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„The role of Interleukin-21 on function and survival of T helper cells and B cells in the germinal center reaction“

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1. Abstract

1.1. Deutsch

Obwohl die Notwendigkeit einer funktionierenden Interleukin-21 Signalkaskade für die adäquate Keimzentrumsformation im Inneren eines Lymphfollikels, sowie einer funktionalen IgG1-Immunantwort allgemein anerkannt ist, bleibt der genaue Mechanismus über den IL-21 seine Funktion ausübt weiterhin unbekannt. Eine genauere Untersuchung der Rolle von IL-21 für die Vorgänge das Keimzentrum betreffend scheint angebracht um ein tieferes Verständnis der Interaktionen die hier am Werk sind zu gewährleisten. In unserer Studie zeigen wir, unter Zuhilfenahme eines pMHCII Tetramers, die starke IL21-Abhängigkeit von Antigen-spezifischen follikulären T-Helferzellen (Tfh) früh in der Keimzentrumsreaktion. Außerdem fanden wir heraus, dass die Zellen welche CXCR5 und PD-1 am meisten produzieren und deshalb als Tfh klassifiziert werden durchaus sehr variabel in ihrer Spezifität für Antigen sind. Durch diesen Fund sind die doch sehr unterschiedlichen Ergebnisse was die Wichtigkeit von IL-21 für Tfh betrifft möglicherweise zu erklären. Des weiteren zeigt unsere Studie eine hohe Aktivierung, jedoch schlechte Überlebensrate für *Il21r-/-* T-Helferzellen welche mittels Überexpression von CXCR5 (CXCR5tg) ins Keimzentrum gezwungen werden. Auch entdeckten wir eine bislang unbekannte schützende Rolle von IL-21 auf IgG1$^+$ B-Zellen die diese vor negativen Auswirkungen durch CD8$^+$ T-Zellen bewahrte, jedoch wenig Effekt auf die Gesamtzahl der B-Zellen hatte.

Dieser mögliche Einfluss von IL-21 auf CD8$^+$ T-Zellen und deren Regulation der IgG1$^+$ humoralen Immunantwort eröffnet eine Reihe neuer Möglichkeiten für die Funktionsweise dieses Zytokins. Die aufgezeigte schützende Rolle von IL-21 vor CD8$^+$ T-Zellen passt überdies gut mit unseren Entdeckungen über die Notwendigkeit von IL-21 für die Differenzierung und das Überleben von T-Helferzellen zusammen. Wir sind uns sicher durch unsere Ergebnisse einen guten Grundstein für weitere Forschung zur Klärung der Rolle von IL-21 im Kontext der CD8$^+$ „suppressor“ T-Zell Hypothese gelegt zu haben.

1.2. English

The importance of functional Interleukin-21 signalling for proper germinal center formation and IgG1 responses is undisputed, however the mechanism how IL-21 executes its function remains uncertain. It has become evident that a closer look on the role of IL-21 on the cells involved in the GC reaction is necessary to further ameliorate our understanding of the intricate interactions at work in this process. In this study we show the strong dependency of Ag-specific T helper cells on IL-21 early in the GC reaction, with the help of a pMHCII tetramer. Additionally we found the CXCR5high/PD-1high Th cell population, traditionally classified as T-follicular helper cells (Tfh), to be quite variable in regard to Ag-specificity, a finding that might somewhat explain the contradicting reports on IL-21 dependency of Tfh cells. We also discovered the drastic effects of channelled translocation of *Il21r-/-* T helper cells into the GC via forced CXCR5 surface-expression, which led to strong activation but poor survival of these cells at the same time. This study also uncovered a novel protective role for IL-21 from $CD8^+$ T cell mediated adverse effects on $IgG1^+$ B cells, with little effect on overall B cell numbers.

A possible involvement of $CD8^+$ T cells in the regulation of the IgG1-switched humoral immune response and the role of IL-21 in this process opens up a new layer of complexity for the functions associated with this cytokine. The suggested protective role for IL-21 from $CD8^+$ T cells also fits well with our findings on the importance of IL-21 for differentiation and survival of T helper cells. We are confident that further studies will build on the findings presented in this study to clarify the functional role of IL-21 in the context of the $CD8^+$ “suppressor” T cell hypothesis.

2. Introduction

2.1. The mammalian immune system in a nutshell

2.1.1. Innate immunity

The mammalian immune system is composed of two distinct defence systems; the innate or non-specific immune system arose early in the evolution and is found in all classes of plant and animal life, whereas the adaptive immune system is more specialized and is found only in vertebrates. The innate immune system recognizes evolutionarily conserved epitopes of pathogens through a limited set of germ-line encoded receptors. These pattern-recognition receptors (PRRs) recognize a group of molecules known as pathogen-associated molecular patterns (PAMPs) such as bacterial cell wall components or double stranded viral RNA (Janeway, 1989). Many cells of the innate immune system (including mast cells, macrophages, neutrophils and dendritic cells) express surface or internal PRRs, such as the Toll-like receptor family (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). The interaction of PRRs with their corresponding PAMPs generally leads to the activation of a signalling cascade and subsequently to the activation of gene expression and onset of an inflammatory reaction (Akira et al., 2006). Because of their quick reaction time, cells of the innate immune system compose an essential first wave of defence against invading pathogens. Phagocytes such as macrophages, dendritic cells (DC) and neutrophils can engulf pathogens they encounter and apart from lysing and thereby neutralizing them they are able to present peptide components of the pathogens on their surface in context of major histocompatibility complex (MHC) class II (Banchereau and Steinman, 1998; Itano and Jenkins, 2003). With the expression of both antigen (Ag) and co-stimulatory molecules these antigen presenting cells (APC) are able to activate the cells of the adaptive immune response and thereby form an important link between adaptive and innate immunity. Another part of the mammalian innate immune response consists of anatomical barriers, such as the protective layer of the skin with its acidic pH or the gastrointestinal tract with its digestive enzymes and the gut flora. The tight junctions formed by dermal keratinocytes pose a physical barrier preventing bacteria and other pathogens from entering the organism, while the

skins acidic pH (pH 5.5), as well as secreted proteases form a very hostile environment for potential invaders (Braff and Gallo, 2006; Jensen and Proksch, 2009; Koziel and Potempa, 2012). The humoral response of the innate immune system consists of chemokines and cytokines released by mast cells and neutrophils, as well as the over 25 proteins of the complement system. The complement system consists of a number of soluble proteins circulating in the blood that get activated through binding to pathogens and proteolytic cleavage of precursor proteins (Muller-Eberhard, 1988). The activation triggers a cascade of further cleavages massively amplifying the response and leading to formation of the membrane attack complex lysing the invading pathogens, as well as attracting macrophages and neutrophils and enhanced clearing of pathogens through their opsonisation (Muller-Eberhard, 1988; Carroll and Sim, 2011).

2.1.2. Adaptive immunity

The second and evolutionary younger part of the mammalian immune system is called adaptive or acquired immunity and it's only found in higher organisms. It takes longer to build up this highly pathogen specific immune response, but a long lasting immunological memory is obtained which enables a quick and strong response upon reinfection. One can broadly divide the adaptive immune system into two compartments, a humoral compartment with B cells as their main effector cells and a cellular compartment where T cells play the main role. The site of early B cell development is the bone marrow, where haematopoiesis takes places, common lymphocyte precursors (CLP) commit to the B cell lineage and become pro B cells (Welner et al., 2008). Rearrangement of the variable (V), diversity (D) and junctional (J) segments of both the immunoglobulin light and heavy chain locus via excision of random gene segments leads to an antigen (Ag) specific B cell receptor (BCR), in a process called VDJ-recombination (Honjo et al., 1981). These immature B cells are then released into bloodstream from where they migrate into the spleen and are termed as transitional B cells. Two different subsets can emerge from these cells, on one hand marginal zone B cells which are only found in the white pulp of the spleen and respond to blood borne antigen. On the other hand follicular B cells travel through the blood and lymph nodes whilst screening for

antigen (Casola, 2007; Pillai and Cariappa, 2009). Because of the random nature of the VDJ-recombination an infinite number of different Ag-specific receptors can be generated, which gives the adaptive immunity considerable advantage over the innate immunity and its limited range of germline encoded PPR.

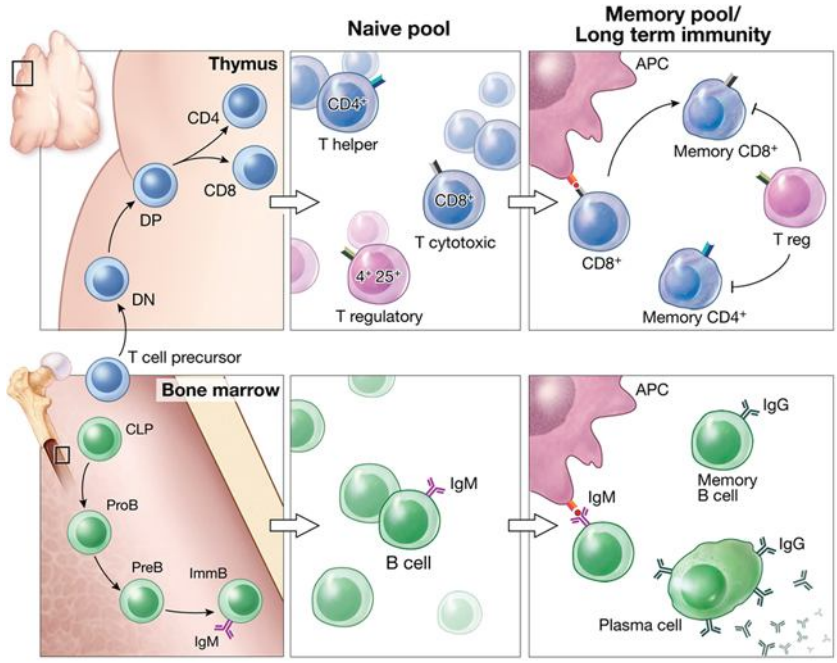

Figure 2-1) Overview T and B cell development
Figure adapted from Aragon-Ching et al., 2007

2.1.3. T cell development

Although T cells, much the same as B cells, originate from haematopoietic stem cells in the bone marrow, they are named after the thymus, because this is the organ where their maturation and differentiation takes place (Miller and Osoba, 1967). The T cell lineage branches into two major groups, $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells (Th). Both start out as CD8-/CD4- double negative (DN) precursors in the thymus, and these immature DN cells develop into $CD8^+/CD4^+$ double positive (DP) immature thymocytes (Bhandoola et al., 2003). The epitope specificity of the T cell receptor (TCR) is determined much in the same way as the BCR through a process called clonal selection, which means that each TCR as well recognizes one particular epitope and that the number of different possible receptors is limitless (Burnet and Holmes, 1965; Cohn et al., 2007). Double positive cells undergo both positive and negative selection, positive selection screens for

cells possessing TCRs capable of interacting with MHC making sure the thymocytes will be able to react to Ag presented in the context of MHC molecules. The cells binding to MHC class I develop into $CD8^+$ single positive cells, the ones binding to MHC class II develop into $CD4^+$ single positive cells, cells binding neither, or only weakly binding die by neglect (Zúñiga-Pflücker et al., 1991). Negative selection on the other hand removes the cells harbouring TCRs binding too strongly to "self" peptides presented to them in context of MHC in the thymus, thereby actively removing self-reactive T cells. Cells surviving the harsh treatment of positive and negative selection receive maturation signals and the mature, naïve T cells are released in the periphery via the lymphatic system in the search for antigen (Starr et al., 2003). The two groups of the T cell lineage possess a distinct set of functions, the $CD4^+$ T cells who recognize their Ag in the context of MHC class II regulate humoral Ab responses against extra-cellular pathogens, as well as regulating cellular immune responses against both extra- and intracellular pathogens. Cytotoxic T cells ($CD8^+$) on the other hand recognize their peptide antigen in the context of MHC class I expressed by almost all cells and tissues of the organism. When $CD8^+$ T cells find their Ag presented through MHC class I on the surface of a cell, they recognize the cell as infected by intracellular pathogens (bacteria or viruses). Upon receiving co-stimulatory signals they then proceed to lyse the infected cells either by releasing perforin, which forms pores in the membrane of the target cell, or via FAS-FASL interaction, also leading to apoptosis of the infected cell (Wong and Pamer, 2003).

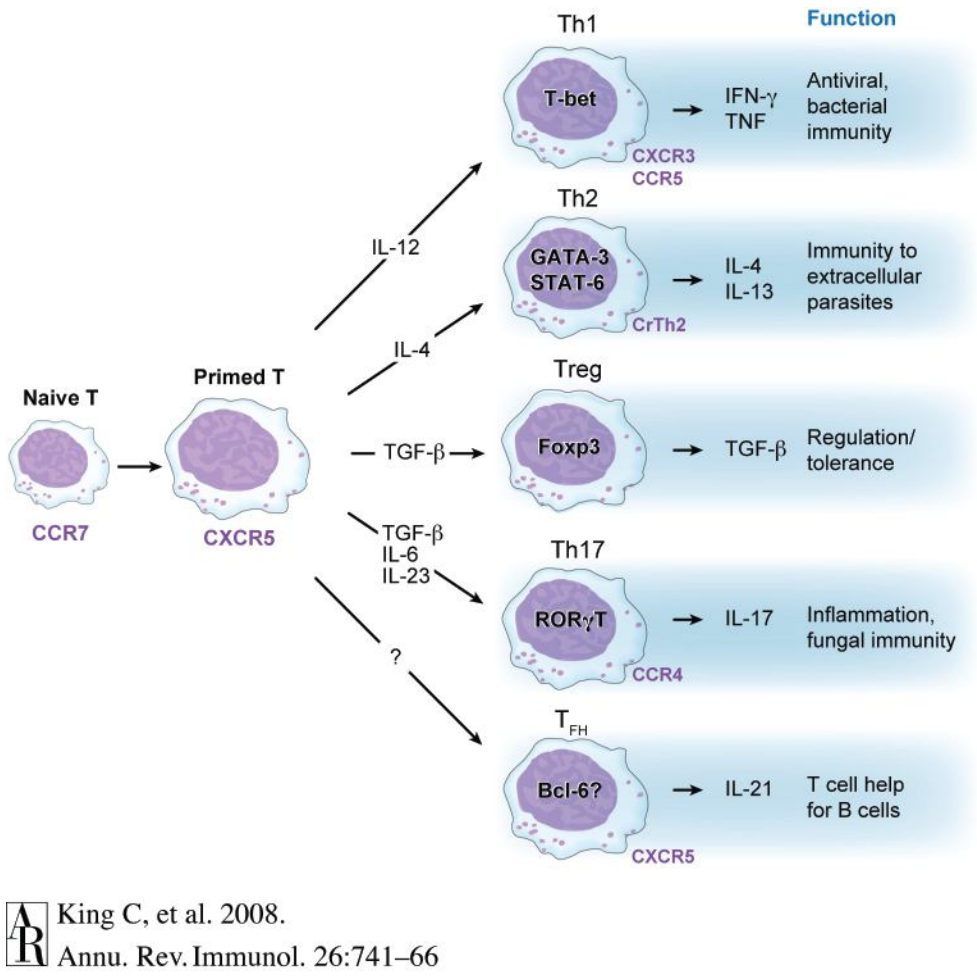

Figure 2-2) Different T helper cell subsets and their signature cytokine expression
Figure adapted from King et al., 2008

2.2. CD4$^+$ T cell subsets

For CD4$^+$ T cells to get activated professional antigen presenting cells (APC) need to present processed peptide-antigen to them in the context of MHC class II. Apart from their cognate antigen T helper cells also need co-stimulatory signals in form of CD80 (B7.1) or CD86 (B7.2) presented from APCs to their CD28-receptor to get activated (Coyle and Gutierrez-Ramos, 2001; Greenwald et al., 2005). In the absence of this second signal the Th cells become anergic and will not respond to any antigen encountered in the future, this mechanism is in place to circumvent the activation of self-reactive Th cells (Bretscher, 1999). After activation the Th cells are allowed to proliferate, they release the growth factor Interleukin-2 (IL-2) which binds on their own IL2-receptor (CD25 or IL2R) and thereby generate an autocrine loop enforcing proliferation. The activated Th cells become T helper 0

(Th0) cells and secrete IL-2, IL-4 as well as interferon gamma (INFγ), depending on their cytokine environment these cells can differentiate to one of several T helper cell subsets (Kim and Broxmeyer, 1999).

2.2.1. Th1 cells

Historically T helper subsets were distinguished by their differential cytokine expression, the first subsets described this way were Th1 and Th2 cells. The subset expressing INFγ and tumor necrosis factor-beta (TNFβ) was named Th1, whereas the IL-4, IL-5 and IL-13 expressing subset was termed Th2 (Mosmann et al., 1986). Further studies revealed that the function of the Th1-subset lies in the activation of macrophages and cytotoxic T cells ($CD8^+$) to facilitate the defence against intracellular pathogens such as Leishmania major, as well as promoting the production of IgG2a by B cells (Heinzel et al., 1989). The Development of Th0 into the Th1 subset is driven by the activation of the transcription factor (TF) STAT1 by the presence of INFγ and IL-12 in the cytokine milieu at the time of activation. The TCR activation and simultaneous STAT1 activation leads to the expression of the transcription factors T-bet and RUNX3, which further prompt the Th1 phenotype by suppressing Th2 transcription factors and gene products via expression of INFγ (Djuretic et al., 2007). Commitment to the Th1 lineage is also reinforced via APC derived IL-12 and its activation of STAT4 signalling in the lymphocytes (Wilson et al., 2009). Apart from the Th1 cells function in defence against different intracellular intruders they also seem to play an important role in the bodies' anti-tumour defence, as has been shown in mouse models (Micallef et al., 1997). As every part of the immune system Th1 cells have to be tightly regulated, when this regulation fails autoimmune diseases such as rheumatoid arthritis (RA) or inflammatory bowel disease (IBD) can be the consequence (Davidson et al., 1996; Leung et al., 2000), Th1 cells also play a role in graft versus host disease (GVHD) in murine transplant models (Hu et al., 1999).

2.2.2. Th2 cells

The presence of IL-4 during thymocyte maturation (TCR activation) leads to the activation of the transcription factor STAT6, which in turn signals together with the

Notch transmembrane receptor to induce GATA3 and MAF expression (Amsen et al., 2007). The activation of this signalling cascade leads to the production of the Th2 cytokines IL-4, 5, 9, 10 and 13, which further push Th2 differentiation and also block the Th1 pathway (Ansel et al., 2006). The function of Th2 and the cytokines produced by this T cell subset lies largely in the activation of the epithelial defence and also to help humoral immunity against large parasites such as helminthes and nematodes (Abbas et al., 1996). Th2 cells mediate their mainly humoral function through stimulation of B cell proliferation and facilitation of antibody class switching to IgE, and also attract eosinophiles to the site of infection (Mosmann et al., 1986; Wan and Flavell, 2009). The involvement of Th2 cytokines in diseases such as asthma or allergy has also been shown (Wills-Karp, 2000).

2.2.3. T regulatory cells

To prevent the immune system from attacking the own body T cells with specificity to "self-Ag" are deleted in the thymus in a process called central tolerance (Rudensky AYu et al., 1991). As reliance on this single mechanism still would lead to autoimmunity through a small number of cells escaping central tolerance, there is a second safeguard mechanism called peripheral tolerance. A subset of the T helper cell lineage called regulatory T cells (Treg) is responsible to induce anergy in self-reactive T cells in the periphery and hereby prevent an autoimmune reaction (Sakaguchi, 2000). These Treg cells execute their function through anti-inflammatory cytokines, cell-to-cell contact as well as through modulation of APCs. They are produced both in the thymus (natural Treg) from T cells with a TCR affinity for self Ag too low to get deleted as potential autoimmune cells, but too high to get selected as effector T cells, and also in the periphery (inducible Treg) in response to tolerating signals such as TGFβ and high levels of IL-2 . Both Treg subsets express CD25 (high affinity IL-2 receptor) and the forkhead box p3 (Foxp3) transcription factor, which inhibits the expression of proinflammatory cytokines in these cells (Sakaguchi et al., 1995; Josefowicz and Rudensky, 2009). The fact that these aforementioned surface markers are general markers of T cell activation, and therefore not strictly limited to Tregs makes this cell population hard to distinguish from other T cell populations. The importance of this subset is shown when

humans or mice have a defect in the Foxp3-locus and therefore no fully functional Treg population is present. Organisms with this defect develop severe autoimmune reactions, ranging from eczematous dermatitis, type 1 diabetes mellitus, autoimmune thrombocytopenia, autoimmune neutropenia, and tubular nephropathy (Bennett and Ochs, 2001; Brunkow et al., 2001).

2.2.4. Th17 cells

Research on murine models for autoimmune disease, namely experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) have led to the discovery of yet another T helper cell subset, the so called T helper 17 cells (Th17) (Harrington et al., 2005). It was previously thought that both of these diseases are caused by uncontrolled Th1 responses, but with the discovery of the IL-12 family member IL-23, which induces the development of Interleukin-17 (IL-17) producing Th17 cells, this view was challenged. Apart from the production of IL-17, that is to say IL-17A-F, this subset is characterized by the expression of IL-21 as well as IL-22. Although the Th17 subset shares compelling developmental similarities with Th1 cells, it is widely accepted that these lineages don't develop from a common precursor, but are distinct, non-overlapping lineages (Steinman, 2007; Stockinger and Veldhoen, 2007). Additional to the similarities between the Th1 and Th17 lines, Th17 also share similarities with the Treg subset. It seems that TGFβ and Interleukin-6 (IL-6) exposure needed for Treg development is also crucial for Th17 development (Weaver et al., 2006). Apart from their role in autoimmune disease the suggested function of Th17 cells lies in the mobilization of neutrophils and inflammatory cytokines which leads to the clearance of pathogens like Listeria, Salmonella, Mycobacterium tuberculosis as well as several fungal species (Ivanov et al., 2009; Pepper et al., 2011).

2.2.5. Th9 cells

An IL-9 expressing T helper cell subset termed Th9 has independently been described by the groups of Kuchroo and Stockinger in 2008 (Dardalhon et al., 2008; Veldhoen et al., 2008). Initially it was thought that the secretion of IL-9 is part of the functional repertoire of Th2 cells, but upon more detailed examination it was

revealed that IL-9 expressing cells down-regulate expression of certain Th2 markers such as IL-4, IL-5 and IL-13 (Veldhoen et al., 2008). These cells also don't express the Th2 transcription factor GATA3, or RORγT and Foxp3 associated with Th17 and Treg cells which further supported the believe these cells form a distinct lineage. However there was no Th9-specific marker known until it was discovered that the expression of the transcription factor PU.1 is necessary for IL-9 expression and at the same time causes the downregulation of other Th2 cytokines (Chang et al., 2010). The differentiation of Th9 cells is dependent on IL-4, much the same as Th2 cells, but unlike the Th2 population they also need TGFβ1 for proper development. The function of this subset is still disputed, but an involvement in what classically was described as "Th2-immunity" seems probable, also a role in allergic inflammation and asthma has been shown (Chang et al., 2010). The description of this lineage and its development has added further complexity to the understanding of the different T helper subset and the plasticity involved in their differentiation.

2.2.6. Tfh cells

B cells need the help of specialized T cells to undergo IgG1 class switching and affinity maturation in the germinal center (GC) to produce a long-lasting, high-affinity Ab-response. It has originally been thought that the Th1 and Th2 populations were the only cells responsible to provide help to B cells (Mosmann et al., 1986), but further studies discovered a specialized subset called T follicular helper cells (Tfh) to be the main binding partners of B cells destined to produce high affinity Ab. These cells have been identified due to their high expression of the CXC chemokine receptor 5 (CXCR5) and their resulting ability to migrate into the B cell follicle (Breitfeld et al., 2000; Schaerli et al., 2000). Apart from CXCR5 Tfh cells also express co-stimulatory molecules such as CD40L, which is important for the interaction CD40 expressed on B cells, or the T cell activation marker ICOS (Hutloff et al., 1999; van Kooten and Banchereau, 2000). The high expression of the inhibitory molecule programmed cell death-1 (PD-1) by Tfh cells might reflect their role as strong B cell interaction partners in the GC (Haynes et al., 2007; Fazilleau et al., 2009). As PD-1 expression on $CD8^+$ cells is associated with chronically

exhausted cells one might speculate that there could be by a similar process going on in $CD4^+$ Tfh cells, but this hasn't been proven yet (Barber et al., 2006; King, 2009). Cytokines influencing B cell antibody production and class switching such as IL-4, IL-10 and IL-21 are known to be produced by Tfh (Chtanova et al., 2004; Haynes et al., 2007; Reinhardt et al., 2009). The cytokine IL-21 is also found to be expressed by Th17 cells but at much lower levels than by Tfh (Chtanova et al., 2004; Nurieva et al., 2007; King, 2009). The IL-21 receptor (IL-21R) is expressed on B cells and on Tfh cells themselves, and considerable debate has been going on whether IL-21 plays his dominant role on one cell type or the other (Nurieva et al., 2008; Vogelzang et al., 2008; Linterman et al., 2010; Zotos et al., 2010). In the search for a master controller for the Tfh gene-expression program the transcription factor B cell lymphoma 6 (BCL-6) was identified in microarray analyses to be expressed in Tfh cells but not in other T helper subsets (Chtanova et al., 2004). Presence of IL-6 and IL-21 in the cytokine milieu leads to the upregulation of BCL-6 and further to the inhibition of the Th2 programme through the blocking of STAT6 binding to DNA. It was shown that overexpression of BCL-6 was able to suppress both Th1 and Th17 differentiation pathways (Nurieva et al., 2009), and also was sufficient to drive Tfh development in $CD4^+$ T cells (Yu et al., 2009). Mice deficient in BCL-6 weren't able to mount a proper GC reaction, if this is due to the known role of BCL-6 in B cells or due to its newly discovered role in Tfh cells remains elusive (Yu et al., 2009). Like other T helper subsets Tfh cells are derived from naïve $CD4^+$ T cells Ag primed by dendritic cells in the T cell follicle of the secondary lymphoid organs (lymph nodes, spleen, and payer's patches). Signalling through the TCR leads to the expression of the early activation marker CD69, as well as downregulation of the sphingosine 1 phosphate receptor 1 (S1PR1) which leads to the detention of the cells in the lymph node. Upregulation of CXCR5 leads to the recruitment of these cells to the border of the B cell zone (T-B border) where they establish interaction with Ag-presenting B cells. Only the $CD4^+$ T cells with the strongest interaction with B cells are able to become Tfh, the rest either leaves the secondary lymphoid organ to become effector T helper cells or to aid antibody production in the extrafollicular foci (Fazilleau et al., 2009). The cells selected to become Tfh downregulate CCR7 and migrate into the B cell follicle to the sites of GC formation, constant interactions with B cells might deliver the final

signals needed for their differentiation into Tfh (Hardtke et al., 2005; Haynes et al., 2007).

2.3. B cell activation

Mature B cells patrol the lymphatic system in the search for foreign Ag, upon Ag encounter in the spleen or lymph node they relocate to the border of the T/B cell zone to get T cell help (Garside et al., 1998; Gatto and Brink, 2010). Following Ag stimulation the B cells can process and present the Ag they encountered in context of MHC class II to $CD4^+$ T helper cells, which in turn are able to stimulate B cell proliferation (Lanzavecchia, 1985; Batista and Harwood, 2009). The stronger binding B cells migrate to extrafollicular areas of the B cell zone and become short lived plasmablasts, producing low-affinity antibody as a first wave of protection (MacLennan et al., 2003; Chan et al., 2009). B cells with lower affinity for antigen migrate to the border of the T/B cell zone, where they receive help from T cells to become GC B cells. Through continuous help from T cells in the GC these cells undergo Ab-class switching and somatic hypermutation (SHM) to become high-affinity Ab producing plasma cells or memory B cells (MacLennan, 1994; Garside et al., 1998). Because of the much larger number of B cells than $CD4^+$ T cells in the GC there is fierce competition between B cells for T cell help. The B cells not receiving enough positive signals undergo apoptosis rather than to differentiate into memory B cells or plasma cells (Allen et al., 2007a, 2007b). The main function of plasma cells is the secretion of antibodies against soluble antigens, which leads to the identification and neutralization of foreign invaders like bacteria and other pathogens in the bloodstream and their subsequent clearance. Memory B cells on the other hand are part of the adaptive immune systems memory response, they circle in the body for a long time and can quickly respond to a second exposure of the same antigen (MacLennan, 1994; Rajewsky, 1996).

2.3.1. The germinal center reaction

Secondary lymphoid organs such as the spleen, lymph nodes or payers patches provide the environment needed for naïve B cells to encounter antigen and make the transition to high-affinity antibody production. After receiving help from T

cells, the activated, Ag-experienced B cells start to proliferate in the primary B cell follicle (MacLennan, 1994). After 3 days distinct structures, named germinal centers, first described by Ian Flemming in 1884 can be observed under the microscope (reviewed by Nieuwenhuis and Opstelten, 1984). The GCs can be divided in dark and light zone, dependent on their haematoxylin and eosin staining. The dark zone is mainly composed of centroblasts, B cells undergoing rapid clonal expansion, whereas the light zone is inhabited by B cells called centrocytes, antigen enriched follicular dendritic cells (FDC) as well as $CD4^+$ helper T cells (Allen et al., 2007a). Clonal selection based on antibody affinity is thought to occur in the light zone, the limiting signal being help from T cells (Allen et al., 2007b). The migration of T helper cells in the GC occurs along a gradient of CXCL13, the ligand for the CXCR5 expressed transiently on all activated $CD4^+$ T cells, but only continuously expressed by Tfh cells (Breitfeld et al., 2000; Schaerli et al., 2000).

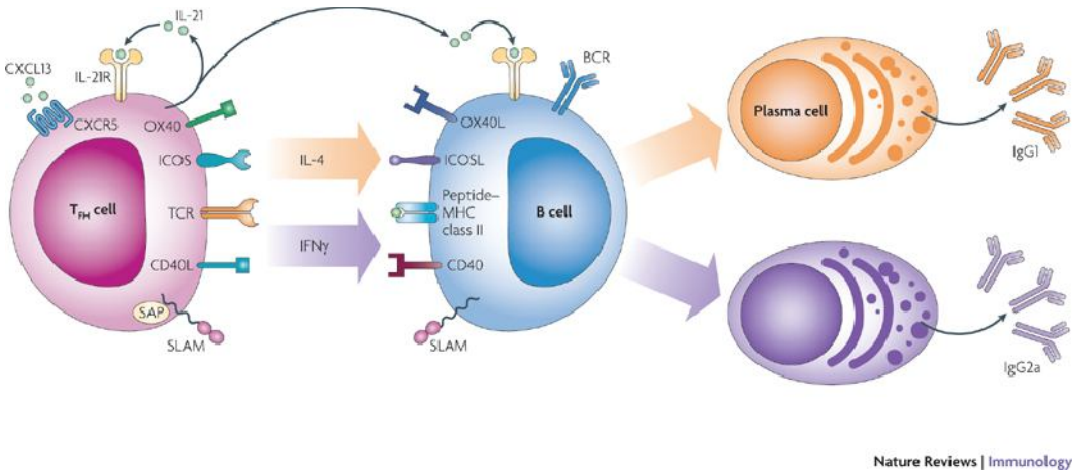

Figure 2-3) Interaction of Tfh cells and B cells via surface molecules and secreted cytokines
Figure adapted from King, 2009

2.3.2. T-B interaction, partners in crime?

Although the formation of GC seems to work independent of T cell derived signals, functional T follicular helper signalling seems to be essential for the maintenance of the GC reaction and SHM (de Vinuesa et al., 2000). The formation of a stable interaction between T follicular helper cells and germinal center B cells is essential for plasma cell and memory B cell formation and the subsequent production of

high-affinity antibody (Foy et al., 1994; Han et al., 1995). The T-B cell interaction is initiated at the border of the T cell zone and the B cell follicle, where the processed Ag in the context of MHC class II presented by B cells is recognized by the TCR of T follicular helper cells, which in turn provide help to B cells in the form of cytokines and co-stimulatory signals (Ron and Sprent, 1987; Garside et al., 1998; Hutloff et al., 1999; van Kooten and Banchereau, 2000) One of the crucial signals is the interaction of CD40 expressed on T cells with its ligand CD40L expressed by B cells, without it a fast dissolution of the newly formed GC can be observed (Han et al., 1995; van Kooten and Banchereau, 2000). It has been proposed that this signalling might allow the selected B cells to re-enter the cell cycle and therefore be important for GC maintenance and affinity maturation (Oprea and Perelson, 1997). Another vital interaction happens through the signalling lymphocytic activation molecule (SLAM) expressed both by B cells and Tfh. In $CD4^+$ T cells the SLAM associated protein (SAP) has been shown to enhance both Th2 differentiation and TCR signals (Wu et al., 2001; Cannons et al., 2004). T cells deficient in SAP can't form stable T-B cell interactions and are therefore unable to support GC formation (Qi et al., 2008). The stimulation of ICOS on Tfh cells by its ligand ICOSL on GC B cells induces the expression of the cytokines IL-2, IL-4 and IL-10, known to help B cells. The importance of ICOS has been shown by disrupted GC reactions in *Icos-/-* mice (Bossaller et al., 2006). Another cytokine with crucial role in GC development and maintenance is the Tfh cell expressed IL-21 (Chtanova et al., 2004). Interleukin-21 is important for proper GC responses in various ways, it has been shown to stimulate B cell proliferation, as well as their differentiation into IgG1 secreting plasma cells and also plays an important role for the production of high affinity antibody (Pène et al., 2004; Ettinger et al., 2005; Bryant et al., 2007). Whether these effects are purely due to the function of IL-21 on B cells or also due to its proposed intrinsic role in Tfh regulation and development remains under discussion (Nurieva et al., 2008; Vogelzang et al., 2008; Linterman et al., 2010; Zotos et al., 2010).

2.4. Interleukin-21

Interleukin-21 (IL-21) is a type I cytokine encoded at the insulin dependent diabetes 3 (Idd3) locus on mouse chromosome 3 in close proximity to IL-2 (Yamanouchi et al., 2007; Datta and Sarvetnick, 2008; Spolski et al., 2008). IL-21 signalling is carried out via the specific receptor IL-21R and the common cytokine receptor gamma chain (yc), shared with several other cytokines such as IL-2, IL-4, IL-7, IL-9 and IL-15 (Ozaki et al., 2000; Asao et al., 2001; Leonard and Spolski, 2005). Downstream of the IL-21R, STAT3 signalling is activated by IL-21, being relevant for a wide range of cell types, such as B cells, T cells, natural killer cells (NK) and dendritic cells (Leonard, 2000; Parrish-Novak et al., 2000; Zeng et al., 2007). Whereas IL-21 can be produced by a wide range of Th cells in smaller amounts, it's most abundantly produced by Tfh cells and constitutes one of their signature cytokines (Chtanova et al., 2004; Nurieva et al., 2007; King, 2009). A mutation in the common gamma chain leads to disrupted signalling and to a failure to generate T cells or NK cells and to non-functional B cells, an immunodeficiency disorder named X-linked severe combined immunodeficiency (X-SCID) is the consequence (Leonard, 2000).

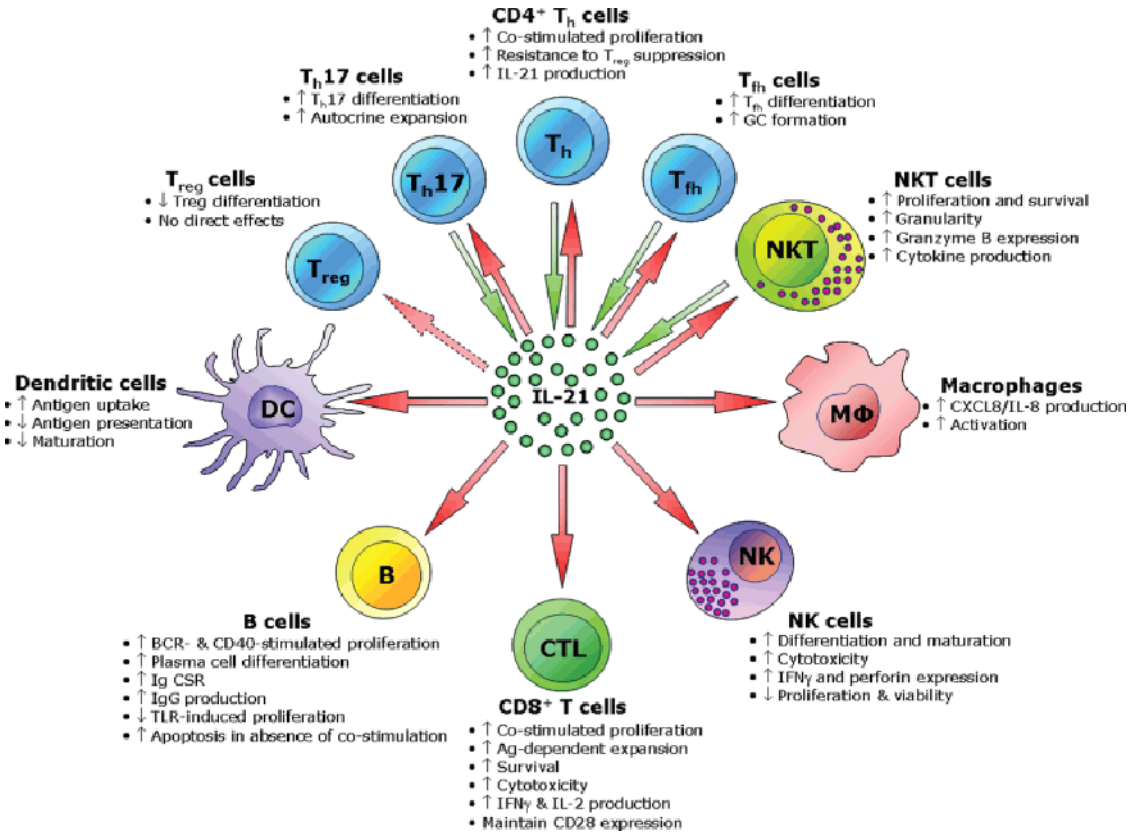

Figure 2-4) The function of Interleukin-21 on different cell types
Figure adapted from Søndergaard and Skak, 2009

2.4.1. IL-21 and T helper cells

Although the IL-21R is expressed in early stages (DP stage) of T cell development in the thymus, normal thymic development has been shown in mice genetically deficient in IL-21R (*Il21r-/-*) (Kasaian et al., 2002; Ozaki et al., 2004). Therefore an early involvement of IL-21 in T cell development seems unlikely, more probable is the function of IL-21 at a later stage during proliferation, differentiation or formation of effector function. Studies on *Il21r-/-* mice revealed a critical role for IL-21 signalling in the differentiation of Th2 cells (Fröhlich et al., 2007). In these mice the response to intracellular pathogens mediated by Th1 activation and interferon gamma (INFγ) secretion remained intact, whereas Th2 responses seemed to be compromised. T helper type 2 cells are produced, but they migrate poorly to the site of infection and also secrete less cytokines as cells from wild type (WT) mice in response to nematode infection. These findings indicate an involvement of IL-21 signals in Th2 responses, but no important role for IL-21 in

Th1 reactions. *In vitro* studies further supported this view by showing an inhibitory role of IL-21 on INFγ production in developing Th1 cells through the inhibition of the transcription factor Eomes (Suto et al., 2006). The IL-17 producing Th subset Th17 is also known to produce considerable amounts of IL-21 in comparison to Th1 or Th2 cells, but not as much as Tfh cells. It has been shown in a range of *in vitro* studies that IL-21 is able to provide the signals for enhancing IL-17 production and to substitute for IL-6 in Th17 differentiation (Korn et al., 2007). But although IL-21 has been shown to be beneficial for Th17 in the absence of IL-6, a redundancy of its signals for *in vivo* Th17 responses has also be shown before (Sonderegger et al., 2008).

2.4.2. Effects of IL-21 on $CD8^+$ T cells

It has been shown, that although IL-21 can't induce $CD8^+$ T cell proliferation without TCR activation or additional co-stimulatory signals, in concert with IL-15 or IL-7 signalling it does augment the proliferation of $CD8^+$ memory T cells (Liu et al., 2007). Interleukin-21 was also shown to prevent IL-15 mediated downregulation of CD28 in naive $CD8^+$ T cells and thereby preserves their ability to respond to co-stimulatory signals upon future encounters with activated APC (Alves et al., 2005). As $CD8^+$ T cell development seems normal in IL-21R deficient mice, it seems that other factors can compensate for its effects on $CD8^+$ T cell growth (Kasaian et al., 2002; Ozaki et al., 2002). Constant antigen exposure in the course of chronic viral infection can inhibit cytotoxic T cell activity, this process is known as "exhaustion". The exhausted $CD8^+$ T cells are unable to destroy infected cells and clear the infection, but it has been shown that $CD4^+$ T cell derived IL-21 can prevent exhaustion and thereby support long-term antiviral responses. Interestingly it also has been shown that these IL-21 signals are redundant in an acute response against the same pathogen (Yi et al., 2009). Additionally IL-21 has been able to increase perforin expression in cytotoxic T cells from HIV positive patients without affecting activation or proliferation, suggesting the ability of IL-21 to ameliorate the cytolytic ability of $CD8^+$ without affecting HIV gene expression or virus production (White et al., 2007). Altogether these studies suggest a co-

stimulatory role of IL-21 for cytotoxic T cell proliferation and function, whether the cytokine acts directly or via additional molecules remains an open question.

2.4.3. IL-21 and B cells

The Interleukin-21 receptor (IL-21R) is expressed on both immature and mature B cells and upregulation can be observed upon antigen binding (Ozaki et al., 2000; Parrish-Novak et al., 2000). Dependent on the combination of received co-stimulatory signals quite opposite effects can be observed. If delivered in the context of BCR activation and CD40-CD40L ligation, IL-21R signalling on B cells leads to augmented proliferation and facilitated antibody production (Parrish-Novak et al., 2000; Jin et al., 2004). The delivery of IL-21R signals together with TLR signals on the other hand leads to apoptosis of the respective B cell (Mehta et al., 2003; Jin et al., 2004; Jin and Malek, 2006). This discrepancy in the function of IL-21R signalling on B cells might be another safeguard mechanism to prevent the production of autoreactive antibody. Activated B cells receiving both IL-21R signals was well as T cell help in form of the CD40-CD40L interaction can be assumed to be directed against a foreign pathogen and therefore get boosted. If the IL-21R signalling is only received in the context of TLR activation on the other hand, potential inappropriate B cell responses must be avoided through the induction of apoptosis (Jin et al., 2004). The forced expression of elevated levels of IL-21 in mice leads to a reduced number of mature B cells, which can be explained by the aforementioned regulatory effect leading to apoptosis. The overexpression of IL-21 leads to increased class-switching to IgG1 antibody and upregulation of the transcription factor BCL-6 in B cells. The forced expression of IL-21 also resulted in upregulation of Blimp-1 levels in B cells and forced them into the plasma cell differentiation program, indicating a B cell intrinsic response to IL-21 (Ozaki et al., 2004). Although the overexpression of IL-21 in *in-vivo* mouse models showed dramatic effects, it has been shown that IL-21 may not be essential for *in vivo* B cell development. The exception being the Ab class-switch to IgG1 isotype, which is heavily dependent on IL-21, but if this is due to B cell intrinsic effects or due to the effects of IL-21 on $CD4^+$ T cells and missing T cell help remains to be investigated (Ozaki et al., 2002).

2.4.4. Effects of IL-21 on NK cells and NKT cells

Natural killer cells are part of the innate immune system and therefore of the bodies first line of defence against pathogens. Their major role is the induction of apoptosis in virus infected cells via release of their effector-proteins perforin and granzyme A & B (Moretta et al., 1996; Biron et al., 1999). The effects of IL-21 on NK cells are variable depending on their stage of activation and maturation, as well as the co-stimulatory signals delivered. An inhibitory effect of IL-21 on the IL-15 mediated expansion of naïve, murine NK cells can be observed, as well as apoptosis inducing effects (Kasaian et al., 2002; Strengell et al., 2002). On the other hand IL-21 stimulation seems to execute positive functions on activated NK cells by enhancing cytotoxicity and INFγ production in concert with IL-15 (Kasaian et al., 2002; Strengell et al., 2002). Interestingly IL-21R deficient mice show normal NK cell development and maturation, this can be explained by the lack of IL-21R expression in NK precursors. They are dependent on IL-15 signalling on the other hand, which induces IL-21R expression leading to accelerated NK cell maturation through IL-21 signals (Parrish-Novak et al., 2000; Sivori et al., 2003; Perez et al., 2006). The T cell subset of natural killer T cells expresses a TCR able to recognize lipid antigens presented by CD1d, therefore they can somewhat bridge the gap between innate and adaptive immunity. Resting NKT cells express the IL-21R and start producing IL-21 in response to anti-CD3 or alfa-galactosylceramide (alfa-GC). Additionally survival and proliferation of NKT cells was improved through IL-21 as well as granzyme B expression was increased, which suggests an autocrine role for IL-21 in those cells (Coquet et al., 2007). An association between decrease in NKT cell function and numbers and the progression of type-1 diabetes mellitus in NOD mice has also been documented (Falcone et al., 1999; Laloux et al., 2001)

2.4.5. IL-21 in autoimmune disease

The term “autoimmune disease” describes a range of conditions where the immune system wrongly identifies Ag expressed by the organisms own cells as foreign Ag, launching an immune response against it, thereby damaging his own cells and tissues. The mechanisms of central and peripheral tolerance are in place to keep T

and B cells with specificity for self-Ag at bay, but a multitude of components consisting of genetic and environmental factors can lead to the breakdown of self tolerance and thereby the onset of autoimmune disease (Forte et al., 2006; Zhou et al., 2007; Chen and O'Shea, 2008). In some autoimmune diseases the response is launched against a self-antigen only expressed in one organ, confining the damage to that specific organ, such as the beta-cells of the pancreas in type-1 diabetes (T1D) or the follicular cells of the thyroid in Graves disease (Green and Flavell, 1999; Prabhakar et al., 2003; Anderson and Bluestone, 2005). In other autoimmune diseases however the antigen is expressed throughout the body, thereby leading to a wide range of pathologies. Patients suffering from systemic lupus erythematosus (SLE) for example can suffer from damage to their skin, muscle, joints, heart, lungs, blood vessels and nervous system as a result of their disease (Crowson and Magro, 2001). Although only 3-8% of the population suffers from autoimmune diseases, the severity of these illnesses makes them an important target for further studies. The involvement of IL-21 has been implicated in an array of these diseases, for example in systemic lupus erythematosus (SLE), type-1 diabetes (T1D) as well as multiple sclerosis (MS) and ulcerative colitis (UC). A wide range of genetic and environmental factors contribute to SLE, genetic studies showed that polymorphisms in human IL-21 and IL-21R genes are associated with SLE (Sawalha et al., 2008; Webb et al., 2009). Studies also showed overexpression of IL-21 in BXSB-Yaa and sanroque mouse strains, both of whom express lupus-like phenotypes (Ozaki et al., 2004; Vinuesa et al., 2005). The blockage of IL-21 signals by IL-21R.Fc reduces disease progression in a lupus-prone mouse model, and IL-21R deficiency seems to completely protect from lupus (Herber et al., 2007; Bubier et al., 2009). Another example for an autoimmune disease linked to IL-21 is type-1 diabetes mellitus (T1D). This disease is caused by the autoimmune destruction of the insulin producing beta-cells in the pancreas, resulting in deregulated blood glucose levels. A commonly used mouse model for T1D is the non-obese diabetic (NOD) mouse, studies have shown overexpression of IL-21 in these mice, as well as their protection from disease onset when made deficient for IL-21R (King et al., 2004; Spolski et al., 2008; Sutherland et al., 2009). Rheumatoid arthritis (RA) is a humoral autoimmune disease affecting many tissues and organs but most prominently the joints. Similarly to T1D linkage of IL-21 to RA has been shown by

genetic association studies, connecting SNPs in the IL-21R containing region to RA (Zhernakova et al., 2007). Overexpression of IL-21 and its receptor has been shown in RA patients and studies examining the therapeutic value of IL-21 modulation have been initiated (Jüngel et al., 2004; Li et al., 2006). Biopsies from patients suffering from ulcerative colitis (UC) or crohn's disease (CD) have been shown to have increased IL-21 transcript levels in inflamed but not unaffected gut mucosa (Fina et al., 2008a, 2008b). Also the *Il2* and *Il21* containing locus Idd3 has been associated with both UC and DC by genome wide studies conducted by Festen et.al. (Festen et al., 2009). In multiple sclerosis (MS) the immune system attacks the myelin coating around nerve cells, thereby resulting in disruption of nerve signals, leading to loss of cognitive and physical function. Mouse models, such as experimental autoimmune encephalomyelitis (EAE), are used to study this autoimmune disease of the central nervous system. Studies of these mouse models support the role of IL-21 predominantly on the onset of the disease process. Whereas the administration of IL-21 before the induction of disease increases severity, there seems to be no effect after the disease has been initiated (Vollmer et al., 2005). Th17 cells play a critical role in EAE and recent studies have linked IL-21 to the induction and expansion of the Th17 population, leading to increased severity of EAE (Nurieva et al., 2007; Chen and O'Shea, 2008). With the demonstrated role of IL-21 in a broad variety of autoimmune diseases, this cytokine becomes an interesting candidate for immunosuppressive therapy. The blockage of IL-21 signalling seems to ameliorate diseases such as T1D or lupus in mouse models and thereby demonstrates a beneficial role for therapeutic inhibition of this cytokine (Herber et al., 2007; Spolski et al., 2008; Bubier et al., 2009; Sutherland et al., 2009). On the other hand one shouldn't underestimate the role of IL-21 in GC formation and high-affinity antibody production as well as control of chronic viral infections through $CD8^+$ T cells. Further studies need to be done to separate the beneficial effects of IL-21 during immunization and infection from its harmful role in autoimmune disease, to enlighten possible therapeutic effects of this cytokine.

2.5. The aim of this study

In this study we tried to elucidate the role of IL-21 on early $CD4^+$ T cell differentiation and survival with the help of a method which allowed us to analyse Ag-specific T helper cells. We followed an Ag-specific T helper response in *Il21r-/-* and WT mice to shed further light on the possibly heterogeneous nature of the CXCR5high/PD1high T helper subset, which is commonly referred to as T follicular helper cells. In a secondary approach we tried to clarify the role of IL-21 for the survival of the Tfh subset at later stages in the germinal center and the function of this cytokine during the GC reaction. Additionally we wanted to expand on existing observations made in our lab (data not shown) concerning the profound effect of IL-21 on $CD8^+$ T cells and their surprising suppressive role on T and B cells during the GC response.

3. Materials and methods

3.1. Buffers and reagents

Table 3-1) Buffers and reagents used

Buffer/Solution	Components	Suppliers
DNA isolation buffer	670mM Tris pH8.8 166mM Ammonium sulfate 65mM Magnesium chloride 10% b-mercaptoethanol 5% Triton X-100 100ug/ml Proteinase K	Gibco Amersham Amersham Gibco Sigma Promega
DNAseI	-	Sigma-Aldrich
EDCI	N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide	Sigma
ELISA buffer	1% BSA 0.1% Tween 20 1X PBS	Gibco Sigma Gibco
Fc-blocking solution	1x RPMI 0.5ng/ml Mouse BD Fc Block 2% mouse serum 2% rat serum	Gibco BD Pharmingen
FACS buffer	0.1% NaN_3 0.5% BSA 1X PBS	Amersham Gibco Gibco
Imject alum	40mg/ml aluminium hydroxide 40mg/ml magnesium hydroxide in H_20	Pierce
Low-tox guinea pig complement	Lyophilized, reconstituted in 1ml H_2O	Cedar Laboratories
Lymphocyte isolation media	1x RPMI 10% BCS	Gibco Gibco
MACS buffer	2mM EDTA 3% BCS 1X PBS	Gibco Gibco Gibco
MACS running buffer	2mM EDTA 0.5% BSA 1X PBS	Gibco Gibco Gibco
MACS rinsing buffer	2mM EDTA 1X PBS	Gibco Gibco
PBS (10X)	3.6% Di-sodium hydrogen orthophosphate (Na_2HPO_4) 0.2% KCl 0.24% KH_2PO_4 8% NaCl	Merck Ajax Finechem Merck Ajax Finechem Merck
RBC lysis solution	8.26g NH_4Cl 1g $KHCO_3$ 0.037g EDTA 1L dH20	Merck Merck Gibco
SRBC	Sheep red blood cells in Alsever's solution	IMVS Adelaide
SRBC-conjugation buffer	0.35M Mannitol 0.01M NaCl	Sigma Merck

3.2. Mice

Il21r-/- mice were kindly provided by Dr Mark Smyth (Peter Mac Callum Cancer Centre, Melbourne) at B6 N6 and backcrossed to N7 for experimental use. Thy1.1^{+} OTII and *Il21r-/-* Thy1.1^{+} OTII mice were bred in house. C57BL/6 and Ly5.1 congenic mice were purchased from the Animal Research Centre in Perth, Australia. SW_{HEL} and *Il21r-/-*, SW_{HEL} mice were kindly provided by Dr Robert Brink (Garvan Institute of Medical Research, Sydney). CXCR5tg mice were kindly provided by Dr Jason Cyster (UCSF) and crossed onto CD4^{+} T cell TCR tg OTII mice in house to generate CXCR5tg OTII mice, including *Il21r-/-* CXCR5tg OTII Th1.1^{+} mice. Animals were housed under specific pathogen-free conditions and handled in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Age matched littermate mice used for experimental purposes were between 7 and 14 weeks of age.

3.3. Flow cytometry

Single cell suspensions of lymph nodes (LN) were obtained by mincing tissue between two frosted slides, splenocytes were teased out of the spleen by working the tissue through a cell strainer with the bung of a 1ml syringe. Cell suspensions of spleen and LN were filtered through 70μm cell strainers in lymphocyte isolation buffer. Red blood cells were removed from spleens using 1ml RBC lysis buffer for 1 minute and 30 seconds on ice before washing in Lymphocyte isolation buffer. 50μl of a single cell suspension at a concentration of $2x10^{7}$ cells/ml from spleen and lymph nodes were stained in FACS buffer containing pre-titred antibodies in 96 well V-bottomed microtitre plates (Nunc, Roskilde, Denmark) at concentrations shown in table 3-2. Cells were acquired using Canto cytometer (BD Biosciences, CA) and analyzed using Flowjo (Treestar, CA). Doublets were excluded by forward scatter height and width.

Table 3-2) Antibody clones and concentrations

Antibody/Reagent	Clone	Label	Dilution
Armenian Hamster IgG1 Isotype control	-	PE	1:200
2W1S-tetramer	-	PE	1:50 (10nM)
B220	RA3-6B2	PE	1:200
		PE-Cy5	1:200
		PE-Cy7	1:200
		PerCP	1:200
		APC	1:200
		APC-Cy7	1:200
		PB	1:200
		biotin	1:200
CD3	145-2C11	PE-Cy7	1:200
	500A2	PB	1:200
CD4	RM4-5	PerCP	1:200
		APC-Cy7	1:200
		PB	1:300
	GK1.5	FITC (IHC)	1:200
		biotin	1:100
CD8	53-6.7	FITC	1:200
		PerCP-C5.5	1:200
		APC	1:200
		PO	1:50
		biotin	1:200
CD11b	M1/70	biotin	1:400
		PB	1:200
CD11c	N418	PB	1:200
CD19	Ebio103	PB	1:200
CD21	7G6	FITC	1:200
CD38	281-2	FITC	1:200
		APC	1:200
	90	PE-Cy5	1:200
CD44	IM7	APC	1:200
		APC-Cy7	1:200
CD45.1	A20	PE-Cy7	1:200
		biotin (IHC)	1:200
CD45.2	104	PerCP	1:200
		PE-Cy7	1:200
		APC-Cy7	1:200
CD69	H1.2F3	PE-Cy7	1:200
CXCR5	2G8	biotin	1:100
Fas	Jo2	FITC	1:200
		biotin	1:100
GL7	GL7	FITC	1:200
HyHEL9	-	Alexa647	1:200

ICOS	C398-47	PE FITC	1:1000 1:300
IgD	11-26c.2a	FITC Alexa647 (IHC) purified	1:200 1:200 1:100
IgG1	A85-1	PE biotin	1:200 1:200
IgM	II/41 R6-60.2	PE-Cy5.5 biotin	1:200 1:100
PD-1	J43	FITC PE	1:200 1:200
PNA	SIGMA	biotin	1:400
Streptavidin	-	PE PerCP-C5.5 APC PB Cy3 (IHC)	1:400 1:500 1:800 1:200 1:400
Syndecan-1	281-2	PE APC	1:200 1:200
Thy1.1	HIS51	PeCy7 Alexa750 biotin	1:300 1:500 1:100

3.4. 2W1S-peptide immunizations

Mice were immunized i.p. with either 50µg 2W1S-peptide (EAWGALANWAVDSA) ordered from Genescript or 100µg 2W1S-peptide conjugated to HEL (CGGEAWGALANWAVDSA) absorbed to alum, subsequently spleen and lymph nodes were analyzed at various timepoints.

3.5. Crosslinking 2W1S-peptide to HEL

Crosslinking of the 2W1S peptide to hen egg lysozyme (HEL) by incubating 1ml HEL [2mg/ml] (Sigma) with 14µl SMPH [100mM] (Thermo Scientific), which represents a 10x molar excess of SMPH, for 45min at RT on a rocking table. Dialysis is carried out over night against 500ml PBS buffer at 4°C to remove the excess SMPH from the solution. The SMPH-HEL [2mg/ml] is incubated with 5x molar excess of the CGG-2W1S peptide [5mM] (CGGEAWGALANWAVDSA), for 4hrs at RT on a shaker. Again the solution is dialyzed over night against 500ml PBS buffer at

4°C to get rid of excess peptide. The coupling efficiency is determined via SDS-PAGE and Coomassie blue staining, aliquots are stored at -80°C.

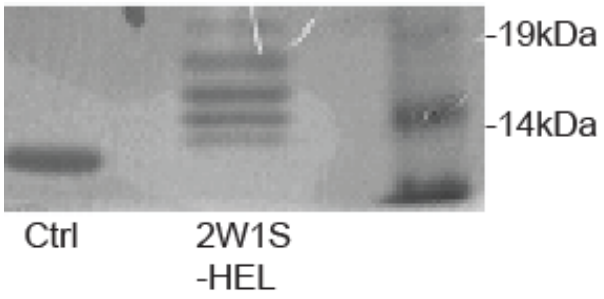

Figure 3-1) Coupling efficiency of 2W1S-HEL
To prove the successful coupling of the 2W1S-peptide to the HEL-protein 5µg sample (2W1S-HEL) was loaded on a polyacrylamide gel next to 5µg of unconjugated HEL as a negative control (Ctrl). After gel electrophoresis the gel was stained with Coomassie blue to detect separated protein bands. As can be seen multiple bands were detected in the sample, indicating varying numbers of peptide bound per HEL molecule. As no leftover unbound HEL can be seen in the sample, the overall coupling efficiency of the 2W1S-HEL conjugation was more than satisfactory.

3.6. Staining with pMHC class II tetramer

Because of the unique nature of the pMHCII tetramer (provided by Mark Jenkins) a special staining and enrichment protocol devised by the Jenkins lab (Moon et al., 2009) had to be followed. Spleen and lymph nodes (inguinal, axillary, brachial, submandibular, and mesenteric) were harvested and homogenized using 70µm cell strainers in FACS buffer. RBCs were removed from spleens using 2ml RBC lysis buffer for 1min 30sec on ice before washing in FACS buffer. After spinning down the buffer was aspirated and replaced with Fc blocking solution (1:200 Mouse BD Fc Block, 2% mouse serum, 2% rat serum), tetramer was added to a concentration of 10nM and incubated at RT for 1hr in the dark. Again the cells were washed in FACS buffer and the pellet was resuspended in a small amount (200µl) of FACS buffer and 50µl of Miltenyi anti-PE microbeads, next the solution was incubated for 30min on ice in the dark. The solution was washed in FACS buffer again and filtered through a 70µm cell strainer before it was applied to a Miltenyi LS column on a MidiMACS magnet. Both the unbound and the bound fractions were collected and washed in FACS buffer. The pellet from the bound fraction was resuspended in 100µl, the pellet from the unbound fraction in 2ml Fc blocking solution and cell

counting was performed for both fractions. From here on staining for Ab of interest for Flow cytometry was carried out on ice as described before.

3.7. $CD4^+$ T cell enrichment through MACS

Homogenized cells from spleens and lymph nodes were taken and enriched for $CD4^+$ T cells via negative selection using the Milentenyi $CD4^+$ T cell Isolation Kit II, in this enrichment protocol cells not labeled with the antibody that coats the magnetic beads do not bind to the column and thus get excluded. About $5x10^7$ cells were resuspended in 40µl FACS buffer and incubated with 2µg of each anti CD8-biotin Ab, anti B220-biotin Ab and anti CD11b-biotin Ab at 4°C whilst shaking for 30min. The cells were washed in MACS buffer twice, dead cells were removed before 15µl of anti-biotin microbeads were added and the solution was incubated again at 4°C whilst shaking for 30min. The cells were washed twice in MACS buffer again and resuspended in 1ml of the same buffer, and separated using the autoMACS Pro Separator (Miltenyi Biotec). Both the bound and unbound fractions were collected and staining for Ab of interest for flow cytometric analysis was carried out like described before.

3.8. $CD4^+$ T cell enrichment through complement depletion

Homogenized cells from lymph nodes and spleens were taken and enriched for $CD4^+$ T cells via complement depletion of B cells. Red blood cells were depleted beforehand from the splenocyte/lymphocyte mixture and dead cells were removed before the cells were incubated with an anti-B220 Ab (RA3-6B2) targeting B cells for 30min on ice. The cells were washed with lymphocyte isolation media and resuspended therein at $5x10^7$ cells/ml. One tenth of Low-Tox Guinea Pig Complement (Cedarlane Laboratories) and 10µg/ml DNAseI (Sigma-Aldrich) was added to the cells and the mixture was incubated for 40min at 37°C in a water bath. To remove the complement the cells were thereafter washed twice with lymphocyte isolation media and resuspended in the same media for immunostaining.

3.9. CXCR5tg adoptive transfer studies

For CXCR5tg transfer experiments 1.5 – 3 x 10^6 CXCR5tg/*Il21r-/-* $CD4^+$ T cells or 1.5 – 3 x 10^6 *Il21r-/-* $CD4^+$ T cells as a control were injected i.v. into CD45.1 congenic mice which were at the same time immunized i.v. with 2x10^8 SRBC. On the day of FACS analysis (either 7 or 14 days after transfer/immunization) spleens were harvested and 5 x 10^6 cells were stained to detect and analyze donor Tfh cells, 1 x 10^6 cells were stained to analyze host germinal centre cells.

3.10. SW_{HEL} adoptive transfer studies

Three days before as well as on the day prior adoptive transfer CD45.1 congenic mice were injected i.p. with 100-500µg anti-CD8 antibody (YTS 169.4 rat IgG2b) for *in vivo* depletion of $CD8^+$ T cells. On the day of transfer a mixture of splenocytes from CD45.2/ SW_{HEL} mice either *Il21r-/-* or WT containing 3 x 10^4 HEL-binding B cells were injected i.v. into recipient mice (CD45.1 congenic mice) together with 30µg HEL chemically conjugated to the OVA-peptide (CGGISQAVHAAHAEINEAGR) and the mice were immunized with 2 x 10^8 SRBC-HEL i.v. at the same time. FACS analysis was carried out 5 and 10 days after transfer/immunization, spleens were harvested and 5 x 10^6 cells were stained to detect surviving HEL-binding donor cells.

3.11. SRBC to HEL conjugation

Approximately 8-10 x 10^9 SRBC were washed 3 times with PBS and once with SRBC-conjugation buffer (0.35M Mannitol, 0.01M NaCl) and resuspended in 0.8ml conjugation buffer, HEL was added to a final concentration of 10µg/ml and the mix was rocked on ice for 10min. EDCI was added to the mixture to a final concentration of 10mg/ml and again rocked on ice for 30min. After 4 washes with PBS the cells were counted and the solution was diluted to a concentration of 1 x 10^9 cells/ml. The efficiency of the conjugation was assessed by comparing the SRBC-HEL conjugated cells to mock-conjugated SRBC via FACS analysis.

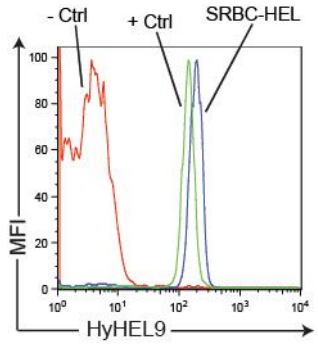

Figure 3-2) Coupling efficiency of SRBC-HEL
Shown in this figure is the successful coupling of sheep red blood cells (SRBC) to hen egg lysozyme (HEL). The leftmost curve shows the negative control (- Ctrl) which consisted of SRBC with no HEL protein bound, the curve tagged "+ Ctrl" shows the positive control, a successful SRBC-HEL conjugation from a previous experiment. The curve tagged "SRBC-HEL" shows our SRBC-HEL conjugation which was a success as well.

3.12. Genotyping PCR

2mm of mouse tail was incubated overnight at 65°C in 200μl DNA isolation buffer with 1.13μl of Proteinase K (10mg/ml, Promega) and 0.5μl of the resulting digest was used as a template in PCR reactions. All primers and probes used for PCR are listed in table 3-3 from 5' to the 3' end. Mice were screened for presence/absence of genes and transgenes by PCR analysis. PCR was conducted using 0.5μl of DNA template, 0.24μM forward and reverse primer (Sigma-Aldrich), 50μM each dNTP (Promega, Madison, WI, USA), 0.625 U Taq polymerase (Promega) and 1X Green GoTaq Reaction Buffer (Promega) in a 25μl reaction volume. All reactions were performed on an iCycler Thermal Cycler (Biorad, CA, USA). PCR products were visualized by electrophoresis on a 1-2% agarose (Sigma) gel containing 10μg/ml ethidium bromide (Sigma-Aldrich). CXCR5tg mice were genotyped via FACS analysis.

Table 3-3) Primer sequences

Primer	Sequence 5' to 3'	Annealing T
IL-21R F neo	ATCGCCTTCTATCGCCTTCTTGACG	60
IL-21R F WT	GACTCTTGGCCTGCAGTTCTGACG	60
IL-21R R common	CCAAAGAGCTCCAGTAAACAG	60
OTII F	GCTGCTGCACAGACCTACT	59
OTII R	CAGCTCACCTAACACGAGGA	59
THY1.1 F	GTGCTCTCAGGCACCCTC	65
THY1.1 R	CCGCCACACTTGACCAGT	65
THY1.2 F	GCGACTACTTTTGTGAGAGCTTCA	65
THY1.2 R	CGCCACTTGACCAGC	65

3.13. Data analysis and statistics

Data were analyzed using Prism software (Graphpad software, CA) to calculate un-paired, two-way Student's T test, with an F test to compare variances.

4. Results

4.1. Analysis of Ag-specific T helper cell populations

4.1.1. Visualizing Ag-specific T helper cells using a MHC class II tetramer

In the past, most *in vivo* studies of antigen-specific T helper cells relied on adoptively transferring large populations of monoclonal TCR transgenic T cells into histocompatible hosts, which could be identified via congenic markers such as Thy1 (Pape et al., 1997; Jenkins et al., 2001). Although this technique has proven immensely useful in the study of the immune system, the effects of the large quantity of uniform cells transferred on the *in vivo* immune response are still largely unknown (Jenkins et al., 2001). The study of Ag-specific $CD8^+$ T cell populations with the help of fluorochrome-labelled MHC class I tetramers has been highly successful (Altman et al., 1996; Busch et al., 1998; Murali-Krishna et al., 1998). By contrast, the manufacture of MHC class II tetramers for the study of Ag-specific $CD4^+$ T cell populations has proven difficult in the past because of the need for a covalent link between peptide and MHC molecule (Kozono et al., 1994; Jenkins et al., 2001). Marc Jenkins and his group successfully developed a method to manufacture a fluorochrome-labelled MHC class II tetramer with a peptide antigen covalently bound and presented by each of the four MHCII molecules of the tetramer (Moon et al., 2009) (Figure 4-1). With a magnetic bead based enrichment protocol, and MHC class II tetramers selected based upon a detectable precursor population in naïve mice, FACS analysis of rare, Ag-specific T helper cell populations in unmanipulated mice has now been made possible (Moon et al., 2007). We used the MHC class II tetramer bound to the 2W1S-variant (Rees et al., 1999) of peptide 52-68 from the I-E alpha chain (Rudensky AYu et al., 1991) in our studies, because out of the three peptide-antigens tested by Moon et.al. this one seemed to have the largest Ag-specific T helper cell population in the C57BL/6 mouse strain.

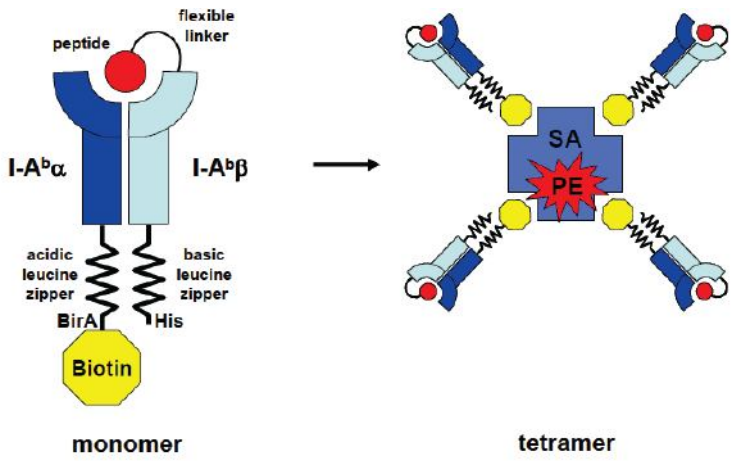

Figure 4-1) Layout of a pMHC class II tetramer
On the left side a single MHC class II molecule can be observed, the α-chain of the MHC molecule is linked to a biotin molecule, whereas the β-chain is covalently bound to the peptide of interest via a flexible linker which holds the peptide in the binding groove. Both chains bind to each other via a leucine zipper motif. On the right side four pMHC class II molecules can be seen binding to a Phycoerythrin-labelled Streptavidin-core, thereby forming a pMHCII tetramer (copyright of the illustration Mark Jenkins).

4.1.2. Early results reveal technical difficulties

We decided that looking at activated Ag-specific T follicular helper cell populations in wild-type C57BL/6 (WT) mice and mice deficient in the IL21-receptor (*Il21r-/-*) with 2W1S:I-A^b tetramer (provided by Marc Jenkins) would help us to further characterize the role of IL-21 on this $CD4^+$ T cell subset and its function in GC formation and IgG1 class switching. To this end, we immunized C57BL/6 mice as well as *Il21r-/-* mice with 50μg of the 2W1S-peptide adsorbed to alum as an adjuvant and after 7 days (height of the immune response as shown by Moon et.al. 2007) we harvested the spleen as well as the lymph nodes of the animals for FACS analysis. Analysis of the approximately $5x10^7$ obtained cells was not feasible because the accumulation of low-frequency background events would obscure our rare population of interest. For this reason, the cells were stained with phycoerythrin-labelled pMHCII tetramer specific for 2W1S-peptide binding cells, after a magnetic bead based enrichment step specific for PE-positive cells, about $5x10^5$ cells were obtained. These cells were analysed via FACS for T cell markers such as CD4, CD3 and CD8, as well as the T cell activation marker CD44, which is a receptor for hyaluronic acid, and surface molecules expressed by Tfh cells such as the chemokine receptor type 5 (*CXCR5*) and programmed cell death-1 molecule

(PD-1). Surface markers not expressed on T cells, such as B220, CD11b and CD11c, were also included in our analysis to eliminate cells that were not of interest to us. Despite our meticulous following of the protocol provided by the Jenkins group we didn't seem to be able to replicate the distinct Ag-specific population they showed in their FACS plots (Moon et al., 2009). Although we did see a difference in the tetramer specific population comparing immunized to unimmunized mice in both WT and *Il21r-/-* mice (data not shown) and there was also a clear difference comparing tetramer stained $CD4^+$ T cells and $CD8^+$ T cells, which served as an internal control, we could only observe a slight population shift in FACS plots indicating the presence of tetramer positive cells and not a distinct Ag-specific population (Figure 4-2A).

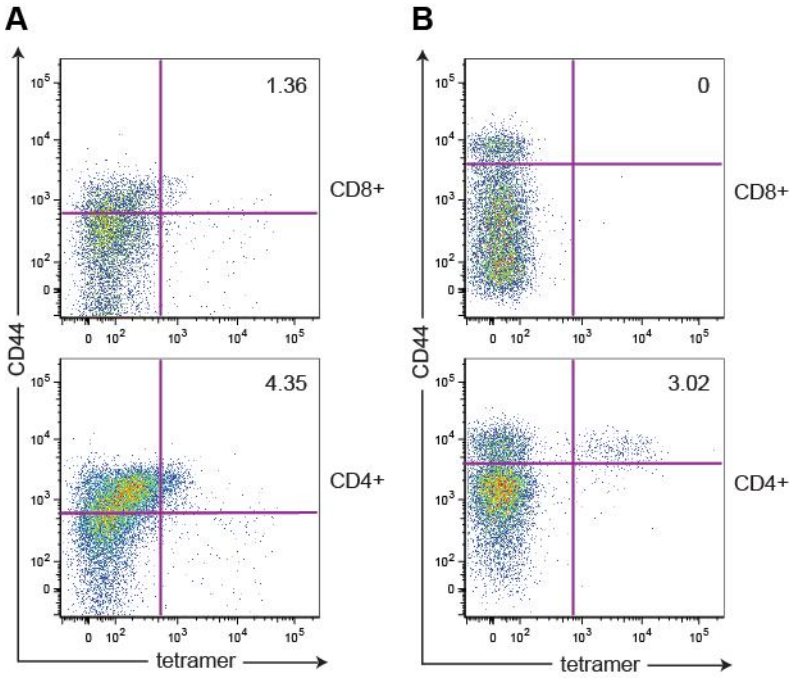

Figure 4-2) Comparison of old and new tetramer staining to negative control
Representative dot plots comparing pMHCII tetramer staining of activated, Ag-specific T helper cells gated on total $CD4^+$ cells (lower plots) to pMHCII tetramer staining of total $CD8^+$ T cells (upper plots) acting as the negative control. All cells were taken from C57BL/6 animals 7 days after 2W1S-peptide immunization. In A) initial staining experiments with an older tetramer batch can be seen, whereas in B) later staining with a fresh batch is shown. Distinct differences in staining intensity and background events can be observed.

Due to the background events, which can be observed in the negative control, the analysis of these experiments was limited to Ag-specific T helper cells, as the noise could be subtracted at this level, but hampered the further analyses of T helper cell

subsets. We tried to ameliorate our immunization protocol by crosslinking our peptide of interest to hen egg lysozyme (HEL), because through the close proximity of HEL-recognizing B cells to our peptide-activated T helper cells and the resulting interaction, a stronger immune response should be formed. Unfortunately neither this, nor the increase of the antigen-dose to 100µg 2W1S-HEL delivered satisfying results. After that we hypothesized that the problem might concern our pMHCII tetramer and we started a new series of experiments after receiving a fresh batch from Marc Jenkins lab. In further experiments we had no trouble detecting a clear, distinct 2W1S-specific CD4+ T cell population (Figure 4-2B), which brought us to the conclusion that the previous tetramer lost much of its conjugated fluorochrome during its storage at 4°C and therefore was not able to stain brightly enough to reveal a distinct population of antigen specific CD4+ T cells.

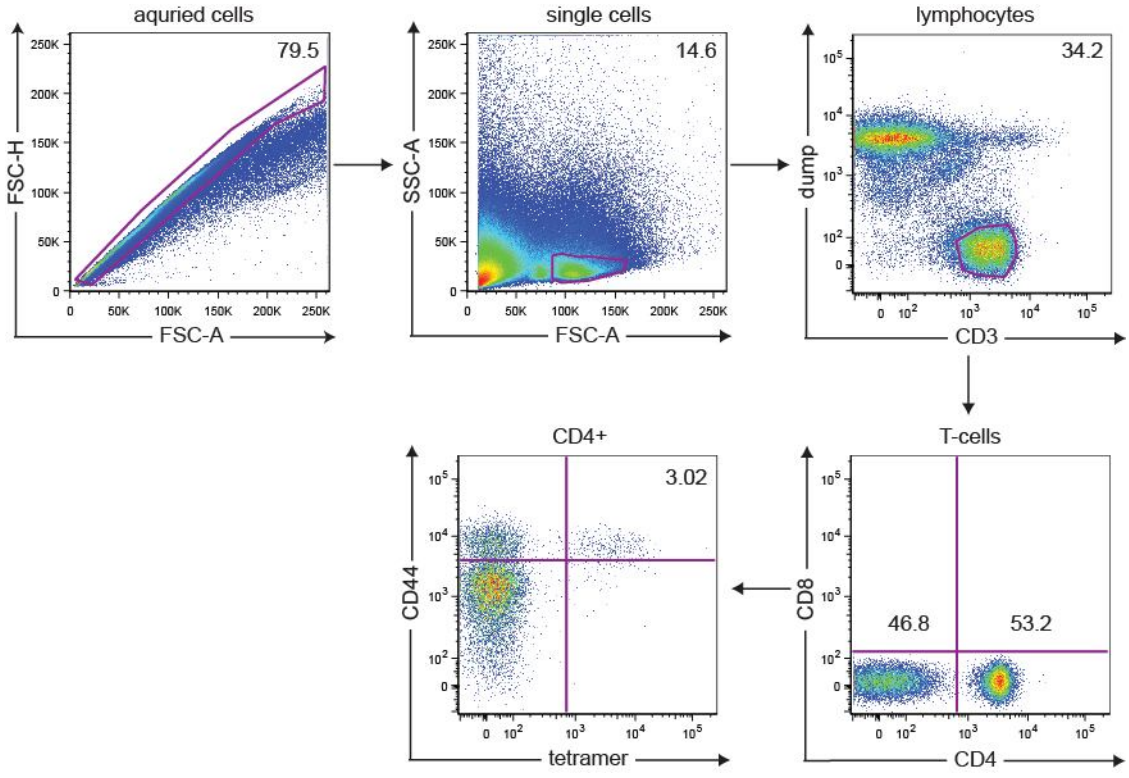

Figure 4-3) Gating strategy of the pMHCII tetramer staining
Shown here are representative dot-plots visualizing the gating strategy applied in all our experiments to obtain our activated, 2W1S-peptide specific T helper cell population of interest. Cells were taken from C57BL/6 animals 7 days after 2W1S-peptide immunization. After gating on single cells, a tight lymphocyte gate was drawn to exclude undesired events. Next CD3+ T cells were separated from dump (B220, CD11b, CD11c) and afterwards CD4+ T helper cells were selected. Finally activated (CD44+) antigen-specific (tetramer+) cells could be shown.

4.1.3. The effect of IL21R-deficiency on T helper cells

As we now could visualize a distinct, activated, Ag-specific $CD4^+$ T cell population with virtually no background events, we could finally start to have a more in-depth look at the effect of IL-21 on different Ag-activated T helper cell subpopulations. The experimental setup remained the same with respect to immunisation as well as tetramer and FACS staining, but we decided to use a complement-based enrichment protocol, as described in the methods section, instead of MACS from now on. As we didn't specifically enrich for pMHCII tetramer positive cells with this step anymore, but got rid of all cells, which were not $CD4^+$ via complement depletion, we got a slightly higher number of cells (about $2x10^7$) after enrichment. In our first analysis of this experiment we focused on overall activated ($CD44^+$), Ag-specific T helper cells from both WT and *Il21r-/-* mice immunized with 100µg 2W1S-HEL and alum, the exact gating strategy can be seen in Figure 4-3. Seven days after immunization, the effect of the deficiency of IL21-signalling in these cells was apparent in the *Il21r-/-* mice as shown in the representative dot-plot of Figure 4-4A. In WT mice, 3.02% of T helper cells were activated Ag-specific cells, whereas only 1.94% $CD44^+$ Ag-specific cells could be detected in *Il21r-/-* mice. Unimmunized mice of the same age and gender were used as controls for both genotypes. By grouping the experimental data from separate experiments into one graph, we found that the experiments conducted with both old and new tetramer batch were quite consistent and revealed a decreased population of activated/Ag-specific T helper cells in *Il21r-/-* mice compared with WT mice, p=0.0209 (Figure 4-4B).

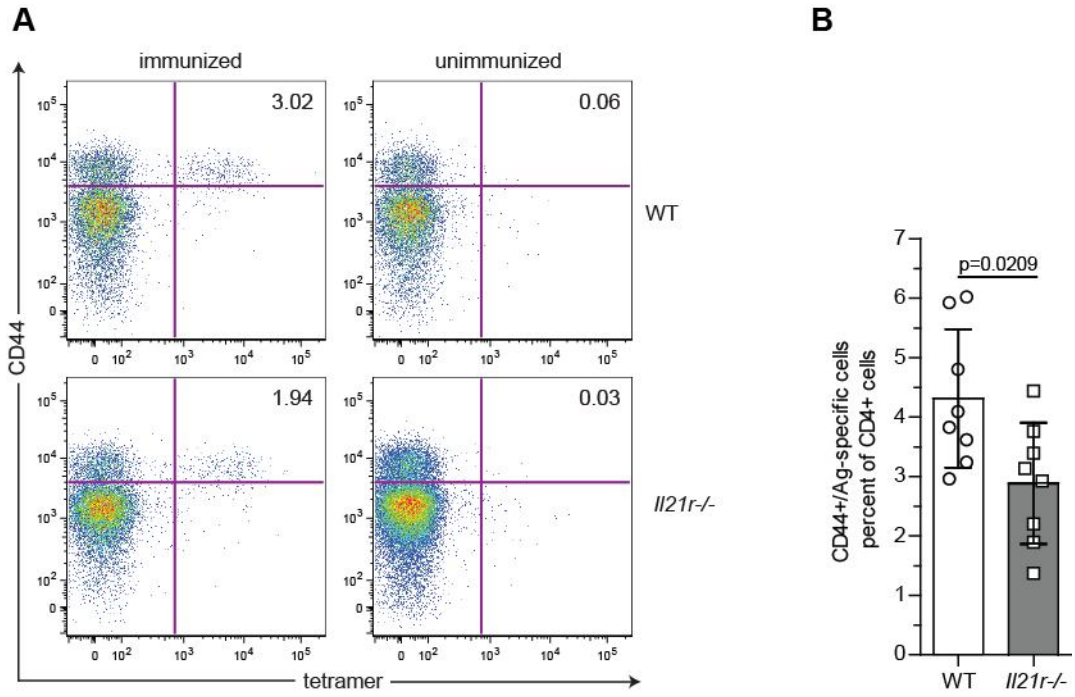

Figure 4-4) Effect of IL-21 on activated, Ag-specific T helper cells
A) Representative dot plots from flow cytometric analysis of activated, Ag-specific T helper cells gated on total $CD4^+$ T cells. Shown are cells from mice 7 days after immunisation as well as cells from unimmunized mice as a control. B) Activated, Ag-specific cells as a percent of total T helper cells are shown here from the same mice as in A), the background was subtracted before plotting. Data is shown as values from individual mice and mean +/- SD, and is derived from 4 experiments with both old and new 2W1S-tetramer where n=8.

4.1.4. The effect of IL21R-deficiency on T follicular helper cells

Seeing the negative effect of the absence of IL-21:IL21R-signalling on overall, antigen specific T helper cells, we next determined whether we could detect a similar influence of IL-21R on the antigen specific T follicular helper (Tfh) cell population. Tfh cells were defined as CXCR5high/PD1high $CD4^+$ T helper cells and although we did see a trend of a decreased CXCR5high/PD1high population in *Il21r-/-* mice (19.2% of activated Ag-specific cells in WT to 16.6% in *Il21r-/-* mice), the observed trend did not reach statistical significance (Figure 4-5). Our data largely points to an overall effect of IL-21 on activated, Ag-specific T helper cells rather than an exclusive effect on Tfh cells (Figure 4-5). However, further experiments to increase sample size may help to clarify this issue.

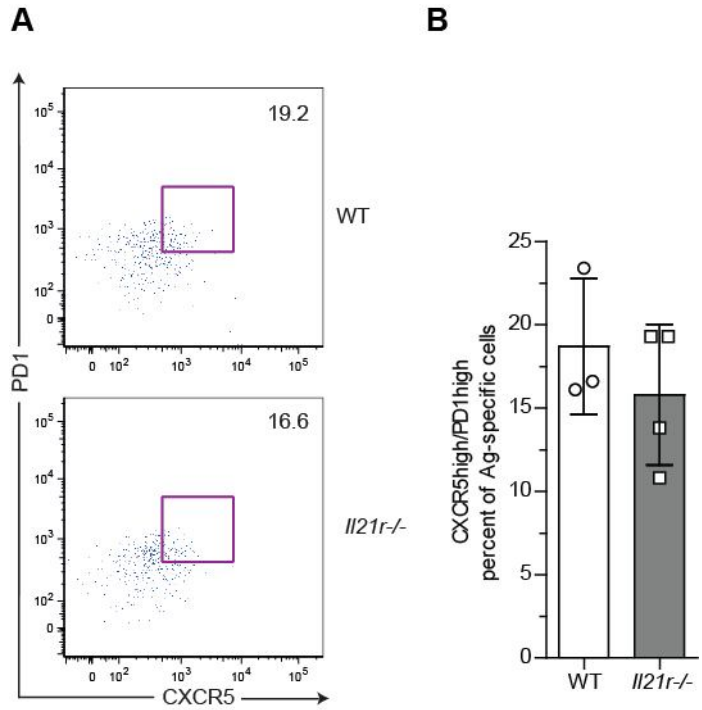

Figure 4-5) Effect of IL-21 on Ag-specific T follicular helper cells
Representative dot plots showing CXCR5high/PD1high cells, gated on Ag-specific cells from WT and *Il21r-/-* mice 7 days after 2W1S-peptide immunization (A). Shown are CXCR5high/PD1high cells from the gate seen in A) as percent of overall activated, Ag-specific cells. Only values from experiments with the new tetramer batch are shown. Data is shown as the mean +/- SD and individual values from 2 experiments where n=3-4 (B).

During our analyses on the effect of the absence of IL-21:IL21R-signalling on Ag-specific Tfh cells we realized that by far not all of the CXCR5high/PD1high T helper cells we observed in our experiments were stained with the pMHCII tetramer and therefore couldn't be considered Ag-specific (Figure 4-6). Although there didn't seem to be a difference between WT and *Il21r-/-* mice and the variability was rather high, our data does suggest that only a highly variable 60 to 90 percent of CXCR5high/PD1high cells generally referred to as T follicular helper cells were actually Ag-specific. This is an important finding since the retained high expression of the surface markers CXCR5 and PD-1 (in addition to the expression of ICOS and IL-21) are surface molecules that are currently used to characterise the Tfh subset (Chtanova et al., 2004; King et al., 2008). Furthermore, the observed heterogeneity of this subset might explain the somewhat contradicting findings in respect to the importance of IL-21 in Tfh cells (Nurieva et al., 2008; Vogelzang et al., 2008; Bessa et al., 2010; Zotos et al., 2010).

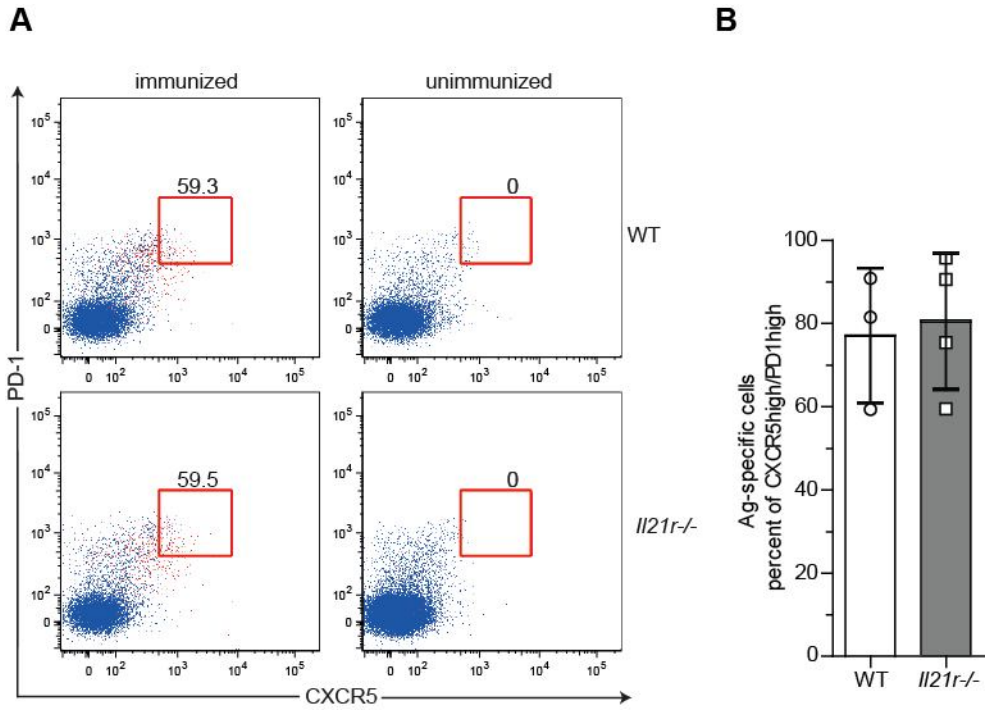

Figure 4-6) The Heterogeneity of the CXCR5high/PD1high T helper cell population
In A) overlaps of overall T helper cells (blue) and activated, Ag-specific T helper cells (red) are shown. The gate shows the CXCR5high/PD1high subpopulation, whereas the values indicate the percentage of Ag-specific cells in this gate. The cells were taken from mice 7 days after 2W1S-peptide immunisation or unimmunized controls. In B) Ag-specific cells as percent of CXCR5high/PD1high cells from the gate seen in A) are shown. Data is shown as the mean +/- SD and individual values from 2 experiments where n=3-4.

4.2. The impact of forced CXCR5 expression on *Il21r-/-* T helper cells

4.2.1. The function of CXCR5 in Tfh cells

The chemokine receptor CXCR5, which is used as a marker for T follicular helper cells, is not limited to these cells, but is expressed on all activated $CD4^+$ T cells, as well as on mature B cells (Förster et al., 1996; Ansel et al., 1999). Most $CD4^+$ T cell subsets however only transiently express CXCR5 (Kim et al., 2004; Hardtke et al., 2005), whereas Tfh cells maintain its expression at a high level longer than 2-3 days after activation (Hardtke et al., 2005; Haynes et al., 2007; King et al., 2008). T follicular helper cells are therefore often identified as the highest CXCR5 expressing cells, together with a high expression of other Tfh markers such as inducible T cell co-stimulator (ICOS) or PD-1 (King et al., 2008). Much like B cells, T follicular helper cells are guided into the B cell follicle of the secondary lymphoid organs (spleen, LN, PP), and subsequently into the germinal centre by surface expressed CXCR5 along a gradient of its ligand CXCL13 secreted by follicular

dendritic cells (Hardtke et al., 2005; Haynes et al., 2007). Although CXCR5 has been shown to be necessary for Tfh entry into the B cell follicle, its forced expression on naive CD4⁺ T cells wasn't sufficient to drive them into the follicle. The simultaneous downregulation of CCR7 and upregulation of CXCR5 was necessary for successful homing of CD4⁺ T cells in the B cell follicle (Haynes et al., 2007). In mice that lacked CXCR5 expression on T cells the GC response was noticeably reduced a phenotype very similar as in mice lacking IL-21:IL21R signalling (Haynes et al., 2007; Vogelzang et al., 2008).

We therefore hypothesized that the reduced expression of CXCR5 expression on *Il21r-/-* T cells (Vogelzang et al., 2008) could be the underlying defect in IL21-deficient mice. To test this hypothesis, we decided to transfer CXCR5tg *Il21r-/-* as well as WT *Il21r-/-* T cells into IL21R-deficient recipient mice. By enforcing expression of CXCR5 on *Il21r-/-* T cells, we argued that the IL21R-deficient T cells might have better access to the GC and thus better access to redundant growth factors such as IL-6. At the time of donor cell transfer we also immunized the host mice with the polyvalent antigen sheep red blood cells to make analysis of the GC-reaction as well as survival studies for the activated Tfh cells possible.

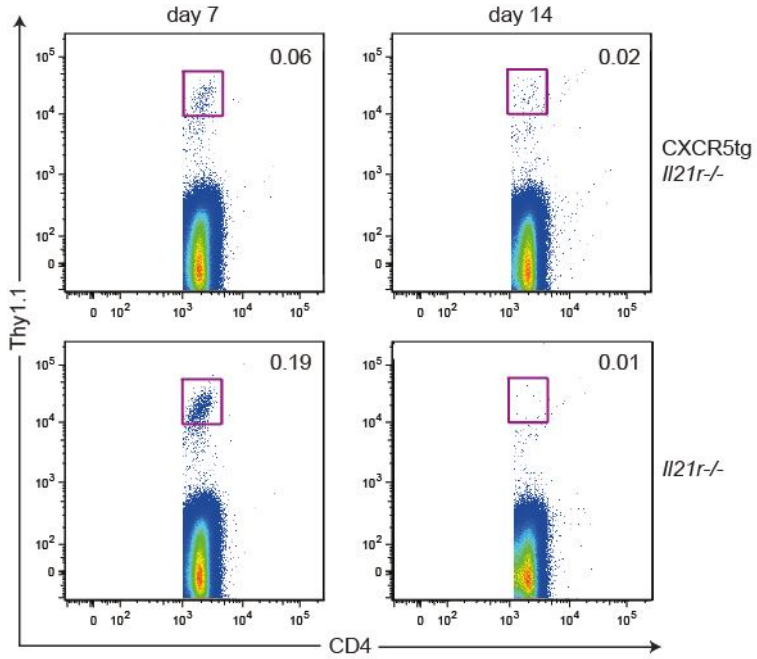

Figure 4-7) Survival of donor *Il21r-/-* T helper cells
Shown here are *Il21r-/-* cells either WT or CXCR5tg gated on total CD4⁺ T cells, these cells were transferred into *Il21r-/-* host mice and analysed 7 or 14 days after injection to evaluate their activation and survival. Looking at the total numbers of donor CD4⁺ T cells identified via the congenic marker THY1.1 the survival of these cells can be tracked over time (n=1).

4.2.2. In vivo survival of IL21-deficient CXCR5tg T helper cells

To get a better understanding on the effect of IL-21R-deficiency on the survival of $CD4^+$ T helper cells *in vivo*, we wanted to determine the influence of forced expression of CXCR5 on these cells. To this end we transferred $3x10^6$ *Il21r-/-* $CD4^+$ T cells either WT or overexpressing CXCR5 (CXCR5tg) into *Il21r-/-* mice to check for their activation and survival at different timepoints. At the same time, we immunized the mice with $2x10^8$ SRBC to stimulate an immune response. We decided to cull two groups of mice 7 and 14 days after transfer/immunisation and extract the spleen for donor T helper cell analysis. We had to stain the relatively large number of $5x10^6$ splenocytes with our T cell markers of interest because the donor T helper cell population was expected to be comparatively small. Although only very few cells from either donor survived up to day 7, it was nevertheless quite interesting and unexpected to see that the *Il21r-/-* donor cells seemed to fare better than the CXCR5tg *Il21r-/-* cells (Figure 4-7). From our CXCR5tg *Il21r-/-* transfer, only about 0.06% of total $CD4^+$ T cells detected on day 7 stem from our donors, whereas it was 0.19% of total T helper cells if we transferred *Il21r-/-* cells. On day 14 even fewer cells from either donor survived, interestingly though about a third of the small CXCR5tg *Il21r-/-* donor $CD4^+$ T cell population was still detectable in comparison to day 7 but the previously larger *Il21r-/-* donor $CD4^+$ T cell population diminished to a barely detectable level (Figure 4-7). Seeing these drastic changes from day 7 to day 14 after cell transfer, with the CXCR5tg *Il21r-/-* population not faring too well at first but surviving better in the long-term we were naturally interested to further characterize these cells. When we looked at the activation of the donor cells with the T cell activation marker CD44 on day 7 as well as day 14 it was interesting to see that most of the CXCR5tg *Il21r-/-* cells were highly activated from day 7 on and remained that way till day 14. Whereas only a small population of the *Il21r-/-* donor $CD4^+$ cells was activated on day 7 (Figure 4-8), and only a few scattered cells could be detected on day 14. To explain the difference in the activation status of our two donor cell populations we hypothesized that the CXCR5tg *Il21r-/-* cells translocated into the GC because of their forced CXCR5 expression where they get highly activated, whereas a higher proportion of the *Il21r-/-* donors remain out of the B cell follicle and therefore do not receive the same high levels of Ag-presentation by B cells. *Il21r-/-* $CD4^+$ T cells

may exhibit reduced survival due to lack of IL-21:IL-21R signalling, as it has been shown before that IL21-signalling in CD8⁺ T cells prevents them from exhaustion (Yi et al., 2009), and a similar mechanism might be at work here. By contrast, increased access to antigen bearing B cells and redundant cytokine growth factors such as IL-6 in the GC might explain the improved survival of the CXCR5tg *Il21r-/-* T cells. Although we were also interested in the expression of Tfh markers such as PD-1 and ICOS in these cells, the small size of the detected donor populations hindered further analyses (data not shown).

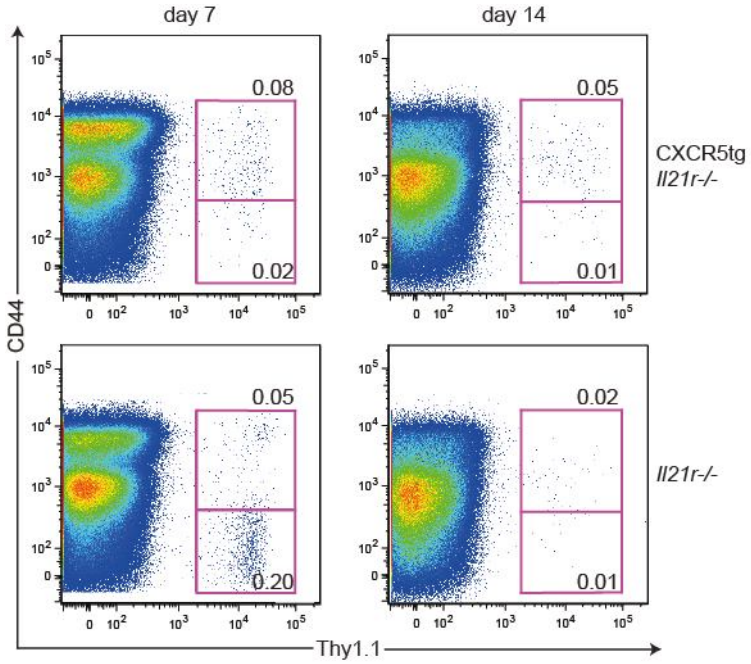

Figure 4-8) Activation of Il21r-/- T helper cells forced into the GC
Shown here are *Il21r-/-* cells either WT or CXCR5tg gated on total CD4⁺ T cells, these cells were transferred into *Il21r-/-* host mice and analysed 7 or 14 days after injection to evaluate their activation and survival. Looking at the total numbers of donor CD4⁺ T cells identified via the congenic marker THY1.1. The activation of the THY1.1⁺ donor cells was analysed with the T cell activation marker CD44, CD44high cells indicating antigen-activated cells, whereas CD44low cells weren't activated (n=1).

4.3. The impact of IL-21 on CD8-mediated effects on B cells

4.3.1. Analysis of Ag-specific B cells with the SW_{HEL} -system

The exact role of IL-21 on GC-formation and maintenance as well as class switch recombination (CSR) remains controversial, whereas some researchers claim IL-21 exerts its effects solely on B cells (Bessa et al., 2010; Linterman et al., 2010; Zotos

et al., 2010), others find it of equal importance for T follicular helper cells in an intrinsic manner (Nurieva et al., 2008; Vogelzang et al., 2008). The degree of discrepancy in this regard questions whether other factors may be involved. Since $CD8^+$ T cells have been shown to influence the survival of both Tfh cells and B cells (Kim et al., 2010; King and Sprent, 2012), we tested whether $CD8^+$ T cells could preferentially influence the survival of growth factor deprived *Il21r-/-* B cells. As we were primarily interested in the function of IL-21 on B cells in the context of CSR and GC formation we decided to follow changes in IgG1 isotype switched B cells, because those were the cells activated in T cell dependent B cell responses. To exclude a possible overall effect on B cells we decided that an analysis of total B cell numbers would also be necessary.

To monitor our cells of interest in an *in vivo* setting we utilized the antigen-specific SW_{HEL}-system (Phan et al., 2003) for further examination of the effect of $CD8^+$ T cell depletion on IL21R-deficient B cells. With the SW_{HEL}-system, B cells specific for the model antigen hen egg lysozyme (HEL) at population sizes approximating naive precursor frequencies could be transferred to the spleen of host mice and subsequently followed via a congenic CD45-marker in FACS and histological analyses. The possibility to analyse low-frequency, antigen-specific B cell populations at early phases in B cell development, as well as to monitor isotype switching and SHM (Paus et al., 2006; Chan et al., 2009) constitute the key advantages of this system.

4.3.2. CD8-mediated effects on *Il21r-/-* B cells

To test the importance of IL21-signalling in the context of $CD8^+$ T cell mediated effects on IgG1 switched B cells, as well as overall B cells we set up two groups of C75BL/6 host mice. The first group was injected i.p. with anti-CD8 antibody (IgG2b, YTS 169.4) one day as well as three days prior to B cell transfer to deplete them of $CD8^+$ T cells, whereas the second group served as a non-depleted control. These two groups were again divided into two sub-groups, the first group was injected i.v. with splenocytes from CD45.2/ SW_{HEL} mice, which were deficient in IL21R, whereas the second group received splenocytes with intact IL21-signalling

capabilities. The number of splenocytes transferred in both groups was calculated to contain approximately $3x10^4$ HEL-binding donor B cells per mouse, since only about 10% of transferred donor B cells successfully migrate into the spleen of the host mice the numbers for HEL-binding B cells were comparable to physiological precursor frequencies (Phan et al., 2005; Chan et al., 2010). At the time of transfer both groups were also immunized i.v. with the strong polyvalent antigen sheep red blood cells (SRBC) conjugated to HEL (SRBC-HEL) to trigger an immune response. Spleens of mice from these groups were taken 5 days as well as 10 days after donor cell transfer, and B cells were analyzed using immunostaining and flow cytometry analyses.

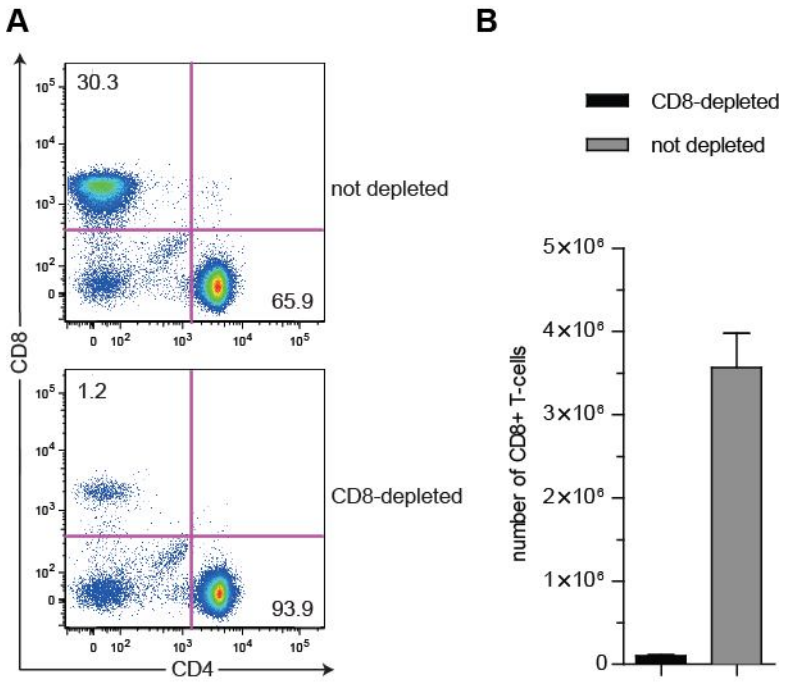

Figure 4-9) Efficiency of CD8⁺ T cell depletion in host mice
Shown is efficiency of CD8⁺ T cell depletion in mice injected with anti-CD8 antibody (IgG2b, clone YTS 169.4) one and three days prior to B cell transfer 13 days after depletion (day 10) as well as non-depleted mice as a control. The dot plots show CD8⁺ and CD4⁺ T cells in WT as a percentage of total CD3⁺ T cells (A). The bar graph shows total CD8⁺ T cell numbers for depleted as well as non-depleted mice (B) (n=2).

We have seen in previous experiments that CD8⁺ T cell numbers were negligible up to 8 days after depletion (data not shown). To test for a successful CD8⁺ T cell depletion on our day 10 timepoint (13 days after second depletion) we included a range of T cell markers in our FACS stains. Shown in Figure 4-9 are the results from

two injections with anti-CD8 Ab, in which largely diminished $CD8^+$ T cell numbers were observed up to 13 days after the second depletion.

4.3.3. Protection of early IgG1-switched B cells by IL-21

The profound effect of CD8-depletion on IgG1 switched B cells from our donor mice was evident 5 days after cell transfer (Figure 4-10). We show the $IgG1^+$ cells as a percentage of total B cells to exclude the simple loss of $CD8^+$ T cells from our gating and analyses. Surprisingly, not only did the cells from our *Il21r-/-* donors fare better in CD8-depleted mice, but even the IgG1 class switched WT population that was IL21-receptor sufficient, was increased by the absence of $CD8^+$ T cells in the host mice. In our non-CD8 depleted mice 27.8% of HEL-binding donor B cells were $IgG1^+$, whereas this population rose to 35.7% of HEL-binders in depleted mice (representative figure of two experiments) (Figure 4-10A). The *Il21r-/-* donor B cells also were also increased on day 5 in the absence of $CD8^+$ T cells; the percentage of IgG1 switched HEL-binding donor B cells rose from 17.3% in non-depleted to 25% in CD8-depleted mice, and therefore reached levels comparable to WT SW_{HEL} B cells transferred to mice with an intact $CD8^+$ T cell population (Figure 4-10A).

Interestingly, the recovery of the $IgG1^+$ donor population on day 5 seemed to be even more accentuated when absolute numbers were compared (Figure 4-10B). On one hand, 5 days after transfer of SW_{HEL} cells, the $IgG1^+$ population derived from WT SW_{HEL} B cells had a size of about $2.8x10^4$ cells with an intact $CD8^+$ T cell population, but increased to $9.6x10^4$ cells in CD8-depleted mice, resulting in a 3.5-fold increase. On the other hand, when we transferred *Il21r-/-* SW_{HEL} B cells, the IgG1 switched population consisted only of about $0.4x10^4$ cells in non-depleted mice, but increased to $2.1x10^4$ cells in CD8-depleted recipient mice, constituting a 5-fold increase (Table 4-1), therefore reaching a population size comparable with the one derived from WT donors in mice with an intact $CD8^+$ T cell population (Figure 4-10B). Comparing the changes of the $IgG1^+$ SW_{HEL} population upon CD8-depletion for WT and *Il21r-/-* donor cells on day 5, one can see that there is a

smaller fold change in WT (3.5x) than in *Il21r-/-* (5x) cells, indicating an involvement of the IL21-signalling cascade in this CD8-mediated effect (Table 4-1).

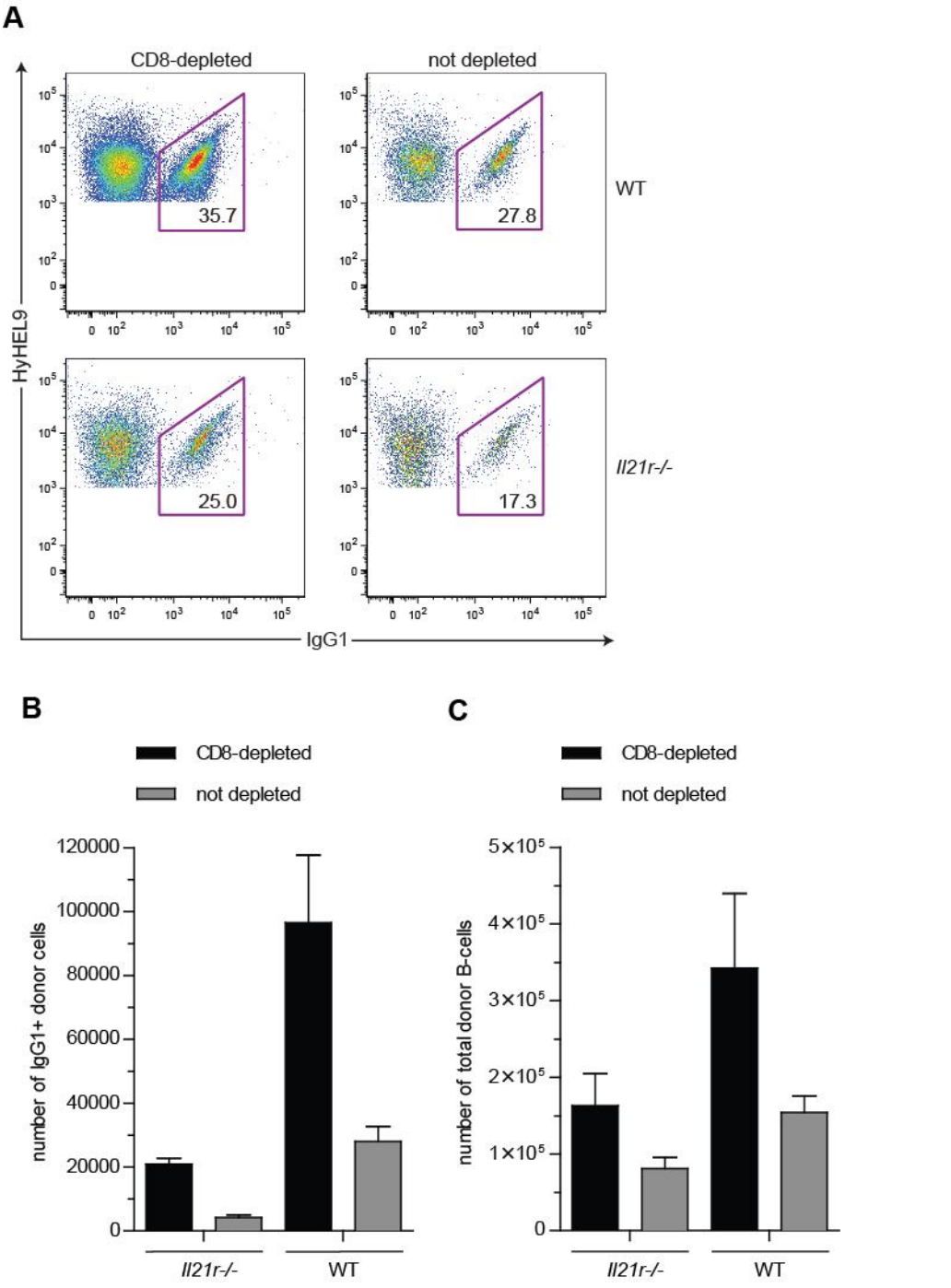

Figure 4-10) Effect of CD8⁺ T cell depletion on *Il21r-/-* and WT donor B cells on day 5
Shown are the effects of CD8-depletion in host mice on IgG1⁺ donor B cells as well as total donor B cell numbers. The dot-plot shows transferred SW_{HEL} donor cells from both *Il21r-/-* as well as WT SW_{HEL} mice, the IgG1⁺ population is indicated as % of total SW_{HEL} donor B cells (A). Total numbers for the cell population indicated in A) are shown in this bar-graph (B). Total donor B cell numbers are shown in C) (n=2).

To determine whether the beneficial effect of CD8-depletion on IgG1$^+$ donor SW_{HEL} B cells we observed on day 5 was due to an effect on overall B cell numbers, we decided also to look at total B cell numbers in our mice (Figure 4-10C). For both *Il21r-/-* as well as WT we could see increased total donor B cell numbers upon CD8$^+$ T cell depletion (when cells were gated on non-CD8$^+$ T cells in all mice), the overall effect was smaller than the effect witnessed on IgG1$^+$ SW_{HEL} donor B cells though (Table 4-1). Total B cell numbers of donors WT for the IL21-receptor were at about 15x10^4 cells in non-depleted mice and increased to about 34x10^4 cells in the mice injected with anti-CD8 antibody. This constituted a 2.2-fold rise from non-depleted to depleted mice in comparison to the larger 3.5-fold increase in IgG1$^+$ HEL-binding B cells seen before. The total donor B cell numbers from *Il21r-/-* mice rose from about 8x10^4 cells in non-depleted to about 16x10^4 cells in CD8-depleted animals (Figure 4-10C). The doubling of B cell numbers seen here comparing depleted versus non-depleted mice was also smaller than the 5-fold increase observed before in IL21R-deficient IgG1$^+$ donor B cells (Table 4-1). As we have seen in Figure 4-10C the depletion of CD8$^+$ T cells in host mice clearly has an effect on total donor B cell numbers, comparison of the fold changes does suggest however that this phenomenon acts equally on B cells from both *Il21r-/-* and WT mice (Table 4-1). In our opinion this effect on total B cell numbers is therefore independent of IL-21 and could possibly be attributed to an expansion of all cells in the spleen due to the absence of the CD8$^+$ T cell population, and increased availability of shared growth factor resources (such as cytokines) utilised by both CD8$^+$ T cells and B cells.

	fold change of IgG1$^+$ cell #		fold change of total donor B cell #	
timepoint	day 5	day 10	day 5	day 10
Il21r-/-	5x	2x	2x	1x
WT	3.5x	1.3x	2.2x	1.2x

Table 4-1) Changes in cell number in reaction to CD8$^+$ T cell depletion
In this table the difference in fold changes of IgG1$^+$ donor B cell numbers, as well as total donor B cell numbers in CD8-depleted vs. non-depleted mice is shown for day 5 as well as day 10 (n=2).

Taken together, these data showed that the impact of CD8-depletion on IgG1$^+$ SW_{HEL} B cells did exceed the effect seen on overall B cells on day 5 and was more prominent on IL-21R-deficient donor cells (Table 4-1). Because this phenomenon specifically targeting IgG1$^+$ B cells was mediated by the ability to react to IL21-signals we inferred a direct or indirect protective role of IL-21 on B cells from these CD8$^+$ T cell dependent adverse effects.

4.3.4. Adverse effects of *Il21r-/-* on IgG1$^+$ B cells at later timepoints

After discovering the negative impact of the absence IL21-signalling on IgG1$^+$ B cells on day 5 after donor cell transfer through an effect mediated by CD8$^+$ T cells, we became interested if we could also detect this effect at later timepoints. We decided to carry out the same analyses on day 10 after donor cell transfer as we performed on day 5 to clarify the impact of defective IL21-signalling on this CD8-mediated effect at later stages of the GC response.

Looking at IgG1-switched B cells as a percentage of total B cells on day 10 it seemed that both the protective effect of functional IL21-signalling as well as the beneficial effect of CD8-depletion on these cells was not evident at this stage. In fact, we did not observe a difference between CD8-depleted versus non-depleted mice for WT or *Il21r-/-* cells (Figure 4-11A). For transferred WT SW_{HEL} donor B cells, the IgG1$^+$ population was around 41% of total donor B cells for both CD8-depleted and non-depleted hosts, whereas the same population for transferred *Il21r-/-* donor cells was 45.3% in CD8$^+$ T cell depleted and 49.9% in non-depleted mice. By contrast, when we analysed the total numbers of the B cell population, the protective effect of intact IL21-signalling although weaker on day 10 than on day 5 was still detectable. In non-depleted mice the IgG1-switched SW_{HEL} B cell population from WT mice consisted of about $6.6x10^4$ cells, while the same population derived from the same donor mice was $8.5x10^4$ cells in mice depleted of CD8$^+$ T cells, which constitutes a minimal 1.3-fold increase (Figure 4-11B). The IgG1$^+$ HEL-binding B cell population derived from *Il21r-/-* donors was $1.3x10^4$ cells in non-depleted mice and reached twice the size in CD8-depleted mice with about $2.6x10^4$ cells. Similar to what we observed on day 5 there was a larger change in

IL21R-deficient B cells than WT cells upon CD8-depletion, on day 10, which further suggests an involvement of IL21-signalling in this effect (Table 4-1).

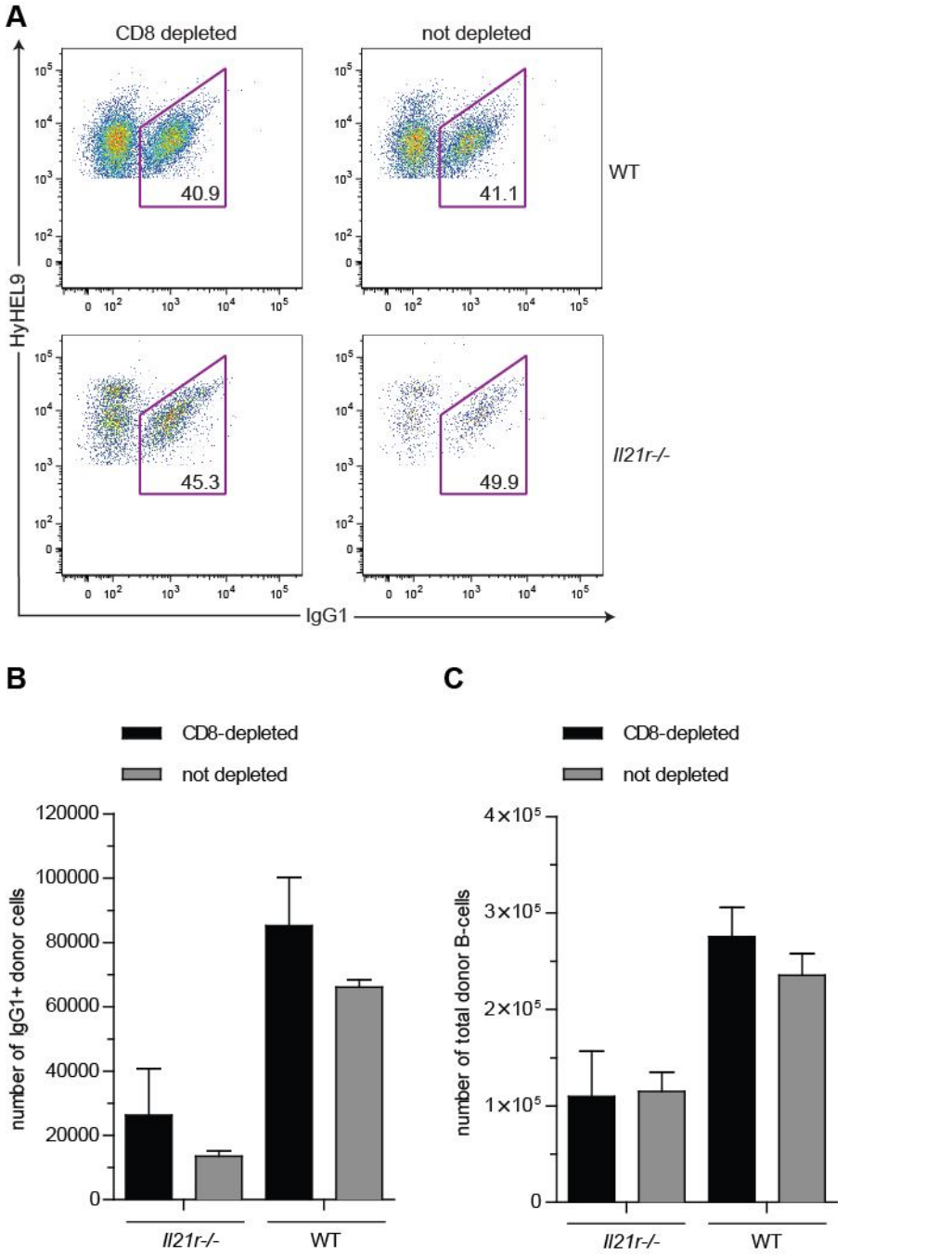

Figure 4-11) Effect of CD8⁺ T cell depletion on *Il21r-/-* and WT donor B cells on day 10
Shown are the effects of CD8-depletion in host mice on IgG1⁺ donor B cells as well as total donor B cell numbers. The dot-plot shows transferred SW_{HEL} donor B cells from both *Il21r-/-* as well as WT SW_{HEL} mice, the IgG1⁺ population is indicated as % of total SW_{HEL} donor B cells (A). Total numbers for the cell population indicated in A) are shown in this bar-graph (B). Total donor B cell numbers are shown in C) (n=2).

To exclude an overall effect of the absence $CD8^+$ T cells on total B cell numbers on day 10 as we did before on day 5, we again checked the impact of the CD8-depletion on the total B cell population. As it turns out, there was less effect of CD8-depletion on total B cell numbers detectable in the later timepoint of our experiment (Figure 4-11C). In non-depleted mice, the WT donor B cell population consisted of about $23x10^4$ cells, whereas in CD8-depleted mice around $27x10^4$ WT SW_{HEL} donor B cells survived, the difference in non-depleted versus depleted mice constituted a marginal 1.2x increase in depleted hosts. By contrast, there was no difference in the total B cell numbers derived from *Il21r-/-* donors upon $CD8^+$ T cell depletion on day 10, the size of the total B cell population was about $11x10^4$ cells in depleted as well as in non-depleted mice. Following the IL21-mediated protection of B cells from adverse $CD8^+$ T cell associated effects a decline at the later timepoint is clearly visible. The positive impact on the SW_{HEL} donor B cells, as well as on total B cell numbers, both clearly visible on the early timepoint (day 5) was reduced by day 10 (Table 4-1). One possible explanation for this apparent time dependency of our observed phenomenon might be, although the $CD8^+$ T cell numbers didn't return to normal levels, a small population of emerging cytotoxic T cells could be observed on day 10. This small population might be more highly activated or better specialized and subsequently lead to less B cell survival than under normal conditions due to the abundance of available CD8-activating signals. The observation that the numbers of $IgG1^+$ B cells in *Il21r-/-* mice were less than WT on day 5 as well as day 10 with and without $CD8^+$ T cell depletion support the important role of IL-21 on survival and differentiation of B cells during the GC reaction.

5. Discussion and outlook

The importance of the interaction of T and B cells for GC formation and CSR to generate a proper IgG1-response has been widely acknowledged in the scientific community (Miller and Mitchell, 1968; MacLennan et al., 1992), in the last years it has become evident that without functioning IL-21:IL21R signalling these responses are critically impaired (Ozaki et al., 2002; Vogelzang et al., 2008). IL21-signalling has become the focus of the work of many researchers around the world, but there is still disagreement in the literature whether the role of IL-21 in the GC reaction is intrinsic to B cells or T follicular helper cells (Nurieva et al., 2008; Vogelzang et al., 2008; Linterman et al., 2010; Zotos et al., 2010). It has become clear that a more detailed examination of the role of IL-21 on the cells involved in the GC reaction will be necessary to further improve our understanding of the intricate interactions at work in this process. In this study we aimed to shed some light on the importance of IL-21 for T helper cell activation and differentiation at early stages of the humoral immune response with the help of a fluorescently labelled pMHCII tetramer to track an Ag-specific Th cell population (Moon et al., 2007). We also used cells overexpressing CXCR5 (CXCR5tg) in transfer experiments to elucidate the role of IL-21 for the migration of Tfh cells into the GC, as well as their activation and survival in this environment at early and late stages. Finally, we followed up on previous findings in our lab (unpublished data) concerning the protective effect of IL-21 on B cells relative to the adverse effects mediated by $CD8^+$ T cells in secondary lymphoid organs, with the help of Ag-specific SW_{HEL} B cells (Phan et al., 2003).

5.1. Negative effects of IL-21 deprivation on T helper cells

An intrinsic role for IL-21 on T follicular helper cells has been shown before (Nurieva et al., 2008; Vogelzang et al., 2008), and although the GC reaction and $IgG1^+$ antibody production is impaired in *Il21r-/-* mice because of the importance of IL-21 for Tfh cells (Vogelzang et al., 2008), the exact mode of action of this cytokine still remains unclear. With this work we tried to gather further information on the exact circumstances in which IL-21 reacts on $CD4^+$ T cells in general and Tfh cells in particular. In our experiments, a strong dependency of T

helper cells on intact IL-21:IL21R signalling early in the GC reaction was evident (Figure 4-4). However, while the reduced Ag-specific T helper population in *Il21r-/-* mice provided evidence for their dependency on IL21-signalling, further experiments are still needed to clarify whether the lack of IL-21 acts on the differentiation or survival of Tfh cells, or indeed on their ability to migrate in the GC. There was a distinctive effect of IL21R-deficiency on activated, Ag-specific T helper cells visible in our experiments. By contrast, the difference between *Il21r-/-* and WT Ag-specific T follicular helper cells did not reach significance (Figure 4-5), indicating that further experiments to increase the sample size are needed to fully clarify this matter. Interestingly we observed that not all of the T helper cells expressing the highest amounts of CXCR5 and PD-1, which are generally classified as T follicular helper cells, actually were Ag-specific (Figure 4-6). It appears unlikely that T cells lacking Ag-specificity are part of the genuine Tfh population, furthermore these cells might influence the calculation of Tfh cell numbers during an immune response. In this manner, the heterogeneity of this Th population might contribute to the contradictory findings on the IL21-dependency of Tfh cells (Nurieva et al., 2008; Vogelzang et al., 2008; Linterman et al., 2010; Zotos et al., 2010). Alternatively, the detection of antigen-specific Th cells by MHC class II tetramers has been shown to have a bias in binding the T cells with the highest affinity for antigen. Thus, the tet- cells in our Tfh cell gate might be Tfh cells with lower affinity for the 2W1S peptide antigen.

When we analysed the role of IL-21 for early CXCR5-mediated migration of Tfh to the T/B cell border and into the GC in secondary lymphoid organs we observed a drastic effect on *Il21r-/-* cells overexpressing CXCR5 (CXCR5tg) (Figure 4-7). Although the T helper cells expressing the CXCR5tg were highly activated in comparison to the WT *Il21r-/-* cells only very few of them were detected on day 7. The high level of activation of the CXCR5tg Th cells suggested migration into the GC where they receive an abundance of Ag-presentation and costimulatory signals from GC B cells, whereas more of the *Il21r-/-* cells that did not overexpress CXCR5 may have remained excluded from the GC (Figure 4-8). However, the translocation to the GC doesn't guarantee the survival of these cells. Indeed, our findings indicated that Ag-stimulation by the GC B cells seems to have adverse effects

without IL-21 signalling, as can be seen by the poor survival of CXCR5tg *Il21r-/-* cells in comparison to WT *Il21r-/-* cells on day 7(Figure 4-7). Taken together these findings indicate an additional role for IL21-signalling in maintaining Tfh survival apart from its importance for high CXCR5 expression on these cells (Vogelzang et al., 2008). The fact that a small population of CXCR5tg *Il21r-/-* Th cells survive up to day 14 after transfer but none of the WT *Il21r-/-* cells hints to additional survival mechanisms present once the Tfh cells enter the GC, which might be explained by a better adaption of the CXCR5tg *Il21r-/-* cells to the GC environment. The conclusions we can draw from these preliminary CXCR5tg experiments is of course limited by the small number of mice we used (n=1), but we remain confident that further repeats will confirm our initial findings.

5.2. Protective effects of IL-21 on IgG1$^+$ B cells

A CD8$^+$ T cell mediated mechanism to regulate humoral Ab-responses was first discovered in the 1970s, a specialized subset of CD8$^+$ T cells termed CD8$^+$ "suppressor" T cells was thought to be responsible for the decrease of proliferation in CD4$^+$ T cells (Gershon and Kondo, 1970; Cantor et al., 1978). The molecular mechanisms explaining CD8$^+$ T cell mediated suppression of the humoral immune responses nevertheless remained controversial for the past 30 years (King and Sprent, 2012). When the sequencing of the I-J region of the MHC, previously reported to code for soluble suppressive factors secreted by the CD8$^+$ "suppressor" T cells, turned out to contain no coding elements the idea of T cell mediated suppression was largely abolished (Steinmetz et al., 1982; Kronenberg et al., 1983). However, more recent findings indicate a renaissance for this unappreciated hypothesis of CD8-mediated regulation of the humoral immune response (Salgame et al., 1991; Basten and Fazekas de St Groth, 2008; Kapp and Bucy, 2008).

When recent findings in our lab (unpublished data) indicated a massive increase in B cell numbers in mice deficient for both IL-2 as well as the IL21-receptor (*Il2-/-*, *Il21r-/-*) upon *in vivo* depletion of CD8$^+$ T cells we became interested to determine whether we could observe a similar effect on *Il21r-/-* B cells. In our analyses of the IgG1$^+$ B cell populations from WT and *Il21r-/-* mice early in the GC response (day 5)

we could indeed notice much larger populations if the host mice were depleted of $CD8^+$ T cells before donor cell transfer (Figure 4-10). As previous studies reported the translocation of $CD8^+$ T cells into the B cell follicle and the GC (Khanna et al., 2007; Quigley et al., 2007), and these cells were able to suppress IgA, IgE as well as IgG responses (Tada, 1975; Moskophidis et al., 1992), we postulated a possible involvement of these cells in the survival of *Il21r-/-* B cells. The observed change upon $CD8^+$ T cell depletion of WT recipient mice was much larger for adoptively transferred *Il21r-/-* $IgG1^+$ antigen specific SW_{HEL} B cells than for adoptively transferred WT antigen specific SW_{HEL} B cells. In fact, the small *Il21r-/-* $IgG1^+$ B cell population in non-depleted mice increased to the same levels upon $CD8^+$ T cell depletion as the WT $IgG1^+$ B cell response in non-depleted mice (Figure 4-10B). These findings further support previous reports on the effect of IL21-deficiency on GC formation, IgG1 isotype switching and successfully T dependent B cell responses (Ozaki et al., 2002; Vogelzang et al., 2008).

When we performed the same analysis later in the GC response (day 10) we could still measure a positive effect of $CD8^+$ T cell depletion on both WT and *Il21r-/-* $IgG1^+$ B cell numbers, but it was much less pronounced than on day 5, the fact that $CD8^+$ T cells begin to leave the secondary lymphoid organs by day 5 after immunization (Khanna et al., 2007) might account for the weaker effect observed at later timepoints (Figure 4-11B). Since the *Il21r-/-* $IgG1^+$ B cell population was still much smaller than the WT $IgG1^+$ B cell population an additional need for IL-21 by these cells can be inferred apart from the discussed protective effects from $CD8^+$ T cell mediated suppression (Figure 4-11B). The depletion of $CD8^+$ T cells also led to larger total B cell populations for both WT and *Il21r-/-* cells early in the GC reaction, when $CD8^+$ T cells were excluded from the analyses (Figure 4-10C), but this effect had vanished at day 10 (Figure 4-11C). The fact that the overall B cell effect was independent of IL21-signalling and also much smaller than the one observed on $IgG1^+$ B cells led us to the conclusion that the loss of $CD8^+$ T cells in these mice may have resulted in an abundance in growth factor cytokines and therefore initiated homeostatic expansion of other cells, as detected in the spleen.

5.3. Influence of IL-21 on suppressor function of CD8$^+$ T cells

Recent reports about the influence of CD8$^+$ "suppressor" T cells on the survival of T helper cells and B cells (Kim et al., 2010; King and Sprent, 2012) as well as the finding that IL-21 in concert with IL-15 regulates expansion and function of CD8$^+$ T cells (Zeng et al., 2005) prompted us to re-examine our findings from this new viewpoint. The MHC class I molecule Qa-1 has been shown to be one of the major target molecules for CD8$^+$ T cell mediated suppression of the humoral immune response (Noble et al., 1998), and surface expression of Qa-1 on target cells was essential for regulatory activity (Cantor et al., 1978). Interestingly recent studies report the high expression of Qa-1 on mature Tfh cells not activated by antigen, whereas other T helper subsets hardly expressed Qa-1 (Kim et al., 2010), which makes T follicular helper cells a major target for suppression by CD8$^+$ T cells (King and Sprent, 2012). Mice with a mutant version of Qa-1 (B6 Qa-1) disrupting the binding of the TCR/CD8 co-receptor and Qa-1 develop spontaneous autoimmune symptoms (Kim et al., 2010) similar to the sanroque and BXSB-Yaa mouse strains (Bubier et al., 2009; Linterman et al., 2009).

In the light of these studies we could propose that lower numbers of Ag-specific T helper cell levels in *Il21r-/-* mice in comparison to WT mice (Figure 4-4) might be influenced by CD8$^+$ T cells acting more harshly on *Il21r-/-* cells than on WT cells. The higher mortality of CXCR5tg *Il21r-/-*cells in comparison to *Il21r-/-* cells (Figure 4-7) might also be explained by the CD8$^+$ "suppressor" T cell hypothesis, as the CXCR5tg cells are forced to translocate into the GC, increasing the chance to encounter CD8$^+$ T cells also residing there (Khanna et al., 2007; Quigley et al., 2007). As a reason for the higher susceptibility of *Il21r-/-* cells to suppression by CD8$^+$ T cells we propose a role for IL-21 in Tfh cells for the regulation of Qa-1 surface expression, however this has to be examined in further studies. Regulation of Tfh cell numbers by CD8$^+$ T cells could also explain the poor IgG1 switched immune response in *Il21r-/-* mice, as suppressed Tfh cells fail to provide help to GC B cells which could result in lower IgG1$^+$ B cell numbers. However the low *Il21r-/-* IgG1$^+$ B cell numbers we observed in our experiments (Figure 4-12 and Figure 4-13) that were transferred into WT mice and therefore should receive help from WT Tfh cells point to an additional, more direct protective effect of IL-21 on

IgG1$^+$ B cells from CD8-mediated adverse effects. Reports in the literature of increased Tfh cell numbers as well as a massive increase in IgG1$^+$ B cell numbers in mice with a mutant version of Qa-1 (Kim et al., 2010) further support the regulation of the IgG1$^+$ B cell response through CD8$^+$ T cells.

To further test our proposed hypothesis on the role of IL-21 on CD8$^+$ T cell mediated suppression of T cell dependent B cell responses it would be interesting to determine the effects of CD8$^+$ T cell depletion on Ag-specific *Il21r-/-* T follicular helper cells, to assess if the effects observed on *Il21r-/-* IgG1$^+$ B cells are more pronounced on B cells themselves or upstream on T helper cells. Also it would be interesting to study Tfh cells and IgG1$^+$ B cells from *Il21r-/-* mice additionally made deficient for perforin (*Prf1-/-*), as CD8-suppression has been reported to be perforin-dependent (Kim et al., 2010). We are confident that the findings presented in this thesis will help to understand the regulation of T follicular helper cells and T cell dependent B cell responses and the role of IL-21 in this process a little better, a topic that remains of immense interest in the research community (King and Sprent, 2012).

6. References

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7. List of abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
APC	Allophycocyanin
BCL-6	B cell lymphoma-6
BCR	B cell receptor
CCR7	Chemokine motif receptor 7
CD	Cluster of differentiation
CD	Crohn's disease
CSR	Class switch recombination
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescence assisted cell sorting
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box p3
GC	Germinal center
HEL	Hen egg lysozyme
ICOS	Inducible co-stimulation factor
ICOSL	ICOS ligand
Idd3	Insulin dependent diabetes susceptibility 3
Ig	Immunoglobulin
IL	Interleukin
IL21R	Interleukin-21 receptor
INFγ	Interferon-γ
LN	Lymph node
MHC	Major histocompatibility complex
NOD	Non-obese diabetic
NK	Natural killer cells
NKT	Natural killer T cells
PAGE	Polyacrylamid gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PB	Pacific blue
PBS	Phosphate buffered saline
PD-1	Programmed cell death-1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
pMHCII	Peptide-bound MHC class II
PP	Payer's patches
RBC	Red blood cell
RA	Rheumatoid arthritis

RT	Room temperature
RNA	Ribonucleic acid
RUNX	Runt-related transcription factor
SAP	SLAM associated protein
SDS	Sodium dodecyl sulfate
SHM	Somatic hypermutation
SLAM	Signalling lymphocytic activation molecule
SLE	Systematic lupus erythematosus
SNP	Single nucleotide polymorphism
SRBC	Sheep red blood cell
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper cell
Tg	Transgene
TGFβ	Tumor growth factor-β
Th	T helper
Treg	Regulatory T cell
TNFβ	Tumor necrosis factor-β
T1D	Type-1 diabetes mellitus
STAT	Signal transducer and activator of transcription
UC	Ulcerative colitis
WT	Wild type

8. List of tables and figures

9. Curriculum vitae

Curriculum vitae

Christoph Jandl

Personal Data:

Date of birth: November 1st 1984

Place of birth: Klagenfurt, Austria

Citizenship: Austria

Education:

Since October 2005:
Diploma studies in Molecular Biology at the University of Vienna
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1999-2003:
Militaerrealgymnasium an der Theresianischen Militaerakademie in Wr. Neustadt
A-levels passed in May 2003

Research Experience:

February 2010 – February 2011
Diploma thesis "The role of Interleukin-21 on function and survival of T helper cells and B cells in the germinal center reaction"
Garvan Institute for Medical Research, Sydney, Australia
Supervised by Cecile King

August 2009 – October 2009
Internship at the Institute for Molecular Biotechnology, Vienna
Supervised by Andrew Pospisilik
Working title: "A two-way approach to relating genome-wide gene expression and chromatin structure changes to obesity and type 2 diabetes mellitus"

February 2009:
Internship at Harvard Medical School, BIDMC at the Department for Surgery, Boston, USA
Supervised by Leo Otterbein
Working title: "Silencing of selected miRNAs in cultured eukaryotic cells via siRNA"

August 2008 – July 2009:
Research Student, part time; Medical University of Vienna at the KIMCL,
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Working title: "Characterization of a specific promoter region involved in the innate immune response"

May 2007 – June 2008:
Research Student, part time; Medical University of Vienna at the Department for Clinical Microbiology;
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Working title: "Extraction of DNA from human whole blood samples under sterile conditions for the detection of Aspergillus and Candida ssp. infection via qPCR, and detection of fungal antigen with ELISA"

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-German (native speaker)

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P u b l i c a t i o n s:

Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, Knapp B, Haas R, Schmid JA, Jandl C, Amir S, Lubec G, Park J, Esterbauer H, Bilban M, Brizuela L, Pospisilik JA, Otterbein LE, Wagner O. The Sedoheptulose Kinase CARKL Directs Macrophage Polarization Through Control of Glucose Metabolism. (2012) Cell Metab. *15*, 813–826.