tfh differentiation is dependent on GC B cells. The first TCR activation of the naive CD4 T cells is performed by a DC. When the naive CD4 T cell is fated to become a Tfh cell, this cell starts to upregulate the expression of CXCR5. CXCR5 promotes homing of the naive Tfh cell at the T-B border. At the T-B border the Tfh cells need the GC B cells to accomplish and maintain their differentiation. In addition, the GC B cells need the Tfh cells at the T-B border to become antibody producing B cells. This two-way activation can be illustrated by the fact that Tfh cells are triggered to upregulate Bcl6 expression by GC B cells and GC B cells are triggered by Tfh cells to upregulate the expression of Bcl6. Bcl6 is important for the differentiation and activation of both cell types[14].

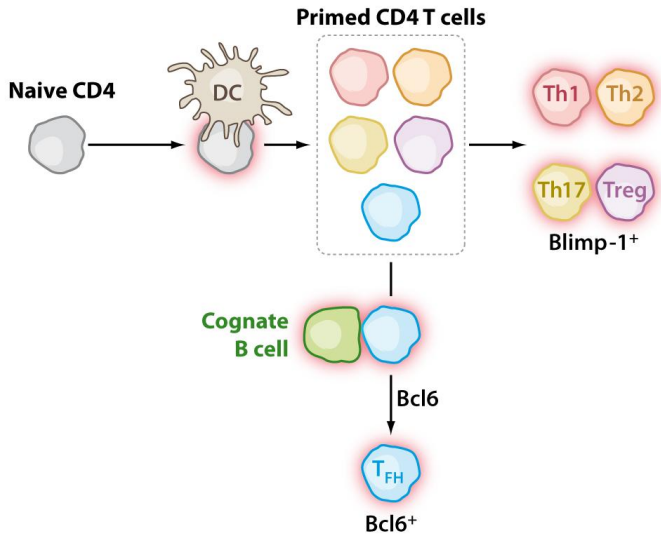

Figure 10. Tfh cell differentiation model 2[4]. Naïve CD4 T cells are activated by DC via protein-MHCII binding on their TCR. Differentiation and maintenance of Tfh cells independent on GC B cells present at the T-B border. Bcl6 is an antagonist of Blimp-1. Differentiation of Th1, Th2, Th17 and Treg occurs under Blimp-1 circumstances.

Model 3 (Figure 11) proposes that The Tfh cell is not a distinct subtype. Tfh cell differentiation occurs via a secondary program. The formation Tfh cells is a phenotypic state of already differentiated Th1, Th2, Th17 or Treg cells. For example, the generation of Tfh cells from Treg cells has been demonstrated by the use of reporter mice with $FOXP3^{GFP}$ (FOXP3 expression marked by a green fluorescent protein). Generation of high levels of Tfh cells from FOXP3+ Treg cells compared to low Tfh cell levels derived from FOXP3- T cells were measured in the Peyer's patches[27]. The Tfh cell is rich in cell surface proteins and cytokine production, resulting in overlap with other Th cell subsets. Even though the Tfh cell has two characteristic markers (CXCR5 and Bcl6), this model assumes that Tfh cells differentiate via a secondary program.

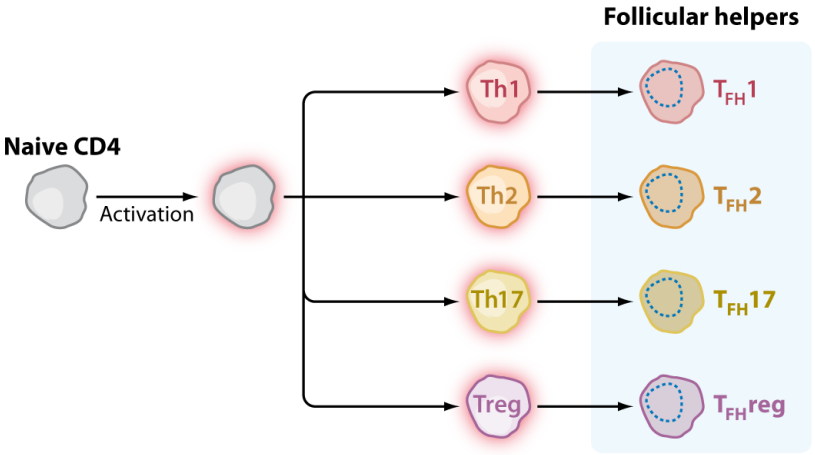

Figure 11. Tfh cell differentiation model 3[4]. The Tfh cell is not a distinct cell subset. Tfh differentiation occurs via a secondary program. Differentiated Th1, Th2, Th17 and Treg cells are able to adopt a phenotypic state of a Tfh cell.

7.3 Reasons behind Tfh cell plasticity

Different models of Tfh cell differentiation are proposed. All models have plausible elements, but the exact way of Tfh differentiation remains unknown. Nevertheless, there is strong evidence of Tfh cell plasticity. Mechanisms like a variety in transcription factor binding, epigenetic modifications and expression of miRNA seem to play a key role in Th cell plasticity[10]. To sum up, differentiation of Tfh cells is a process depending on different microenvironmental situations that modulate the particular transcriptional program. This Tfh transcription program is bivalent confirming the plasticity among CD4 T cell subsets.

8. Tfh cell function in autoimmune pathology

8.1 Tfh cell and autoimmunity

The high amount of membrane proteins and cytokines related to the Tfh cell make this cell subtype a complex subject to study. Recent studies are often concentrated on the presence of a system in which Tfh cells are able to differentiate into another cell type. This Tfh cell plasticity is related to microenvironmental conditions modulating a distinct transcription program. Although the existence of this Tfh cell plasticity has been demonstrated in different experimental settings the exact way in which Tfh cells differentiate remains elusive[28].

When looking at the function of the Tfh cell, the Tfh cell has turned out to be a key player in the GC reactions and the differentiation of B cells into high-affinity antibody producing plasma cells and memory B-cells. The production of antibodies must be regulated very tight to prevent the change of the production of autoantibodies. There are always some autoantibodies circulating through the body[29]. However, when the number of autoantibodies increases, a development of autoimmune pathology may appear. Knowing that the Tfh cell is essential in the process of antibody production there might be a correlation between the Tfh cell and the increased production of autoantibodies. Maybe changes in Tfh cell plasticity contribute to a disturbed interaction between Tfh cells and GC B-cells leading to the formation of autoantibodies. The relationship between Tfh cells and the increased production of autoantibodies is still unknown. However, it is plausible that a change in the number or activity of Tfh cells affects GC B-cells. In other words, a disturbed collaboration between Tfh cells and GC B-cells may result in an increased production of autoantibodies. This supports the idea of incorrect somatic hypermutation contributing to an increased production of autoantibodies[28].

Taking previous points in consideration, there are some studies providing matter from which possible explanations of the relationship between Tfh cells and the increased production of autoantibodies can be drawn. These studies are performed in patients with Systemic Lupus Erythematosus (SLE) or in sanroque mice with SLE like phenotypes. The next paragraph focuses on the role of the Tfh cell in SLE, providing a foundation for the discussion in which the potential roles of Tfh cells in the production of autoantibodies are discussed.

8.2 Tfh cells and Systemic Lupus Erythematosus

The autoimmune disease Systemic Lupus Erythematosus (SLE) is a chronic inflammatory disease. Different organs can be affected making the clinical manifestations of SLE highly variable. Periods of remission and acute or chronic relapse are seen in patients with SLE. The disease is characterised by the high amount of immune complexes and autoantibodies moving to the end-organs (e.g. skin, joints and kidneys). The autoantibodies are mainly directed to cell nuclear components, for example against double-stranded DNA (dsDNA), histones and ribonuclear proteins[30]. The US prevalence of SLE is stated on 161,000 adults with definite SLE and 322,000 adults with probable SLE[31]. No cure for SLE has been found yet. Current treatments mainly encompass the administration of immune suppressive agents. Unfortunately, these therapies are inadequate since there is still progression of autoantibodies to end-stage organs causing damage to these organs. In addition, current therapies are not free of side affects. The first step towards the development of more specific

treatments is the identifications of biomarkers or cell subsets contributing to the pathophysiology of SLE phenotypes[32]. More knowledge about the pathophysiology of SLE also provides better opportunities for accurate and early diagnoses for the different SLE phenotypes. The production of dsDNA antibodies is a constant factor of the SLE phenotype. For this reason, fundamental failures resulting in this over production of autoantibodies are thought to be informative for understanding the mechanism of the disease. Tfh cells are involved in the production of (auto)antibodies and therefore one of the cell subsets of interest when looking for new therapy targets[32].

As described before, Tfh cells are essential in the selection process of high affinity antibody producing GC B cells. When the GC B cell is self reactive or not able to express high affinity antibodies the B cell is excluded from Tfh cell help and becomes apoptotic. In Sanroque mice with SLE like phenotypes defects in Tfh cell accumulation in GC are seen, this resulted in a wrong collaboration between Tfh cells and GC B cells. Finally the number of autoantibodies did increase which may indicate that under certain circumstances Tfh cells are stimulating self reactive B cells and low affinity B cells as well[32].

In the GCs high numbers of tingible body macrophages are present, these large phagocytic cells are responsible for the clearance of apoptotic material in the GCs. In a sub-group of SLE patients the tingible body macrophages do not clear apoptotic material in a proper way. This results in an increased amount of nuclear autoantigens binding to FDCs which in turn may provide activation of self reactive B cells to produce autoantibodies[33]. This pathway illustrates one way of autoantibody production in the GCs. This literature study focuses on the role of Tfh cells in the production of autoantibodies. As described before, this role is still unknown. However, there are designations leading to some possible explanations.

Different points of view can be taken when discussing the role of Tfh cells in the production of autoantibodies. With the use of two recent research papers about the increased level of CXCL13 in children and adults with SLE the possible Tfh cell roles in the production of autoantibodies are discussed[34]. In the end, approaches for future research are put forward. These approaches may help in the development of targeted therapies against SLE.

8.3 Increased CXCL13 levels in SLE

Two recent studies are focused on the level of CXCL13 in patients with SLE. These research papers are of interest when looking at the role of Tfh cells in the production of autoantibodies. One study is performed in children younger than 16 years with SLE[34]. 15-20% of all SLE patients consists of children younger than 16 years. The other study is performed on adults with SLE[35]. Differences are seen in SLE phenotype between children with SLE and adults with SLE. For example, in children with SLE a higher prevalence of progression to end-stage renal disease is seen. Both studies have one common outcome concerning the role of chemokine CXCL13 in SLE. Data of serum CXCL13 levels measured with ELISA indicated that increased levels of CXCL13 are seen in SLE.

In both studies the level of CXCL13 correlates with the activity and severity of the disease. CXCL13, mainly produced by macrophages and dendritic cells, attracts the chemokine receptor CXCR5. Since CXCR5 is expressed on both B-cells and Tfh cells these cells are attracted to the lymph nodes and in addition, more GCs are formed within the lymph nodes[34, 35].

Since increased levels of CXCL13 are seen in SLE patients it is useful to know the triggers of CXCL13 expression. CXCL13 expression can be triggered after bacterial exposure by the high amount of inflammatory cytokines[35]. Another trigger of CXCL13 expression is the proteoglycan biglycan. Proteoglycan biglycan is able to trigger CXCL13 expression via TLR2 and 4 in macrophages and dendritic cells. Mice with proteoglycan biglycan deficiency demonstrated to have lower levels of autoantibodies compared to mice with SLE like phenotypes[31].

In SLE patients the level of CXCL13 equals with disease severity[34]. During periods of remission CXCL13 levels decrease and during periods of relapse CXCL13 levels increase. Since CXCL13 is a chemokine responsible for homing of Tfh cells in the GC the accumulation of Tfh cells alters during periods of remission and relapse as well. The mechanisms behind this altered Tfh cell accumulation remains unclear but this might be related with the amount of autoantibodies produced during SLE. This possible relation between Tfh cell accumulation at the GC and the production of autoantibodies is described below.

9. Discussion

In this literature study the different characteristics of the follicular T helper cell are described. This overview forms a good basis for a discussion based on new insights into the function of the Tfh cell. Especially investigation of the Tfh cell role in autoimmune pathology is of high interest regarding to the development of more specific treatments.
This leads to the main question of this literature study:

What are the potential roles of follicular T helper cells in autoimmune pathology and what potential targets can be used to develop new therapeutics to restore this immune disturbance?

Tfh cells are essential in the stimulation of GC B cells to differentiate into high-affinity antibody producing plasma cells and memory B cells. When this process is disturbed an increased production of autoantibodies can be measured[28]. It is plausible that Tfh cells play an important role in this increased production of autoantibodies. The exact role of the Tfh cell in the production of autoantibodies remains elusive but different roles can be put forward. These roles provide new ideas for future research that may lead to the development of better treatments against distinct autoimmune diseases. The different roles of Tfh cells in autoantibody production are discussed using the autoimmune disease SLE.

9.1 Different roles Tfh cell in autoantibody production

The different possible roles of Tfh cells in the stimulation of autoantibody production are thought to depend on the chemokine CXCL13 present in the GC. When the expression of CXCL13 increases an altered accumulation of Tfh cells at the GC appears. Following this assumption, the severity of SLE correlates with GC levels of CXCL13. CXCL13 can be triggered by among others different inflammatory cytokines and proteoglycan biglycan. In SLE patients proteoglycan biglycan is a trigger for increased CXCL13 expression[31]. In addition, an unknown genetic or environmental trigger might be responsible for the increased production of CXCL13. After an increased expression of CXCL13 in the GC, the accumulation of Tfh cells to the GC changes.

In this discussion three possible ways of Tfh cell accumulation are put forward that might lead to an increased production of autoantibodies (Figure 12). First, an increased number of Tfh cells localising in the GC might lead to an increased production of autoantibodies. When the level of CXCL13 in the GCs increases a stronger CXCL13 gradient in and around the GCs is present. This strong gradient is thought to attract both Tfh cells with low CXCR5 expression and Tfh cells with high CXCR5 expression. It is assumed that under normal circumstances Tfh cells with low CXCR5 expression are less likely to be attracted to the GCs and only Tfh cells with high CXCR5 expression are attracted to the GCs. When this increased level of CXCL13 attracts more Tfh cells it is likely that there are more interactions with GC B cells. In SLE an increased amount of Tfh cell help to self reacting B cells and low affinity B cells is seen[32]. This might be a result of the increased amount of Tfh cells in the GC. An interesting point to consider is whether the Tfh cells with low expression of CXCR5 are self reacting or low affinity Tfh cells which stimulate the corresponding B cells which might lead to the production of autoantibodies. To sum up, this assumption is based on an increased amount of Tfh cells migrating to the GC resulting in more self reactive and low affinity Tfh cell – B cell interaction leading to a higher number of autoantibodies.

Second, Tfh cells might be captured in the GCs for a longer period before they migrate back into the periphery, supporting Tfh cells to activate several B cells. The amount of Tfh cells entering the GC is thought to remain the same but the high level of CXCL13 in the GC may provide a longer stay of these CXCR5+ Tfh cells in the GC´s resulting in a disturbed balance between incoming and outgoing Tfh cells. The longer stay of Tfh cells in the GC may provide increased B cell activation by the Tfh cell. This increased B cell activation might lead to incorrect somatic hypermutation and in this way an increased production of autoantibodies[28].

Third, a combination of the two possible mechanisms behind a disturbed Tfh cell accumulation described above might lead to an increased production of autoantibodies. Taking previous points in consideration, in all three assumptions the altered accumulation of Tfh cells to the GC leads to more crowded GCs. In the more crowded GCs the flow of Tfh cells is disturbed, that may lead to incorrect interactions between Tfh cells and B cells.

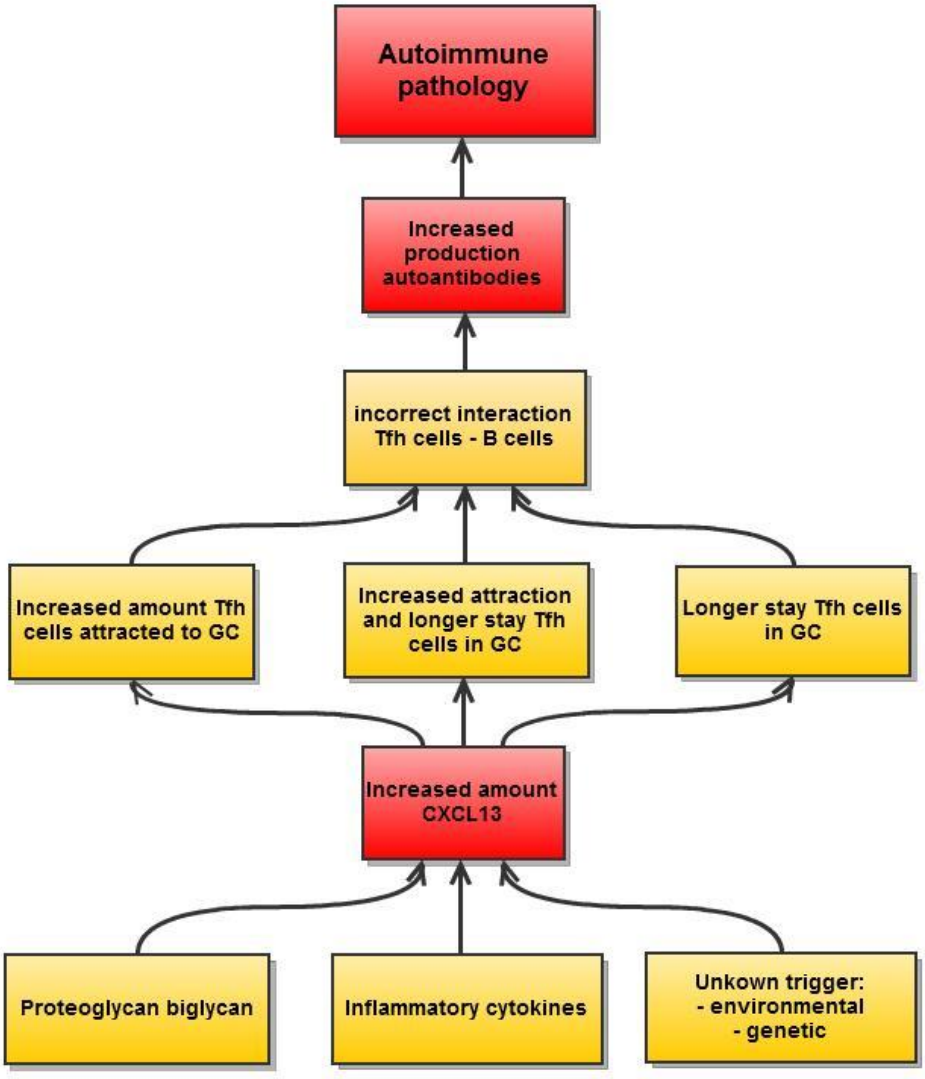

Figure 12. Three assumptions for the mechanisms behind a disturbed Tfh cell accumulation leading to the production of autoantibodies. CXCL13 can be triggered by different agents. The increased amount of CXCL13 binds chemokine receptor CXCR5 present on Tfh cells. This diagram illustrates three possibilities for a disturbed Tfh cell accumulation. An altered accumulation of Tfh cells might lead to autoantibody production. The exact role of Tfh cells in the production of autoantibodies remains unknown.

9.2 Future approaches

In autoimmune diseases like SLE high numbers of autoantibodies are responsible for end-organ damage. In SLE it would be very helpful to be able to detect the mechanisms that are responsible for the high number of autoantibodies since the characteristic autoantibody pattern in SLE can be detected before the disease manifestation appears[29]. Biomarkers or cell subsets contributing to the pathophysiological process of the disease form interesting targets for new therapies. The Tfh cell is a key player in the increased production of autoantibodies in SLE. The assumptions of an altered Tfh cell accumulation described before might form one factor leading to an increased production of autoantibodies. However, more research on the exact role of Tfh cells in the production of autoantibodies is needed. One can think of an assay in which the differences between Tfh cells with low expression of CXCR5 and Tfh cells with high expression of CXCR5 are sorted on the basis of different markers. These differences may form a starting point for the development of Tfh cell specific treatments. Unfortunately, there is a big chance of side effects when depleting distinct Tfh cells. An increased risk of infections may appear. Human tonsils are suitable organs to study the altered accumulation of Tfh cells to GCs in SLE. However, to obtain these organs from SLE patients is difficult.

Another possibility for future drug development might be focused on CXCL13. During periods of remission and relapse in SLE the levels of CXCL13 increase and decrease respectively. It is not known whether these periods of decrease and increase in CXCL13 are a cause or a consequence of disease remission and relapse. This forms a disadvantage for current research. Maybe the amount of proteoglycan biglycan varies which influences the levels of CXCL13. Blocking of proteoglycan biglycan might be interesting for future drug development as well.

10. Concluding remarks

Taking previous points in consideration, it is obvious that the Tfh cell plays a key role in the stimulation of autoantibody production by activating GC B cells in an incorrect way. This literature study underlines the importance of CXCL13 for an altered accumulation of Tfh cells to the GC. It is assumed that this altered accumulation of Tfh cells forms the basis for an incorrect B cell activation that results in an increased production of autoantibodies. The mechanism behind the formation of the characteristic autoantibody pattern in SLE contains several biomarkers and cell subsets that form interesting targets for the development of new treatments. According to this literature study CXCL13 and proteoglycan biglycan seem to be promising biomarkers for future research. Since these biomarkers regulate the migration of immune cells there may be less severe side effects when depleting these biomarkers compared to the depletion of cell subsets like Tfh cells to combat SLE. More specific treatments against the formation of autoantibodies might have a milder effect on SLE. However, these specific treatments may have less severe side effects than current immunosuppressive treatments. In conclusion, future research focused on Tfh cells and other biomarkers and subsets involved in the production of autoantibodies is of high importance. This might result in more specific treatment strategies against the formation of autoantibodies in the early onset of autoimmune pathology. Progression in treatment strategies will improve the life of many patients suffering from autoimmune diseases like SLE.

List of abbreviations

AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
BCA-1	B-cell attracting chemokine 1
BCL6	B-cell lymphoma 6
BCR	B-cell receptor
CD	Cluster of differentiation
dsDNA	double stranded DNA
FDC	Follicular dendritic cell
GC	Germinal centre
ICOS	Inducible co-stimulator
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-21R	Interleukin-21 receptor
MHC	Major histocompatibility complex
NO	Nitric oxid
PD-1	Programmed death-1
PP	Peyer's patches
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
STAT3	Signal transducer and activator of transcription 3
TCR	T-cell receptor
Tfh	T follicular helper cell
Th	T helper cell
TNF-α	Tumor necrosis factor- α
Treg	Regulatory T cell

11. Acknowledgements

First of all, special thanks to my supervisor Theo van den Broek for all his support and enthusiasm. I also thank Berent Prakken for giving me the opportunity to write my Bachelor thesis at the Laboratory of Translational Immunology, Chantal Duurland for her help during my practical period, and the colleagues of the Prakken laboratory for contributing to this educational period.

12. References

1. Janeway, C. A., *et al.* *Janeway's immunobiology,* Garland Science, 2008.

2. Kindt, T. J., *et al.* *Kuby immunology,* Freeman, 2007.

3. King, C. New insights into the differentiation and function of T follicular helper cells. *Nat. Rev. Immunol.* 9, 2009, 757-766.

4. Crotty, S. Follicular Helper CD4 T Cells (T(FH)). *Annu. Rev. Immunol.* 29, 2011, 621-663.

5. Hernandez, J. B., *et al.* Life and death in the thymus--cell death signaling during T cell development. *Curr. Opin. Cell Biol.* 22, 2010, 865-871.

6. Romagnani, S. Th1/Th2 cells. *Inflamm. Bowel Dis.* 5, 1999, 285-294.

7. Bi, Y., Liu, G. & Yang, R. Reciprocal modulation between TH17 and other helper T cell lineages. *J. Cell. Physiol.* 226, 2011, 8-13.

8. Dong, C. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat. Rev. Immunol.* 8, 2008, 337-348.

9. Zhou, L., *et al.* Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30, 2009, 646-655.

10. O'Shea, J. J. & Paul, W. E. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* 327, 2010, 1098-1102.

11. Kim, C. H., *et al*. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J. Exp. Med.* 193, 2001, 1373-1381.

12. Breitfeld, D., *et al*. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* 192, 2000, 1545-1552.

13. Zhu, J., *et al.* Differentiation of effector CD4 T cell populations. *Annu. Rev. Immunol.* 28, 2010, 445-489.

14. Johnston, R. J., *et al*. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325, 2009, 1006-1010.

15. Nurieva, R. I., *et al*. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29, 2008, 138-149.

16. Yu, D., *et al*. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31, 2009, 457-468.

17. Tsuji, M., *et al*. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* 323, 2009, 1488-1492.

18. Hutloff, A., *et al*. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397, 1999, 263-266.

19. Eto, D., *et al*. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One* 6, 2011, e17739.

20. Cannons, J. L., *et al*. SAP regulates T cell-mediated help for humoral immunity by a mechanism distinct from cytokine regulation. *J. Exp. Med.* 203, 2006, 1551-1565.

21. Good-Jacobson, K. L., *et al*. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat. Immunol.* 11, 2010, 535-542.

22. Junqueira, C. & Carneiro, J. *Basic histology,* McGraw-Hill Education, 2005.

23. Gatto, D. & Brink, R. The germinal center reaction. *J. Allergy Clin. Immunol.* 126, 2010, 898-907.

24. Haynes, N. M., *et al*. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J. Immunol.* 179, 2007, 5099-5108.

25. de Villartay, J. P., *et al.* The mechanisms of immune diversification and their disorders. *Nat. Rev. Immunol.* 3, 2003, 962-972.

26. Zotos, D., *et al*. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J. Exp. Med.* 207, 2010, 365-378.

27. Tsuji, M., *et al*. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* 323, 2009, 1488-1492.

28. Gomez-Martin, D., *et al.* Follicular helper T cells poise immune responses to the development of autoimmune pathology. *Autoimmun. Rev.* 10, 2011, 325-330.

29. Fattal, I., *et al*. An antibody profile of systemic lupus erythematosus detected by antigen microarray. *Immunology* 130, 2010, 337-343.

30. Linterman, M. A., *et al*. Follicular helper T cells are required for systemic autoimmunity. *J. Exp. Med.* 206, 2009, 561-576.

31. Moreth, K., *et al*. The proteoglycan biglycan regulates expression of the B cell chemoattractant CXCL13 and aggravates murine lupus nephritis. *J. Clin. Invest.* 120, 2010, 4251-4272.

32. Simpson, N., *et al*. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum.* 62, 2010, 234-244.

33. Baumann, I., *et al*. Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum.* 46, 2002, 191-201.

34. Ezzat, M., *et al.* Elevated production of serum B-cell-attracting chemokine-1 (BCA-1/CXCL13) is correlated with childhood-onset lupus disease activity, severity, and renal involvement. *Lupus,* 2011, 845-854.

35. Schiffer, L., *et al*. Elevation of serum CXCL13 in SLE as well as in sepsis. *Lupus* 20, 2011, 507-511.

36. adapted from http://www.mda-sy.com/pathology/HISTHTML/NORMAL/NORM027.HTM, available on may 21

13. Lab report

After lymphocytes are isolated from peripheral blood, the amount of CD4-CXCR5 positive cells is measured using FACS. CD4 and CXCR5 are markers for follicular T helper (Tfh) cells. Samples of healthy controls with different ages (<1 month, between 6 and 12 months and between 20 and 30 years) are measured. The amount of CD3+CD4-CXCR5 positive cells is increasing during ageing.

Introduction

This lab report describes the procedure and results of CD3+CD4+ CXCR5+ immunofluorescent cell staining and flow cytometric analysis by using Fluorescence-activated cell sorting (FACS). This experiment is part of a study on the influence of a neonatal thymectomy on the maturation and behaviour of T lymphocytes.

Neonates born with heart failures often get cardiac surgery during the first weeks of their life. To get better access to the heart and the great vessels the thymus often has to be removed partially or completely, this is called a thymectomy[1]. The amount of immune cells after a neonatal thymectomy changes highly. Since the thymus is responsible for the maturation of T cells a drop in peripheral T cell populations is seen in the short- as well as long-term after neonatal thymectomy. This high drop in peripheral T cells causes a lymphopenic situation. In addition, the lack of naïve T cells derived from the thymus causes premature immunosenescence[1]. Immunosenescence is the natural degeneration of the immune system during aging, immunosenscence after thymectomy occurs already during early life. One would expect an increased risk of infectious diseases and immune disturbances (e.g. auto-immune diseases) after neonatal thymectomy. However, until now this has not been reported. A suspicious observation is the normal level of IgG after thymectomy. On the contrary, significant lower levels of IgA and IgG1 are measured in thymectomized patients[2]. Since the follicular T helper (Tfh) cells play a key role in the antibody production there might be a correlation between the level of IgG and the presence of Tfh cells after neonatal thymectomy. When this relationship is understood it might clarify the absence of infectious diseases and immune disturbances after neonatal thymectomy. In addition, it might be possible to find other ways to prevent these patients for getting premature immunosenescence or other immune disturbances during their further life. Taking previous thing in consideration, immunofluorescent staining and flow cytometric analysis of Tfh cells are performed to measure the amount of Tfh cells at different time points before and after neonatal thymectomy. Referring to the short amount of time of this practical period, this lab report only describes the measurement and interpretation of four healthy control samples taken at different ages.

Peripheral blood samples from different healthy controls are used to collect lymphocytes and monocytes. The isolation of these lymphocytes and monocytes is obtained using density centrifugation. The peripheral blood is put on a ficoll solution (Figure 1A). After centrifuging different layers with different cell types can be distinguished (Figure 1B). Lymphocytes have a low density compared to the other cell types. For this reason, the lymphocytes are found in a thin layer between the plasma cells and the ficoll. Afterwards, the lymphocytes are isolated and exposed to washing steps for several times to obtain the pure lymphocyte solution.

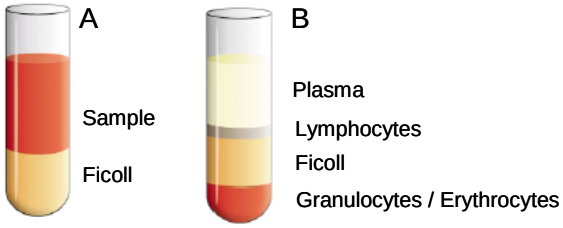

Figure 1. Separation of different cell types using ficoll[3]. Figure A illustrates the cell division layers before centrifugation. Figure B illustrates the cell division layers after centrifugation.

When the lymphocytes are obtained immunofluorescent staining is performed to be able to analyse the amount of CD4+CXCR5+ lymphocytes present. The cells are first treated with mouse serum to prevent a-specific bindings. Than the cells are stained with fluorochrome-conjugated monoclonal antibodies specific for among others the cell surface antigens CD3, CD4 and CXCR5. By using the fluorenscence-activated cell sorter (FACS) machine the individual cells are characterised and separated. The cells pass different photodetectors in a stream. In this way the fluorescence due to bound fluorescent antibodies is measured. This technique is efficient since individual cells are simultaneously analysed for several parameters in a short time.

During this experiment the amount of CD3+CD4+CXCR5 positive cells is measured in healthy controls. These markers are present on Tfh cells. The healthy controls included in this experiment do still have a functional thymus. Having said this, an increase in the amount of CD4-CXCR5 positive cells is expected during ageing.

Materials and methods

lymphocyte isolation

For the composition of the different reagents and the list of equipment and accessories tools see the protocol 'Isolation of human mononuclear cells from blood'.

To be able to perform this experiment blood samples of healthy controls with different ages are collected. The blood samples are centrifuged for 10 minutes at 1200 rpm. Afterwards, plasma can be collected and used for other purposes. A 15 ml sterile tube is filled with 4 ml ficoll (density = 1077 g/L). All blood samples are transferred to sterile 50 ml tubes and diluted with an equal volume of basic culture medium. A minimum of 4 ml diluted blood is slowly layered onto the ficoll. It is essential to work slowly, to prevent mixing of the ficoll sample with the diluted blood. Centrifuge the tubes for 20 minutes at 2300 rpm at room temperature (RT). On this point, the different cell layers are formed. Collect the lymphocyte fraction out of the 15 ml tube and transfer the cells to a 50 ml tube containing 10 ml wash medium. Fill 50 ml tube with containing the lymphocytes up to 50 ml with wash medium. Centrifuge the tubes for 10 minutes at 1600 rpm at RT. After centrifuging the supernatant is removed and the cells are resuspended. Remove the cellular debris within the pipet by keeping it in an angel of 90° and empty slowly. Fill the tube up to 50 ml with wash medium and centrifuge 10 minutes at 1600 rpm at RT. Remove the supernatant and resuspend the cells. 10 µl cell suspension is diluted with 90 µl counting solution (Trypan-blue or Turk). Trypan-blue is used when the cells were previously frozen, and when the viability has to be assessed. Turk is used when the cells are freshly isolated. Turk lysis red blood cells, this promotes the counting of lymphocytes. Afterwards, the cells are counted using a hemacytometer. In the end, the cells are equally divided in different tubes. The cells can be frozen with the use of freezing medium in Nunc cryovials.

FACS staining

For the composition of the different reagents and the list of equipment and accessories tools see the protocol 'Immunofluorescent staining and flow cytometric analysis of cells'.

When frozen cell samples are used the samples are first briefly thawed in a 37°C water bath. Pellet the samples for 5 minutes at 1600 rpm at +4°C. Discard the supernatant carefully and wash the cells by resuspending the pellet in cold FACS buffer. Pellet the samples again for 5 minutes at 1600 rpm at +4°C. Discard the supernatant carefully. Resuspend $\pm 2.0 \cdot 10^5$ Cells in 25 µl FACS buffer containing 10% mouse serum. Incubate for 5 minutes at +4°C. Mix 10% mouse serum with the pre-titrated optimal concentration for cytometric analysis of cells of

fluorochrome-conjugated monoclonal antibody specific for a cell surface antigen. Different fluorochrome-conjugated monoclonal antibodies are used (Table 1). A 96 wells plate is used, 25 µl per well is added. Incubate the plates for 20 minutes at +4°C in the dark at a plate shaker, shake slowly. Wash the cells with 150 µl cold FACS buffer and pellet the cells 5 minutes at 1600 rpm at +4°C. After centrifuging, discard the cells carefully. Wash the cells in 200 µl cold FACS buffer. Pellet the cells afterwards again 5 minutes at 1600 rpm at +4°C. Discard the supernatant carfully. Transfer the cell samples with 150 µl cold FACS buffer to a Nunc Micronic tube.

Fluorochrome	Antibody against
Fitc	CXCR5 (0.25µl)
PE	CD31 (2µl)
PerCP (Cy5.5)	CD8 (6µl)
PeCy7	CD3(0.5µl)
Alexa 647	PD-1 (2 µl) CD279
V450	Foxp3 (1µl)
APC-Cy7	CD4 (4µl)

Table 1. Different fluorochrome-conjugated monoclonal antibodies. By using FACS the different surface molecules on the lymphocytes are measured due to the binding of the fluorescent antibodies. Amount of µl presented in the table is calculated for a total end solution of 50ul staining volume.

When the samples are successfully stained the fluorescence of the different fluorochrome types is measured with the use of a FACS machine. In figure 2 the amount of FITC-A CXCR5 is counted. A peak is seen around 10^2, this is the population that does not express CXCR5 (negative peak). The peak seen at approximately 10^3 are the cells that express CXCR5. First the cells are selected with the use of a lymphocyte gate. Afterwards, CD3+ and CD4+ positive cells are selected. Within the CD3+CD4+ cell population the level of CXCR5 expression is measured. The obtained dotplots are analysed with FlowJo V7.6.1 and the amount of different surface proteins is quantified.

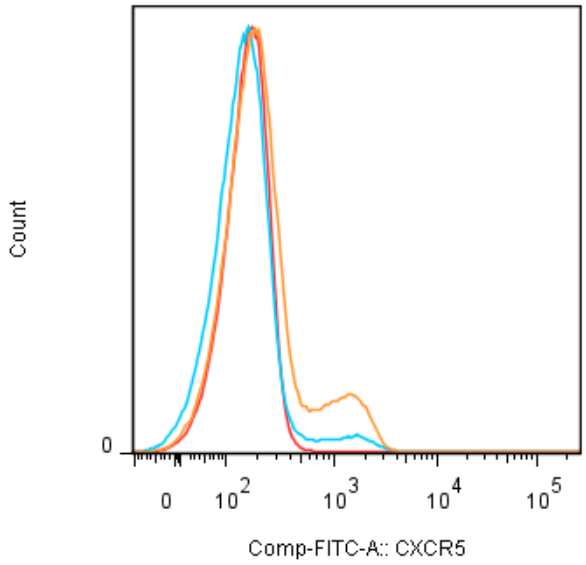

Figure 2. FITC-A CXCR5 counting. A CXCR5 negative peak is seen around 10^2. Around 10^3 a CXCR5 positive peak is seen.

Results

Different dotplots are made after lymphocyte staining. In this lab report the dotplots of healthy controls at different ages are gated for CD3+CD4+CXCR5. The healthy control aged <1 month only contains a low percentage of CD4+CXCR5 positive cells (0.362%). When looking at the dotplots of the healthy controls aged between 6 and 12 months and aged between 20 and 30 years the amount of CD4+CXCR5 positive cells is increasing (respectively 4.44% and 13.2%). Since CD4 and CXCR5 are markers for Tfh cells the previous results confirm the growing population of Tfh cells between the age of 0 and the age of 30.

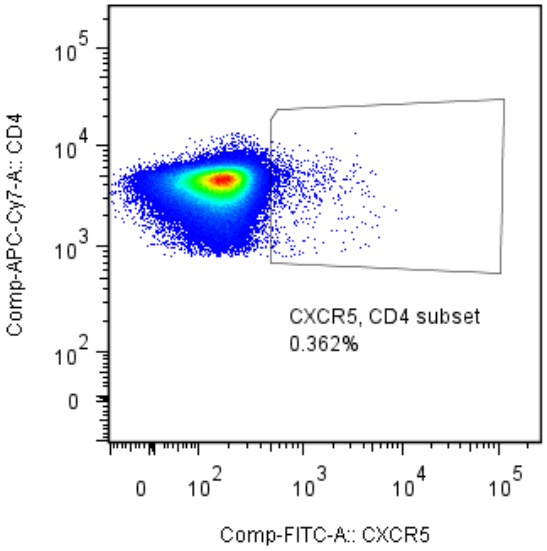

Figure 3. Dotplot of lymphocytes isolated from healthy control aged < 1 month. A low percentage (0.362%) of the total amount of cells is CD4+CXCR5 positive.

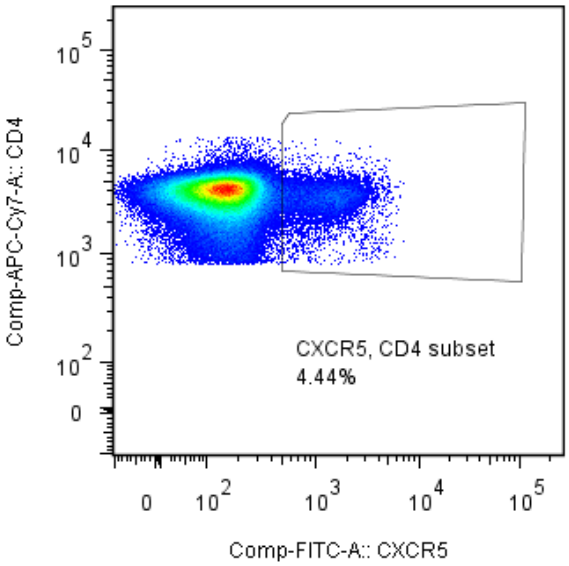

Figure 4. Dotplot of lymphocytes from healthy control aged between 6 and 12 months. 4.44% of the total amount of cells is CD4+CXCR5 positive.

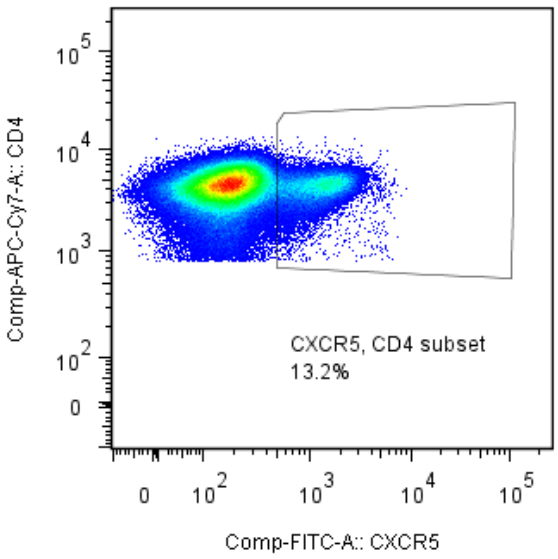

Figure 5. Dotplot of lymphocytes from healthy controls aged between 20 to 30 years. 13.2% of the total amount of cells is CD4+CXCR5 positive.

Discussion

This lab report describes a small part out of a comprehensive thymectomy study. Several markers are measured to study the amount of different T cell subsets before and after neonatal thymectomy. This lab report only encompasses the measurement of CD4+CXCR5 positive cells in differently aged healthy controls. CD4 is a marker present on all T helper cells. The chemokine CXCR5 is a characteristic marker for the Tfh cell, this chemokine is responsible for homing of the Tfh cell in the lymph node Germinal Centres. Since the thymus is still present in all the healthy controls the production of T cells is expected to increase between age 0 and age 30. This is confirmed when looking at the percentages of CD4+CXCR5 positive cells at different ages. At age <1 month the percentage of CD4+CXCR5 positive cells is only 0.362% compared to 13.2% between the age of 20 to 30 years.

The results of this experiment confirm the increasing production of Tfh cells between the age of 0 and the age of 30. When performing the same experiment with differently aged thymectomy patients the amount of CD4+CXCR5 positive cells is expected to be less. Although, the normal amounts of IgG after neonatal thymectomy might indicate that there are still Tfh cells present when the thymus is absent. Another possibility might be that the Tfh cell activity to stimulate B cells to become antibody producing plasma cells is taken over by other immune cells.

Up till now no increased rate of infections is seen in neonatal thymectomy patients. It is of high importance to study the T cell pattern before and after neonatal thymectomy. These studies may lead to the development of drugs that prevent patients from premature immunosenescence and possible infections during later life.

References

1. Afifi, A., *et al.* For neonates undergoing cardiac surgery does thymectomy as opposed to thymic preservation have any adverse immunological consequences? *Interact cardiovasc thorac surg.*11, 2010, 287-291.

2. Zlamy, M., *et al*. Antibody dynamics after tick-borne encephalitis and measles-mumps-rubella vaccination in children post early thymectomy. *Vaccine* 28, 2010, 8053-8060.

3. Protocol: Isolation of human mononuclear cells from blood. *Department of Pediatric Immunology WKZ version 9*, 2008.