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Dahlgren, Madelene

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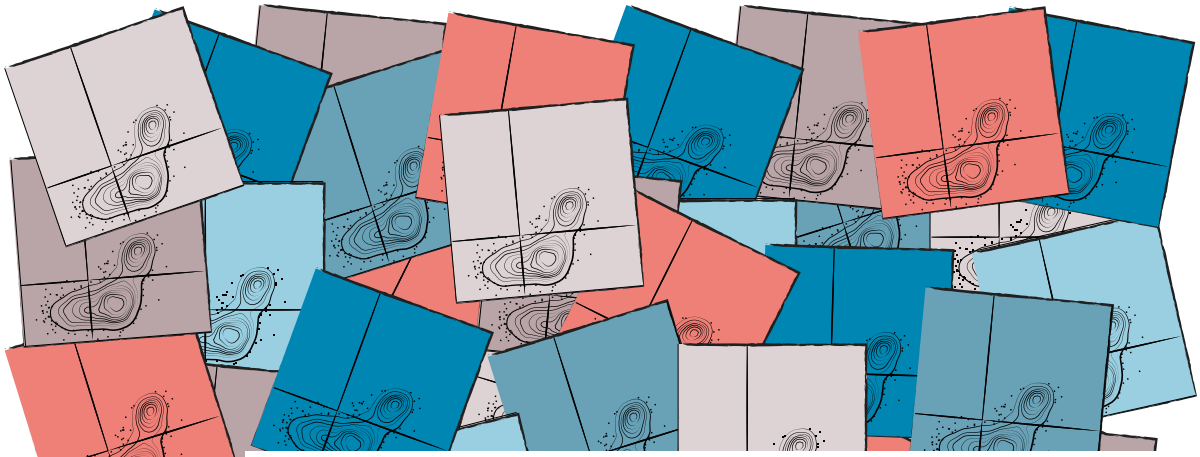

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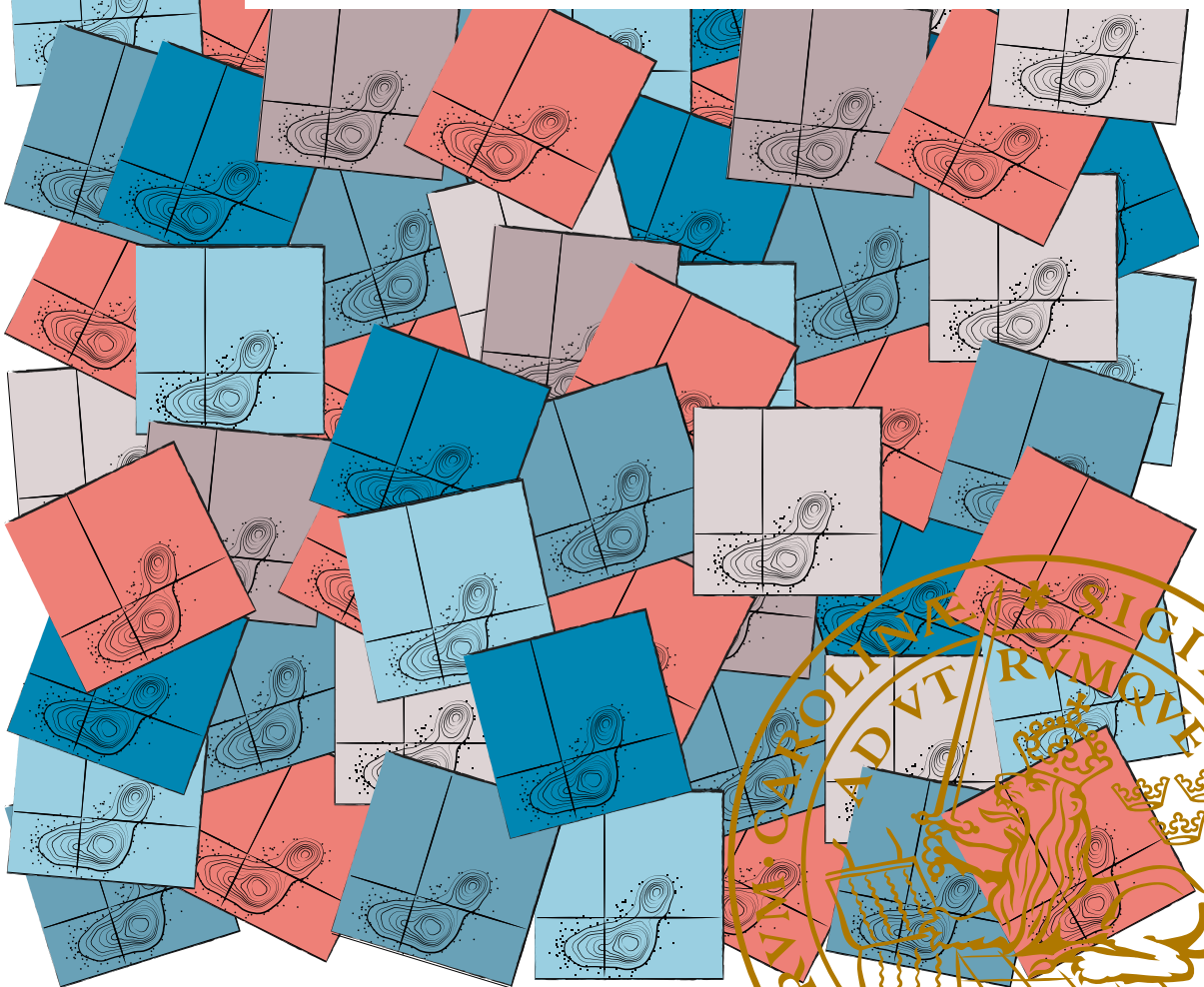
MADELENE DAHLGREN
FACULTY OF MEDICINE | LUND UNIVERSITY 2016



Regulation of the Germinal Center Response by T Follicular Helper Cells and Type I Interferons

Madelene Dahlgren

LUND
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Abstract

The protective effect of most currently available vaccines is dependent on high-affinity antibodies and long lived B cell memory, which develop within organized structures of secondary lymphoid organs called germinal centers (GCs). GC formation is supported by T follicular helper (Tfh) cells, a subset of $CD4^+$ T cells that develop in parallel to other effector T cells and specifically localizes to B cell follicles. The aim for the work presented in this thesis was to define how the GC response and Tfh cell differentiation are regulated by innate effectors, such as conventional dendritic cells (cDCs) and type I interferons (IFNs), in response to protein immunization adjuvanted by the dsRNA-analogue poly(I:C).

In paper I, we show that Tfh cells and GC B cell responses of the IgG1 isotype can develop normally in the absence of cDCs when a sufficient amount of antigen, allowing initial T cell priming, is provided. In contrast, the concurrent Th1 cell differentiation is impaired together with a selective loss of IgG2c production. We also find that B cells, monocytes and possibly plasmacytoid DCs (pDCs) redundantly can prime $CD4^+$ T cells in the absence of cDCs, and thereby support early expression of the Tfh cell-associated chemokine receptor CXCR5. In paper II we find that type I IFNs predominantly promote $IgG2c^+$ GC B cell differentiation and in this context function through both B cell intrinsic and extrinsic signaling. While we provide evidence for that direct IFN-γ signaling in B cells controls switching to IgG2c, our results indicate that type I IFNs regulate IgG2c-associated GC responses beyond isotype switching, as the magnitude of the GC B cell response is reduced when type I IFNs, but not when IFN-γ, are absent. In contrast to IgG2c, switching to IgG1 is unaffected by type I IFN deficiency, although the overall magnitude and quality of the IgG1 response also is affected when type I IFN signaling is abrogated. Additionally, the formation of B cell memory appears to be impaired in the absence of type I IFNs. In paper III, we demonstrate how clonal competition selectively affects Tfh cell differentiation, GC responses and generation of $IgG1^+$ GC B cells, while the generation of $IgG2c^+$ GC B cells appears to be less affected.

Taken together, the work included in this thesis increase our understanding of how GC B cell responses and Tfh cell development are regulated, and furthermore, it suggests that switching to IgG1 and IgG2c associated GC B cell fate commitment may be differentially dependent on Tfh cells.

Key words Germinal Center, T follicular helper cell, type I interferon, dendritic cell, adjuvant, differentiation, IgG.

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Section for Immunology
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Faculty of Medicine, Lund University

Cover

Modified flow cytometry contour plots of CXCR5 versus Bcl6 expression by OT-II cells eight days after immunization with poly(I:C).

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Till mormor

"Everything should be made as simple as possible, but not simpler"

Albert Einstein

Preface

The reason to why I became interested in immunology is quite simple; my own immune system had been bothering me for many years. Somehow, my immune cells thought that horses, or more specifically the dander they produce, was something I should be protected from. My parents might have agreed on that, but my interest for horses remained and although worse at times, the allergy was manageable. On top of this, all kinds of pollen were fought off with power, making spring and early summer periods when my consumption of antihistamines spiked. I was certainly not thinking about immunology at that time, but it was likely these experiences that eventually made me curious as to why I produce antibodies towards seemingly harmless proteins.

The work presented in this thesis does not specifically handle allergic responses, but it does focus on the cellular events leading to production of high-affinity antibodies in response to immunization (the more scientific word for vaccination), which occurs in the Germinal Center response. Production of antibodies is a shared feature between allergy, autoimmunity and vaccination, and a further understanding of this process may have bearing on all three entities.

I have definitely learned a lot during my time as a PhD student, but for each new insight I have also realized how much there is left to learn about immunology. Another realization has been how fascinating the immune system actually is. In many ways it is difficult to understand how this complex network of cells and mediators has developed and, most often, functions without complications.

For most readers (who will rapidly skip to the acknowledgement section), I would like to recommend the first part of the 'Vaccination' chapter, which will give an explanation of the picture on the back of this book.

Happy reading!

Madelene Dahlgren
Lund, Mars 2016

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Original papers and manuscripts

Paper I

T Follicular Helper, but Not Th1, Cell Differentiation in the Absence of Conventional Dendritic Cells

Madelene W. Dahlgren, Tobias Gustafsson-Hedberg, Megan Livingston, Helena Cucak, Samuel Alsén, Ulf Yrlid and Bengt Johansson-Lindbom

*The Journal of Immunology, 2015 Jun 1; 194(11): 5187-5199. **

Paper II

Complementary roles for type I and II interferons in IgG2c germinal center B cell fate commitment

Madelene W. Dahlgren and Bengt Johansson-Lindbom

In manuscript

Paper III

Clonal competition impairs T follicular helper cell differentiation and reveals separable T helper cell requirements for IgG1 and IgG2c associated germinal center B cell responses

Madelene W. Dahlgren and Bengt Johansson-Lindbom

In manuscript

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Papers not included in this thesis

Direct interaction between cholera toxin and dendritic cells is required for oral adjuvant activity

Tobias Gustafsson, Yeu-Jiann Hua, Madelene W. Dahlgren, Megan Livingston, Bengt Johansson-Lindbom and Ulf Yrlid

European Journal of Immunology, 2013 Jul; 43(7): 1779-1788.

Extracellular histones induce chemokine production in whole blood ex vivo and leukocyte recruitment in vivo

Johannes Westman, Praveen Papareddy, Madelene W. Dahlgren, Bhavya Chakrakodi, Anna Norrby-Teglund, Emanuel Smeds, Adam Linder, Matthias Mörgelin, Bengt Johansson-Lindbom, Arne Egesten, Heiko Herwald

PLoS Pathogens, 2015 Dec; 11(12): e1005319.

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
Ascl-2	Achaete-scute homologue 2
Bcl6	B cell lymphoma 6
BCR	B cell cell receptor
Blimp-1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
CCR	C-C chemokine receptor
CXCR	Chemokine (C-X-C motif) receptor
CSR	Class switch recombination
DC	Dendritic cell
DT	Diphtheria toxin
DZ	Dark zone
GC	Germinal center
ICOS	Inducible co-stimulator
IL	Interleukin
Ig	Immunoglobulin
IFN	Interferon
IFNAR	IFN alpha-beta receptor
IFNγR	IFN gamma receptor
IRF	Interferon regulatory factor
LZ	Light zone

MHC	Major histocompatibility complex
MPLA	Monophosphoryl lipid A
MZ	Marginal zone
NLR	NOD-like receptor
NP	4-hydroxy-3-nitrophenylacetyl
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PC	Plasma cell
PD-1	Programmed death-1
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
SHM	Somatic hypermutation
SLAM	Signaling lymphocyte activation molecule
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
STAT	Signal transducer and activator of transcription
Tfh	T follicular helper
Th	T helper
TI	T cell-independent
TCR	T cell receptor
TLR	Toll-like receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β

Introduction

Our immune system essentially has two tasks. First, it should protect us against disease, either by fighting off potentially harmful microbes to prevent infection, or by recognizing aberrant cells to prohibit tumor progression. In addition, it needs to stay tolerant to our own tissue in order to prevent autoimmune disease. This balance is maintained by a network of different cell types and organs that together found the basis of our immune system, generally divided into innate and adaptive immunity. These systems have distinct features but are nonetheless equally important. Moreover, the communication between innate and adaptive cells is central to mount an effective immune response.

The innate immune system is our first line of defense and consists of both physical barriers, such as our skin and epithelial cells lining the gut and lungs, and cells residing in the underlying tissue with the ability to rapidly respond if the barrier is breached. Cells belonging to the innate defense recognize different types of conserved patterns associated with danger, collectively called pathogen associated molecular patterns (PAMPs). These cells have the ability to prevent dissemination of the pathogen as well as recruiting and activating cells of the adaptive arm of the immune system. Innate immune cells include monocytes, macrophages, granulocytes, mast cells, natural killer (NK) cells and dendritic cells (DCs).

In contrast to the immediate response by innate immune cells, the adaptive response takes days before it has differentiated into functional effector cells. B and T cells are the effector cells of the adaptive response, which develop in the bone marrow and thymus, respectively. Instead of recognizing conserved patterns, B and T cells express receptors that recognize a wide variety of antigens. This enables them to respond to essentially any type of pathogen. These receptors are generated by a random assembly of different variable (V), diversity (D) and joining (J) gene segments during lymphocyte development, producing a highly diverse repertoire of T cell receptors (TCRs) and B cell receptors (BCRs). These receptors are then tested for recognition of autoantigens, which in most cases leads to clonal deletion, but also development of lymphocytes with regulatory properties.

A major difference between B and T cells is that B cells recognize native antigens, such as folded proteins, small molecules or glycosylated structures, while T cells recognize processed peptides presented on major histocompatibility complexes (MHCs) by

antigen presenting cells (APCs). Cells that are particularly good at priming T cells are referred to as professional APCs and include DCs, B cells, monocytes and macrophages. Grossly, T cells are divided into two subsets based on the expression of CD4 and CD8. $CD8^+$ T cells recognize peptides in the context of MHC class I and, upon activation, differentiate into cytotoxic T cells with the ability to immediately kill infected cells. On the other hand, $CD4^+$ T cells binds peptides presented on MHC class II and, following activation, support macrophage activation and antibody production by B cells (humoral immunity). Therefore, this subset is also called T helper (Th) cells. Depending on the type of immune response, Th cells obtain different properties and are further divided into subsets based on their function.

Importantly, adaptive immune cells tailor the response to the invading pathogen by selective amplification of cells that recognize the specific antigen and B cells can edit their receptors to improve their binding ability. In many cases, this is required to efficiently clear the infection. However, the utmost significant feature of adaptive immunity is its ability to form memory, which means that during secondary exposure to the same antigen the response will be more rapid and of higher quality.

The work included in this thesis mainly focuses on the adaptive immune response, and more specifically, how T cell- dependent B cell responses are regulated by innate cells and mediators.

Humoral immunity

A central aspect of the adaptive response is humoral immunity, mediated by Immunoglobulins (Igs) or antibodies. The activity of antibodies was observed already in 1890 by Behring and Kitasato who found that blood had the ability to neutralize diphtheria toxin, demonstrating that serum inhabiting anti-toxin activity could protect animals exposed to the same toxin [1]. Antibodies are heterodimeric glycoproteins circulating in body fluids that are small enough to enter and scavenge extracellular spaces, with the ability to neutralize toxins and mediate antibody-dependent cellular cytotoxicity (ADCC). Antibodies are produced by plasma cells (PCs), which are differentiated B cells specialized in producing high amounts of antibodies. The antibody molecule is composed of two light (L) and two heavy (H) chains, both with variable (V) regions that together forms two identical antigen-binding sites and determines the antigen specificity of the antibody. The constant (C) region of the H chain, designates the isotype subclass that determines the effector function of the antibody (Figure 1). This is regulated by binding to different types of Fc-receptors (FcR) expressed on distinct immune cells, or complement proteins, with varying affinity [2]. Simply put, Igs are the adaptors of adaptive immunity.

Immunoglobulins

Five different isotypes of Igs are found in both mice and human; IgM, IgD, IgA, IgE and IgG. The first isotype expressed during B cell development is IgM, and together with IgD, IgM function as the native BCR of all mature naïve B cells egressing from the bone marrow [1]. IgM is secreted as a pentameric molecule and initially dominates the primary humoral response until other isotypes have been generated. IgM has the ability to activate the classical complement pathway and contributes to the repertoire of natural antibodies thought to be involved in, for example, the clearance of apoptotic cells [3]. IgD is probably the least studied isotype of all Igs, although expressed on all mature naïve B cells. In addition to serving as a surface receptor, soluble IgD has the ability to bind basophils and is suggested to be involved in the defense against pathogens present in the upper respiratory tract [4]. IgA is the predominant isotype in mucosal secretions and breast milk, primarily secreted as dimers. Hence, IgA is part of the immunity against virus, bacteria and parasites at mucosal surfaces such as the gut and

vaginal tract. Two subclasses of IgA are present in humans, but only one is present in mice. IgE is involved in responses against parasites, but is also associated with aberrant responses to harmless environmental antigens in atopic allergy. Very low levels of free IgE is present in blood, partially due to its high affinity to the FcεRI expressed on mast cells, basophils, and eosinophils [5].

IgG is the predominant isotype found in blood and extravascular compartments, compromising up to 75% of the total Ig in the blood. Serum IgG has a half-life of approximately 23 days, which is the longest of all Igs. An important feature of the IgG molecules is their ability to cross the placenta, contributing to the initial protection of the newborn. There are four different IgG subclasses in humans; IgG1, IgG2, IgG3 and IgG4, named after their relative concentration in serum [6]. Mice instead produce the IgG subclasses IgG1, IgG2a, IgG2b and IgG3. Certain inbred mouse strains (C57Bl/6, C57Bl/10, SJL and NOD mice) do not express IgG2a but instead have an equivalent isotype named IgG2c, whereas some outbred mice strains can produce both IgG2a and IgG2c isotypes [7,8]. Mouse IgG subclasses are however not homologues of the human IgG isotypes [9]. Functionally, IgG3 and IgG1 of the human isotypes are known as efficient activators of the complement system, while IgG2a and IgG2c are the superior isotypes for this function in mouse [10].

Antibodies of the IgG isotype represent an important hallmark of immunological memory and notably, the protective effect of most currently available vaccines relies on the generation of long-lived, high affinity IgG-responses [11].

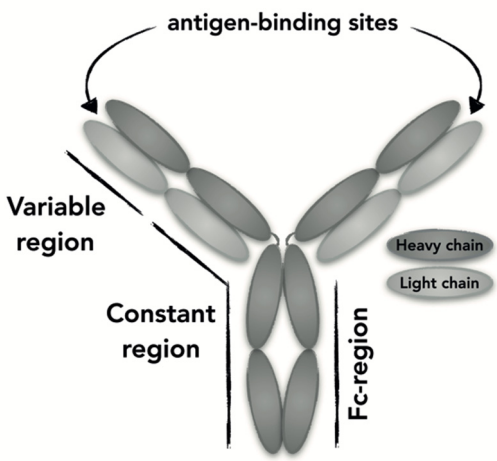

Figure 1. Schematic overview of an immunoglobulin molecule (antibody)
An antibody is composed of two heavy (IgH) and two light chains (IgL). Both IgH- and IgL-chains have a variable region that together forms the antigen-binding sites. The two constant IgH-chains form the Fc-region and determine the isotype of the antibody.

Functional differences between IgG subclasses

While the variable regions determine the antigen specificity, the Fc-region (made up of the two identical C_H chains) of the antibody determines which type of response that should be elicited. The major players involved in IgG mediated effector responses are the complement system and FcγRs.

The classical pathway of the complement system comprises a group of plasma proteins that is activated by binding to antibodies of the IgM and IgG isotypes. This pathway is an essential part of the innate response that mediates rapid responses to infection. Although all IgG subclasses can bind to the complement protein C1q [12], and IgG2a does so with greatest affinity [10], this is not the single mechanism by which IgGs can mediate effector functions.

In addition to having varying affinities for complement proteins, the functionality of IgG subclasses is linked to their ability to bind specific FcγRs. The FcγRs also differs between mice and human. In this section, I will refer to the mouse system. Mice express four different FcγRs, of which FcγRIIb is inhibitory, and FcγRI, FcγRIII and FcγRIV are activating [2]. These receptors are associated to signaling subunits containing either immunoreceptor tyrosine-kinase inhibition motifs (ITIMs) or immunoreceptor tyrosine-kinase activation motifs (ITAMs), respectively. Of the activating receptors, IgG1 exclusively binds FcγRIII and does so with low affinity, whereas IgG2a binds all receptors with high (FcγRI), intermediate (FcγRIV) or low (FcγRIII) affinity. IgG2b behaves similar to IgG2c, but does not bind FcγRI. All isotypes bind the inhibitory FcγRIIb with low affinity [2].

The activities of specific IgG subclasses have been assessed by the use of switch variants (identical antigen binding clones fused to alternate C_H chains). These studies have identified IgG2a and IgG2b as the most effective subclasses to mediate ADCC [13] as well as host responses to bacterial and viral infections [14-16]. Indeed, IgG2a is the predominant subclass produced in response to viral infections [17]. In further support of this, the efficacy of antibody-mediated depletion of B cells with anti-CD20 monoclonal antibodies (mAbs) is highest when antibodies of the IgG2a subclass are used [18,19]. Due to the variable effects on FcγR- mediated effector functions of IgG subclasses, Ravetch and Nimmerjahn has suggested that the outcome of the IgG-activity can be predicted by the ratio of activating/inhibitory signals. Hence, as all subclasses bind the inhibitory receptor with low affinity, IgG1 is more sensitive to inhibitory signals than IgG2a and IgG2b due to its low affinity binding only to the activating FcγRIII, whereas IgG2a and IgG2b bind to two or more activating receptors with higher affinity [2].

The involvement of FcγRs and complement in antibody-mediated responses have been investigated in multiple models of infection, ADCC and auto inflammatory disease using mice genetically deficient in either FcγRs [20], or components of the complement

system [21]. Some general conclusions can be drawn from these studies. First, deficiency in the inhibitory FcγRIIb increases the resistance to various types of infections but also susceptibility to autoimmune disease [20]. Second, complement is not required for IgG2a mediated depletion of B cells during anti-CD20 immunotherapy [18,22], ADCC [13,23] or auto inflammatory conditions [24,25]. Instead, specific FcγRs appear to be involved in these IgG2a-dependent processes. Third, the complement system appears to play a prominent role during infection [21]. Some of the observed effects in studies using complement deficient mice could however be due to early IgM mediated complement activation and not specifically due to the effect of IgGs. Nevertheless, the protective effect of an IgG2a mAb during Vaccinia virus (VACV) infection is at least partially dependent on complement [26]. Presumably, the high affinity binding to several activating FcγRs, in combination with a greater ability to fix complement proteins, at least partially explain why antibodies of the IgG2a subclass have a higher potency than antibodies of the IgG1 subclass.

Class switch recombination

Depending on the type of antigen and route of infection, specific isotypes of antibodies are required to efficiently clear the invading pathogen. Class switch recombination (CSR) is the process when antigen experienced B cells change their isotype expression from the natively expressed IgM and IgD to IgA, IgE or IgG, contributing to the adaptation of the response as the different isotypes mediate distinct effector functions. CSR events depend on proliferation and involve recombination of the Ig heavy chain (*Igh*) locus containing C_H exon clusters encoding all Ig isotypes. Specifically, recombination events occur between switch (S) regions located upstream of the C_H genes. Consequently, the μ and δ C_H chain regions are deleted and replaced by α, ε or γ C_H regions, a process guided by germline (GL) transcripts, also referred to as sterile or switch transcripts. T cell-dependent CSR requires interaction between CD40L on T cells and CD40 on B cells, which together with IL-4 signaling induce expression of activation-induced cytidine deaminase (AID) [27]. This enzyme converts cytosine to uracil in S-regions resulting in double strand DNA breaks. The central function of CD40 ligation is evident from patients suffering from hyper IgM syndrome, characterized by increased IgM and greatly reduced IgG, IgA and IgE levels caused by genetic deficiencies in CD40 or CD40L [28]. CSR can also occur in the absence of T cell help, then requiring other CSR-inducing stimuli either by B cell activating factor (BAFF) or TLR-ligands, which in combination with BCR crosslinking also can stimulate this event [29].

Regulation of IgG isotype switching

Switching to specific isotypes are guided by cytokines, generally thought to be Th cell derived [30], which induce transcription from GL promoters with cytokine responsive elements located upstream of the Ig S-regions. The GL transcripts subsequently guide AID to specific S-regions [27]. Due to the scope of this thesis I will mainly focus on the subclass specific switching to IgG1 and IgG2a.

IgG1and IgG2a

GL transcripts for Cγ1 can be detected as early as 12 h post-challenge during secondary responses [31]. During primary responses, the highest levels of total Cγ1 and Cγ2a GL transcript is found in spleens around day 4-5 post immunization, closely following the production of IL-4 and interferon (IFN) -γ transcripts [31-33]. To be able to monitor early class-switched B cells in vivo, Jenkins and colleagues transferred BCR transgenic 3-83 B cells and could detect $IgG2a^+$ antigen-specific B cells already by day 2 post-immunization [34]. Although these studies indicate that CSR is initiated prior to GC formation, there is also GL transcripts present in sorted human tonsillar GC B cells, suggesting that CSR also occurs at later time points [35]. However, since the amount of GL transcripts *per* antigen-specific cell is approximately 100-fold lower day 4 than day 1 after immunization [31], CSR activity most likely peaks before GC entry.

IL-4 has for a long time been known to induce switch to IgG1 as well as IgE [36-39]via signal transducer and activator of transcription (STAT) 6 signaling [40]. Later, IL-21 was identified to block sequential switching from IgG1 to IgE by inhibition of Cε transcription [41,42]. Consequently IL-21R deficient mice display decreased IgG1 and high levels of IgE [43]. Indeed, patients with loss of function mutations in IL-21R have reduced IgG and increased IgE serum levels in comparison to healthy donors [44]. The effects of IL-21 signaling on IgG1-switching has been shown to be both B cell intrinsic [45,46] and T cell intrinsic [47,48], where the loss of T cell intrinsic IL-21 signaling has been associated with a reduced T follicular helper (Tfh) cell response [46,47] (further described in a following chapter) and impaired IL-4 production [48].

Already 30 years ago, Snapper et. al. could demonstrate a reciprocal regulation between IgG1 and IgG2c. Whereas IL-4 had the ability to induce IgG1 and IgE by LPS stimulated B cells in vitro, addition of IFN-γ blocked the production of both isotypes in a dose-dependent manner and instead, stimulated the production of IgG2a [49]. The role for IFN-γ in IgG2a-responses has since then been confirmed in vivo using viral infection models, displaying reduced IgG2a and increased IgG1 serum titers in the absence of IFN-γ signaling [50-52]. However, there is also evidence for IFN-γ independent IgG2a production [53-57] that, at least partially, can be explained by type I interferons (further discussed in a separate chapter).

Another alternative mechanism for CSR to IgG2a is engagement of TLRs directly on B cells. In a B cell transfer model, using virus like particles (VLPs) loaded with either ssRNA or CpG, Jegerlehner et al. demonstrated that TLR9 expression by B cells were essential to mount TD IgG2a responses independently of IFN-γ [57]. However, in another study using mixed bone marrow (BM) chimeras, impaired IgG2c production in the absence of B cell-intrinsic MyD88 signaling was associated with a loss of IFN-γ signaling by $CD4^+$ T cells [58]. The role for IFN-γ in IgG2a switching is further strengthened by its involvement in the production of autoreactive IgG2a antibodies in different lupus models [59,60].

B cell intrinsic expression of T-bet, encoded by the *T box transcription factor 21* (*Tbx21*) gene, appears to be a common feature for all IgG2a responses, where T-bet has been described to be critical for both induction [61,62] and maintenance of IgG2a-switched memory cells [63]. Whereas both IFN-γ and IL-27 induce IgG2a via T-bet expression in a STAT1- dependent manner [64], CpG-induced IgG2a switching appears to involve IL-12 [65].

IgG2b and IgG3

Antibodies of the IgG3 subclass is generally directed against TI-antigen, such as carbohydrates and repetitive epitopes [66,67], whereas IgG2b can be produced in response to both TI- and TD- antigens [68,69]. Without addition of other stimuli, B cells activated by LPS in vitro primarily produce IgG2b and IgG3 [70,71]. Addition of IL-4 or IFN-γ to these cultures instead induce IgG1 or IgG2a production, respectively, and inhibits production of both IgG2b and IgG3 [71-73]. In contrast, addition of low concentration (<1ng/ml) TGF-β1 to the LPS-activated B cells has been demonstrated to stimulate IgG2b production [74], whereas higher concentrations of TGF-β1 (>1ng/ml) primarily augment IgA production [74]. During in vivo responses to adenoviral infection, production of virus-specific IgG2b appear to be dependent on type I IFN signaling in both B and T cells, similar to the IgG2a subclass [75].

The Germinal Center response

Germinal centers (GCs) were originally described by Walther Flemming in 1885, who noticed compartments within secondary lymphoid organs (SLOs) that were enriched for mitotic cells. GCs were, at that time, suggested to be the origin of lymphocyte development and Flemming consequently named them "Germinal Centers". Even though this initial hypothesis turned out to be wrong, the function of these structures is fundamental to the adaptive immune response. We now know that GCs are sites where antigen-specific B cells undergo extensive proliferation and random somatic hypermutation (SHM) of their BCR genes. Subsequently, B cells are selected based on BCR-antigen-affinity by helper T cells. These events ultimately lead to the generation of B cells expressing BCRs with an improved affinity for the antigen, which eventually differentiates into antibody secreting plasma cells and memory B cells, which together founds the basis of long-lived serological immunity.

B cell subsets

The major B cell subset that participate in GC responses are follicular (FO B) cells, that primarily responds to T cell dependent (TD) antigens. FO B cells are the dominant B cell population in SLOs and constitute approximately 70% of all B cells found in the spleen [76]. FO B cells continuously recirculate between follicles of SLOs [77] and respond to monovalent protein antigens in a TD-manner. FO B cells follow one of three pathways: differentiation into short-lived extra follicular PCs, emerge as early IgM^+ memory B cells, or enter the GC reaction that ultimately supports the development of long-lived PCs and memory B cells with an improved affinity [78] (described in a following section). The extra follicular PC response is initiated in the T cell zone, but subsequently moves into the red pulp or extramedullary chords where the activated PCs continue to proliferate and differentiate. This type of FO B cell-derived PC response is primarily characterized by production class-switched antibodies with low levels of mutations and have a limited ability to contribute to the pool of long-lived plasma cells [77,79].

In contrast to the FO B cells, B1 and marginal zone (MZ) B cells are classified as innate-like B cells as the targets for their responses are generally restricted to evolutionary conserved patterns, the so called T cell independent (TI) antigens [80,81]. TI antigens are further classified by their ability to stimulate polyclonal responses (TI-1) or to repetitive epitopes (TI-2), such as polysaccharides produced by encapsulated bacteria

or viral capsid proteins that has the ability to crosslink BCRs [1]. B1 and MZ B cells primarily contribute to the short-lived plasma cell response that predominantly produces low affinity antibodies. In line with this, B1 and MZ B cells have a lower activation threshold than FO B cells, and this is associated to higher levels of IgM and CD21 (only MZ B cells) expression in combination with lower levels of IgD [77]. Although B1 and MZ B cells share some innate-like features they are found in distinct sites. B1 B cells are primarily located in the peritoneal and pleural cavities, while MZ B cells are strategically sited to capture blood borne antigens in the outer white pulp of the spleen, between the marginal sinus and the red pulp.

Onset of the GC response

During steady-state conditions, B and T cells are segregated in separate areas of SLOs, B cell follicles and T cell zones. This compartmentalization is maintained by distinct stromal cell populations, follicular dendritic cells (FDCs) and fibroblastic reticular cells (FRCs), producing chemotactic signals attracting either B cells expressing CXCR5, or T cells expressing CCR7 [82]. However, upon immunization with TD-antigen, the activated antigen-specific B and T cells change their preferences for chemotactic signals, promoting a synchronized movement towards the interfollicular region, which facilitates the encounter of cognate T and B cells. Activated B cells start to proliferate already by day 2 post-antigen exposure, which is concurrent with the formation of visible T-B conjugates [83-86]. Following interaction with T cells, the pool of activated antigen-specific B follows one of three different pathways. Some of the B cells, preferentially the ones with the highest affinity for the antigen, differentiates into short-lived extra follicular PCs with a life span of approximately 3 days [87,88]. Another population of the activated B cells have been shown to form GC-independent memory B cells, emerging previous or simultaneous to GC formation [89,90]. Among the cells following the GC pathway, there is further selection of cells that eventually enter the GC reaction, favoring cells expressing BCRs with the highest affinity. Using transgenic B cells with defined affinities to the hapten 4-hydroxy-3-nitrophenylacetyl (NP), Schwickert et al. could show that this step was regulated by competition for cognate T cell help [91].

Bcl6

GC B cell commitment is controlled by expression of the transcriptional regulator B cell lymphoma 6 (Bcl6) [92], which is detected around day 2-3 post immunization [93]. First, it controls a transcriptional program that is required for the activated B cells to reach the central part of the follicles. Importantly, Bcl6 down-regulates expression of the G-coupled receptor 183, also known as Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2), which together with CXCR5 and CCR7 orchestrates the directional migration of B cells towards the FDC-rich center of the follicles [94]. Secondly, it counteracts PC differentiation by repression of the transcriptional regulator

B lymphocyte-induced maturation protein-1 (Blimp-1). Additionally, Bcl6 keeps the cells in a pro-apoptotic state by silencing Bcl-2, but also make them tolerant to DNA damage by repression of pro-apoptotic genes such as p53 [95]. How Bcl6 is regulated in GC B cells is not entirely clear, but IL-21 has been shown to induce Bcl6 protein expression in B cells via JunD/AP-1 and activation of STAT3 [96]. Notably, the same study found that although IL-6 also signals via STAT3, it does not upregulate Bcl6. Furthermore, they show that IL-4/STAT6 can induce Bcl6, although to a lower degree than IL-21 [96].

T cell-mediated regulation

The idea that GCs were the site where B cells undergo somatic mutation of their Ig-variable region genes and are selected based on improved affinity for antigen (based on interactions with immune complexes on FDCs) were forecasted already in 1986 [97], and followed by a number of publications supporting this hypothesis [98-100]. It was also suggested that not only BCR-signals via antigen binding, but also T cell derived signals, were involved in affinity maturation [101,102]. A specific subset of $CD4^+$ T cells have been ascribed to this role, named T follicular helper (Tfh) cells, and these will be discussed in more detail in a separate chapter. Here, I will briefly describe the most important T cell derived signals that have been shown to influence the GC reaction.

CD40L

It is important to remember that the default mode of a GC B cell is apoptosis. Consequently, it is not a matter of inducing death, but rather a positive selection by induced survival. CD40 was one of the first molecules that was identified in this process, demonstrated in experiments where sorted tonsillar GC B cells were rescued from apoptosis when BCR-stimuli was added in combination with an anti-CD40 antibody [101]. The ligand for CD40 was characterized by Armitage et. al., found to primarily be expressed by activated T cells and capable of inducing B cell proliferation [102]. Furthermore, expression of CD40L was localized to areas of cognate T-B cell interaction [103]. The central role for CD40-CD40L in GCs were assessed in studies using anti-CD40L or soluble mCD40, demonstrating blocked TD (but not TID) B cell responses [104], abrogated GC formation [105], and impaired memory B cell development [106].

CD40L belongs to the tumor necrosis factor (TNF) superfamily and is induced on $CD4^+$ T cells within a few hours after TCR engagement [107]. By binding to CD40 expressed on B cells, it promotes clustering of CD40, which via TNFR-associated factors (TRAFs) and JAK proteins activate multiple signaling pathways that induce proliferation and inhibit apoptosis of activated B cells [108].

ICOS

Inducible co-stimulator (ICOS) was identified in 1999 by Hutloff et. al. as a fourth sibling to the membrane bound T cell-specific cell-surface receptors CD28, programmed death-1 (PD-1) and CTLA-4 (B7-CD28 family), known for their ability to modulate T cell activation. Of particular interest, ICOS was found to be expressed at high levels on T cells localized in the apical light zone of tonsillar GCs [109]. Two years later, three parallel studies using ICOS deficient mice reported impaired GC and IgG responses, although different down-stream mechanisms were proposed to account for these effects in the absence of ICOS. While the study by Dong et.al. deduced defective cytokine production (primarily IL-4) by $CD4^+$ T cells to cause the reduced GC formation and IgG production [110], McAdams et. al. ascribed this phenotype to impaired CD40L expression by T cells, and could rescue the IgG production by injection of a stimulatory anti-CD40 mAb [111]. The third study by Tafuri et. al. took both mechanisms into account as they observed both reduced CD40L expression and IL-4 production by T cells [112]. Nevertheless, the phenotype in ICOS deficient mice appears to be mostly attributed to T cell intrinsic defects, which is also suggested by the more recent studies addressing the role for ICOS-ICOSL interactions in the context of Tfh cell differentiation (described in the next chapter).

IL-4 and IL-21

Both IL-4 and IL-21 belongs to the type I cytokine family that share the common γ-chain (γc) receptor, also including IL-2, IL-7, IL-9 and IL-15, but signals via distinct pathways due to paring with specific cytokine binding receptors. When IL-21 is bound, the IL-21R activates STAT1, STAT3, and to a lesser extent STAT5 [113,114]. In contrast, IL-4R signaling is critically dependent on STAT6 activation [40]. In addition to their co-operative role in balancing class-switch to IgG1 and IgE, both IL-4 and IL-21 regulates the magnitude of B cell responses. Some features are shared, such as their ability to stimulate B cell activation and proliferation [40,115], also demonstrated by the more pronounced effects on IgG production and GC formation found in mice deficient in both cytokines [43].

In comparison to IL-21, IL-4 appears to have a more evident role in CSR as it is involved in the induction of AID [116]. However, the role for IL-4 in GC formation has been somewhat controversial and both reduced [117,118] and normal [119,120] responses have been reported in the absence of IL-4. A recent study could demonstrate that following immunization with type 2-biased responses (parasite infection and alum immunization), the combined deficiency of both IL-4 and IL-13 led to impaired GC responses, whereas the GC responses to viral infections (LCMV and MCMV) was unaffected [121]. This study also showed that intrinsic STAT6 signaling in B cells was required for expression of *Bcl6* and *Aicda* (encoding AID) in GC B cells following parasite infection [121].

IL-21 is associated with both GC B cell and PC differentiation, as it has the ability to induce Bcl6 as well as Blimp-1 expression [96,114,122]. The influence of IL-21 on GC responses is evident by reduced GC formation [45,46] and weakened affinity maturation [123] in mice deficient in the IL-21 receptor. Interestingly, despite the reduced GC responses, generation of early memory B cells (defined as antigen-binding, $CD38^{+}GL\text{-}7^{+}$) is accelerated in the absence of IL-21 signaling [46,123]. However, these putative memory cells appear to be functionally impaired, as IL-21R deficient mice do not respond to challenge 5 weeks post primary immunization [124].

Affinity maturation

The main function of the GC reaction is to amplify and tailor the response towards the foreign antigen by supporting clonal expansion and improving the BCR affinity of the responding B cells. This is obtained by a stepwise process of SHM and subsequent selection of clones with the highest affinity. SHM is, similar to CSR, also dependent on AID expression. However, the activity of AID is now directed to the V-regions of the Ig-genes. GCs are visible by histology around 5-6 days after primary immunization, after which they grow in size and are polarized into a dark zone (DZ) and a light zone (LZ) [125]. In the DZ, centroblasts divide intensively with a generation time of 6-7 hours with a mutational rate of their variable genes at 10^{-3} mutations per base pair per generation; approximately 10^{6} times higher than the normal rate of somatic mutation [126]. In contrast, LZ centrocytes are in a quiescent, non-proliferating state and express surface Ig. Expression of the chemokine receptor CXCR4 by centroblasts is critical for the compartmentalization of DZ and LZ [127]. The mutational rate is overall lower in CXCR4-deficient GC B cells, although transition between DZ and LZ phenotypes still occur, indicating that the spatial organization is functionally important for the GC [128].

The relation between centrocytes and centroblasts was described already in 1964, when Hanna et al. suggested that centrocytes were progeny of the dividing centroblasts. However, it was not until a few years ago that the movement between LZ and DZ was described in more detail using live-imaging of photoactivatable reporter mice. This approach allowed tracing of the LZ- and DZ- residing B cells over an extended time period. While interzonal migration had been demonstrated before [129], Nussenzweig and colleagues could quantify the rate at which cells exchanged between LZ and DZ areas of the GCs, and determine that ~30% of the LZ B centrocytes return to the DZ for additional cycles of proliferation and SHM [130]. Additionally, they show that BCR-dependent uptake of the antigen and subsequent presentation to T cells controls re-entry into the DZ, and thereby mediates affinity maturation of the response (defined

by accumulation of the high-affinity mutation W33L). From these results, they conclude that T cell help is the limiting factor for B cell selection in GCs.

Output of the GC response

The main products from a robust GC response are memory B cells and long-lived PCs with the ability to rapidly respond to re-infection, or continuously produce high affinity class-switched antibodies, respectively. Both populations contributes to the long term protection we aim for following vaccination, and in some situations, they have been shown to last for a life time [131].

A memory B cell can be defined as a B cell that, after an initial response, has the ability to survive for a long time period without the support of persisting T cells or access to cognate antigen [79]. Already in 1983, Thorbecke and colleagues demonstrated that memory B cells mainly derive from GCs. By adoptive transfer of PNA^+ and PNA^- B cells at the peak of the GC response (10 days after immunization) they could conclude that most of the secondary response in recipients was derived from PNA^+ B cells, but also noted that there was some contribution from the PNA^- population [132]. The presence of GC-independent memory has indeed been demonstrated to both TI [133] and TD antigens [89,90]. Nonetheless, GC derived memory B cells are generally of higher affinity due to accumulated mutations in their V-regions.

In contrast to memory B cells, long-lived PCs are terminally differentiated cells that reside in the BM with the ability to sustain antibody levels in serum over several years, sometimes for life. It is currently unclear how GC B cells, that recycle between the DZ and LZ for an unknown number of times, are finally instructed to differentiate into either PCs or memory B cells. Notably, Shlomchik and colleagues recently demonstrated that the output from GCs changes over time. Using pulse-labeling of B cells, they elegantly demonstrate how antigen specific IgM^+ and IgG^+ memory B cells, as well as long lived PCs, form over a time period of 40 days. From these results, they suggest that B cells of a memory phenotype, both IgM^+ and $IgG1^+$, are emerging at an earlier time point compared to PCs, which instead are generated to a lager extent during the later phase of the response [78]. Furthermore, depletion of GCs from day 12 and onwards had little effect on the memory B cells while $IgG1^+$ high affinity PCs were virtually absent.

The signals that promote the different fates are not entirely clear, although some have been suggested. Both IL-6 and IL-21 have been shown to promote PC differentiation [134], and PD-1 has also been suggested to promote GC B cell maintenance and PC differentiation, possibly via IL-21 produced by Tfh cells [135]. Interestingly, prolonged dwell time in the LZ when B cells lack expression of CXCR4 has also been shown to

increase the generation of memory B cells [128]. The authors speculate that the increase in memory cell generation could be explained by T cell-mediated rescue from deletion, but this T cell help is not sufficient to drive further participation in the GC when B cells are deficient in CXCR4 expression [128].

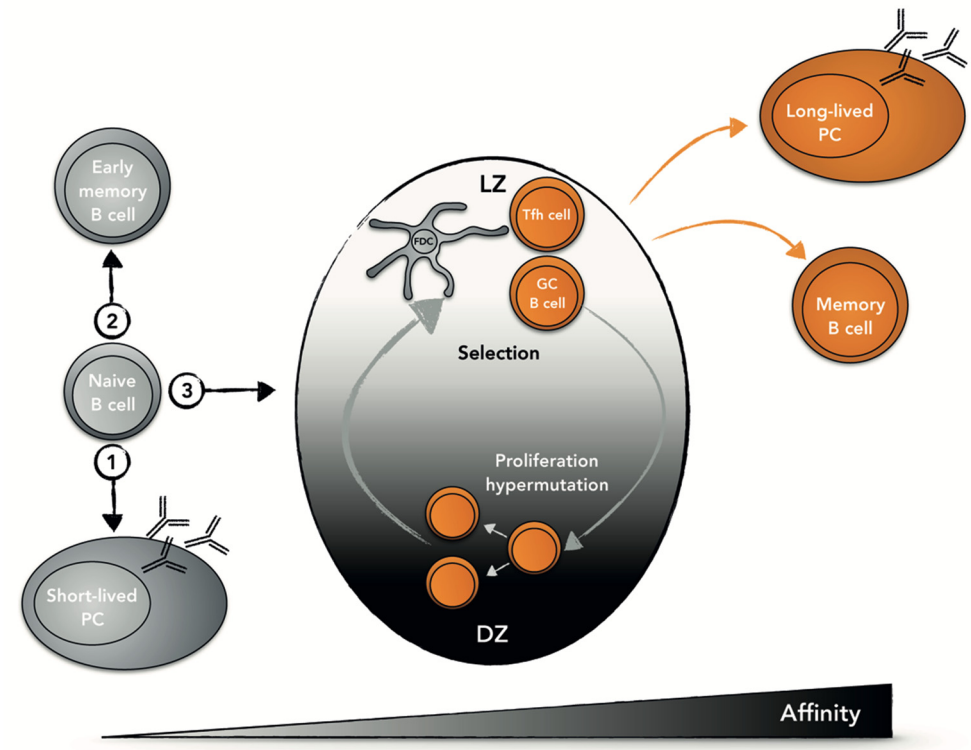

Figure 2. Overview of follicular and extra follicular T- dependent B cell differentiation
Upon exposure to antigen, naïve B cells differentiate into extra follicular PCs (1), emerge as early memory B cells (2) or enter the GC reaction (3). In the GC, B cells undergo rounds of proliferation in the dark zone (DZ) and hypermutate the variable regions of their Ig-genes. Subsequently, the B cells are exposed to immune complexes on FDCs and B cells expressing high-affinity BCRs are positively selected by Tfh cells. B cells emerging from the GC, either as plasma cells or a memory B cells, will presumably be of higher affinity than originally. Adapted from Victora et. al. 2015 [136].

T follicular helper cells

T follicular helper (Tfh) cells are a subset of $CD4^+$ T cells shown to be specialized B cell helpers and responsible for supporting GC B cell responses and production of high affinity antibodies. As described in the previous chapter, the concept of T cell help to B cells in the selection process of high affinity clones was discussed already over thirty years ago, but the designation "follicular B helper-T cells" was coined in 2000 [137,138]. Due to the direct implication of this subset in both vaccine development and other contexts involving antibody-mediated function, characterization of Tfh cells has been under intense investigation for the past years.

Tfh cells were first described in human tonsils as $CXCR5^+$ memory ($CD45RO^+$) $CD4^+$ T cells with an enhanced ability (in comparison to their $CXCR5^-$ counterparts) to support IgG and IgA production from co-cultured B cells. Prior to this, CXCR5 expression had been observed on a subset $CD4^+$ T cells [139] and found to be upregulated on these cells after in vivo activation [140] in a OX40L-dependent manner [141,142]. The OX40L responsible for initial T cell accumulation in follicles was demonstrated to be provided by DCs, and the expression of OX40L was shown to be induced via CD40 signals [143,144]. Expression of CXCR5 (a chemokine receptor otherwise associated with B cells) facilitate the localization of Tfh cells to B cell follicles [145], which logically situates B cell helping T cells at the right place. This population of GC residing T cells were also shown to express a distinct transcriptional profile from classical Th1 and Th2 cells [146,147], and furthermore, Tfh cells was demonstrated to develop independently of other Th subsets (Th1, Th2, Th17) [148]. This observation was closely followed by the identification of the transcriptional repressor Bcl6 as a critical regulator for Tfh cell development reported by three individual groups [149-151]. With these findings, Tfh cells were recognized and widely accepted as a separate subset of $CD4^+$ T cells.

The above mentioned findings have literally led to an explosion of Tfh-related publications, which also have created some perplexity in the field. In this section I will summarize some of the most important observations and discuss the relation of Tfh to other populations of Th cells.

Characteristics

Tfh cells are primarily identified by CXCR5 expression, most often in combination with Bcl6 or the T cell activation marker programmed cell detah-1 (PD-1). Also, downregulation of the signaling lymphocyte activation molecule (SLAM), P-selectin glycoprotein ligand 1 (PSGL1) or Ly6C are used to distinguish Tfh cells. In humans, Tfh cells can be identified by their expression of high levels of ICOS, but this is not equally useful in mice as all activated murine $CD4^+$ T cells appear to express ICOS to some extent. It is however important to point out that different types of nomenclature are used to define the different stages of Tfh development. The most commonly used notions are the following; pre-Tfh, Tfh and GC Tfh. However, it is generally a matter of two stages that need to be separated; (1) the GC-residing population and (2) the stage prior to GC formation when $CXCR5^+$ cells with high levels of $Bcl6^+$ and PD-1 expression have not yet developed. In the papers included in this thesis, we have used the term 'Tfh' for cells that reside within GCs and accordingly display high levels of CXCR5 in combination with either Bcl6 or PD-1 (at day 8 following immunization expression of Bcl6 and PD-1 is almost exclusively overlapping). For analyses performed at earlier time points of T cell dependent B cell responses, we have used the term "early Tfh development", and in this context defined early Tfh cell development based on CXCR5 expression only.

Localization

A key feature of Tfh cells is their re-localization from T cell areas to GCs, that develop within B cell follicles. CXCR5, CCR7 and P-selectin glycoprotein ligand-1 (PSGL-1) are the main receptors that in a synchronized manner initially facilitates to the movement of primed T cells to the interfollicular zone and later into follicles [145,152,153]. CXCR5 is upregulated on T cells following their initial activation in the T cell zone and mediates migration towards CXCL13 produced by FDCs in the B cell follicle. In contrast, CCR7 together with PSGL-1 are down-regulated upon activation [145,153]. As these receptors anchors T cells to T cell areas, where their ligands CCL19 and CCL21 are present, this receptor down-modulation is also important for the directional migration of $CXCR5^+$ T cells towards the B cell area [145,152].

Function

Many of the phenotypic characteristics of Tfh cells are directly connected to their function. PD-1 belongs to the B7-CD28 family and is an inhibitory receptor that attenuates T cell proliferation by reducing TCR-mediated signals [154]. Interaction with PD-L1 limits the expansion of the GC-residing T cells [155,156], that will be exposed to continuous antigen presentation. ICOS is another member of the same receptor family as PD-1 that takes part in the differentiation and function of Tfh cells

by promoting both IL-4 [110,112,120] and IL-21 expression via induction of the transcription factor c-maf [157]. Production of IL-21 is a signature for Tfh cells, and this cytokine mediates its effects through both autocrine and paracrine signaling to establish the Tfh phenotype and provide B cell help, respectively. Additionally, ICOS expression is required not only for Tfh development as it induces Bcl6 expression [158], but also for its ability to affect $CD4^+$ T cell motility and subsequent recruitment into follicles [159]. The SLAM family of receptors include CD150 (SLAM1), CD84 and Ly108 (SLAM6), are expressed on both Tfh cells and GC B cells and function as self-ligands [160]. Importantly, these receptors bind to the cytoplasmic protein SLAM associated protein (SAP), which is upregulated in Tfh cells. Mutations in the *SAP* gene have been linked to a number of immunodeficiency disorders, and expression of SAP specifically in T cells has been shown to be responsible for the loss of long-lived humoral immunity in SAP deficient mice [161]. More recently, it was shown that SAP mediates the adhesion of T cells to GC B cells and in its absence, Tfh cells are not retained within the GCs [162] and cannot produce IL-4 [163].

Differentiation and transcriptional regulation

One of the first factors shown to influence Tfh differentiation was TCR-affinity, elegantly shown by McHeyzer-Williams and colleagues using the pigeon cytochrome c (PCC) antigen. By identification of T cells expressing low and high affinity TCRs, they could demonstrate that T cells with a higher affinity for the antigen preferentially differentiated into CXCR5 high expressing cells [164]. Additionally, increasing the availability of antigen increase the number of Tfh as well as GC B cells [165]. More recently, the TCR-p:MHCII dwell-time was shown to bias the fate of Tfh vs. T effector differentiation, adding information on how TCR signal strength can influence separate T cell fates [166].

The discovery of Bcl6 as a critical factor for Tfh cell differentiation firmly established Tfh cells as a distinct subset of $CD4^+$ T cells. Although initially suggested to be both necessary and sufficient for Tfh development, it has become clear that differentiation into a fully functional Tfh cell is a multifactorial process that is dependent on Bcl6 expression, but Bcl6 cannot alone complete the program. Although a number of both positive and negative regulators has been identified, the lack of a comprehensive understanding of the factors promoting Tfh differentiation is evident by the numerous failed attempts to develop a reproducible protocol for in vitro generation of Tfh cells [134,148,151,167,168]. Neither in our lab, despite numerous attempts and modifications, we have been able to develop a protocol that supports Tfh differentiation.

The reciprocal regulation of the transcriptional repressors Bcl6 and Blimp-1 is decisive in the process of becoming an effector T or Tfh cell. A number of studies using Bcl6 deficient T cells in either conditional knock-out models, mixed chimeras or adoptive transfer experiments, have uniformly demonstrated a lack of Tfh development and impaired GC responses when T cells lack Bcl6 [149-151,169]. Consistent with this, forced expression of Bcl6 in T cells subsequently transferred in vivo strongly enhances CXCR5 expression and Tfh development [149] and limits production of cytokines associated with alternate T effector cell fates in vitro [150]. Conversely, overexpression of Blimp-1 instead potently represses Tfh differentiation, GC responses and IgG production [149]. Although Bcl6-independent induction of CXCR5 has been demonstrated [170], Bcl6 is closely linked to CXCR5 expression in a number of different models [158,171-173].

As previously mentioned, ICOS signaling has been shown to support Tfh development by induction of Bcl6 expression [158]. Currently, co-stimulation via ICOS is probably one of the most distinctive signals that instructs Tfh development. ICOS also induce c-Maf, which regulates IL-21 production in both Tfh and Th17 cells [157]. c-Maf is regulated by BATF, and expression of c-Maf together with Bcl6 can partially rescue the defective Tfh differentiation in BATF deficient T cells [174].

Recently, ICOS was also shown to block the activity of Krüppel-like factor 2 (Klf2). By negative regulation of Klf2, ICOS was shown to promote Tfh development by down-regulation of PSGL-1, CCR7 and CD62L expression in combination with induction of CXCR5 [175]. Additionally, expression of non-Tfh related transcription factors such as Blimp-1, T-bet and GATA3 are induced by Klf2 [176]. Similar activities are reported in mice deficient in the gene encoding the Forkhead box (FOX) protein Foxo1, which also is negatively regulated by ICOS and promotes expression of PSGL-1 in combination with reduced CXCR5 and Bcl6 expression [177]. However, unlike Klf2, expression of Foxo1 appears to be required at a later time point for full Tfh and GC development.

Foxp1 is another member of the FOX-family that recently was shown to negatively regulate Tfh differentiation and subsequent GC responses. In contrast to Foxo1, Foxp1 (in particular the isoform Foxp1D) is not regulated by ICOS, but is instead involved in the regulation of ICOS expression following TCR-stimulation. Notably, conditional knock-out of Foxp1 in $CD4^+$ T cells increase CXCR5 expression similar to forced expression of Bcl6 [178]. Indeed, the importance of stringent regulation of ICOS expression is apparent also from earlier studies by Goodnow and colleagues, where the sanroque mouse strain presents with an autoimmune phenotype due to excessive Tfh differentiation. This was caused by a mutation in the Roquin protein, a negative regulator of ICOS [179].

Achaete-scute homologue 2 (Ascl2) was recently identified as a transcription factor that had the ability to induce CXCR5 in vitro, independently of Bcl6 expression [170].

Ascl2, induced by the Wnt-signaling pathway, was identified to be highly expressed in both human and mouse Tfh cells. Most importantly, in vitro CXCR5 expression was reported when an inhibitor of GSK-3 (a negative regulator of Wnt-signaling) was added to mouse $CD4^+$ T cells activated with antibodies to CD3 and CD28. Furthermore, retroviral expression of Ascl2 appeared to induce CXCR5 expression at levels detectable by flow cytometry [170]. However, in our hands, the same GSK-3 inhibitor does not induce CXCR5 expression above background (compared to an isotype control) (unpublished observation). Ascl2 may indeed be involved in Tfh cell development and CXCR5 expression, however, if ectopic Ascl2 expression is sufficient to induce CXCR5 by in vitro activated $CD4^+$ T cells remains to be confirmed by additional studies.

An activator of the Wnt-signaling pathway, TCF-1, was by two individual groups recently reported to positively regulate Tfh cell development [180,181]. By screening genes that are selectively expressed in Tfh cells, Choi et. al. identified TCF-1 and LEF-1 as potential candidates for specifying Tfh cell development. Although not absolutely required, both TCF-1 and LEF-1 augment Tfh cell differentiation, and the effect is more pronounced if both factors are absent. This effect was ascribed to reduced levels of ICOS and IL-6Rα expression, and Tcf7/Lef1 double-deficient GC Tfh cells expressed increased levels of Blimp-1 and decreased levels of Ascl-2. Wu et. al. additionally demonstrated that Blimp-1 negatively regulated TCF-1 expression, but also conversely, that TCF-1 inhibited Blimp-1 and IL-2Rα expression. Consequently, decreased Tfh development in TCF-1 deficient mice could be rescued by Bcl6 overexpression [181].

STAT proteins

Several studies have investigated the role for IL-6 and IL-21 in Tfh cell differentiation reporting absent [182], reduced [148,183,184] and unchanged [134,153] Tfh cell development when IL-6 or IL-21 is absent. Deficiency in both cytokines results in a more pronounced phenotype [134,183], indicating that these cytokines might have overlapping roles. IL-6 also stimulates IL-21 production in vitro, but cannot induce CXCR5 or Bcl6 on its own [134].

Both IL-6 and IL-21 signal via STAT1 and STAT3, and the early induction of Bcl6 and CXCR5 following LCMV infection appear to be critically dependent on STAT1 signaling in T cells and to a lesser extent STAT3 [182]. In further support of this, type I IFNs, which are produced at high levels following viral infection, were demonstrated to induce STAT1 binding to promoter elements of the Bcl6 loci [185]. Notably, type I IFNs has previously been demonstrated to instruct early Tfh development by signaling in DCs, possibly via IL-6 production [186], indicating that STAT1 signaling in both DCs and T cells promotes Tfh differentiation.

A study by Craft and colleagues recently addressed the role of STAT3 signaling using conditional knock-out mice, and found that STAT3 activation in $CD4^+$ T cells

counterbalances the Th1 cell response driven by type I IFN signaling during LCMV infection [187]. As STAT3 activation appears to be required for down-regulation of the IL-2Rα [182,187] , this could at least partially explain how IL-6 and IL-21 promote Tfh cell differentiation. IL-2 signaling has previously been shown to induce Blimp-1 expression via STAT5 activation [188], which means that an increased ability to respond to IL-2 signaling would skew the T cell response away from the Tfh cell fate. Hence, IL-6 and IL-21, via STAT3 activation, make T cells less sensitive to IL-2 signals and thereby appears more prone to follow the Tfh differentiation pathway.

Apparent from the studies discussed above, regulation of Tfh cell localization and differentiation involves a complex network of factors. However, the core activity appears to be to the reciprocal regulation of Bcl6 and Blimp-1, and further to this, the ability of Bcl6 to block differentiation into 'non-Tfh' effector T cells.

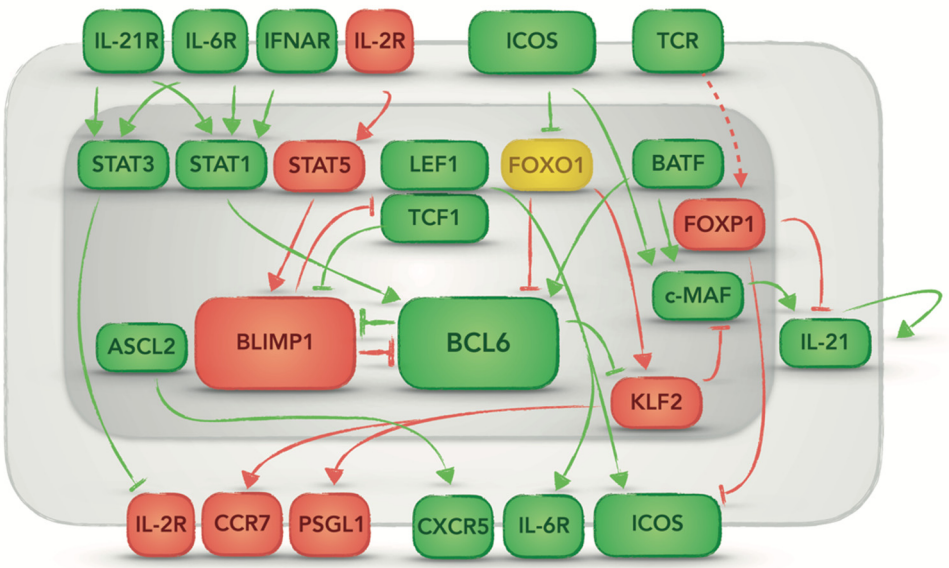

Figure 3. Transcriptional regulation of Tfh cells
Simplified schematic overview of the transcriptional network that controls Tfh cell differentiation. Transcription factors that positively (green) or negatively (red) regulate Tfh differentiation are shown (a dual role is indicated by yellow). Adapted from Vinuesa et. al. 2016 [189].

Regulation of Tfh cell development by DCs and B cells

The cellular interactions that underlie successful Tfh cell differentiation are not fully understood. Currently, mainly conventional DCs (cDCs) and B cells have been suggested to be involved in a sequential manner, where cDCs are responsible for the initial priming and support of initial $Bcl6^{+}CXCR5^{+}$ Tfh cell commitment and B cells are required for completion of this process. However, whereas the specific role for cDCs in Tfh cell differentiation has not yet been firmly established, our results indicate that cDCs are redundant for Tfh cell development (paper I).

Dendritic cells

cDCs are APCs that express a wide range of pattern recognition receptors (PRRs), including toll-like receptors (TLRs), C-type lectin receptors (CLRs) NOD-like receptors (NLRs) and RIG-1-like receptors (RLRs). Collectively, these receptors enable them to respond to virtually any types of PAMPs [190]. The main function of cDCs is to continuously sample the environment and present peptides in the context of MHC class I and II. When sensing danger, cDCs readily get activated and mature into professional APCs through upregulation of MHC expression and co-stimulatory molecules. Determined by the type of pathogen, cDCs also secrete distinct cytokines to polarize and tailor the immune response.

Already over ten years ago, T cell accumulation in B cell follicles were shown to be driven by OX40L-expression by DCs [143,144]. It was also demonstrated that CXCR5 was induced on the antigen specific T cells in a CD40-dependent manner [144]. Since then, the specific role for cDCs in Tfh development has been assessed in a number of studies [158,171,186,191,192]. When antigen presentation is restricted to cDCs in vivo, early Tfh differentiation is supported [171] and clearly dependent on ICOS-ICOSL interaction [158]. However, full differentiation cannot be completed in the absence of B cells [191]. In the absence of cDCs, we observe that overall T cell priming is compromised. This is evident by the lack of CD62L down-regulation and reduced expansion of the antigen-specific T cells [192]. However, this defect in T cell priming can be overcome by providing a higher antigen dose, possibly by allowing other types of APCs to pick up and present antigen more efficiently, and both Tfh cell and GC development are supported under these conditions [192]. From these observations, it appears that (1) cDCs are essential for T cell priming when antigen is limited, (2) antigen presentation by cDCs can allow initial upregulation of CXCR5 and Bcl6 expression in vivo, however (3), cDCs do not uniquely provide any signals required for Tfh development.

cDCs are further subdivided into distinct populations, and the two main subsets are separated based on CD8α expression. $CD8\alpha^{+}$ and $CD8\alpha^{-}$ cDCs develop from a common precursor (pre-DC) but can be separated by their dependency on distinct transcription factors. IRF8, Batf3 and Id2 are required for $CD8\alpha^{+}$ cDCs [193-195],

whereas CD8α⁻ cDCs are primarily dependent on IRF4 [196]. CD8α⁻ cDCs represents a CD11b⁺ subset of cDCs that heterogeneously express CD4, and this population is localized to the MZ bridging channels while CD8α⁺ cDCs mainly resides in the T cells zones [197].

One approach to assess the ability of CD8α⁺ cDCs has been to target antigen to the CLR Clec9a (also known as DNGR-1) that is expressed at high levels by CD8α⁺ cDCs and to some extent plasmacytoid DCs (pDCs) [198]. By injection of anti-Clec9a both Tfh development [199] and humoral responses [198,200,201] are enhanced in comparison to isotype control, most strikingly when delivered in the absence of adjuvant [201]. However, with the use of antibodies targeting either DEC205 (expressed by CD8α⁺ cDCs) or DCIR2 (also known as 33D1 and expressed by CD8α⁻ cDCs) conjugated to protein, Shin et. al. recently demonstrated a superior ability of CD8α⁻ cDCs to support Tfh cell, GC B cell and IgG responses when delivered together with an adjuvant, and this was linked to higher expression of both ICOSL and OX40L by CD8α⁻ cDCs [202].

Conditional knockout models can also be used to further evaluate specific differences between cDC subsets. Studies using mice that specifically lack CD8α⁻ cDCs have reported impaired Th2 responses, to both lung [203] and skin [204,205] infection, while Tfh cell development and GC responses appears to be unaffected in the same model [205]. However, in a study by Cyster and colleagues, antigen-specific B and T cell responses were attenuated when cDCs were deficient in EBI2-expression, resulting in impaired positioning to bridging channels and a specific reduction in CD8α⁻ cDC numbers [197].

Beyond cDCs, also monocyte-derived DCs (mDCs), or inflammatory monocytes, have been shown to enhance the Tfh response when the TLR9 agonist CpG was used as an adjuvant [206]. We also find that mDCs induce MHCII expression and high levels of ICOSL upon polyinosinic-polycytidylic acid (poly(I:C)) injection, and in vivo expansion of antigen-specific CD4⁺ T cells appears to be redundantly supported by cDCs, mDCs and B cells [192]. However, whether CXCR5 expression is directly induced by these individual cell types is still an open question.

B cells

Although a role for B cells in supporting T cell responses was noted already 1981 [207], B cells has received relatively little attention for their capacity to activate and influence CD4⁺ T cell responses, and we mostly discuss the reverse dependence: how T cells help B cells. It has, however, been demonstrated that B cells indeed are required to support robust proliferation and expansion already by day 3 post immunization [208], and also to induce effector functions and generation of T cell memory [209-213]. Additionally, the follicle has been shown to be the major site for expansion of antigen-specific T cells following immunization with alum-precipitated protein [214].

An important role for B cells in Tfh cell development is supported by several studies. However, when B cell interaction is required and what type of signals they provide are still under investigation. The requisite for B cells in Tfh development have been shown in models displaying varying B cell abnormalities, such as μMT (lacking B cells) [145,153,215], CD19-deficient mice (largely normal B and T cell numbers, but are deficient in MZ B cells and display impaired GC formation [216]) [145,153] and mice that express an irrelevant BCR for the immunizing agent (cannot form cognate interactions with T cells) [86,153]. From these studies, it is clear that T cells with a Tfh cell phenotype ($CXCR5^{hi}PD\text{-}1^{hi}$) do not develop in the absence of B cells [145,153], and cannot be maintained in the absence of cognate B cell interactions [86].

Studies of SLAM and SAP signaling during B-T cell interactions have provided further insight as to how B cells regulate Tfh development. Homotypic interactions between the SLAM-family of receptors have been shown to stabilize the interaction between B and T cells [217]. While T cell-intrinsic expression of the adaptor protein SAP is required for B-T cell interaction [161,162], the formation of DC-T cell conjugates is unaffected in the absence of SAP [162]. The roles for individual SLAM receptors in this process are not entirely clear, as deficiency of some receptors (CD84 and Ly108), but not all (CD150), affects Tfh cell differentiation, while SAP expression appears to be overall important [163,217,218]. One mechanism that, at least partially, explain the relationship between SAP and associated SLAMs, is that SAP prevents binding of the inhibitory phosphatase SHP-1 to Ly108, and SAP instead mediates adhesion and helper functions when bound to Ly108 [219].

Functionally, CD150 regulates IL-4, but not IL-21 expression, whereas SAP deficient Tfh cells display impaired expression of both cytokines [163], indicating that B cell interactions are required for efficient cytokine production. Indeed, it has been shown that antigen-presentation by B cells induce IL-4 production by T cells [220]. This B cell-induced IL-4 production appears to be mediated by IL-6 and IL-10 [221]. Additionally, the frequency of IL-4 and IL-21 double-producing T cells are enhanced when antigen presentation by B cells is augmented [222]. This, however, does not have to be a consequence of increased ICOS-ICOSL interaction, as ICOSL expression by cognate B cells is not required for IL-4 or IL-21 production [223].

Further, an important role for GC B cells in Tfh cell development has been demonstrated by us and others, using systems where GC B cells are selectively depleted by day 4-6 [165,192]. We do, however, observe a $CXCR5^{hi}PD\text{-}1^{hi}$ subset also in the absence of GC B cells when mice are given a high antigen dose, but these Tfh cells do not express Bcl6 or IL-21 at the same levels as cells that develop in the context of GCs. Interestingly, Deenick et. al. have suggested that the $CXCR5^{hi}PD\text{-}1^{hi}$ phenotype is not absolutely dependent on cognate T-B cell interaction, as Tfh cell development appears to be supported also in mice lacking MHCII specifically on B cells when they are boosted with additional peptide on day 3 to extend the ag-presentation by other cells

[224]. Moreover, if SAP-deficient T cells are co-transferred with wt T cells, which have the ability to support GCs, in combination with a peptide boost, also the SAP-deficient T cells acquire a $CXCR5^{hi}PD\text{-}1^{hi}$ phenotype and express both Bcl6 and IL-21. Collectively, these results imply that the $CXCR5^{hi}PD\text{-}1^{hi}$ phenotype is most likely a reflection of ongoing antigen presentation, which under normal conditions is restricted to GCs, but can be extended by increasing the antigen dose or by giving an additional boost of antigen. This is also consistent with the loss of PD-1 expression by previously activated $CXCR5^{hi}PD\text{-}1^{hi}$ T cells after transfer into naïve mice [165]. Nevertheless, a combination of prolonged TCR-signaling and GC environment appears to be required to maintain Bcl6 expression and IL-21 production. It should therefore be noted that cells displaying a $CXCR5^{hi}PD\text{-}1^{hi}$ phenotype may not necessarily under all conditions equal functionally normal Tfh cells.

Relation to other T helper subsets

Based on their ability to produce distinct cytokines, $CD4^+$ T helper cells were originally divided into Th1 and Th2 by Mossman and Coffman in 1986. Th1 and Th2 cells displayed mutually exclusive expression of IL-2/IFN-γ and IL-4, where the Th2 were found to have the ability to support IgG1 and IgE production [225]. Also, these cells maintained their identity over several months, indicating that these two cell types were indeed functionally distinct. Now, almost exactly 30 years later, the dogma of functionally distinct Th cell subsets still holds true, although now further extended to include additional subsets, such as Th17, Tfh and regulatory T cells (Treg). Generally, one can assign individual Th subsets to different types of immunity: Th1 cells and intracellular viral and parasite infections; Th2 cells and extracellular immunity to parasites and nematodes and; Th17 cells and extracellular bacterial or fungal infections. In contrast, Tfh and Treg cells participate in all types of immunity and are instead primarily classified based on their functional sub-specialization within a given response: supporting B cell responses or restraining excessive immunity/preventing autoimmune responses, respectively.

Although Tfh can develop independently of other Th cell subsets, the notion that Tfh cells inherit traits of other Th cell subsets have been suggested by a number of studies. In this section, I will focus on their relation to Th1 and Th2 cells.

Tfh vs Th2

Many characteristics originally ascribed to Th2 cells have instead been shown to apply to Tfh cells since their recognition as an individual subset. Tfh and Th2 cells are now separated into distinct populations based on their localization to lymphoid versus non-lymphoid tissue, and functionally, based on the ability of Tfh cells to support follicular B cell responses, while Th2 cells are responsible for recruitment of effector cells to the

site of infection and allergic inflammation. Both Tfh and Th2 produce IL-4, but IL-5 and IL-13 expression is restricted to Th2 cells and closely associated to expression of high levels of GATA3. This functional distinction between Tfh and Th2 cells was demonstrated by Liang et. al., who reported that the majority of IL-4 producing cells in draining lymph nodes do not express GATA3, whereas the lung-resident cells that produce IL-4 and/or Il-13 following helminth infection are almost exclusively positive for GATA3 [226]. Other studies also support that Tfh cells are the dominant source of IL-4 in LNs [163,215,227]. Furthermore, Liang et. al. showed that $CD4^+$ T cells in LNs exclusively express GATA3 or Bcl6, and additionally, while GATA3-associated IL-13 production is critical for cell recruitment and worm expulsion, it is not required for IgE production [226].

The fact that Tfh, but not Th2, cell development is normal in STAT6 deficient mice [148], and vice versa when $CD4^+$ T cells lack Bcl6 expression [151], strongly support that these subsets are developmentally distinct. However, whether or not there is plasticity between the two subsets is somewhat more controversial. Glatman-Zaretsky et. al. initially showed that Tfh cells can develop from Th2 cells, based on adoptive transfer experiments where IL-4 expressing $CXCR5^-PD\text{-}1^-$ non-Tfh cells had the ability to differentiate into $CXCR5^+PD\text{-}1^+$ Tfh cells upon secondary immunization [215]. Reversely, a more recent study from Léon and colleagues suggest that "type 2" Tfh cells generated in response to house dust mite (HDM) during the sensitization phase efficiently differentiate into Th2 effector cells upon challenge [228]. On the contrary, Coquet et. al. does not observe efficient Th2 responses following transfer of either IL-21 producing or non-producing Tfh cells [229]. It remains to be clarified if this is due to differences in experimental systems. Thus, to what extent interconversion between Tfh and Th2 occurs remains an open question.

Tfh vs Th1

Both Tfh and Th1 cell responses are robustly induced during to viral infections, and the developmental relationship between these two subsets has been investigated in a number of studies. Th1 cells are dependent on the transcription factor T-bet, which also has the ability to bind Bcl6 and regulate both Th1 and Tfh associated genes, such as *Ifng* and *Tcf7* [230]. Tfh cells develop independently of T-bet, and in some models, Tfh differentiation is even enhanced in the absence of T-bet expression [168]. Tfh cells can, however, express T-bet to a certain extent during type 1 immunity, and they may also have the ability to produce IFN-γ [163,231]. A clear distinction between Th1 and Tfh cells however appears to be an early divergence of IL-2Rα and Bcl6 expression. Jenkins and colleagues demonstrate that the $CXCR5^-$ cells generated following Listeria infection express high levels of IL-2Rα. These $CXCR5^-$ cells are selectively reduced in the absence of IL-2Rα, and are enriched for cells that express high levels of T-bet. Reversely, $CXCR5^+$ cells are instead dependent on Bcl6 and do not express elevated levels of IL-2Rα [173], consistent with the negative regulation of Bcl6 via IL-2 induced

Blimp-1 expression [232]. Further to this, it has been reported that Th1 cells has a proliferative advantage over Tfh cells due to enhanced metabolic function driven by IL-2 induced mTOR activation. This is suggested to be a complementary way by which IL-2 signaling favors Th1 and suppresses Tfh cell responses [233]. The imprinting of these properties appears to occur early, as primed cells sorted and transferred already day 3 post LCMV infection appears to maintain their identity as either Th1 or Tfh cells in their new hosts [172].

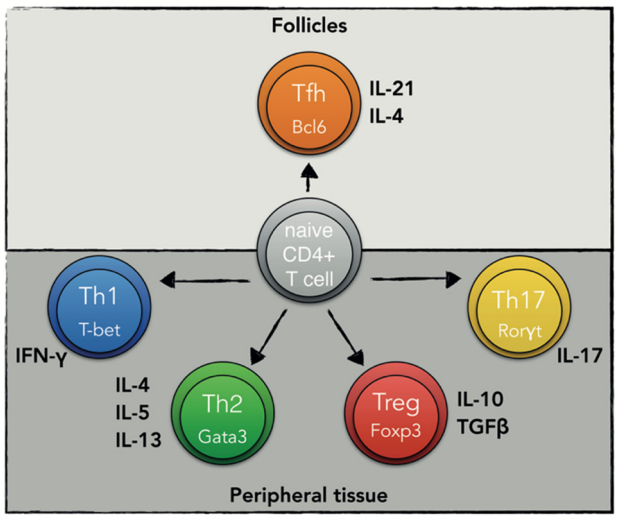

Figure 4. CD4⁺ T helper cell subsets
Schematic overview of Th cell subsets. In contrast to effector Th cells, which preferentially localize to peripheral non-lymphoid tissue, Tfh cells home to B cell follicles of SLOs to support GC B cell responses.

Tfh cell memory

As mentioned before, a key feature of the adaptive immune response is the generation of memory cells that have the ability to respond rapidly upon re-infection. Memory T cells are persisting in at least two forms following the primary response: T central memory (Tcm) and T effector memory (Tem) cells. Tem cells are identified as $CD44^{hi}$ $CD62L^{-}$ $CCR7^{-}$ and are strategically localized in peripheral tissues, ready to mount an immune response upon antigen encounter. Tcm cells are also $CD44^{hi}$, but in contrast to Tem cells, they express CD62L and CCR7 consistent with their localization to lymphoid tissue. Tcm cells appear to inhabit a greater proliferative potential than Tem cells [234]. Hence, upon antigen recognition, Tcm cells undergo clonal expansion and give rise to secondary effector cells [234].

The relation or contribution of Tfh cells to the memory pool has been assessed over the last years by different approaches. It is becoming increasingly clear that the $CXCR5^+$ antigen-specific cells, that are generated during a range of different responses, contain a population of $CCR7^+$ cells that give rise to Tcm cells [173]. Upon challenge, this population differentiates into T cells with an increased ability to support B cell responses in comparison to the Tem cells deriving from the $CXCR5^-$ population [235]. Additionally, the $CCR7^+CXCR5^+$ population appears to have a broader potential to differentiate into both $CXCR5^+$ Tfh and $CXCR5^-$ effector cells, while the $CXCR5^-$ cells were more restricted and did not give rise to Tfh cells, consistent with their respective classification as Tcm and Tem [173,236,237].

These studies have largely assessed the individual contribution of $CXCR5^-$ and $CXCR5^+$ cells to memory responses. Approximately 50-60% of the ag-specific CD4 T cells are $CXCR5^+$ in LNs around day 8 post immunization depending on the type of the response. Of these, approximately 20% are GC Tfh cells distinguished by PD-1 expression. Other studies have specifically assessed the ability of GC-derived Tfh cells to engage in memory responses. These studies show that the transferred $CXCR5^+$ $PD\text{-}1^+$ cells can participate in recall responses, and although Bcl6 expression is reduced during the resting phase, these cells appear to have a preference to differentiate into Tfh cells when challenged [231,238-240].

Human Tfh cells

The phenotype of human Tfh cells has been further explored since their initial definition as $CD45RO^+CXCR5^+$ cells in tonsils [137,138]. In blood, $CD4^+$ T cells with the ability to support antibody production appears to be enriched among a population of cells that are defined as $CXCR5^+CXCR3^-PD\text{-}1^+$, $CXCR5^+CXCR3^+ICOS^+$ or alternatively $CXCR5^+CCR7^-PD\text{-}1^+$ [241-243]. Perhaps most importantly, these subpopulations of circulating $CXCR5^+$ cell have been identified in HIV infected individuals [241], influenza vaccinated subjects [242], as well as patients suffering from the autoimmune disease Systemic Lupus Erythematosus (SLE) [243], and are associated to development of broadly neutralizing antibodies (bnAbs), antibodies to influenza antigens, or high titers of anti-dsDNA antibodies, respectively. Importantly, the same association to antibody titers was not observed with the total population of circulating $CXCR5^+$ memory cells [241]. Apparent from the above mentioned studies, the use of CXCR3 as a phenotypic marker of circulating Tfh cells is not entirely consistent. As suggested by Yu and colleagues, the preferential expression of the chemokine receptor CXCR3 could be influenced by the type of response. By using two different adjuvants and parallel analyses of the response in lymphoid tissue and blood in mice, they could show that although both adjuvants provoke Tfh responses to a similar degree, the

fraction of $CXCR5^+CXCR3^+$ cells in blood is preferentially generated during type 1, and not type 2 immunity [243]. Although results are not entirely unanimous, these studies on circulating human $CXCR5^+$ Th cells collectively highlight the heterogeneity among $CXCR5^+$ $CD45RA^-$ memory cells found in peripheral blood.

Consistent with the murine system, human Tfh cells localized to GCs are distinguished by high levels of CXCR5, PD-1 and Bcl6 expression [244,245], and their differentiation can be augmented by retroviral expression of Bcl6 and c-maf [174,245]. Nevertheless, there are clear differences between the two species as well. Tfh differentiation in mice is mostly influenced by STAT3, STAT1 and to some extent STAT4. In contrast, human Tfh differentiation mainly appears to be be driven by STAT4, but is also influenced by STAT3. Accordingly, IL-21 production is promoted by IL-6 in mice and by IL-12 in humans [246]. The function of IL-12 in humoral responses has been demonstrated in individuals lacking the IL-12R subunit, which display attenuated GC formation and produce tetanus-specific antibodies with a lower affinity [247]. Also, whereas in vitro induced expression of CXCR5 by $CD4^+$ T cells in the murine system is challenging, human T cells upregulate CXCR5 upon stimulation with anti-CD3+ anti-CD28 antibodies, and is further enhanced by IL-12 and TGF-β [248].

Vaccination

The first steps towards what we now refer to as vaccination were taken already 300 years ago. At that time, the *Variola* virus was spreading over the world causing smallpox, which killed 30% of infected individuals. To prevent spreading of this disease, the concept of "Variolation" was introduced. In principle, pustule material from infected patients was inoculated in the skin of uninfected individuals, and although not without risk, it generally caused a less severe disease than the natural infection and conferred immunity to the virus. The concept of vaccination was introduced by Edward Jenner in 1796, who instead of using material from smallpox-pustules, used cowpox-infected human material. Cowpox is caused by a virus similar to Variola, the Vaccinia virus, which trigger a smallpox like disease in cows, hence the name cowpox. Also humans could be infected with this virus, although contracting milder symptoms, and it was being noted that people exposed to cowpox were subsequently protected against smallpox. By inoculation of cowpox infected material, Jenner could show that it caused an initial local infection, but also produced a long-term (over 5 years) protection against smallpox challenge. Jenner called the inoculum Vaccinia from the Latin word for cow, "Vacca", and accordingly, the process for "Vaccination" [1].

Although we no longer use crude preparations from pustules, we still use the principle of vaccination to protect from and prevent spreading of many life threatening diseases. Most impressively, a global vaccination campaign succeeded to eradicate smallpox infection in 1967, and the last case of naturally contracted smallpox was reported in 1979, now over 30 years ago. Most of the currently available vaccines are protective through their ability to stimulate generation of long lived antibody responses of sufficient quantity and quality. Remarkably, Amanna et. al. showed that ag-specific antibody titers to a number of either naturally contracted infectious agents, or proteins introduced by vaccination, were stable over a time period of 25-years with calculated half-lives ranging from 11 to over 10 000 years [249]. However, even if our knowledge of how vaccines work have increased enormously since Jenners first experiments, there are numerous details that still needs further exploration. The most obvious example of this is probably the many attempts to develop a protective HIV vaccine without success.

Most vaccine preparations include some sort of adjuvant that will, similar to an intact pathogen, stimulate the immune system to produce a protective response. The most prevalently used adjuvant in current vaccines is aluminum precipitated salts (alum). While alum has proven its efficacy and safety over the years of use, the exact mechanism

by which it stimulates the immune system is still debated [250]. Alternates to alum are used, either alone or in combination with alum, and additional adjuvants are under development. Many of these are TLR agonists that stimulate the immune response in different ways by binding to distinct receptors, expressed by distinct cells.

Adjuvants

Alum

As previously mentioned, alum is the most widely used adjuvant. The discovery that toxins precipitated with aluminum potassium sulfate provoked stronger humoral responses than toxins alone was done already in 1926. Only six years later, the same effect was observed in humans and alum was subsequently used to enhance the efficacy of Diphtheria and Tetanus vaccines [251]. Exactly how alum mediates is adjuvanticity has been a longstanding question. First, alum was suggested to prolong the time that the toxoid remained in the body by forming antigen-depots that, in comparison to soluble protein, would release the antigen over an extended time period. This might be part of the explanation, however, other mechanisms have been suggested, although not entirely coherent. Alum appears to function independently of TLR-signaling. This have been shown in mice lacking the adaptor proteins MyD88 and TRIF, which are used by all currently known TLRs [252]. NLRs have been proposed as a possible mediator, which has the ability to sense PAMPs but also endogenous danger signals. Although it is clear that alum can induce production of pro-inflammatory mediators by NLR-signaling, other in vivo studies have questioned whether or not NLR-signaling is needed for the adjuvanticity of alum with regards to humoral responses [253,254].

Something that has been known for a long time is that alum promotes a classical Th2 response, inducing a strong $CD4^+$ cell response with high levels of IL-4 production [255] that predominately supports an IgG1 response in mice. Thus, despite the fact that alum can induce protective immunity following vaccination it would be interesting to use other adjuvants that promote a more Th1-biased immunity and hence the production of IgG2a antibodies (or the seemingly functionally analogous subclasses IgG1 and IgG3 in humans), as this could have more potent antiviral effects.

TLR-based adjuvants

As alternatives or complement to alum, TLR agonist-based adjuvants are eligible candidates due to their well recognized ability of TLRs to trigger robust immunity [256,257]. TLRs signal via the adaptor proteins MyD88 and TRIF, which

preferentially activate NF-κB and interferon regulatory factors (IRFs), respectively. Subsequently, this leads to the production of pro-inflammatory cytokines and type I IFNs. Due to the scope of this thesis, I will briefly discuss three TLR agonists that stimulate production of high levels of type I IFNs and are prospective candidates for use as vaccine adjuvants.

TLR3 (Poly(I:C))

In the studies included in this thesis, we have almost exclusively used poly(I:C), which is an adjuvant known to stimulate high levels of type I IFNs. Poly(I:C) is a dsRNA analogue that mimics infection by dsRNA viruses and signals via endosomal TLR3 and cytosolic MDA5 receptors via adaptor proteins TRIF [258] and IPS-1 [259], respectively. Although using different adaptor proteins, both pathways eventually leads to IRF3 and IRF7 mediated IFN production as well as NFκB activation [257,260,261]. While TLR3 are predominantly expressed by $CD8\alpha^+$ cDCs and possibly to a lesser extent also $CD8\alpha^-$ $CD4^-$ cDCs [262], MDA5 has a broader expression pattern. Poly(I:C) induces a rapid burst of type I IFNs , with serum levels of both IFN-α and -β peaking already after 4-6 hours and essentially undetectable within 24 hours after injection [263,264]. The type I IFN response to poly(I:C) is primarily MDA5-dependent [263]. The major subsets responsible for production of type I IFNs are radio-resistant stromal cells, $CD8a^+$ cDCs and monocytes, while pDCs do not seem to be involved [264,265]. Although dispensable for type I IFN production, TLR3 and TRIF signaling appear to be more important for maturation (increased expression of MHCII and CD86) of B cells following poly(I:C) stimulation [258]. However, the generation of ovalbumin (OVA)-specific IgG2a, following immunization with antigen in combination with alum and poly(I:C), is completely reliant on MDA5 while TRIF only augments the response [263].

Notably, poly(I:C) has been implicated in clinical use in both conventional and peptide-based cancer vaccines due to its immunostimulatory effects [266]. Although promising results were obtained from mice studies [267], the presence of nucleases in sera limited the efficacy of poly (I:C) in primates (including humans) [268], and higher doses led to severe side effects in phase I-II clinical trials [269]. To circumvent these unwanted effects, poly(I:C) derivatives have been generated to stabilize the structure [266]. At least one of these derivatives is still in clinical trials as an intranasal influenza vaccine adjuvant.

TLR4 (MPLA)

In terms of clinical use, the most outstanding candidate of all TLR agonists is the TLR4 agonist monophosphoryl lipid A (MPLA), which is already a component of the clinically approved adjuvant system (AS) 04 [270]. MPLA is a derivative from lipopolysaccharide (LPS), which is found on gram-negative bacteria and probably one of the most widely studied TLR-agonists [256]. In relation to LPS, MPLA is less toxic

but still holds its immunostimulatory capacity and potently skew the response towards a Th1-biased response with increased IgG2a as compared to alum [271]. TLR4 is expressed on the cell surface, signals both via MyD88 and TRIF adaptor proteins and thus, induces type I IFN production via IRF3 and IRF7 transcription factors [257].

TLR9 (CpG ODN)

TLR9 is primarily expressed on B cells and pDCs in the endo-lysosomal compartment and recognizes unmethylated CpG motifs present in bacterial, but not mammalian, DNA. The immunostimulatory capacity of TLR9 has been explored by using different types of synthetic CpG oligodeoxynucleotides (ODNs), which have been shown to mediate a range of biological effects by preferentially stimulating TLR9 on B cells or pDCs. CpG ODNs have been utilized as adjuvant in clinical trials, both against infectious disease and cancer treatment [272]. The progression of these substances are however unclear, although beneficial effects of CpG ODNs as vaccine adjuvant have been reported [273]. TLR9 signals exclusively via MyD88 but in contrast to other TLRs, it has through this pathway the ability to induce high levels of type I IFN production by pDCs via direct activation of IRF7[257].

Type I interferons

Type I IFNs are a family of immunomodulatory proteins comprising one IFN-β and fourteen IFN-α proteins [274]. The activity of type I IFNs was discovered already in 1957 by Isaacs and Lindenmann, who found that a product derived from virus-infected chick embryo cells had the ability to induce resistance to viral infection [275]. Due to its potential use as an antiviral drug, this discovery took 'Interferons' all the way into a comic strip of the popular Flash Gordon magazine, where it was used in a space ship to cure a patient infected with a mysterious extraterrestrial virus. Despite its potential 'superpowers' it took another twenty years before the genes encoding type IFNs were identified, which also led to a wider scientific acceptance [276].

Signaling and immunomodulatory functions

Type I IFNs are produced by many different cell types in response to viral, bacterial and parasite infections through activation of both TLR-dependent and -independent pathways. Particularly, pDCs are known to be high-level producers of type I IFNs in response to ssRNA or CpG DNA via endosomal expression of TLR7 and TLR9 [257]. Although other cell types express these TLRs, only pDCs have the capacity to directly activate IRF7, leading to instant production of IFNα proteins [257]. In contrast, type I IFNs are primarily induced by conventional DCs (cDCs), macrophages, and epithelial

cells following TLR3 and TLR4 activation [277]. These receptors initially induce production of type I IFNs via IRF3, due to their association with the adaptor protein TRIF, which subsequently stimulates activation of IRF7 in a positive feedback loop [278]. Non-TLRs involved in type I IFN production include retinoic-acid-inducible protein I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), stimulator of IFN genes (STING) and DNA-dependent activator of IRFs (DAI). These cytoplasmic sensors are expressed by multiple cell types and recognize ssRNA, dsRNA and cytosolic DNA [279].

All type I IFNs signals via the heterodimeric IFNα/β receptor (IFNAR), composed of the two subunits IFNAR1 and IFNAR2. The IFNAR is constitutively associated to Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) and upon activation, STAT proteins are phosphorylated, primarily STAT1, STAT2 and STAT3 but in lymphocytes also STAT4 and STAT6 [280,281]. pSTAT1 and 2 proteins dimerize and translocate to the nucleus, where they together with IRF9 form IFN-stimulated gene factor 3 (ISGF3) that induce transcription of IFN-stimulated genes (ISGs). To date, over 400 ISGs have been identified [277], which signifies how type I IFNs must be regulated in a context dependent manner.

Since the initial discovery of its anti-viral activity type I IFNs have been shown to have multiple immunomodulatory effects, not always in favor of the host. Indeed, in the context of bacterial infection, type I IFN appears to impair clearance of L. Monocytogenes [282] and Mycobacterium tuberculosis [283], and IFNAR deficient mice are instead resistant against infection [282]. One possible mechanism for this is that type I IFNs suppress production of IL-17 [284], which is important for the clearance of extracellular bacteria. The complex role of type I IFNs also expands to viral infection. Two parallel studies were recently published showing that neutralization of prolonged type I IFN signaling were beneficial for clearance of the otherwise chronic LCMV (clone 13) infection [285,286]. Additionally, the same effect was seen during plasmodium infection [287]. In at least two of these studies, prolonged type I IFN signaling were shown to stimulate increased levels of IL-10 and block of IFN-γ production by $CD4^+$ T cells, and this phenotype was reversed by neutralization with an anti-IFNAR1 mAb [285,287].

Nevertheless, the anti-viral properties of type I interferon are essential to control acute viral infections and support both innate and adaptive responses. Direct effects of type I IFNs on B and T cells include increased proliferation, survival, co-stimulatory function and sensitivity to external signals [288-290]. In terms of specific effector functions, it is involved in IFN-γ production by $CD4^+$ T cells, both directly [281] and indirectly via DCs [264]. Additionally, type I IFNs have a major effect on humoral responses, which will be further discussed below.

Type I IFNs in the regulation of humoral responses

Due to their ability to modulate the immune response, type I IFNs have been under intense investigation since their discovery and used in clinical practice since 1986. Whereas recombinant type I IFN proteins have been proved to have beneficial effects in multiple sclerosis (MS), hepatitis and cancer therapy, one of the side effects of long term treatment is the generation of neutralizing anti-IFNβ Abs [291].

The influence of type I IFNs on the generation of antigen-specific antibodies of the IgG subclasses were recognized already in 1991 by Gause and colleagues, demonstrating increased IgG2a and decreased IgG1 serum levels following injection of increasing doses of IFN-α together with an anti-IgD mAb [53]. While the decrease in IgG1 was eliminated when a neutralizing anti-IFNγ mAb was injected in parallel, the level of IgG2a was lower but still increased with higher doses of IFN-α, indicating that the stimulating effects of IFN-α was not merely due to its ability to induce IFN-γ production. Although these serum analyses were done 8 days after IFN-α injection and likely reflects a TI extra follicular PC response, another study using mice deficient in IFNAR, IFNγR or both demonstrated that the absence of both IFNAR and IFNγR expression completely blocked production of antigen specific IgG2a 4 weeks after LCMV infection. Mice lacking the IFNγR only, displayed a 50% reduction of IgG2a [54]. Hence, these studies show that type I IFN can stimulate IgG2a responses independently of IFN-γ. Additionally, in the absence of IFNAR, IFNγR or both, IgG1 responses were increased in comparison to wt controls, most significantly in mice deficient in the IFNγR [54]. While the majority of the studies addressing IgG2a responses report reduction or absence of this isotype when mice lack IFNAR expression, IgG1 levels have been shown to be either reduced [292] or increased [54,293] in these mice. A possible explanation for this discrepancy might be the type of stimuli that has been used, as IgG1 levels appear to be higher in response to live viral infections than in immunization models. However, the IgG1 generated in the absence of type I IFNs is of lower affinity [186], presumably a consequence of aberrant Tfh cell development [186] and attenuated GC responses [75].

Some of these studies also address the individual contribution of IFNAR expression on DCs, T cells, B cells or both T and B cells, either in the absence of receptor signaling or with type I IFNs added as an adjuvant. In response to adenoviral vectors, transfer of WT DCs was not sufficient to restore IgG2a production in IFNAR deficient mice [75]. In this system, the IFNAR instead appears to be engaged in T as well as B cells to mount IgG2a responses and this isotype was essentially absent when both subsets lacked IFNAR expression [75]. In contrast, when type I IFN were used as an adjuvant, signaling in DCs alone was able to support IgG2a production, although at lower levels than observed in wt controls[294]. However, in a follow-up study, the ability of type I IFNs to enhance IgG2a responses was primarily assigned to T cells [295]. Clearly, type I IFNs are involved in humoral responses, by mediating both basic signals such as expansion, survival and activation of both T and B cells, but also more selective effects, which bias the response towards production of IFN-γ and IgG2a.

Present investigation

Aims of the thesis

The overall aim of this thesis was to study the role for innate mechanisms in the development of T follicular helper cell development and germinal center responses in the context of protein immunization adjuvanted by poly(I:C).

Specifically, the aims of the included studies were:

I. To determine the role for conventional dendritic cells in Tfh cell development and associated GC responses.

II. To dissect the effects of type I and II interferons on GC formation and GC-dependent IgG1 and IgG2c responses.

III. To assess how T cell precursor numbers influence Tfh cell differentiation and IgG1 versus IgG2c associated GC B cell responses.

Results and discussion

Paper I

T follicular helper, but not Th1, cell differentiation in the absence of dendritic cells

Although Tfh cells were recognized as a distinct subset of $CD4^+$ T cells, the cues that instructed Tfh differentiation was, in the beginning of this study, largely unknown. Our group had previously shown that type I IFN signaling in cDCs enhanced Tfh cell development, possibly via IL-6 signaling [186], while others had suggested that both IL-6 and IL-21 as well as ICOSL signaling, provided by B cells, were involved [148]. As DCs are considered to be the main APC to prime $CD4^+$ T cells, we wanted to investigate whether $CD11c^+$ cDCs provide any unique signals required for Tfh differentiation. To this end, we took advantage of the CD11c-DTR mouse model [296], which allows selective in vivo depletion of CD11c expressing cells. To study the ag-specific $CD4^+$ T cell response we transferred OVA-specific OT-II cells that can be tracked in the recipient mice by congenic CD45 expression. These mice were then used to study CD4 T cell differentiation and GC development in response to systemic protein immunization adjuvanted by poly(I:C) in the presence, or absence, of cDCs.

Results in short

- Administration of a high antigen dose can overcome the initial requirement for cDCs during T cell priming in cDC-depleted mice.
- Tfh differentiation and GC development can develop independently of cDCs, although, cDCs are required for concurrent Th1 development.
- B cells, monocytes, and possibly pDCs, can redundantly prime $CD4^+$ T cells and thereby support early CXCR5 expression in vivo, in the absence of cDCs.
- cDC-depleted mice produce ag-specific IgG1 responses of unaltered quality and quantity, while IgG2c responses are selectively impaired in these mice during both primary and secondary responses.

T cell priming in the absence of cDCs – antigen dosing

There is no doubt that cDCs are efficient APCs, especially with regards to their ability to capture, digest and present essentially all types of antigen. cDCs are also strategically localized beneath epithelial surfaces, and in the case of a breached epithelial barrier, cDCs mature and transports antigen to the sentinel LN where they immediately enter the T cell zone to prime and activate ag-specific T cells. In our study, we found that $CD4^+$ T cell priming was impaired in the absence of cDCs in response to 300 μg of antigen delivered by intraperitoneal injection (i.p.). As we aimed to study the accessory

function of cDCs, beyond ag-presentation, we approached this by giving a 10-fold higher ag-dose (3mg) to allow other, presumably less efficient APCs, to prime T cells in the absence of cDCs. By doing this, we observed comparable priming and activation in both cDC sufficient and deficient animals and could accordingly separate the APC function of cDCs from their accessory signals. This, in many people's eyes, exceptionally high ag-dose did however not bypass the known requisite for ICOSL-mediated signals, thus indicating that the Tfh cells that develop under these conditions still follow the same rules as Tfh cells that develop in response to lower ag-doses.

Although 3 mg is a lot of antigen, this is a dose that we deliver via i.p. injection that will be systemically distributed through peritoneal lymphatic draining, eventually reaching the blood. This should be put in relation to studies that use subcutaneous ag-doses of in the range of hundreds of μg, which presumably leads to an even higher local antigen concentration in draining LNs as compared to the amount of antigen accumulating in the spleen after high dose i.p. injections. However, in most cases I think that using lower, perhaps more physiologically relevant, doses are preferable. However, in our study the high antigen dose represents a technical approach to evaluate the accessory functions of cDCs.

CD11c-DTR system – off target effects

The CD11c-DTR mice express the human receptor for Diphtheria Toxin (DT) under control of the murine *Cd11c* promoter. Since mice normally lack expression of the DTR, this means that only the CD11c expressing cells will be sensitive to DT injections. The CD11c-DTR mouse was generated in 2002 by Jung et. al. in 1992 [296], and has been used in various studies to assess the function of $CD11c^+$ cDCs. The DTR-DT system has indeed provided a useful tool to selectively deplete specific cell populations in vivo, however, it also requires careful examination of which cells that are *specifically* depleted. During the course of this study it became apparent to us that also GC B cells, similar to plasma cells [297], started to express CD11c and hence, became sensitive to the DT injections. This was also published by Sallusto and colleagues [165]. Consequently, we also used a complimentary mouse model (described in paper I) in which we could repeatedly inject DT to ensure persistent cDC-depletion without risking any direct effects on B cells.

Another population of cells that has been reported to be depleted in the CD11c-DTR mouse is the MZ macrophages that resides in the MZ of the spleen [298]. MZ macrophages have previously been shown to be involved in trapping antigen, but not T cell priming or induction of antigen specific IFN-γ production to Listeria monocytogenes [299]. Although this study indicates that MZ macrophages are dispensable for the priming of IFN-γ producing T cells, it is a possibility that we cannot entirely exclude in our study.

Although the DT-system were suggested to work by apoptosis and thus, could be applicable also to an immunological steady state context [300], this has later been shown to not be entirely accurate. Depletion of $CD11c^+$ cells by DT have been shown to induce neutrophilia in the blood [301], and we observed an increased frequency of granulocytes as well as monocytes in the spleen following depletion. This influx of cells could however be counteracted by injection of an anti-Gr1 mAb, targeting both Ly6G (neutrophils) and Ly6C (monocytes).

During the course of this study, other models of DC-depletion have been developed. Except from the previously mentioned conditional CD11c-cre- based systems to delete specific subsets of cDCs, expression of the Zbtb46 transcription factor was suggested to be more restricted to cDCs as compared to CD11c, and a Zbtb46-DTR mouse was generated [302]. Although this mouse possibly will present less off-target effects than the CD11c-DTR model, it will presumably still display similar DT-induced effects on of inflammatory myeloid cell populations. Also, it has been shown that $CD206^+$ monocyte-derived DCs upregulate Zbtb46 expression upon maturation following LPS injection [303], which further emphasizes the importance of carefully monitoring the 'specific' depletion of cells.

To be, or not to be… a Tfh cell?

Quite remarkably, we found that many cell types that can support T cell priming associated with early CXCR5 induction when they have access to sufficient amounts of antigen. Strictly, it is in one way a bit redundant to discuss Tfh cell development in a context where cognate B-T cell interactions cannot occur. Whether or not the definition of Tfh cells is based on their localization to GCs, the ultimate function of a Tfh cell is to support B cells, and the B-T cell interaction is clearly not a one-way type of communication. The current view on Tfh differentiation is, however, that DCs instructs early cell fate commitment [158,171,191,304]. This view is at least partially based on the mutually exclusive subsets of IL-$2R\alpha^+$ and $Bcl6^+$ T cells that can be detected as early as day 2 days post-infection with LCMV, corresponding to a time point when the primed T cells have undergone only two rounds of cell division [158]. Also in support of this notion is that ag-presentation by DCs alone is sufficient to induce CXCR5 expression in vivo [158,171]. An important role for DCs in the directional migration of activated $CD4^+$ T cells towards the follicles is also evident from earlier observations [143,144].

DCs may indeed facilitate B-T cell interaction, but they do not necessarily specify commitment to the Tfh cell fate. An alternative way of viewing Tfh cell fate commitment would be the engagement with a cognate B cell, as antigen presentation by B cells also has been demonstrated to be an early event [86]. Soluble antigens reach the follicles within minutes after injection, and Pape et. al. show that the majority of ag-specific B cells have captured antigen within 4 hours [305]. This means that cognate interactions between B and T cells can occur within the first 24-48 hours, which also

have been visualized by intravital microscopy [86]. Thus, as we observe Tfh cell development also in the absence of cDCs, an alternative suggestion is that Tfh cell commitment instead occurs when T cells form stable cognate interactions with B cells at the follicular border.

Paper II

Complementary roles for type I and II interferons in IgG2c germinal center B cell fate commitment

Type I IFNs mediate a broad range of effects in various different cell types. Previous work on type I IFNs in the context of humoral immunity has demonstrated that type I IFN signaling enhance production of antigen-specific IgG [292,294] and in its absence, the most pronounced effect is an impaired production of the IgG2a/c subclass [292]. Type I IFN signaling in DCs has also been shown to support both Tfh and Th1 development [186,264], and our previous study (paper I), revealed a distinctive effect on Th1 cell development and associated IgG2c responses in the absence of cDCs. Others did, however, report that type I IFN signaling had an overall negative effect on Tfh differentiation and subsequent IgG production [187]. In this study, we therefore set out to comprehensively assess how type I IFNs influence primary T and B cell responses that give rise to long-lived humoral immunity and B cell memory.

Results in short

- Type I IFNs enhance titers and affinity maturation of ag-specific IgG1, and is essential for the production of antibodies of the IgG2c subclass
- GC B cell responses are overall enhanced by type I IFN signaling.
- Generation of IgG2c$^+$, but not IgG1$^+$, GC B cells are attenuated in the absence of type I IFN signaling.
- Tfh cell development is augmented by, but does not require, type I IFN signaling in hematopoietic cells, while concurrent Th1 cell development is obstructed in the absence of type I IFNs.
- Type I IFN signaling within the first 24 hours is sufficient to induce IFN-γ production and IgG2c-switching.
- Direct IFN-γ signaling in B cells is dispensable for GC responses, but is required for CSR to IgG2c.
- Direct type I IFN signaling augments GC B cell development through a selective effect on IgG2c$^+$ GC B cells.

Type I IFNs and Tfh cell development – intrinsic and extrinsic effects

From our results, it is clear that type I IFNs are needed to kick-off robust poly(I:C)-induced immune responses that are characterized by production of high affinity antibodies, IFN-γ and efficient switching to IgG2c. Evidently, in our study type I IFN signaling also augments Tfh differentiation. The somewhat contradictory conclusions by Craft and colleagues [187], suggesting that type I IFN signaling negatively regulate Tfh differentiation, highlights the importance of considering the multiple effects of type I IFNs. Consistent with Ray et. al., we also observed slightly elevated levels of Bcl6 when T cells lack IFNAR expression, which might indeed indicate that T cell intrinsic type I IFN signaling counteracts Tfh cell differentiation. However, this rather subtle effect of T cell intrinsic signaling is clearly inferior to the overall augmenting effect of type IFN signaling in hematopoietic cells, in terms of both Tfh and Th1 induction. Rather than claiming that type I IFN signaling has a negative effect on Tfh differentiation, it would probably be more correct to state that STAT3 counteracts Th1 differentiation.

Type I IFNs and control of B cell responses

B cell intrinsic type I IFN signaling enhances both BCR and TLR mediated activation, including survival, proliferation and expression of co-stimulatory molecules such as CD86, and in some cases also directly regulates the level of TLR expression [290,306]. Direct IFNAR signaling has been demonstrated to regulate production of IgG2c in response to TI antigen adjuvanted with poly(I:C) [66]. Our results show that type I IFN signaling in B cells enhances the generation of $IgG2c^+$ GC B cells, but switching to this isotype appears to be ultimately regulated by IFN-γ in response to TD-antigen. How type I IFNs mediate this enhancing effect is however not yet clear to us. One possibility might be impaired autocrine signaling, as B cells themselves have the capacity to produce IFN-γ [307]. However, B cell derived IFN-γ were not necessary for IgG2c production in response to LPS [58]. Alternatively, type IFNs could regulate the levels of IFNγR expression on B cells and hence, regulate the sensitivity of the B cells to IFN-γ. Lastly, loss of intrinsic IFNAR signaling and reduced expression of co-stimulatory signals could impair the ability of the receptor deficient B cells to efficiently interact with $CD4^+$ T cells.

The good and bad of type I IFNs – a matter of timing

One of the challenges of studying type I IFNs is the many versatile effects they have on various cells of the immune system. In addition to that, there is the immunological context that changes during the progression of an immune response, and continuously alters the responsiveness to type I IFNs of individual cell types.

In our study, in the context of protein immunization, type I IFNs appear to rely on a distinct burst produced within 24 hours from immunization. This is consistent with the early peak and rapid decline of type I IFNs in the serum of poly(I:C)-injected mice

[263]. This early burst of type I IFNs thus governs both IFN-γ production and switching to IgG2c. In contrast, models based on chronic infections show that type I IFNs have additional effects due to prolonged, or a secondary wave of type I IFN production. These later effects appear to be of an anti-inflammatory character, where type I IFNs instructs cDCs or mDCs to go into a more regulatory mode manifested by expression of higher levels of PD-L1/PD-L2 and IL-10 production, which in turn inhibits IFN-γ production by $CD4^+$ T cells [285,286,308]. The early effects of type I IFNs do however control viral titers during the initial phase of infection, implicating a brief pro-inflammatory role during viral infections while sustained levels instead mediate anti-inflammatory effects. This may reflect an intrinsic regulatory mechanism to prevent excessive immune responses induced by prolonged type I IFN signaling that might lead to autoimmune responses.

Notably, multiple sclerosis (MS) patients treated with IFN-β frequently present with production of antibodies against various types of autoantigens [309], but one of the ameliorating effects of the treatment are indeed IL-10 production [277]. The harmful effects of type I IFN is evident in the autoimmune disorder systemic lupus erythematosus (SLE), which is associated with high serum levels of type I IFNs [310]. One of the hallmarks for SLE is the production of autoantibodies to nuclear antigens that eventually cause the formation of immune complexes, which in turn can give rise to severe complications such as renal failure.

The notion that type I IFNs suppress IL-17 production falls in line with the ameliorating effects of IFN-β treatment, as MS and animal models of the disease at least partially are characterized by the presence of Th17 cells [277,311]. On the other hand, the treatment augments production of autoantibodies. IFN-β has, at least in vitro, been associated with a Th2-skewing (increased IL-4 and IL-10 production) of the autoreactive T cells from MS patients [312]. Together with the association of autoantibodies and systemic levels of type I IFNs in SLE-patients, this could imply that excessive type I IFN signaling may be involved the production of autoantibodies.

Paper III

Clonal competition impairs T follicular helper cell differentiation and reveals separable T helper cell requirements for IgG1 and IgG2c associated germinal center B cell responses

Adoptive transfer of transgenic $CD4^+$ T cells is a widely used method to study antigen specific T cell responses, due to the low number of naïve T cells specific for a certain antigen among the endogenous repertoire. Although the transgenic T cells in many aspects mirror endogenous T cell responses, previous studies have shown that increased numbers of precursor cells have a negative impact on expansion [313], and differentiation into IFN-γ producing cells [314]. From our previous work, Paper I, it

was apparent that the number of transferred OT-II cells also influenced Tfh cell differentiation. To further explore how the precursor number affects concurrent Tfh and Th1 cell differentiation, as well as GC B cell responses, we transferred increasing numbers of OT-II cells, ranging from 5000 to 10^6, and again assessed the response to immunization with OVA plus poly(I:C).

Results in short

- A high precursor number limits T cell expansion, Tfh differentiation and GC formation.
- IFN-γ production is not influenced by the number of precursor cells.
- Generation of $IgG1^+$, but not $IgG2c^+$, GC B cells is impaired when a high number of precursors are transferred.

Why are Tfh cells particularly sensitive to clonal competition?

Although we were expecting the precursor number to have an impact on Tfh differentiation, we were perhaps not anticipating that the effects would be as strong as they eventually turned out to be. Low precursor numbers clearly favored Tfh differentiation, whereas high numbers (over $5x10^5$) impaired the ability of the T cells to differentiate into Tfh cells.

Tfh cells have been shown to preferentially develop from T cells expressing a high affinity TCR [164]. The role for TCR signaling strength in Tfh cell differentiation is further supported by the findings that prolonged TCR-p:MHCII dwell time favors Tfh cell development [166] and that an increased amount of antigen can augment the generation of Tfh cells [165]. Other molecules that have been suggested to be involved in Tfh cell differentiation beyond TCR signaling include ICOS [158], IL-6 and IL-21[134,148], which all co-stimulates TCR-dependent T cell activation.

In this study we used TCR-transgenic OT-II cells, which means that all T cells have identical TCR affinities for the p:MHCII complexes presented. However, increased competition for antigen at higher numbers of antigen-specific precursors would presumably affect the TCR-signaling strength, and therefore also Tfh cell differentiation. This was addressed in one experiment using two antigen doses, 300μg or 3mg. At lower precursor numbers ($5x10^4$), the 10-fold higher antigen dose led to a marginal increase of the frequency of Tfh cells. However, increasing the amount of antigen could not rescue the impaired Tfh differentiation observed when a high number of cells (10^6) was transferred (unpublished observation), indicating that the ag-dose is not the limiting factor for Tfh cell differentiation under these conditions.

The seemingly unchanged frequencies of IFN-γ producing cells, irrespective of T cell precursor numbers, indicate that Th1 cell development is less sensitive to clonal competition than Tfh cell development. The results from this study stands in contrast

to the first paper included in this thesis, where Tfh cells but not Th1 cells develop, in that context due to the absence of cDCs [192]. Although purely speculative, a preferential requisite for distinct APCs could at least partially explain the selective impairment of either Tfh or Th1 cell differentiation in the respective studies. In essence, clonal competition for cognate interaction with B cells at high numbers of precursor T cells might interfere with the stability of the B-T cell interactions, which in turn would lead to impaired Tfh differentiation. On the other hand, clonal competition does not appear to interfere with the T cells' ability to produce IFN-γ, a feature that previously have been shown to be dependent on cDCs [192,264].

Although the total numbers of B cells are higher than the total numbers of cDCs, the number of cDCs that can present OVA-derived peptides are most likely higher than the number of B cells with the same ability at the time of T cell priming. Thus, the number p:MCHII$^+$ cDCs is not as limiting as the number of cognate B cells, which possibly could explain why IFN-γ production appears to be less sensitive to clonal competition. Differential regulation of Th cell responses by distinct APCs has indeed been suggested in studies demonstrating that antigen presentation by B cells preferentially inhibit IFN-γ and induce IL-4 production by cognate T cells in vitro [220,221].

Divergent regulation of switching to IgG1 and IgG2c

In addition to the effect on Tfh cell differentiation, the precursor number clearly influenced GC development, presumably a consequence of the impaired Tfh cell differentiation. Surprisingly, the precursor number also affected the polarization between IgG1$^+$ and IgG2c$^+$ cells within the GCs. The percentage of IgG1$^+$ GC B cells was favored at low and, conversely, impaired at higher precursor T cell numbers, similar to Tfh cell differentiation. In contrast, the percentage of IgG2c$^+$ GC B cells appeared stable irrespective of precursor number.

Distinct factors regulate CSR to IgG1 and IgG2c. While Tfh cells have been shown to produce IFN-γ [163,231], they are mainly associated with production of IL-4 and IL-21 that promote switching IgG1 [39,43,315]. Although we show that IFN-γ regulate switching to IgG2c during TD responses to poly(I:C) (paper II), we do not know whether Tfh cells or other cell type(s) are the source of the IFN-γ that controls the IgG2c-switch. Switching to IgG2c can indeed be induced by factors that are not linked to Tfh cells, such as type I IFNs and TLR-ligands [57,65,66].

The strikingly similar effect of precursor numbers that we find on the generation of IgG1$^+$, but not IgG2c$^+$, GC B cells and Tfh cell development, indicates that Tfh cells are specifically involved in the switching to the IgG1 subclass and to a lesser extent in the switching to IgG2c. However, as the number of Tfh cells display a strong correlation with the number of total GC B cells, the number of IgG2c$^+$ GC B cells is also influenced by the precursor number. This suggests that although Tfh cells might

not be involved in the switch to IgG2c, they are likely to support the expansion of the IgG2c$^+$ GC B cells.

Concluding remarks and future perspectives

The work included in this thesis has investigated basic mechanisms that underlies the generation and composition of germinal centers, including differentiation of Tfh cells and effects of type I IFNs. Furthermore, we report additional novel findings regarding the switch to IgG1 and IgG2c subclasses that appear to be differentially associated with Tfh cell development and function.

High affinity antibodies and memory B cells are products of the GC response that contribute to the long term protection we acquire following vaccination. The impact of vaccination is indeed impressive, and the world health organization (WHO) estimates that vaccination prevents 2.5 million deaths per year. Vaccine regimens have also been able to eradicate the natural occurrence of smallpox [316]. The requisite for vaccine development is however still urgent, as protective vaccines against the human immunodeficiency virus (HIV) and malaria have not yet been produced. The constant emergence of new viruses also highlights the never ending need of new vaccines. Last year, the Ebola virus outbreak in West Africa caused over 10 000 deaths within less than one year. Impressively, a vaccine candidate against the Ebola virus was rapidly produced after the outbreak, and this has already shown protective effects against the Ebola virus disease in endemic areas [317]. An even more recent example, against which there is not yet a vaccine, is the Zika virus. Infection with the Zika virus usually causes mild symptoms in the infected individual. However, there is a potential association between Zika virus-infected women and the birth of children with the Guillain-Barré syndrome as well as microcephaly, which both cause severe neurological symptoms.

A deeper understanding of the mechanisms behind differential IgG class-switching would be applicable to both vaccines and autoimmune diseases. Interestingly, in mouse models, IgG2a/c is often referred to as the "pathogenic" IgG subclass in the autoimmune setting [318], whereas it is the most "protective" in terms of viral clearance [55] and "efficient" with regards to anti-tumor cytotoxicity [13]. This highlights the value of understanding how to regulate the generation of this particularly potent Ig. The human equivalents of IgG2a/c subclass appear to be IgG1 and IgG3. Similar to the mouse IgG2a/c subclass, these human IgG subclasses display a broader range of Fc-receptor binding as well as a higher capacity to activate the complement system [319]. Although functionally similar, much less is known with regards to the signals that regulate IgG1 and IgG3 specific switching in human B cells. In contrast to mouse IgG2a/c class switch, which includes type I IFN, TLR and IFN-γ signaling, IL-10 and IL-21 have been suggested to be involved in production of IgG1 and IgG3 antibodies

by human B cells [320,321]. However, these cytokines also appear to partially stimulate the production of IgM and IgG4 isotypes, respectively. Broadening our understanding of how IgG1 and IgG3 subclasses are specifically regulated could potentially help us in future development of vaccines, and also, treatment of antibody-mediated autoimmune disorders.

Evidently, although our knowledge about the GC reaction and associated Tfh cell differentiation has increased vastly over the past years, there are still many details that remain unknown. This thesis work has hopefully added another piece to that puzzle.

Populärvetenskaplig sammanfattning

Varje gång vi drabbas av en förkylning, eller ännu värre, åker på influensan så gör vårt immunförsvar sig påmint. Vad vi däremot inte tänker på är alla de gånger som detta nätverk av celler arbetar på i tysthet, utan att orsaka varken feber eller snuva. Utan vårt immunförsvar hade vi stått skyddslösa mot alla de potentiellt sjukdomsalstrande bakterier och virus som finns runtomkring oss. Vårt immunsystem är uppbyggt av ett stort antal av specialiserade celler som tillsammans arbetar för att skydda oss mot yttre, men även inre hot. Bland de yttre fienderna känner vi igen just virus, bakterier och olika typer och parasiter. De inre hoten orsakas faktiskt av våra egna celler, som i vissa fall börjar bete sig olämpligt och i kan ge upphov till cancer. Även dessa känns igen av immunceller och omedelbart tas omhand innan de kan orsaka någon skada.

Genom att vaccinera oss ger vi vårt immunförsvar ett försprång gentemot en riktig infektion. Detta gör vi för att skydda oss från allvarliga sjukdomar som t ex. stelkramp, difteri, och mässling, som före upptäckten av respektive vaccin fick svåra följder. Vid en vaccination är att man injicerar man en del av, eller hela döda, virus eller bakterier. Detta är ungefär som att publicera en bild på en förbrytare för att uppmärksamma människor på att det är en farlig individ om de skulle stöta på personen i fråga. Våra immunceller får därför en chans att träna upp sitt försvar mot just den typen av mikrob som vi vaccinerar oss emot, och vid en riktig infektion så har kroppen skaffat sig ett överläge.

I vårt immunförsvar finns det även en repertoar av olika typer av celler som känner igen ett väldigt stort antal olika motiv, men det betyder också att varje enskild celltyp är väldigt ovanlig. Detta är för att vi i princip ska ha möjlighet att försvara oss mot alla typer av mikrober. När vi vaccinerar oss kan de, från början väldigt få, celler som känner just det motivet dela sig och snabbt gå från runt hundra till tusentals. En del av dessa lever sedan kvar och nästa gång de känner igen samma motiv reagerar de snabbare och mer kraftfullt. Detta är en process som kallas immunologiskt minne.

En annan del av vårt immunförsvar som utvecklas vid en vaccination är den humorala immuniteten, så kallade antikroppar. Antikroppar är små molekyler som cirkulerar i våra kroppar och kan ta sig fram i små utrymmen där inte ens celler kan ta sig fram. Antikroppar har två huvudfunktioner. De kan både binda upp och oskadliggöra lösliga protein, som t ex toxin från bakterier, men kan även binda till och markera hela mikrober så att immunceller förstår att de ska oskadliggöras och förhindra att de sprider

sig. Antikroppar produceras av B celler, och för att förbättra kvalitén på antikroppar av bättre kvalitet (med andra ord, hur starkt de kan binda) ska bildas så behöver B cellerna hjälp av en annan typ av cell som kallas för T celler. Detta sker i våra lymfkörtlar, i en process som kallas ”germinal centra reaktioner” där B celler som producerar antikroppar av bättre kvalitet väljs ut av T celler. Dessa B celler kan sedan bidra till vårt immunologiska minnet, men även utvecklas till plasma celler. Plasma celler är specialiserade på att producera stora mängder antikroppar och är en del av orsaken till att vi har skydd under en lång tid efter vaccination, i vissa fall livet ut.

I mitt avhandlingsarbete har jag undersökt hur just germinal centra reaktionen regleras och hur olika typer av antikroppar bildas. För att kunna utveckla effektiva vaccin mot olika typer av infektioner måste vi förstå i detalj *vilka* celler som är involverade i den här processen, och även *hur* dessa celler kommunicerar med varandra. Detta har jag gjort genom att använda musmodeller där vi kan förändra olika egenskaper i specifika typer av celler, medan andra fortfarande fungerar som vanligt. I en av våra studier har vi t ex funnit att en signalsubstans som kallas typ I interferon, som naturligt produceras i stora mängder vid virus infektioner, är viktigt för att germinal centra reaktioner ska bildas.

Förhoppningsvis har våra studier lagt en bit i det enorma pussel av faktorer och celler som immunologi består av, och därmed också ökat vår förståelse för hur vi bör tänka när vi utvecklar nya typer av vaccin. Trots att vi har vaccin för att skydda oss mot många av de tidigare stora folksjukdomarna finns där fortfarande luckor som behöver fyllas i. Det tydligaste exemplet är förmodligen vår oförmåga att skapa skyddande vaccin mot humant immunbristvirus (HIV) och malaria, men även plötsliga utbrott av dödliga virus, som t ex Ebola, belyser det ständiga behovet av nya vaccin.

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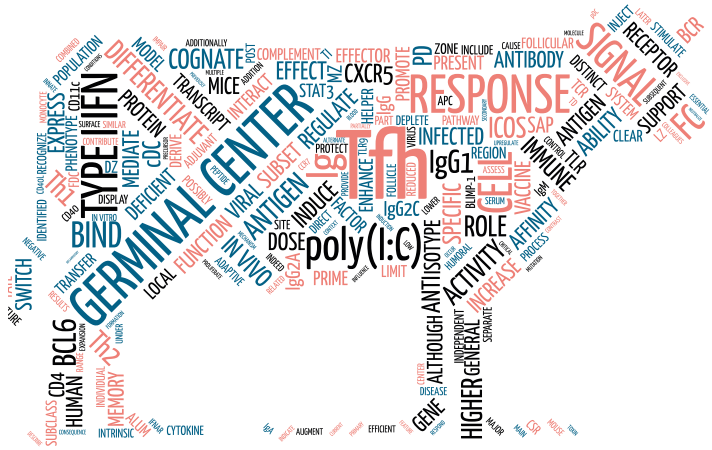

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