Generation, maintenance, and fate of T follicular helper cells

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1. Abstract/Zusammenfassung

1.1 Abstract

The humoral immune response is mediated by B cells that aid the elimination of pathogens through the secretion of antibodies and are therefore the basis of current vaccination strategies. Activated B cells mature in germinal centres (GCs) and abnormal germinal centre responses have been reported to be causative of autoimmune diseases. T follicular helper (TFH) cells represent the subpopulation of $CD4^+$ T cells which provides help for B cells in the GC response. In order to optimise vaccination strategies and develop better therapeutic approaches against autoimmune diseases, it is necessary to gain a deeper understanding of the differentiation and maintenance of TFH cells. Underlying molecular requirements were analysed in this thesis using an adoptive transfer system.

The co-stimulatory molecules ICOS and CD28 are both important for TFH cells, yet their specific roles for TFH cells are unknown. The data presented here attribute to each molecule an exclusive role at different stages of humoral immune responses: while CD28 regulated early expression of the TFH master transcription factor Bcl-6, ICOS co-stimulation was essential to maintain the TFH phenotype and thereby GC reactions.

It has been a long-standing question whether TFH cells contribute to the $CD4^+$ memory pool after the GC response has been terminated. To answer this question, a TFH re-transfer system of antigen-specific T cells was established, in which antigen-specific TFH and non-TFH effector cells were sorted from ongoing GC responses and transferred into naïve mice, where they could be analysed separately. The populations of transferred cells rapidly contracted in the absence of antigen with a small population of both TFH and non-TFH cells surviving as memory cells for at least four weeks. TFH cells strongly downregulated signature molecules in the memory phase but maintained a distinct phenotype from non-TFH memory cells. Upon *in vivo* re-challenge with antigen, TFH memory cells preferentially re-adopted the TFH phenotype. An enhanced potential to produce IL-21, paired with higher expression of CXCR5 and lower expression of CCR7, should enable them to be effective B cell helpers in secondary immune responses.

My thesis presents evidence for functional differences between the structurally related proteins CD28 and ICOS, which may be a step-stone to better treatment of autoimmune diseases caused by aberrant germinal centre reactions. The data further demonstrate that TFH cells can develop into memory cells, presenting an interesting target for vaccine development.

1.2 Zusammenfassung

Humorale Immunreaktionen werden durch Antikörper-sezernierende B-Zellen vermittelt, die die Grundlage heutiger Impfstrategien sind. Aktivierte B-Zellen reifen in Keimzentren und abnormale Keimzentren werden mit vielen Autoimmunerkrankungen in Verbindung gebracht. Follikuläre T-Helferzellen (TFH) sind die CD4$^+$-T-Helferzellen, die B-Zellen in der Keimzentrumsreaktion unterstützen. Um bessere Impfstoffe und therapeutische Ansätze gegen Autoimmunerkrankungen entwickeln zu können, ist es notwendig, die Differenzierung und Aufrechterhaltung von TFH-Zellen besser zu verstehen. Zugrundeliegende molekulare Mechanismen wurden in dieser Arbeit in einem adoptiven Transfersystem analysiert.

Die Kostimulatoren ICOS und CD28 sind wichtig für TFH-Zellen, allerdings ist ihre spezielle Rolle für diese Zellen bisher nicht bekannt. Die hier präsentierten Daten weisen jedem der Moleküle eine exklusive Rolle in verschiedenen Stadien der humoralen Immunreaktion zu: CD28 regulierte die frühe Expression des TFH-Mastertranskriptionsfaktors Bcl-6, wohingegen ICOS-Kostimulation für die Aufrechterhaltung des TFH-Phänotyps und daher auch der Keimzentrumsreaktionen nötig war.

Es war lange offen, ob TFH-Zellen nach Beendigung der Keimzentrumsreaktion zu CD4$^+$-Gedächtniszellen werden. Um diese Frage zu beantworten, wurde ein Retransfersystem für TFH-Zellen entwickelt, in dem antigenspezifische TFH- und nicht-TFH-Effektorzellen aus Keimzentrumsreaktionen isoliert und sortiert wurden. Anschließend wurden die Zellen in naive Mäuse transferiert, wo sie unabhängig voneinander analysiert werden konnten. Die retransferierten Zellpopulationen kontrahierten schnell ohne Antigen, jedoch blieb eine kleine Population TFH- und nicht-TFH-Gedächtniszellen für mindestens vier Wochen in der Maus vorhanden. TFH-Zellen regulierten für ihre Identifikation und Funktion typische Moleküle herunter, behielten jedoch einen anderen Phänotyp als nicht-TFH-Gedächtniszellen. Nach Antigenstimulation *in vivo* adaptierten ehemalige TFH-Zellen präferentiell den TFH-Phänotyp. Ein erhöhtes Potential IL-21 zu sezernieren, gepaart mit erhöhter Expression von CXCR5 und geringerer Expression von CCR7, sollte diese Zellen zu effektiven B-Zell-Helfern in sekundären Immunreaktionen machen.

Meine Arbeit zeigt funktionelle Unterschiede der strukturell verwandten Moleküle CD28 und ICOS auf und könnte daher ein Mosaikstein für die Suche nach besseren therapeutischen Ansätzen zur Bekämpfung von Autoimmunkrankheiten sein. Die Daten zeigen weiterhin, dass TFH-Zellen zu Gedächtniszellen werden können, welche ein interessanter Schwerpunkt zukünftiger Impfstrategien sein können.

2. Introduction

2.1 The adaptive immune system

The adaptive immune system of vertebrates allows for a tailored immune response against infectious agents. Large numbers of naïve cells, each usings antigen recognition molecules with a different specificity, allow for a wide range of potential attacks to be thwarted. The adaptive immune system has additional mechanisms in play that allow for the development of an immunological memory to fight off recurring infections more easily and cost-efficiently.

The humoral immune response is mediated by B cells, which use B cell receptors (BCRs), membrane-bound immunoglobulins (Igs), as antigen recognition molecules. B cells secrete soluble Igs – or antibodies – which bind to specific epitopes of an antigen and thereby aid the elimination of intruders mediated by other components of the immune system. After activation via their BCR, B cells can differentiate quickly into short-lived plasma blasts producing a first burst of antibodies. Alternatively, B cells enter a germinal centre (GC) reaction (further described below), where they develop into high-affinity plasma cells or long-lived memory B cells, providing a lasting protection to the host by the production of neutralising antibodies.

The subset of T cells characterised by the expression of the surface molecule CD4 provides help to effectors of immune responses. Naïve $CD4^+$ T cells further differentiate into functional $CD4^+$ T helper subsets depending on the co-stimulatory signals they receive and the cytokine milieu present during primary activation. To date, several subsets of $CD4^+$ T helper cells are discriminated according to their cytokine secretion profile and the master transcription factor needed for their differentiation. The major T helper (Th) subsets are Th1, Th2, Th17, regulatory T cells, and T follicular helper (TFH) cells with their specific effector cytokines IFN-γ, Interleukin(IL)-4/IL-5, IL-17, IL-10/TGF-β, and IL-21, and the master transcription factors T-bet, GATA3, RorγT, FoxP3 and Bcl-6 (B cell lymphoma-6), respectively. Originally, the helper subsets seemed to be stably maintained; however, recent evidence caused debates about the stability and inter-convertability of T helper subsets (Nakayamada *et al.*, 2012).

2.2 T cell co-stimulation

$CD4^+$ T cells receive a "first" signal via their epitope-specific T cell receptor (TCR) to get activated. Dendritic cells are the main professional antigen-presenting cells (APCs) in the early immune response that present a peptide of the antigen to the TCR of $CD4^+$ T cells; later on, B cells can act

as APCs. Further to the TCR signal, "second" co-stimulatory signals amplifying or counteracting the TCR signals are provided. These co-stimulatory signals are believed to prompt the CD4$^+$ T cell specialisation by initiating a cytokine milieu that stabilises T helper subsets in a self-reinforcing manner (Murphy and Reiner, 2002; Takatori *et al.*, 2008). The second signal is also important to select between activation (in the case of a positive co-stimulatory signal) of T cells or their attenuation, or tolerance induction (both mediated by co-inhibitors) of T cell responses. Most co-signalling molecules for conventional T cells are members of the CD28 family and tumor necrosis factor (TNF) superfamily of receptors (Kroczek *et al.*, 2004; Zhu *et al.*, 2011).

The best-studied co-stimulatory molecules of the CD28 family are CD28 and CTLA-4 (cytotoxic T lymphocyte–associated antigen 4), which both engage B7-1 (CD80) and B7-2 (CD86) on the APC (Greenwald *et al.*, 2005). CD28 delivers signals for activation and survival, whereas CTLA-4 inhbits T cell responses and can regulate peripheral T cell tolerance (Greenwald *et al.*, 2005). While CD28 is constitutively expressed on naïve T cells, another member of the CD28 familiy, the inducible co-stimulator (ICOS), is only expressed at low basal levels and is upregulated by both TCR and CD28 signals (McAdam *et al.*, 2000). However, CD28 signalling is not essential for functional ICOS expression, as blocking of ICOS in CD28 knockout (KO) mice interferes with Th1 differentiation (Kopf *et al.*, 2000). The members of the CD28 superfamily do not only play a role in the initial T cell activation, as CTLA-4 terminates T cell responses (Sharpe and Freeman, 2002) and ICOS plays an important role in the longevity of effector T cells (Burmeister *et al.*, 2008).

CD28 and ICOS are structurally related molecules of the B7 family activating the Akt pathway via phosphatidylinositol-3-kinase (PI3K; Rudd and Schneider, 2003). Missing signalling of either molecule has similar impacts on the humoral immune response: reduced Ig levels and isotype class switch, and abnormally small germinal centers upon immunisation (Shahinian *et al.*, 1993; Akiba *et al.*, 2005). Nonetheless, distinctive cytoplasmic tails of the molecules result in functional differences: ICOS, in contrast to CD28, does not induce IL-2 production (Hutloff *et al.*, 1999). IL-2 transcription is mediated by Grb-2, which can bind to the YMNM motif of CD28 but not to the YMFM motif of ICOS (Harada *et al.*, 2001). On the other hand, signalling via ICOS results in a stronger phosphorylation of PI3K than signalling via CD28 (Okamoto *et al.*, 2003). The finding that ICOS/CD28 double-knockout mice show lower levels of isotype class switch than CD28 KO mice is a further indication of non-redundant signalling of both molecules (Suh *et al.*, 2004).

After their initial activation, some CD4$^+$ T cells will start to provide help to B cells in the form of co-stimulatory signals, which can be cell–cell contact signals (e.g. via CD40L on T cells and CD40 on B cells), as well as signals transmitted via cytokines like IL-4 and IL-21, which are important for B cell expansion and maturation (King and Mohrs, 2009; Spolski and Leonard, 2008).

2.3 T follicular helper cells in the germinal centre reaction

GCs are micro-environments of secondary lymphoid tissues fundamental to the humoral immune response in which B cells undergo somatic hypermutation (SHM) and isotype class switch. A continuous cycle of SHM, proliferation, and consecutive selection is the basis of increased affinity of the B cell receptor and hence the specificity of protective antibody responses. B cells can enter a GC reaction after activation and leave it either as high-affinity plasma cells or as long-lived memory B cells, providing a lasting protection to the host by the production of high-affinity neutralising antibodies and the potential for quick re-activation. GC B cells depend on the expression of the master transcription factor Bcl-6 and have sugar structures on their surface that make them identifiable in flow cytometry and histology by staining with peanut agglutinin (PNA) and the GL7 antibody clone.

Specialised stroma cells residing in GCs, follicular dendritic cells, present immune complexes to the maturing B cells, which take up the antigen and present it to specialised $CD4^+$ T cells, thus competing for survival signals (Vinuesa *et al.*, 2010). These specialised $CD4^+$ T helper cells located in the B cell follicle are called TFH cells and provide the survival signals by secretion of IL-21, expression of CD40L, and other factors (Crotty, 2011).

Follicular homing of $CD4^+$ T cells is a two-step chemotactic process: naïve T cells are retained in the T cell zone based on their expression of C-C chemokine receptor type (CCR7), whose ligands CCL19 and CCL21 are produced in T cell zones of lymph nodes and spleen. Upon activation, $CD4^+$ T cells downregulate CCR7, and some cells upregulate C-X-C chemokine receptor type 5 (CXCR5), the receptor for CXCL13, a chemokine abundant in B cell zones (Ansel *et al.*, 1999; Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). This allows T cell migration to the interfollicular zone, from where T cells migrate to the B cell follicle, differentiating to TFH cells (Kerfoot *et al.*, 2011). TFH cells are routinely identified by their high expression of programmed death-1 (PD-1; Haynes *et al.*, 2007), CXCR5, and their master transcription factor Bcl-6 (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009).

Development of TFH and GC B cells is interdependable: GC B cells do not develop without sufficient T cell help (Yu *et al.*, 2009). Prolonged antigen presentation, which under physiological, antigen-limiting conditions can only be provided by B cells, has been shown to be necessary for TFH cell differentiation (Haynes *et al.*, 2007; Deenick *et al.*, 2010; Goenka *et al.*, 2011). The interaction of T and B cells is stabilised by co-stimulatory molecules, such as those of the Slam family. Mice with Slam-associated protein (SAP) deficiency do not develop TFH cells (Qi *et al.*, 2008). As mentioned above, the frequency of TFH cells is also strongly reduced in mice deficient in ICOS or CD28, but no convincing description of a mechanism has been provided to date. One study has

suggested that induction of the TFH master transcription factor Bcl-6 is dependent on ICOS (Choi *et al.*, 2011; however, this claim will be challenged in this thesis. A detailed study on the influence of CD28 on TFH-characteristic molecules has also not been published to date.

Although early TFH development has been studied in detail, the focus of research seldom extends beyond the early phases of the GC development. Hence, relatively little is known about the signals necessary for TFH maintenance or about whether the pool of TFH cells is constantly replenished by non-TFH effector cells or if TFH cells are a self-renewing population. Further, the fate of TFH cells once a GC reaction has been terminated, had not been determined when this study was started: do TFH cells die or do they develop – similarly to B cells exiting the GC reaction – into long-lived memory cells supporting secondary immune reactions?

2.4 CD4$^+$ T cell memory

B cell memory is essential to provide immunity by the secretion of neutralising antibodies. However, about 10 % of CD4$^+$ T cells survive as predominantly quiescent cells after clearance of antigen, forming memory cells (Pepper and Jenkins, 2011). CD4$^+$ memory cells are proposed to exist in at least two distinct, heterogeneous subsets: effector memory (CD44^{hi} CD62L^{lo}) and central memory cells (CD44^{hi} CD62L^{hi}). Effector memory cells are thought to be immediate cytokine-secreting responders residential in nonlymphoid sites, providing protective immunity like long-lived plasma blasts, whereas central memory cells patrol the body and proliferate upon antigen encounter, providing protective immunity similarly to memory B cells (Sallusto *et al.*, 2004).

It has been shown with transfer experiments that Th1 cells can become memory cells, partly maintaining, partly showing flexibility in, their cytokine response – depending on the type of the secondary response (Löhning *et al.*, 2008). IFN-γ-producing Th1 cells mainly maintained the effector phenotype, whereas IFN-γ-negative effector cells equally adopted an effector memory and a central memory phenotype. The generation of memory cells from Th1 cells was further demonstrated with a polyclonal system (Pepper *et al.*, 2010); the same has been done for IL-4$^+$ Th2 cells, whereas there is little evidence for the progression of Th17 cells into the memory pool (reviewed in Pepper and Jenkins, 2011). It has been an open discussion if TFH cells can progress to become memory cells, as data in humans suggest, where CXCR5$^+$ CD4$^+$ T cells circulate in peripheral blood (Chevalier *et al.*, 2011; Morita *et al.*, 2011). However, no convicing evidence had been provided that these cells develop from TFH cells, neither in man, nor in mice (Crotty, 2011; Pepper and Jenkins, 2011).

One key characteristic of memory cells is their expression of the receptor for IL-7 (IL-7R), as IL-7 or TCR signals are essential for memory cell maintenance (Seddon *et al.*, 2003). Memory cells have mostly been isolated and studied from secondary lymphoid organs such as lymph nodes and spleens. These cells were slowly cycling *in vivo*, and the memory cell populations were decaying slowly with a half-life of 25–70 days (Pepper and Jenkins, 2011). It has been suggested that, like B cells, truly stably maintained CD4$^+$ memory cells are situated in the bone marrow, where they are never lost (Tokoyoda *et al.*, 2009).

2.5 Objective

TFH cells are pivotal in humoral immune responses, which are at the basis of vaccination strategies as well as the cause of many autoimmune diseases. In order to optimise vaccination strategies and develop better therapeutical approaches against autoimmune diseases, it is necessary to gain a deeper understanding of B cells and their interaction partner TFH cells. In this study, the differentiation of TFH cells was investigated using an adoptive transfer system. The molecular dependencies on the main co-stimulatory molecules CD28 and ICOS were analysed in the generation of TFH cells, as well as during their maintenance and therefore that of the GC reaction. A TFH re-transfer system of antigen-specific T cells was established, allowing the separate analysis of non-TFH and TFH effector cells. This system further allowed the analysis of the TFH cell fate after a GC reaction, as well as an in-depth characterisation of the newly described TFH memory cells.

3. Materials and Methods

3.1 Mice

OT-II (Barnden *et al.*, 1998) TCR-transgenic mice were crossed to B6PL mice (Thy1.1$^+$) to track cells in adoptive transfer experiments and further to ICOS KO (Özkaynak *et al.*, 2001) or CD28 KO (Shahinian *et al.*, 1993) mice to analyse the role of these molecules. For co-transfer experiments, OT-II and C57BL/6 (BL/6) mice were crossed, and cells isolated from the F1 generation (Thy-1.1$^+$ Thy-1.2$^+$) were used. T cells from Smarta (Oxenius *et al.*, 1998) TCR-transgenic mice (also crossed to B6PL mice) were used for experiments in which transgenic T cells stayed longer than 2 weeks in the recipients. B1-8i (Sonoda *et al.*, 1997) mice, whose B cells carry a BCR specific for the hapten nitrophenol (NP), were crossed with CκT mice (Zou *et al.*, 1993) and Ly-5.1 mice (CD45.1$^+$) to investigate antigen-specific B cells. All strains had been back-crossed at least 10 times to the BL/6 strain and were bred under specific pathogen–free conditions. Mice were used for experiments at an age of 8–12 weeks.

3.2 Adoptive Transfer Experiments

For primary transfers, transgenic splenic T cells were isolated and in some experiments enriched for naïve CD62L$^+$ cells by magnetic-activated cell sorting (MACS) using CD62L microbeads (Miltenyi Biotec). The relative frequency of ovalbumin (OVA)-specific T cells was determined by flow cytometry and 10^6 (analysis day 0 or 1) or 2.5×10^5 (all other analysis times) transgenic CD4$^+$ T cells were adoptively transferred by intravenous (i.v.) injection into BL/6 mice. Similarly, transgenic B cells were isolated and transferred when the antigen-specific B cell reaction was analysed. Cells were counted before transfer using Viacount reagent and a PCA capillary flow cytometer (Millipore).

For sorting of TFH and non-TFH cells, transgenic T cells from inguinal lymph nodes of a pool of antigen-challenged mice were first enriched by MACS using Thy-1.1 microbeads (Miltenyi Biotec) followed by sorting on a FACSAria II flow sorter (BD Biosciences). Transfer into second recipients was performed as above and cells from lymph nodes or spleen were analysed.

For *in vivo* proliferation analysis of sorted effector cells, sorted cells were mixed with 10^6 BL/6 splenocytes and labelled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) according to standard methods prior to adoptive transfer. Upon cell division, the CFSE is segregated equally between daughter cells (Lyons and Parish, 1994) resulting in a reduction of the CFSE signal.

16–24 hours after transfer, recipient mice were injected subcutaneously (s.c.) at the tail base with 20–50 µg cognate antigen, which was either NP-coupled (Biosearch Technologies) OVA (Sigma-Aldrich, abbreviated NP-OVA) for OT-II mice or Smarta protein (LCMV $GP_{61\text{-}80}$ peptide (Genscript) chemically coupled to mouse serum albumin (Calbiochem), abbreviated SMA) or NP-SMA for Smarta mice. Keyhole limpet hemocyanin (KLH; Pierce Biotechnology) was used as non-cognate antigen. The antigen was emulsified with Complete Freund's Adjuvant (CFA; Sigma-Aldrich) as adjuvant.

3.3 *In Vivo* Blockade

150 µg of the blocking anti-ICOS-L antibody MIL-5733 (rat IgG2a; Frey *et al.*, 2010) were injected into the intraperitoneal cavity (i.p. injection) of mice to achieve systemic distribution and block signal transduction via ICOS. 240 µg of the chimeric protein CTLA-4-humanIg (CTLA-4-Ig), a high-avidity ligand of B7, were injected to block signal transduction via CD28 (Linsley *et al.*, 1991; Linsley *et al.*, 1992). Injection was repeated the next day for MIL-5733 and daily for CTLA-4-Ig. MIL-5733 was free of endotoxins (< 0.5 pg/mg) and CTLA-4-Ig was either free or had low levels of endotoxins (< 14 pg/mg). Equal amounts of a rat IgG2a antibody (clone 1D10, which does not bind any epitope in the mouse) or human Ig (Kiovig; Baxter Deutschland GmbH) were injected as control where appropriate.

3.4 Flow Cytometry

Single cell suspensions from lymph nodes or spleen were stained with different combinations of the following monoclonal antibodies conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll, phycoerythrin-cyanine dye-7 (PE-Cy7), Alexa Fluor 647, allophycocyanin, Alexa Fluor 700, allophycocyanin-Cy7, Pacific Blue, Pacific Orange, Cascade Yellow, or Brilliant Violet 711: KT3 (anti-CD3), YTS 191.1 or RM4-5 (anti-CD4), 53-6.72 (anti-CD8), 5C6 (anti-CD11b), IM7.8.1 (anti-CD44), RA3-6B2 (anti-B220), MEL-14 (anti-CD62L), OX-7 (anti-Thy-1.1), HO13 or 53-2.1 (anti-Thy-1.2), 2G8 (anti-CXCR5), J43 or 29F.1A12 (anti-PD-1), 4B12 (anti-CCR7), A7R34 (anti-IL-7R), MIC-2043 (anti-ICOS) (Burmeister *et al.*, 2008), and PNA and GL7 (anti-GC B cells). Streptavidin-PE-Cy7 was used as secondary reagent for biotinylated antibodies. Fc receptors were blocked with 100 µg/ml 2.4G2 (anti-CD16/32) prior to staining. Antibodies were bought from BioLegend, eBioscience, BD Biosciences, or were purified from hybridoma supernatants and coupled to fluorophores by standard procedures. For intracellular staining of Bcl-6 and CD40L, FITC-conjugated or Alexa Fluor 647–conjugated GI191E/A8 (BD Biosciences), PE-conjugated MR1 (BioLegend), and the FoxP3 Staining Buffer Set from eBioscience were used.

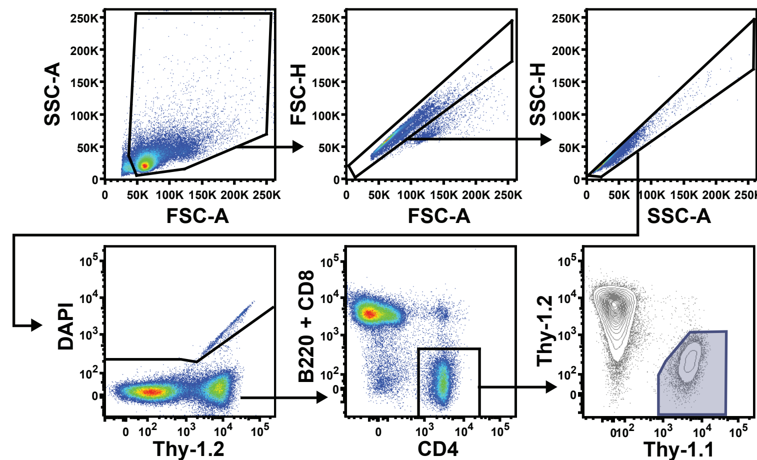


Figure 1 Exemplary gating strategy for the identification of transgenic T cells. Upper panel: Identification of cells according to size (forward scatter; FSC-A) and granularity (side scatter; SSC-A) and gating on singlets. Lower panel: Gating on live ($DAPI^-$) cells, followed by identification of $CD4^+$ T cells ($B220^-$ $CD8^-$ $CD4^+$) and, among these, transgenic T cells (Thy-1.1^+ Thy-1.2^-, blue).

To discriminate dead cells, either 0.33 µM 4',6-diamidino-2-phenylindole (DAPI; Merck) was added to live cells immediately before analysis or cells were incubated on ice for 25 min with 1.34 µM Pacific Orange succinimidyl ester (Invitrogen) prior to fixation (modified protocol from Perfetto *et al.*, 2006). Up to 2.5×10^7 events were analysed on an LSR II flow cytometer, FACSAria II flow sorter (405 nm, 488 nm, and 633 nm excitation) or LSR Fortessa (additionally 561 nm excitation; all BD Biosciences). Analysis gates were set on live cells defined by scatter characteristics and exclusion of DAPI or Pacific Orange–positive cells (Figure 1). Data were further analysed with FlowJo Software (Treestar).

3.5 Analysis of Cytokine Production

For analysis of cytokine production, cells were re-stimulated *in vitro* in $R10F^+$/β-ME (RPMI1640 (Biochrom) + 10 % FCS (Gibco) + 100 µg/ml Penicillin and Streptomycin (PAA Laboratories GmbH) + 50 µM β-Mercaptoethanol) with 10 ng/ml PMA and 10 µg/ml ionomycin (both Sigma-Aldrich) for 2–4 hours or 10 µg/ml $OVA_{323\text{-}339}$ peptide for 2 hours with addition of 5 µg/ml brefeldin A (Sigma-Aldrich) for the last 2–3 hours. After staining of cell surface antigens and live/dead stain with Pacific Orange or Alexa 700, cells were fixed with 2 % paraformaldehyde (Merck) for 20 minutes and permeabilised with 0.5 % saponin (Sigma-Aldrich). Unspecific binding sites were blocked with 100 µg/ml 2.4G2 and 50 µg/ml purified rat Ig (Nordic), and cells were stained intracellularly with the following fluorophore-conjugated mAb: PE-Cy7-conjugated BVD6-24G2

(anti-IL-4), Alexa Fluor 700–conjugated TC11-18H10 (anti-IL-17), PE-conjugated AN18.17.24 (anti-IFN-γ), or an IL-21R-Fc chimera (R&D Systems), followed by Cy5-conjugated or Alexa Fluor 647–conjugated goat anti-human IgG serum (Jackson ImmunoResearch). Cells were then analysed by flow cytometry as described above.

3.6 Histology

Lymph nodes were embedded in TissueTek OCT (Sciences Services) compound, frozen in liquid nitrogen and 8 µm cryostat sections were prepared. After fixation in acetone and blocking of unspecific binding sites with Casein Solution (Vector Laboratories) supplemented with 2.4G2 (100 µg/ml), sections were stained with FITC-coupled OX-7 (anti-Thy-1.1), digoxigenin-coupled GL7 (anti-GC B cells), and Alexa Fluor 594–coupled 11-26c (anti-IgD) or RA3-6B2 (anti-CD45R/B220). Signals for Thy-1.1 and GL7 were amplified with peroxidase-coupled anti-FITC and anti-digoxigenin followed by Alexa Fluor 488–coupled or Alexa Fluor 647–coupled tyramid (Invitrogen). Prior to and between the two amplification steps, peroxidase was inactivated with H_2O_2 (generated *in situ* with glucose and glucose oxidase). Nuclei were counterstained with DAPI (100 ng/ml). Images were captured on an LSM 780 with ZEN2010 imaging software (Carl Zeiss).

3.7 Statistical Analysis

All data were analysed using GraphPad Prism 5. Differences between the means of groups were calculated using two-tailed Student's t-tests. A p-value below 0.05 was considered as significant.

4. Results

4.1 Activated effector T cells express TFH hallmark proteins before the emergence of TFH cells

Antigen-specific T cells are rare in naïve mice and in the early initiation phases of immune reactions. To gain a better understanding of the generation of TFH cells, expression kinetics of TFH hallmark proteins were established. To analyse early activation signals, antigen-specific T cells had to be increased in frequency. To this end, naïve transgenic $CD4^{+}$ T cells from OT-II mice were transferred into immunocompetent BL/6 recipients, which were subsequently immunised with an emulsion of the adjuvant CFA and NP-OVA, the cognate antigen for $CD4^{+}$ T cells from OT-II mice, and the draining lymph nodes were harvested at different times after injection of antigen. Antigen-specific T cells expanded (data not shown) and showed an activated phenotype by day 1, as indicated by the upregulation of PD-1 (Figure 2A). 6 days after antigen challenge, double-positive cells for PD-1 and CXCR5 appear as a separate population, which increases in size by day 8. These surface molecules have routinely been used as surrogate markers for TFH cells. It is therefore possible to generate antigen-specific TFH cells in the adoptive transfer system with $CD4^{+}$ T cells from OT-II mice.

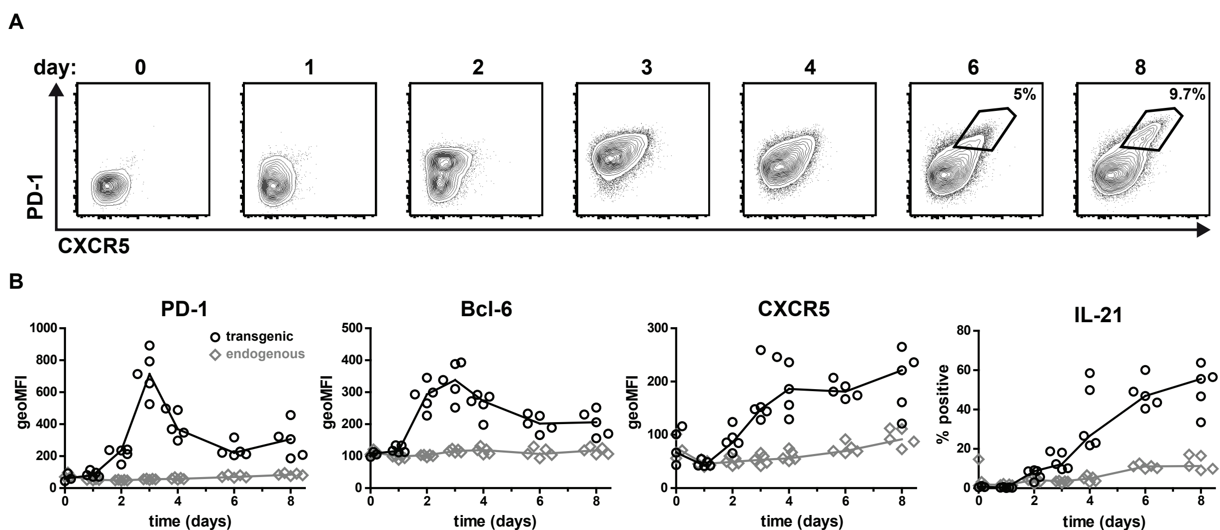


Figure 2 Expression kinetics of TFH hallmark proteins. Naïve OVA-specific T cells were transferred into BL/6 mice subsequently injected with NP-OVA in CFA. **A** Flow cytometric plots show transgenic $CD4^{+}$ T cells at indicated days after antigen challenge. Numbers in the plots indicate the percentage of cells within the gates. **B** The geometric MFI and percentage of positive transgenic (black circles) and endogenous (grey diamonds) T cells is shown for each protein. IL-21 expression was analysed after two hours *in vitro* re-stimulation with PMA + ionomycin. Each symbol represents one mouse (n = 5), lines connect medians. Data are representative of 2–3 independent experiments.

Bcl-6 has been identified as the master-transcription factor of TFH cells, being both necessary and sufficient for the generation of TFH cells (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009), and in early 2011 staining of Bcl-6 in flow cytometry became possible. IL-21 is a cytokine necessary for the generation of TFH cells, as well as B cell proliferation and hypermutation in germinal centres, and it has been shown that TFH cells express the highest levels of IL-21 mRNA compared to other T helper cells (Spolski and Leonard, 2008); however, no flow cytometric data of IL-21 protein expression by TFH cells *in vivo* had been published. After establishing staining procedures for Bcl-6 and IL-21, it became possible to follow the expression kinetics of these TFH hallmark proteins during the differentiation of TFH cells. Therefore, I analysed the expression of PD-1, CXCR5, Bcl-6, and the capacity to produce IL-21 of transgenic T cells at different days after antigen challenge *in vivo*.

All analysed markers showed an increased expression 2 days after antigen challenge (Figure 2B), with expression patterns differing from there on: PD-1 and Bcl-6 peaked at day 3, thereafter the expression was reduced to an intermediate level but stayed higher than on naïve or endogenous T cells. The expression of CXCR5 and the potential to secrete IL-21 was gradually increased on the total transgenic T cell population throughout the time period observed (Figure 2B). Taken together, these data show that some of the molecules specifically expressed by TFH cells at day 8 can be highly expressed by activated T cells at day 3 of an immune reaction.

Since more transgenic T cells had acquired the potential to produce IL-21 by day 8 than there are transgenic TFH cells at that time, TFH (PD-1$^+$ CXCR5$^+$ CD4$^+$ T cells, further verified by staining Bcl-6, Figure 3A) and antigen-specific non-TFH effector cells (PD-1$^-$ CXCR5$^-$) were analysed separately on day 8: as shown in Figure 3B, there are only marginal differences in the potential to produce IL-21 between transgenic TFH and non-TFH cells when cells are re-stimulated with

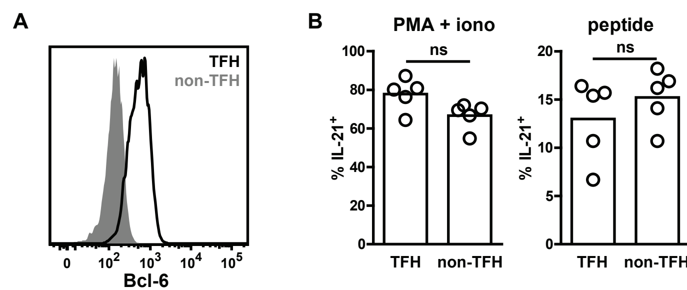


Figure 3 Expression of Bcl-6 and IL-21 by TFH and non-TFH transgenic CD4$^+$ effector cells. Naïve OVA-specific T cells were transferred into BL/6 mice subsequently injected with NP-OVA in CFA and cells from draining lymph nodes were analysed on day 8 after antigen challenge. **A** Bcl-6 expression by TFH cells (PD-1/CXCR5 double-positive, black line) and non-TFH effector cells (PD-1/CXCR5 double-negative, grey shaded). **B** Percentage of IL-21$^+$ T cells within TFH or non-TFH effector cells defined as in (A). Cells were re-stimulated with PMA + ionomycin (left) or OVA-peptide (right) for two hours. Each symbol represents one mouse (n = 5), bars indicate the mean. Data are representative for over five (A) or were obtained from one experiment (B).

PMA and ionomycin, and there are no differences when cells are re-stimulated more physiologically with peptide via their TCRs.

The data characterise the adoptive transfer system as a useful tool in analysing the differentiation of TFH cells. They further show that TFH hallmark proteins are broadly upregulated on transgenic T cells two days after encounter of antigen and that expression of Bcl-6 peaks at day 3, many days before a TFH population is established. The data further call into question the specificity of IL-21 secretion by TFH cells, which had previously only been analysed on an mRNA level in the literature.

4.2 Signalling via CD28 and its family member ICOS is essential for the development of a TFH cell population

CD28 and ICOS are co-stimulatory molecules expressed by – and important for the activation of – T cells. Mice with a deficiency in the expression of CD28 or ICOS have been described to have the same phenotypic defect after challenge with antigen: small or absent germinal centres, reduced Ig levels, and strongly reduced frequencies of polyclonal TFH cells (Walker *et al.*, 1999; Linterman *et al.*, 2009; Dong *et al.*, 2001; Akiba *et al.*, 2005; Bossaller *et al.*, 2006). The aim of our group was to delineate the precise role of these two molecules in the generation of a stable TFH cell population. After having established the adoptive transfer of OT-II T cells as a suitable model for investigating TFH cell generation, the second step was to verify that the defect in the generation of a TFH cell population was reproducible when the molecules were missing on transgenic T cells only.

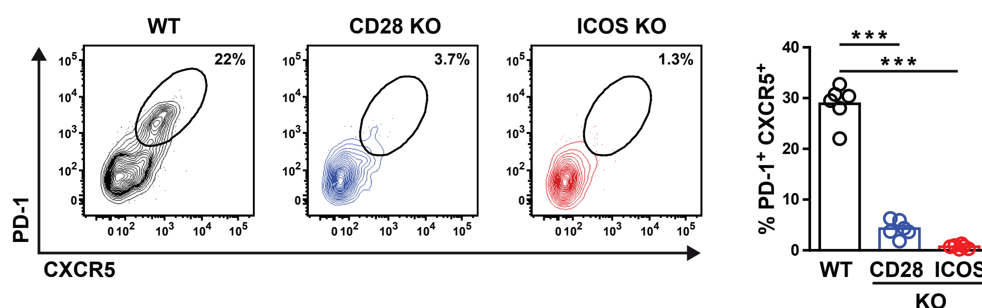


Figure 4 CD4+ T cells lacking ICOS or CD28 do not develop into a stable TFH cell population. 2.5×10^5 CD62L^{hi} OT-2 WT (black), OT-2 CD28 KO (blue) or OT-2 ICOS KO (red) T cells were transferred into naïve BL/6 mice subsequently injected with NP-OVA in CFA. Cells were isolated from draining lymph nodes eight days after antigen challenge. Representative plots of flow cytometric analysis of transgenic CD4+ T cells and statistical evaluation are shown. Numbers in the plots indicate the percentage of cells in the gates. Each symbol represents one mouse, bars indicate the mean. Data are representative of two independent experiments (n = 5–6). *** = $p < 0.001$

I therefore transferred transgenic T cells from either wild-type ("WT") OT-II mice or from OT-II mice with a knockout of the CD28 ("CD28 KO") or ICOS ("ICOS KO") encoding gene into naïve BL/6 recipients. Eight days after antigen challenge, the draining lymph nodes were harvested and the phenotype of transgenic T cells was analysed. A substantial fraction of the transferred WT OT-II $CD4^+$ T cells adopted the TFH phenotype according to the co-expression of PD-1 and CXCR5 (Figure 4, black), whereas OT-II CD28 KO or OT-II ICOS KO $CD4^+$ T cells developed a much smaller population of TFH cells (Figure 4, blue and red, respectively). Analysis of additional TFH markers confirmed the reduced frequency of TFH cells when CD28 or ICOS signalling were missing (data not shown). Hence, the adoptive transfer system of T cells from knockout OT-II mice is suitable to investigate the role of CD28 and ICOS in the generation of TFH cells.

4.3 The defect in the generation of a TFH population is T cell–intrinsic in CD28 KO and ICOS KO T cells

Since TFH cells need signals from activated B cells for their differentiation (Haynes *et al.*, 2007; Deenick *et al.*, 2010; Goenka *et al.*, 2011), the defect of the formation of TFH cells when ICOS or CD28 are missing can have two explanations. The co-stimulatory molecules can either be directly necessary for the generation of TFH cells via a T cell–intrinsic effect. Alternatively, the lack of CD28 or ICOS could cause T cells to develop a diminished early B cell helper capacity, resulting in a lack of B cell activation, formation of germinal centres, and hence diminished antigen presentation and provision of activation signals from B cells to T cells. In this scenario, the effect would be indirectly mediated via B cells and therefore be T cell–extrinsic. To answer the question of the dependence on adequate B cell help for the generation of TFH cells, I have used a co-transfer system in which germinal centres develop normally due to the presence of WT OT-II cells (R. Franke, personal communication) and the analysis of T cells with a specific molecular defect is still possible. Instead of transferring WT and KO T cells into separate hosts, equal numbers of $CD4^+$ T cells from WT and KO OT-II mice were co-transferred into the same hosts. WT OT-II mice had previously been crossed with BL/6 mice, so that they co-expressed Thy-1.1/Thy-1.2. In this system, transgenic WT and KO cells could be discriminated eight days after antigen challenge (Figure 5A) and WT OT-II cells generated substantial populations of PD-1/CXCR5 double-positive cells (black, Figure 5B), showing that TFH cells can develop in this system. Nonetheless, OT-II cells deficient in CD28 (upper panel, blue) or ICOS (lower panel, red) failed to generate comparable TFH cell populations (Figure 5B), pointing at T cell–intrinsic roles for both molecules.

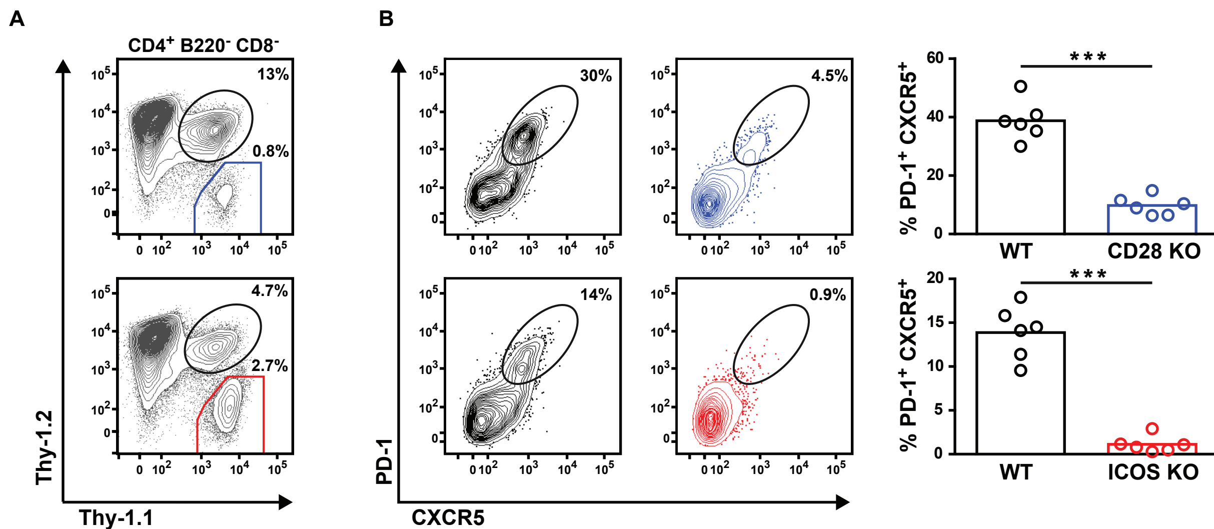


Figure 5 ICOS and CD28 have a T cell–intrinsic effect on the generation of a stable TFH cell population. Co-transfer of naïve WT (black) with either naïve CD28 KO (blue) or naïve ICOS KO (red) OVA-specific $CD4^+$ T cells into BL/6 mice subsequently injected with NP-OVA in CFA. Cells were isolated from draining lymph nodes eight days after antigen challenge. **A** Flow cytometric analysis showing the discrimination between WT (black), KO (blue or red) and endogenous (not gated, $Thy\text{-}1.1^-$ $Thy\text{-}1.2^+$) $CD4^+$ T cells. **B** Flow cytometric analysis of transgenic $CD4^+$ T cells as gated in (A). Representative plots and statistical evaluation are shown, numbers indicate the percentage of cells in the gates. Each symbol represents one mouse, bars indicate the mean. Data are representative of at least two independent experiments. *** = $p < 0.001$

4.4 ICOS, in contrast to CD28, is not essential for the induction of Bcl-6 and molecules characteristic for B cell help

A T cell–intrinsic effect of ICOS and CD28 on the generation of a TFH cell population could either be caused by a defect in the differentiation towards the TFH phenotype or by a defect of its maintenance. To investigate the role of CD28 and ICOS in the differentiation of TFH cells, the expression of molecules characteristic for TFH cells at the early peak response (day 3, as determined in Figure 2) was analysed in the co-transfer system of WT and KO OT-II CD4⁺ T cells.

Bcl-6 expression was independent of ICOS signalling in the early immune response, as ICOS KO and WT OT-II cells expressed the TFH master transcription factor at similar levels (black and red lines in the upper panel of Figure 6A). In contrast, CD28 (lower panel, blue) was necessary for proper induction of Bcl-6 (Figure 6A). Similarly, molecules essential for B cell help (CD40L,

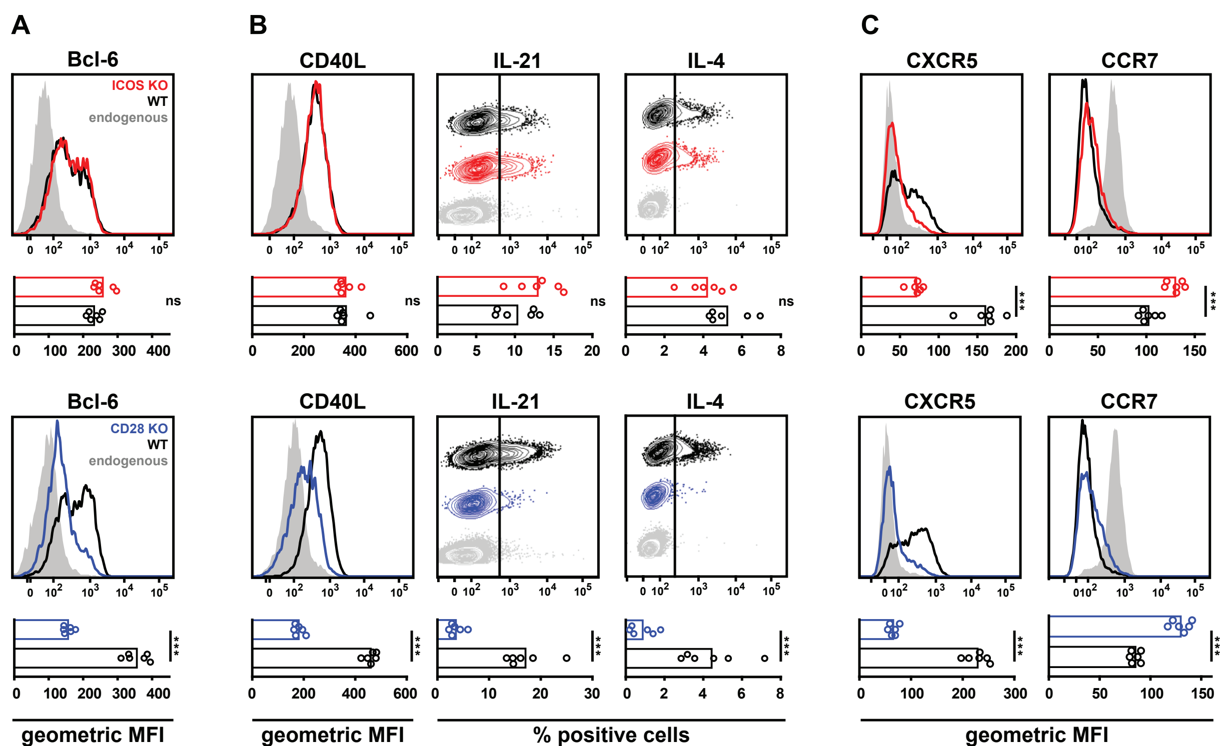


Figure 6 CD28 and ICOS have distinctive roles in the generation of TFH cells. Co-transfer of naïve WT (black) with either naïve ICOS KO (red) or naïve CD28 KO (blue) OVA-specific CD4⁺ T cells into BL/6 mice subsequently injected with NP-OVA in CFA. Cells were isolated from draining lymph nodes three days after antigen challenge. Endogenous T cells are represented as a control in grey. Flow cytometric analysis of transgenic or endogenous CD4⁺ T cells for **A** Bcl-6, **B** molecules important for B cell help, and **C** chemokine receptors. Plots show concatenated samples of all mice per group. Where histograms are shown, the geometric MFI is plotted, otherwise the percentage of positive cells according to the gate indicated by the vertical line is shown. Cells were re-stimulated with OVA-peptide for two hours for the analysis of IL-21 and IL-4 producing T cells. Each symbol represents one mouse, bars indicate the mean. Data are representative of 2–5 independent experiments. *** = $p < 0.001$, ns = not significant

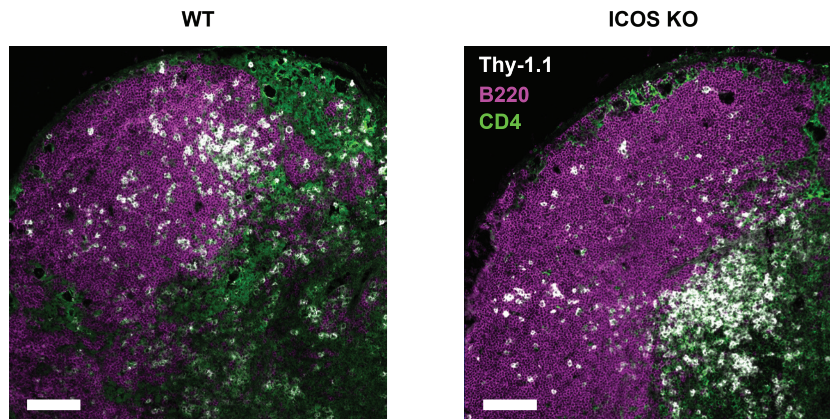


Figure 7 ICOS-deficient T cells can locate to the B cell follicle. Transfer of WT (left) or ICOS KO (right) OVA-specific $CD4^+$ T cells. Lymph nodes were harvested three days after antigen challenge with NP-OVA in CFA and analysed by fluorescence microscopy. Localisation of of transgenic $CD4^+$ T cells is visualised by staining transgenic T cells (white, Thy 1.1), the B cell zone (magenta, B220), and the T cell zone (green, CD4) with fluorophore-coupled antibodies. Confocal image (20 × magnification), scale bar indicates 100 µm.

IL-21 and IL-4) were equally expressed by ICOS KO and WT OT-II T cells; however, they were not properly induced in the CD28 KO cells (Figure 6B). Only the upregulation of the chemokine receptor CXCR5 and downregulation of the chemokine receptor CCR7 were both influenced by missing CD28 signalling as well as missing ICOS signalling (Figure 6C).

Since CXCR5 and CCR7 are important for T cell positioning within the lymph node, localisation of ICOS KO and WT OT-II T cells was compared in histology three days after antigen challenge. Histological analysis of whole lymph node sections revealed that both WT and ICOS KO $CD4^+$ T cells located mainly at the border between the T cell zone and the B cell zone (data not shown), and cells from both groups could be found in similar proportions in the B cell follicles (Figure 7). The impaired regulation of CXCR5 and CCR7 did therefore not result in defective positioning of ICOS KO T cells.

The data suggest that CD28 is an essential molecule for the induction of the TFH phenotype, since $CD4^+$ T cells defective for CD28 fail to induce the TFH master transcription factor Bcl-6 and do not upregulate IL-21, IL-4 and CD40L to become B cell helpers. On the other hand, ICOS KO T cells should be able to become TFH cells since they express Bcl-6, IL-21, IL-4, and CD40L to a smilar extent as wild-type cells, and their impaired regulation of CXCR5 and CCR7 is not reflected in a physiological positioning defect within the lymph node.

Thus, CD28 and ICOS seem to have different roles in the establishment of a TFH population: CD28 is important for the induction of TFH cells, whereas ICOS is not. Without a role of ICOS in the induction of TFH cells, the molecule was likely to play a role in the maintenance of the TFH phenotype. In order to investigate the role of ICOS (and CD28) in the maintenance of TFH cells, a system was needed in which TFH cells could develop normally and signalling via CD28 and ICOS could be effectively interrupted at a given time.

4.5 Blocking of ICOS and CD28 signalling via MIL-5733 and CTLA-4-Ig is as effective as using knockout mouse strains

The ligands of ICOS and CD28 are known: ICOS-L and B7.1/B7.2 (CD80/CD86), respectively (Greenwald *et al.*, 2005). Our lab has developed the blocking anti-ICOS-L antibody MIL-5733 (Frey *et al.*, 2010). When this antibody is applied, ICOS-L can no longer bind to ICOS and no further signals are transmitted into the T cell (Figure 8A). A similar system is available to block signalling via CD28 using the high-avidity B7-ligand CTLA-4. When the chimeric protein CLTA-4-Ig is applied, B7 on APCs is saturated and no more signals are transmitted via CD28 into T cells (Figure 8A).

To test the efficacy of the blocking, transgenic T cells were transferred and mice challenged with antigen as before. Either blocking agent was injected thrice: at the day of transfer, the day of antigen challenge and one day later to interfere with signalling via ICOS and CD28 during initial T cell activation. The effects on T cell expansion were similar between the transfer of KO OT-II cells (Figure 8B) and the system where signalling was interrupted using the blocking agents (Figure 8C): CD28 signalling was essential for proper expansion of transgenic T cells and a lack of ICOS signalling had only minor effects on T cell expansion at day 3. The regulation of a range of activation markers was also very similar in both systems, as can be seen by comparing panel D (KO transfer) with panel E (blockade) in Figure 8.

Blocking with MIL-5733 or CTLA-4-Ig is thus similarly effective in abolishing signalling via ICOS or CD28 as is a knockout of the genes ICOS and CD28. Blocking can therefore be used to abrogate signalling at later times when the initial T cell activation has occurred normally and TFH cells have developed.

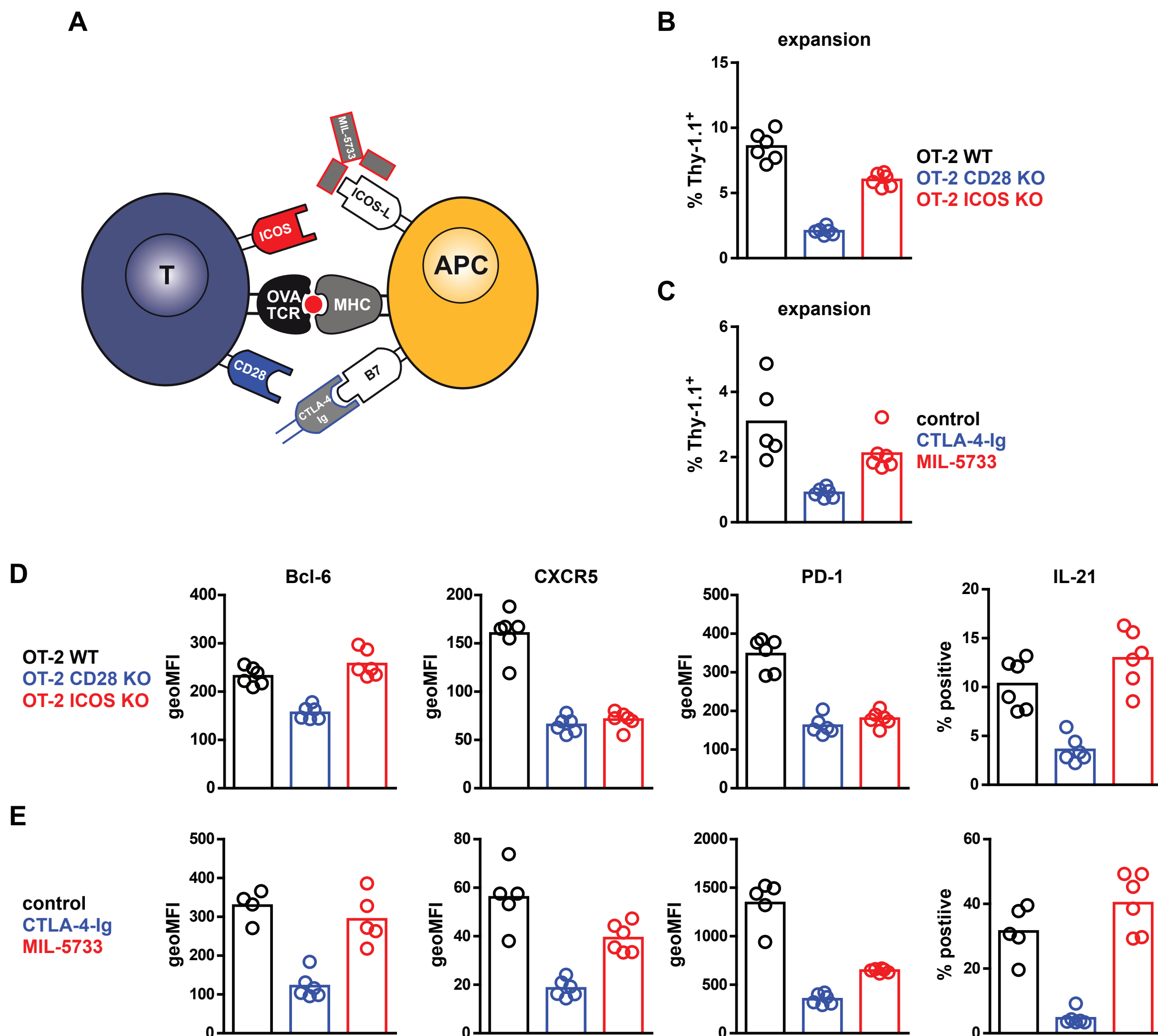
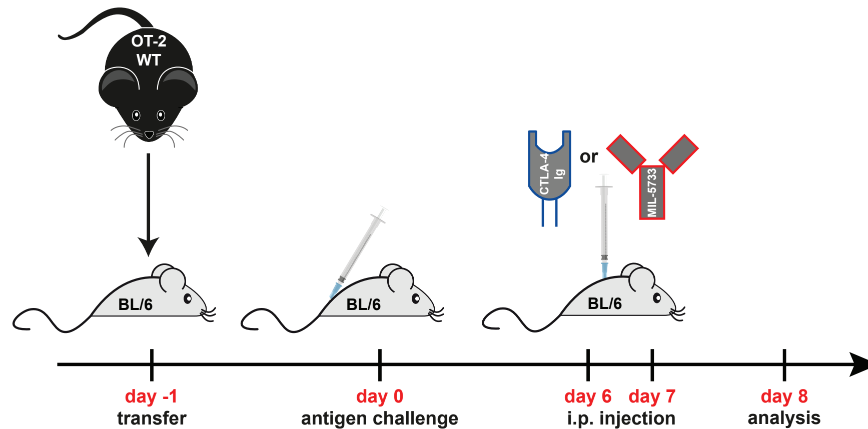


Figure 8 Application of MIL-5733 and CTLA-4-Ig effectively blocks signalling via ICOS and CD28. **A** Schematic representation of the blocking reagents binding to their targets. **B** and **D** Co-transfer of WT (black) with either ICOS KO (red) or CD28 KO (blue) OVA-specific CD4⁺ T cells into BL/6 mice subsequently injected with NP-OVA in CFA. **C** and **E** Transfer of OVA-specific WT CD4⁺ T cells into BL/6 mice challenged as above. At the day of transfer, day of antigen challenge, and day 1 of the immune reaction, 240 µg CTLA-4-Ig (blue) or 150 µg MIL-5733 (red) were injected i.p., black shows the not-injected control. Cells were isolated from draining lymph nodes three days after antigen challenge. (B) and (C) show the frequency of transgenic T cells relative to all CD4⁺ T cells; (D) and (E) show the geometric MFI or percentage of positive transgenic cells. Each symbol represents one mouse, bars indicate the mean. Data are representative of two independent experiments.

4.6 Blocking ICOS signalling reduces the frequency of transgenic T cells with the TFH phenotype

After having established that CD28 signals are essential in the development of TFH cells, but having found that Bcl-6 induction is ICOS-independent, we used the blocking systems to specifically look at the role of CD28 and ICOS in the maintenance of fully differentiated TFH cells. 6 days after injection of antigen into mice which had received an adoptive transfer, TFH cells started to develop (see Figure 2A). At this time, the blocking agents were administered twice and draining lymph nodes were analysed 48 hours after beginning of blocking (Figure 9A).

A



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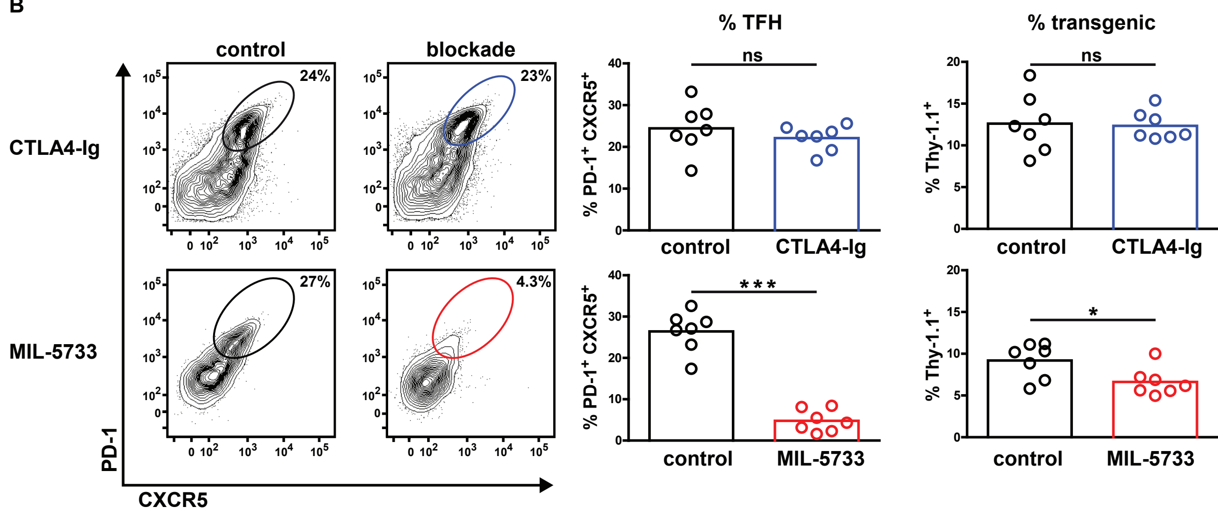

Figure 9 Application of the ICOS-L-blocking antibody MIL-5733 reduces the frequency of transgenic T cells with the TFH phenotype. **A** Schematic representation of the experimental system. **B** TFH phenotype of transgenic T cells on day 8 of the immune reaction, 48 hours after the first injection of blockade reagents. From left to right: representative flow cytometric plots (numbers indicate the percentage of cells within the gates), statistical analysis of the frequency of TFH ($PD\text{-}1^+$ $CXCR5^+$) cells within the transgenic T cells, frequency of transgenic T cells among isolated $CD4^+$ T cells. Each symbol represents one mouse (n = 7), bars indicate the mean. Data are representative of at least two independent experiments. *** = $p < 0.001$, * = $p < 0.05$, ns = not significant

Injection of CTLA-4-Ig did not have an effect on neither the frequency of PD-1⁺ CXCR5⁺ cells amongst transgenic T cells, nor on the frequency of total transgenic T cells (Figure 9B, upper panel, blue). In contrast, application of MIL-5733 resulted in a strong decrease of transgenic T cells with the TFH phenotype and in a moderate reduction of total transgenic T cells (Figure 9B, lower panel, red). The phenomena observed were not only due to a decreased expression of PD-1 and CXCR5, TFH cells also got lost according to a number of other hallmark proteins (data not shown). The reduction of the TFH phenotype was not transient and could still be observed four and six days after the blockade (Figure 10A), although it was less apparent, as the TFH cell population was contracting at that time. In parallel, the frequency of germinal centre B cells, defined as the percentage of PNA^+ $GL\text{-}7^+$ cells amongst antigen-specific B cells, was also reduced (Figure 10B).

CD28 therefore does not play a role in the maintenance of TFH cells once that these have developed. ICOS, on the other hand, is important for maintaining germinal centres, as TFH and GC B cells are both reduced when ICOS signalling is abolished. To investigate whether the reduction of TFH cells is due to a loss from the lymph node of those transgenic T cells exhibiting the TFH phenotype or whether TFH cells reverse their phenotype, an experimental system to separately investigate the effect of blocking ICOS signalling on TFH and non-TFH cells was needed.

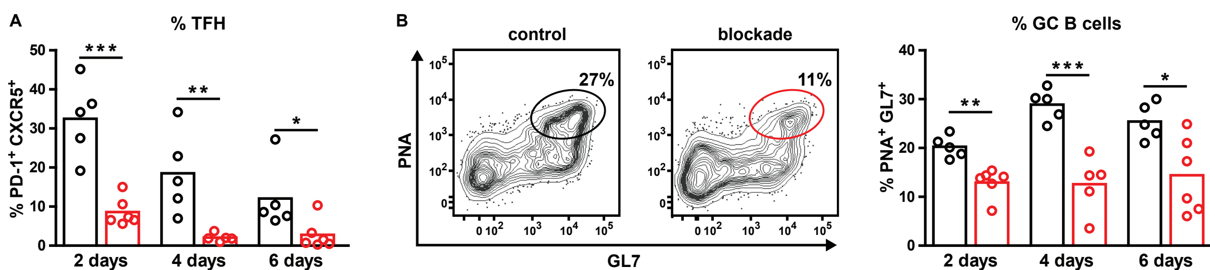

Figure 10 Effects observed after blocking ICOS signalling on TFH and GC B cells are not transient. **A** Analysis for TFH frequency as in Figure 9 at indicated times after beginning of blocking. **B** Experimental system as in (A), except that 10^6 transgenic B cells from B1-8i mice specific for nitrophenol were co-transferred on day -1. Statistical analysis of germinal centre B cells (PNA^+ $GL7^+$) among transgenic B cells is shown at indicated times after beginning of blocking. Each symbol represents one mouse (n = 5-6), bars indicate the mean. Data are representative of at least two independent experiments. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

4.7 Establishment of the re-transfer of *in vivo* generated transgenic T follicular helper cells

To analyse TFH and non-TFH cells separately, antigen-specific TFH and non-TFH effector cells had to be isolated, purified and re-transferred in high enough cell numbers to allow following their fate. Hence, transgenic TFH cells were generated *in vivo* as before. On day 7, draining lymph nodes were harvested and the cell suspension was enriched for transgenic T cells by MACS. This cell suspension was stained with fluorophore-coupled antibodies and submitted to FACS, separating

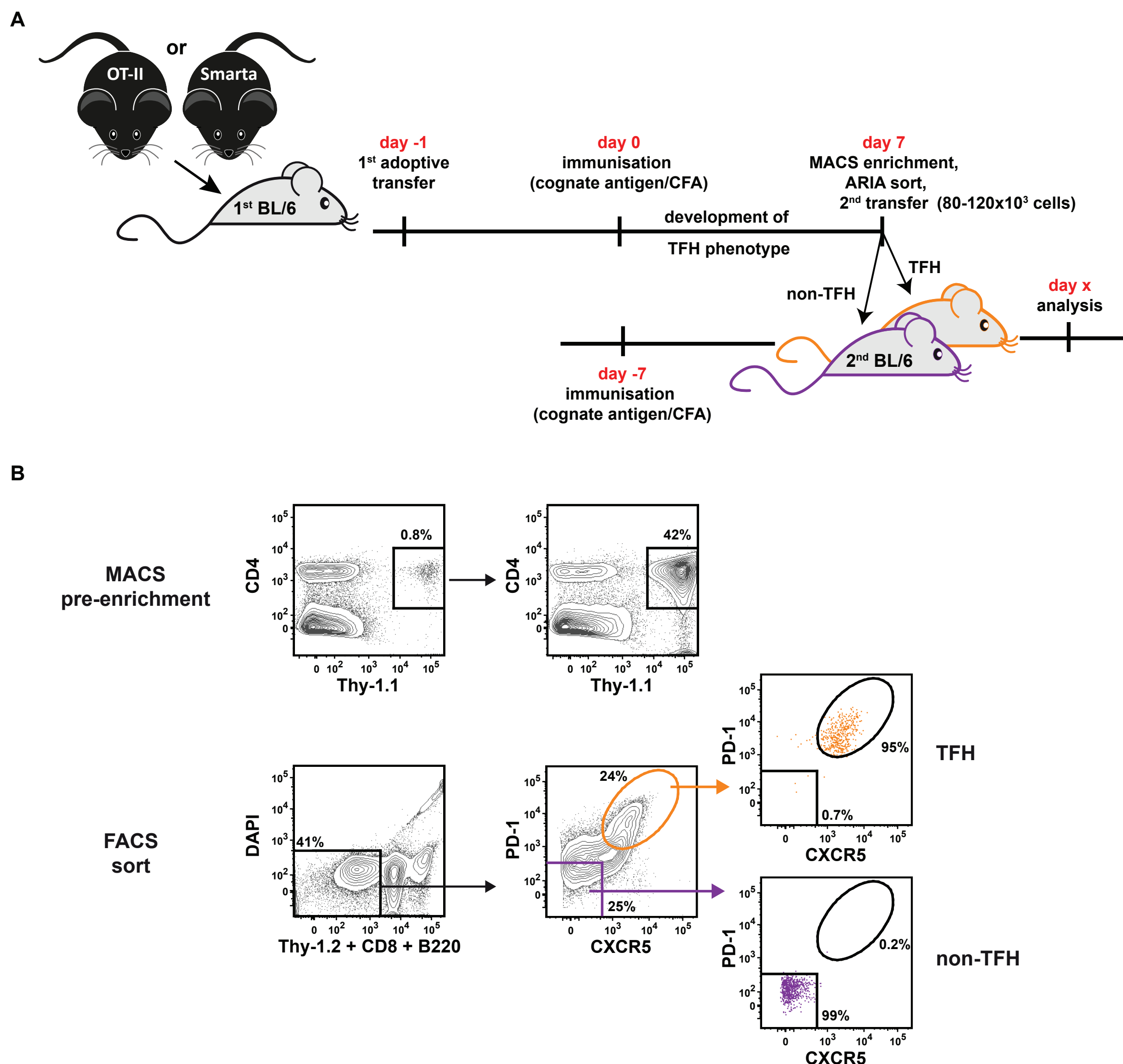

Figure 11 Re-transfer of *in vivo*–generated transgenic TFH cells. **A** Schematic representation of the *in vivo* generation of transgenic TFH cells and their re-transfer into secondary pre-immunised hosts. **B** Representative plots of the MACS enrichment procedure for Thy-1.1-positive cells and the FACS sort on day 7, sorting transgenic TFH (PD-1$^+$ CXCR5$^+$, orange) and non-TFH (PD-1$^-$ CXCR5$^-$, purple) effector cells. Numbers indicate the percentages of cells in the gates.

transgenic TFH and non-TFH cells (Figure 11A). Both populations could be sorted to above 95 % purity (Figure 11B). The purified TFH and non-TFH cells were adoptively transferred into separate mice which had been immunised in parallel with the donor mice to have ongoing germinal centre reactions to accommodate the re-transferred TFH cells (Figure 11A).

4.8 Distribution of re-transferred T effector cells in pre-immunised hosts

Activated T cells and naïve T cells differ in their expression of chemokine receptors and cell adhesion molecules, which potentially results in different homing characteristics. In order to investigate the distribution of the re-transferred effector T cells, TFH and non-TFH cells were transferred and recipients were sacrificed two days after transfer.

Although they are an extremely rare population, re-transferred effector T cells can be identified with confidence in flow cytometry (Figure 12A). The frequency of transgenic T cells in draining lymph nodes and in the spleen was double that of cells in the blood or isolated from the lungs (Figure 12B). When accounting for the cellularity of the analysed organs, it becomes apparent that re-transferred effector T cells are enriched in the draining lymph nodes and the spleen of recipient mice (Figure 12C). Added together, the analysis of draining lymph nodes and spleen account for approximately 15 % of all transferred cells, which is in accordance with the approximately 10 % of cells found in lymphatic organs after transfer of naïve T cells into naïve hosts (data not shown). It has to be considered, however, that antigen was present in this system. Thus, T cells would have proliferated and should have been most numerous in the draining lymph nodes, where antigen was abundant due to the subcutaneous injection.

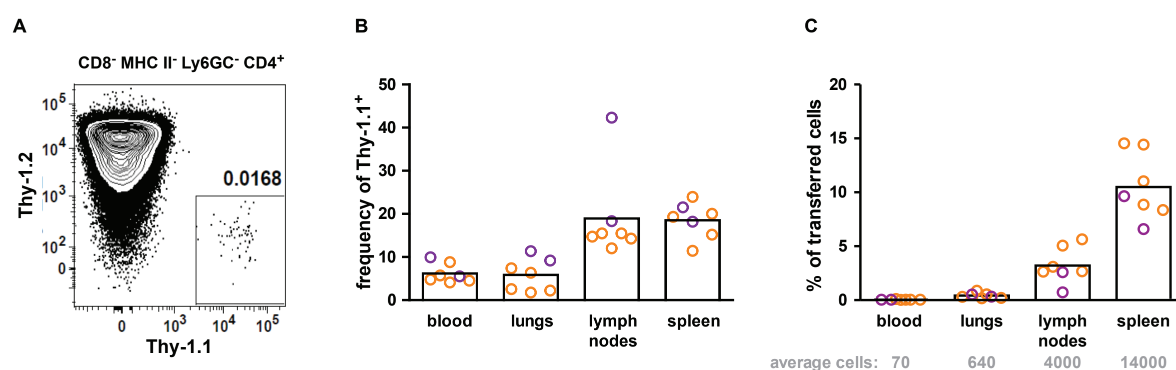

Figure 12 Distribution of re-transferred effector cells in pre-immunised hosts. Transfer as described in Figure 11. **A** Representative flow cytometric plot of re-transferred (non-TFH) effector cells in the spleen of pre-immunised mice on day 2 after transfer. **B** Flow cytometric analysis of the frequency of transgenic $CD4^+$ T cells as gated in (A) per 10^5 live cells. **C** Distribution of transgenic T cells expressed as percentage of re-transferred effector cells. Numbers in grey indicate an approximation of the absolute number of transgenic T cells present in the analysed organs. Each symbol represents one mouse (n = 7). Data were obtained from one experiment.

4.9 Re-transferred TFH and non-TFH effector cells differ in phenotype and proliferation

Instead of analysing the total transgenic T cell population, the re-transfer system allows for separate analysis of populations with certain characteristics at a given time. It was thus possible to investigate for the first time the proliferation of TFH and non-TFH effector cells separately, and to address the question if non-TFH cells continuously differentiate towards the TFH phenotype and thereby replenish the pool of TFH cells, or whether TFH and non-TFH populations exist independently of each other.

Transgenic TFH and non-TFH cells were labelled with the cell proliferation dye CFSE and transferred into pre-immunised recipients. Cells were analysed three days after transfer for their proliferation and expression of PD-1 and CXCR5 as markers for the TFH phenotype. TFH cells were mostly non-proliferating with about 90 % being undivided after three days (Figure 13A). Interestingly, only 40–80 % of re-transferred TFH cells exhibited the TFH phenotype, and the expression of PD-1 and CXCR5 correlated with proliferation (Figure 13B).

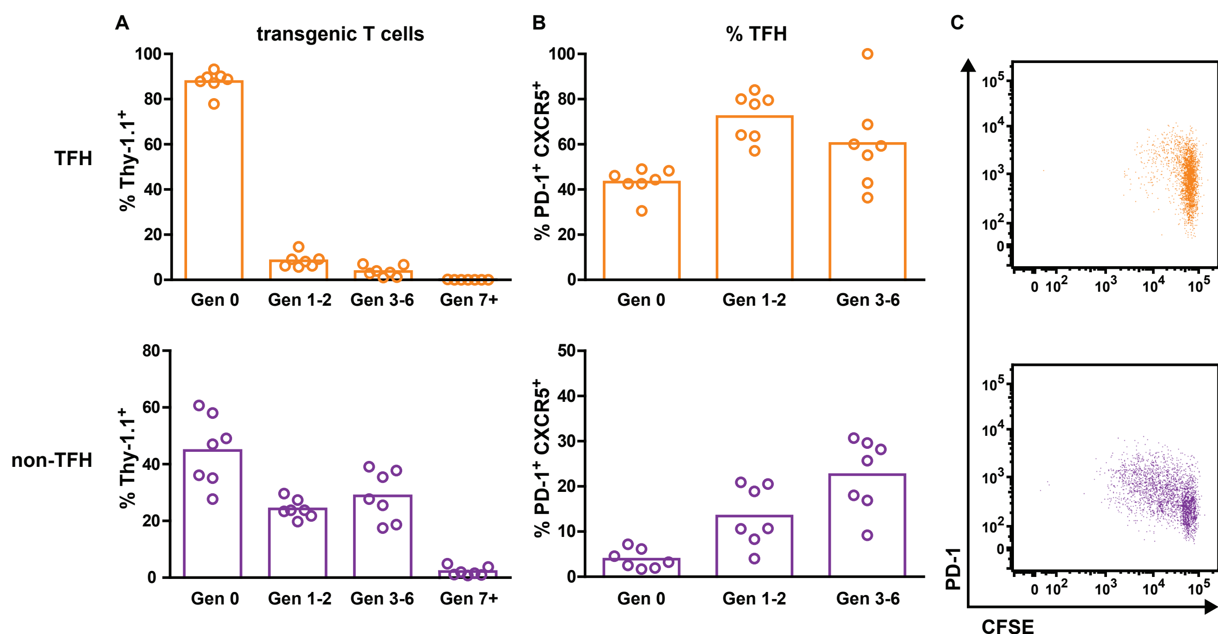

Figure 13 Proliferation and analysis of the TFH phenotype of re-transferred TFH and non-TFH effector cells. Transfer as described in Figure 11, with labelling of cells with CFSE before transfer. Cells from draining lymph nodes were analysed three days after re-transfer. Flow cytometric analysis of transgenic T cells with the TFH phenotype (upper panel) or the non-TFH effector phenotype (lower panel). **A** Proliferation pattern of transgenic CD4$^+$ T cells as determined by CFSE dilution assay. Numbers indicate the percentage of cells in a given generation (Gen). **B** Percentage of transgenic CD4$^+$ T cells with the TFH phenotype (PD-1$^+$ CXCR5$^+$) according to proliferation status. **C** Flow cytometric plot from concatenated data from 7 animals showing 3,000 events. Analysis of TFH phenotype was omitted for generations 7+ in (B) due to low cell numbers. Each symbol represents one mouse (n = 7), bars indicate the mean. Data were obtained from one experiment.

In contrast, 50 % of transgenic non-TFH effector cells had proliferated within three days, as indicated by strongly decreased CFSE levels within the cells (Figure 13C). Non-TFH effector cells also upregulated PD-1 and CXCR5 (Figure 13B). This acquisition of the TFH phenotype seemed to correlate with proliferation: cells that had proliferated more had a higher frequency of the TFH phenotype (Figure 13B), and Figure 13C illustrates that those cells with the lowest CFSE signals (cells which had proliferated the most) have the highest expression levels of PD-1 (and CXCR5, data not shown).

The data on non-TFH effector cells could be an indication that the pool of TFH cells in an ongoing germinal centre reaction is constantly being replenished by non-TFH effector cells adopting the TFH phenotype. Unfortunately, not all TFH cells maintain their phenotype upon re-transfer. This loss of phenotype could either be a representation of the contraction phase or an artefact caused by the stress from the isolation procedure, sort, and re-transfer. Although this points at a weakness in the re-transfer system, the difference in phenotype between cells from a TFH or non-TFH effector cell origin is significant. If both populations maintain further functional characteristics, the system should be suitable to investigate the populations separately.

4.10 Re-transferred TFH cells maintain functional characteristics of TFH cells

By definition, T cells that locate in the B cell follicle and provide B cell help are T follicular helper cells (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). It was therefore mandatory to test if re-transferred TFH cells fulfil these two criteria. Two days after re-transfer of TFH and non-TFH cells into pre-immunised recipients, draining lymph nodes were harvested and subjected to confocal microscopy to analyse the localisation of the re-transferred effector cells. Re-transferred TFH cells were preferentially located in the IgD$^+$ B cell follicle (Figure 14A, left panel), whereas non-TFH cells located predominantly in the IgD-negative T cell zone (Figure 14A, right panel).

To test if the re-transferred TFH cells are superior in providing B cell help, both cell types were transferred into CD28 KO mice, in which endogenous T cells would not provide B cell help (Linterman *et al.*, 2009). Naïve NP-specific B cells were transferred and mice were injected with NP-coupled antigen. Four days after antigen challenge, more non-TFH than TFH cells could be found in draining lymph nodes (Figure 14B), consistent with previous data. However, B cells transferred into the mice which had received TFH cells were more numerous than those in presence of non-TFH cells (Figure 14C), suggesting that re-transferred TFH cells are more effective at supporting B cell expansion than non-TFH cells. Hence, TFH cells migrate into the B cell follicle after re-transfer and are capable to provide better help to B cells than non-TFH cells, even when present in smaller numbers.

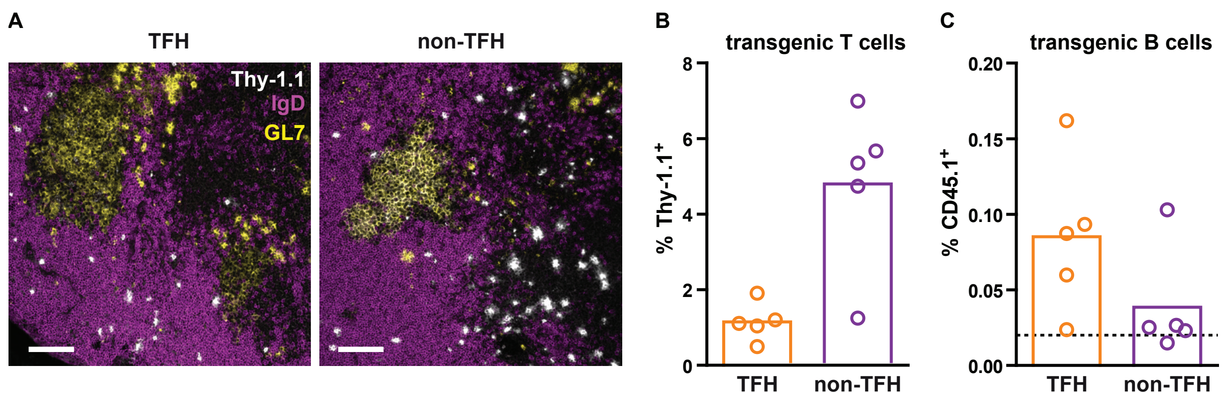

Figure 14 Functional characteristics of re-transferred TFH and non-TFH effector cells. **A** Transfer as described in Figure 11. Draining lymph nodes were harvested two days after transfer and analysed by fluorescence microscopy. Localisation of re-transferred TFH (left) and non-TFH (right) effector cells is visualised by staining transgenic T cells (white, Thy 1.1), the B cell zone (magenta, IgD), and GC (yellow, GL7) with fluorophore-coupled antibodies. Confocal image (20 × magnification), scale bar indicates 100 µm. (B) and (C) 1.3×10^5 TFH or non-TFH cells were transferred into naïve CD28 KO mice. Four days after transfer, mice additionally received 2×10^5 NP-specific B cells from B1-8i mice and were challenged with NP-coupled antigen in CFA. **B** Flow cytometric analysis of frequencies of transgenic T cells relative to all CD4$^+$ T cells and **C** frequencies of transgenic B cells relative to all B220$^+$ cells four days after antigen challenge. The dashed line represents the frequency of B cells in a mouse with no T cell transfer. Each symbol represents one mouse (n = 5), bars indicate the mean. Data were obtained from one experiment.

4.11 Blocking of ICOS signalling causes reversion of the TFH phenotype

Since the majority of adoptively re-transferred transgenic TFH and non-TFH cells maintain their phenotype and function, the system can be used to separately investigate the role of ICOS signalling in the maintenance of TFH and non-TFH cells. Transgenic TFH and non-TFH effector cells were generated and re-transferred into pre-immunised hosts. Cells were allowed to settle for one day, then ICOS blocking was started, and two days after beginning of the blocking, draining lymph nodes were analysed (Figure 15A).

In accordance with the analysis of total transgenic T cells (Figure 9), blocking ICOS signalling on re-transferred TFH cells resulted in a strong reduction of cells with the TFH phenotype (PD-1$^+$ CXCR5$^+$ cells in the left panel of Figure 15B, other markers not shown). However, the frequency of transgenic T cells – now all originally with the TFH phenotype – was not reduced (Figure 15B, right panel). These results indicate that TFH cells reverse their phenotype when ICOS signalling is interrupted and that the cells neither leave the lymph node nor show enhanced cell death. Blocking ICOS signalling on re-transferred non-TFH cells also reduced the frequency of cells with the TFH

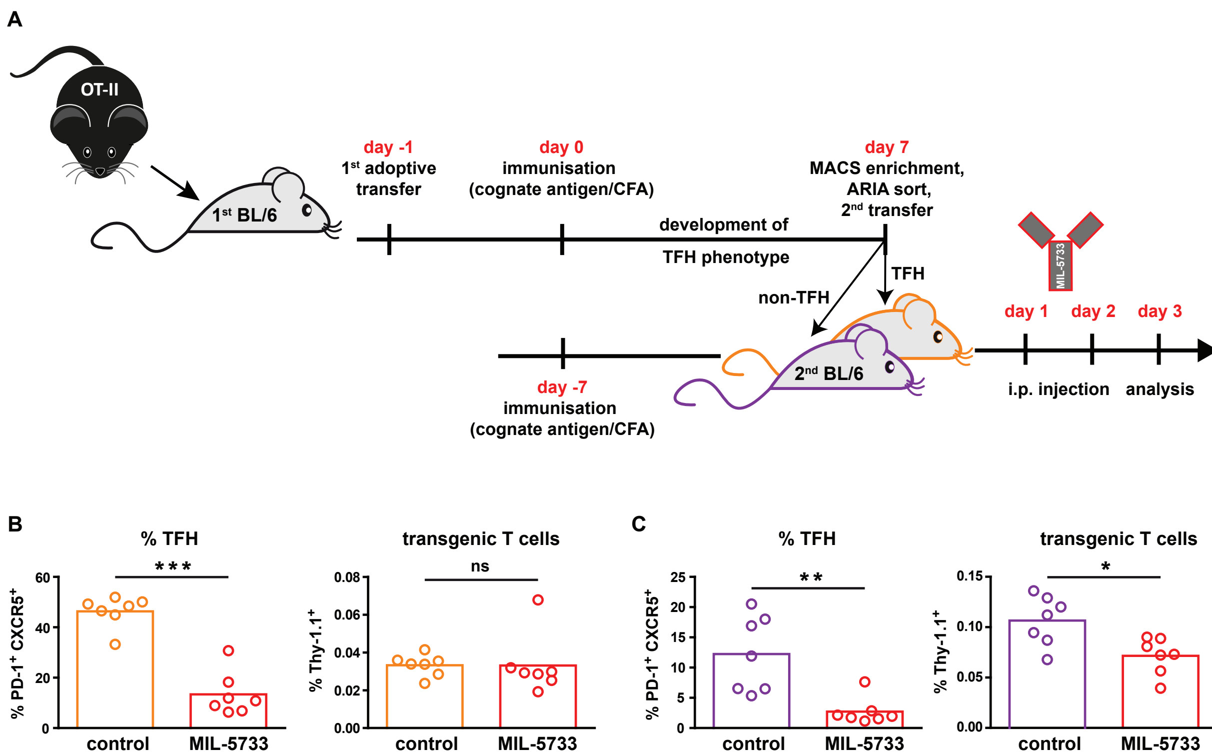

Figure 15 Blocking ICOS signalling causes reversion of the TFH phenotype. **A** Schematic representation of the experimental setup. Flow cytometric analysis of re-transferred **B** TFH and **C** non-TFH transgenic T cells two days after the beginning of blocking. Frequencies of transgenic T cells with the TFH phenotype (PD-1$^+$ CXCR5$^+$) and the percentage of Thy-1.1$^+$ transgenic T cells relative to all CD4$^+$ T cells are shown. Each symbol represents one mouse (n = 7), bars indicate the mean. Data are representative of two independent experiments. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant

phenotype (Figure 15C). This is either an effect on short-term maintenance of the TFH phenotype or indicates a role of ICOS in the late stages of TFH differentiation.

When ICOS signalling was interrupted on all transgenic T cells, a reduction of the frequency of total transgenic T cells was observed (see Figure 9). Since the frequency of transgenic T cells was not reduced when ICOS signalling was blocked on re-transferred TFH cells, this effect must originate from non-TFH cells. Indeed, the frequency of transgenic T cells was reduced when ICOS signalling was interrupted on re-transferred non-TFH cells (Figure 15C).

These results show that ICOS signalling is important for the maintenance of the TFH phenotype and may have a role in the final differentiation steps of non-TFH cells towards the TFH phenotype. Furthermore, application of MIL-5733 seems to have an effect on the frequency of non-TFH, rather than TFH cells. Whether this is due to an egress, cell death, or reduced proliferation of effector T cells is not known.

4.12 Blocking of ICOS signalling does not interfere with cell proliferation

To investigate if application of MIL-5733 reduces proliferation of effector T cells, sorted TFH and non-TFH cells were labelled with CFSE, re-transferred, and analysed two days after beginning of injection of MIL-5733. Since there was no difference in the percentage of cells within each generation according to CFSE dilution (Figure 16), application of MIL-5733 did not seem to result in reduced proliferation of TFH or non-TFH cells. This hints at a role of ICOS in either retaining effector cells in the lymph node or prolonging their survival. There are no data available for or against the first hypothesis; however, the role of ICOS for the survival of activated $CD4^+$ T cells has previously been published by our lab (Burmeister *et al.*, 2008).

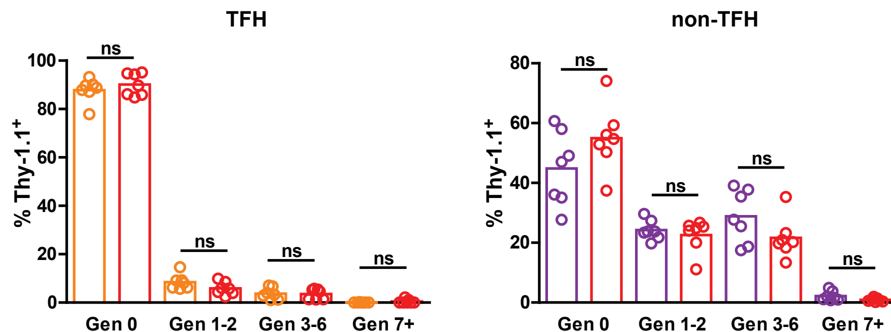

Figure 16 Blocking ICOS signalling does not interfere with proliferation of effector cells. Experimental system as represented in Figure 15A with additional labelling of transgenic T cells with CFSE before transfer. Flow cytometric analysis three days after re-transfer of TFH (left) and non-TFH (right) transgenic T cells into mice injected with 1D10 (orange for TFH, purple for non-TFH) or MIL-5733 (red). The data indicate the distribution of transgenic T cells into generations (Gen) according to CFSE dilution. Each symbol represents one mouse (n = 7), bars indicate the mean. Data were obtained from one experiment. ns = not significant

I have shown so far that abrogation of ICOS signalling has several effects on transgenic T cells: TFH cells lose their phenotype and the frequency of non-TFH cells is reduced. When considering that specific signals are necessary to maintain TFH cells throughout the germinal centre response, it is a logical follow-up question to ask whether TFH cells also persist past the GC reaction and are maintained as memory cells to provide help in secondary infections.

4.13 TFH memory cells can persist in the absence of antigen

Memory has been defined as the persistence of activated T cells in the absence of antigen (Kaech *et al.*, 2002). To generate TFH cells in these studies, I have injected CFA, an adjuvant that is known to slowly release the antigen over a long period of time. In fact, naïve antigen-specific T cells proliferated upon adoptive transfer up to 100 days after the injection of antigen with CFA, showing that at that time antigen was still present in the mouse (data not shown). It is therefore unpractical to analyse the memory response in such a system. However, an adoptive re-transfer of effector T cells into naïve mice is an effective system to separate effector T cells from their cognate antigen. It further allows looking at the fate of specific T cell subsets – in this work, the fate of TFH and non-TFH cells was analysed.

I first tested whether TFH and non-TFH effector cells needed antigen or germinal centre structures for survival, or whether they could survive in the absence of an ongoing immune reaction. To this end, TFH and non-TFH cells were transferred into naïve hosts as well as into mice pre-immunised with cognate antigen and non-cognate antigen, respectively. Nine days after transfer, transgenic T cells from TFH and non-TFH effector cell origin were detected in all three

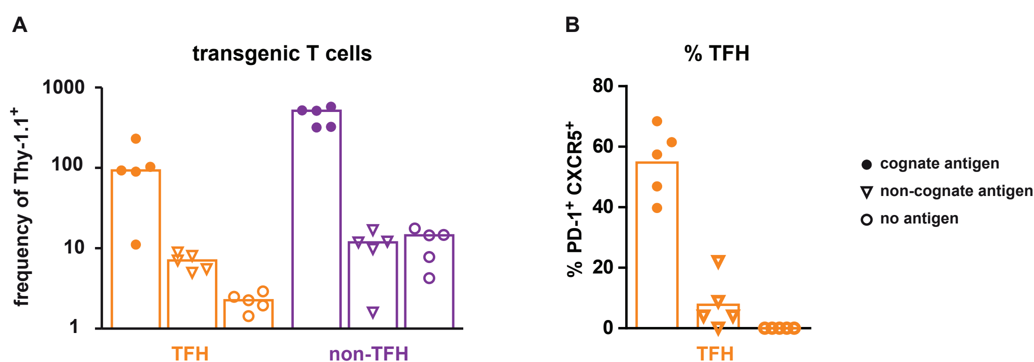

Figure 17 TFH cells can survive in the absence of antigen. 1.2×10^5 sorted OT-II TCR-transgenic TFH or non-TFH cells were transferred into BL/6 mice which had been injected seven days earlier with the cognate antigen (OVA in CFA), a non-cognate antigen (KLH in CFA), or which were left unimmunised. Draining lymph nodes (or a pool of peripheral lymph nodes for the unimmunised recipients) were analysed by flow cytometry nine days after re-transfer for **A** the presence of transgenic T cells (shown as number of transgenic T cells per 10^6 live cells) and **B** the percentage of cells with the TFH phenotype (PD-1$^+$ CXCR5$^+$) amongst the re-transferred TFH cells. Each symbol represents data from one mouse (n = 5), bars indicate the mean. Data were obtained from one experiment.

situations, although in lower frequencies when no cognate antigen was present (Figure 17A). This is probably due to the fact that the effector cells do not proliferate without cognate antigen (data not shown). After transfer of TFH cells, higher frequencies of transgenic T cells and cells with the TFH phenotype were observed in mice with non-cognate germinal centres compared to naïve mice (Figure 17B). This suggests that antigen-independent signals provided by germinal centre structures maintain TFH cells.

To test if the few cells remaining after the contraction phase survived as long-term memory cells, we transferred TFH and non-TFH cells into naïve mice and challenged these with antigen 28 days after transfer and analysed the draining lymph nodes seven days later. Figure 18 shows that although they represented a rare population, TFH cells and non-TFH cells could be unambiguously identified when re-challenged after four weeks in the absence of cognate antigen – when no transgenic T cells were transferred, no Thy-1.1^{+} Thy-1.2^{-} events were detected (data not shown).

Hence, antigen seems to be necessary for maintaining the TFH phenotype, although non-cognate germinal centre structures also seem involved in the maintenance of phenotype and survival of TFH cells. Although few cells remained detectable after the contraction phase in the absence of antigen, these cells persisted for four weeks and probably represent TFH memory cells.

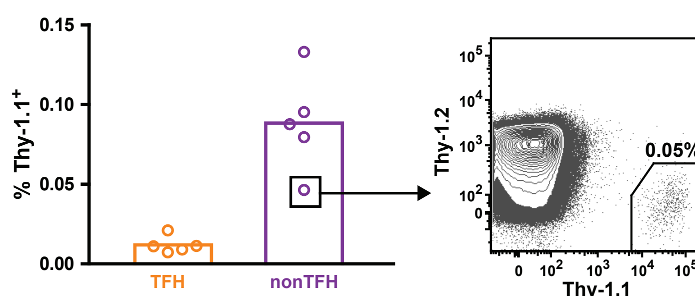

Figure 18 Memory cells survive for at least four weeks in the absence of antigen. 10^4 Smarta TCR-transgenic TFH and non-TFH cells were sorted as shown in Figure 11 and transferred into naïve hosts. 28 days later, recipient mice were challenged with SMA in CFA. Cells from draining lymph nodes were analysed by flow cytometry on day 35. Data indicate the frequency of Thy-1.1^{+} T cells among total CD4^{+} T cells. Each symbol represents data from one mouse (n = 5), bars indicate the mean. The plot shown represents data obtained from the re-transfer of non-TFH cells. Data are representative of two independent experiments.

4.14 TFH and non-TFH memory cells have distinctive phenotypes

After having shown that there is a population of memory cells originating from TFH cells, the question arose whether these cells are phenotypically different from non-TFH memory cells and to which extent the TFH-specific phenotype is maintained in the memory phase.

To analyse resting TFH memory cells, re-transferred transgenic TFH and non-TFH cells were analysed fourteen days after transfer into naïve recipients and their phenotype was compared to that of effector cells on day 8 of a primary immune reaction. As depicted in Figure 19, TFH memory cells strongly downregulated Bcl-6, PD-1, and CXCR5 compared with the effector phase, although the expression was higher than on non-TFH memory and naïve T cells. TFH memory cells maintained a higher potential to secrete IL-21 after re-stimulation with PMA and ionomycin than non-TFH memory cells; however, both populations were not able to produce IL-4 upon re-stimulation *in vitro* and had similar expression levels of CCR7.

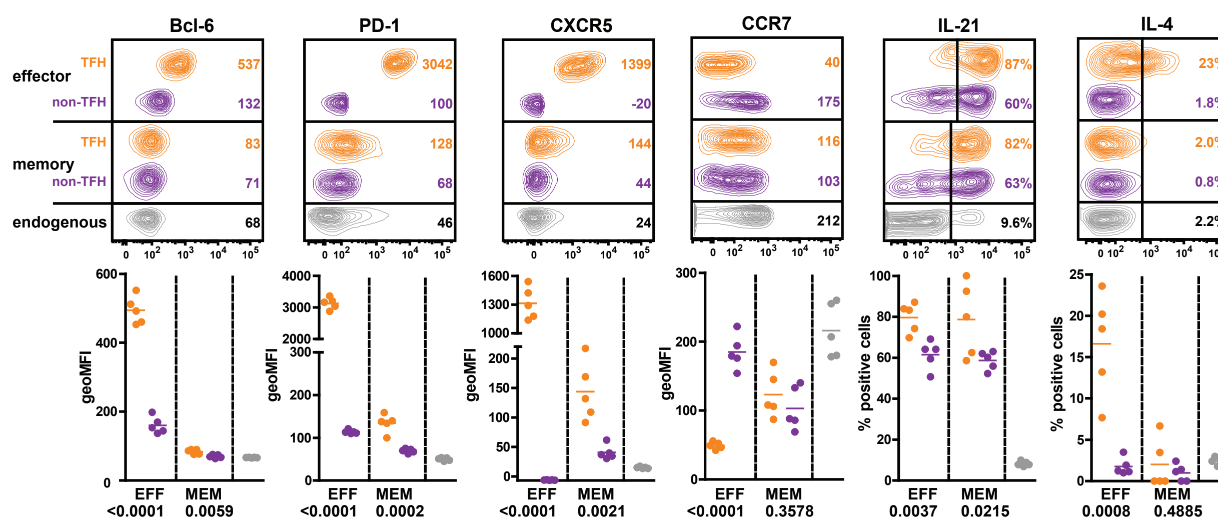

Figure 19 TFH memory and non-TFH memory cells differentially express TFH-characteristic molecules. T cells from Smarta TCR-transgenic mice were transferred into BL/6 mice which were injected s.c. with NP-SMA in CFA. The phenotype of antigen-specific TFH (orange) and non-TFH (purple) cells in the draining lymph nodes (defined by gating on CXCR5/PD-1 double-positive or negative $CD4^+$ T cells) was analysed by flow cytometry on day 8 (effector phase). In a second experiment, TFH and non-TFH cells were sorted on day 8 and transferred into naïve recipients ($2–2.5 \times 10^5$ cells/mouse). Fourteen days later (memory phase), cells were isolated from spleen and peripheral lymph nodes. The expression of the same markers as in the effector phase was analysed. For intracellular staining of cytokines, cells were stimulated with PMA and ionomycin for four hours. The graphs are showing transgenic $CD4^+$ T cells. For comparison, staining of non-transgenic (endogenous) T cells is shown in grey. Where vertical lines indicate gates, the numbers on the graph represent the percentage of cells positive for the respective marker. Otherwise, the geometric MFI of the whole population is shown. To give a representative picture, concatenated flow cytometric data from five animals are shown in the upper panel and statistical analysis in the lower panel, where numbers indicate the p-values for the differences between TFH and non-TFH cells within their respective group (primary effector (EFF) response or resting memory (MEM) phenotype). Each symbol represents data from one mouse ($n = 5$), horizontal lines indicate the mean. Data are representative of two independent experiments.

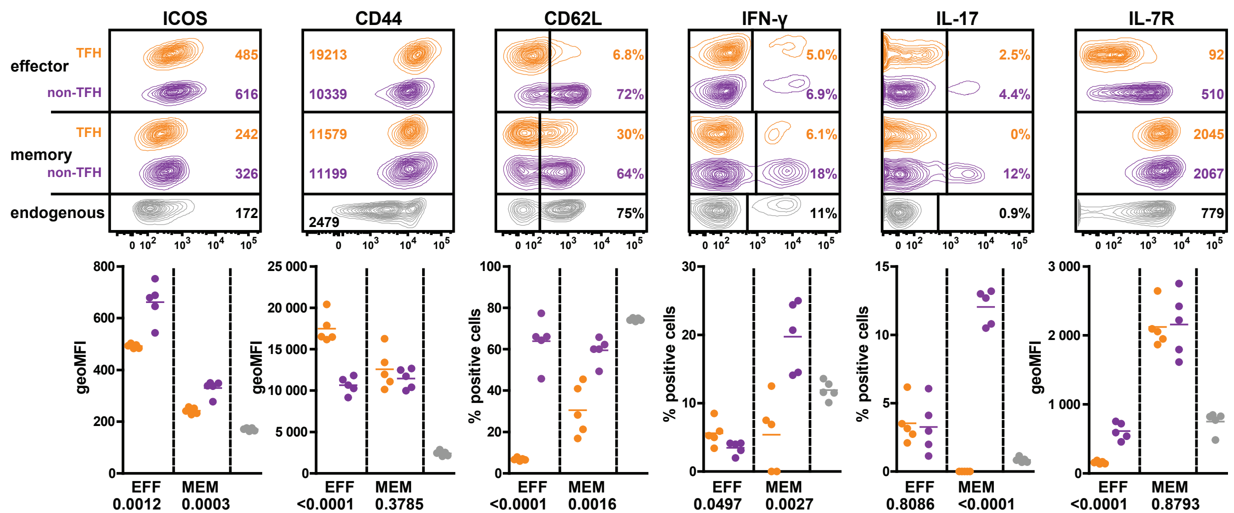

Figure 20 TFH memory and non-TFH memory cells differentially express activation markers. Analysis of additional markers with the same experimental system as in Figure 19.

Figure 20 shows that non-TFH effector and memory cells had a slightly higher expression of ICOS than TFH effector cells, whereas TFH effectors showed a more activated phenotype, since they expressed higher levels of CD44 and lower levels of CD62L. Additionally, non-TFH memory cells produced more of the inflammatory cytokines IFN-γ and IL-17 after *in vitro* re-stimulation, and both memory populations highly expressed the memory marker IL-7R. Hence, TFH and non-TFH memory cells share the expression of the memory markers CD44 and IL-7R, but they have different expression profiles of other activation markers and different capacities to secrete cytokines. Although it is possible to discern differential phenotypes for TFH and non-TFH memory cells, these differences are too small to discriminate the populations in flow cytometric analyses without further means.

4.15 Re-activation of TFH memory cells shows their superior potential for B cell help

After having shown that TFH cells can develop into a memory population and that this population is phenotypically distinct from other memory cells, the questions arose whether TFH memory cells could be re-activated *in vivo* and participate in secondary immune responses where they fulfil a different role from non-TFH memory cells.

To test the capacity to participate in a secondary immune reaction, transgenic TFH and non-TFH cells were rested for fourteen days in naïve mice before being challenged with cognate antigen. Upon re-challenge, both memory cell populations re-expanded, as shown in Figure 21.

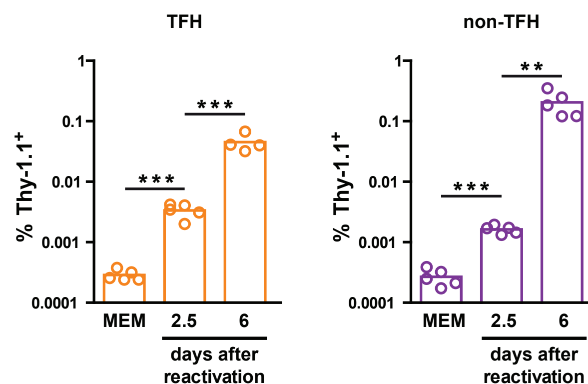

Figure 21 TFH memory cells re-expand after antigen challenge *in vivo*. Recipients of TFH or non-TFH cells (5-120 × 10^4 per mouse) were injected with NP-SMA in CFA 14 days after transfer. Analysis of cells from peripheral lymph nodes before antigen challenge (memory, MEM) and draining lymph nodes 2.5 days and 6 days after antigen challenge. Frequencies of antigen-specific TFH and non-TFH cells relative to all live cells are shown linearly normalised to a transfer of 5 × 10^4 cells per mouse. Each symbol represents data from one mouse (n = 5), bars indicate the mean. Data were obtained from three experiments. *** = $p < 0.001$, ** = $p < 0.01$

To test on a functional level whether the TFH memory cells behaved like memory cells, their activation profile 2.5 days after re-challenge was compared with that of primary responding transgenic T cells 2.5 days after antigen challenge. Figure 22 and Figure 23 show that the memory cells had a generally increased activation profile, since they expressed higher levels of Bcl-6, PD-1, CXCR5, and CD40L and lower levels of IL-7R and CCR7 than cells from a primary immune reaction. Further, TFH memory cells showed an increased expression of TFH-typical markers compared to non-TFH memory cells: heightened expression of CXCR5 and reduced expression of CCR7 gave them the potential to quickly migrate to the B cell follicle; increased potential to secrete IL-21 and IL-4 might have made them the better B cell helpers there. The only exception to this was an equal expression of the B cell co-stimulatory molecule CD40L, which was equally expressed on re-activated TFH and non-TFH memory cells (Figure 23). As in the memory phase, more non-TFH memory cells produced IFN-γ and IL-17 after *in vitro* re-stimulation, clearly distinguishing this population from that of re-activated TFH memory cells.

When additionally looking at 6 days after re-activation, it can be summarised that more TFH memory cells re-adopt the TFH phenotype and maintain it to a higher proportion than non-TFH memory cells (Figure 24).

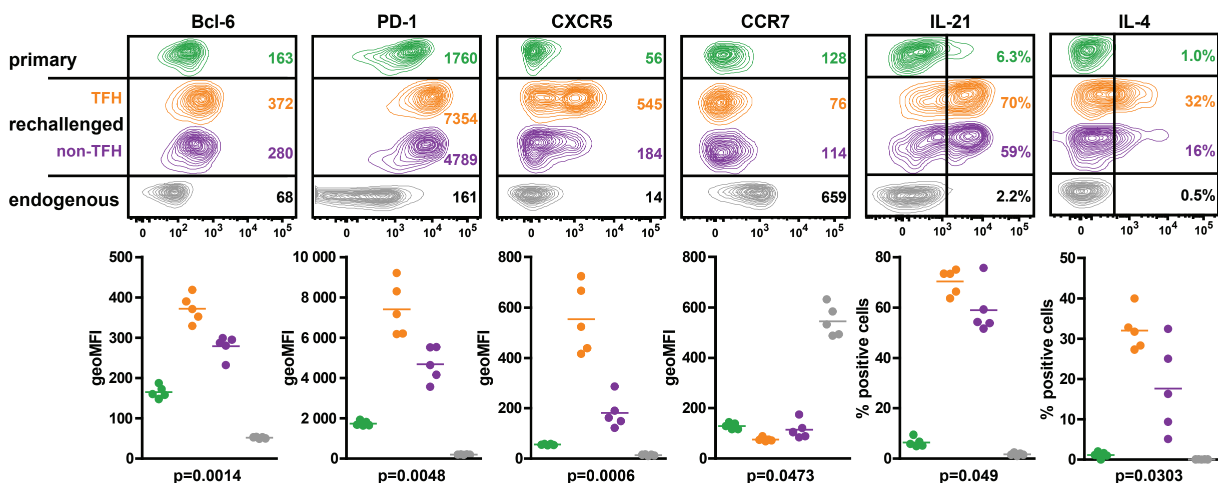

Figure 22 TFH memory cells show an enhanced TFH phenotype after re-activation *in vivo*. Recipients of TFH or non-TFH cells (5–8 × 10^4 per mouse) were challenged with NP-SMA in CFA fourteen days after transfer. Comparison of transgenic T cells from day 2.5 of a primary response (green) and re-activated TFH (orange) or non-TFH (purple) memory cells at day 2.5 after *in vivo* challenge with antigen. Non-transgenic (endogenous) T cells are shown in grey. Expression of cytokines and cell surface markers was analysed as in Figure 19. To give a representative picture, concatenated flow cytometric data from five animals are shown in the upper panel and statistical analysis in the lower panel, where numbers indicate the p-values for the differences between re-activated TFH and non-TFH memory cells. Each symbol represents data from one mouse (n = 5), horizontal lines indicate the mean. Data are representative of two independent experiments.

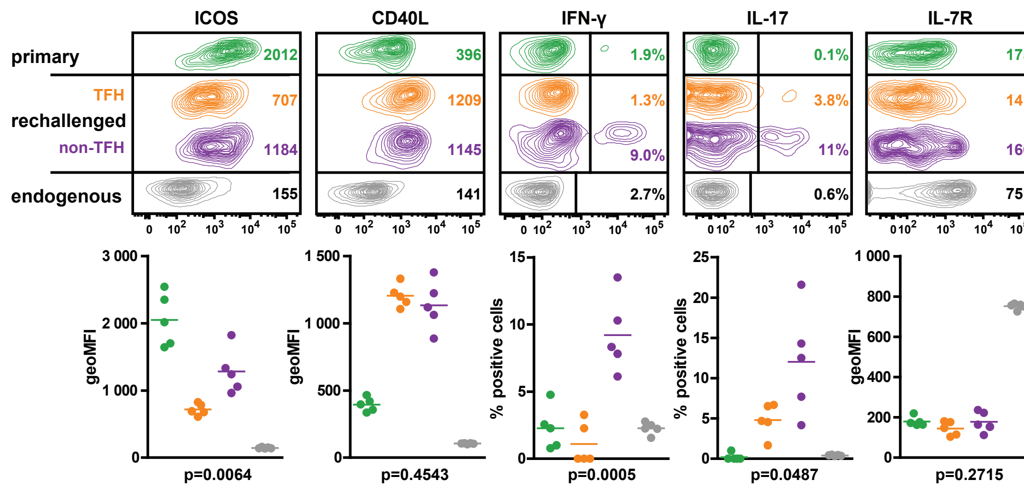

Figure 23 TFH memory and non-TFH memory cells differentially express activation markers after re-activation *in vivo*. Analysis of additional markers with the same experimental system as in Figure 22.

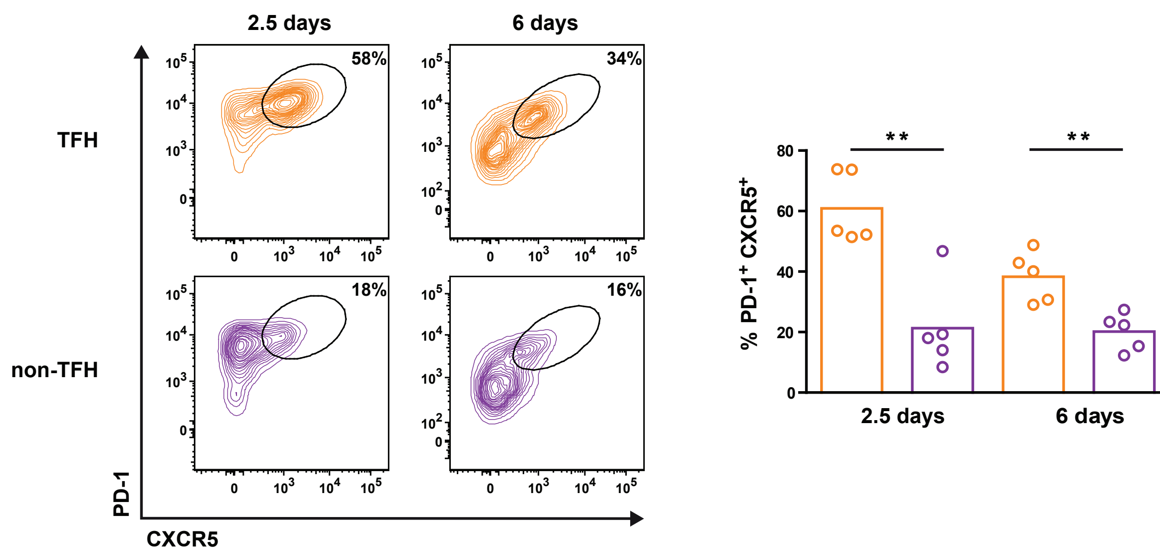

Figure 24 TFH memory cells preferentially re-adopt and maintain the TFH phenotype after re-challenge with antigen *in vivo*. Recipients of TFH or non-TFH cells (5–8 × 10^4 per mouse) were injected with NP-SMA in CFA fourteen days after transfer. Analysis of cells from draining lymph nodes 2.5 days and 6 days after antigen challenge. Expression of CXCR5 and PD-1 on antigen-specific T cells is shown in flow cytometric plots of concatenated data obtained from five mice per group. Each symbol represents data from one mouse (n = 5), bars indicate the mean. Data are representative of two independent experiments. ** = $p < 0.01$, * = $p < 0.05$, ns= not significant

I have therefore shown that TFH cells generate a distinct memory population from non-TFH cells and that both populations are maintained in the absence of antigen with distinct memory phenotypes. TFH memory cells can be re-activated *in vivo*, proliferate quickly, re-adopt the TFH phenotype, and show the potential to provide enhanced B cell help in a recall immune reaction.

5. Discussion

5.1 Merits and dearths of the adoptive transfer system

The first experiments presented in this thesis were designed to evaluate the adoptive transfer of antigen-specific CD4⁺ T cells to study the differentiation of TFH cells. OVA-specific CD4⁺ T cells from OT-II mice were transferred into immunocompetent recipient mice which were subsequently injected subcutaneously with OVA-protein emulsified with the adjuvant CFA. A discrete population of TFH cells was generated by day 6 in the draining lymph nodes and the peak response was observed at day 8 (Figure 2). Other studies using the OT-II transfer obtained comparable results (Baumjohann *et al.*, 2011). However, the immune response differs in amplitude, kinetics, and frequency of TFH cells depending on the number of transferred cells (Foulds and Shen, 2006; personal communication, R. Franke), the adjuvant used (Fazilleau *et al.*, 2009 and data not shown), and the administration route of the antigen (data not shown). Even with constant experimental settings, immune responses in individual mice varied, and even larger variations were observed between experiments. These variations make it imperative to compare experimental groups from within the same experiment only and verify the findings by separate experiments.

Adoptive transfer systems have advantages as well as disadvantages. A disadvantage is the monoclonality of adoptively transferred T cells, which all have the same TCR with the same avidity to the antigen. An alternative approach to study antigen-specific T cells is their identification from an endogenous immune response via staining with tetramers (Nepom, 2012). The analysis of such a polyclonal immune response will yield more physiological results, as TCR avidity has been suggested to influence the fate of the T cell: T cells with the highest avidity have been reported to preferentially differentiate to TFH cells (Fazilleau *et al.*, 2009). Yet this would suggest an equal fate (either no or all CD4⁺ T cells become TFH cells) in a monoclonal response, an observation we did not make. A stronger TCR activation was presumed to be determining preferential TFH cell differentiation, however the higher avidity for antigen may simply result in a competitive advantage for limited antigen. Although the analysis of the more physiological endogenous antigen-specific response seems desirable, today's tetramer conjugates do not provide a sufficient signal to identify rare antigen-specific T cells during early immune responses in flow cytometry. In contrast, the adoptive transfer approach allows analysis at very early times, amongst other advantages: endogenous T cell pools differ in size between mice (Moon *et al.*, 2007) and these cells may have different phenotypes and states of activation. The adoptive transfer allows a defined starting frequency of T cells that can be sorted to have a homogeneous phenotype at the beginning of the experiment, as done

throughout this work with the MACS sort for naïve CD62L^{hi} T cells. Additionally, the adoptive transfer allows transferring T cells with a gene knockout into mice providing a wild-type environment, which allows studying cell type–specific functions of molecules.

We have immunised our mice with antigen in the form of purified protein. It is also possible to elicit a more physiological immune response by infecting mice with living pathogens like bacteria (Pepper *et al.*, 2011) or viruses. The use of lymphocytic choriomeningitis virus (LCMV), for example, allows to either study a monoclonal response using the adoptive transfer of T cells from Smarta mice or the polyclonal response using tetramers (Johnston *et al.*, 2009). When working with replicating pathogens, disease progression is more stochastic and antigen cannot be dosed as easily as in protein immunisation strategies. When comparing data obtained in different studies or by different laboratories, it is important to keep these differences between experimental systems in mind and consider how they could affect the experimental outcomes.

One example of the importance of properly establishing a system and afterwards using constant experimental settings – especially in adoptive transfer experiments – is provided by the co-transfer experiments presented in this study. Wild-type T cells were co-transferred with either ICOS KO or CD28 KO T cells. On day 3, CD28 KO T cells had hardly expanded. Co-transferred ICOS KO and WT T cells expanded to similar degrees, albeit much less than WT cells co-transferred with CD28 KO T cells (data not shown). However, the sum of ICOS KO + WT T cells was equal to the sum of CD28 KO + WT T cells (data not shown). This is an indication that the expansion of WT T cells was inhibited by the expansion of ICOS KO T cells – a limit for total transgenic T cell numbers was reached. The over-saturation of the system was reflected in WT T cells co-transferred with CD28 KO T cells expressing more Bcl-6, CXCR5, and other activation markers than those co-transferred with ICOS KO T cells (Figure 6). As a consequence, fewer WT T cells, whose activation was reduced due to the presence of ICOS KO T cells, adopted the TFH phenotype (Figure 5). It would have been better to transfer lower numbers of antigen-specific T cells to avoid competition for antigen or co-stimulatory factors. This serves as an example why experimental optimisation should be prioritised, and it shows that data can only be compared within an experimental group (here: WT vs. ICOS KO or WT vs. CD28 KO, as done in Figure 6) and that it should be avoided to compare groups that were not explicitly designed to be compared (WT vs. WT from both groups or ICOS KO vs. CD28 KO should not be compared).

5.2 Thou canst be placed in a new home, TFH!

Although the response of 10^3 to 10^5 re-transferred antigen-specific T effector cells scales linearly (data not shown), analysis of the memory phase is impractical when less than 10^5 cells are transferred, due to their extremely low frequencies after contraction in the memory phase. Hence, high numbers of purified, functional TFH cells were needed for analysis, making the establishment of the re-transfer system of TFH cells technically challenging. The yield of the sort for antigen-specific TFH cells was about 10 %, meaning that for every 10^6 transgenic TFH cells isolated from a pool of mouse draining lymph nodes, about 10^5 purified transgenic TFH cells could be obtained for the re-transfer (data not shown). This yield could only be achieved by using antibodies providing the best discrimination for PD-1, even though these have been described to block ligand binding to PD-1 (Ansari *et al.*, 2003). It has recently been proposed that blocking PD-1 signalling on human TFH cells ameliorates antibody responses against HIV (Cubas *et al.*, 2013). The study shows that signalling via PD-1 reduces *in vitro* proliferation of TFH cells. This suggests that re-transferred TFH cells with the blocking antibody on their surface (Figure 11) could be more proliferative than normal TFH cells. However, we observed little proliferation of these cells (Figure 13) and could not detect any of the antibody used for sorting two days after re-transfer (data not shown). Hence, it is unlikely that using the blocking anti-PD-1 antibody is affecting the re-transferred TFH cells.

I further made sure that re-transferred TFH cells were functional by verifying their localisation to the B cell follicle and their B cell helper capacity (Figure 14). When analysing the organ homing of re-transferred activated T cells, few cells were found in the lungs and peripheral blood (Figure 12). Unexpectedly, more cells were retrieved from the spleen than from draining lymph nodes. This would be expected in naïve recipients, as the spleen is the immune organ filtering the blood and therefore an organ that re-transferred cells are likely to end up in. Furthermore, the spleen contains small germinal centres even in naïve mice (personal communication, A. Hutloff), and germinal centres are beneficial for TFH cell survival (Figure 17, further discussed below). However, the transfer was performed into mice that had been immunised subcutaneously with cognate antigen, so that proliferation of cells should have resulted in more cells in draining lymph nodes than in spleen, due to the presence of antigen (Figure 12). Comparison of the proliferation status of re-transferred effector T cells in different organs may provide insights into whether they preferentially home to the spleen or whether their accumulation in the spleen is due to a re-distribution of proliferated cells after antigen encounter.

As $CD4^+$ T cells from OT-II mice are slowly lost from BL/6 mice due to their recognition of a superantigen (Barnden *et al.*, 1998; Bouillet *et al.*, 2002), they were unsuitable for long-term transfers. Hence, the transfer of transgenic T cells from Smarta mice was used in the second part of this study to analyse the memory phase.

5.3 Thy coming of age

The kinetics analysis in Figure 2 shows that only a subpopulation of transgenic T cells expressed the TFH hallmark protein PD-1 on day 2, and this expression correlates with Bcl-6 (data not shown). However, PD-1 was upregulated on all transgenic T cells by day 3 (Figure 2), whereas Bcl-6 and CXCR5 were still expressed only by a subpopulation of transgenic T cells (Figure 6). This raises the question whether early Bcl-6^{hi} cells are TFH precursors or whether the differential expression is due to stochastic effects caused by competition for activation signals. A recent study has sorted and re-transferred early-activated T cells according to their Bcl-6 expression. In these experiments, Bcl-6^{hi} cells from day 3 of an LCMV infection are more likely to become TFH cells than Bcl-6^{lo} cells, suggesting that TFH fate determination occurs during early T/DC interactions and that TFH precursors can be identified via their Bcl-6 expression (Choi *et al.*, 2013).

In our experimental system, Bcl-6 is expressed before CXCR5, a temporal relationship also observed by others. However, Crotty and colleagues also suggested this to be a causative relationship, e.g. that Bcl-6 is important for the induction of CXCR5 (Choi *et al.*, 2011). Other groups either see expression of both molecules starting at the same time (Kerfoot *et al.*, 2011; Kitano *et al.*, 2011) or CXCR5 expression before the induction of Bcl-6, using the same OT-II transfer system as we did (Liu *et al.*, 2012). This latter study also reported that CXCR5 is induced normally in Bcl-6 KO mice, rejecting the notion that Bcl-6 is necessary for the induction of CXCR5. The authors propose instead that Bcl-6 is important for the maintenance of CXCR5 expression. This Bcl-6-dependent maintenance of CXCR5 has also been suggested by others, who reported that Bcl-6 suppresses the expression of the miRNA cluster 17–92, which usually suppresses CXCR5 expression (Yu *et al.*, 2009). Taken together with the proposed role of B cells in the maintenance of Bcl-6 expression (Poholek *et al.*, 2010), it seems that Bcl-6 does not induce but instead maintains CXCR5 expression.

The studies described above hint at a model where TFH cells are derived from identifiable precursors which express Bcl-6. When non-TFH cells, expressing slightly higher levels of Bcl-6 than naïve T cells (compare non-TFH effectors vs. endogenous in Figure 19), were re-transferred into mice with an ongoing immune reaction, I observed that the non-TFH cells are highly proliferative and that a significant fraction adopts the TFH phenotype (Figure 13). Similarly, it has been observed that in chronic LCMV infections, CD4^{+} T cells with the Th1 phenotype adopt the TFH phenotype over time (Fahey *et al.*, 2011). Further, IL-4-producing non-TFH (probably Th2) cells raised in response to helminth antigens adopt the TFH phenotype when re-transferred and challenged with the same antigen (Saretzky *et al.*, 2009) and Th17 cells have been reported to adopt the TFH phenotype in Peyer's patches (Hirota *et al.*, 2013). These data point at a model where non-TFH cells are constantly renewing the pool of TFH cells, regardless of their primary pheno-

type, as long as sufficient antigen is present to drive this differentiation. Another study has evaluated the relationship between TFH cells and antigen availability, concluding that antigen availability is a limiting factor in TFH generation (Baumjohann *et al.*, 2013). These observations further indicate that prolonged TCR stimulation is necessary for TFH cell differentiation, rather than a strong TCR signal provided by high avidity TCRs (Fazilleau *et al.*, 2009). They further suggest that TFH cells do not derive from specific precursor cells but instead that T cells from various specialisation can adopt the TFH phenotype.

5.4 Thy truthful face: are thou the only giver of IL-21, and do thou divide?

TFH cells have been described to be the main $CD4^+$ T helper subset producing IL-21, so that IL-21 has been designated a TFH hallmark protein (Spolski and Leonard 2010; Crotty, 2011). This view often excludes that Th17 cells are also potent producers of IL-21 (Suto *et al.*, 2008; Spolski and Leonard, 2008) and that the IL-21 production of TFH cells *in vivo* has so far been shown on the mRNA level only. When staining IL-21 protein, 50 % of the transgenic T cells expressed IL-21 at a time when about 10 % of transgenic T cells had the TFH phenotype (Figure 2). Subsequent analysis revealed that considerable proportions of non-TFH cells could express IL-21 (Figure 3 and Figure 19) in apparent contradiction to our own (personal communication, M. Al Baz) and published mRNA data. One potential explanation of these findings is that the non-TFH cells in this system are predominantly Th17 cells; however, few non-TFH cells produced the Th17 hallmark cytokine IL-17 (Figure 20) and attempts to stain for the Th17 master transcription factor RorγT were unsuccessful (data not shown). It is therefore unlikely that the discrepancy stems from unidentified Th17 cells.

To date, most IL-21 mRNA data have been generated by quantitative RT-PCR on sorted cell populations. The recent development of an IL-21 reporter mouse, that expresses green fluorescent protein (GFP) under the control of the IL-21 promoter, allows analysing the IL-21 production of living cells (Lüthje *et al.*, 2012). Upon immunisation of the reporter mouse, GFP was not expressed by non-TFH cells and only by a third of the TFH cells. However, the mouse is a transcriptional reporter of IL-21 mRNA and not a translational reporter of the actual protein, which could explain why similar results to previously published mRNA data were obtained.

One main advantage of the reporter mouse is the possibility to analyse living cells directly after isolation, providing data on actual mRNA production on a single-cell level. In contrast, analysis of protein production, as performed in this thesis, requires re-stimulation of cells *in vitro*, thus resulting in data on the potential to secrete IL-21 upon stimulation, rather than actual *in vivo* protein production. Further, the frequency of potential IL-21 producers is dependent on the re-stimulation conditions (Figure 3 and data not shown).

The different data in mRNA transcription and potential to secrete the protein could either result from increased mRNA levels due to the *in vitro* re-stimulation or from post-transcriptional regulation of IL-21 production. The latter is implausible, since protein production needs mRNA and we observed protein in *more* cells than there are cells positive for IL-21 mRNA. The reporter could, however, be underestimating IL-21 production due to different half-lives of IL-21 mRNA and GFP protein. To conclusively settle the relation between IL-21 mRNA levels and protein production, the protein needs to be stained and correlated with GFP signal in the reporter mouse. Until this has not been done, the data presented in this thesis call into question the importance of TFH cells as the sole producers of IL-21 protein.

The data obtained in the re-transfer experiments suggest that non-TFH cells are constantly renewing the pool of TFH cells, whereas TFH cells are non-proliferating, indicating that they have reached an endpoint of differentiation (Figure 13). Final differentiation also implies a stably imprinted phenotype, which, however, was not observed for re-transferred TFH cells, as half of the cells lost the TFH phenotype when re-transferred into mice with ongoing immune reactions against cognate antigen (Figure 13B and Figure 15B). Opposing data were published very recently: TFH cells were found to be proliferative and to maintain their phenotype stably upon re-transfer (Choi *et al.*, 2013). However, proliferation was assessed *in vitro* after stimulation of the TCR with monoclonal antibodies and not *in vivo*. Yet another study claimed that TFH cells were highly proliferative, although here polyclonal T cells were compared without staining for antigen-specific T cells. This resulted in a comparison of antigen-specific TFH cells (as there are no TFH cells without immunisation) with a pool of antigen-specific and antigen-unspecific non-TFH effector cells and non-responding naïve cells (Lüthje *et al.*, 2012). In such a comparison, groups containing mainly cells that do not react to the antigen will usually proliferate less in response to the antigen than antigen-specific T cells.

When TFH cells were transferred into differently treated recipient mice, more TFH cells maintained their phenotype in mice with an ongoing immune reaction against a non-cognate antigen than in naïve mice (Figure 17A). The presence of germinal centres or an inflammatory environment seems to provide antigen-independent signals to TFH cells to promote survival and, to a lesser degree, to maintain their phenotype (Figure 17B). Non-TFH cells, on the other hand, did not benefit from these antigen-independent signals: their frequency was identical in immunised mice and naïve mice (Figure 17A). Thus TFH cells may be more dependable on survival signals than non-TFH cells, possibly due to their high expression levels of the inhibitory molecule PD-1. Blocking PD-1 signalling *in vivo* and comparing the survival of re-transferred TFH cells in immunised or naïve mice could shed light on this issue.

5.5 Thy needed friends CD28 and ICOS

5.5.1 Thy childhood friend CD28

One of the striking features of CD28 KO mice is their reduced Ig levels and isotype class switch upon antigen challenge (Shahinian *et al.*, 1993; Linterman *et al.*, 2009). It was shown that CD28 signalling is necessary for the formation of $CXCR5^+$ $CD4^+$ T cells in spleen upon antigen challenge by using a CTLA-4-Ig-transgenic mouse, in which B7 signalling is constantly blocked: only after administration of a stimulating anti-CD28 antibody, $CXCR5^+$ T cells in these mice could develop (Walker *et al.*, 1999). Since germinal centres persisted after the antibody was cleared, CD28 seems not essential for the maintenance of TFH cells in this polyclonal system (Walker *et al.*, 2003).

Using the adoptive transfer system, I could confirm the dependency of TFH cells on CD28 signalling in an antigen-specific system and investigate the underlying mechanisms. Although CD28 KO cells could expand (data not shown) and showed other signs of activation like the down-regulation of CCR7, they failed to properly induce any of the analysed markers important for B cell help or the differentiation towards TFH cells, including Bcl-6 (Figure 6).

Using a CTLA-4-Ig fusion protein as blocking agent with a short half-life in mice (Linsley *et al.*, 1992), a time-specific abolishment of CD28 signalling, which was as effective as a genetic knockout (Figure 8), could be achieved. Administration of the blocking agent at different time points revealed that CD28 signalling is not only important during early T/DC but also during T/B interactions (data not shown). Blocking the signals on established TFH cells by day 6, however, had no immediate effect on frequencies of PD-1/CXCR5 double-positive T cells (Figure 9). Still, suppression of CD28 signalling reduced the expression of Bcl-6 and ICOS by TFH cells, which eventually resulted in a delayed loss of PD-1/CXCR5 double-positive cells (data not shown).

It has been suggested that a B cell–specific expression of CD86 (B7-2) is necessary for both GC B cells and TFH cells to develop in response to a viral infection (Salek-Ardakani *et al.*, 2011). The same publication shows that the dependency on CD86 decreases at later time points but persists. Another study suggests a role for CD80 (B7-1) on B cells (Good-Jacobson *et al.*, 2012), which the first study excluded. Good-Jacobson *et al.* try to resolve this by attributing the role of CD80 to the late GC response, although effects are also visible at day 7 in their data set. These data are in accordance with our data, as we also see that CD28 blocking becomes less effective the later it is started, although it still has a (delayed) effect on established germinal centres. This indicates that CD28 signalling is primarily important during early TFH development but can have a role in later phases of the immune reaction, possibly by regulating ICOS expression.

5.5.2 Thou canst not thrive without thine ICOS

CD28 and ICOS are two structurally closely related costimulatory molecules signalling via the Akt pathway after recruitment of PI3K (Rudd and Schneider, 2003). Although CD28 is essential during the induction phase of TFH cells, ICOS KO T cells normally induced TFH hallmark proteins including Bcl-6 and molecules necessary for providing B cell help at day 3 of an immune response (Figure 6). The only difference between WT and ICOS T cells was reduced induction of CXCR5 and downregulation of CCR7, yet this abnormal regulation of chemokine receptors had no influence on T cell positioning in the lymph node (Figure 7). These data contradict published data that suggest that ICOS is essential for the induction of the TFH phenotype by inducing Bcl-6, which in turn is necessary for induction of CXCR5 (Choi *et al.*, 2011). However, the authors compared Bcl-6 expression levels between CXCR5^{+} WT cells and total ICOS KO T cells. Since CXCR5 and Bcl-6 are co-expressed in WT T cells and CXCR5^{+} T cells are strongly reduced in ICOS KO T cells, this comparison is misleading, and CXCR5 regulation cannot be excluded as the primary defect causing apparently lower Bcl-6 levels.

Although induction of TFH cells seems independent of ICOS, ICOS KO mice show a pronounced decrease in the number of TFH cells; hence, the role of ICOS on the maintenance of TFH cells was investigated. Blocking of ICOS signalling by application of a blocking anti-ICOS-L antibody resulted in a rapid loss of PD-1/CXCR5 double-positive T cells and a slight reduction of the overall number of transgenic T cells (Figure 9) with subsequent collapse of germinal centres (Figure 10). Blocking of ICOS signalling on re-transferred TFH cells revealed that the loss of TFH cells is due to a reversion of the phenotype (Figure 15), which could be observed as early as 20 hours after beginning of blocking (data not shown). The dependency of TFH cell maintenance on ICOS signalling was very recently confirmed by another group (Baumjohann *et al.*, 2013), although the authors do not observe a reduction of antigen-specific T cells.

The small reduction of antigen-specific T cells in my system was caused by a reduction of antigen-specific non-TFH cells (Figure 15) that is independent of proliferation (Figure 16) and likely to be due to the role of ICOS in the longevity of effector T cells (Burmeister *et al.*, 2008). It was further observed that fewer non-TFH cells had adopted the TFH phenotype when ICOS signalling was missing. Either the cells adopted the phenotype and lost it immediately, or it may indicate that ICOS also has a role in the late differentiation from non-TFH to TFH cells.

5.5.3 One friend's dusk is another friend's dawn

Despite their structural relationship, a functional division between CD28 and ICOS in the humoral immune response could be identified. How is this division of labour orchestrated? One possible explanation for the early dependence on CD28 and not ICOS could be based on their expression pattern: CD28 is constitutively expressed on naïve T cells, whereas ICOS needs to be upregulated from its low basal expression during T cell activation. However, this explanation is unsatisfactory, since ICOS upregulation happens very fast, with peak expression 24 hours after activation (Burmeister *et al.*, 2008), and the lack of ICOS signalling was not without consequences, as ICOS KO cells had defective regulation of the chemokine receptors CXCR5 and CCR7 at day 3 of the immune response (Figure 6). Although CD28 is essential for the induction of the TFH phenotype, aberrantly high ICOS expression can override CD28 deficiency in sanroque mice and leads to normal TFH cell formation (Linterman *et al.*, 2008), which further indicates that ICOS has the potential to influence early activation events. A more likely explanation is that CD28 signalling can substitute for ICOS signals in early T/DC interactions, due to the high expression of its ligands on activated dendritic cells. Interestingly, even the few TFH cells developing from CD28 KO mice seem to depend on ICOS for function, as mice lacking ICOS additionally to CD28 have a stronger defect in isotype switch than CD28 KO mice (Suh *et al.*, 2004). It may be interesting to block ICOS signalling on established CD28 KO TFH cells to verify this role of ICOS.

Dependence on CD28 decreases and the necessity for ICOS signalling increases once the T cell is inside the B cell follicle, as ICOS-L is highly expressed on activated B cells receiving co-stimulatory signals via CD40 (Liang *et al.*, 2002) which are provided by CD40L on activated T cells (see primary response in Figure 23). Further, CTLA-4 is highly expressed on TFH cells (data not shown), competing with CD28 for the shared ligands. The more important role of ICOS during T/B than T/DC interaction is also highlighted by the fact that a B cell–specific knockout of ICOS-L is sufficient to significantly reduce TFH cell frequencies (Nurieva *et al.*, 2009; Pepper *et al.*, 2011). ICOS signalling may be important for either correct positioning of TFH cells or for assuring lasting B cell contacts, a function that has been proposed for the co-stimulatory molecule Slam (Qi *et al.*, 2008). In both cases, ICOS deficiency reduces B cell contacts which are important to maintain Bcl-6 expression (Poholek *et al.*, 2010). Which mechanism is in play could be investigated with *in vivo* imaging studies.

The exclusive role of ICOS for the maintenance of the TFH phenotype is also interesting in the context of the treatment of many autoimmune diseases which are linked to an exaggerated TFH response (Craft, 2012). So far, blocking of CD28 signalling using CTLA-4-Ig has been a powerful treatment option (Gizinski *et al.*, 2010). Blocking the ICOS pathway, on the other hand, might

be a more specific way to interrupt an ongoing autoimmunity-associated GC reaction with less interference on the initiation of protective immune responses.

5.6 Thou lookest different, olde friend!

TFH cells persist for prolonged times in draining lymph nodes in the presence of antigen (Fazilleau *et al.*, 2007). The authors postulate that these cells are memory cells, although T cell memory is usually defined as the survival of activated T cells in the absence of antigen (Kaech *et al.*, 2002). A more recent study used a polyclonal LCMV infection model to investigate which CD4⁺ effector T cells progress to the memory phase and found antigen-specific CD4⁺ T cells up to 150 days after infection when the virus – and therefore the antigen – had been cleared (Marshall *et al.*, 2011). When comparing the composition of effector cells at the peak of the response and during the memory phase, the authors presented circumstantial evidence for memory TFH cells: TFH cells expressed low levels of PSGL-1 at the peak of the response, and cells with this phenotype could also be identified in the memory phase. The authors admit, however, that these cells do not have other typical TFH markers in the memory phase, and a precursor–progeny link between effector and memory cells was not investigated further.

It has been difficult to study TFH cell memory, since it was not known whether the cells would maintain the TFH-characteristic phenotype in the memory phase, which made their identification difficult. In humans, a CXCR5⁺ T cell subset in peripheral blood has been described as TFH-like memory cells with high B cell helper capacity (Morita *et al.*, 2011; Chevalier *et al.*, 2011). However, the precursors of these cells are not identifiable in the human system. In mice, CXCR5⁺ T memory cells have been sorted and shown to have superior B cell helper capacity (MacLeod *et al.*, 2011) similar to the human circulating CXCR5⁺ T cells. Without providing any evidence, the authors assume that these cells were derived from TFH cells. Another study has opposed this view, suggesting that CXCR5⁺ memory cells are Th1 central memory cells and TFH cells cannot survive as memory cells (Pepper *et al.*, 2011).

To settle the question whether TFH cells can give rise to memory cells, TFH cells from an ongoing germinal centre reaction were transferred into naïve recipients and it was thus shown that TFH cells could persist in the absence of antigen for at least 4 weeks (Figure 18). Although minor phenotypical differences between memory cells from TFH or non-TFH cell origin could be identified, these differences are too small to allow for a discrimination of the memory cell subsets by flow cytometric analysis alone (Figure 19 and Figure 20). It is especially noteworthy that the TFH memory cells were not discriminable on the basis of CXCR5 expression.

To date, four independent groups have shown that TFH memory cells can develop using an adoptive transfer of TFH cells (Weber *et al.*, 2012; Lüthje *et al.*, 2012; Liu *et al.*, 2012; Choi *et al.*, 2013). Apart from our lab's study, only one group has attempted to characterise the phenotype of memory TFH cells in the resting phase, and they analysed CXCR5 expression only (Choi *et al.*, 2013). Early TFH cells from day 3 of an LCMV infection were transferred into infection-matched mice and analysed 45 days later, when the virus should be cleared. In contrast to our data, CXCR5 expression was maintained on the majority of TFH cells in the memory phase. This is an indication that the existence of CXCR5$^+$ memory cells is limited to immune responses to viral infections. Their work further suggests that TFH cells are the main precursors to memory CD4$^+$ T cells in LCMV infections (Choi *et al.*, 2013). This stands in apparent contradiction to the finding that CXCR5$^+$ memory T cells are mainly Th1 central memory cells (Pepper *et al.*, 2011), although the latter study used a bacterial infection model and looked at a polyclonal response. It may be that the infection model has a considerable impact on which cells progress to become memory cells and on their phenotype. Hence, it is unfortunate that the other groups have not provided more extensive characterisations of their memory cells in the resting phase, which would have provided a more comprehensive spectrum of memory CD4$^+$ T(FH) cell phenotypes.

Radbruch and colleagues have suggested that true memory T cells, similarly to memory B cells, are maintained only in bone marrow (Tokoyoda *et al.*, 2009). Yet, we and others have identified memory TFH cells in the resting phase in peripheral lymph nodes and spleen (Figure 21; Lüthje *et al.*, 2012; Choi *et al.*, 2013). When analysing bone marrow, liver, and gut tissues, no memory cells could be found (data not shown). However, the frequency and absolute cell number of memory cells was very low in our experimental system and lymphocyte extraction from these organs was less effective, so that we were unable to analyse enough events to exclude the possibility that TFH memory cells are maintained in low frequencies in other organs than lymph nodes and spleen. It is also possible that our memory cells had not yet progressed towards their final localisation in the bone marrow, as antigen-specific T cells have been reported to start accumulating in the bone marrow only after around four weeks after the peak of the immune response (Tokoyoda *et al.*, 2009).

In lymph nodes and spleen, TFH memory cells upregulated CCR7 and downregulated CXCR5 to levels similar to naïve T cells (Figure 19). It is therefore likely that TFH memory cells are located within the T cell zone, although a corresponding histological analysis has not been carried out yet.

5.7 Thy strength was never any greater than now

To test the TFH memory cells on a functional level, we assessed their phenotype after re-activation *in vivo* (Figure 22 and Figure 23). Since TFH memory cells expand after antigen challenge (Figure 21), the re-activation is easier to analyse than the resting phase with its small cell numbers; hence, two of the other studies describing TFH memory cells have also analysed this part of the immune response (Lüthje *et al.*, 2012; Liu *et al.*, 2012). While TFH and non-TFH memory cells are very similar during the memory phase (Figure 19 and Figure 20), their phenotypic differences are substantial after re-activation (Figure 22 and Figure 23). This indicates lineage-specific differences of TFH and non-TFH cells are maintained during the memory phase and that TFH memory cells may have a specific role in recall responses.

However, TFH and non-TFH cells show considerable plasticity during the secondary challenge: although TFH memory cells preferentially re-adopt the TFH phenotype, not all do, and some non-TFH memory cells become PD-1/CXCR5 double-positive (Figure 24; Lüthje *et al.*, 2012). Nonetheless, depending on the experimental systems, non-TFH cells may not adopt the TFH phenotype (Liu *et al.*, 2012). The high expression of PD-1/CXCR5 looks like that observed on day 8 of the primary response, although it could be argued that upregulation of PD-1 and CXCR5 is simply due to cell activation, as observed on day 3 of a primary response (Figure 2).

Directly showing superior B cell help from TFH memory cells was unsuccessful due to technical reasons (data not shown). However, an increased potential to provide B cell help was shown by comparing the activation profile of TFH memory cells to that of naïve T cells from a primary response (Figure 22 and Figure 23). Due to higher expression of CXCR5, lower expression of CCR7, and higher expression of IL-4, IL-21, and CD40L, re-activated TFH memory cells have the potency to be very efficient B cell helpers in a recall response.

Doubt about the function of TFH memory cells has been raised in an editorial published together with the data presented in this thesis (Barr and Gray, 2012). The authors mainly question the short resting phase of 28 days that TFH cells were submitted to and speculate that the memory cells will disappear shortly afterwards, especially since their number has already gone down drastically compared to the effector phase. Yet, this retraction of cell numbers was observed almost immediately after the transfer (Figure 17), and in consideration of the data published by Nutt and colleagues (Lüthje *et al.*, 2012), who analysed memory cells after up to 55 days in absence of antigen, little doubt should be left that TFH cells develop into long-term memory cells.

The data presented in this thesis provide evidence for the functional differences between the structurally related proteins CD28 and ICOS, which was suggested nearly a decade ago (Kroczek *et al.*, 2004), and these findings may be a step-stone to better treatment of autoimmune diseases caused by aberrant germinal centre reactions. The data further demonstrate that TFH cells can develop into memory cells, the cells are phenotypically characterised, and their potential to be potent responders upon re-challenge is highlighted. The importance of this work is stressed by the recent suggestion that the TFH memory could be an important tool for rational vaccine design (Streeck *et al.*, 2013).

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B List of publications

Publications related to this study:

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C Abbreviations

APC	antigen-presenting cell
Bcl-6	B cell lymphoma-6
BCR	B cell receptor
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
CFA	Complete Freund's Adjuvant
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CTLA-4	cytotoxic T lymphocyte–associated antigen 4
CXCR5	C-X-C chemokine receptor type 5
Cy	cyanine dye
DAPI	4',6-diamidino-2-phenylindole
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GC	germinal centre
geoMFI	geometric mean fluorescence intensity
GFP	green fluorescent protein
i.v.	intravenous
ICOS	inducible co-stimulator
ICOS-L	ICOS ligand
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KLH	keyhole limpet hemocyanin
KO	knockout
MACS	magnetic-activated cell sorting
MFI	mean fluorescence intensity
mRNA	messenger ribonucleic acid
NP	nitrophenol
OVA	ovalbumin
PD-1	programmed death-1
PE	phycoerythrin
PI3K	phosphatidylinositol-3-kinase
PMA	phorbol 12-myristate 13-acetate
PNA	peanut agglutinin
RT-PCR	reverse transcription polymerase chain reaction
s.c.	subcutaneously
SAP	Slam-associated protein
SHM	somatic hypermutation
SMA	Smarta protein
TCR	T cell receptor
TFH	T follicular helper
Th	T helper
WT	wild type

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